Vascular Endothelial Growth Factor Synergistically Enhances Induction of E-Selectin by Tumor Necrosis Factor-α

Anita K. Stannard, Rohit Khurana, Ian M. Evans, Vassiliki Sofra, David I.R. Holmes, Ian Zachary

Objective.—The regulation of endothelial cell adhesion molecules (CAMs) by vascular endothelial growth factor (VEGF) was investigated in cell cultures and in a rabbit model of atherogenic neointima formation.

Methods and Results.—VEGF regulation of vascular CAM-1 (vascular cell adhesion molecule), intercellular CAM-1 (intercellular adhesion molecule), and E-selectin were investigated in human umbilical vein endothelial cells using quantitative polymerase chain reaction, enzyme-linked immunosorbent assay, and flow cytometry, and in the rabbit collar model of atherogenic macrophage accumulation by immunostaining. VEGF alone caused no significant induction of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, or E-selectin compared with tumor necrosis factor-α. In both hypercholesterolemic and normal rabbits, adenoviral VEGF-A165 expression caused no increase in endothelial vascular cell adhesion molecule-1 or E-selectin. In contrast, pretreatment of human umbilical vein endothelial cells with VEGF significantly increased E-selectin expression induced by tumor necrosis factor-α, compared with tumor necrosis factor-α alone, whereas vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 were unaffected. VEGF similarly enhanced IL-1β-induced E-selectin upregulation. VEGF also synergistically increased tumor necrosis factor-α-induced E-selectin mRNA and shedding of soluble E-selectin. Synergistic upregulation of E-selectin expression by VEGF was mediated via VEGF receptor-2 and calcineurin signaling.

Conclusions.—VEGF alone does not activate endothelium to induce CAM expression; instead, VEGF “primes” endothelial cells, sensitizing them to cytokines leading to heightened selective pro-inflammatory responses, including upregulation of E-selectin. (Arterioscler Thromb Vasc Biol. 2007;27:494-502.)

Key Words: cell adhesion molecules • endothelial cells • IL-1β

The endothelium has several important functions that include providing a nonadhesive, nonthrombotic barrier between the blood and the underlying tissues. In atherosclerosis, or in response to injury or inflammatory cytokines such as tumor necrosis factor-α (TNF-α), the endothelium becomes activated and cell adhesion molecules (CAMs) are rapidly induced. In particular, members of the immunoglobulin superfamily of CAMs, such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as the selectin family members, E-selectin and P-selectin, have crucial roles in the adhesion and migration of monocyte/macrophage infiltration into atherosclerotic lesions during the early and subsequent stages of atherosclerosis in a variety of animal models.

Recent findings suggest that the angiogenic cytokine, vascular endothelial growth factor (VEGF or VEGF-A) may act as a proinflammatory cytokine by increasing ICAM-1, VCAM-1, and E-selectin mRNA in cultured endothelial cells through activation of nuclear factor-κB (NF-κB), an important transcription factor mediating CAM gene expression. So far, however, the relevance of VEGF-induced surface CAM expression for endothelial function has not been clarified. Given that VEGF is both a major focus of interest as a therapeutic angiogenic cytokine for coronary artery disease, and, paradoxically, has been reported to enhance atherosclerotic lesion formation, elucidation of the effects of VEGF on endothelial CAM expression has important practical, as well as biological, implications.

VEGF elicits an array of biological effects on endothelial cells in vivo and in vitro including survival, proliferation and migration, nitric oxide and prostacyclin (PGL2) production, and increased vascular permeability. VEGF exerts its actions by binding two cell surface protein kinase receptors, VEGF receptor (VEGFR)/KDR/Flk-1 and VEGFR1/Flt-1. Most biological responses triggered by VEGF in endothelial cells are mediated primarily by VEGFR2 and activation of multiple early signaling cascades, including phosphatidylinositol 3'-kinase (PI3K)-dependent Akt/PKB, phospholipase...
C-γ, mitogen-activated protein/extracellular signal-regulated kinase and calcineurin/nuclear factor of activated T-cells pathways.\textsuperscript{13–17}

In the present study, we investigated the ability of VEGF to stimulate CAM expression in human umbilical vein endothelial cells (HUVECs) and in vivo. VEGF induced minor changes in CAM mRNA, which were insufficient to increase CAM expression at the cell surface as determined by sensitive flow cytometry, although TNF-α strongly induced CAM mRNA and cell surface expression. Adenoviral overexpression of VEGF also had no effect on endothelial VCAM-1 and E-selectin expression in a rabbit model of neointima formation and neointimal macrophage accumulation. Surprisingly, when HUVECs were pre-incubated with VEGF before TNF-α treatment, E-selectin mRNA and cell surface expression were synergistically increased as compared with TNF-α alone, whereas other CAMs were unaffected. VEGF also synergistically enhanced TNF-α-induced shedding of soluble E-selectin. These findings demonstrate that VEGF is not able to stimulate endothelial CAM expression alone, but interacts with TNF-α to synergistically and selectively enhance E-selectin expression.

