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Vasopressin induces human mesangial cell growth via induction of vascular endothelial growth factor secretion

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ABSTRACT

Vasoactive hormones, growth factors, and cytokines are important in promoting mesangial cell growth, a characteristic feature of many glomerular diseases. Vascular endothelial growth factor (VEGF) is an endothelial mitogen and promoter of vascular permeability that is constitutively expressed in human glomeruli, but its role in the kidney is still unclear. In the present study, we investigated the ability of vasopressin (AVP) to stimulate VEGF secretion by and correlation with AVP-induced cell growth in human mesangial cells. AVP caused time- and concentration-dependent increases in VEGF secretion from human mesangial cells, which was in turn potently inhibited by a V_{1A} receptor-selective antagonist, confirming that this secretion is a V_{1A} receptor-mediated event. VEGF also induced mesangial cell growth which was completely inhibited on administration of an anti-VEGF neutralizing antibody. Further, AVP-induced mesangial cell growth was completely abolished by the V_{1A} receptor-selective antagonist and partially inhibited by an anti-VEGF neutralizing antibody. These results suggest that AVP stimulates VEGF secretion by human mesangial cells via V_{1A} receptors. This secreted VEGF may function as an autocrine hormone to regulate mesangial cell growth, a mechanism by which AVP might contribute to progressive glomerular diseases such as diabetic nephropathy.

1. Introduction

Vascular endothelial growth factor (VEGF), previously known as vascular permeability factor or vasculotropin, is a 35–45 kDa heparin-binding dimeric glycoprotein that functions as an endothelial-specific growth factor and promotes endothelial cell proliferation, differentiation, and survival, mediates endothelium-dependent vasodilation, induces microvascular hyperpermeability, and participates in interstitial matrix remodeling (Neufeld et al., 1999). Alternative splicing of the VEGF mRNA generates at least six isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$), of which $VEGF_{165}$ is the predominant isoform, secreted by a variety of normal and transformed cells (Tischer et al., 1991; Neufeld et al., 1999). These various VEGF isoforms bind to two major tyrosine-kinase receptors: VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) (De Vries et al., 1992; Millauer et al., 1993; Terman et al., 1992). However, the specific function of each receptor subtype remains poorly defined, and the signal transduction pathways activated on ligand–receptor interaction are largely unexamined.

Production of VEGF has been demonstrated in a variety of mammalian tissues, including the kidney (Monacci et al., 1993; Brown et al., 1992), where it is hypothesized to be important in maintaining blood vessel differentiation (Ferrara and Davis-Smyth, 1997). However, the precise role of VEGF in the kidney is unknown. While renal biopsy studies have indicated that VEGF receptors are predominantly expressed on glomerular endothelial cells, faint expression has also been detected in the mesangial area, and expression has also been demonstrated in cultured rat and human mesangial cells (Takahashi et al., 1995; Thomas et al., 2000). In addition, human and rat mesangial cells have been proven to produce VEGF, synthesis of which is stimulated by cytokines that are important regulators of mesangial cell functions during development of glomerulopathies such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (Takahashi et al., 1995; Iijima et al., 1993; Finkenzeller et al., 1992). Known to be a potent promoter of vascular permeability (Collins et al., 1993), VEGF induces proteinuria in rats (Iijima et al., 1992) and has been implicated in the pathogenesis of minimum change disease (Brenchley, 1996). These findings suggest that VEGF may play an important role in controlling endothelial development and postinjury regeneration in the renal glomeruli, as well as performing a potential role in enhancing glomerular permeability under conditions such as nephrotic syndrome. Mesangial cells activated during glomerulopathies may thus represent an additional source of VEGF production.

