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AP-1 transcription factor mediates VEGF-induced endothelial cell migration and proliferation

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Abstract

VEGF, upon binding to its endothelial cell specific receptors VEGF-R1 and VEGF-R2, can induce endothelial cell migration, proliferation and angiogenesis. However, the molecular mechanism of these effects still remains unclear. In this study, we investigated whether VEGF promotes human umbilical vascular endothelial cell (HUVEC) migration and proliferation through activator protein-1 transcription factor (AP-1) family. We first showed that VEGF induces immediate-early genes AP-1 family gene expression differentially with the profound induction of JunB (both mRNA and protein) under various conditions (PBS, DMSO or control adenoviruses). The increase in AP-1 mRNA expression occurs primarily at the transcriptional level. Inhibition of AP-1 DNA binding activity by adenovirus expressing a potent dominant negative form of c-Fos (Afos) significantly attenuated VEGF-induced HUVEC migration and proliferation and cyclin D1 expression. Knockdown of JunB with adenovirus expressing JunB shRNA reduces VEGF-induced JunB expression and attenuated HUVEC migration. However the shJunB-expressing virus has no effect on VEGF-induced cyclin D1 protein expression and proliferation. These results suggest that VEGF-induced endothelial migration is mediated primarily by induction of JunB whereas the promotion of endothelial proliferation by VEGF is mediated by JunB-independent AP-1 family members.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

Endothelial cells; Angiogenesis; VEGF; AP-1

Introduction

Vascular endothelial growth factor A (or VEGF) is critical for the vascular development and formation of new blood vessels (angiogenesis) (Dvorak et al., 1995; Senger et al., 1993). VEGF-induced angiogenesis in adults plays important roles in many pathological processes including acute/chronic inflammation and tumor growth as the neutralization of VEGF by its specific antibody dramatically improves the outcome of this disease (Dvorak et al., 1995; Senger et al., 1993; Zachary and Glick, 2001). Signal transduction pathways involved in VEGF-induced endothelial cell migration and proliferation have been extensively studied in cultured cells (Qin et al., 2006; Vieira et al., 2010; Zachary and Glick, 2001; Zeng et al., 2002a, 2002b, 2003). In contrast, limited information is available in the transcriptional mechanisms regulating the various effects of VEGF. VEGF stimulates NF- κ B-dependent inflammatory gene expression to mediate its inflammatory effects (Kim et al., 2001). In addition, we and others showed that VEGF induces Nur77 family of orphan nuclear transcription factors to regulate the proliferative effect of VEGF (Liu et al., 2003; Zeng et al., 2006; Zhao et al., 2011). The most notable transcriptional factors known to be critical for cell growth and differentiation are the immediate-early gene AP-1 family of transcriptional factors (review Jochum et al. (2001)). However, little is known about the expression and roles of AP-1 family in VEGF angiogenic response although several studies showed that AP-1 plays important role in regulating VEGF gene expression in response to different cytokines in various cell lines (Chang et al., 2006; Chua et al., 2000; D'Angelo et al., 2000; Mar et al., 2015).

The AP-1 complexes are heterodimers consisting of one Jun family member (c-jun, JunB and JunD) and one Fos family member (c-fos, FosL1, FosL2 and FosB) (Chinenov and Kerppola, 2001; Hess et al., 2004; Jochum et al., 2001). Jun family members also form homodimers to interact with AP-1-binding sites with many gene promoters whereas Fos family members cannot form homodimers (Chinenov and Kerppola, 2001; Hess et al., 2004; Jochum et al., 2001). Transcriptional activity of AP-1 is primarily mediated by induction of their expression and also regulated by post-translational modification, particularly their phosphorylation by MAP kinase family members ERK, p38 and JNK. However, very little is known about whether VEGF affects the expression of AP-1 family members in endothelial cells. One study has shown that VEGF potently induces c-fos mRNA expression but have moderate effect on c-jun mRNA levels in HUVEC (Armesilla et al., 1999). Another work indicated that VEGF weakly induces c-jun and JunB protein in placental artery endothelial cells (Mata-Greenwood et al., 2010). Nevertheless, surprisingly nothing is known whether these AP-1 family members play role in VEGF functions. In the present study, we sought to examine whether VEGF upregulates the expression of AP-1 family proteins in endothelial cells and if so, further determined its function in VEGF-induced angiogenic response.