**Experimental Procedures**

**Materials and Methods**

For sources of cytokines, antibodies, and other reagents see supplemental experimental procedures (http://atvb.ahajournals.org).

**Cell Culture**

HUVECs were cultured as described online in the supplement.\textsuperscript{17}

**Collar Placement and Adenoviral Gene Transfer**

Animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Animal Care and Ethics Guidelines of University College London, UK. Maintenance of New Zealand White rabbits on a high-cholesterol diet, collar placement around the carotid artery, and delivery of adenoviruses (Ark Therapeutics Ltd, Kuopio, Finland) encoding VEGF\textsubscript{A\_{165}} (Ad.VEGF\textsubscript{A\_{165}}) or LacZ (Ad.LacZ) were performed as described.\textsuperscript{11}

**RNA Isolation, Reverse-Transcription Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA isolation from rabbit carotid arteries and HUVECs, reverse-transcription polymerase chain reaction, and quantitative real-time polymerase chain reaction were performed as described (supplemental Table I, available online at http://atvb.ahajournals.org).\textsuperscript{11,17,18}

**LacZ Staining and Immunohistochemistry**

Detection of β-galactosidase and immunostaining of VCAM-1, E-selectin, macrophages, CD31, and VEGF were performed as described.\textsuperscript{11}

**Morphometry and Image Analysis**

Intima/media ratios, and staining of CD31 (endothelial neovascularization), RAM-11 (macrophages), VCAM-1, and E-selectin in collared rabbit carotid arteries were quantified as previously described using Image J.\textsuperscript{11,18}

**Enzyme-Linked Immunosorbent Assay**

Analysis of total (cell surface and intracellular) VCAM-1 expression was performed as described.\textsuperscript{19} E-selectin, ICAM-1, and von Willebrand factor were analyzed by substituting anti-VCAM-1 with anti-E-selectin (5 μg IgG/mL), anti-ICAM-1 (1 μg IgG/mL), or anti-von Willebrand factor (1.2 μg IgG/mL).

**Flow Cytometry**

Cell surface VCAM-1, E-selectin, ICAM-1, and PECAM-1 was measured in confluent HUVECs as described.\textsuperscript{19}

**Soluble E-selectin Immunoassay**

Human soluble E-selectin (sE-selectin) was measured by commercial immunoassay in HUVEC culture supernatants.

**Statistical Analysis**

Differences between different treatment groups in rabbits were evaluated by ANOVA and Bon Ferroni correction (SPSS). Statistical analysis of cell culture experiments was performed using Student t test or 1-way or 2-way ANOVA with post hoc analysis by Fisher PLSD, when appropriate. Results are shown as means±SE or mean±SD, and \( P<0.05 \) was considered significant.

**Results**

**VEGF Does Not Upregulate Endothelial Protein Expression of E-selectin, ICAM-1, or VCAM-1**

The effects of VEGF on CAM mRNA levels were measured in HUVECs by quantitative real-time reverse-transcription polymerase chain reaction and normalized to GAPDH expression (Figure 1A). Tissue factor (TF) mRNA, previously shown to be markedly upregulated by VEGF,\textsuperscript{17} was increased \( \approx 70 \)-fold 90 minutes after addition of VEGF and returned to near control levels by 24 hours. VEGF increased E-selectin, VCAM-1, and ICAM-1 mRNA expression to maximum levels of, respectively, 7-fold after 45 minutes, 29-fold after 3 hours, and 2-fold after 1.5 hours (Figure 1A). Levels of PECAM-1 (CD31) mRNA, constitutively expressed by HUVECs, were unchanged by VEGF incubation (data not shown). To assess the biological relevance of VEGF-induced increases in CAM expression, we compared the effects of VEGF with those of the inflammatory cytokine, TNF-α. The results of this comparison showed that the modest inductions of CAMs by VEGF were negligible compared with the strong induction of E-selectin, ICAM-1, and VCAM-1 by TNF-α alone: \( \approx 900-, 300-, \) and 800-fold increases in mRNA, respectively (Figure 1B).

