Angiotensin II Stimulates the Synthesis and Secretion of Vascular Permeability Factor/Vascular Endothelial Growth Factor in Human Mesangial Cells

CINZIA PUPILLI,* LAURA LASAGNI,* PAOLA ROMAGNANI,* FRANCESCA BELLINI,* MASSIM0 MANNELLI,* NADIA MISCIGLIA,* CARMELO MAVILIA,† UGO VELLEI,* DONATA VILLARI,‡ and MARIO SERIO*

*Department of Clinical Physiopathology, Endocrinology Unit, †Institute of Internal Medicine and Immunoallergology, and ‡ Clinic of Urology, University of Florence, Italy.

Abstract. The aim of the present study was to evaluate the role of angiotensin II (AngII) in regulating both the gene expression and secretion of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) in human mesangial cells (HMC) in culture. Densitometric analysis of Northern blot experiments demonstrated that AngII increases VPF/VEGF mRNA in a dose-dependent manner. The levels of VPF/VEGF mRNA in HMC exposed for 3 h to 10 nM, 100 nM, and 1 μM AngII were, respectively, 1.5-, 2.3-, and 1.6-fold higher than control cells (P < 0.05, P < 0.0001, and P < 0.05, respectively). This effect was blocked by the pretreatment with losartan (1 μM) (P < 0.005), a selective antagonist of the AngII AT1 receptor. Reverse transcription-PCR performed in HMC using oligonucleotide primers specific for all VPF/VEGF mRNA splicing variants detected three bands corresponding to VEGF 189, 165, and 121. Exposure of the cells to 100 nM AngII resulted in an increase of all the mRNA transcripts. Furthermore, in situ hybridization experiments showed that the levels of hybridization signals for VPF/VEGF mRNA resulted consistently higher in HMC exposed for 3 h to AngII (100 nM) than in control cells. The effects of AngII on the secretion of VPF/VEGF peptide in the culture medium of HMC were assessed using an enzyme-linked immunosorbent assay. When different concentrations of AngII were tested in 3-h stimulation periods, the percentage of increase in the levels of released VPF/VEGF was significantly higher than control cells for AngII concentrations of 100 nM (62 ± 11% mean ± SD, P < 0.0001) and 1 μM (17.3 ± 10.9%, P < 0.01). The pretreatment of HMC with losartan (1 μM) prevented the increase of VPF/VEGF secretion induced by AngII (100 nM) (AngII 54.7 ± 3.9 pg/μg DNA versus AngII + losartan 37.8 ± 3.6 pg/μg DNA, mean ± SD, P < 0.005). VPF/VEGF protein was time dependently released in the culture medium under basal, steady-state conditions. Compared with control cells, AngII (100 nM) caused a significant increase in the levels of released VPF/VEGF after 3 and 6 h (control 33.8 ± 1.7 pg/μg DNA at 3 h, 42.1 ± 1.1 at 6 h, and 117.7 ± 10 at 24 h; AngII 54.7 ± 3.9 at 3 h, P < 0.0001, 61.6 ± 8.7 at 6 h, P < 0.05, and 144.7 ± 22.7 at 24 h, NS; mean ± SD). According to the results obtained from enzyme-linked immunosorbent assay experiments, Western blot analysis showed that the intensity of the 19-kD band corresponding to VPF/VEGF was 1.5-fold higher in AngII (100 nM)-treated HMC than in control cells. Similarly, immunocytochemistry on HMC demonstrated an increase in intracellular VPF/VEGF immunostaining in response to AngII treatment (100 nM) compared with control cells. This study demonstrated that in HMC, AngII augmented the levels of VPF/VEGF gene expression and stimulated the synthesis and secretion of its peptide by activating AT1 receptors. Through these mechanisms, AngII may affect the functions of endothelial cells during the development of renal diseases involving the glomerulus.

Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), is composed of a group of four glycoproteins (in humans VPF/VEGF 206, 189, 165, and 121 amino acids) that originate from alternative splicing of mRNA (reviewed in reference (1)). VPF/VEGF is produced by mesenchymal and epithelial cells and acts selectively on endothelial cells through activation of two specific high-affinity receptors, flt-1 and KDR/flk-1, belonging to the family of tyrosine kinase-type receptors (1–3).

