Angiotensin II Stimulates Expression of the Chemokine RANTES in Rat Glomerular Endothelial Cells

Role of the Angiotensin Type 2 Receptor

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Abstract

Glomerular influx of monocytes/macrophages (M/M) occurs in many immune- and non-immune-mediated renal diseases. The mechanisms targeting M/M into the glomerulus are incompletely understood, but may involve stimulated expression of chemokines. We investigated whether angiotensin II (ANG II) induces the chemokine RANTES in cultured glomerular endothelial cells of the rat and in vivo. ANG II stimulated mRNA and protein expression of RANTES in cultured glomerular endothelial cells. The ANG II–induced RANTES protein was chemotactic for human monocytes. Surprisingly, the ANG II–stimulated RANTES expression was transduced by AT_2 receptors because the AT_2 receptor antagonists PD 123177 and CGP-42112A, but not an AT_1 receptor blocker, abolished the induced RANTES synthesis. Intraperitoneal infusion of ANG II (500 ng/h) into naive rats for 4 d significantly stimulated glomerular RANTES mRNA and protein expression compared with solvent-infused controls. Immunohistochemistry revealed induction of RANTES protein mainly in glomerular endothelial cells and small capillaries. Moreover, ANG II–infused animals exhibited an increase in glomerular ED-1–positive cells compared with controls. Oral treatment with PD 123177 (50 mg/liter drinking water) attenuated the glomerular M/M influx without normalizing the slightly elevated systolic blood pressure caused by ANG II infusion, suggesting that the effects on blood pressure and RANTES induction can be separated. We conclude that the vasoactive peptide ANG II may play an important role in glomerular chemotaxis of M/M through local induction of the chemokine RANTES. The observation that the ANG II–mediated induction of RANTES is transduced by AT_2 receptors may influence the decision as to which substances might be used for the therapeutic interference with the activity of the renin–angiotensin system. (*J. Clin. Invest.* 1997. 100:1047–1058.) Key words: angiotensin II • RANTES • glomerular endothelial cells • macrophages/monocytes

Introduction

Glomerular infiltration with macrophages/monocytes (M/M)[1] is a common feature in many immune- and non-immune-mediated glomerular diseases of the kidney (1–5). It is believed that the glomerular influx of M/M is crucial in the progression of renal disease towards the irreversible structural changes of glomerulosclerosis (3, 6). Glomerular M/M are locally activated mononuclear cells that produce an array of cytokines, growth factors, reactive oxygen species, proteases, eicosanoids, coagulation products, and nitric oxide, which may all induce tissue injury and stimulate resident glomerular cells to synthesize extracellular matrix proteins (6, 7). Thus, it is of considerable interest to identify the mechanism of glomerular M/M recruitment. Although several factors, including interleukin-1, TNF-α, platelet activating factor, leukotrienes, and complement components are all chemotactic for M/M, a novel class of proinflammatory chemoattractant cytokines called chemokines, has been identified and characterized in the past few years (8–10). Interleukin-8 and monocyte chemoattractant protein-1 (MCP-1) are probably the most thoroughly studied members of this superfamily (11, 12). RANTES is a member of the C-C-chemokine subfamily with chemoattractant properties for M/M, eosinophil and basophil granulocytes, and for T lymphocytes (13, 14). TNF-α and LPS stimulate RANTES expression in the kidney as well as in cultured renal proximal tubular and mesangial cells (15, 16). It remains unclear, however, whether other cell types in the kidney may express RANTES.

We have had a long interest in the nonhemodynamic mechanisms of how angiotensin II (ANG II) may be involved in the progression of chronic renal disease (17). The observation that angiotensin-converting enzyme (ACE) inhibitors prevent the renal influx of M/M in some models of chronic renal disease prompted us to study potential effects of ANG II on RANTES expression in cultured glomerular endothelial cells (18–20).

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1. *Abbreviations used in this paper:* ACE, angiotensin-converting enzyme; ANG II, angiotensin II; GER, glomerular endothelial cells; MC, mesangial cells; MCP-1, monocyte chemoattractant protein-1; M/M, macrophages/monocytes; PRA, plasma renin activity.

This cell type was selected since a transendothelial gradient of soluble chemokines is most likely responsible for the glomerular influx of M/M (21). Moreover, haptotaxis in response to immobilized chemoattractant molecules on the endothelial surface may be important in this process (21). Our studies demonstrate that ANG II stimulates RANTES protein and mRNA expression in cultured glomerular endothelial cells isolated from rats (GER). Furthermore, the secreted RANTES is chemotactic for M/M as assessed in a Boyden chamber. The induction of RANTES by ANG II is surprisingly mediated by AT_2 receptors. Short-term ANG II infusion for 4 d in rats with osmotic minipumps stimulates RANTES protein and mRNA expression in isolated glomeruli, but not in other renal structures. These data demonstrate for the first time that the vasoactive peptide ANG II may have immunomodulatory properties in the kidney through the induction of the chemokine RANTES.

Methods

Cell culture. GER are a nontransformed glomerular endothelial cell line isolated from adult Sprague-Dawley rats. These cells have been previously characterized in detail, and exhibit positive staining for the endothelial markers Factor VIII, CD 31, endothelial leukocyte adhesion molecule-1, and the lectin BS1 (22). Cells were routinely carried in Dulbecco's modified Eagle's medium (DMEM with 450 mg/dl glucose; GIBCO BRL, Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% heat-inactivated FCS at 37°C in 5% CO_2. GER were passaged twice a week by light trypsinization. All experiments were performed at passage 15–25. For some experiments, primary culture of rat mesangial cells (MC) were used. MC were isolated from outgrowth of isolated glomeruli obtained from Sprague-Dawley rats by differential sieving as previously described (23). MC stained positive for the Thy-1 antigen, but failed to bind the anti-Factor VIII antibody. MC showed contractions after treatment with ANG II, indicating expression of functional ANG II receptors. MC were used at passage 5–8.

ANG II receptor expression. We have previously shown that GER express high-affinity receptors for ANG II (22). The present receptor binding studies were performed to characterize the expression of ANG II-receptor subtypes. Binding studies were performed on 5×10^4 cells grown to subconfluence in 24-well culture plates (Nunc Inc., Naperville, IL) in assay buffer consisting of 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.1), 5 mM EDTA, 0.7% bovine serum albumin, 0.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride on a shaking platform at 22°C. For displacement experiments, 0.5 pM [^{125}I][Sar^1, Ile^8]ANG II (2,000 Ci/mmol, Amersham Buchler GmbH, Braunschweig, Germany) was incubated in the presence or absence of various concentrations of the AT_1 receptor antagonist losartan (gift of Merck, Sharp & Dohme, Munich, Germany) and the AT_2 blocker PD123177 (gift of Parke-Davis, Warner Lambert Co., Ann Arbor, MI). After an incubation period of 2 h, cells were washed three times with ice-cold PBS to remove unbound [^{125}I][Sar^1, Ile^8]-ANG II. The cells were dissolved in 1 N NaOH, and the amount of radioactivity was counted in a gamma scintillation counter. Nonspecific binding was determined in the presence of 10^{-3} M nonradioactive [Sar^1, Ile ^{8}]ANG II (Sigma Chemical Co., St. Louis, MO) and was less than 10% of total binding. Specific binding of [^{125}I][Sar^1, Ile^8]-ANG II in the absence of competitors was considered 100%. Experiments were repeated four times with duplicate measurements for each experiment.

To gain insight into whether GER express mRNA for AT_2 receptors, cDNA amplification after reverse transcription was performed because abundance of transcripts was too low by Northern hybridization (data not shown). Total RNA was isolated by repeated phenol–chloroform extractions and isopropanol precipitation as described by Chomczynski and Sacchi (24). A total of 10 μg RNA was reverse transcribed using 0.7 μg of poly-d(T)primer (Pharmacia Diagnostics AB, Uppsala, Sweden) in the presence of 500 U of Maloney murine leukemia virus reverse transcriptase diluted in 50 μl of a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 500 μM dNTP (25). After incubation for 90 min at 37°C, the reaction was precipitated with 25 μl 7.5 M ammonium acetate and 50 μl isopropanol, and pellets were recovered by centrifugation. After washing in 70% ethanol, pellets were resuspended in 50 μl distilled water. For the polymerase chain reaction, 5 μl of cDNA was combined with a total of 0.15 μg of each of the following primers specific for AT_2 receptor: sense 5′GGGGATGGAGCGAGCACAGAATTG3′, antisense 5′AGTCTATCTATAAGAGTAATAGG3′ (26). Amplification reactions were performed with the GeneAmp™ kit (Perkin Elmer Cetus, Ueberlingen, Germany) using 200 μM of each dNTP, 2.5 U of Amplitaq polymerase in 100 μl PCR buffer (20 mM Tris–HCl, pH 8.3; 25 mM KCl, 2.0 mM $MgCl_2$, and 0.05% Tween 20). A total of 40 cycles was performed with an annealing temperature of 60°C for 1.5 min, an extension step at 72°C for 1.5 min, and a denaturation step at 92°C for 1 min (25, 27). 15 μl of the reaction product was separated in a 1.9% agarose gel containing 0.5 μg/ml ethidium bromide. The correct identity of the amplification product was tested by sequencing after ligation into the TA II vector (Invitrogen Corp., San Diego, CA) and comparison with the published sequence. As an additional control, cDNA amplification was performed after reverse transcription of total RNA from isolated glomeruli of Sprague-Dawley rats.

