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Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney

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Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney.

Background. Emerging evidence suggests that angiotensin II (Ang II) is not only a vasoactive peptide, but also a true cytokine that regulates cell growth, inflammation and fibrosis. Many studies have demonstrated that this peptide plays an active role in the progression of renal injury. Some of Ang II-induced effects are mediated by the production of a large array of growth factors. The aim of this study was to investigate whether Ang II could regulate the expression of cytokines and chemokines in the kidney and its correlation with the Ang II-induced renal damage.

Methods. The model of Ang II-induced renal damage was done by systemic Ang II infusion into normal rats (50 ng/kg/min; subcutaneous osmotic minipumps). In addition, the implication of Ang II was investigated in a model of immune complex nephritis in rats treated with the angiotensin converting enzyme (ACE) inhibitor quinapril. The mRNA expression was analyzed by RT-PCR and/or Northern blot, and protein levels by Western blot and/or immunohistochemistry.

Results. Rats infused with Ang II for 3 days caused elevated renal expression of tumor necrosis factor-α (TNF-α; gene and protein levels). TNF-α positive cells were observed in glomeruli (mainly in endothelial cells), tubules and vessels. In rats with immune complex nephritis, the renal overexpression of TNF-α was diminished by the ACE inhibitor quinapril. Systemic infusion of Ang II also increased renal synthesis of cytokines (interleukin-6, IL-6) and chemokines (monocyte chemoattractant protein-1; MCP-1) that were associated with elevated tissue levels of activated nuclear factor-κB (NF-κB) and the presence of inflammatory cell infiltration.

Conclusions. Ang II in vivo increases TNF-α production in the kidney. Ang II also up-regulates other proinflammatory mediators, including IL-6, MCP-1 and NF-κB, coincidentally associated to the presence of glomerular and interstitial inflammatory cells in the kidney. All these data further strengthen the idea that Ang II plays an active role in the inflammatory response in renal diseases.

Key words: angiotensin, cytokines, inflammation, kidney, interleukin-1, tumor necrosis factor-α, monocyte chemoattractant peptide-1, nuclear factor-κB.

Angiotensin II (Ang II), the main peptide of the renin-angiotensin system (RAS), is considered a growth factor that regulates cell proliferation, apoptosis and fibrosis [1–3]. Emerging information has contributed to enlarge the present view of Ang II as a proinflammatory mediator that participates in the inflammatory response in several pathological processes [1]. In models of renal injury, the blockade of Ang II actions, by angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor (AT) antagonists, decreases proteinuria, inflammatory cell infiltration, fibrosis, and gene expression of matrix proteins and growth factors [1–3]. RAS blockers are commonly used in the treatment of hypertension and proteinuria in humans [4].

Tumor-necrosis factor (TNF-α) is a proinflammatory cytokine with a wide variety of biological effects. TNF-α is produced by various cell types, including proximal tubules [5], mesangial cells [6], glomerular epithelial cells [7], and endothelial cells [8], but the primary source of this cytokine is monocytes/macrophages [9]. Elevated levels of renal production of TNF-α have been described in human and experimental nephritis [7, 10–14]. This cytokine regulates several cellular responses, including cell proliferation and production of other cytokines and proinflammatory mediators (chemokines and adhesion molecules) [9, 10, 15, 16].

Some data suggest that Ang II could regulate TNF-α production. ACE inhibitors suppress TNF-α synthesis both in vitro and in vivo [17]. In hypertensive patients, peripheral blood monocytes are activated presenting elevated levels of proinflammatory cytokines, such as TNF-α and interleukin-1 (IL-1) [18]. In the kidney, TNF-α and Ang II appears to contribute to pathophysiological events leading to renal fibrosis [14]. Ang II induces TNF-α production in isolated tubules from rat medullary thick ascending limb [19]. In macrophages, Ang II up-regulates TNF-α and IL-6 gene expression [20]. In mesangial cells Ang II increases IL-6 mRNA expression and synthesis [21], but whether it could regulate TNF-α has not been

addressed. The role of Ang II in the inflammatory response has been recently demonstrated [1]. Ang II produces several proinflammatory mediators, including adhesion molecules and chemokines, and also may modulate some responses of immune and inflammatory cells, such as chemotaxis, proliferation and the differentiation of monocytes into macrophages [1].

Systemic Ang II infusion causes renal damage [22–24]. We have previously demonstrated that Ang II increased the activity of the nuclear factor-κB (NF-κB) in the kidney; however, the NF-κB-related genes induced by Ang II in this organ have not been determined. Our current study investigated whether Ang II regulates the expression of some NF-κB–related cytokines (in particular TNF-α) and chemokines in the kidney.

METHODS

Experimental design

Systemic infusion of Ang II was done into female Wistar rats (subcutaneously by osmotic minipumps; Alza Corp., Palo Alto, CA, USA), at the dose of 50 ng/kg/min. Animals were sacrificed at three days ($N = 8$ rats in each group). Control animals of the same age also were studied. Immune complex nephritis was induced according to a previously described protocol [25]. When proteinuria appeared, animals were randomly distributed into two groups: untreated and quinapril-treated (ACE inhibitor; 100 mg/L in the drinking water) and studied three weeks later.

Renal histopathological studies

The presence of cytokines (TNF-α, and IL-6) and chemokines, monocyte chemoattractant protein-1 (MCP-1) and interferon producing protein-10 (IP-10) were determined by immunohistochemistry. The antibodies anti-rat TNF-α and IL-6 were from Santa Cruz Technology (tested by Western blot with blocking peptides; Santa Cruz, CA, USA). Paraffin-embedded renal tissue sections (4 μm) were deparaffinized and rehydrated, endogenous peroxidase was blocked by incubating in 3% H_2O_2/methanol (1:1) at 25°C for 30 minutes. The slides were subsequently incubated in phosphate-buffered saline (PBS) with 6% serum in 4% bovine serum albumin (BSA) for one hour at 37°C to reduce nonspecific background staining, and then incubated overnight at 4°C with primary antibodies in PBS containing 1% serum and 4% BSA. After being washed with PBS, the sections were incubated with secondary antibodies diluted 1:200 in 4% BSA/PBS for 30 minutes, and after washing, they were revealed by standard techniques. In each experiment, negative controls without the primary antibody, or using an unrelated antibody, were included to check for nonspecific staining.

The inflammatory cell infiltration was evaluated with an anti-CD43 antibody (marker of monocytes, macrophages, natural killer cells, and T cells) and DNA-binding activity of transcription factors by Southwestern histochemistry [24]. The immunostaining was scored by semiquantitative determination and graded as follows: 0 = no staining; 1+ = mild staining; 2+ = moderate staining; 3+ = marked staining [24]. Identification of different cell types was based on topographical criteria. The mean number of positive cells per glomerular cross section was determined by evaluating 10 to 15 glomeruli. The whole interstitium was examined from each animal. Evaluations were performed by two independent observers in a blinded fashion, and the mean score value was calculated for each rat.

Cell cultures

Mesangial cells were cultured from isolated rat glomeruli by sequential sieving and differential centrifugation, and characterized as described [25].