VPF/VEGF is a potent mitogen for endothelial cells (1,4), increases capillary permeability (5,6), and affects the remodeling of extracellular matrix by endothelial cells (1,7,8). Due to these biologic properties, VPF/VEGF has been demonstrated to be an important factor in stimulating angiogenesis during fetal development (9–11), in wound healing (12), and in the growth of different tumors (1,13). The production of VPF/VEGF has been demonstrated also in normal mammalian tissues, including the kidney (14,15), where it might be important
for the maintenance of the differentiated state of blood vessels (1). In normal human kidney, VPF/VEGF mRNA and protein have been demonstrated in glomerular visceral epithelial cells and in collecting ducts (15). However, human and rat mesangial cells in culture have been proved to produce VPF/VEGF, whose synthesis is stimulated by cytokines deeply involved in the development of glomerulosclerosis, such as platelet-derived growth factor (PDGF) and transforming growth factor-β1 (16,17). These findings suggested that, in vivo, mesangial cells activated during glomerulopathies might become an additional source of VPF/VEGF production.

AngII plays an important role in the development of glomerulosclerosis, as supported by numerous studies indicating that pharmacologic blockade of AngII synthesis or AngII AT₁ receptors retards the progression of glomerulosclerosis in both experimental animal models and human renal diseases (18). Indeed, AngII acts on mesangial cells not only causing their contraction, but also stimulating their growth and production of extracellular matrix (19–21). These data, together with the recent findings that AngII stimulates VPF/VEGF production in other cells phenotypically similar to mesangial cells such as vascular smooth muscle cells (22), prompted us to investigate whether AngII regulates the production of VPF/VEGF in human mesangial cells (HMC).

**Materials and Methods**

**Cell Culture Reagents and Antisera**

AngII and recombinant human VPF/VEGF (rhVPF/VEGF) 165 were obtained from Calbiochem (La Jolla, CA). Collagenase type IV was purchased from Worthington Biomedical (Freehold, NJ). Losartan was a generous gift from Merck Sharp and Dohme (Rahway, NJ). PD123177 was a gift from Parke-Davis (Ann Arbor, MI).

Cell culture reagents, including RPMI 1640, Hepes, glutamine, and streptomycin, were obtained from Life Technologies Laboratories (Gaithersburg, MD); nonessential amino acids solution and sodium pyruvate were from HyClone (Oud-Beijerland, Holland); transferrin, insulin, sodium selenite were from Sigma (Saint Louis, MO); fetal calf serum (FCS) was from Biological Industries (Haemek, MO); mouse monoclonal antibodies antihuman macrophages (clone LN-5), myosin (clone hSM-V), pan-cytokeratin (clone C-11), α-smooth muscle actin (clone 1A4), and VPF/VEGF (clone 26503.11) were obtained from Sigma; mouse monoclonal anti-swine vimentin (clone DE-R-11) and rabbit polyclonal antibody for human vimentin were a generous gift from Dako (Glostrup, Denmark); and rabbit polyclonal antibody for VPF/VEGF was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Isolation of Human Glomerular Mesangial Cells**

To establish HMC in culture, macroscopically normal kidney specimens were obtained from patients undergoing nephrectomy for localized kidney tumors. The cortex was separated from the medulla and minced, and glomeruli were isolated by a standard sieving technique. The specimens were then cultured in RPMI 1640 supplemented with 17% FCS, 25 mM Hepes, 2 mM glutamine, nonessential amino acids, 2 mM sodium pyruvate, 5 μg/ml insulin, 5 ng/ml transferrin, 5 ng/ml sodium selenite, 100 U/ml penicillin, and 100 U/ml streptomycin. Glomeruli were maintained in culture in a humidified environment of 5% CO₂/95% air at 37°C, and the medium was changed three times a week. HMC were cultured in normoxia conditions (21% O₂) and were used between passages 4 and 8.

Before the treatment with AngII, cells were kept in resting conditions for 48 h in a basal medium of RPMI 1640. When the effects of losartan (1 μM) or PD123177 (1 μM) were tested, these compounds were added to HMC 1 h before AngII treatment.