Materials and methods

Materials

Human recombinant VEGF protein was purchased from R&D Systems (Minneapolis, MN). Rabbit anti-human c-fos (sc-52), c-jun (sc-1694), and cyclin D1 (sc-718) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human JunB (Cat. No. 3755) and JunD (Cat. No. 5000) were purchased from Cell Signaling (Danvers, MA). Mouse monoclonal anti-Flag antibody was from Sigma. Actinomycin D were purchased from EMD Millipore (Billerica, MA)

Cell culture

Human umbilical vein endothelial cells (HUVECs) that were acquired from Lonza (Allendale, NJ), were cultured in 3 mg/ml collagen type I (Advanced BioMatrix, Carlsbad, CA) coated plates with EBM-2 medium (Lonza) supplemented with bullet kit as recommended. Cells were subcultured after trypsinization in a 0.25 mg/ml Trypsin-EDTA purchased from Lonza. Cells of passages 4 to 6 were used for the experiments.

RNA isolation and quantitative real-time PCR

Total RNAs were extracted using the RNeasy mini kit (Qiagen, Valencia, CA) and reverse-transcribed by oligo (dT) priming using the iScript cDNA synthesis kit following the manufacturer's instructions (New England Bio Labs, Ipswich, MA). Semi-quantitative real-time PCR analyses were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green PCR Master Mix (Applied Biosciences). Primers used in real-time PCR were purchased from Qiagen. Oligonucleotide primers of human c-fos, c-jun, JunB, JunD, FosL1, FosL2 and FosB were from Origene Co. (Rockville, MD).

Western blot analysis

Treated cells were lysed in cold lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris; pH 8.0) containing a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). After removing the cell debris by centrifugation at 13,000 rpm for 5 min, the protein concentration was determined by the Bradford assay. Equal amounts of cell extracts were separated by SDS-PAGE (10%), and proteins were transferred onto NC membrane (Santa Cruz Biotechnology, CA) at 350 mA for 90 min at 4 °C. Membranes were blocked in 5% nonfat dried milk in Tris-buffered saline/Tween (50 mM, Tris (pH 7.5), 0.15 M, NaCl, 0.05% Tween 20) and then incubated with each primary antibody overnight at 4 °C, then washed in washing buffer (TBS + 0.1% Tween 20) once for 10 min followed by two rinses for 5 min. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins on each blot were visualized with Renaissance® Western Blot Chemiluminescence Reagent (Fisher-Pierce Co., Rockford, IL). Blots were stripped and reprobed with an antibody against human β -actin to demonstrate uniform loading of proteins.

Adenovirus construction and transduction

Adenoviruses expressing a dominant negative form of the c-fos proto-oncogene, Afos (Olive et al., 1997) was constructed by following the instruction of ViraPower Adenovirus Expression System from Invitrogen (Fisher-Invitrogen, Grand Island, NY). Briefly, the Afos fragment in CMV-Afos plasmid (Addgene Co, Cambridge, MA) was subcloned into pENTR-1A shuttle vector and then transferred into a pAd/CMV/V5-DEST Gateway vector by homologous recombination. To construct shJunB-expressing adenoviruses, the oligonucleotide sequences targeting human JunB (Kanno et al., 2012) were synthesized, annealed, ligated into shRNA-expressing shuttle vector (Fisher-Invitrogen) before transferring to pAd/Block-It-DEST vector (Fisher-Invitrogen). Adenoviruses expressing Afos (Ad-Afos), lacZ, or shJunB or shGFP were produced in HEK293 cells and purified by cesium chloride (American Bioanalytical, Natick, MA) gradient centrifugation according to standard methods. The HUVECs were transduced with recombinant adenovirus at virus-particles per cell (VP/cell) of 100– 1000. After transduction, the growth medium was removed and the cells were washed once then replaced with serum-free medium. Virus infection was performed for 24 h at 37 °C then harvested for subsequent experiments.

Cell migration assay

HUVECs were cultured in a 6-well culture plate. When cells achieved 80 to 90% confluence, a wound was made in the cell area using a sterile yellow tip. Cells were washed with PBS and incubated with serum-free medium for 24 h before treated with VEGF (50 ng/ml) for 16–24 h. Cells were washed twice with PBS, fixed with 4% formalin. Cell that migrated into the scratched area was photographed at a magnification of $\times 20$. Cell migration was quantified by calculating the migrated cell numbers in the wounded area. Data were expressed as a percentage of the migrated cells relative to those in the untreated endothelial cells.