RNA studies

Total RNA was isolated using a standard method with Trizol reagent (Gibco-BRL, Grand Island, NY, USA) and mRNA expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and/or Northern blot [25]. DNA probes used were rat MCP-1 [25, 26], rat IP-10 [16], cyclophilin B (CyPB, a gift from SmithKline Beecham) [26] and TNF-α [7, 10]. 28S was used as control of RNA loading. For RT-PCR the specific primers used were: TNF-α (sense: 5′-ATGAGCACAG AAAGCATGATCC-3′, antisense: 5′-GAAGATGATC TGAGTGTG-3′) [20] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense: 5′-AATGCATCCTG CACCACCAA-3′, antisense: 5′-GTAGCCATATTCA TTGTCATA-3′), which yields a 251 bp and 515 bp product, and PCR (1 min at 94°, 1 min at 60°/54°C and 2 min at 68°; 32/25 cycles, respectively).

TNF-α assay

Supernatants were tested for the presence of TNFα using a colorimetric assay for L929 cell viability, and results are calculated as described [6, 7, 27]. Briefly, L929 cells, in 96-well flat-bottomed plates, were incubated for 24 hours with three different dilutions of supernatants containing actinomycin D (1 μg/mL). Then, medium was removed and cells were stained with 0.2% crystal violet in 12% formalin and 10% ethanol. The cells were then washed with water and solubilized with ethanol in phosphate buffer, and absorbance was measured at 550 nm. Recombinant TNF-α standard was also used. The addition of the anti–TNF-α antibody for two hours at 37°C caused more than an 88% inhibition of the observed cytolytic effect in the lipopolysaccharide (LPS) samples. However, this bioassay did not differentiate between the various forms of TNF (that is, TNF-α, TNF-β).

Western blot

Samples from renal cortex were homogenized in lysis buffer [170 mmol/L Tris-HCl, 22% glycerol, 2.2% sodium dodecyl sulfate (SDS), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1μg/mL pepstatin A] and then separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoresis, samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 0.01 mmol/L Tris pH 7.5 and 0.4 mol/L NaCl containing 0.1% Tween-20, and 5% dry skimmed milk for one hour at 37°C, and then incubated in the same buffer with specific anti–rat TNF-α antibody for 18 hours at 4°C. After washing, detection was made by incubation with peroxidase-conjugated secondary antibody, and developed using an enhanced chemiluminescence (ECL) kit (Amersham).

Statistical analysis

Results are expressed as mean ± SEM of the experiments made. Significance was established by the GraphPAD Instat using the Student *t* test (GraphPAD Software), Mann Whitney test (non-parametric *t* test) and analysis of variance (ANOVA) nonparametric (Kruskal-Wallis test). Differences were considered significant if the *P* value was less than 0.05.

RESULTS

Angiotensin II infusion increases TNF in the kidney

Total proteins from renal cortex were isolated and TNF-α protein levels were analyzed by Western blot. In control rats, renal TNF-α levels were almost undetectable, while samples from Ang II-infused rats showed a band of around 26 kD (Fig. 1A), corresponding to the apparent molecular weight of integral transmembrane TNF-α molecule, which derives to soluble 17 kD TNF-α by proteolytic cleavage [15]. The specificity of the antibody was confirmed by competition studies with a blocking peptide (not shown). These data demonstrate that in vivo Ang II increases renal TNF-α production. To localize the cells responsible for Ang II-induced TNF-α overexpression, immunohistochemistry was done (Fig. 1B). In controls, TNF-α staining was almost unappreciated as described in human samples [11, 12]. After infusion of Ang II for three days, a marked increase in TNF-α staining was observed in glomeruli, located in endothelial cells, and in tubular cells. TNF-α positivity also increased in renal arteries, in both endothelial and vascular smooth muscle cells. The controls of the technique are shown in Figure 1C. The semiquantitative score, determined as described in the **Methods** section, is shown in Figure 1D.

The biosynthesis of TNF-α is believed to be regulated primarily at the translational level [28], because TNF-α

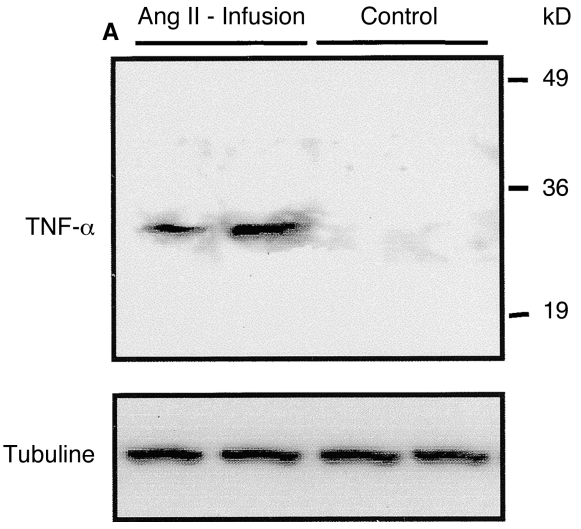

Fig. 1. Systemic infusion of angiotensin II (Ang II) increases renal production of tumor necrosis factor-α (TNF-α). (*A*) Rats were infused with Ang II (50 ng/kg/min) for 3 days, and total proteins were analyzed. In control animals no detectable levels of TNF-α were found, but in Ang II-infused rats, a band of around 26 kD, corresponding to the integral transmembrane TNF-α was detected. A representative Western blot shows two different animals of each group; α-tubuline was used as a loading control.

mRNA is constitutively expressed by a variety of tissues [29]. By RT-PCR, we have observed that Ang II-infused rats presented elevated TNF-α mRNA levels in the kidney compared to control animals (Fig. 2). We also evaluated whether RAS blockers could regulate renal TNF-α. In rats with immune-complex nephritis, renal TNF-α is overexpressed [10]. In this model, we have observed that the ACE inhibitor quinapril diminished proteinuria, inflammatory cell infiltration and chemokine overexpression [25]. As shown in Figure 2, nephritic animals presented elevated renal TNF-α mRNA levels that were diminished by ACE inhibition.

