PRO-INFLAMMATORY CYTOKINES AND INTERLEUKIN 6 IN THE RENAL RESPONSE TO BACTERIAL ENDOTOXIN

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Pro-inflammatory cytokines, including tumour necrosis factor α (TNF-α), interleukin (IL)-1 and IL-6 are thought to play important roles in the pathophysiology of chronic kidney disorders, including glomerulonephritis. In particular, IL-6 has received considerable attention as it appears at high concentrations to promote the progression of renal disease while at lower levels may be involved in regulating repair mechanisms. As such, cytokine profiles have been examined in the kidney by either examining secretion from isolated kidney cells or quantitating plasma and urinary levels in experimental models of glomerulonephritis. To examine the cytokine responses within the kidney, without the contribution of other organ systems, we used semi-quantitive polymerase chain reaction (RT-PCR) analysis and a recently developed kidney slice culture model from tissues of mice treated with combinations of endotoxin and neutralizing antibodies against TNF-α. The expression of IL-6, in addition to other pro-inflammatory cytokine genes, was increased by endotoxin treatment and reduced by pretreatment with neutralizing antibodies to TNF-α. Immunohistochemical staining revealed that IL-6 was expressed primarily in mesangial cells. Urinary IL-6 was also increased in endotoxin-treated mice and was inhibited by treatment with neutralizing TNF-α antibodies. Kinetics of the kidney-specific cytokine responses indicated that increase in TNF-α occurred initially, followed by IL-1β and finally IL-6. Furthermore, addition of TNF-α to glomerular mesangial cells induces IL-6 secretion. Taken together, these studies indicate that, like in the liver, a cytokine response occurs in the kidney from bacterial endotoxin and that TNF-α acts as a primary cytokine capable of stimulating additional cytokines, including IL-6.

Pro-inflammatory cytokines are produced in response to a variety of stimuli including bacterial endotoxin, pro-oxidants, and certain environmental agents. The pathophysiological responses associated with inflammatory cytokines in renal diseases are currently being characterized. For example, inflammatory cytokines exacerbate many types of glomerulonephritis including mesangial IgA disease, myasthenia gravis, systemic lupus erythematosus and systemic vasculitis as well as rejection of renal transplants.[1–5] In addition to glomerulonephritis, they are thought to play a role in ischaemia and toxic chemical injury in renal tissue.[6] This response can be broadly defined as part of the acute phase response which, at least in the liver, represents a co-ordinated set of changes including the altered rate of synthesis of certain plasma proteins such as C-reactive protein.[7] Interleukin 6 (IL-6) a 26-kDa protein produced by a variety of cell types including kidney mesangial cells,[8] can mediate most if not all of the acute phase response. In the kidney, IL-6 is involved in mesangial proliferative nephritis,[5] as well as renal tubular epithelial cell regeneration.[9] While IL-6 is commonly found in the kidney, TNF-α has been the most studied as it is thought to be involved in glomerulonephritis and inflammatory renal diseases. More recently, attention has also focused on the role of intraglomerular growth factors and cytokines involved in glomerular cell proliferation and extracellular matrix expansion that can accompany progressive glomerular diseases.[4]

Although considerable information is available on the types and kinetics of cytokine responses in

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Received 8 November 1996; revised and accepted for publication 27 March 1997

