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Vascular Endothelial Growth Factor Upregulates Follistatin in Human Umbilical Vein Endothelial Cells

In Suk Oh and Hwan Gyu Kim*

Division of Biological Sciences, Institute for Molecular Biology & Genetics, Chonbuk National University, Chonju, Chonbuk 561-756, Korea

Abstract Vascular endothelial growth factor (VEGF), plays a key role in angiogenesis. Many endogenous factors can affect angiogenesis in endothelial cells. VEGF is known to be a strong migration, sprouting, survival, and proliferation factor for endothelial cells during angiogenesis in endothelial cells. Searching for novel genes involved in VEGF signaling during angiogenesis, we carried out differential display polymerase chain reaction on RNA from VEGF-stimulated human umbilical vein endothelial cells (HUVECs). In this study, follistatin (FS) differentially expressed in VEGF-treated HUVECs, compared with controls. Addition of VEGF (10 ng/mL) produced an approximately 11.8-fold increase of FS mRNA. FS or VEGF produced approximately 1.8- or 2.9-fold increases, respectively, in matrix metalloproteinase-2 (MMP-2) secretion for 12 h, compared to the addition of a control buffer. We suggest that VEGF may affect the angiogenic effect of HUVECs, through a combination of the direct effects of VEGF itself, and the indirect effects mediated via induction of FS *in vitro*.

Keywords: VEGF, follistatin, DD-PCR, MMPs

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a 46-kDa dimeric peptide that is essential for the induction of angiogenesis and drives both endothelial cell proliferation and migration [1,2]. VEGF is produced early in the angiogenic cascade and is responsible for the initial activation of endothelial cells [3]. The biological effects of VEGF are mainly regulated by two tyrosine kinase receptors, Flt-1 and Flk-1/KDR [4,5], the latter being important in cell proliferation, antiapoptosis and cell survival in endothelial cells [1,6]. The angiogenic effects of VEGF have been used clinically, to stimulate collateral artery formation in regions of tissue with vascular deficits, which occurs with ischemic heart disease and peripheral vascular disease [7].

VEGF gene expression is tightly regulated, mostly at the transcriptional level. Hypoxia is the major regulator, via binding of hypoxia-inducible transcription factors (HIF-1 and HIF-2) to the hypoxia-responsive element (HRE), located in the VEGF promoter [8]. Other mechanisms regulating VEGF transcription include several growth factors, such as, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), tumor necrosis factor-α (TNF-α), transforming growth factors (TGF-α, TGF-β) and inflammatory cytokines [9].

***Corresponding author**
Tel: +82-63-270-3352 Fax: +82-63-270-3362
e-mail: hgkim@moak.chonbuk.ac.kr

Follistatin (FS) is a glycosylated monomeric protein, that was originally identified in follicular fluid, as an inhibitor of FSH secretion [10,11]. The FS gene is known to be expressed, not only in the ovary and testis, but also in most, if not all, extragonadal tissues. It influences a variety of biological processes in addition to reproduction, including neural and mesodermal morphogenesis, angiogenesis, inflammation and wound repair [12,13].

Angiogenesis is a process involving cell attachment, basement membrane degradation, migration, proliferation, and cell differentiation, as well as formation of new capillary structures [14,15]. Emerging evidence suggests that matrix metalloproteinases (MMPs) play an important role in angiogenesis [16]. In order to migrate and sprout, endothelial cells should secrete protease to dissolve the adjacent extracellular matrix (ECM). During inflammatory angiogenesis, a variety of toxins, growth factors, and cytokines, exert their functions through autocrine, paracrine or endocrine action [15]. However, little is known about the effects of these molecules on MMPs secretion.