Effects of Ang II on TNFα mRNA expression and production in cultured mesangial cells

Northern blots showed that growth-arrested mesangial cells expressed low TNF-α mRNA levels, as previously described [6]. In mesangial cells treated with Ang II (10^{-7} mol/L) for six hours, TNF-α mRNA was weakly expressed (Fig. 3). In contrast, in LPS-activated cells TNF-α gene expression was characterized by two hybridizing bands: a strongly hybridizing band and a second weaker band, which could represent a precursor to the main TNF-α mRNA [6]. To further investigate this point, we have measured whether Ang II could stimulate TNF-α production. Mesangial cells were incubated with Ang II (10^{-7} to 10^{-10} mol/L) from 0 to 24 hours, conditioned media was collected and TNF-α bioactivity was determined by L929 bioassay. LPS (1 μg/mL) was used as positive control [6, 27]. As shown in Figure 3, at different

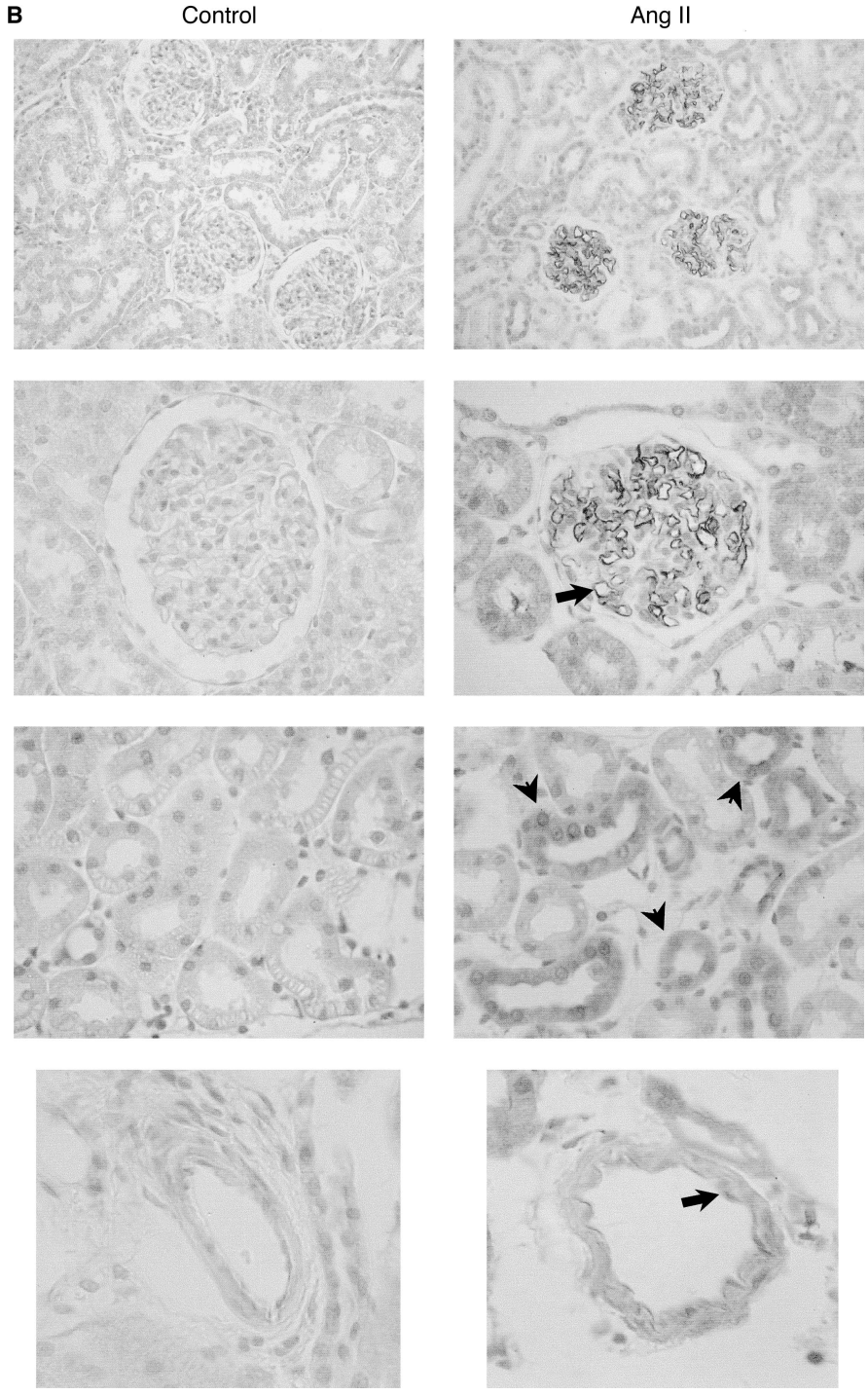

Fig. 1. (Continued) (*B*) Localization of TNF-α immunostaining in Ang II-infused rats. In control rats, TNF-α staining was almost undetectable in the glomeruli and renal arteries, and was weak in the renal tubules. In Ang II-infused rats at day 3, TNFα immunostaining was clearly shown in the glomerulus (localized to endothelial cells; arrow) and renal arteries (mainly in endothelial cells; arrow). There was a marked increase in the cytosolic staining of renal tubules following Ang II infusion (arrowhead). (Magnification, ×200 and ×400).

time points studied there were no differences between control and Ang II-treated mesangial cells, while in LPS-stimulated cells TNF-α–dependent cytotoxicity was increased from three to nine hours, and decreased progressively thereafter. These data show that Ang II did not increase TNF-α bioactivity in mesangial cells. Since TNF-α production was measured using a bioassay, we could not exclude the possibility that Ang II increased levels of immunoreactive TNF-α that was bound by soluble TNF-α receptors and hence biologically inactive.

Ang II in vivo increases renal cytokine and chemokine production

We also evaluated the expression of other cytokines and chemokines in the kidney of these animals using immunohistochemistry. As shown in Figure 4, in rats in-

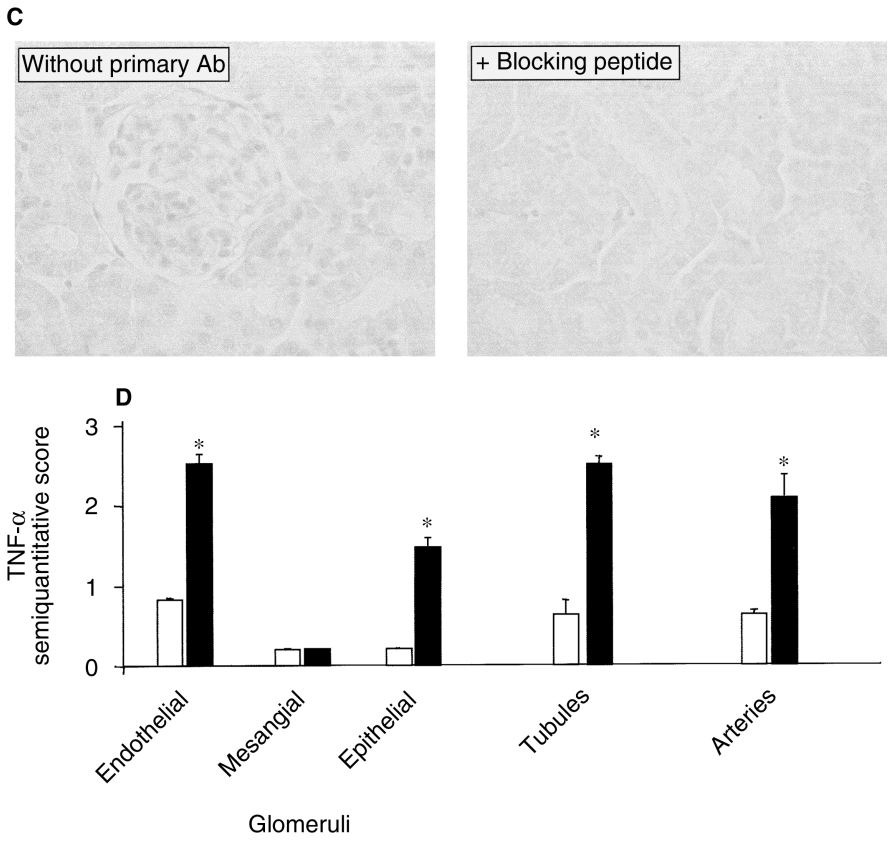

Fig. 1. (Continued) (*C*) The negative control of the immunostaining, as described in **Methods** section. Magnification, ×200. (*D*) Semiquantitative score of TNF-α immunostaining in renal tissue. Symbols are: (□) control animals, (■) animals infused with Ang II for 3 days. Results are means ± SEM of 8 animals per group. $*P < 0.05$ vs. control.