Because VEGF has previously been reported to stimulate protein expression of CAMs in endothelial cells,\textsuperscript{8,9} we examined the possibility that VEGF might increase CAM expression despite the relatively small effect of VEGF on mRNA induction compared with TNF-α. Although TNF-α treatment induced increases in E-selectin (Figure 1C), ICAM-1 (Figure 1D), and VCAM-1 (Figure 1E) of 6-fold, 5.6-fold, and 2-fold above basal unstimulated levels, respectively, VEGF failed to induce any significant increase in CAM expression after any of the incubation times tested (Figure 1C through 1E). In each experiment, von Willebrand Factor, which is known to be increased by VEGF,\textsuperscript{20} was significantly increased after 24-hour incubation with VEGF by \( 44.3\%\) (\( P<0.05, n=3, \) results not shown). Measurement of cell surface CAMs using flow cytometry confirmed the results of enzyme-linked immunosorbent assays, and was unable to detected a subpopulation of cells with increased CAM expression in response to VEGF; fluorescence-activated-cell sorter profiles provided clear evidence that CAM expression on the cell surface was
not increased by VEGF, whereas TNF-α strongly upregulated CAM expression (data not shown).

**Effect of VEGF on VCAM-1 and E-selectin Expression in a Model of Atherogenic Macrophage Accumulation**

Because of the key role thought to be played by upregulation of endothelial VCAM-1 in neointimal macrophage infiltration during the early stages of atherosclerosis, the effects of high-efficiency adenoviral VEGF-A165 (Ad.VEGF) expression on VCAM-1 expression were investigated in a model of neointimal thickening and neointimal macrophage accumulation induced by placement of an inert silastic collar around the carotid artery of rabbits fed a high-cholesterol diet. Staining for β-galactosidase in arteries transduced with Ad.LacZ revealed abundant strongly stained cells in the adventitia consistent with a high efficiency of gene transfer (≈5% to 10%) (supplemental Figure IA).

Expression of the VEGF transgene after periadventitial delivery of Ad.VEGF to carotid arteries was confirmed by reverse-transcription polymerase chain reaction (supplemental Figure IB). No VEGF transgene expression was detected in Ad.LacZ-transduced arteries, in segments of the transduced carotid arteries distal to the collared arterial region, in contralateral noncollared control arteries, or in nontargeted tissues (results not shown), indicating that the perivascular collar localized transgene expression to the collared region of the artery. Immunostaining of sections of transduced arteries with a specific antibody to VEGF showed strong expression of VEGF in the adventitia of Ad.VEGF-transduced arteries (supplemental Figure IC).

Consistent with previous findings,11 immunostaining with macrophage-specific RAM11 and specific VCAM-1 antibodies showed that neither neointimal macrophage accumulation nor endothelial VCAM-1 expression occurred in the collared arteries of rabbits fed a normal diet. Ad.VEGF delivery to collared arteries in rabbits on a normal diet significantly increased neointima formation and adventitial neovascularization, but had no effect on either neointimal macrophage accumulation or VCAM-1 expression (supplemental Figure ID; results not shown). However, as compared with Ad.lacZ, Ad.VEGF delivery had no significant neointima-increasing effect in the collared arteries of cholesterol-fed rabbits (Figure 2A). Collar placement in hypercholesterolemic rabbits induced lesions containing RAM11-positive macrophages and marked endothelial VCAM-1 and E-selectin expression (Figure 2B and 2C), but Ad.VEGF caused no significant increase in either neointimal macrophage accumulation or VCAM-1 expression (supplemental Figure ID; results not shown). However, as compared with Ad.lacZ, Ad.VEGF delivery had no significant neointima-increasing effect in the collared arteries of cholesterol-fed rabbits (Figure 2A). Collar placement in hypercholesterolemic rabbits induced lesions containing RAM11-positive macrophages and marked endothelial VCAM-1 and E-selectin expression (Figure 2B and 2C), but Ad.VEGF caused no significant increase in either neointimal macrophage accumulation or VCAM-1 expression (supplemental Figure ID; results not shown). However, as compared with Ad.lacZ, Ad.VEGF delivery had no significant neointima-increasing effect in the collared arteries of cholesterol-fed rabbits (Figure 2A).