CCK-8 cell proliferation assay

Cell proliferation was determined by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, cells were transduced with indicated adenoviruses and then serum starved overnight before stimulation with VEGF (50 ng/ml) for 2 days. Medium was aspirated and fresh medium was added along with CCK-8 reagent (10:1 ratio). Cell culture medium and CCK-8 reagent were added to four wells without cells to serve as a background control. Plates were then incubated for an additional 4 h at 37 °C. After incubation, 100 μ l of solution from each well was transferred into a separate 96-well plate, where the absorbance of the samples against a background control was measured at a wavelength of 450 nm on SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Results are presented as mean $\pm$ SD. Student's t-test was used to determine statistical significance. p values less than 0.05 were considered to be statistically significant.

Results

VEGF upregulates AP-1 family gene expression in HUVECs

We first determined whether expression of AP-1 family member protein and mRNA is affected by VEGF in human endothelial cells. As shown in Fig. 1, VEGF significantly enhances mRNA expressions of c-jun, JunB, JunD, c-fos, FosB and FosL1 in a time-dependent manner (Fig. 1a–f, left panel) but had little effect on FosL2 mRNA levels (Fig. 1g). Enhancement effects of c-jun, JunB, c-fos and FosB mRNAs were peaked at 0.5 h then decreased thereafter, whereas the effects of VEGF on JunD and FosL1 mRNAs were peaked at 1 h then decreased thereafter. We then further examined the effect of VEGF on their protein levels. VEGF enhances protein expression of c-jun, JunB and c-fos in a time-dependent manner, but there was little effect on JunD protein expression (Fig. 1c, right panel). Enhancement effect of c-fos, c-jun, JunB were peaked at 2 h after VEGF treatment (Fig. 1d, a, b, right panel).

In order to see whether VEGF induces these mRNAs at transcriptional levels, quiescent HUVECs were pretreated with actinomycin D, a DNA intercalator which can block the progression of RNA polymerases and then stimulated with VEGF. The data show that pretreatment with actinomycin D completely blocked VEGF-induced expression of c-fos, c-jun and JunB mRNA (Fig. 2a–c), indicating that VEGF affects these AP-1 family gene expressions primarily at the transcriptional levels.

Effect of Afos on VEGF-induced endothelial proliferation and cyclin D1 expression

As VEGF induces expression of different members of AP-1 family gene expression, we sought to block all the AP-1 family protein functions by utilizing a dominant negative form of c-fos (Afos), Afos which forms very strong heterodimers with all AP-1 family proteins therefore prevent them from binding to AP1-binding sites (Olive et al., 1997). Adenoviruses expressing Afos were generated as described in Methods and used to examine its effect on VEGF-induced HUVEC proliferation and migration. HUVECs were transduced with viruses expressing control lacZ or Flag-tagged Afos and overexpression of Afos protein was confirmed by western blotting using anti-Flag antibody (Fig. 3a). HUVECs seeded on a 96-well plate were transduced with different doses of adeno-LacZ or adeno-Afos, serum-starved and then stimulated with VEGF for 48 h. Cell proliferation was measured by CCK-8 cell proliferation kit as described in Methods. The data showed that compared to control cells, Afos completely blocked VEGF-induced HUVEC proliferation (Fig. 3b).

Next we determined whether AP-1 activation is required for VEGF-induced cell cycle protein expression. Cyclin D1 is critical for cell cycle progression of endothelial cells and AP-1 modulates cell migration and proliferation through regulating cyclin D1 (Ansari et al., 2008), therefore, cyclin D1 expression was examined for this study. HUVECs were transduced with viruses expressing control lacZ or Afos, serum starved and then stimulated with VEGF for the indicated time period. Total RNA and cellular protein were subjected to qPCR and western blot analysis using cyclin D1-specific primers and antibody, respectively. The data indicate that Afos overexpression significantly inhibited VEGF-induced cyclin D1 mRNA and protein expression (Fig. 4a and b).

VEGF-induced endothelial cell migration is inhibited by Afos overexpression

We further investigated if VEGF promoted endothelial cell migration is mediated through AP-1 transcription factor. HUVEC migration was measured by scratch wound assay. Stimulation of HUVEC with VEGF for 16 h caused significant cell migration into scratched area compared to vehicle-treated cells at the presence of control lacZ-expressing virus infection (Fig. 5a and b). When HUVECs were transduced with adenoviruses expressing Afos, cell migration was significantly reduced as compared to that in cells transduced with control-lacZ (Fig. 5a and b). Our observation is that VEGF induces significant endothelial proliferation in 48 h, whereas its effect on migration can be obvious 6–16 h after VEGF stimulation, indicating that the effect of Afos on VEGF-induced proliferation most likely is not due to its effect on the migration.