1043–4666/97/090688 + 8 $25.00/0/ck970214

KEY WORDS: kidney cytokines/renal sepsis/urinary IL-6

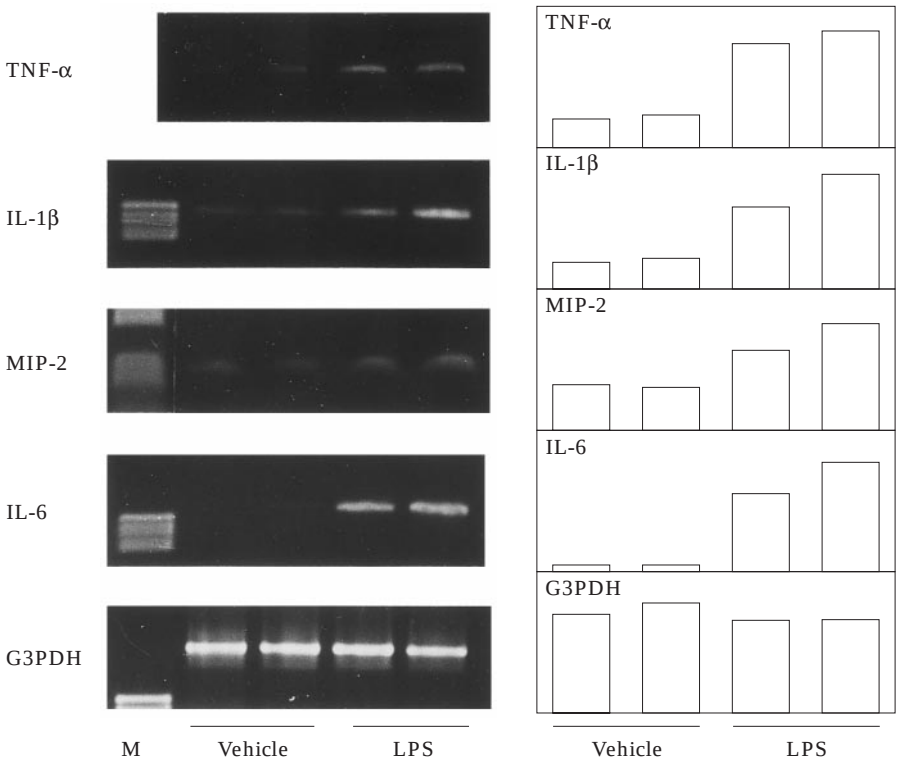

Figure 1. Amplification of cytokine mRNA transcripts by RT-PCR for mouse kidney RNA.

Mice were treated with vehicle (0.9% saline) or LPS (2 mg/kg, dissolved in saline) 1 h prior to collection of kidneys for processing as described in Materials and Methods. Molecular weight marker (pBR322/HaeIII digest) is represented in lane M. The density of each amplified cDNA band for cytokine message was normalized relative to the density of each corresponding band for G3PDH. The amplified PCR products from cytokine mRNA were 692 base pairs (bp) for TNF-α, 563 bp for IL-1β, 638 bp for IL-6, 259 bp for MIP-2, and 983 bp for G3PDH. The gels were scanned with a computerized laser densitometer and the area under the curve expressed as percentage of control.

animal models of glomerulonephritis, most of these studies have examined responses in plasma or in isolated glomeruli, mesangial or renal epithelial cells. While such studies are critical in establishing the composite and individual cell responses, they do not provide information on organ contributions in which both soluble and cellular interactions control the expression, release and regulation of pro-inflammatory mediators. Since endotoxin plays a major role in septic acute renal failure and LPS is known to induce the production of a number of cytokines, then, to examine the characteristics of cytokine expression and production that occur specifically in the kidney from bacterial endotoxin, a liver slice culture system was adapted for kidneys and cytokine expression examined. This was complemented with a semi-quantitative polymerase chain reaction (PCR) analysis to determine transcriptional events.

RESULTS

Relative changes in cytokine mRNA transcripts were examined in kidneys of lipopolysaccaride (LPS)-treated and control mice by RT-PCR. In addition to IL-6, the expression of TNF-α, IL-1α, IL-1β and

TABLE 1. TNF-α and IL-6 secretion from kidney slices of mice treated with LPS

Treatment	TNF-α (U/ml)	IL-6 (ng/ml)	IL-1β (pg/ml)	IL-1α (pg/ml)
Vehicle	0.05 ± 0.01	2.0 ± 0.4	10.2 ± 2.3	<1
LPS	0.29 ± 0.04*	6.2 ± 0.2*	36.2 ± 7.6*	<1

Mice were administered LPS (2 mg/kg) i.v. one hour before removal of kidneys. Tissue slices were prepared and incubated as indicated in Materials and Methods. Each value represents the mean ± SE of replicate cultures from four animals.
*Slightly different from vehicle control at $P < 0.05$.

macrophage inflammatory protein 2 (MIP-2) were examined. As shown in Figure 1, LPS administration induced a relative increase in TNF-α, IL-1β, MIP-2, and IL-6 mRNA transcripts. IL-α was not detected in kidneys of vehicle of LPS-treated mice (data not shown). In order to determine whether increased cytokine mRNA expression in the kidney is associated with cytokine secretion, kidney slices from LPS-injected mice were prepared, cultured for 6 h and supernatants examined. Similar to mRNA expression, increased secretion of TNF-α, IL-6, and IL-1β, but not IL-1α, could be detected in the supernatants in response to LPS (Table 1).