RANTES mRNA expression. A total of 10^7 GER or MC were made quiescent in DMEM without serum for 24 h, and were subsequently incubated for another 24 h with 10^{-8}–10^{-6} M ANG II (Sigma Chemical Co.). For time-course experiments, 10^{-7} M ANG II was given for 6–24 h. ANG II was free of measurable endotoxin concentrations (endotoxin < 0.001 ng/ml, endotoxin kit from Sigma Chemical Co.). Additional cells were treated with ANG II and either 10^{-6} M losartan or PD 123177. Some cells were also incubated with 1 μg/ml LPS from *Escherichia coli*, serotype 02:B6 (endotoxin $>$ 10,000 U/mg LPS; Sigma Chemical Co.). At the end of the stimulation period, cell layers were washed twice with RNAse-free PBS, and total RNA was isolated as described above. The quantity and purity of the preparations were assessed by measuring absorption at 260 and 280 nm. Total cellular RNA (20 μg/lane) was denatured in formamide-formaldehyde, and was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde (27). After completion of electrophoresis, RNA was vacuum-blotted onto a nylon membrane (Zetabind; Cuno Inc., Meriden, CT), and short-wave UV cross-linked. All hybridization steps were carried out in a rotating drum in a temperature-controlled oven. The Rapidhyb™ system (Amersham Buchler GmbH) was used as recommended by the manufacturer. An EcoRI-XhoI insert from the murine RANTES clone pMuR3 (16) and a 2.0-kb cDNA insert of the plasmid pMC1 encoding the murine 18S ribosomal RNA band were radioactively labeled using hexamer primers with 5 μCi [^{32}P]deoxyadenosine 5′-triphosphate (3,000 Ci/mmol; New England Nuclear, Boston, MA). Prehybridization was performed at 70°C for 2 h. Hybridization was done with 2 ng radioactive probe per ml hybridization fluid for 2 h at 70°C. After hybridization, the membrane was washed at room temperature in 2× SSC (20 × SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% SDS, followed by two washes (each wash for 15 min) at 65°C in 0.5× SSC with 0.1% SDS. Autoradiography was performed with intensifying screens for 48 h (RANTES cDNA) and 30 min (18S cDNA). Blots were stripped in 5 mM Tris-HCl, 0.2 mM EDTA, 0.5% sodium pyrophosphate, and 5× Denhardt's (50× Denhardt's: 1% ficoll, 1% polyvinylpyrrolidone, 1% BSA, 50% formamide, and 0.1% SDS) solution for 3 h at 65°C, and subsequently were rehybridized with a cDNA probe for the 18S ribosomal RNA to account for small loading and transfer variations. Exposed films were scanned with a laser densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA) connected to a computer system, and the area under the curves was determined by

Gaussian integration with the computer program GS 365W (Hoefer). Relative changes in RNA were calculated after assigning hybridization in control lanes a relative value of one (27). Samples were normalized for the signal intensity of the 18S hybridizations. All Northern blot experiments were repeated three times with similar results.

RANTES ELISA. RANTES protein production was measured in cell culture supernatants with a commercial solid-phase ELISA assay that uses two antibodies specific for RANTES (R&D Systems, Minneapolis, MN). For this assay, 10^6 GER or MC were made quiescent in serum-free DMEM, and were subsequently stimulated with different concentrations of ANG II (10^{-8}–10^{-6} M) in the presence or absence of 10^{-6} M losartan, 10^{-6} M PD 123177, or 10^{-7} M CGP-42112A (Neosystem, Strasbourg, France) for 24 h. Additional cells were treated with 1 μg/ml LPS. At the end of the experiment, supernatants were harvested, and RANTES protein concentrations were directly measured in culture supernatants with the ELISA as recommended by the manufacturer. Cells were released from the plates with trypsin/EDTA, and were counted in a hemocytometer. Concentrations were expressed as fg RANTES/10^4 cells. Experiments were independently repeated six times with duplicate measurements.

Monocyte chemotactic assay. Monocyte chemotactic activity was determined in modified Boyden chambers (NeuroProbe, Cabin John, MD) by using freshly prepared human peripheral blood mononuclear cells exactly as previously described (28–30). In brief, 10^4 GER were plated in 24-well plates, stimulated as indicated, and medium was collected after 24 h, centrifuged at 10,000 *g* for 5 min, and stored at 70°C until assay. After a 30-min incubation at 37°C with or without 30 μg/ml neutralizing polyclonal goat anti-RANTES antibody (R&D Systems), different dilutions in serum-free DMEM were assayed for monocyte migration using 3.5×10^6 monocytes/ml, freshly prepared from human blood by ficoll gradient centrifugation (28). As additional control, 10^{-6} M ANG II was directly added to nonconditioned DMEM, and the chemotaxis assay was performed. Chemotactic activity was expressed as the mean number of monocytes migrating per field in 10 high-power fields (29). Background migration in response to nonconditioned medium (only serum-free DMEM) was 1.06 ± 0.35 ($n = 10$) and was subtracted from all values. Chemotactic assays were independently performed six times with duplicates for each experiment.

Animal experiments. To test the effect of ANG II delivery in vivo, male Sprague-Dawley rats (SAVO-Ivanovas, Kissleg, Germany) with a body weight of 150 g were anaesthetized by ether, and osmotic minipumps (Model 2002; Alzet, Palo Alto, CA) were intraperitoneally placed under sterile conditions. These pumps delivered 500 ng ANG II dissolved in 0.9% NaCl per h with a pumping rate of 0.5 μl/h. Control animals received only 0.9% NaCl. As an additional positive control, a limited number of rats ($n = 3$) were infused with 500 ng of LPS from *E. coli*, serotype 02:B6 (endotoxin > 10,000 U/mg LPS; Sigma Chemical Co.) per h. Some animals were directly treated after pump implantation with the AT_2 receptor antagonist PD 123177 in the drinking water (50 mg/liter) ad libitum. After 4 d, systolic blood pressures were measured by tail plethysmography under light ether anesthesia, blood was collected in EDTA tubes on ice by puncturing the aorta, and samples were stored at −70°C after plasma separation. Plasma renin activity (PRA) was measured as the generation of ANG I/ml/h with RIA using commercially available reagents (Sorin Biomedica, Saluggia, Italy). Kidneys were removed, and glomeruli were isolated by differential sieving with ice-cold Krebs-Ringer buffer as previously described (23). Purity of the preparations was assessed by light microscopy. Part of the glomeruli was directly lysed in 10 ml of a buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium lauroyl sarcosinate, and 0.7% β-mercaptoethanol. Total RNA was prepared, and Northern blots for RANTES hybridization were performed as described above. In addition to glomeruli, the remnant renal tissue was also collected for RNA isolation. Some glomeruli were not lysed, but incubated in serum-free DMEM at 37°C for 2 h in a shaker. After centrifugation, the protein content of the glomeruli was determined by the Lowry method (31), and chemotactic activity of the supernatants was measured in various dilutions as described above. As an additional control, 30 μg/ml neutralizing polyclonal goat anti-RANTES antibody or control IgG were added to some supernatants before the chemotactic assay was performed. Isolated glomeruli from control animals and ANG II–infused rats were also lysed in disruption buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM dithiothreitol) and the protein content was measured by a modification of the Lowry method, which is insensitive to the used concentrations of SDS and dithiothreitol (31). Equal amounts of protein (100 μg/lane) were loaded onto a denaturing 15% SDS-polyacrylamide gel. Low molecular weight Rainbow™ markers (2,350–46,000 kD; Amersham Buchler GmbH) were used as standards. Proteins were electroblotted onto nitrocellulose (Hybond-N; Amersham Buchler GmbH), and membranes were stained with 0.2% Ponceaus S (Sigma Chemical Co.) to test for complete protein transfer. Detection of RANTES was performed exactly as previously described (16) using a 1:500 dilution of a mouse monoclonal anti–human RANTES antibody (R & D Systems). The secondary antibody was a rabbit horseradish peroxidase-conjugated anti–mouse IgG in a 1:1000 dilution (Transduction Laboratories, Lexington, KY). Peroxidase labeling was detected with luminescence immunodetection (ECL; Amersham Buchler GmbH) according to the manufacturer's recommendations.