**Figure 1.** VEGF does not increase CAM protein or mRNA levels in HUVECs. HUVECs were treated in triplicate with 25 ng/mL VEGF for the times indicated, or with 100 U/mL TNF-α for 6 hours. CAM mRNA level was measured by quantitative real time reverse-transcription polymerase chain reaction (A,B). CAM protein expression was analyzed by cell-bound enzyme-linked immunosorbertent assay (C to E). A, VCAM-1 ( ), yellow) and E-selectin ( , red) mRNA levels were modestly increased by 3 hours after addition of VEGF, whereas ICAM-1 ( , green) mRNA levels were virtually unchanged. TF mRNA levels increased as expected ( , blue). Reverse-transcription polymerase chain reaction results are expressed as fold-increases ±SD above the control level (in basal unstimulated cells) normalized to GAPDH expression. B, Levels of VCAM-1 (open bars), ICAM-1 (gray bars), and E-selectin (black bars) mRNA were measured in HUVECs treated for 1.5 hours (ICAM-1 and E-selectin) or 3 hours (VCAM-1) with 25 ng/mL VEGF-A (V), or for 6 hours with 100 U/mL TNF-α (T). VEGF failed to increase E-selectin (C), ICAM-1 (D), or VCAM-1 (E) protein levels, whereas TNF-α stimulated CAM expression in each case. Representative experiments are shown and findings were confirmed in 3 independent assays using different batches of HUVECs.
VEGF and TNF-α Synergize to Upregulate E-Selectin Expression

We next investigated the possibility that VEGF could modulate CAM expression induced by TNF-α. VEGF pretreatment of HUVECs for 24 hours before TNF-α stimulation for the last 6 hours of incubation increased E-selectin expression by 61.7 ± 5.8% from the level induced by TNF-α alone (Figure 3A; P = 0.004, n = 3). In contrast, TNF-α-induced ICAM-1 expression was not enhanced with VEGF pretreatment (Figure 3A).

Enzyme-linked immunosorbent assay data were confirmed by flow cytometry. Figure 3B shows a representative 2-dimensional fluorescence-activated cell sorter overlay histogram for cell surface E-selectin expression in HUVECs. The fluorescein isothiocyanate fluorescence profile showed that basal E-selectin expression in unstimulated cells was negligible (Figure 3B), similar to the profile of cells incubated with VEGF alone or to isotype-treated controls (data not shown). TNF-α treatment upregulated cell surface E-selectin, whereas VEGF pretreatment before TNF-α incubation further increased cell surface E-selectin expression by 77.2 ± 15.0% (Figure 3C, P < 0.005, n = 3). In parallel, VEGF preincubation did not significantly increase either ICAM-1 or VCAM-1 above TNF-α-induced levels (Figure 3C). Constitutive levels of PECAM-1 (ie, without TNF-α stimulation) were not altered significantly by VEGF (data not shown).

Synergistic upregulation of E-selectin expression by VEGF and TNF-α also occurred at 10 U/mL (0.5 ng/mL) TNF-α (Figure 3D). Indeed, pretreatment with VEGF before addition of 10 U/mL TNF-α induced a greater synergistic effect than 100 U/mL TNF-α (143% versus 71%, respectively, above the corresponding TNF-α controls), as shown by enzyme-linked immunosorbent assay.

IL-1β and TNF-α stimulated E-selectin protein expression to a similar extent, ≈ 25-fold above the level in control unstimulated cells (data not shown). IL-1β synergized with VEGF to enhance E-selectin expression (48.2 ± 10.5% increase from level of IL-1β control, P = 0.005, n = 3; Figure 3E), although in parallel cell cultures, TNF-α caused a greater synergistic effect with VEGF than IL-1β (77.2 ± 15.0% enhancement).