**Characterization of Cultured Mesangial Cells**

Cells used in this study were identified as mesangial cells on the basis of morphologic features and immunocytochemical characterization. HMC exhibited typical stellate morphology when subconfluent, while in long-term culture they piled up and formed “hillocks” (23). Immunocytochemistry was performed on 4% paraformaldehyde-fixed cells by the avidin-biotin-peroxidase complex method using a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) as described previously (24). HMC did not show positive immunostaining for von Willebrand factor and pan-cytokeratin, indicating that they are neither endothelial nor epithelial cells; furthermore, they did not express human macrophage marker. These cells positively stained for myosin, α-smooth muscle actin, and vimentin. Fibroblast contamination was excluded by the ability of the cells to grow in D-valine-containing selective media (23).

**Northern Blot Analysis**

Northern blot analysis was performed as described previously (24). Total RNA was isolated from HMC using guanidine isothiocyanate-phenol-chloroform method. Total RNA (20 to 30 μg) was size-fractionated by electrophoresis on 1.2% agarose/formaldehyde gel containing 0.5 μg/ml ethidium bromide, and transferred to GeneScreen Plus nylon membranes (NEN-Du Pont, Paris, France). The membranes were subsequently hybridized for 16 h at 65°C to a reverse transcription-PCR (RT-PCR)-generated 204-bp human VPF/VEGF cDNA probe labeled with [α-32P]dCTP (3000 Ci/mmol, Amersham International, Buckinghamshire, United Kingdom) by the nick translation method using a commercial labeling kit (Boehringer Mannheim, Mannheim, Germany). The uniformity of loading was verified by hybridizing the same nylon membranes to human cyclophilin cDNA probe. The relative amount of VPF/VEGF mRNA was quantified by densitometric analysis.

The 204-bp VPF/VEGF cDNA was generated from HMC total RNA by RT-PCR as described below. The sense primer was 5'-CGAAGTTGAGATTCGTGATG-3', and the antisense primer was 5'-TCTTCTGATACGTACTCTTCCGTATGAC-3' (22). The PCR program consists of 2 min at 95°C followed by 40 cycles (1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and final extension for 7 min at 62°C). The resulting 204-bp cDNA was subcloned into the BamHI and EcoRI sites of pGEM-4Z (Promega, Madison, WI). To confirm the identity of the cloned human fragment, representative PCR products subcloned in pGEM-4Z were sequenced using the CY5 AutoRead sequencing kit (Pharmacia Biotech, Piscataway, NJ), according to the manufacturer’s specifications, followed by electrophoresis on an automated ALFexpress DNA sequencer (Pharmacia). The sequence obtained was identical to the reported human VPF/VEGF sequence (25).

**Reverse Transcription-PCR**

To investigate the effect of AngII on the expression of different VPF/VEGF mRNA splicing variants, RT-PCR was performed using specific oligonucleotides that detect all four VPF/VEGF transcripts.

The sense primer was 5′-CCTGGTGACATCTTCAGGAGTACC-3′, and the antisense probe was 5′-CTCACCCTCTGCTTGTCA-3′ (26). RT-PCR was made using a commercially available kit (GeneAmp RNA PCR, Perkin Elmer, Roche Molecular System, Branchburg, NJ) according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed with an oligo dT16 primer in the presence of MuLV reverse transcriptase. The resulting cDNA was amplified using a 480 Thermal cycler from Perkin Elmer-Cetus programmed with an initial incubation at 95°C for 2 min followed by 30 cycles (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C) and an additional extension step for 7 min at 62°C at the end of the last cycle. Primers for β-actin were used to normalize the results. Antisense primer: 5′-CTTCTGCTTGTGATCCACAT-3′; sense primer: 5′-AACCGCGAAGATGACCCAG-3′ (27).