Immunohistochemistry for M/M. To analyze the infiltration of M/M into glomeruli, a separate series of animals consisting of five rats per group were treated as described above. Kidneys were perfused in situ with ice-cold 0.9% NaCl, and tissues were fixed in Carnoy's solution. Kidney tissue was cut and stained with a monoclonal antibody directed against the rat monocyte-specific marker ED-1 (Chemicon International, Inc., Temecula, CA). The stainings were developed using the alkaline phosphatase anti-alkaline phosphatase technique. All quantitative morphologic analyses were performed in a blinded fashion. Evaluation of ED-1–positive cells was performed by counting positive cells in 30 glomeruli in each section from five (three LPS-infused rats) individual animals.

RANTES immunohistochemistry. Pilot experiments revealed that frozen sections of rat kidney may be successfully used to identify RANTES protein expression in ANG II–infused animals, but that this procedure is suboptimal for delineation of structural details. Additional pilot studies demonstrated that fixation in Carnoy's solution or buffered formaldehyde destroyed RANTES epitopes (data not shown). A separate series of animals consisting of three rats per group were infused with ANG II or vehicle as described above. Kidneys were in situ perfused with ice-cold 0.9% NaCl followed by 2% glutaraldehyde in cacodylate buffer for 2 min. Tissues were stained using a modified avidin–biotin complex technique with Tyramide Signal Amplification system (Renaissance; DuPont-NEN, Boston, MA). Before staining, 5-μm sections were post-fixed with 1% glutaraldehyde for 15 min at room temperature. Steam antigen retrieval pretreatment was performed using 1× H.E.I.R. 10 mM citrate buffer (Ventana-Bioteck; Tucson, AZ), pH 6.0 at 90°C for 20 min. After pretreatment, sections were reacted with a murine monoclonal anti-human RANTES antibody (Biosource International, Camarillo, CA) at 0.2–0.5 μg/ml. After primary antibody reaction, the sections were treated according to a modification of the Ventana-Biotek protocol, which included staining enhancement using biotin–tyramide. Slides were counterstained with hematoxylin. For quantification of positive staining, an investigator unaware of the origin of slides counted 100 glomeruli and classified each glomerulus as negative or capillary lumen positive. As positive control, NIH-3T3 fibroblasts were treated for 24 h with TNF-α (400 U/ml; Sigma Chemical Co.) in culture to stimulate RANTES expression. Cytospin sections were then stained as described for kidney tissue. To ensure that the anti-RANTES antibody specifically recognizes RANTES, the antibody was combined with recombinant human RANTES protein (R&D Systems) at a 1:2 molar ratio with continous shaking for 24 h at 4°C. The mixture was ultracentrifuged and the supernatant was used for staining.

Statistical analysis. All values are presented as means±SEM. Statistical significance among multiple groups was tested with nonparametric Kruskal-Wallis test. A P value of < 0.05 was considered significant.

Results

ANG II receptor subtypes. We have previously shown that GER expressed high affinity receptors for ANG II (22). The present studies were performed to characterize possible ANG II receptor subtypes. Fig. 1 *A* demonstrates that competition experiments with the AT_1 antagonist losartan replaces ~ 80% of the tracer [^{125}I][Sar^1, Ile^8] ANG II. The AT_2 receptor blocker PD 123177 appears to have at lower concentrations a slightly higher affinity to the ANG II receptor compared with losartan, and replaces up to 30% of the radioactive tracer (Fig. 1 *A*). The complex competition characteristics are compatible with expression of both AT_1 and AT_2 receptors on GER. The presence of specific transcripts for the AT_2 receptor was detected by cDNA amplification after reverse transcription (Fig. 1 *B*). In addition to the predicted size and selecting primers that span introns, the identity of the band was confirmed by dideoxynucleotide chain termination sequencing after cloning in the TA II vector, and comparison with the published sequence (26). As an additional control, cDNA amplification for AT_2 receptor mRNA was performed with RNA from isolated glomeruli obtained from normal male Sprague-Dawley rats (Fig. 1 *B*). Northern blots with 20 μg total RNA, however, failed to detect a discrete signal, probably due to the low abundance of AT_2 receptor transcripts.

RANTES production in GER. We first used a sandwich ELISA to quantify RANTES protein in cell culture supernatants of GER after stimulation with ANG II. To control for any growth stimulatory effects of ANG II on GER, RANTES protein secretion was normalized to total cell counts. As shown in Fig. 2, a single dose of 10^{-8}–10^{-6} M ANG II for 24 h significantly stimulated secretion of RANTES from GER. This effect was specific for these cells because syngeneic mesangial cells failed to secrete RANTES after ANG II treatment (Fig. 2). No endotoxin could be detected in the used ANG II preparation, indicating that the induced RANTES secretion is not due to contamination with endotoxins. 1 μg/ml LPS for 24 h, however, strongly stimulates RANTES secretion in both glomerular cell types in accordance with previous observations (15, 32). This response appears to be specific for RANTES because ANG II failed to stimulate the release of MCP-1, another chemokine of the C-C family (data not shown). To characterize the subtype of the ANG II receptor which is responsible for signal transduction of this response, GER were stimulated in the presence of 10^{-6} M of the AT_1 antagonist losartan, or 10^{-6} M of the AT_2 blocker PD 123177. In addition, 10^{-6} M of the peptide AT_2 antagonist CGP-42112A with some agonistic properties was also used. ANG II–stimulated RANTES secretion was almost completely abolished in the presence of the AT_2 antagonists PD 123177 or CGP-42112A, whereas the AT_1 blocker losartan had no significant effect (Fig. 3).

We next tested whether secreted RANTES induced by ANG II treatment is chemotactic for human monocytes. Human monocytes were used because they can be easily isolated in large quantities, and samples from the same donor are a reliable system for multiple analysis (29). As shown in Fig. 4 *A*, conditioned supernatant from ANG II–treated GER in vari-