VEGF and TNF-α Synergize to Upregulate E-Selectin mRNA Levels

Quantitative real-time reverse-transcription polymerase chain reaction showed that VEGF and TNF-α synergize to increase E-selectin transcription (Figure 3F). The induction in E-selectin mRNA increased from ≈ 830-fold above the control unstimulated level with TNF-α alone to 1500-fold with VEGF and TNF-α (81% increase, P = 0.05, n = 3). Neither ICAM-1 nor VCAM-1 mRNAs were significantly enhanced by the combination of VEGF and TNF-α above the levels induced by TNF-α alone (data not shown). Consistent with previous findings, VEGF and TNF-α synergized to upregulate TF mRNA expression (P < 0.05), whereas VEGF/TNF-α caused no synergistic upregulation of COX-2 (supplemental Figure III). Although VEGF induces rapid upregulation of COX-2, COX-2 expression declines to basal levels after 6 hours and is not detectable after 24 hours.16,17

VEGF/TNF-α Synergy Requires Preincubation With VEGF

HUVECs were preincubated with VEGF for various times (0 to 24 hours) before TNF-α stimulation and then analyzed for E-selectin expression by flow cytometry to find the minimum preincubation time needed with VEGF for synergy to occur. The maximum synergistic effect of VEGF/TNF-α on E-selectin required at least 4 hours pretreatment with VEGF (Figure 4A).

The possibility that VEGF might enhance E-selectin expression via secretion of a soluble factor was tested by transferring media from VEGF-treated cells to another set
Figure 3. VEGF and TNF-α synergize to upregulate E-selectin expression. HUVECs were pretreated in triplicate with 25 ng/mL VEGF for 24 hours and then with 100 U/mL TNF-α for 6 hours (gray bars) or treated with TNF-α alone (black bar). A, E-selectin, determined by specific enzyme-linked immunosorbent assay, was increased by 61.7±5.8% (**P<0.01). Results show values for CAM expression above the basal unstimulated level normalized to the corresponding TNF-α control (both 100±4.2%, black bars), obtained from 3 independent experiments performed in triplicate (means±SE using different cell batches. B, Anti-E-selectin binding to HUVECs was detected using a secondary fluorescein isothiocyanate-conjugated antibody before fixation in 1% paraformaldehyde and flow cytometric analysis. The 2-dimensional overlay histogram shows a 3-decade-log fluorescence scale against the number of gated intact cells with corresponding fluorescein isothiocyanate fluorescence values from a representative experiment. Basal E-selectin expression was negligible (solid gray line), TNF-α (6 hours) upregulated cell surface E-selectin (dashed gray line), and VEGF preincubaton before TNF-α further upregulated E-selectin expression (solid black line). C, Cell surface E-selectin determined by fluorescence-activated cell sorter analysis. The 2-dimensional overlay histogram shows a 3-decade-log fluorescence scale against the number of gated intact cells.

of cultures that were then immediately incubated with TNF-α for 6 hours. The results showed that media from VEGF-treated cells did not enhance E-selectin expression (Figure 4B), suggesting that VEGF does not induce production of a diffusible secreted factor that mediates synergy with TNF-α. In the wells where the VEGF-containing media had been removed after incubation, addition of fresh media without VEGF but containing TNF-α still caused synergy (Figure 4B). These experiments confirmed that pre-incubation with VEGF is needed for synergy with TNF-α, although VEGF does not need to be present when cells are stimulated with TNF-α.
VEGF/TNF-α Synergy Is Mediated by VEGFR2 and the Calcineurin/Nuclear Factor of Activated T-Cells Signaling Pathway

We examined the receptor and second messenger mechanisms mediating the VEGF/TNF-α synergistic effect on E-selectin. Placental growth factor (PIGF), a ligand for VEGFR1 but not for VEGFR2, failed to synergize with TNF-α to enhance E-selectin expression, and PIGF had no effect on the VEGF/TNF-α synergy when HUVECs were preincubated with a combination of PIGF and VEGF (Figure 5A), as shown by flow cytometry. Preincubation with the specific VEGFR2 inhibitor, SU5614, almost completely blocked the synergistic effect on E-selectin expression (84.6±8.1% inhibition, \(P=0.005\)), indicating that VEGFR2 mediated this effect (Figure 5B).

Cyclosporin A (0.2 μmol/L), an inhibitor of the protein phosphatase activity of calcineurin/protein phosphatase 2B, partially blocked the VEGF/TNF-α synergism implicating the involvement of the calcineurin/nuclear factor of activated T-cells signaling pathway (59.3±0.8% inhibition \(P=0.03, n=3\); Figure 5C). Inhibition of either the extracellular signal-regulated kinase 1/2 pathway using the specific MAP kinase kinase (mitogen-activated protein/extracellular signal regulated kinase) inhibitor, U0126 (10 μmol/L), or PI3K-
E-selectin Shedding

