

Vasopressin regulates rat mesangial cell growth by inducing autocrine secretion of vascular endothelial growth factor

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Abstract Mesangial cell growth is a key feature of several glomerular diseases. Vascular endothelial growth factor (VEGF) is a potent mitogen of vascular endothelial cells and promoter of vascular permeability. Here, we examined the ability of vasopressin (AVP), which causes mesangial cell proliferation and hypertrophy, to stimulate VEGF secretion from cultured rat mesangial cells. AVP potently induced a time- and concentration-dependent increase in VEGF secretion in these cells, which was then inhibited by a V_{1A} receptor-selective antagonist, confirming this is a V_{1A} receptor-mediated event. VEGF also induced hyperplasia and hypertrophy in mesangial cells, which was completely abolished by an anti-VEGF antibody. In addition, AVP-induced hyperplasia and hypertrophy were completely inhibited by the V_{1A} receptor-selective antagonist and partially abolished by the anti-VEGF antibody. These results indicate that AVP increases VEGF secretion in rat mesangial cells via V_{1A} receptors and modulates mesangial cell growth not only by direct action but also through stimulation of VEGF secretion. This autocrine mechanism might contribute to glomerulosclerosis in renal diseases such as diabetic nephropathy.

Keywords Vasopressin · Vascular endothelial growth factor · V_{1A} receptors · Mesangial cells

Introduction

Vascular endothelial growth factor (VEGF) is a 35–45 kDa heparin-binding homodimeric glycoprotein that functions as an endothelial cell mitogen and stimulator of angiogenesis [1]. Alternative exon splicing of a single VEGF gene results in at least six different isoforms containing 121, 145, 165, 183, 189, and 206 amino acids (VEGF_{121–206}) in humans, with rodent forms one amino acid shorter each [2]. VEGF_{165/164} (human/rodent homologue) is the predominant isoform secreted by a variety of normal and transformed cells [3]. Furthermore, at some 50,000 times more potent than histamine, VEGF is one of the most potent vascular permeability-enhancing factors identified to date [4]. This potent action of VEGF makes it an attractive candidate as a mediator of normal and pathological changes in vascular permeability. VEGF is produced by mesenchymal and epithelial cells and acts selectively on endothelial cells through activation of two high-affinity transmembrane tyrosine kinase receptors, previously described as fms-like tyrosine kinase (Flt-1) and fetal liver kinase 1 (Flk-1/KDR) and now designated as VEGFR-1 and VEGFR-2, respectively [5–7]. However, the specific function of each receptor subtype remains poorly defined, and the signal transduction pathways for ligand-receptor interaction are largely unknown.

Production of VEGF has also been demonstrated in mammalian tissues, including the kidney [8, 9], where it is hypothesized to be important in maintaining blood vessel differentiation [10]. In the human kidney, VEGF mRNA and protein have been identified in glomerular visceral epithelial cells and collecting ducts [9]. Further, human and rat mesangial cells in culture have been proven to produce VEGF, synthesis of which is stimulated by cytokines involved in the development of glomerulosclerosis, such as

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platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β) [11, 12]. VEGF has also been reported to induce proteinuria in rats [13] and has been implicated in the pathogenesis of minimal change disease [14]. These previous findings suggest that mesangial cells activated during renal diseases may constitute an additional source of VEGF production.

Vasopressin (AVP) plays an important role in the development of glomerulosclerosis, as supported by several studies indicating that pharmacologic blockade of V_{1A} receptors retards the progression of nephropathy and glomerulosclerosis in experimental models and human patients with renal disease [15–17]. Further, AVP acts on V_{1A} receptors on mesangial cells, not only causing their contraction but also stimulating their growth and production of the extracellular matrix [18–20]. These previous data, together with recent findings that AVP stimulates VEGF production in vascular smooth muscle cells phenotypically similar to mesangial cells [21], prompted our investigation into whether or not AVP regulates the production of VEGF in rat mesangial cells. To assess VEGF's potential role in mesangial cell pathophysiology, we investigated the ability of AVP to stimulate secretion of VEGF from rat mesangial cells.