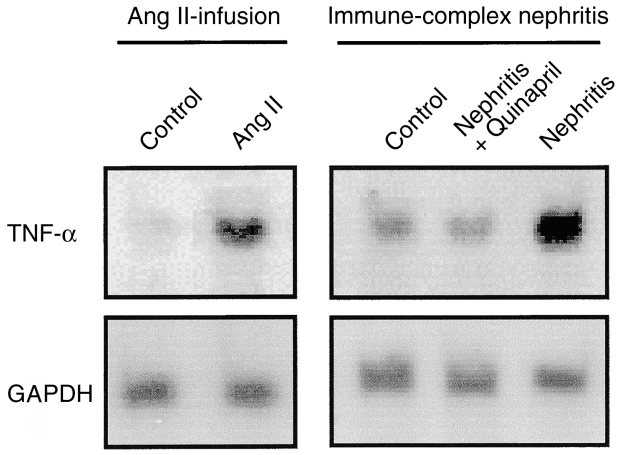

Fig. 2. Systemic infusion of Ang II increases renal TNF-α mRNA expression (left). Ang II (50 ng/kg/min; $N = 8$) was infused into rats for 3 days, and the total cortex RNA was extracted and analyzed by RT-PCR. (Right) ACE inhibition diminishes renal TNF-α overexpression in a normotensive model of immune-complex nephritis in rats. Total cortex RNA was analyzed by RT-PCR. Some animals were treated with the ACE inhibitor quinapril (100 mg/L; $N = 8$). A representative RT-PCR experiment of one animal from each group is shown. GAPDH gene expression was used as control.

fused with Ang II for three days there was a marked increase in IL-6 immunostaining, mainly in glomeruli (mesangial, endothelial and epithelial cells), some tubuli and renal arteries (located mainly in vascular smooth muscle cells). Some chemokines also were evaluated; MCP-1 was markedly up-regulated in glomerular and tubular cells of Ang II-infused rats, while IP-10 immunostaining was not modified (Fig. 5).

Systemic infusion of Ang II for three days into normal rats caused a slight elevation in blood pressure within the normotensive range (121 ± 9 mm Hg, compared to 100 ± 2 in controls; $P < 0.05$, $N = 8$). These animals presented activated NF-κB complexes located mainly in the glomeruli (mesangial, endothelial and epithelial cells), and in tubulointerstitial areas (Fig. 5), as previously described [24]. In Ang II-infused rats the presence of inflammatory cells (CD43 positive cells) in glomeruli and in tubulointerstitial areas was also observed (Fig. 5), but fibrosis was not found (not shown), as described [22–24]. These data show a correlation between cytokine and chemokine overexpression, activation of NF-κB and the presence of inflammatory cells in the kidney of Ang II-infused rats.

Ang II up-regulates chemokine mRNA expression in rat mesangial cells

Renal cells can produce cytokines and chemokines in response to different stimuli, such as LPS and TNF-α [6, 10, 16, 26]. By Northern blot, we have studied in mesan-

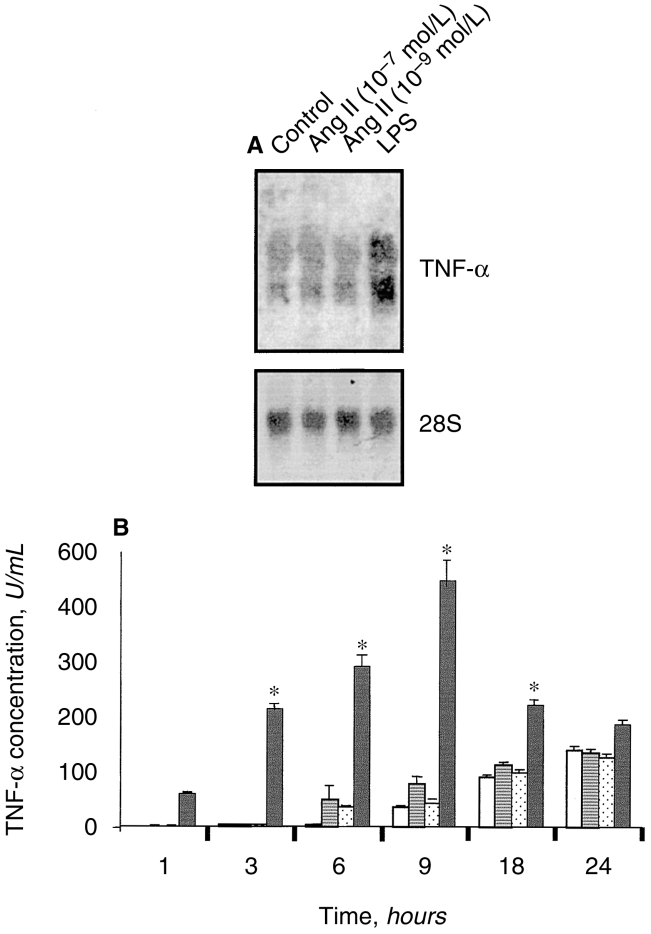

Fig. 3. Regulation of TNF-α by Ang II in mesangial cells. (*A*) Growth-arrested mesangial cells were stimulated with 10^{-7} mol/L Ang II for 6 hours. A known TNF-α activator, 1 μg/mL lipopolysaccharide (LPS) was used. A representative Northern blot of two experiments is shown, with 28S as the loading control. (*B*) TNF-α production after stimulation with Ang II and LPS. Mesangial cells were stimulated by indicated times and TNF-α production in supernatants was determined using the cytotoxic activity assay on L929 cells. Symbols are: (□) control; (■) LPS; (▤) 10^{-7} mol/L Ang II; (▩) 10^{-10} mol/L Ang II. Data are means ± SEM for 6 experiments. $^*P < 0.05$ vs. control.

gial cells the effect of Ang II on the gene expression of some chemokines. Stimulation of mesangial cells with Ang II for six hours increased mRNA levels of MCP-1, CyPB (3- and 2-fold vs. control, respectively), but not IP-10 (Fig. 6). As a positive control recombinant human TNF-α (100 U/mL) was used.