Increasing evidence suggests that autocrine endothelial cell activity contributes significantly to the angiogenic cascade, once the endothelial cells are initially activated by exogenous stimuli. To identify genes regulated via VEGF signaling in HUVECs, a differential display polymerase chain reaction [17] was utilized. In this study, FS was expressed differentially in VEGF-treated HUVECs compared with controls. Data from induction of FS and MMPs, indicates that VEGF may affect the angiogenic effect of HUVECs, through a combination of the direct effects of VEGF itself and indirect effects mediated via

induction of FS *in vitro*.

MATERIALS AND METHODS

Materials

Recombinant human $VEGF_{165}$ was purchased from R&D systems (Minneapolis, MN). Recombinant human MMP-1, MMP-2, MMP-3, and MMP-9 enzyme immunoassay kits, were purchased from Fuji Chemical Industries (Toyama, Japan). Media and sera were obtained from Life Technology, Inc. (Gaithersburg, MD, USA). An MMP standard was purchased from Calbiochem (La Jolla, CA, USA). Most other biochemical reagents, including recombinant human $follistatin_{300}$, dexamethasone (Dex), gelatin, fibrinogen, giemsa staining solution, antibiotics, antimycotics, and trypsin-EDTA, were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Cell Culture

HUVECs were prepared from human umbilical cords by collagenase digestion as previously described [18]. The endothelial origin of the cultures was confirmed by immunofluorescent staining with an anti-von Willebrand factor antibody; acceptable cultures had > 95% fluorescent cells. These endothelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% (v/v) heat-inactivated fetal bovine serum, at 37°C in 5% CO_2. The primary cultured cells used in this study were taken between passages 2 and 4.

RNA Isolation

Total RNA was isolated from VEGF-treated HUVECs, using Trizol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's recommendations. Messenger RNA was isolated using a Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden), containing oligo (dT)-cellulose, according to the manufacturer's instructions. The concentration and purity of the total RNA and the mRNA were calculated with absorbance at 260 and 280 nm, respectively, using a spectrophotometer.

Differential Display-Polymerase Chain Reaction (DD-PCR)

A DD-PCR was performed according to the method described by Liang and Pardee [17] using the RNAimage kit (Genhunter Corp., Nashville, TN, USA). Briefly, 100 ng of the isolated mRNA was reverse transcribed in reverse transcriptase buffer (25 mM Tris-Cl, pH 8.3, 37.6 mM KCl, 1.5 mM $MgCl_2$ and 5 mM DTT), containing 5 unit/μL of MMLV-reverse transcriptase, 20 μM dNTP mix and 0.2 μM of each one-base-anchored oligo (dT) primer (G, C or A). Subsequent PCR was performed in a PCR buffer (10 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin), containing 2 μM dNTP, 0.2 μL of one-base-anchored oligo (dT) primer, 0.2 μM of arbitrary primer, 0.2 μL of α-[^{32}P]dATP (2000 Ci/mmol) and 0.05 unit/μL of AmpliTaq DNA polymerase (Perkin-Elmer). The thermocycler (GeneAmp PCR System 9700, Perkin-Elmer) was programmed as follows; 40 cycles at 94°C for 30 s, 42°C for 2 min, and 72°C for 30 sec, and terminated with a final extension at 72°C for 5 min. ^{32}P-labeled PCR products were separated on 6% denaturing polyacrylamide gel for 3.5 h at 60 W constant power. The blotted gel on a piece of 3 M paper was dried under vacuum at 80°C for 1 h. The autoradiogram, oriented with the dried gel, was exposed and developed. The bands of interest were cut from the dried gel, eluted by boiling in water and reamplified by PCR, with the same set of primers in the same conditions as used previously. The reamplified cDNA fragments were cloned in pCR2.1 vector (Invitrogen Corp, San Diego, CA, USA) according to the manufacturer's instructions. The sequence alignment was aligned in all EMBL libraries.