Materials and methods

Materials

AVP and angiotensin II were obtained from Peptide Institute (Osaka, Japan). Rat VEGF₁₆₄ and anti-rat VEGF₁₆₄-neutralizing antibody were obtained from R&D Systems (Minneapolis, MN, USA). The V_{1A} receptor-selective antagonist YM218 [22] and the V_2 receptor-selective antagonist SR121463A [23] were synthesized at Astellas Pharma (Ibaraki, Japan). These nonpeptide antagonists were initially dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and diluted to the desired concentration using assay buffer. Fetal calf serum (FCS) and trypsin-EDTA were obtained from Gibco Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA) was obtained from Nacalai Tesque (Kyoto, Japan).

Cell culture

Mesangial cells were isolated from glomeruli of kidneys of 7- to 10-week-old-male Wistar rats as previously described [24]. Briefly, kidneys were excised from rats, and cortical tissues were minced and passed through series of stainless steel sieves of decreasing pore size (212, 104, and 75 μ m). The sedimentation step was repeated three times using Hank's balanced salt solution. The glomeruli were then

treated with 5 mg/ml of collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) for 10 min and plated into flasks maintained in RPMI-1640 supplemented with 20% FCS and insulin-transferrin-selenium-G supplement (ITS Premix) (Gibco Invitrogen), penicillin 100 U/ml, and streptomycin 100 μ g/ml at 37°C in 5% CO₂ and 95% atmospheric air. By 2–3 weeks after plating, only mesangial cells with strap-like outgrowth in interwoven bundles remained and grew to confluence. Experiments were performed using cells from passages 5 through 10, and subculture was performed via trypsin-EDTA treatment.

VEGF secretion by mesangial cells

Mesangial cells were seeded into 12-well culture plates at 80% confluence, washed with phosphate-buffered saline (PBS), and incubated for 24 h in culture medium containing 0.5% FCS. The cultures were then incubated in conditioned medium containing 0.5% FCS, ITS Premix, and 0.1% BSA with vehicle alone or various concentrations of AVP, antagonists/antibody, or both for 24 h. After incubation, the medium was collected and stored at -40°C until assay. To determine total protein content of cells per culture well, the cell layers were washed with PBS and scraped from the plates. The suspensions were homogenized, and protein was determined using the Coomassie blue method (Bio-Rad Laboratories, Hercules, CA, USA), with BSA as a standard. VEGF concentration in the medium was determined via a quantitative enzyme-linked immunosorbent assay (ELISA) system using anti-mouse VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results were normalized per cellular protein content.

Hyperplasia and hypertrophy of mesangial cells

Mesangial cells were seeded into 48-well culture plates at 50% confluence, washed with PBS, and incubated for 48 h in RPMI-1640 medium supplemented with 0.5% FCS. The cultures were then incubated for a further 72 h in conditioned medium containing 0.5% FCS, ITS Premix, and 0.1% BSA with vehicle alone or VEGF/AVP, antibody/antagonists, or both. To determine the number of cells per culture well, the cells were treated with AlamarBlue (Asahi Techno Glass, Tokyo, Japan) [25] during the final 3 h of incubation, and the absorbance of each well at 570 and 600 nm was measured using a SPECTRAMax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Control experiments showed a linear relationship between absorbance and cell number up to a cell density of 100,000 cells/well. To determine the total protein content of cells per culture well, the cell layers were washed three times with PBS and scraped from the plate.

The suspensions were then homogenized, and protein content was determined as described above.