**In Situ Hybridization**

In situ hybridization was performed as described previously (24). Briefly, HMC grown on glass slides were fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were subsequently treated with 0.2N HCl for 20 min, 0.125 mg/ml Pronase in PBS for 10 min, 0.1 M glycine in PBS for 30 s, and 4% paraformaldehyde for 20 min. Thirty microliters of hybridization solution 40% formamide, 4× SSC, 10 mM dithiothreitol, 1× Denhardt’s solution, 10% dextran sulfate, 0.1 mg/ml sheared herring sperm DNA, and 1 mg/ml yeast tRNA(9) containing 8 × 105 cm of 35S-labeled human VPF/VEGF RNA probes, synthesized as described below, were applied to cells and covered with Parafilm®. Hybridization was carried out at 52°C for 16 h. Removal of the nonspecifically bound probe by RNase digestion, subsequent washing steps, and autoradiography were described as performed elsewhere (24). Cells were subsequently counterstained with hematoxylin-eosin-phloxine and mounted with Permount. Negative controls consisted of hybridization to a sense RNA probe, synthesized as described below. A minimum of two slides was analyzed for each experimental point, and two independent sets of experiments were performed.

Human VPF/VEGF RNA sense and antisense probes were synthesized from the RT-PCR-generated 204-bp VPF/VEGF cDNA that had been subcloned in pGEM-4Z plasmid vector (Promega). The cDNA was subsequently linearized with BamHI or EcoRI restriction enzymes followed by phenol-chloroform extraction and ethanol precipitation. Thereafter, sense and antisense RNA radiolabeled probes were synthesized using SP6 or T7 RNA polymerases as appropriate (Riboprobe Gemini System, Promega), in the presence of 35S-α-thio-UTP (1300 mCi/mmole, NEN DuPont). RNA probes were extracted using phenol-chloroform, ethanol-precipitated, and subsequently subjected to alkaline digestion to obtain probes of 150 nt.

**Enzyme-Linked Immunosorbent Assay**

The amount of VPF/VEGF protein released by HMC in culture medium was measured using a commercially available immunoenzymatic assay (R&D Systems, Abingdon, United Kingdom). This solid-phase enzyme-linked immunosorbent assay (ELISA) method was designed to measure the levels of soluble VPF/VEGF 165 and 121. For cell culture media, a sensitivity of 5 pg/ml could be achieved. The intra-assay coefficient of variation was 4.6%; the interassay coefficient of variation was 6.7%. HMC were seeded in 6-well dishes at a density of 1 × 10⁶ cells/well and allowed to grow to near confluence in complete medium, followed by 48 h of quiescence in serum-deprived culture medium. At the end of the treatment periods, conditioned media were collected, centrifuged at 2000 × g for 10 min, and stored at −20°C in the presence of 1% FCS. The levels of VPF/VEGF released were normalized to the DNA content of each well. DNA was measured according to the Burton method (28).

**Immunocytochemistry**

Immunocytochemistry for VPF/VEGF was performed as described previously (24) on 4% paraformaldehyde-fixed cells by the avidin-biotin-peroxidase complex method using a polyclonal anti-VPF/VEGF antibody (diluted 1:400), which recognized three VPF/VEGF isoforms (121, 165, and 189 amino acids). All of the steps were performed at room temperature, and 3-amino-9-ethyl carbazole was used as peroxidase substrate. As negative control, the primary antibody was replaced by rabbit IgG. The effects of AngII on VPF/VEGF immunostaining was assessed in three separate experiments.

**Western Blotting**

Cells from three experiments were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM phenylmethlysulfonylfluoride, and 0.25% NP-40). Fifty micrograms of protein were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide under denaturing conditions and electrotransferred to nitrocellulose membranes (Sigma). Nonspecific protein binding was blocked by incubating the membranes with blocking solution (1× PBS, 0.1% Tween 20, and 5% nonfat dried milk) overnight at 4°C. Monoclonal antibody specific for VPF/VEGF (1:250 in PBS containing 2.5% bovine serum albumin) was applied to the membrane for 60 min at room temperature. After rinsing with washing buffer (1× PBS, 0.15% Tween 20), peroxidase-conjugated anti-mouse IgG antibody diluted 1:4000 (Sigma) was applied to the membrane for 60 min at room temperature. The detection of specific signals was performed using the ECL detection system (Amersham) according to the instructions of the vendor. Proteins were measured by the method of Bradford (Bio-Rad).