DISCUSSION

Our current study demonstrates that infusion of Ang II for three days caused an elevation in renal production of TNF-α. The role of TNF-α during renal injury has already been demonstrated [7, 10, 14]. Elevated renal mRNA expression, glomerular and tubular production, and urine TNF-α levels have been found in several models of renal injury, including immune-complex nephritis, lupus nephritis, anti-glomerular basement membrane and puromycin nephrosis [7, 10, 14]. In some patients with renal diseases, elevated TNF-α levels in serum or supernatants from peripheral blood mononuclear cells have also been described [10]. Some data suggest a potential relation between Ang II and TNF-α during renal injury. Treatment with ACE inhibitors is associated with lower plasma TNF-α in patients with advanced chronic renal failure [13]. In renal damage induced by endotoxemia, RAS inhibitors completely suppressed LPS-induced IL-6 and TNF-α mRNA levels in the kidney [30]. In rats with immune complex nephritis, we have observed that renal overexpression of TNF-α was diminished by the ACE inhibitor quinapril. A previous study has shown that Ang II increased TNF-α production in isolated tubules from rat medullary thick ascending limb [19]. We have observed that tubular cells from Ang II-infused rats presented elevated TNF-α immunostaining, demonstrating that in vivo Ang II increased TNF-α production in tubular cells. Although in mesangial cells several factors elicit the synthesis of TNF-α, we did not find a significant increase in TNF-α release in response to Ang II, neither in vivo nor in vitro. Since Ang-II induces prostaglandin E_2 (PGE_2) production, and PGE_2 inhibits LPS-stimulated TNF-α release [9], this could be a potential explanation for this negative result.

We have observed that in Ang II-infused rats, TNF-α is expressed in glomerular endothelial cells and renal arteries. By immunohistochemistry high levels of TNF-α have been found in endothelial cells of human atheroma plaques [31]. Some data suggest that endothelial cells can produce TNF-α. In human umbilical vein endothelial cells, LPS or IL-1 induced, in a dose-dependent manner, TNF-α mRNA, but only IL-1 produced TNF-α protein [8]. Most of the TNF-α protein in cell lysates was found in the membrane fraction. In paraformaldehyde-fixed endothelial cells a diffuse pattern over the cell surface was observed by fluorescence, suggesting that TNF-α may be anchored in the membrane or may be processed and bound back to the cell surface [8]. The transmembrane form of TNF-α is reported to be superior to soluble TNF-α in T-cell activation, thymocyte proliferation, and production of granulocyte-macrophage colony-stimulating factor [32]. By Western blot in renal samples from Ang II-infused rats we have detected the 26 kD TNF-α corresponding to transmembrane form. Our data suggest that in response to Ang II, renal endothelial cells can produce TNF-α that may act in a juxtacrine fashion to activate target cells on the endothelial surface. In addition, secreted TNF-α may be involved in the autocrine activation of neighboring cells. Therefore, TNF-α may mediate, in part, the inflammatory responses elicited by Ang II in endothelial cells.

Interactions between TNF-α and Ang II are not limited to the kidney. In Ang II-induced hypertension anti-TNF antisera increases mean arterial pressure, suggesting that TNF-α participates in a counter-regulatory

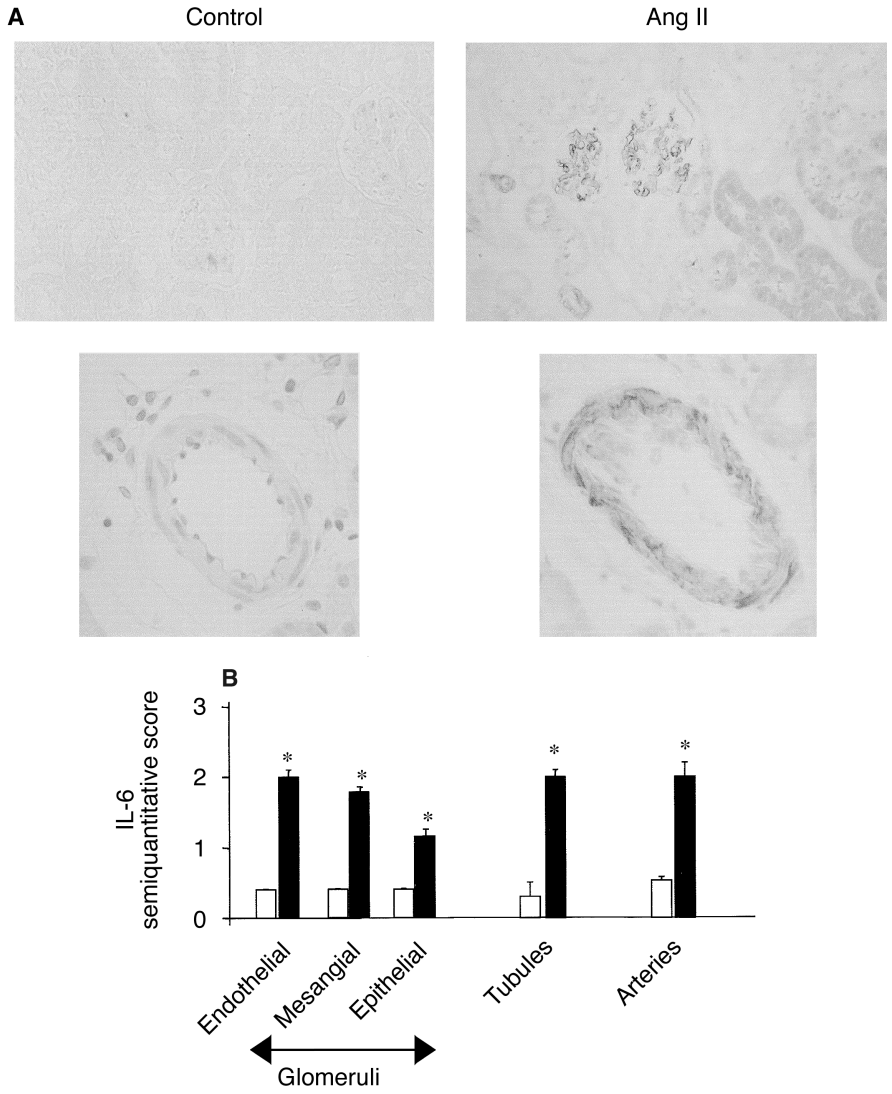

Fig. 4. Systemic infusion of Ang II increases renal production of IL-6. (*A*) Photomicrographs show representative immunostaining of kidney sections of control and an Ang II-infused rat for 3 days, which were stained with a specific antibody to rat IL-6. In control rats, IL-6 staining was almost undetectable. There was no staining in the negative controls included in each experiment (not shown). (Magnification ×100) (*B*) Semiquantitative score of IL-6 immunostaining. Symbols are (□) control animals; (■) animals infused with Ang II for 3 days. Results are means ± SEM of 6 animals per group. $*P < 0.05$ vs. control.

mechanism that opposes the pressor effects of Ang II [33]. It is now well established that activation of the circulating and tissue RAS is involved in the pathophysiology of heart failure. In patients with end-stage cardiac disease, circulating TNF-α concentrations are increased associated with marked activation of RAS [34]. Recent reports have demonstrated that the cardiac myocyte may be an important source of TNF-α production in response to LPS stimulation or pressure overloading [35, 36]. In cardiac fibroblasts, but not in myocytes, Ang II stimulates TNF-α mRNA expression and the production of biologically active TNF-α [37]. However, the amount of TNF-α produced by stimulation with Ang II was less than that induced by LPS. The TNF-α level elicited by stimulation with Ang II was similar to the TNF-α concentrations reported in various human cardiac diseases [34, 38]. All these data suggest that TNF-α production in cardiac fibroblasts may act on cardiac myocytes in a paracrine fashion in the setting of congestive heart failure or cardiac hypertrophy.