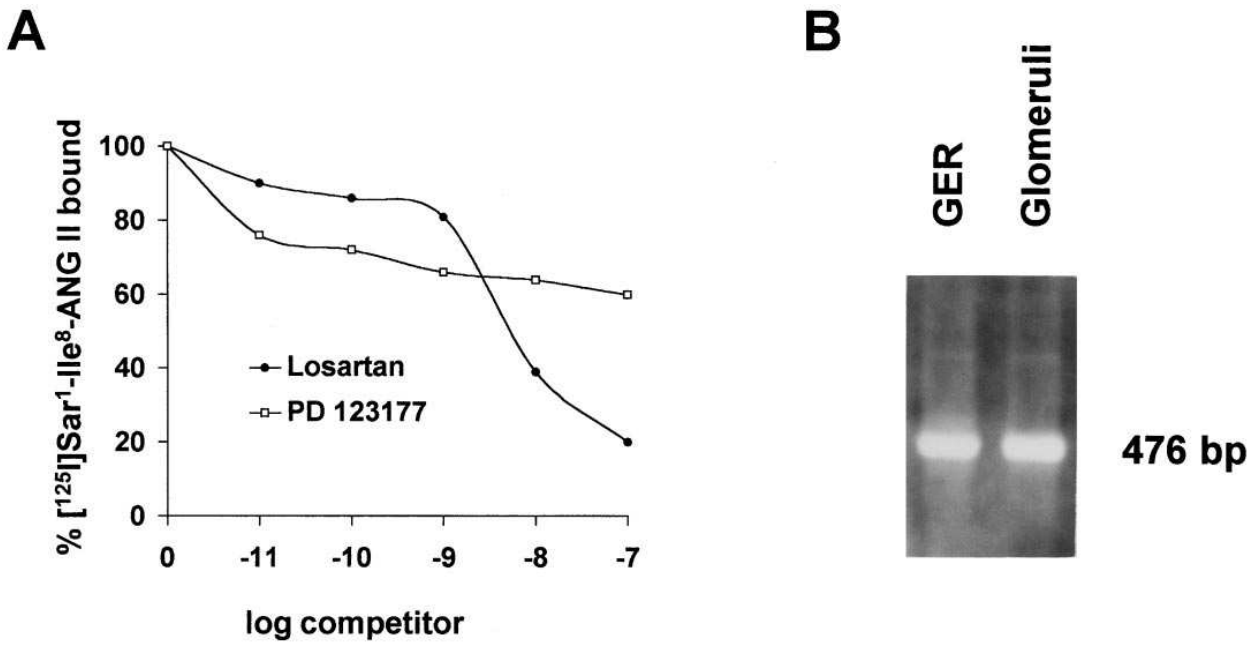

Figure 1. ANG II-receptor expression in GER. (*A*) Competition of the AT_1 receptor antagonist losartan and the AT_2 blocker PD 123177 for [^{125}I][Sar^1-Ile^8] ANG II binding sites on GER. GER express AT_1 and AT_2 receptors with complex binding characteristics. Mean values of four independent binding experiments with duplicates for each measurement. (*B*) cDNA amplification of reverse-transcribed RNA isolated from GER and normal rat glomeruli using specific primers for the rat AT_2 receptor. The predicted band at 476 bp indicates the presence of AT_2 receptor transcripts in GER and whole glomeruli. The identity of the bands was confirmed by sequencing the cDNA amplification products. A total of 40 cycles was performed with an annealing temperature of 60°C for 1.5 min, an extension step at 72°C for 1.5 min, and a denaturation step at 92°C for 1 min.

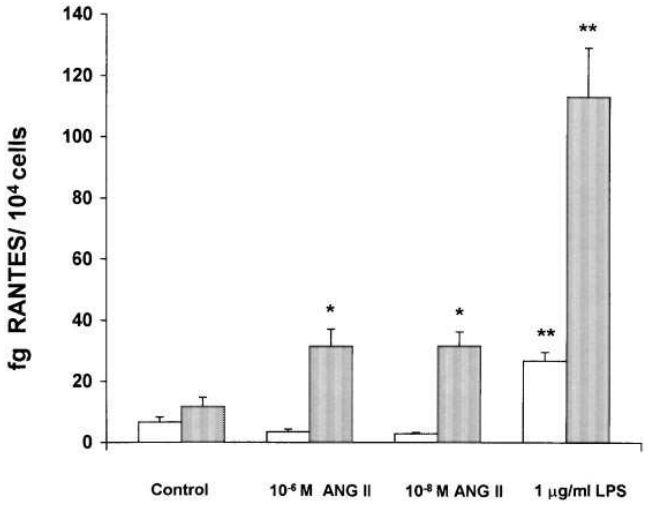

Figure 2. RANTES secretion in culture supernatants. A single dose of 10^{-8}–10^{-6} M ANG II for 24 h significantly stimulated RANTES protein secretion in GER, but not in syngeneic MC as measured by a sandwich ELISA. LPS stimulated RANTES secretion in both cell cultures. $n = 6$ independent stimulation experiments with duplicate measurements; $^{*}P < 0.01$ versus control; $^{**}P < 0.001$ versus controls. *White bars*, MC; *striped bars*, GER.

ous dilutions has significantly more chemotactic activities for human monocytes than culture supernatant from control GER that were not treated with ANG II. A maximal monocyte migration was observed at a 1:2 dilution of the conditioned supernatant. A bell-shape activity curve with an optimal monocyte migration at a distinct dilution is typical for chemotactic assays, and represents a genuine chemotactic response (33). A

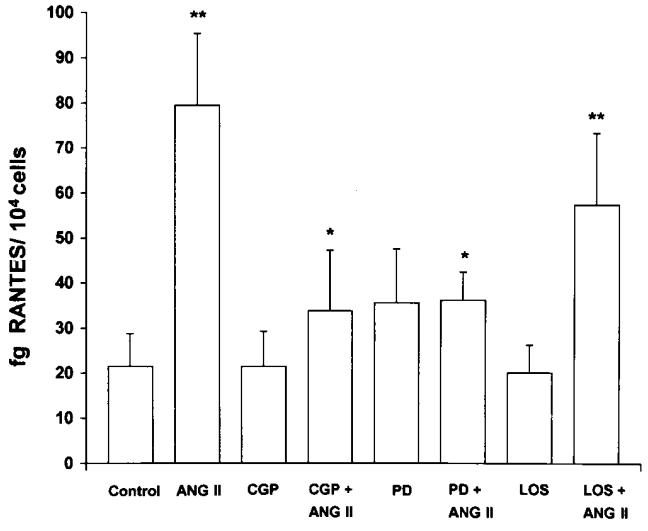

Figure 3. Effect of ANG II receptor blocker on RANTES secretion. Quiescent GER were stimulated for 24 h with 10^{-6} M ANG II in the presence or absence of 10^{-6} M of the receptor antagonists. The ANG II–stimulated RANTES secretion was attenuated by the AT_2 receptor antagonists PD 1231777 (*PD*) or CGP-42112A (CGP), but not by the AT_1 antagonist losartan (*los*). $n = 6$ independent stimulation experiments with duplicate measurements; $^{**}P < 0.01$ versus unstimulated controls; $^{*}P < 0.05$ versus cells treated with ANG II only.

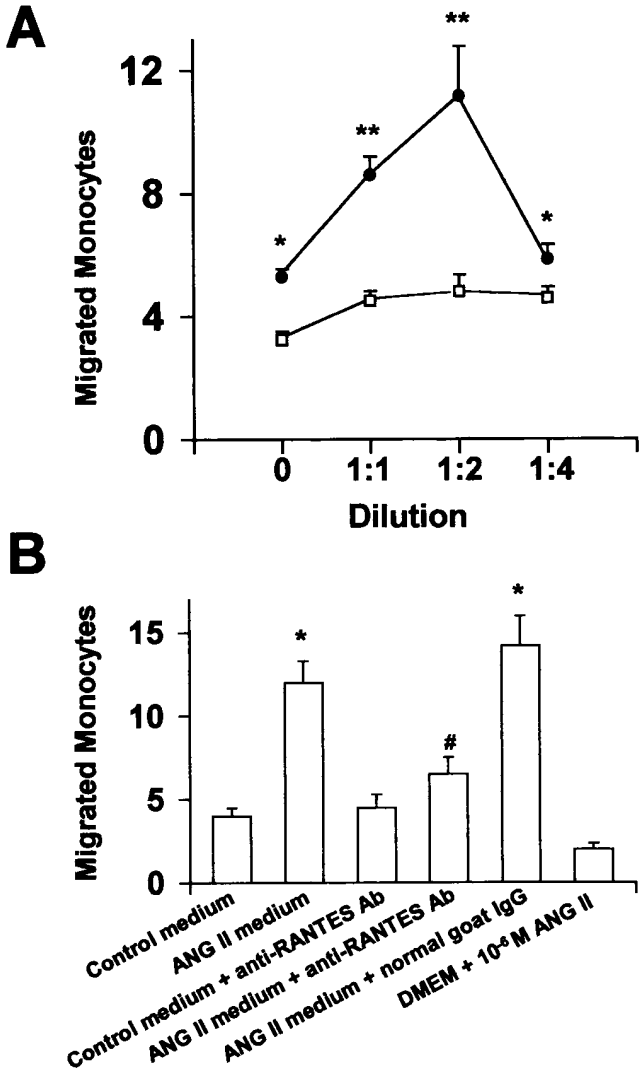

Figure 4. Chemotactic assay for human monocytes. Conditioned medium from GER treated for 24 h with a single dose of 10^{-6} M ANG II or supernatant from GER incubated with solvent (0.9% NaCl) were diluted with endotoxin-free sterile PBS and used for the chemotactic assay in a modified Boyden chamber. Migrated monocytes were quantitated by counting after staining of filter. (*A*) 1:2 dilution of conditioned medium from ANG II–treated cells showed the maximal chemotactic activity. All other dilutions of conditioned supernatants from ANG II–incubated GER, however, exhibited more chemotactic activity than control supernatants. Such a bell-shape dilution curve is typical for chemotactic assay. $n = 6$ separate GER stimulation experiments with subsequent chemotactic assays in duplicate. $^{*}P < 0.05$ versus control medium; $^{**}P < 0.001$ versus control medium. *White squares*, control medium; *black circles*, ANG II medium. (*B*) 1:2 dilution of conditioned supernatant from ANG II–treated GER was supplemented with 30 μg/ml of a neutralizing goat anti-RANTES antibody (*anti-RANTES Ab*) or normal goat IgG. The neutralizing anti-RANTES antibody almost completely abolished the chemotactic activity in conditioned supernatant from ANG II–treated cells, indicating that the chemotactic factor for human monocytes is RANTES. Control goat IgG did not influence the chemotactic activity of conditioned supernatant obtained from ANG II–treated GER. Direct addition of 10^{-6} M ANG II in serum-free DMEM into the Boyden chamber exhibited no significant chemotactic activity, suggesting that ANG II alone is not chemotactic. $n = 6$ separate GER stimulation experiments with subsequent chemotactic assays in duplicate. $^{*}P < 0.01$ versus control medium; $^{*\#}P < 0.05$ versus ANG II medium without antibody.