Synergistic Increases in E-selectin Shedding

VEGF/TNF-α Synergism Increases E-selectin Shedding

After endothelial activation, surface E-selectin undergoes shedding to release soluble isoforms. To test the effect of VEGF on TNF-α-induced shedding, culture media were removed after VEGF pretreatment and subsequent incubation with TNF-α for 6 hours and replaced with fresh media not containing VEGF or TNF-α. Soluble E-selectin (sE-selectin) shed into the media was measured using a sensitive sandwich enzyme-linked immunosorbent assay (Figure 6). TNF-α-induced sE-selectin expression in HUVECs was detectable after 2 hours and increased up to 24 hours (Figure 6). While VEGF alone did not cause any detectable increase in the level of sE-selectin produced by HUVECs, VEGF preincubation synergistically increased TNF-α-induced shedding of sE-selectin at all time points, an effect that was statistically significant (P<0.05) after 6, 20, and 24 hours (Figure 6).

Discussion

The role of VEGF in atherosclerosis is contentious because studies have reached differing conclusions as to whether VEGF can either inhibit or promote intimal thickening and atherosclerotic lesion formation in a variety of animal models. A proatherogenic role of VEGF is supported by reports that VEGF upregulates expression of inflammatory CAMs in endothelial cells, including VCAM-1, ICAM-1, and E-selectin. Uprogulation of endothelial CAMs, particularly VCAM-1, is recognized to play a key role in the activation of endothelium in inflammation, and a key mediator of monocyte/macrophage adhesion and infiltration in early and later atherosclerotic lesions responsible for the excessive accumulation of macrophages in the atherosclerotic plaque. Whether or how VEGF regulates endothelial CAM expression is therefore a question of central importance for the pathophysiology of this cytokine, which also has ramifications for the therapeutic potential of VEGF in ischemic heart disease.

The first major finding of this article is that VEGF alone is unable to induce protein expression of endothelial VCAM-1, ICAM-1, and E-selectin in endothelial cells expressing high levels of VEGFR2/KDR. In addition, adenoviral overexpression of VEGF in a model of atherogenic neointima formation and macrophage accumulation also had no significant effect on endothelial VCAM-1 and E-selectin expression in vivo. Our previous findings showed that liposome-mediated plasmid VEGF gene delivery to the collared arteries of hypercholesterolemic rabbits, reduced neointima formation, neointimal macrophage accumulation, and endothelial VCAM-1 expression, but this study did not examine the effects of high-efficiency adenoviral VEGF gene delivery. Importantly, neither low- nor high-efficiency VEGF expression caused any increase in VCAM-1 expression. The different effects of plasmid and adenoviral VEGF on intimal thickening, macrophage accumulation, and VCAM-1 expression in the collar model may be attributable to an arterioprotective effect of low intra-arterial VEGF concentrations that is impaired at the higher local concentrations of VEGF produced by adenoviral expression. Interestingly, Ad.PIGF gene delivery in the same model in parallel with the study of Ad.VEGF presented here, increased neointimal thickening, macrophage accumulation and endothelial VCAM-1 expression in collared arteries in hypercholesterolemic rabbits. The present results therefore indicate a striking contrast in the biological effects of VEGF and PIGF in atherogenic lesion formation in rabbits.

Whereas VEGF significantly increased VCAM-1, ICAM-1, and E-selectin mRNA expression in endothelial cells, these effects were extremely small compared with the effect of TNF-α. Kim et al reported that VEGF increased VCAM-1, ICAM-1, and E-selectin protein expression, as determined by Western blot. In contrast, we found that VEGF had no significant effect on CAM protein expression as judged by sensitive and quantitative enzyme-linked immunosorbent assays of total cellular CAM expression or measurement of cell surface CAM expression by flow cytometry. The lack of CAM upregulation as determined by flow cytometry also precluded the possibility that there was a subpopulation of cells that could upregulate CAM expression in response to VEGF.