TNF-α and IL-1 are considered primary cytokines in that they stimulate the secretion of other cytokines, such as IL-6 and MIP-2. To determine whether such responses occur in the kidney, mice were pretreated with neutralizing dose of anti-TNF-α antibodies prior to LPS administration and cytokine mRNA transcripts for IL-6 and MIP-2 quantitated by competitive RT-PCR. As shown in Figure 2, the quantitative increase in IL-6 and MIP-2 message that occurs following LPS treatment was almost completely prevented by pre-treatment with TNF-α antibodies, indicating that TNF-α helps mediate these activities. Furthermore, since elevated urinary IL-6 has been reported to occur in many chronic inflammatory diseases and to determine whether urinary IL-6 is present in sepsis and is regulated by TNF-α, urinary IL-6 concentrations were determined in endotoxin-treated mice pretreated with vehicle or

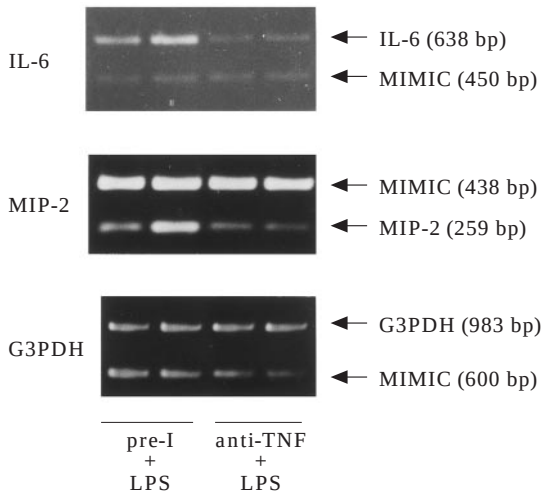

Figure 2. Quantitative analysis of IL-6 and MIP-2 mRNA transcripts by competitive RT-PCR from mouse kidney RNA.

Mice were pretreated with either pre-immune sera or sera containing neutralizing anti-TNF-α antibodies 1 h prior to LPS (2 mg/kg) administration and 2 h prior to killing. cDNA equivalents of 10 ng mRNA were amplified for 30 cycles in the presence of 10^{-1} attomol of appropriate competitor. Each lane represents PCR products amplified from the mRNA of representative animals.

TABLE 2. Urinary IL-6 levels following LPS and anti-TNF antibody treatments

Treatment	Urinary IL-6 (ng/ml creatinine)*
Pre-immune sera plus vehicle	0.2 ± 0.1
Pre-immune sera plus LPS	0.7 ± 0.2†
Anti-TNF sera plus LPS	0.2 ± 0.2

Mice were injected i.p. with 0.2 ml of either preimmune serum or anti-TNF antiserum 2 h prior to injection of LPS (2 mg/kg) or vehicle. Twenty-four hour urine samples were collected and IL-6 levels quantified by ELISA. *Values were standardized by urine creatinine concentrations. Each value represents the mean ± SE of 4–5 animals. †Significantly different from vehicle control at $P < 0.05$.

neutralizing TNF-α antibodies (Table 2). IL-6 was detectable at low concentrations in the urine of vehicle-treated mice while LPS induced a three-fold increase. Pretreatment of mice with neutralizing TNF-α antibodies prior to LPS treatment prevented the IL-6 increase.