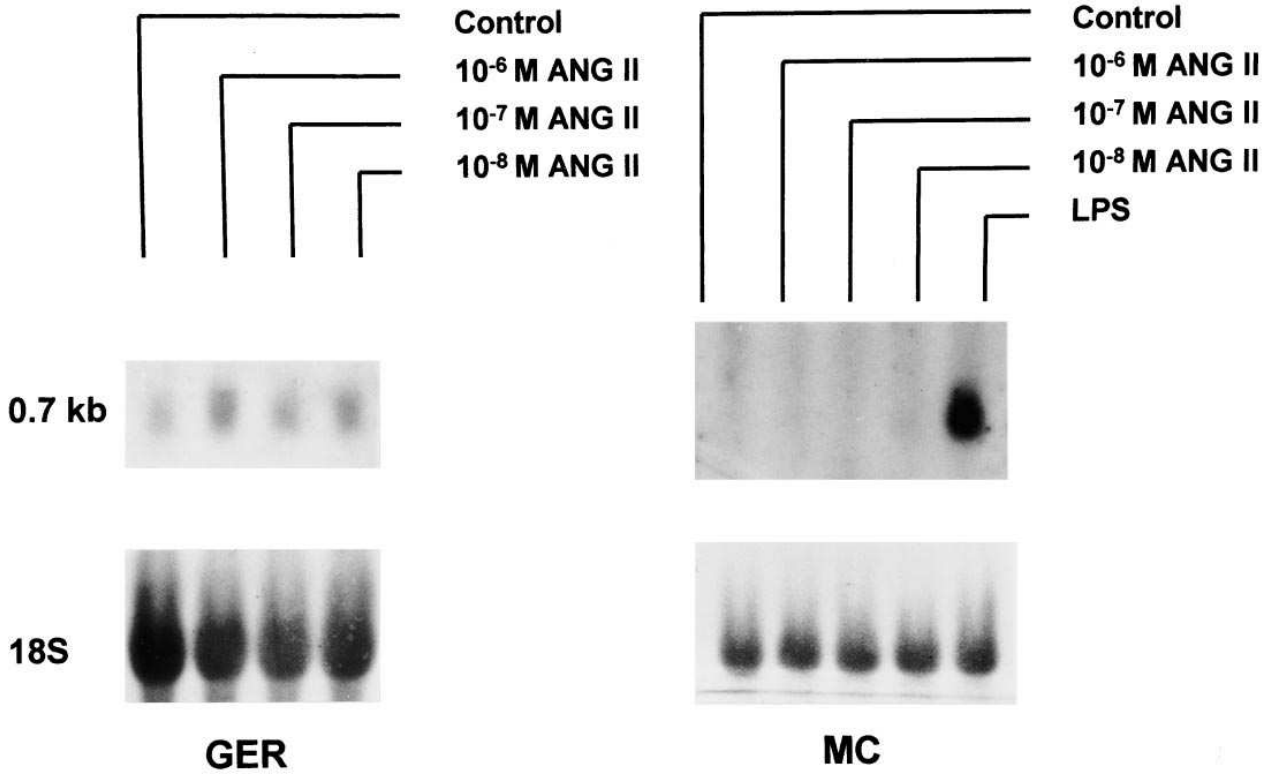

Figure 5. RANTES mRNA expression. GER or MC were stimulated for 24 h with a single dose of 10^{-8}–10^{-6} M ANG II. A total of 20 μg total RNA were used for the Northern blot. Expression of RANTES transcripts increased in GER stimulated with ANG II. The vasoactive peptide did not induce RANTES expression in MC. 1 μg/ml LPS, however, induced RANTES transcripts in MC. The blots were stripped and rehybridized with a cDNA probe for the 18S ribosomal RNA to adjust for small variations in RNA loading and transfer to the membrane. This blot is representative of three independent experiments with similar results.

neutralizing goat anti-RANTES antibody, but not normal goat IgG, blocked the chemotactic activity in culture supernatant from ANG II–treated GER, indicating that the response is due to the secretion of RANTES and not of other chemokines (Fig. 4 *B*). Direct addition of 10^{-6} M ANG II to serum-free DMEM failed to induce any significant chemotaxis (Fig. 4 *B*). This observation strongly suggests that the vasoactive peptide per se is not chemotactic for monocytes.

As demonstrated in Fig. 5, treatment of quiescent GER with 10^{-8}–10^{-6} M ANG II stimulated the expression of RANTES mRNA (control: 1.00; 10^{-8} M ANG II: 3.15; 10^{-7} M ANG II: 3.22; 10^{-6} M ANG II: 3.65 relative changes in mRNA expression). ANG II, however, failed to induce RANTES transcripts in syngeneic MC. As predicted, RANTES mRNA can be induced in MC by LPS (Fig. 5). Time-course experiments revealed maximal induction of RANTES mRNA 24 h after stimulation with 10^{-7} M ANG II, although expression was already detectable after 6 h of incubation (Fig. 6).

In vivo induction of RANTES. To confirm our cell culture observations in vivo, rats were intraperitoneally infused for 4 d with ANG II. This time course was selected to investigate early potential changes in the expression of RANTES, because longer infusion of ANG II induces irreversible structural changes in the kidney (34). ANG II–infused rats had slightly but significantly elevated systolic blood pressure (control-infused: 73±1.6; ANG II-infused: 97±3.3 mmHg, $P < 0.001$, $n = 6$). Treatment with the AT_2 antagonist PD 123177 in the drinking water did not significantly lower systolic blood pressure (ANG II-infused + oral PD 123177: 100±3.4 mmHg, not significant versus ANG II–infused only, $n = 6$). ANG II–infused rats had a significantly lower PRA compared with controls (control-infused: 13.4±2.7; ANG II–infused: 6.8±1.2 ANG I/ml/h, $P < 0.05$, $n = 6$).

Isolated glomeruli from ANG II–infused animals and controls were incubated for 2 h on a shaker at 37°C with serum-free DMEM. After incubation, the medium was assayed for monocyte chemotaxis, and the protein content of the glomeruli was measured. As shown in Fig. 7 *A*, DMEM incubated with glomeruli from ANG II–infused animals exhibited significantly more chemotactic activity than medium which was conditioned by incubation with glomeruli from control-infused rats. Addition of 30 μg/ml of the neutralizing anti-RANTES antibody significantly inhibited this activity (conditioned medium from ANG II-infused rats + anti-RANTES antibody: 6.2±0.5 migrated monocytes, $P < 0.01$ versus conditioned medium without anti-RANTES antibody, $n = 8$). Electrophoresis of 100 μg glomerular protein with subsequent Western blotting applying an anti-RANTES antibody revealed an 8-kD band only in the glomeruli obtained from ANG II–infused, but not from control-infused rats (Fig. 7 *B*).

Isolated glomeruli from ANG II–infused animals expressed more RANTES mRNA than control rats infused with 0.9% NaCl only (control-infused: 1.00; ANG II-infused: 1.97 relative changes in mRNA expression [see Fig. 8]). Although there was some variation in the expression of RANTES transcripts in controls that may be due to some stress response as-

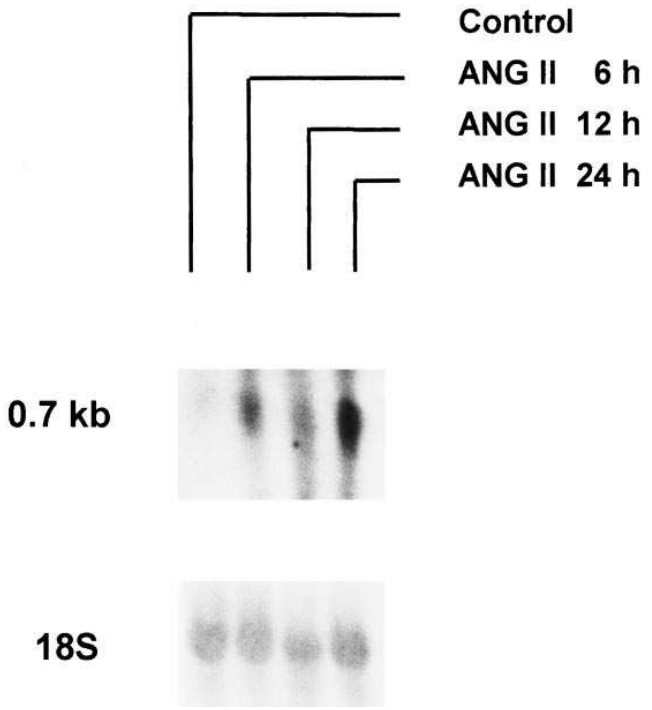

Figure 6. Time course of RANTES mRNA expression. Stimulation of GER with a single dose of 10^{-7} M ANG II induced RANTES transcripts after 6 h, although maximal expression was observed after 24 h of incubation. This blot is representative of three independent experiments with similar results.