Interleukin-6 is a multifunctional cytokine exerting a wide variety of biologic responses that participate in the pathogenesis of mesangial proliferative glomerulonephritis [39]. Ang II increased IL-6 release and mRNA expression in cultured mesangial and vascular smooth muscle cells [21, 40]. We have observed that Ang II-infused rats presented elevated production of IL-6, expressed in glomerular cells (mainly in mesangial cells), tubular cells and renal arteries (mainly in vascular smooth muscle cells), demonstrating that Ang II in vivo increases IL-6 production. The blockade of IL-6 signaling with anti–IL-6 receptor antibody abolished Ang II-induced cell proliferation [21]. Ang II also regulates the production of several growth factors, such as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), parathyroid hormone-related protein (PTHrP) involved in cell growth and extracellular matrix accumulation regulation [3, 41]. Thus, some effects of Ang II may be medi-

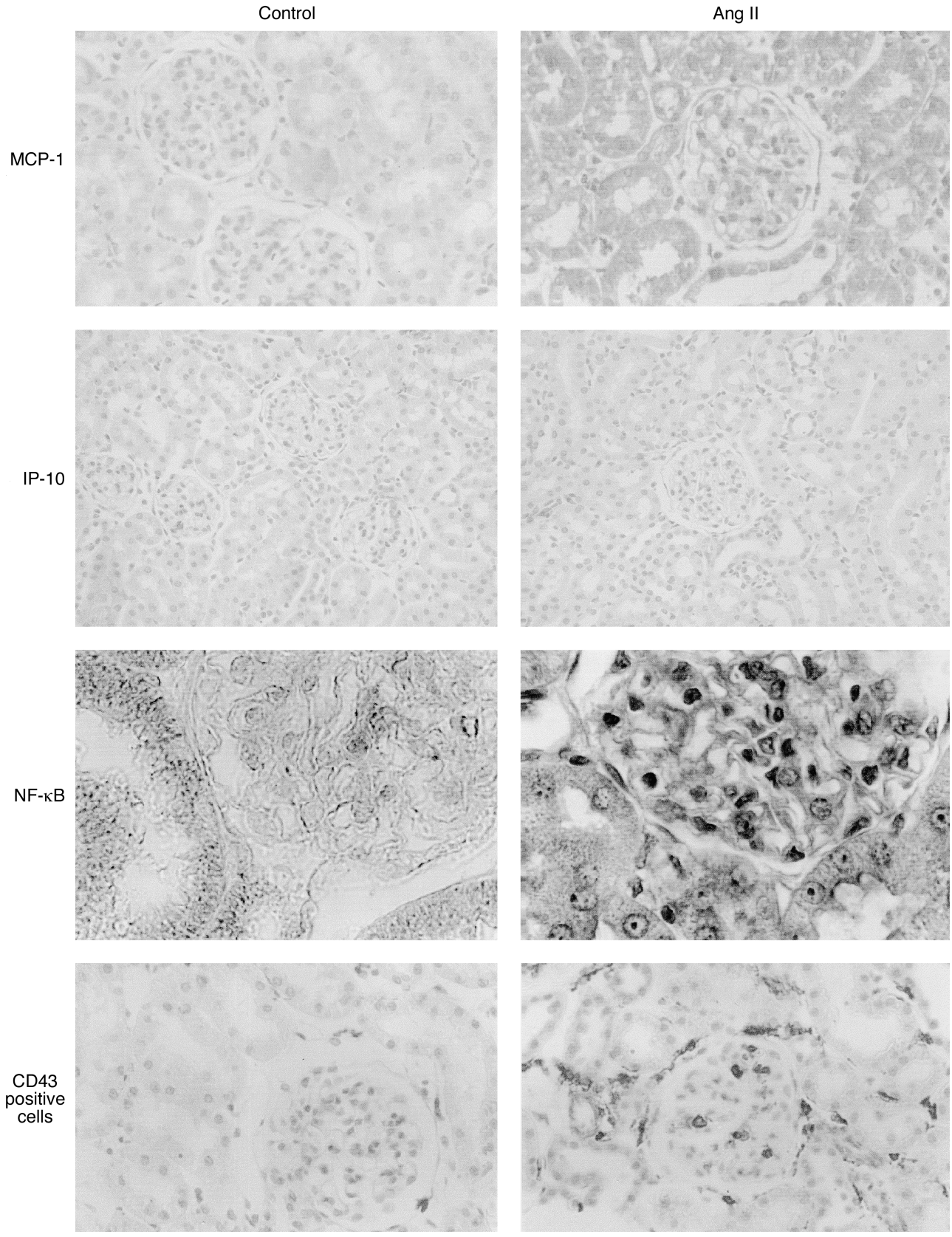

Fig. 5. Ang II-infused rats presented elevated renal production of chemokines, activation of the transcription factor NF-κB, and the presence of inflammatory cells. Rats were infused with Ang II (50 ng/kg/min) for 3 days. Photomicrographs show a representative immunostaining of kidney sections with specific antibodies to rat MCP-1 and IP-10. In control rats, no staining for chemokines was found. Control animals did not present staining for NF-κB. After Ang II infusion a clear nuclear staining for NF-κB was seen in the glomeruli, located in mesangial, epithelial and endothelial cells. Ang II-infused animals had inflammatory cells expressing CD43 antigen in glomeruli and interstitium, distributed in a focal manner. There was no staining in the negative controls included in each experiment (not shown). Figures show a representative animal of each group of four studied.

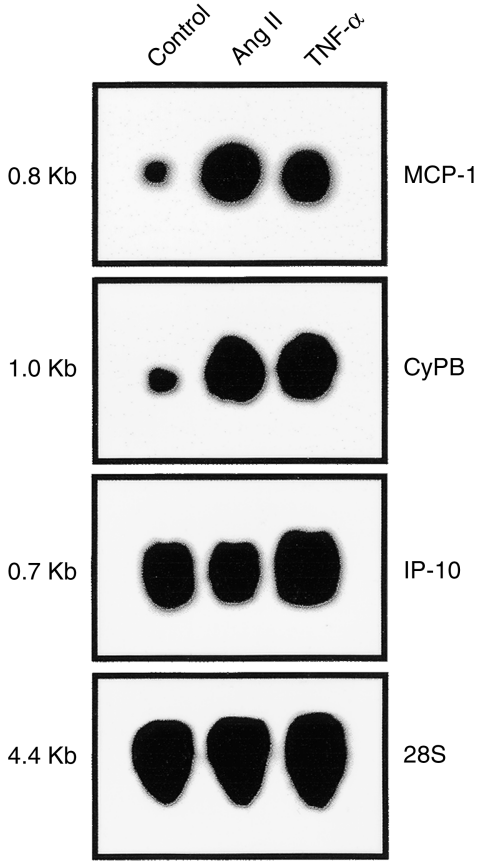

Fig. 6. Effects of Ang II on mRNA expression of chemokines in rat mesangial cells. Quiescent cells were treated with 10^{-7} mol/L Ang II for 6 hours, and gene expression of MCP-1, CyPB and IP-10 was analyzed by Northern blot. As positive control TNF (100 U/mL) was used. 28S was used as an internal control. A representative Northern blot of 3 different experiments is shown.

ated, either directly or indirectly, by cytokines produced in response to this peptide.