**Statistical Analyses**

All values were expressed as mean ± SD. When appropriate, statistical analysis was performed using t test for unpaired data considered significant at the level of P < 0.05.

**Results**

**Effects of AngII on VPF/VEGF Gene Expression**

**Northern Blot Analysis.** Northern blot analysis, performed with a cDNA probe specific for and complementary to all known alternative splicing products of VPF/VEGF mRNA, showed that quiescent HMC expressed an apparent single transcript of approximately 3.7 kb (Figure 1A). When solvent or different concentrations of AngII (from 0.1 nM to 1 μM) were added to HMC for a period of 3 h, an increase in VPF/VEGF mRNA compared with control cells was present for an AngII concentration of 1 nM, with the maximal effect observed for an AngII concentration of 100 nM. For AngII concentration of 1 μM, VPF/VEGF mRNA levels were still higher than controls but lower than 100 nM AngII (Figure 1, A and B). Densitometric analysis of VPF/VEGF hybridization signals from repeated experiments, normalized to those for cyclophilin, demonstrated that VPF/VEGF mRNA levels were, respectively, 1.5-, 2.3-, and 1.6-fold higher in HMC treated with 10 nM AngII (P < 0.05), 100 nM AngII (P < 0.0001), and 1 μM AngII (P < 0.05) than in control untreated cells (Figure 1B). The stimulatory effect of AngII on VPF/VEGF mRNA levels was not longer detected after 24 h of treatment.
To clarify the AngII receptor subtypes involved in AngII-induced upregulation of VPF/VEGF gene expression in HMC, cells were preincubated with losartan, a selective antagonist of AngII AT1 receptor. Supplementation of culture medium with losartan (1 μM) alone had no effect on the basal expression of VPF/VEGF mRNA levels in HMC (Figure 2, A and B). On the contrary, the increase in VPF/VEGF mRNA levels induced by AngII (100 nM) in HMC was abolished by pretreatment with 1 μM losartan (AngII versus AngII plus losartan, P < 0.005) (Figure 2, A and B). PD123177 (1 μM), an AT2 receptor antagonist, did not affect per se the levels of VPF/VEGF mRNA in HMC and did not modify the effect of AngII on VPF/VEGF (data not shown).

Reverse Transcription-PCR. RT-PCR performed in HMC using oligonucleotide primers specific for all VPF/VEGF mRNA splicing variants detected three bands of 479, 407, and 275 bp corresponding to VEGF 189, 165, and 121, respectively (Figure 3).

Exposure of HMC to 100 nM AngII for 3 h resulted in an increase of the level of all the mRNA transcripts. The intensity of each VPF/VEGF transcript was quantified by densitometric analysis and normalized to the signals of β-actin. VPF/VEGF 189, 165, and 121 mRNA were, respectively, 1.6-, 2-, and 3.5-fold higher in AngII-treated HMC than in control cells (n = 2).

In Situ Hybridization. Since AngII exhibited growth-promoting properties on HMC (19–21), the effects of this
peptide on VPF/VEGF mRNA were further evaluated at the level of single cells using in situ hybridization. VPF/VEGF mRNA appeared as a small cluster of silver grains localized in each HMC after 3 h of exposure to solvent (Figure 4, A and B). However, after 3 h of exposure to AngII (100 nM), the number of silver grains present in each single HMC increased compared with controls in all of the slides analyzed (Figure 4, C and D). A more intense effect was observed after 3 h of exposure to 10% FCS (Figure 4E). Sections hybridized to sense RNA probe did not show specific hybridization signals (Figure 4 F).