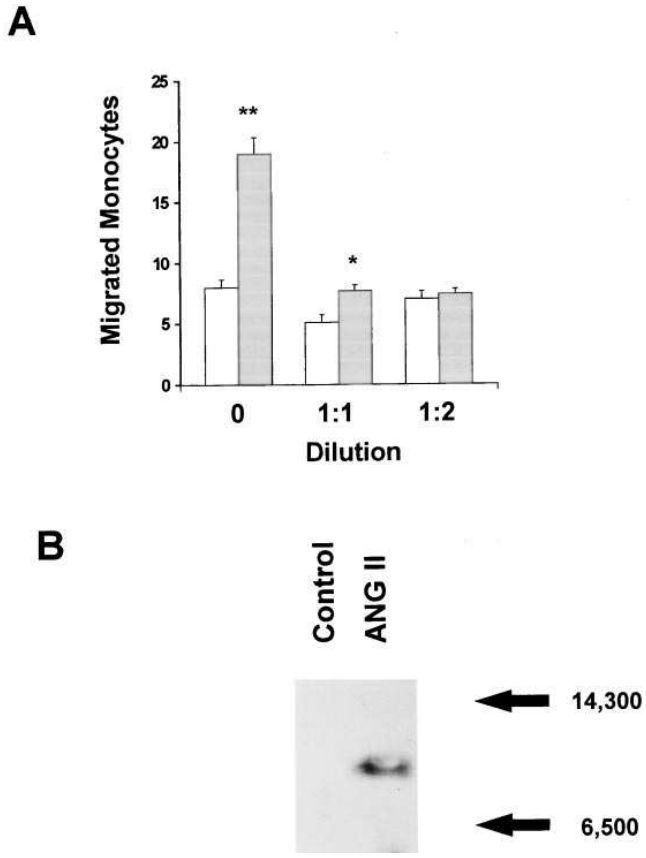

Figure 7. RANTES protein expression in isolated glomeruli from rats infused with ANG II. (*A*) Isolated glomeruli (200 mg) from ANG II-infused animals or solvent-infused animals (pooled from four animals in each group) were incubated for 2 h in serum-free DMEM at 37°C. After incubation, glomeruli were removed by centrifugation, and the supernatants were assayed for chemotactic activity in a modified Boyden chamber. The conditioned medium from ANG II-treated animals revealed a significantly higher chemotactic activity for monocytes compared with the medium that was incubated with glomeruli from control-infused animals. The figure shows pooled data from two series of animal experiments with four rats in each group. Glomeruli from each series were independently incubated four times with subsequent chemotactic assay. $^{**}P < 0.001$ versus control-medium; $^{*}P < 0.05$ versus control-medium. *White bars*, control medium; *gray bars*, ANG II medium. (*B*) Western blot of 100 μg glomerular protein obtained from ANG II–infused or control-infused rats. The blot was incubated with an anti-RANTES antibody. Molecular weight markers are shown on the right side. A single band of $\sim$ 8 kD is detectable in the glomerular lysates isolated from ANG II–infused rats (ANG II), but not from control-infused animals. This blot was repeated twice with similar results. *White bars*, control medium; *shaded bars*, Ang II medium.

sociated with peritoneal surgery and wound healing, there was in all three series (with four rats in each series) always a higher RANTES mRNA expression in isolated glomeruli from ANG II rats compared with control-infused animals. No significant RANTES mRNA expression, however, was detected in the remaining renal tissue after removal of glomeruli (Fig. 8). Oral treatment with the AT_2 receptor antagonist PD 123177 (50 mg/liter drinking water ad libitum) attenuated the increase in RANTES transcripts in ANG II–infused rats (control: 1.00; ANG II–infused: 1.70; ANG II–infused + PD 123177: 0.98 relative changes in mRNA expression (Fig. 9).

We could not detect RANTES protein expression using a polyclonal or a monoclonal antibody in Carnoy's solution or buffered formaldehyde, suggesting that the specific epitopes are destroyed by the fixation process (data not shown). Frozen sections exhibited unsatisfactory preservation of morphology. Thus, we had to rely on glutaraldehyde perfusion-fixed renal tissue which, in combination with biotin–tyramide staining enhancement, offers an excellent sensitivity and exact morphological localization of staining. To test further the specificity of the used monoclonal anti-RANTES antibody, we used fibroblasts challenged with TNF-α as a control system. As shown in Fig. 10 *A*, treatment of NIH-3T3 fibroblasts with TNF-α for 24 h induced a strong cytoplasmic staining for RANTES protein. In contrast, no specific staining was observed in TNF-α–treated cells when the used antibody was preabsorbed with recombinant RANTES, indicating the specificity of this antibody (Fig. 10 *B*). Although there was some limited tubular staining, glomeruli were only occasionally positive for RANTES protein in animals infused with vehicle (Fig. 10 *C*). A strong specific staining for RANTES protein, however, was observed in ANG II–infused rats (Fig. 10 *D*). This staining was principally localized to small capillaries and glomerular endothelium with some glomerular epithelial cells showing also a positive reaction (Fig. 10 *D*). Control sections that were stained only with the secondary antibody or irrelevant control sera showed no staining indicating the specificity of the response (data not shown). Quantification of positive glomerular endothelium in 100 glomeruli from a total of three rats in each group revealed

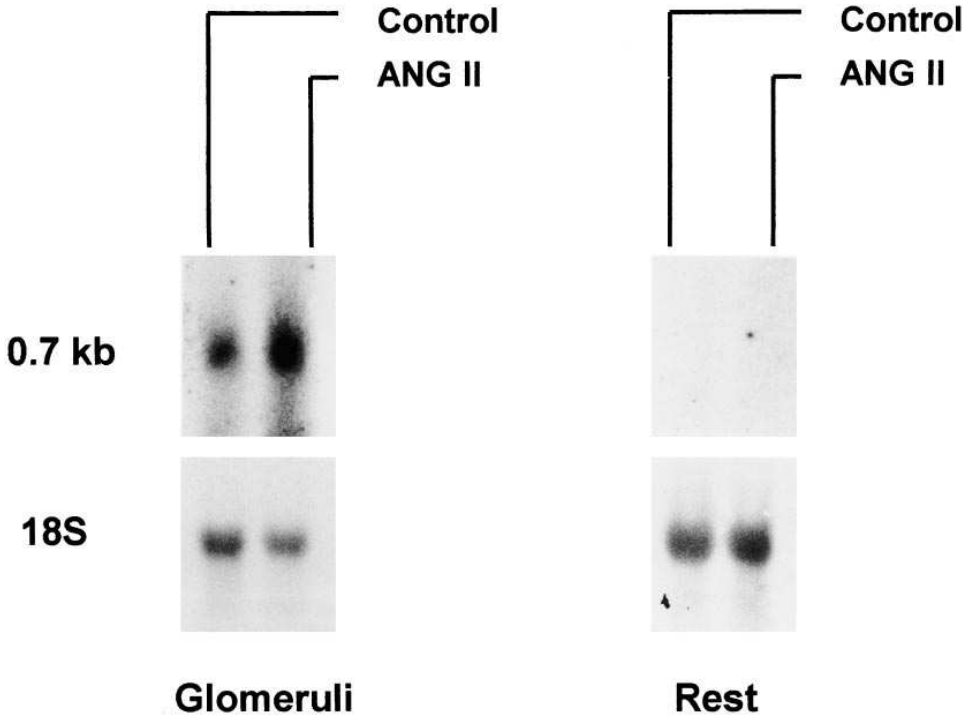

Figure 8. Northern blot analysis of RNA from rats infused for 4 d with ANG II (*ANG II*) or control-solvent (*Control*). The expression of RANTES mRNA was significantly higher in ANG II–infused animals compared to control-infused rats. No RANTES transcripts were detectable in RNA isolated from the rest tissue after glomeruli were removed by sieving. Although there was some variation in the intensity of the hybridization signals that may be due to RANTES induction by operative stress, RANTES transcripts were always more abundant in the ANG II–infused animals in all three series consisting of four animals in each group. This blot is representative for three independent experiments.

an increase from 1% in control-infused rats to 26% in ANG II–treated animals. There was no change in the slight staining of tubular structures by ANG II infusion. Moreover, ANG II–infused animals exhibited significantly more glomerular ED-1–positive cells compared with control-infused rats (Table I). ANG II–infused animals had almost as many ED-1–positive cells as LPS-infused rats, which served as a positive control (Table I). Oral treatment with the AT_2 receptor antagonist PD 123177 reduced this glomerular influx of ED-1–positive cells (Table I).