Studies were conducted to determine whether addition of LPS to the kidney slice cultures stimulated IL-6 secretion. The LPS concentration response of IL-6 is shown in Figure 3 with maximum IL-6 secretion occurring at a concentration of 25 μg/ml of LPS. Subsequent experiments were conducted to determine the kinetics of the LPS-induced cytokine response from the kidney. LPS was added to kidney slice cultures and cytokine secretion monitored over a 6 h time period (Fig. 4). Increases in TNF-α were found in supernatants within 1 h following addition of LPS, while IL-1β could be detected within 3 h. In contrast, increases in IL-6 were not detected until 6 h following LPS addition.

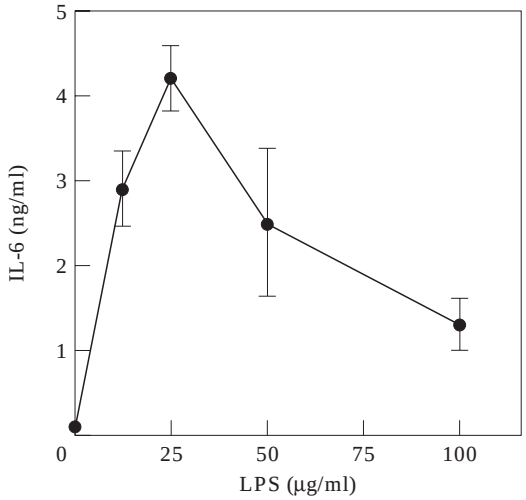

Figure 3. IL-6 secretion in mouse kidney slices in the presence of increasing concentrations of LPS.

Supernatants were collected after 6 h of culture. Each value represents the mean ± SD of culture supernatants from 4 animals.

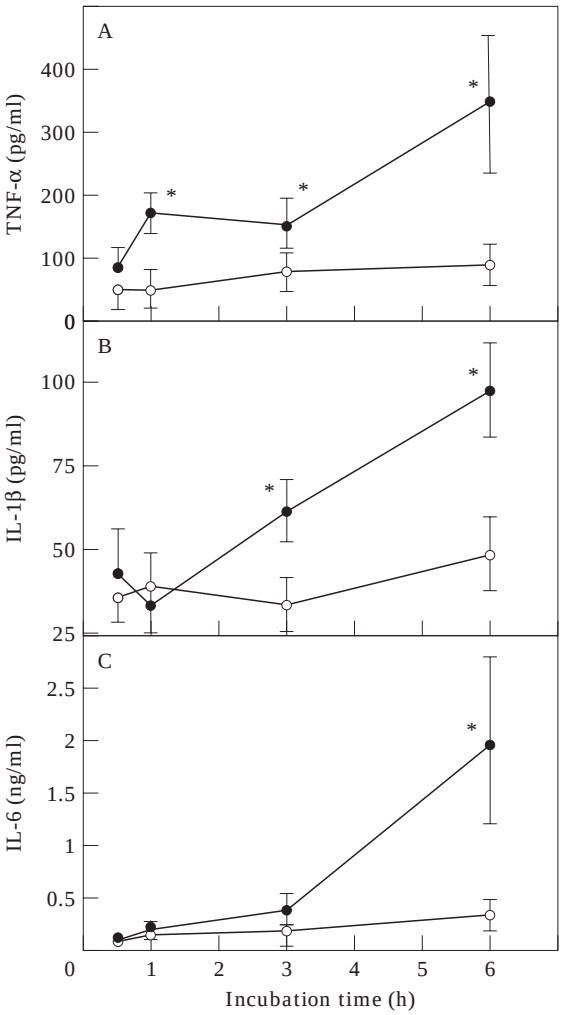

Figure 4. Kinetics of cytokine secretion from mouse kidney cultures.

Kidney slices were prepared as described in Materials and Methods and incubated in the presence of 25 μg/ml of LPS (●) or vehicle (○). The data are expressed as mean ± SD of 4 culture supernatant. Data are representative of 3 separate experiments. *Significantly different from vehicle control at $P < 0.05$.