The recruitment of monocyte-macrophages plays a crucial role in the onset and progression to irreversible structural renal changes [1]. Ang II induces the production of chemotactic factors by resident renal cells. We have observed that in Ang II-infused rats MCP-1 staining was increased in glomeruli and tubular cells. In cultured mesangial cells, we have previously observed that Ang II is a potent activator of MCP-1, with a magnitude comparable to inflammatory cytokines [25]. Interestingly, an anti–MCP-1 antibody did not completely block the chemotactic effects of Ang II [42], suggesting that other chemokines may be involved. RAS blockers also diminished the expression of several chemokines in damaged vessels and kidney. Thus, in a model of atherosclerosis ACE inhibitors decreased IL-8 [1]. Fractalkine, a novel CX3X chemoattractant and adhesion molecule, was diminished by AT_1 antagonist in uninephrectomized anti-Thy nephritis [43]. Ang II in glomerular endothelial cells elicited the synthesis of the chemokine RANTES (normal T cell expressed/secreted) [23] and in vascular smooth muscle cells the mRNA expression of IL-8 and IP-10 [1]. In vivo, Ang II infusion into normal rats increased glomerular expression of RANTES [23] and tubular overexpression of osteopontin (OPN) [44]. Renal IP-10 overexpression has been found in renal diseases [16], but, at least at times and doses employed in our study, Ang II did not regulate IP-10 in vivo and in cultured mesangial cells. CypB is a cyclosporine A (CsA)-binding protein that exhibits chemotactic activity for polymorphonuclear leukocytes and monocytes [45]. Elevated levels of CyPB have been found in proximal tubules of hypertensive rats [46], renal interstitial fibroblasts in response to MCP-1 and IP-10 [26], and in mesangial cells stimulated with Ang II, as described in our current study. All these data strongly suggest that Ang II, through the chemokine production by resident renal cells, may play a central role in the regulation of inflammatory cell recruitment in renal pathology.

The NF-κB pathway plays an important role in the progression of kidney damage [47]. Emerging data suggest a potential role of NF-κB as a mediator of Ang II-induced inflammatory process. Ang II increases proinflammatory genes under NF-κB control, including cytokines (IL-6, and as shown in this study, TNF-α), chemokines (MCP-1 and RANTES), adhesion molecules and angiotensinogen [1]. We have observed that systemic infusion of Ang II caused NF-κB activation associated with overexpression of cytokines (TNF-α and IL-6) and chemokines (MCP-1). In some models of renal injury, ACE inhibitors diminished renal NF-κB activity, chemokines (MCP-1, RANTES and OPN) and adhesion molecules, correlated with a diminution in the mononuclear cell infiltration in the kidney [1]. In this sense, we have observed that quinapril diminished renal TNF-α. Another ACE inhibitor, enalapril, protects from pulmonary hypertension by inhibiting TNF-α–mediated activation of NF-κB [48]. These data are consistent with a role for NF-κB in transcriptional activation of those genes, as shown in cultured cells [47]. Depending on the cell type, different Ang II receptors are involved in NF-κB pathway [1]. Moreover, AT_1 and AT_2 receptors seem to participate in the inflammatory response in renal and vascular tissues. In this regard, future studies are needed to define the Ang II receptor subtypes implicated in gene/NF-κB regulation in each tissue and pathological condition.

On the whole, Ang II infusion into normal rats causes elevation of renal production of TNF-α and other proinflammatory mediators, including IL-6 and MCP-1, and activation of the transcription factor NF-κB associated with the presence of inflammatory cells. Our data show that Ang II acts as a true cytokine regulating many factors that contribute to the inflammatory response in renal diseases.