Northern Blot Analysis

Twenty micrograms of total RNA were denatured and electrophoresed in 1% agarose/formaldehyde gel and then transferred to the Hybond N^+ membrane (Amersham Pharmacia, Buckinghamshire, England) by a capillary transferrance method. Following blotting, the membrane was UV-crosslinked by a FUNA-UV linker (Funakohi, Tokyo, Japan). FS cDNA probes were labeled with ^{35}S and then hybridized with the membrane RNA. For RNA loading and transfer variation control, filters were routinely rehybridized with a glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Enzyme Immunoassay of MMPs

HUVECs were seeded to 24-well plates at a density of 5×10^4 cells/cm^2 and were grown for 24 h in DMEM supplemented with 20% serum. Then, confluent HUVECs were incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with fresh medium, a control buffer or indicated reagents were applied for 12 h. The actual quantities of the MMPs were assayed by enzyme immunoassay, according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan).

Zymography

HUVECs were seeded to 24-well plates at a density of 5×10^4 cells/cm^2 and were grown for 24 h in DMEM supplemented with 20% serum. Then, confluent HUVECs were incubated in serum-and phenol red-free DMEM for 12 h. After the cells were washed with fresh medium, a control buffer or the indicated reagents were applied for 12 h. The hydrolytic activities of MMPs were measured by gelatin zymography [19]. Samples were mixed with 5X sample buffer (4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol and 0.1% bromophenol blue) and were applied to a 10% SDS-PAGE containing 0.1% gelatin. Reference

Table 1. Results of the DD-PCR product sequences in HUVECs treated with VEGF

Primers		Expression Types	
Anchored primers[a]	Arbitrary primers[b]	Up-regulation	Down-regulation
H-T_{11}G	H-AP1	human homeobox	
H-T_{11}G	H-AP2	RNA helicase I	
H-T_{11}G	H-AP3	tissue plasminogen activator	
H-T_{11}G	H-AP6	follistatin	
H-T_{11}A	H-AP1	melanin concentrating hormone	
H-T_{11}A	H-AP3	human ADP-ribosylation factor 4	
H-T_{11}A	H-AP5	Gu protein	
H-T_{11}A	H-AP5	p62	
H-T_{11}A	H-AP6	human focal adhesion kinase	
H-T_{11}A	H-AP7	clathrin coat assembly protein	
H-T_{11}A	H-AP8	ran GTP binding protein 5	
H-T_{11}C	H-AP2	ribosomal protein S14	
H-T_{11}C	H-AP8		C_2H_2-type zinc finger protein

[a] H-T_{11}G; AAGCTTTTTTTTTTTG, H-T_{11}A; AAGCTTTTTTTTTTTA, H-T_{11}C; AAGCTTTTTTTTTTTC.
[b] H-AP1; AAGCTTGATTGCC, H-AP2; AAGCTTCGACTGT, H-AP3; AAGCTTGGTCAG, H-AP5; AAGCTTAGTAGGC, H-AP6; AAGCTT GCACCAT, H-AP7; AAGCTTAACGAGG, H-AP8; AAGCTTTTACCGC.

standards were MMP-2 and MMP-9 (Chemicon International, Inc., Temecula, CA, USA). After running, gels were incubated in 2.5% Triton X-100 for 1 h and incubated in enzyme buffer (0.05 M Tris-Cl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35) for 24 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue 250 solution and destained with several changes of 30% methanol and 10% acetic acid.

Data Analysis

Data is expressed as means ± standard deviation (SD). Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Differential Display of the mRNA Species in HUVECs

Using specific arbitrary primers, several cDNA fragments that display differential expression from VEGF-treated HUVECs, were identified. Of these, 35 bands (94%) were upregulated. DNA sequencing revealed 13 genes that have been described already (Table 1). It was found that RNA helicase I, tissue plasminogen activator, follistatin, melanin concentrating hormone, ADP-ribosylation factor 4 and focal adhesion kinase genes, were increasingly expressed, while the C_2H_2-type zinc finger gene was decreasingly expressed. Most of the fragments showed no homology or were identified as expressed sequences tags (ESTs), with no known function. Further analysis was carried out with the FS gene (Assesion # AH001463) (Fig. 1).