As IL-1 and/or TNF-α can induce IL-6 expression in the liver, studies were conducted to determine whether IL-1 or TNF-α secretion was responsible for kidney IL-6 secretion. Kidney slices obtained from naive mice were cultured in the presence of LPS and neutralizing antibodies to mouse TNF-α and/or IL-1β. Following 6 h of culture, supernatants were collected and IL-6 concentrations determined (Fig. 5). Addition of LPS caused a significant increase in IL-6 secretion. The increase in IL-6 was partially abrogated in the presence of antibodies against TNF-α or IL-β. IL-6 levels were significantly decreased from those of the LPS-alone treatment group in the presence of the combination of both antibodies to mouse TNF-α and IL-1β as opposed to either antibody alone.

To determine the cellular origin of IL-6, immunohistological studies were conducted (Fig. 6).

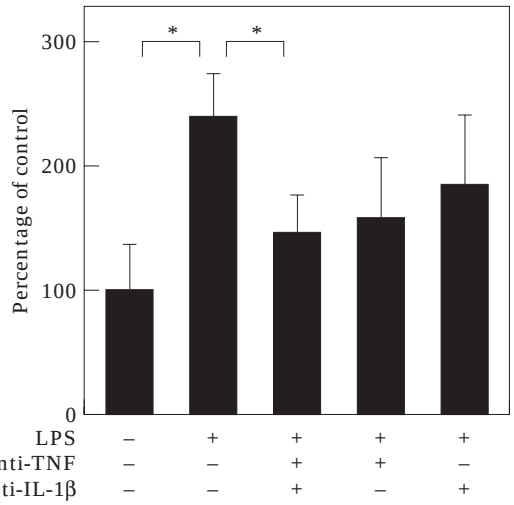

Figure 5. IL-6 levels following neutralization of TNF-α or IL-1β cytokines in kidney slice cultures.

Kidney slices were prepared from naive mice as described in Methods and incubated in the presence of LPS (25 μg/ml) and/or neutralizing TNF-α and IL-1β antibodies (10 ng/ml) for 6 h. The data are expressed as mean ± SD of 4 culture supernatants. Data are representative of 3 separate experiments. *Significantly different from vehicle control at $P < 0.05$.

While mice pretreated with neutralizing antibodies to TNF-α prior to LPS administration did not demonstrate mesangial cell staining (A), within 6 hrs following LPS administration alone IL-6 was evident in glomerular mesangial cells (B). There was no staining evident in renal tubular or epithelial cells.

DISCUSSION

Pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6, participate in both physiological and pathological conditions. Increasing evidence suggests that secretion of these products at low levels following tissue injury participate in cellular repair mechanisms while, when produced in excess, pathological sequelae may ensue such as atherosclerosis[10] and chronic liver disease.[11,12] In this respect, overexpression of cytokines in the kidney are features of both acute and progressive glomerular disease.[1–4,13–15] In particular, TNF-α released from either infiltrating or intrinsic glomerular cells is thought to contribute to glomerular injury in experimental models of toxic or immunologic-mediated renal diseases as well as ischaemia.[6] This has been suggested by several lines of evidence including: (1) TNF-α can be detected locally in a fashion that parallels the development of glomerular injury; (2) addition of TNF-α to glomerular cells promotes similar changes observed in glomerular disease; (3) neutralization of TNF-α activity in vivo

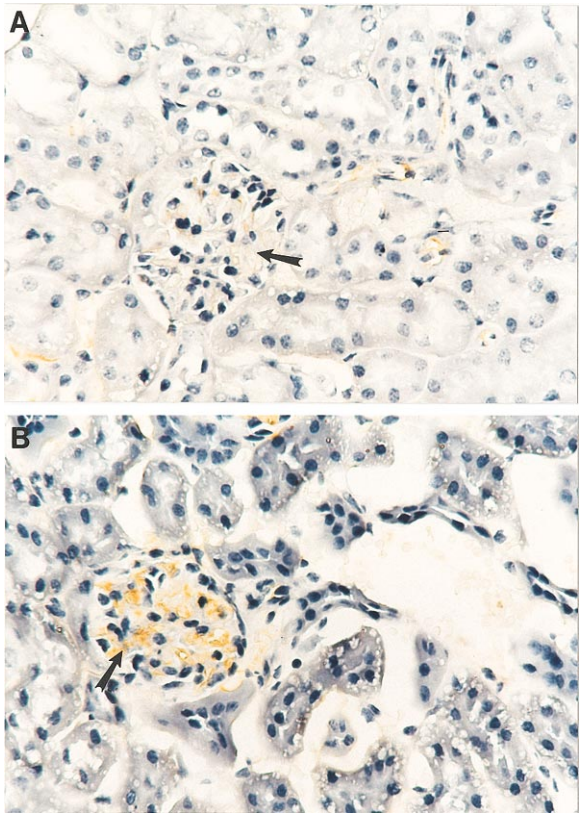

Figure 6. Immunostaining for IL-6.