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REFERENCES

1. Ruiz-Ortega M, Lorenzo O, Suzuki Y, *et al*: Proinflammatory actions of angiotensin II. *Curr Opin Nephrol Hypertens* 10:321–329, 2001
2. Wolf G, Neilson EG: Angiotensin II as a renal growth factor. *J Am Soc Nephrol* 3:1531–1540, 1993
3. Mezzano SA, Ruiz-Ortega M, Egido J: Angiotensin II and renal fibrosis. *Hypertension* 38:635–638, 2001
4. Andersen S, Tarnow L, Rossing P, *et al*: Renoprotective effects of angiotensin II receptor blockade in type 1 diabetic patients with diabetic nephropathy. *Kidney Int* 57:601–606, 2000
5. Ferreri NR, Sarr T, Askenase PW, Ruddle NH: Molecular regulation of tumor necrosis factor α and lymphotoxin production in T cells. *J Biol Chem* 267:9443–9449, 1992
6. Baud L, Oudinet JP, Bens M, *et al*: Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. *Kidney Int* 35:1111–1118, 1989
7. Bustos C, González-Cuadrado S, Ruiz-Ortega M, *et al*: Cyclosporin A modulates the glomerular production of inflammatory mediators and proteoglycans in experimental nephrosis. *Clin Exp Immunol* 102:608–613, 1995
8. Imaizumi T, Itaya H: Fujita K, Kudoh D, Kudoh S, Mori K, Fujimoto, K, Matsumiya T, Yoshida H, Satoh K: Expression of tumor necrosis factor-α in cultured human endothelial cells stimulated with lipopolysaccharide or interleukin-1. *Arterioscler Thromb Vasc Biol* 20:410–415, 2000
9. Pennica D, Nedwin GE, Hayflick JS, *et al*: Human tumor necrosis factor: Precursor structure, expression and homology to lymphotoxin. *Nature* 312:724–728, 1984
10. Egido J, Góme-Chiarri M, Ortiz A, *et al*: Role of tumor necrosis factor-α in the pathogenesis of glomerular diseases. *Kidney Int* 43 (Suppl 39):S59–S64, 1993
11. Neale TJ, Ruger BM, Macaulay H, *et al*: Tumor necrosis factor-α is expressed by glomerular visceral epithelial cells in human membranous nephropathy. *Am J Pathol* 146:1444–1454, 1995
12. Malide D, Russo P, Bendayan M: Presence of tumor necrosis factor-α and interleukin-6 in renal mesangial cells of lupus nephritis patients. *Hum Pathol* 26:558–564, 1995
13. Stenvinkel P, Andersson P, Wang T, *et al*: ACE-inhibitors suppress tumour necrosis factor-α production in advanced chronic renal failure? *J Intern Med* 246:503–507, 1999
14. Guo G, Morrissey J, McCracken R, *et al*: Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy. *Am J Physiol* 277:F766–F772, 1999
15. Jäättelä M: Biologic activities and mechanisms of action of tumor necrosis factor-α/cachectin. *Lab Invest* 64:724–742, 1991
16. Gómez-Chiarri M, Ortiz A, Serón D, *et al*: The intercrine superfamily and renal disease. *Kidney Int* 43(Suppl 39):S81–S85, 1993
17. Fukuzawa M, Satoh J, Sagara M, *et al*: Angiotensin converting enzyme inhibitors suppress production of tumor necrosis factor-α in vitro and in vivo. *Immunopharmacology* 36:49–55, 1997
18. Dorffel Y, Latsch C, Stuhlmuller B, *et al*: Preactivated peripheral blood monocytes in patients with essential hypertension. *Hypertension* 34:113–117, 1999
19. Ferreri NR, Escalante BA, Zhao Y, *et al*: Angiotensin II induces TNF production by the thick ascending limb: Functional implications. *Am J Physiol* 274:F148–F155, 1998
20. Nakamura A, Johns EJ, Imaizumi A, *et al*: Effect of beta(2)-adrenoceptor activation and angiotensin II on tumour necrosis factor and interleukin 6 gene transcription in the rat renal resident macrophage cells. *Cytokine* 11:759–765, 1999
21. Moriyama T, Fujibayashi M, Fujiwara Y, *et al*: Angiotensin II stimulates interleukin-6 release from cultured mouse mesangial cells. *J Am Soc Nephrol* 6:95–101, 1995
22. Johnson RJ, Alpers CE, Yoshimura A, *et al*: Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19:464–474, 1994
23. Wolf G, Ziyadeh FN, Thaiss F, *et al*: Angiontensin II stimulates expression of the chemokine RANTES in rat glomerular endothelial cells. Role of the angiotensin type 2 receptor. *J Clin Invest* 100:1047–1058, 1997
24. Ruiz-Ortega M, Lorenzo O, Ruperez M, *et al*: Systemic infusion of angiotensin II into normal rats activates nuclear factor-κB and AP-1 in the kidney. Role of AT_1 and AT_2 receptors. *Am J Pathol* 158:1743–1756, 2001
25. Ruiz-Ortega M, Bustos C, Hernández-Presa MA, *et al*: Angiotensin II participates in mononuclear cell recruitment in the kidney through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 gene expression. *J Immunol* 161:430–439, 1998
26. Gonzalez-Cuadrado S, Bustos C, Ruiz-Ortega M, *et al*: Expression of leukocyte chemoattractants by interstitial renal fibroblast: Up-regulation by drugs associated with interstitial fibrosis. *Clin Exp Immunol* 106:518–522, 1996
27. Green LM, Reade JL, Ware CF: Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 70:257–268, 1984
28. Han J, Brown T, Beutler B: Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. *J Exp Med* 171:465–475, 1990
29. Tovey MG, Content J, Gresser I, *et al*: Genes for IFNγ-2 (IL6), tumor necrosis factor-α, and IL1 are expressed at high levels in the organs of normal individuals. *J Immunol* 141:3106–3110, 1988
30. Niimi R, Nakamura A: Yanagawa Y. Suppression of endotoxin-induced renal tumor necrosis factor-α and interleukin-6 mRNA by renin-angiotensin system inhibitors. *Jpn J Pharmacol* 88:139–145, 2002
31. Barath P, Fishbein MC, Cao J, *et al*: Detection and localization of tumor necrosis factor in human atheroma. *Am J Cardiol* 65:297–302, 1990
32. Grell M, Douni E, Wajant H, *et al*: The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83:793–802, 1995
33. Ferreri NR, Zhao Y, Takizawa H, McGiff JC: Tumor necrosis factor-alpha-angiotensin interactions and regulation of blood pressure. *J Hypertens* 15:1481–1484, 1997
34. Levine B, Kalman J, Mayer L, *et al*: Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 323:236–241, 1990
35. Kapadia S, Lee J, Torre-Amione G, *et al*: Tumor necrosis factor gene and protein expression in adult feline myocardium after endotoxin administration. *J Clin Invest* 96:1042–1052, 1995
36. Kapadia SR, Oral H, Lee J, *et al*: Hemodynamic regulation of tumor necrosis factor-gene and protein expression in adult feline myocardium. *Circ Res* 81:187–195, 1997
37. Yokoyama T, Sekiguchi K, Tanaka T, *et al*: Nagai R Angiotensin II and mechanical stretch induce production of tumor necrosis factor in cardiac fibroblasts. *Am J Physiol* 276:H1968–H1976, 1999
38. Doyama K, Fujiwara H, Fukumoto M, *et al*: Tumour necrosis factor is expressed in cardiac tissues of patients with heart failure. *Int J Cardiol* 54:217–225, 1996
39. Ooi BS, Cohen DJ, Veis JH: Biology of the mesangial cell in glomerulonephritis–Role of cytokines. *Proc Soc Exp Biol Med* 213: 230–237, 1996
40. Han Y, Runge MS, Brasier AR: Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-kappa B transcription factors. *Circ Res* 84:695–703, 1999
41. Lorenzo O, Ruiz-Ortega M, Esbrit M, *et al*: Angiotensin II

increases parathyroid hormone-related protein (PTHrP) and the type I PTH/PTHrP receptor in the kidney *J Am Soc Nephrol* 13:1595–1607, 2002
42. Ruiz-Ortega M, Lorenzo O, Egido J: Angiotensin III increases MCP-1 and activates NF-kappaB and AP-1 in cultured mesangial and mononuclear cells. *Kidney Int* 57:2285–2298, 2000
43. Ito Y, Kawachi H, Morioka Y, *et al*: Fractalkine expression and the recruitment of CX3CR1+ cells in the prolonged mesangial proliferative glomerulonephritis. *Kidney Int* 61:2044–2057, 2002
44. Yu XQ, Wu LL, Huang XR, *et al*: Osteopontin expression in progressive renal injury in remnant kidney: Role of angiotensin II. *Kidney Int* 58:1469–1480, 2000
45. Sherry B, Yarlett N, Strupp A, Cerami A: identification of cyclophilin as a proinflammatory secretory product of lypopolisaccharide-activated macrophages. *Proc Natl Acad Sci USA* 89:3511–3515, 1992
46. Kainer DB, Doris PA: Cyclophilin B expression in renal proximal tubules of hypertensive rats. *Hypertension* 35:958–964, 2000
47. Guijarro C, Egido J: Transcription factor κB (NF-κB) in renal disease. *Kidney Int* 59:415–424, 2001
48. Ortiz LA, Champion HC, Lasky JA, *et al*: Enalapril protects mice from pulmonary hypertension by inhibiting TNF-mediated activation of NF-kappaB and AP-1. *Am J Physiol (Lung Cell Mol Physiol)* 282:L1209–L1221, 2002