A prominent role for angiotensin II in the development of glomerulosclerosis has been suggested in light of experimental and

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clinical evidence indicating that angiotensin-converting enzyme inhibitors and angiotensin receptor blockers have renoprotective effects (Leehey et al., 2000). In addition, AVP has also been suggested to affect the progression of chronic renal disease by increasing intraglomerular capillary pressure and stimulating mesangial cell proliferation, hypertrophy, and production of extracellular matrix, indicating a potential important role in glomerular fibrosis (Edwards et al., 1989; Wolthuis et al., 1992; Tahara et al., 2006; 2008). In fact, AVP receptor antagonists attenuate the progression of nephropathy and glomerulosclerosis in experimental models and human renal diseases (Kurihara et al., 1996; Nishikawa et al., 1996; Okada et al., 1996). These previous data, together with the recent findings that AVP stimulates VEGF production in vascular smooth muscle cells phenotypically similar to mesangial cells (Tahara et al., 1999), prompted our investigation into whether or not AVP regulates secretion of VEGF in human mesangial cells. To assess VEGF's potential role in pathogenesis of glomerular diseases, we examined the ability of AVP to stimulate secretion of VEGF in human mesangial cells.

2. Materials and methods

2.1. Materials

AVP and angiotensin II were obtained from Peptide Institute Inc. (Osaka, Japan). The V_{1A} receptor-selective antagonist YM218 (Tsukada et al., 2005) and V_2 receptor-selective antagonist SR 121463A (Serradeil-Le Gal et al., 1996) were synthesized at Astellas Pharma Inc. (Ibaraki, Japan). These nonpeptide antagonists were initially dissolved in dimethyl sulfoxide 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 Corporation (Grand Island, NY). Bovine serum albumin (BSA) was obtained from Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Cell culture

Human mesangial cells purchased from Clonetics (San Diego, CA) were grown at 37 °C in Mesangial Basic Medium (MsBM; Clonetics) supplemented with 5% FCS and antibiotics (GA-1000) in a humidified atmosphere of 5% CO_2 in air. The cultures were subcultured every 7 days in a 150-cm^2 culture dish using 0.05% trypsin–0.53 mM EDTA, with culture medium changed every 3 days. The cells were stellate or fusiform in appearance, grew in multilayers, formed hillocks in long-term culture, and stained for α-smooth muscle actin with direct immunofluorescence. Cells did not stain for cytokeratin, factor VIII, common leukocyte antigen. Studies were performed using cells from passages 4 though 10, the period during which the cells retained all of the morphologic and immunoflorescent features described above.

2.3. VEGF secretion by mesangial cells

Mesangial cells were seeded into 12-well culture plates at 70% 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 and 0.1% BSA with vehicle alone or various concentrations of AVP, antagonists/antibody, or both for 48 h. After incubation, culture supernatants were collected, centrifuged to remove cell debris, and stored at −40 °C until analysis. To determined 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 (BioRad Laboratories, Hercules, CA), with BSA as a standard. VEGF concentrations in the samples were determined using a highly sensitive enzyme-linked immunosorbent assay (ELISA) with anti-human VEGF antibodies MA-851 (Austral Biologicals, San Ramon, CA) and A-20 (Santa Cruz Biotechnology, Inc., CA). Results were normalized per cellular protein contents.

2.4. 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 culture medium supplemented with 0.5% FCS. The cultures were then incubated for 72 h in conditioned medium containing 0.5% FCS 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) (Ahmed et al., 1994) 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). 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 thee times with PBS and scraped from the plate. The suspensions were then homogenized, and protein content was determined as described above.

2.5. 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, 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. The culture supernatant was collected, and an equal volume of a mixture of proteinase inhibitors was added; the final concentrations of the components of this mixture were: 10 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM *N*-ethyl maleimide and 25 mM EDTA. These preparations were 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 PMSF, 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-human type IV collagen antibodies #1340-01 (Southern Biotechnology Associates, Inc., Birmingham, AL) and #68124 (ICN Biomedicals, Inc., Costa Mesa, CA). Results were normalized per cellular protein contents.

2.6. Statistical analysis

Data are expressed as means ± SE or mean with 95% confidence limits. Statistical comparison was conducted via one-way analysis of variance (ANOVA) followed by Dunnett's multiple-range test. Comparison between two different groups was done using the Student's unpaired *t*-test. Significance was set at $P < 0.05$.

3. Results

3.1. Effect of AVP on VEGF secretion

Time-dependent secretion of VEGF by human mesangial cells stimulated with AVP and angiotensin II are shown in Fig. 1 and Table 1. When mesangial cell monolayers were incubated with conditioned medium, VEGF secretion increased in a time-dependent