Frozen kidney sections were prepared from mice treated 6 h earlier with anti-TNF antibodies plus LPS (A) or LPS alone (B) as described in Methods. Note staining in glomerular mesangial cells (arrow). There was no staining evident in kidney from naive mice (not shown).

protects from kidney injury,[1,16] and; (4) TNF-α exacerbates proteinuria and accelerates the course of glomerular injury in experimental models of nephritis.[5,17] While numerous studies have shown changes in cytokine levels in serum during nephritis, few studies have examined kidney-specific expression and regulation of cytokines. The present studies indicate that like other organs, the kidney responds to bacterial endotoxin by producing a pro-inflammatory cytokine response. The earliest response observed includes IL-1β and TNF-α secretion followed by IL-6, and presumably other inflammatory mediators such as chemokines.[1]

Increases in IL-6 occur in many disease states including bacterial and viral infections, autoimmune diseases, malignancies and inflammatory diseases (reviewed in Ref. 18). Its role in these pathologies is not clearly established but IL-6 can induce an acute phase response and has growth promoting activity for various cells. In the kidney IL-6 is most closely associated with mesangial proliferative glomerulonephritis[19] and may also be involved in tubular epithelial cell regeneration following renal damage.[20] Mesangial cells play a prominent role in inflammatory renal diseases, as they can produce as well as respond to inflammatory cytokines.[8,21–23] Glomerular epithelial and tubular epithelial cells, as well as infiltrating mononuclear cells, may also contribute to the IL-6 pool in inflammatory kidney diseases[13,14] It was observed that urinary IL-6 concentrations were increased by LPS administration and reduced by neutralizing antibodies to TNF-α. Urinary IL-6 is believed to be derived from the kidney as increased levels do not always coincide with serum IL-6 concentrations, but do correlate with local inflammatory responses such as proteinuria, hematuria and leukocytopenia.[25] The cellular source of urinary IL-6 has not been identified. While, we and others[26] have shown that IL-6 is secreted from mesangial cells, a contribution by non-mesangial kidney cells, such as monocytes and fibroblasts cannot be excluded. In this respect, IL-6 may serve as an indicator of active inflammatory processes within the renal parenchyma.[27–29]

In summary, we have demonstrated that bacterial endotoxin exposure is associated with gene expression and secretion of inflammatory cytokines in the kidney, particularly IL-6. IL-6 can also be found in the urine and similar to hepatic sepsis, primary cytokines such as TNF-α help mediate the IL-6 response. The exact role of IL-6 in nephrotoxicity has not been established but its presence in the urine may serve as a marker for local inflammatory disease. In this respect, urinary IL-6 has been used to monitor kidney infection, pyelonephritis and nephrotoxicity.[26,29]

MATERIALS AND METHODS

Animals and reagents

Adult female B6C3F1 (C57BL/6 × C3H) mice (Charles River, Portage, MI, and Charles River, Tokyo, Japan) were provided sterile food (NIH 31, Ziegler Bros., Inc., Gardner, PA or CE-2, Clea Japan Inc., Tokyo) and deionized water *ad libitum*. Endotoxin from *Escherichia coli* lipopolysaccharide (LPS; 0111:B4 phenol extract; Sigma, St Louis, MO), dissolved in saline, was administered at a dose of 2 mg/kg intravenously (i.v.) 1 h prior to killing of mice. Euthanasia was performed by CO_2 asphyxia using NIH-approved guidelines for the humane treatment of laboratory rodents. Urine was collected over a 24-h period in metabolic cages. Recombinant human IL-6 (rhIL-6), monoclonal anti-mouse IL-1β antibodies and anti-mouse TNF-α antibodies were purchased from Genzyme (Cambridge, MA).