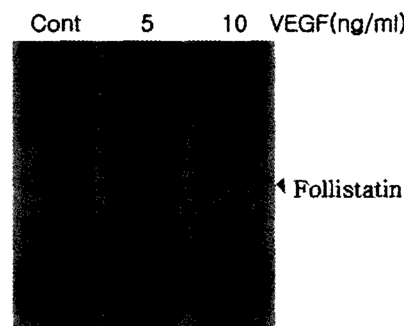

Fig. 1. Gel analysis of differentially displayed mRNA from VEGF-treated HUVECs. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Control buffer (C) and VEGF (5, 10 ng/mL) were added to 0.5 mL of culture medium. Messenger RNAs were reverse transcribed and the products were run on 6% non-denaturing gels. The arrowhead on the right indicates the FS cDNA fragment. Sequences of DDRT-PCR primers for specifying FS were H-T_{11}G, 5'-AAGCTTTTTTTTT TTG-3' and H-AP6, 5'-AAGCTTGCACCAT -3'.

FS mRNA Expression Associated with VEGF

A purified FS cDNA fragment was used as a probe in a

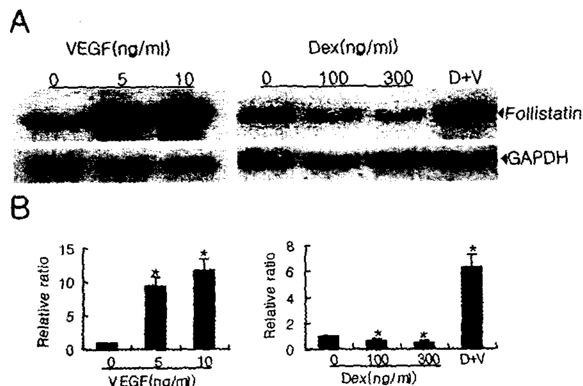

Fig. 2. Northern blot analysis of FS mRNA from VEGF and Dex treated HUVECs. (A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Control buffer (C), VEGF (5, 10 ng/mL), Dex (100, 300 ng/mL) and Dex (300 ng/mL) plus VEGF (5 ng/mL) (D+V) were added to 0.5 mL of culture medium. Total RNA (20 μg) was fractionated on a 1% agarose/formaldehyde gel and Northern blot analysis was carried out as described in Materials and Methods. The upper panel shows the FS expression, and the lower panel indicates the internal control, GAPDH. (B) The relative densitometry values are shown.

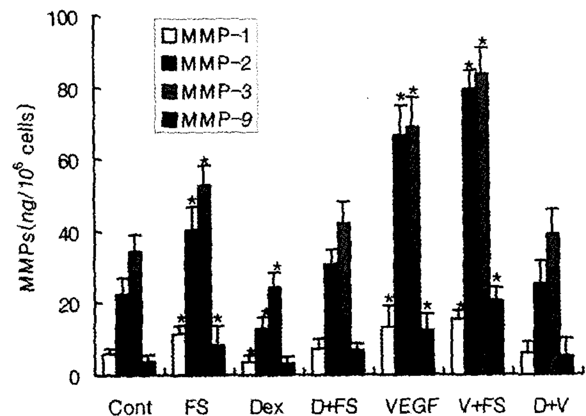

Fig. 3. Effects of FS, Dex and VEGF on the secretion of MMPs in HUVECs. . Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of a control buffer (Cont), FS (50 ng/mL), Dex (300 ng/mL), Dex (300 ng/mL) plus FS (50 ng/mL) (D + FS), VEGF (10 ng/mL), VEGF (10 ng/mL) plus FS (50 ng/mL) (V + FS) and Dex (300 ng/mL) plus VEGF (10 ng/mL) (D + V), and the media were quantitatively assayed by enzyme immunoassay. Bars represent the means ± SD from three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Students *t* test. $^*P < 0.05$ versus control buffer.