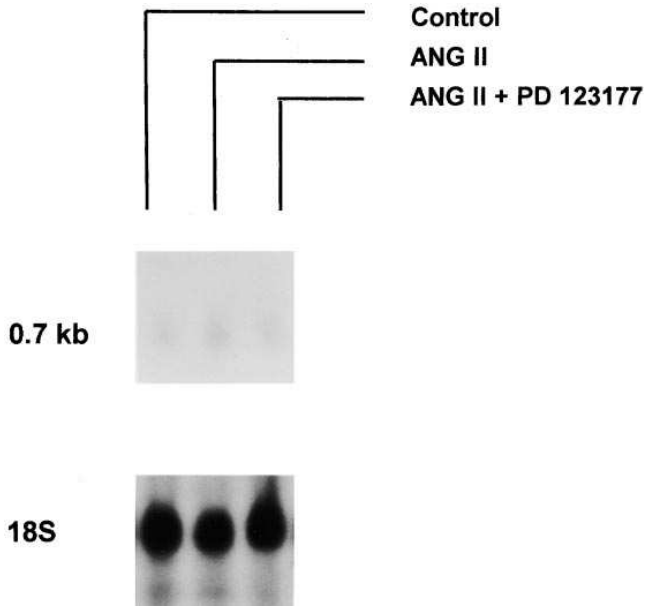

Figure 9. Northern blot analysis of isolated glomeruli from ANG II or control-infused rats treated with PD 123177 in the drinking water. ANG II infusion stimulated expression of RANTES mRNA in isolated glomeruli. Oral treatment with the AT_2 receptor blocker PD 123177 completely abolished this increase. This blot is representative of two independent series of four animals in each group.

Discussion

Glomerular influx of M/M is a common feature of immune-mediated glomerular injury (1–3). In addition, glomerular infiltration with M/M occurs early even in renal diseases that are traditionally considered to be of nonimmune origin, such as diabetic nephropathy and glomerulosclerosis subsequent to renal ablation (4, 5). Release of various cytokines, proteinases, and toxic oxygen species by M/M clearly contributes to the initiation and progression of renal failure in such pathophysiological situations (6). ACE inhibitors and to some extent ANG II receptor blockers reduce glomerular injury in various models of chronic renal failure (35–38). There exists suggestive evidence that these effects are independent of the reduction in systemic blood pressure, and they may not even be associated with a decrease in glomerular capillary pressure (17, 19, 39). Treatment with ACE inhibitors also reduces glomerular and tubulointerstitial infiltration with M/M in different immune and nonimmune models of renal disease (18, 19, 36, 39).

Although there is some evidence that other chemotactic factors such as osteopontin or lipid factors may be involved in ANG II–mediated invagination of M/M into the tubulointerstitium (40), the role of glomerular chemokine expression in the recruitment of M/M is only incompletely understood. We have, for example, recently shown that glomerular MCP-1 expression is enhanced in a model of proliferative glomerulonephritis (41). This increased MCP-1 expression was associated with an increase in glomerular influx of M/M. In contrast, expression of RANTES occurs later in this model and may not

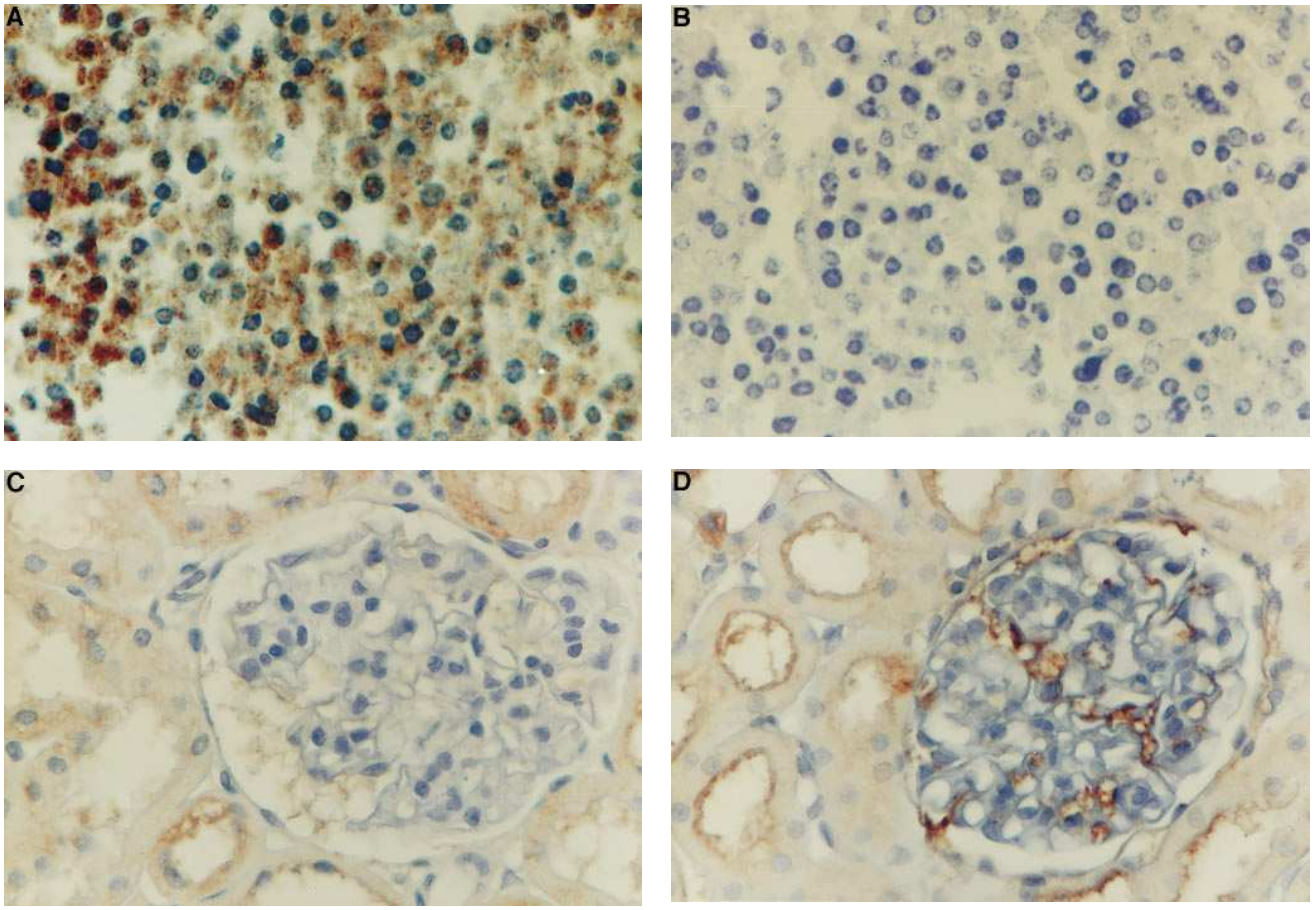

Figure 10. Immunohistochemistry for RANTES protein. (*A*) NIH-3T3 fibroblasts stimulated for 24 h with TNF-α revealed positive cytoplasmic staining for RANTES protein using a monoclonal antihuman RANTES antibody. (*B*) Preabsorption of the antibody with recombinant RANTES, and subsequent staining of TNF-α–challenged 3T3 cells revealed no positive staining indicating the specificity of this antibody. (*C*) Kidney section of animals infused with solvent for 4 d revealed only a slight staining of tubular cells with no detection of RANTES protein in the glomerular tuft. (*D*) Infusion of ANG II for 4 d, however, induced RANTES staining which appeared to be principally localized in glomerular endothelial cells and small capillaries. See Methods for details. ×200.

play a significant role in the early wave of glomerular M/M invagination (Wolf et al., unpublished data.)