The preparation of polyclonal rabbit anti-mouse TNF antisera and specificity testing were descibed elsewhere.[20] Each mouse was injected intraperitoneally with 0.2 ml of filtered preimmune sera (pre-I) or an equal volume of anti-TNF antisera (representing 250 000 units). This dose effectively inhibits increases in serum TNF-α following endotoxin administration. (Luster, unpublished observations).

Reverse transcriptase polymerase chain reaction (RT-PCR) amplification

Approximately 100 mg samples of kidney tissue were homogenized in 2 ml of RNAzol B™ solution (Biotecx Laboratories, Inc. Houston, TX) and total cellular RNA was extracted according to the manufacturer's instructions. RNA was dissolved in Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and the mRNA fraction was enriched by binding to oligo d(T) cellulose spin columns (Invitrogen Corp., San Diego, CA). For the synthesis of cDNA, 0.1 μg of mRNA from each sample was resuspended in a 20-μl final volume of the reaction mixture [25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP (Perkin Elmer Cetus, Norwalk, CT) and 0.5 μg oligo (dT)12-18 primer (BRL, Gaithersburg, MD)]. After the reaction mixture reached 42°C, 400 U reverse transcriptase (BRL, Gaithersburg, MD, 200 U/μl) was added into each tube and incubated for 30 min at 42°C. Reverse transcription was stopped by denaturing the enzyme at 99°C for 5 min. The reaction mixture was diluted with distilled water to 50 μl final volume and either commercial (Clontech Laboratories, Inc., Palo Alto, CA) or prepared PCR primer pairs were added. PCR MIMICs™ (Clontech) were used as an internal standard for competitive PCR amplification. PCR primers for macrophage inflammatory protein-2 (MIP-2) were prepared at Duke University DNA Synthesis Unit (Durham, NC). Competitors for MIP-2 were produced using the PCR MIMIC™ Construction Kit (Clontech). The amplified PCR products from cytokine mRNA were 563 base pairs (bp) for IL-1β, 692 bp for TNF-α, 259 bp for MIP-2, 638 bp for IL-6, and 983 bp for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), whereas the competitors were 450 bp for IL-6, 438 bp for MIP-2, and 600 bp for G3PDH. The primers contained the following sequences:

IL-1α (sense 5′-ATGGCCAAAGTTCCTGACTTGTTT-3′,
anti-sense 5′-CCTTCAGCAACACGGGCGCCGC-3′),
IL-1β (sense 5′-ATGGCCAAAGTTCGAGACATGTTTG-3′,
anti-sense 5′-GGTTTTCCAGTATCTGAAAGTCAGT-3′),
TNF-α (sense 5′-ATGAGCACAGAAAGCATGATCCGC-3′,
anti-sense 5′-CCAAAGTAGACCTGCCCGGACTC-3′),
MIP-2 (sense 5′-CAGAGCTTGAGTGTGACG-3′
anti-sense 5′-GAGGCACATCAGGTACGA-3′)
IL-6 (sense 5′-ATGAAGTTCCTCTGCAAGAGACT-3′,
anti-sense 5′-CACTAGGTTTGCCGAGTAGATCTC-3′)
G3PDH (sense 5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′,
anti-sense 5′-CATGTGGGCCATGAGGTCCACCAC-3′).

Amplification was performed as described previously[20] using a GeneAmp PCR System 9600 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, the samples were incubated for 7 min at 72°C. For each set of primers, a dilution of cDNA were amplified for 20, 23, 25, 28, 30, 33, 35 and 38 cycles to define optimal conditions for linearity and to permit semi-quantitative analysis of signal strength. When necessary, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA which generates fragments of the expected size (data not shown). Quantitative analysis of cytokine message was conducted by competitive PCR using 10^{-1} attomol of artificial competitor (PCR MIMIC™) as a template, which contains conplementary sequences to the cytokine primers but different-sized PCR products. Aliquots of cDNA, equivalent to 10 ng mRNA were amplified for 30 cycles with the cytokine primers in the presence of respective competitors.