JunB induction is involved in VEGF-induced cell migration but not proliferation

We found that among three Jun family members (c-jun, JunB and JunD), increased JunB expression is more profound and consistent when HUVECs were treated with VEGF both in the absence or presence of adenovirus infection (Fig. 1). For the reason, we focused our attention in JunB. Adenoviruses expressing JunB shRNA and GFP control shRNA were generated as in Methods. First we tested whether the JunB shRNA-expressing viruses can efficiently knockdown VEGF-induced JunB protein expression. Fig. 6a indicates that JunB shRNA inhibits VEGF-induced JunB protein expression. Then we determined the effect of JunB knockdown on VEGF-induced HUVEC migration. The image data showed that compared to control GFP shRNA, expression of JunB shRNA has little effect on basal cell migration in the absence of VEGF stimulation (Fig. 6b and c) and also significantly attenuated VEGF-induced cell migration (Fig. 6b and c). The quantification of the numbers of migrated cells in Fig. 6b was showed in Fig 6c. Then we examined whether JunB shRNA affects VEGF-induced cyclin D1 expression and cell proliferation. HUVECs were transduced with GFP shRNA or JunB shRNA and stimulated with VEGF. Cyclin D1 expression and cell proliferation were determined. The data showed that compared to GFP shRNA, JunB shRNA had no significant effect on VEGF-increased cyclin D1 expression and proliferation (data not shown).

Discussion

AP-1 transcriptional factor plays critical roles in mediating transcription of numerous genes involved in cell growth, differentiation and cell–cell interaction and its function in regulating tumor cell growth has also been extensively studied (Chinenov and Kerppola, 2001; Hess et al., 2004; Jochum et al., 2001). Surprisingly, little is known about its role in VEGF-induced angiogenesis. Our present study demonstrates for the first time that VEGF stimulates endothelia cell migration and proliferation by activating AP-1 transcription factor. AP-1 activation is one main transcriptional mechanism that drives VEGF expression in response to different stimuli in various tumor cell lines and endothelial cells as well (Chang et al., 2006; Chua et al., 2000; D'Angelo et al., 2000; Lee et al., 2006; Mar et al., 2015), and as shown here, it further mediates VEGF-triggered angiogenic response. This suggests that targeting AP-1 activation may represent more effective approach to treatment of cancer via inhibition of both the upstream and downstream of VEGF signaling.

The effect of VEGF on all AP-1 family members (c-jun, JunB, JunD and c-fos, FosB, FosL1, FosL2) for both mRNA and protein expression was examined in detail. Their mRNA levels appear to be upregulated to different degrees when HUVECs were stimulated with VEGF under the physiological condition in the absence of stress such as DMSO or adenoviral infection). c-jun, JunB and c-fos mRNAs are induced to their maximal levels 30 min after VEGF stimulation and their proteins are also induced under the physiological condition (Fig. 1). To our surprise, JunD protein cannot be induced although its mRNA is induced 1 h after VEGF treatment, the cause for which remains to be investigated. Also when HUVECs were treated with VEGF in the presence of 0.1% DMSO (vehicle for pharmacological inhibitors) or control adenovirus, the levels of c-jun protein cannot be further increased by VEGF since DMSO or viruses are able to induce c-jun protein (data not shown), consistent with the fact that c-jun is a stress-inducible gene. In contrast, JunB mRNA and protein can be consistently induced by VEGF either in the absence or in the presence of DMSO or adenoviruses (data not shown). For these reasons, JunB might play a major role in VEGF-induced HUVEC responses under our experimental conditions with inhibitor treatment or viral infection.

Our results by using a dominant negative form of c-fos (Afos) indicate that the prevention of Jun family members from binding to the AP-1 sites of downstream target genes completely inhibits VEGF-induced HUVEC migration and proliferation, suggesting that Afos may be used therapeutically for targeting tumor angiogenesis there for tumor growth. Although it may not indicate from the results that each Jun family member plays a similar role in VEGF angiogenic responses, it still shows the importance of them in the response. Further, our data from JunB knockdown clearly indicate that the member of Jun family plays a major role in the angiogenic response of VEGF at least under our experimental conditions. It is known that c-jun^{-/-} mice (E12.5) and JunB^{-/-} mice (E8.5–10) die during early embryogenesis (Jochum et al., 2001). The embryonic phenotypes suggest that JunB might play relatively more important role for vasculogenesis when compared to c-jun because JunB-null embryos die during early vessel development (Jochum et al., 2001). Consistent with our observation that JunB knockdown inhibits VEGF-induced endothelial cell migration not proliferation, JunB deficiency results in defective neovascularization which is obviously not from defective vascular endothelial proliferation (9). The exact mechanism by which AP-1 transcription factor regulates endothelial cell functions remains to be investigated further.