Northern blot analysis (Fig. 2). The results are consistent with the mRNA differential display spectra (Fig. 1). FS was increased in a dose-dependent manner. Addition of VEGF (10 ng/mL) produced an approximately 11.8-fold increase of FS mRNA (Fig. 2). Dex has been shown to inhibit the expression of VEGF [20]. Clerch *et al.* [21] showed that Dex-induced inhibition of angiogenesis due to downregulation of VEGF receptor-2. In the present experiment, addition of Dex (300 ng/mL) producued an approximately 42% decrease of FS mRNA. The addition of Dex (300 ng/mL) produced approximately 53% suppression of VEGF-induced FS mRNA induction. This data suggests that FS expression in HUVECs may be regulated by Dex-induced VEGF downregulation. FS is now thought to function as a diverse regulator of morphogenesis, vascular remodeling, inflammation, and wound repair [12]. These observations are consistent with previous studies concerning other cell types [22,23]. The suppression of FS expression by Dex, and its upregulation by VEGF, attests to the precise balance that appears to exist among these factors. In fact, exogenous recombinant FS induced proliferation and angiogenesis of HUVECs [23].

Secretion of MMPs from HUVECs Treated with FS and VEGF

One of the steps in angiogenesis is the degradation of the underlying basement membrane via proteases [24]. Endothelial cells release proteinases to degrade the ECM for their migration and proliferation *in vivo*. One family of such proteinases is the MMPs. The role of MMPs in angiogenesis has been demonstrated *in vitro* [25]. In culture, endothelial cells secrete MMP-2, which can disrupt the ECM and enable their migration and tube formation [26]. MMPs have been implicated in endothelial cell migration induced by VEGF [27]. There is evidence for a reciprocal relationship between VEGF and MMPs in different cell types. VEGF treatment accelerated the migration of vascular smooth muscle cells through Matrigel and enhanced their production of MMPs [28]. Using the submaximal doses defined above, the effect of FS, Dex, and VEGF on the secretory activity of HUVECs was assessed. Enzyme immunoassay showed that culture media from HUVECs contained marked amounts of MMP-2 and MMP-3, whereas MMP-1 and MMP-9 levels were low (Fig. 3). The addition of FS (50 ng/mL) for 12 h produced approximately 1.6-, 1.6-, 1.4-, and 2.7-fold increases in MMP-1, MMP-2, MMP-3, and MMP-9 secretion, respectively, compared with addition of a control buffer. The addition of VEGF (10 ng/mL) for 12 h produced approximately 2.2-, 3.2-, 2.4-, and 4.1-fold increases in MMP-1, MMP-2, MMP-3, and MMP-9 secretion, respectively, compared with the addition of a control buffer. Combination of the submaximal doses of FS and VEGF, produced an enhanced effect on the induction of MMPs secretion. The effect of the anti-inflammatory Dex, on the production of MMPs, was examined. The addition of Dex (300 ng/mL) for 12 h produced approximately 28-, 44-, and 29% suppression of MMP-1, MMP-2, and MMP-3 secretion, respectively, compared with addition of a control buffer. Preincubation with Dex (300 ng/mL) produced complete inhibition of FS-induced MMP-1, MMP-2, and MMP-3 secretion in HUVECs (Fig. 3). In fact, Dex inhibited the angiogenic ability of endothelium