The PCR products were visualized by UV illumination following electrophoresis through 2.5% agarose (UltraPure, Sigma) at 60 V for 80 min and stained in Tris Borate–EDTA buffer containing 0.5 μg/ml ethidium bromide. A molecular weight marker (pBR322 Hae III digest) was purchased from Sigma. The gels were photographed with type 55 positive/negative film (Polaroid; Cambridge, MA). The films were scanned with a computerized laser densitometer (LKB) and the area under the curve normalized for G3PDH content. The relative amounts of mRNA transcripts were determined using an Eagle Eye II video system™ (Stratagene, Inc., La Jolla, CA) and densitometric analysis of the captured image was performed using the NIH image 1.54 analysis software.

Tissue slice preparation

The kidneys were removed and 6 mm diameter cylindrical cores were prepared for placement into a Krumdieck tissue slicer (Alabama R&D Corp., Munford, AL). Two-hundred mm thick sections (c.a. 10 mg wet weight) were cut and collected in cold Hanks balanced saline solution. Individual sections were cultured in 24-mm transwell culture dishes (8.0 μm pose; Costar, Cambridge, MA) containing 0.5 ml of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 10 μg/ml bovine pancreatic insulin and 2 mM L-glutamine. The slices were incubated at 37°C in 95% O_2 and 5% CO_2. After an initial 30 min of incubation, the culture medium was collected, replaced with fresh medium, and slices were incubated for an additional 6 h. There was no indication of cytotoxicity during the incubation periods as determined by lactate dehydrogenase (LDH) release into the supernatant. Preliminary experiments indicated that the cytokine responses between perfused and non-perfused kidneys were similar. Hence, all studies reported used non-perfused kidneys.

Cytokine assays

TNF-α activity in culture supernatants from kidney slices was measured by quantitating cytolytic activity against an L929 target cell.[30] Immunoreactive murine TNF-α and IL-1β were quantitated by commercial ELISA systems (Genzyme, Cambridge, MA) while murine IL-6 was quantitated by ELISA as previously described.[31] Briefly, microtitre plates were coated with rat anti-mouse IL-6 (clone MP5-20F3; Pharmingen, San Diego, CA). After washing and blocking non-specific binding sites with phosphate-buffered saline containing 0.05% FBS, test samples or purified rat IL-6 (R&D Systems, Minneapolis, MN) were added to the wells. After overnight incubation at 4°C, the plates were washed to remove unbound material and a biotinylated polyclonal rat anti-mouse IL-6 antibody (Pharmingen), which binds captured IL-6, was added. After washing to

remove unbound material, a streptavidin–alkaline phosphatase conjugate was added to each well followed by *p*-nitrophenyl phosphatase (Sigma) substrate solution. The reaction was stopped by adding 10 mM diethanolamine solution and the absorbance at 405 nm was measured in a microplate reader. For urinary IL-6, urine samples were collected at the indicated time points in metabolic cages (Model T-488; Tokima Inc., Tokyo, Japan) installed with a cooling device. Urinary creatinine concentrations were determined as previously described.[9]

Histology

The kidneys were removed and transverse sections prepared and fixed overnight in 10% buffered formaldehyde. Paraffin-embedded tissue sections (5–6 μm thickness) were prepared and stained with haematoxylin and eosin. For immunohistochemical staining, samples of kidney were fixed in 10% buffered formaldehyde for 1 h, soaked in gum acacia solution overnight, and then frozen in liquid nitrogen and stored at -70°C. Frozen sections (6 μm) were prepared and incubated for 4 h at 4°C in a 1:50 dilution of rat anti-mouse IL-6 (Pharmingen) followed by incubation with peroxidase-conjugated anti-rat immunoglobulin (Vector, Burlingame, CA).

Statistics

Experiments shown are representative of at least three separate studies. Multiple comparisons utilizing the Bonferroni adjustment of the Student *t*-test were performed. Statistically significant differences were reported at $P < 0.05$.

Acknowledgements

This work was supported, in part, by Grant for promotion of occupational health of University of Occupational and Environmental Health and National Institute of Environmental Health Sciencecs.

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