**Effect of AngII on VPF/VEGF Protein Synthesis and Release**

**ELISA Assay.** To confirm that the upregulation of the levels of VPF/VEGF mRNA induced by AngII resulted in an increase in the synthesis of its protein, the amount of VPF/VEGF released by HMC was measured using an ELISA technique. When different concentrations of AngII were tested in 3-h stimulation periods, the percentage of increase in the levels of released VPF/VEGF was significantly higher than control for AngII concentrations of 100 nM (62 ± 11%, P < 0.0001) and 1 μM (17.3 ± 10.9%, P < 0.01) (Figure 5). Figure 6 shows the time course of VPF/VEGF protein release in culture medium by untreated or AngII-treated HMC. VPF/VEGF protein was time dependently released in culture medium under basal, steady-state conditions (Figure 6). Compared with control cells, AngII (100 nM) caused a significant increase in the levels of released VPF/VEGF after 3 and 6 h (control 33.8 ± 1.7 pg/μg DNA at 3 h, 42.1 ± 1.1 pg/μg DNA at 6 h, and 117.7 ± 10 pg/μg DNA at 24 h; AngII 54.7 ± 3.9 pg/μg DNA at 3 h, P < 0.0001, 61.6 ± 8.7 pg/μg DNA at 6 h, P < 0.05, and 144.7 ± 22.7 pg/μg DNA at 24 h, NS).

Similar to the results obtained for VPF/VEGF gene expression, the pretreatment of HMC with losartan (1 μM) prevented the increase of VPF/VEGF secretion induced by AngII (100 nM).
nM) after 3 h (AngII 54.7 ± 3.9 pg/μg DNA versus AngII + losartan 37.8 ± 3.6 pg/μg DNA, P < 0.005) (Figure 7). Losartan alone did not modify VPF/VEGF levels. The exposure to 5% FCS caused an increase in the release of VPF/VEGF (Figure 7).

Western Blot Analysis. VPF/VEGF protein in HMC lysate was detected as a single band of approximately 19 kD (Figure 8). This band disappeared when the antibody was preincubated overnight at 4°C with 1 μg/ml antigen peptide (data not shown). After 3 h stimulation of HMC with 100 nM AngII, VPF/VEGF protein concentration increased (Figure 8). Densitometric analysis of the specific bands showed that the intensity of VPF/VEGF after 100 nM AngII treatment was 1.5-fold higher compared with controls (n = 3). As positive control, 50 ng of rhVPF/VEGF 165 was processed together with the cell samples.

Immunocytochemistry. To confirm VPF/VEGF protein production by HMC, a specific polyclonal anti-VPF/VEGF antibody was used in immunocytochemical staining. In untreated HMC, a faint immunostaining for VPF/VEGF was observed (Figure 9A), whereas after 3 h treatment with AngII (100 nM), the signal increased in all three experiments performed (Figure 9B). A more intense signal was observed after 3 h stimulation with 10% FCS (Figure 9C). Absence of reactivity by the same cells was obtained when rabbit IgG replaced the primary antibody (negative control) (Figure 9D).

Discussion

In the study presented here, we demonstrated that AngII stimulates the synthesis and secretion of VPF/VEGF in HMC through the activation of the AT₁ receptor. The finding that cultured HMC constitutively synthesize VPF/VEGF is in agreement with previous results obtained in human and rat cultured mesangial cells, in which both VPF/VEGF mRNA and protein immunostaining were detected (16,17). In addition to these findings, the results reported herein demonstrated that quiescent HMC are able to secrete VPF/VEGF in the culture medium.

The glomerular production of VPF/VEGF in the normal mammalian kidney appeared to be limited to visceral epithelial cells, as documented by in situ hybridization and immunohistochemistry (14,15). However, it is generally accepted that cultured mesangial cells are more similar to the activated mesangial cells than to the quiescent cells observed in the normal glomerulus (23). Therefore, it seems reasonable to hypothesize that in the settings of in vivo pathologic processes affecting the glomerulus, mesangial cells may represent an other source of VPF/VEGF production. The synthesis of VPF/VEGF by mesangial cells may be relevant from a pathophysiologic point of view because of the absence of a continuous glomerular basement membrane at the endothelial–mesangial interface (29).

Northern blot analysis, RT-PCR, and in situ hybridization consistently demonstrate that AngII induces an increase in the levels of VPF/VEGF mRNA in HMC. Densitometric analysis of Northern blot experiments have shown that the effect of AngII on VPF/VEGF mRNA was maximal at the concentration of 100 nM. In close agreement with these results, we also observe that 100 nM AngII maximally stimulated the secretion of VPF/VEGF peptide from these cells. The concentration of AngII required for the maximal effects on VPF/VEGF mRNA and protein secretion are consistent with those used to elicit biologic activity in cultured smooth muscle and mesangial cells (19,22). The action of AngII on VPF/VEGF secretion from HMC paralleled the increase in VPF/VEGF content demonstrated in these cells by two complementary techniques such as immunocytochemistry and Western blotting.