The present study was undertaken to test the hypothesis that ANG II may influence the expression of the chemokine RANTES in GER. Our data collectively indicate that ANG II induces mRNA and protein expression of RANTES in cultured GER. In addition, the induced RANTES is chemotactic for monocytes as measured in a chemotactic assay. The observation is surprising, however, that the signal transduction of ANG II occurs through the AT_2 receptors because two different AT_2 receptor antagonists, but not the AT_1 receptor blocker losartan, inhibit the ANG II-induced RANTES expression. Almost all effects of ANG II in the kidney are transduced by the seven-membrane–spanning, G-protein–coupled AT_1 receptors (42, 43). The mechanism of signal transduction through the AT_1 receptor is a topic of current research; activation of the AT_1 receptor causes phosphorylation of various cellular proteins, although the receptor itself has no kinase activity (44). Furthermore, the AT_1 receptor couples to multiple other effector systems such as phospholipase C, D, and A_2, adenylate cyclase, and ion channels in distinct cells (44). Much less is known, however, about the AT_2 receptor and its signal transduction pathways. The recently cloned AT_2 receptor also has the putative seven-transmembrane domain structure, but with very little homology with the AT_1 receptor (45, 46). The AT_2 receptor may be involved in the regulation of intracellular phosphotyrosine phosphatases (47, 48). Although the AT_2 receptor is abundantly expressed in the fetal kidney, only 5–10% of ANG II receptors in the adult kidney are of the AT_2 sub-

Table I. ED-1–Positive Cells per Glomerular Cross-section 4 d After Infusion

Infusion	No. of cells
Control (solvent)	0.25±0.04
Control (solvent) and oral treatment with PD 123177	0.18±0.04
ANG II	0.46±0.03*
ANG II and oral treatment with PD 123177	0.24±0.02‡
LPS	0.48±0.02*

A total of 20 glomeruli were counted from each individual animal ($n = 3$ for LPS infusion, $n = 5$ for all other groups). *$P < 0.01$ versus control infusion; ‡$P < 0.01$ versus infusion of ANG II only.

type (49). Autoradiography studies demonstrate expression mainly in preglomerular vessels, the renal capsule, and only little in the glomerular tufts (42, 43, 50). Which effects of ANG II in the kidney are actually transduced by AT_2 receptors is currently incompletely understood, but it has been recently described that activation of AT_2 receptors blunt pressure-induced natriuresis (49, 51). Our GER express AT_1 and AT_2 receptors with the AT_1 subtype being much more abundant. This is in good agreement with recent studies demonstrating an AT_1 to AT_2 ratio of 80:20 in cultured rat coronary endothelial cells (52). We have previously demonstrated that ANG II stimulates a slight, but significant proliferation of GER, a response that is solely mediated by AT_1 receptors, and is associated with stimulation of mitogen-activated protein kinases (22). In contrast to observations in cultured microvascular cells from coronary arteries (52), we do not observe an inhibition of proliferation associated with activation of the AT_2 receptor. So far, the only known effect that is transduced through AT_2 receptors in GER is the stimulation of RANTES expression. The ANG II–stimulated increase in RANTES mRNA and protein is specific for GER because syngeneic mesangial cells showed no response after treatment with ANG II. Whether this failure of RANTES induction in mesangial cells is due to the absence of AT_2 receptors in these cells remains to be investigated.

There exists some evidence that angiotensin III may be chemotactic for polymorphonuclear neutrophils, but chemoattraction for M/M has not been tested (53). Moreover, it has been reported that ANG II activates M/M under certain circumstances (54, 55). In our experiments, ANG II alone was not chemotactic for human monocytes and the chemotactic activity of conditioned supernatants from ANG II–treated GER was abolished in the presence of the neutralizing anti-RANTES antibody, strongly indicating that induction of RANTES is necessary, and ANG II per se is not chemotactic for monocytes in our experimental setting. Interestingly, a previous study reported release of a neutrophil chemoattractant factor from human and bovine arterial endothelial cells after treatment with ANG II (56). This factor was not an eicosanoid or a phospholipid (56). Although the nature of this factor is not known, it is possible that some of the chemoattractant activity may be due to chemokine induction by ANG II.

Our data from our in vivo infusion experiments clearly demonstrate that glomeruli from ANG II–infused rats produce more RANTES compared with control-infused rats. We selected a short-time infusion period with lower concentrations of ANG II than previously used because we wanted to study early changes before structural alterations such as glomerulosclerosis or interstitial fibrosis occurred (34). Since we studied isolated glomeruli, we do not currently know whether glomerular endothelial cells are indeed the only source of the ANG II–mediated increase in RANTES mRNA production. We tried very hard to evaluate RANTES protein expression by immunhistochemistry through the application of different approaches. Unfortunately, routinely applied fixation procedures such as Carnoy's solution or formaldehyde fixation also destroyed the specific RANTES epitopes. Thus, we had to rely on glutaraldehyde-perfused kidneys with some particular modifications of the staining procedure to localize RANTES protein expression. This method was highly sensitive and reproducible in our hands. Additional experiments with preabsorbed antibody on TNF-α–treated fibroblasts confirmed the specificity of the staining method. Immunohistochemistry experiments using a specific anti-RANTES antibody revealed a positive glomerular staining in ANG II–infused rats, whereas no RANTES protein expression could be detected in control-infused animals. The positive staining may be principally localized to small capillaries and glomerular endothelium. We cannot, however, rule out that other glomerular structures such as epithelial cells may also show positive staining for RANTES, although quantification revealed that this staining did not increase in ANG II–infused rats. Along this line, it may be very difficult to demonstrate convincingly chemokine expression by immunohistochemistry since the small molecules may be cleared rapidly and removed from the tissue. Moreover, only very limited information hitherto exists in the literature demonstrating positive renal immunohistochemistry for RANTES (57, 58). The positive RANTES immunohistochemistry in ANG II–infused animals does not necessarily imply that RANTES protein is produced there, because circulating RANTES may simply adhere specifically or nonspecifically to the endothelium. Since the glomerular localization of M/M was different from RANTES staining, however, it is not likely that a primary influx of M/M may subsequently release RANTES protein. Treatment in vivo with the AT_2 receptor blocker PD 123177 partly abolished the ANG II–stimulated increase in RANTES expression in isolated glomeruli without influencing the systolic blood pressure. In addition, we have obtained preliminary evidence from pilot experiments that losartan, although lowering the ANG II–mediated increase in blood pressure, does not influence glomerular RANTES expression (our unpublished observations).

Glomerular ED-1 positive cells were significantly increased in rats infused for 4 d with ANG II compared with controls. The AT_2 receptor blocker PD 123177 abolished this glomerular influx of M/M. Although we cannot totally rule out that ANG II per se in the absence of RANTES induction may stimulate glomerular M/M influx, these data combined with the observed RANTES expression are highly suggestive that the ANG II–mediated glomerular infiltration of M/M depends on RANTES expression. Interference with RANTES expression using neutralizing antibodies or antisense technology, however, is necessary to demonstrate unequivocally a causal relationship between ANG II, RANTES expression, and glomerular M/M influx. In comparison with our studies, Johnson et al. (34) infused a higher dose ANG II into rats for 14 days and observed a higher systolic blood pressure associated with mild tubulointerstitial fibrosis and interstitial monocyte infiltration but only little glomerular infiltration of M/M. Since glomerular infiltration of M/M is a dynamic process, it is possible that earlier time-points after ANG II infusion such as selected in our study may show a higher degree of glomerular M/M infiltration.

In the glomerulus, emphasis has been placed on the study of mesangial cell biology and less on the role of glomerular endothelial cells in the initiation and progression of glomerular injury. Lee and coworkers (59) have recently provided convincing evidence that an early alteration in glomerular endothelial cell biology in the renal ablation model, which is characterized by an early glomerular influx of M/M, is associated with an increase in synthesis of angiotensinogen, TGF-β_1, and extracellular matrix proteins. These observations suggest that glomerular endothelial cells play an initiating role in the development of glomerular sclerosis in this model. It is therefore

possible that ANG II–stimulated endothelial cells may synthesize chemoattracting factors such as RANTES, which attract M/M into the glomerular tuft.

Dietary protein restriction reduces the glomerular influx of M/M in several models of immune- and non-immune-mediated glomerular disease (60). Since reduction of dietary protein intake also downregulates renin transcription (61), it is tempting to speculate that a decrease in intrarenal ANG II concentration which in turn reduces RANTES expression is responsible for some of the immune-protective effects associated with protein restriction.

Taking into consideration that RANTES induction in GER is mediated by AT_2 receptors, ACE inhibitors or a combination of AT_1 and AT_2 receptor antagonists may be more effective in preventing chemokine induction than an AT_1 receptor blocker only.

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