Type IV collagen production by mesangial cells

Mesangial cells were seeded into 24-well culture plates at 50% confluence, washed with PBS, and incubated for 48 h in culture medium supplemented with 0.5% FCS. The cultures were then incubated in conditioned medium containing 0.5% FCS, ITS Premix, 0.1% BSA, 0.4 mM β -aminopropionitrile, and 0.3 mM ascorbic acid with vehicle alone or VEGF/AVP, antibody/antagonists, or both for 72 h. Medium was stored at -40°C until assay. To solubilize cell-associated matrix proteins, cells were washed with PBS, incubated with 0.5 N NaOH containing 10 mM phenylmethylsulfonyl fluoride, sonicated, and stored at -40°C . Total protein content of cells was determined as described above. Type IV collagen concentrations in the samples were determined via a highly sensitive ELISA using anti-mouse type IV collagen antibody (LSL, Tokyo, Japan), which cross-reacts with rat type IV collagen. Results were normalized per cellular protein contents.

Statistical analysis

Data are expressed as mean $\pm$ SE or mean with 95% confidence limits. Significant differences between pairs of groups were determined using Student's *t* test, while those between multiple groups were determined using Dunnett's multiple range test. Significance was set at $P < 0.05$. All analyses were conducted using the SAS 8.2 software package (SAS Institute Japan, Tokyo, Japan) and Prism program (GraphPad Software, San Diego, CA, USA).

Results

Effect of AVP on VEGF secretion

Time-dependent secretion of VEGF by rat mesangial cells stimulated by AVP is shown in Fig. 1. When mesangial cell monolayers were incubated with conditioned medium, VEGF secretion by mesangial cells increased in a time-dependent manner. Incubation with AVP (100 nM) significantly enhanced VEGF secretion in a time-dependent manner compared to conditioned medium-treated cells. While this increase in VEGF secretion was not observed up to 3 h after stimulation by AVP, it was clearly detected from 6 h on. Further, AVP-induced VEGF secretion increased almost linearly over the first 24 h and then gradually for up to 72 h. Angiotensin II (100 nM) and FCS (20%) also induced VEGF secretion from mesangial cells with similar time course changes (data not shown). In total,

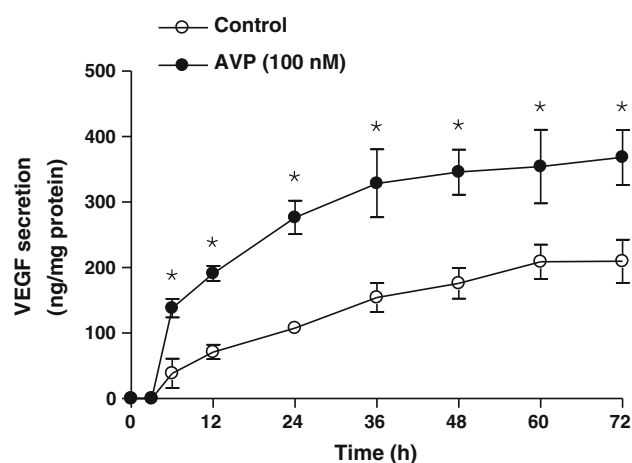


Fig. 1 Time-dependent secretion of VEGF from rat mesangial cells stimulated with AVP. Cells were incubated with AVP (100 nM) for the indicated duration. Values are expressed as the mean $\pm$ SE of four independent experiments. * $P < 0.05$ versus control

VEGF secretion was stimulated by AVP, angiotensin II, and FCS at respective rates of 260, 340, and 360% over that in conditioned medium-treated cells at 24 h.

Concentration-dependent increases in VEGF secretion from mesangial cells stimulated with various concentrations of AVP for 24 h are shown in Fig. 2. The minimum stimulatory effect of AVP on VEGF secretion from mesangial cells was observed at a concentration of 3 nM, with an EC_{50} value of 5.50 (2.98–10.2) nM and maximum stimulation 320% that of conditioned medium-treated cells (control: 8.1 ± 1.5 ng/ml; AVP 100 nM: 27.1 ± 1.3 ng/ml). In addition, AVP significantly and concentration-dependently increased total protein content of cells, suggesting stimulation of mesangial cell growth (data not shown).