VPF/VEGF is composed of four isopeptides, originating from alternative RNA splicing, which possess different bioavailability due to differences in the isoelectric point and affinity for heparin (1). In agreement with previous studies, RT-PCR demonstrated that HMC express mRNA splicing variants corresponding to 189, 165, and 121 amino acid isoforms (17). All of the three VPF/VEGF mRNA appeared to be upregulated by AngII. Thus, these findings seem to indicate that AngII acts on freely soluble VPF/VEGF isoform (VPF/VEGF 121) as well as on those partially (VPF/VEGF 165) or almost completely (VPF/VEGF 189) sequestered in the extracellular matrix (1).

The effects exerted by AngII on VPF/VEGF mRNA and secretion in HMC were abolished by losartan, demonstrating the involvement of AT₁ receptor. The AngII AT₁ receptor mediates all of the known actions of AngII, and only the AT₁
receptor was demonstrated in HMC in vitro (30). In the present study, we did not investigate the mechanism through which the AngII-induced activation of AT₁ receptors leads to the increase in VPF/VEGF gene expression. This effect could be due to a stimulation of the transcription rate of VPF/VEGF mRNA and/or to an increase in its stability. In this regard, it is interesting that the sequence within the 3' untranslated region of rat VPF/VEGF mRNA contains a number of motifs involved in the regulation of mRNA stability (31) and that the increase in the gene expression of VPF/VEGF induced by hypoxia in

Figure 4. In situ hybridization showing the effects of AngII on VPF/VEGF mRNA levels in HMC. HMC, grown on sterile glass microscope slides, were exposed to AngII (100 nM) for 3 h, after 48 h of quiescence in serum-free medium. Dark-field (white grains; magnification, ×100) and bright-field (black grains; magnification, ×250) photomicrographs show that the levels of hybridization signals corresponding to VPF/VEGF mRNA are higher in AngII-treated HMC (C and D) compared with control cells (A and B). Note the marked increase in VPF/VEGF mRNA induced by 10% fetal calf serum (FCS) (E). Hybridization with sense probe does not show specific hybridization signals (F).
human epithelial cells seems to be mediated by both an increase in mRNA transcription and mRNA stability (32).

The findings that AngII stimulates the gene expression and secretion of VPF/VEGF in cultured HMC suggest that this peptide might be important in inducing the synthesis and secretion of VPF/VEGF in HMC during renal diseases affecting the glomerulus. Indeed, it has been extensively demonstrated that AngII plays a pathophysiologic role in the progression of glomerular lesions in experimental animal models and human chronic renal diseases (18). In addition, it has also been reported that PDGF and transforming growth factor-β1 increase the synthesis of VPF/VEGF in rat and HMC (16,17). Like AngII, these cytokines are important regulators of mesangial cell functions in the developing of glomerulopathies (33). The pathophysiologic relevance of AngII regulation of VPF/VEGF in HMC is further supported by recent studies demonstrating that AngII upregulated the gene expression of VPF/VEGF in cultured smooth muscle cells through activation of AT1 receptor (22). These cells share several phenotypic features with mesangial cells and represent a critical element in the development of vascular injury (34). In apparent contrast to the

**Figure 5.** Effects of AngII on the release of VPF/VEGF protein from HMC. Cells were exposed to different concentrations of AngII for 3 h, after 48 h of quiescence in serum-free medium. VPF/VEGF was measured in culture medium using an enzyme-linked immunosorbent assay (ELISA) method, which detects the 165 and 121 amino acid isoforms of the peptide. Data are expressed as percentage of control ± SD. *P < 0.0001 versus control; **P < 0.01 versus control.

**Figure 6.** Time course of the VPF/VEGF protein release from untreated (■) or AngII-treated (□) HMC. Cells were exposed to AngII (100 nM) after 48 h of quiescence in serum-free medium. VPF/VEGF was measured in culture medium using an ELISA method, which detects the 165 and 121 amino acid isoforms of the peptide. Data are expressed as mean ± SD. *P < 0.0001; **P < 0.05.