In summary, our results showed that VEGF differentially regulates AP-1 family gene expression in endothelial cells and inhibition of AP-1 transcriptional activation by a potent AP-1 inhibitory protein Afos blocks VEGF-induced endothelial cell migration and proliferation. This study suggests that Afos can be used as a therapeutic agent for inhibition of tumor angiogenesis and tumor growth.

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Fig. 1.

Effect of VEGF on AP-1 family gene expression. HUVECs were starved for 24 h, and then incubated with VEGF (50 ng/ml) for various time periods as indicated. No VEGF treatment was used as a control. On the left panels, RNAs were harvested and subjected to real-time PCR using specific primer sets for human c-jun (a), JunB (b), JunD (c), c-fos (d), FosB (e), FosL1 (f), FosL2 (g), or internal control GAPDH. Histograms represent quantification of RT-PCR corrected with GAPDH. Results are expressed as fold of control and are expressed as mean $\pm$ SD. * and # denote $p < 0.01$ and $p < 0.05$ versus control, respectively. On the

right panels, cell lysates were prepared and protein levels of c-jun (a), JunB (b), Jun D (c) and c-fos (d) were monitored by immunoblotting. Reprobing blots against β -actin was performed to ensure equal protein loading. Data are representative of three independent experiments.

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Fig. 2.

AP-1 family gene regulation by VEGF occurs at the transcriptional level. Serum-starved HUVECs were pretreated with actinomycin-D (5 μ g/ml) or control vehicle for 30 min and then stimulated with VEGF (50 ng/ml) or control for 30 min. RNA was harvested and subjected to RT-PCR using specific primer sets for human c-fos (a), c-jun (b) and JunB (c). Histograms represent quantification of RT-PCR corrected with GAPDH. Results are expressed as fold of control and are expressed as the mean $\pm$ SD. * denotes $p < 0.001$ vs DMSO with VEGF. Data are representative of three independent experiments.

Fig. 3.

Afos overexpression decreases VEGF-induced HUVEC proliferation. (a) HUVECs were transduced with adenoviruses expressing control lacZ or Flag-Afos at the indicated doses (VP/cell) and expression of Afos was determined by western blotting using Flag antibody. (b) Transduced cells were starved for 24 h and then treated with VEGF (50 ng/ml) for 48 h. Cells were then subjected to CCK-8 cell proliferation assays. Results are expressed as fold of control. The signs * represent $p < 0.01$ versus VEGF without Afos. Data are representative of three independent experiments.

Fig. 4.

Afos overexpression decreases VEGF regulated cyclin D1 expression. HUVECs transduced with adenoviruses expressing control lacZ or Afos were starved for 24 h then treated with VEGF (50 ng/ml) or PMA (1 μ M). RNA and total cell protein were subjected to qPCR and immunoblotting to determine the levels of cyclin D1 mRNA (a) and protein (b), respectively. The signs * represent $p < 0.01$ versus VEGF with lacZ. Data are representative of three independent experiments.

Fig. 5.

Overexpression of Afos decreases VEGF-induced HUVEC migration. HUVECs were transduced with adenoviruses expressing control lacZ or Afos at the doses of 200 VPs/cell, starved for 24 h. Scratch wounds were created and then treated with VEGF (50 ng/ml) for 16 h. Representative images were taken (a). Quantification of cells that migrated into wounded area was calculated (b). Results are expressed as mean $\pm$ SD (b). The signs * represent $p < 0.01$ versus lacZ with VEGF. Data are representative of three independent experiments.

Fig. 6.

JunB knockdown attenuates VEGF-induced cell migration. (a) HUVECs were transduced with adenovirus expressing GFP shRNA or JunB shRNA and then stimulated with VEGF (50 ng/ml) for 2 h. JunB expression was examined by immunoblotting as described in Fig. 1. (b) HUVECs were transduced with adenovirus expressing GFP shRNA or JunB shRNA with a dose of 1000 VPs/cell. Wounds were created and stimulated with VEGF (50 ng/ml) for 24 h. Images were taken and shown here. (c) Quantification of cell migration was done as in Fig. 4. Data are representative of three independent experiments.