We also examined the effects of AVP receptor antagonists using preparations stimulated with 100 nM AVP (Fig. 3). The $\text{V}_{1\text{A}}$ receptor-selective antagonist YM218 potently and concentration-dependently inhibited AVP-stimulated VEGF secretion with an IC_{50} value of 1.75 (0.83–3.69) nM. This antagonist also potently inhibited AVP-induced mesangial cell growth (data not shown). In contrast, the V_2 receptor-selective antagonist SR121463A did not potently inhibit AVP-stimulated VEGF secretion, showing an IC_{50} value of 7,770 (4,700–12,800) nM.

Effect of VEGF on proliferation, hypertrophy, and type IV collagen synthesis

Addition of VEGF caused a concentration-dependent increase in cell number and protein content of mesangial cells (Fig. 4), effects that were significant at concentrations of 100 ng/ml or higher; maximum stimulations over vehicle-treated control were 1.4- and 1.7-fold, respectively.

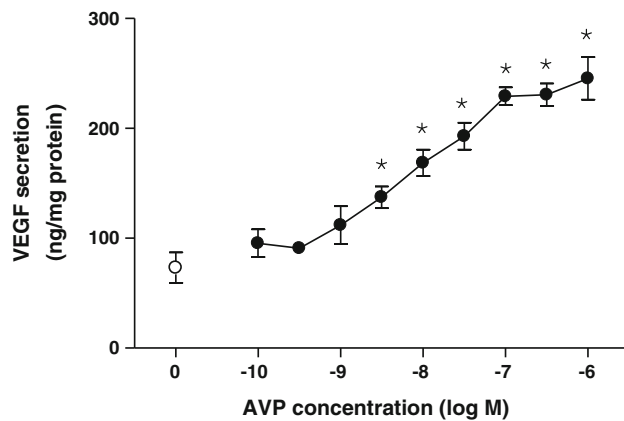


Fig. 2 Concentration-dependent secretion of VEGF from rat mesangial cells stimulated with AVP. Cells were incubated with various concentrations of AVP for 24 h. Values are expressed as the mean $\pm$ SE of four independent experiments. * $P < 0.05$ versus control

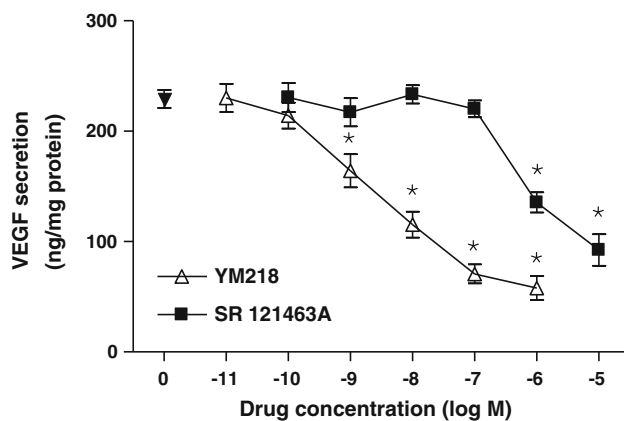


Fig. 3 Inhibitory effects of AVP receptor antagonists on AVP-stimulated VEGF secretion in rat mesangial cells. Cells were incubated with AVP (100 nM) and antagonists for 24 h. Each point represents the mean $\pm$ SE of four independent experiments. * $P < 0.05$ versus AVP

In addition, VEGF caused a concentration-dependent increase in both secreted and cell-associated type IV collagen production by rat mesangial cells. This effect was also significant at concentrations of 100 ng/ml or higher, and the maximum stimulation over vehicle-treated control was 1.8-fold.