**Figure 7.** Effects of losartan, a specific antagonist of AngII AT1 receptor, on AngII-stimulated release of VPF/VEGF from HMC. Cells were exposed for 1 h to losartan (1 μM) before AngII treatment. VPF/VEGF was assayed in culture medium using an ELISA method, which detects the 165 and 121 amino acid isoforms of the peptide. Data are expressed as mean ± SD. *P < 0.0001 versus control; **P < 0.005 versus AngII; ***P < 0.005 versus control.

**Figure 8.** Representative Western blot analysis showing the effect of 100 nM AngII on the cell content of VPF/VEGF in HMC. Fifty micrograms of total proteins were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions and electrotransferred to nitrocellulose membranes. VPF/VEGF protein was revealed by a specific monoclonal antibody (1:250) and ECL detection. Fifty nanograms of recombinant human VPF/VEGF 165 were used as positive control.
aforementioned results, in vivo studies performed in biopsies from patients with a variety of glomerular diseases suggest that the production of VPF/VEGF in sclerotic glomeruli is limited to viable podocytes, while mesangial cells did not appear to express VPF/VEGF (36,37). However, in the experimental glomerulonephritis induced by the injection of anti-Thy 1 antibody, VPF/VEGF mRNA was detected not only in epithelial cells, but also in cells localized in the mesangial regions, suggesting that they are mesangial cells (38). The same authors also observed that the upregulation of the gene expression of VPF/VEGF in glomerular cells is limited to the early stages of glomerular damage, suggesting that mesangial production of VPF/VEGF may be transient and therefore not easily detectable.

The results reported herein that AngII stimulates the production of VPF/VEGF in HMC raise intriguing questions regarding the role of AngII in the pathogenesis of glomerular injury. VPF/VEGF is a potent mitogen for endothelial cells (1,4), and it has been demonstrated to be involved not only in the processes of angiogenesis (9–13), but also in promoting reendothelialization in animal models of arterial injury (39). Thus, the AngII-induced VPF/VEGF production in HMC could represent a compensatory mechanism directed to the replacement of damaged glomerular endothelium during pathologic processes. In keeping with this hypothesis, it has been reported recently that AngII stimulated a mild but significant proliferation of cultured rat glomerular endothelial cells through activation of AT1 receptor (40). VPF/VEGF is capable also of inducing an increase of vascular permeability (5,6). Although the glomerular proteinuria and macromolecular flux induced by AngII in animals seem to be due mainly to its hemodynamic effects (21), it is tempting to speculate that the modifications of the filtration properties of the glomerular barrier induced by AngII in some proteinuric renal diseases such as diabetic nephropathy (41) may be mediated by the stimulation of VPF/VEGF production.

In addition to the above-mentioned paracrine actions, VPF/VEGF produced by mesangial cells in response to AngII stimulation might also act in an autocrine manner. In fact, the gene expression of VPF/VEGF receptor flt-1 has been demonstrated

Figure 9. Immunocytochemistry showing the effect of AngII on the VPF/VEGF protein production in HMC. HMC, grown on sterile glass microscope slides, were exposed to AngII (100 nM) for 3 h, after 48 h of quiescence in serum-free medium. Photomicrographs show that VPF/VEGF immunostaining (red color) is higher in AngII-treated HMC (B) compared with control cells (A). A marked increase in VPF/VEGF protein was induced by 10% fetal calf serum (C). No immunoreactivity was observed when rabbit IgG replaced the primary antibody (negative control) (D). Magnification, ×250.
in cultured rat mesangial cells, where it is selectively increased by PDGF (16), a well known stimulator of mesangial cell proliferation (42), raising the possibility that VPF/VEGF produced by mesangial cells may exert autocrine actions on these cells.

In summary, we demonstrated that in HMC AngII augmented the levels of VPF/VEGF gene expression and stimulated the synthesis and secretion of its peptide through the activation of AT1 receptor. These findings represent a mechanism whereby AngII may affect the functions of endothelial cells during the development of renal diseases affecting the glomerulus.

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References