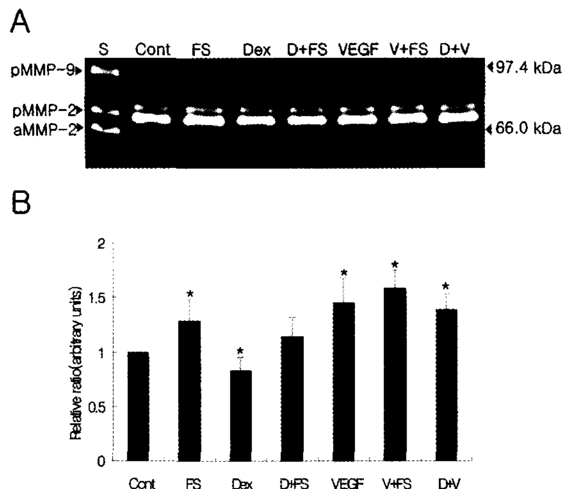

Fig. 4. Gelatin zymography of MMP-2 in culture medium of HUVECs treated with FS , Dex and VEGF. (A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, control buffer (Cont) or indicated reagents were added to 0.5 ml culture medium, incubated for 12 h. Equal amounts of proteins (10 μg/lane), from supernatants, were loaded into each lane. Lane S contains standards of MMP-9 and MMP-2. (B) Densitometric analyses of the zymographs are presented, as the relative ratio of induction of the MMP-2 by addition of indicated reagents. The MMP-2 secretion, by addition of a control buffer for 12 h, is arbitrarily presented as 1. Data are mean ± SD from three experiments. Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. $^*P < 0.05$ versus control buffer.

[29]. Combination of the submaximal doses of FS and VEGF, produced an enhanced effect on the induction of MMPs. Since the treatment of VEGF already induced FS, the change by the treatment of VEGF could be due to both VEGF itself and FS induction, it is hard to separate the effect of VEGF from that of FS. These results suggest that FS in synergy with VEGF, induces angiogenesis.

FS Can Act as a Direct or Indirect Angiogenic Factor

MMP-2 secretion was confirmed by gelatin zymography. FS or VEGF produced approximately 2.2- or 1.8-fold increases, respectively, in MMP-2 secretion for 12 h, compared to the addition of a control buffer (Fig. 4). Culture media from HUVECs treated with FS or VEGF had clearly increased ~68 kDa gelatinolytic bands (MMP-2), compared with the cells treated with buffer alone. Consistent with the enzyme-immunoassay data (Fig. 3), Dex significantly reduced the levels of MMP-2 in HUVECs treated with FS. This suggests that FS and VEGF may enhance angiogenesis, in part, by stimulating MMP-2 production. It was reported that exogenous recombinant FS induced proliferation of HUVECs. *In vivo*, FS was moderately angiogenic in the rabbit cornea [23]. FS might be expected to play a prominent role in endothelial cell proliferation [30]. The notable finding in this study, is that FS differentially expressed in VEGF-treated HUVECs. VEGF and FS treatment increased the production of MMP-2 which may degrade the basement membrane during angiogenesis.

In summary, this study provides further insight into the cellular mechanism of VEGF in primary cultured HUVECs. Since VEGF-dependent up-regulation of FS was observed at the level of both mRNA and protein, it is probable that angiogenesis in HUVECs is due in part, to enhanced MMP-2 secretion through FS up-regulation. Further analysis of the signaling pathways responsible for initiating the responses of proliferation and proteolysis during VEGF-induced angiogenesis will be carried out.

CONCLUSION

VEGF is essential for the induction of angiogenesis and drives both endothelial cell proliferation and migration. In this study, FS was expressed differentially in VEGF-treated HUVECs compared with controls. The addition of VEGF (10 ng/mL) for 12 h produced approximately 2.2-, 3.2-, 2.4-, and 4.1-fold increases in MMP-1, MMP-2, MMP-3, and MMP-9 secretion, respectively, compared with the addition of a control buffer. Combination of the submaximal doses of FS and VEGF produced an enhanced effect on the induction of MMPs secretion. The addition of Dex (300 ng/mL) for 12 h produced approximately 28-, 44-, and 29% suppression of MMP-1, MMP-2, and MMP-3 secretion, respectively, compared with addition of a control buffer. The notable finding in this study, is that FS differentially expressed in VEGF-treated HUVECs. VEGF and FS treatment increased the production of MMP-2 which may degrade the basement membrane during angiogenesis.

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