Involvement of VEGF in AVP-induced mesangial cell growth

VEGF-induced hyperplasia, hypertrophy, and type IV collagen production were completely abolished on addition of an anti-VEGF antibody but were not affected by AVP receptor antagonists (Fig. 5). In contrast, AVP-induced cell growth effects were completely abolished on addition of

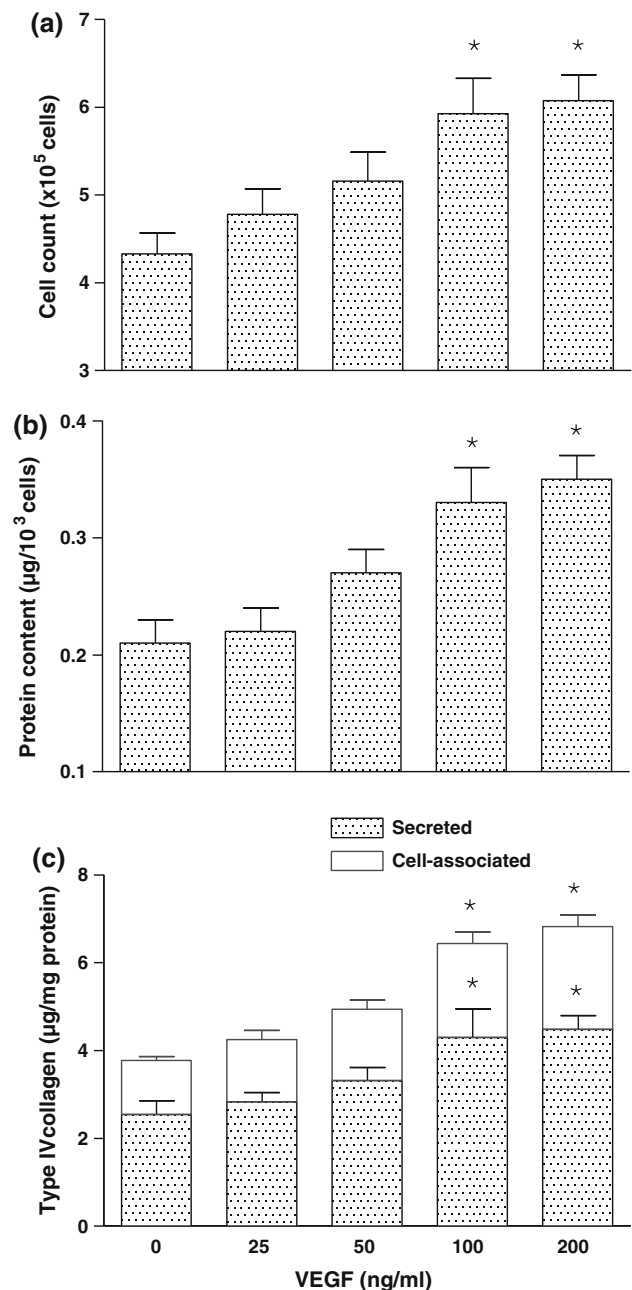


Fig. 4 Concentration-dependent **a** hyperplasia, **b** hypertrophy, and **c** type IV collagen production in rat mesangial cells stimulated with VEGF. Cells were incubated with various concentrations of VEGF for 72 h. Values are expressed as the mean $\pm$ SE of four independent experiments. * $P < 0.05$ versus control

the V_{1A} receptor-selective antagonist YM218 and partly but significantly inhibited on addition of an anti-VEGF antibody (Fig. 6). Addition of AVP receptor antagonists or anti-VEGF antibody to mesangial cell cultures not treated with VEGF or AVP did not significantly affect mesangial cell growth (data not shown).

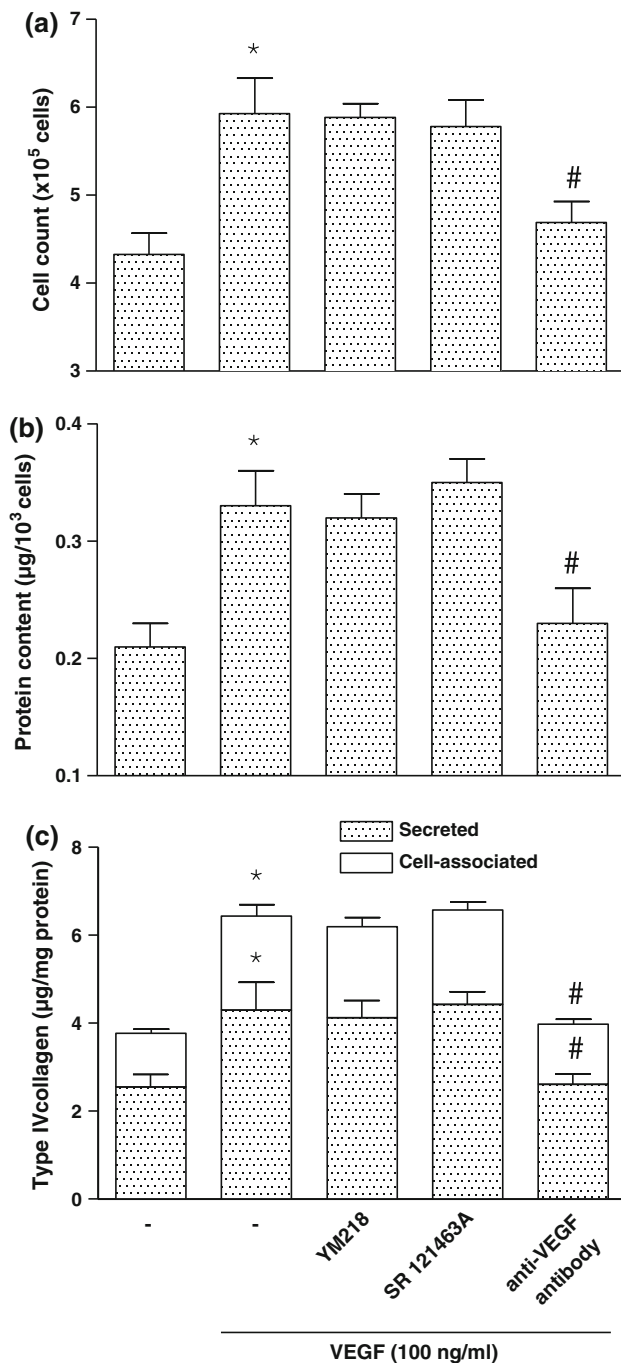


Fig. 5 Effect of AVP receptor antagonists and anti-VEGF antibody on VEGF-induced **a** hyperplasia, **b** hypertrophy, and **c** type IV collagen production in rat mesangial cells. Cells were incubated with VEGF (100 ng/ml) and either YM218 (1 μM), SR 121463A (1 μM), or anti-VEGF antibody (10 μg/ml) for 72 h. Values are expressed as the mean ± SE of four independent experiments. **P* < 0.05 versus control, #*P* < 0.05 versus VEGF

Discussion

Mesangial cells are thought to regulate both glomerular microcirculation and inflammatory responses to glomerular

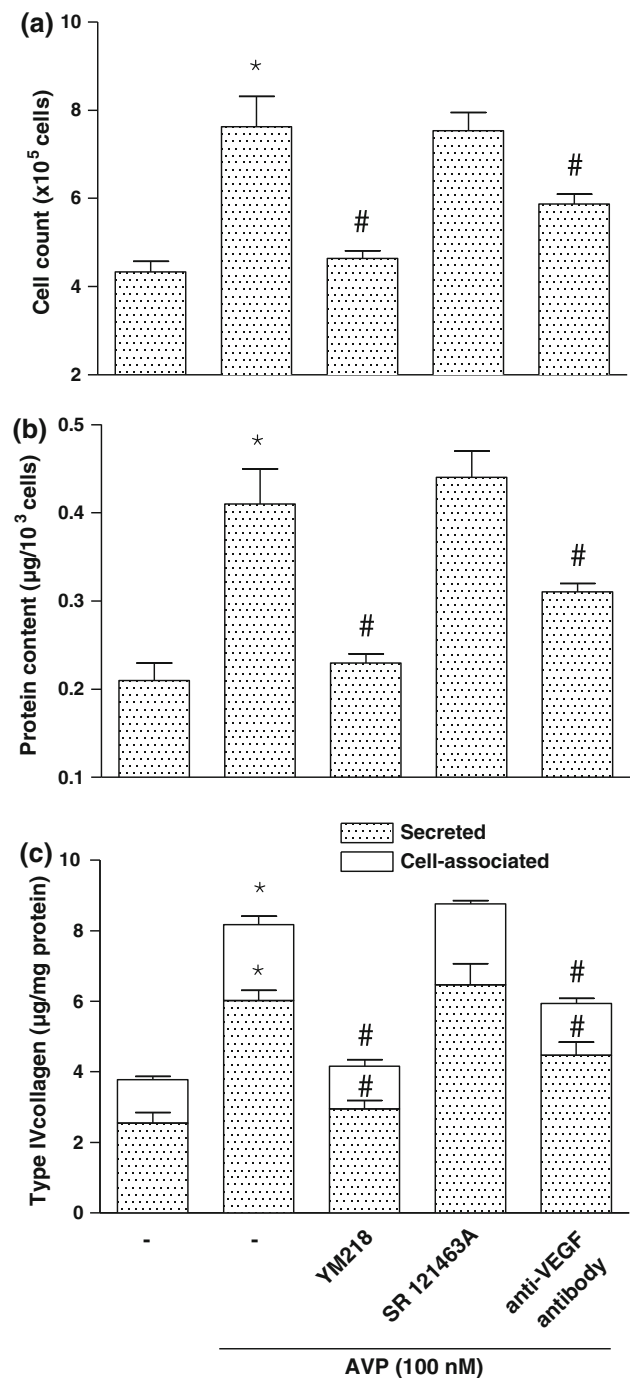


Fig. 6 Effect of AVP receptor antagonists and anti-VEGF antibody on AVP-induced **a** hyperplasia, **b** hypertrophy, and **c** type IV collagen production in rat mesangial cells. Cells were incubated with AVP (100 nM) and either YM218 (1 μM), SR 121463A (1 μM), or anti-VEGF antibody (10 μg/ml) for 72 h. Values are expressed as the mean ± SE of four independent experiments. **P* < 0.05 versus control, #*P* < 0.05 versus AVP

damage. Additionally, contraction, hyperplasia, and hypertrophy of glomerular mesangial cells are associated with the appearance of proteinuria, glomerulosclerosis, and progressive renal failure [26]. A number of cytokines,

growth factors, and vasoactive agents have been found to induce physiologic and pathophysiologic responses, including mitogenesis, in mesangial cells [27]. Among these factors, AVP potently induces hyperplasia and hypertrophy in, and production of extracellular matrix by, mesangial cells that express only the AVP V_{1A} receptor subtypes, which may consequently contribute to the development of glomerular injury [19, 20, 28]. Indeed, administration of V_{1A} receptor antagonists has been found to inhibit AVP-induced mesangial cell growth and attenuate glomerular sclerosis and improve renal function in animal models of chronic renal failure [15, 19, 29]. These observations further suggest that AVP may also play a crucial role in the pathophysiology of renal diseases.

In the present study, we demonstrated that AVP stimulates secretion of VEGF from mesangial cells concomitant with cell growth via the V_{1A} receptor. Our finding that cultured mesangial cells constitutively synthesize VEGF is in agreement with previous results obtained in rat- and human-cultured mesangial cells, in which both VEGF mRNA and protein secretion were detected [11, 12]. VEGF secretion has been found to be potently increased in response to hypoxia [30], vasopressor hormones such as angiotensin II [31, 32], cytokines (i.e., TGF- β and interleukin-1) [33, 34], and growth factors (i.e., tumor necrosis factor, fibroblast growth factor, and PDGF) [35–37]. VEGF secretion has also been found to be stimulated when mechanical stretching is applied to mesangial cells, suggesting that hemodynamic insult to the glomerulus may activate production of VEGF [38]. With specific relevance to diabetic mellitus, elevated glucose concentrations have also been found to increase VEGF expression [39]. Indeed, VEGF and its receptors are up-regulated in experimental animal models and humans with types 1 and 2 diabetes [40–42]. Thus, there are many stimuli that are known to be deeply involved in the pathogenesis of renal diseases that could act independently or in combination to upregulate VEGF production by mesangial cells.

In the human kidney, VEGF is known to be abundantly expressed in the podocytes of glomeruli, important cell constructions of the glomerular filter barrier that express VEGF receptors [2, 43]. Diabetic animal studies using inhibition of VEGF activity by neutralizing antibodies or small molecule inhibitors of VEGF receptor kinase signaling have demonstrated marked amelioration of albuminuria/proteinuria [44–46], suggesting that VEGF is directly involved in the albuminuria/proteinuria noted in diabetic nephropathy. Stimulation of VEGF secretion by podocytes is thus able to increase macromolecular permeability of the glomerular capillary by affecting blood flow and glomerular endothelial cell function as well as possibly exerting an autocrine effect to alter podocyte synthesis of

the glomerular basement membrane constituents and foot processes [43]. AVP V_{1A} receptor antagonists have been reported to attenuate proteinuria in several renal disease models [15, 47]. Thus, VEGF secreted by mesangial cells in response to AVP might act in a paracrine manner to induce podocyte dysfunction—in particular, the induction of albuminuria/proteinuria.

VEGF receptors are expressed not only in endothelial cells but also mesangial cells of glomeruli [48]. In the present study, VEGF induced hyperplasia, hypertrophy, and production of type IV collagen in rat mesangial cells. These findings, which are consistent with previously reported results [48–50], suggest that VEGF acts as a mitogen not only for endothelial cells but also mesangial cells and directly influences their function. A significant effect on mesangial cell growth by VEGF was seen at concentrations of approximately 100 ng/ml, a level higher than that found to induce endothelial cell proliferation [51] but comparable to that which induces monocyte migration and pericyte proliferation [52, 53]. In addition, AVP stimulated secretion of VEGF at concentrations of this order (approximately 30 ng/ml), suggesting that these concentrations may be pathophysiologically relevant. In addition, mesangial cell growth induced by AVP via V_{1A} receptors was somewhat inhibited on administration of an anti-VEGF antibody. Taken together with the above-mentioned observations regarding paracrine activity, these findings suggest that VEGF secreted by mesangial cells in response to AVP may also act in an autocrine manner.

We previously reported that AVP stimulated secretion of TGF- β , which induces an increase in the production of extracellular matrix proteins such as type IV collagen via V_{1A} receptors in cultured rat mesangial cells [20]. A recent study confirmed that TGF- β in turn stimulates VEGF synthesis and secretion from mesangial cells [54]. Furthermore, in our preliminary examination, anti-TGF- β antibody significantly inhibited AVP-induced VEGF secretion from rat mesangial cells. It is therefore conceivable that AVP-induced VEGF secretion may be mediated, at least in part, via AVP-induced release of TGF- β . AVP stimulates the secretion of VEGF and TGF- β , which then stimulates mesangial matrix expansion, thus fostering the development of glomerulosclerosis.

In summary, we demonstrated that AVP stimulated the secretion of VEGF through V_{1A} receptors in rat mesangial cells, modulating mesangial cell growth via direct action as well as through VEGF secretion. Interaction between AVP and VEGF may be involved in regulating mesangial cell growth in patients with renal diseases.

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