Previous studies examined effects of VEGF in freshly isolated HUVECs cultured in M199 medium with serum, whereas our studies used commercially available HUVECs cultured in supplemented EBM. However, we conclude that differences in the source and culture of HUVECs are unlikely to account for our failure to observe an effect of VEGF alone on CAM expression. TNF-α and IL-1β strongly upregulated CAM expression at protein and mRNA level indicating that the cellular mechanisms mediating transcriptional upregulation of CAMs are not impaired in commercial HUVECs. We show here and in previous studies that VEGF strongly induces an array of signaling and cellular...
responses in commercially obtained HUVECs, including upregulation of multiple genes.\textsuperscript{17,25} Furthermore, we have confirmed that several key signaling and biological responses to VEGF are preserved in low-passage number freshly isolated and in commercial HUVECs.\textsuperscript{20,25} Moreover, our previous findings together with the results of adenoviral VEGF overexpression presented here also indicate that VEGF is unable to significantly enhance endothelial VCAM-1 and E-selectin expression in vivo, even under inflammatory hypercholesterolemic conditions favoring CAM upregulation. Zhang and Isssekutz also showed that VEGF, in contrast to TNF-\( \alpha \), had no effect on ICAM-1, VCAM-1, and E-selectin expression in HUVECs as determined by enzyme-linked immunosorbent assay, although this study examined effects of a 3-day treatment.\textsuperscript{26} We provisionally conclude that differences between our findings and those of other researchers may be attributable to the different approaches used to measure CAM expression, although more work is needed to establish more precisely the reasons for these differences.

The second major finding of this paper is that whereas VEGF alone had no significant effect on CAM expression, it synergistically enhanced the induction of E-selectin mRNA and cell surface protein expression by TNF-\( \alpha \). This was a selective synergistic interaction because VEGF did not enhance TNF-\( \alpha \)-induced expression of VCAM-1 or ICAM-1. VEGF also synergized with TNF-\( \alpha \) to enhance shedding of the soluble extracellular domain of E-selectin. VEGF and TNF-\( \alpha \) have previously been shown to synergistically increase functional TF expression in endothelial cells, but it has been unclear whether cooperative interactions between these 2 cytokines are relevant for other endothelial responses. Synergistic enhancement of E-selectin expression by VEGF was mediated by VEGFR2/KDR; in addition, the inhibition of the effect by cyclosporin A, a specific inhibitor of calcineurin, indicates that a calcineurin-dependent pathway also plays a major role in this response. It has been reported that mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal-regulated kinase is the convergence point of the VEGF and TNF-\( \alpha \) signaling for the synergistic upregulation of TF, which leads to activation of the transcription factor early growth response-1.\textsuperscript{27} However, inhibition of mitogen-activated protein/extracellular signal regulated kinase did not block the synergistic enhancement of E-selectin. Early growth response-1 elements have been found in some CAM promoters but are unknown for E-selectin. The nuclear factor of activated T-cells/calcineurin pathway has been implicated in expression of E-selectin\textsuperscript{28} and VEGF-induced upregulation of TF,\textsuperscript{29} and Down syndrome critical region protein 1.\textsuperscript{17} Cyclosporin A has been reported to suppress E-selectin, but not VCAM-1, induction in HUVECs by TNF-\( \alpha \), even though the E-selectin promoter is activated by NF-\( \kappa \)B rather than nuclear factor of activated T-cells.\textsuperscript{28}

E-selectin has been implicated in angiogenesis in vitro\textsuperscript{30,31} and in vivo,\textsuperscript{32} although the underlying mechanisms remain to be defined. Synergistic augmentation of TNF-\( \alpha \)-regulated E-selectin expression by VEGF might therefore be relevant for neovascularization in an inflammatory milieu, as for example in pathophysiological settings such as atherosclerotic disease, cancer, and rheumatoid arthritis. E-selectin is proteolytically shed by an unknown mechanism with the resulting sE-selectin, lacking transmembrane and cytoplasmic domains, being biologically active and playing a role in angiogenesis through the Src-PI3K pathway.\textsuperscript{33,34} VEGF-dependent augmentation of sE-selectin generation induced by inflammatory cytokines may contribute to the pathogenesis of cardiovascular disease, possibly through enhanced angiogenesis within atherosclerotic lesions, a phenomenon that has been hypothesized to promote intraplaque hemorrhage and destabilization leading to rupture. However, E-selectin-deficient mice develop normally and exhibited no impairment in experimentally induced angiogenesis,\textsuperscript{35} indicating that VEGF-enhanced shedding of sE-selectin is unlikely to play a key role in developmental angiogenesis, although it is not precluded that it may contribute to pathophysiological angiogenesis in disease-specific settings. The role of sE-selectin in atherosclerosis is less clear because, whereas it has proinflammatory effects on neutrophil function,\textsuperscript{36} induces monocyte chemotaxis,\textsuperscript{37} and is significantly increased in patients with coronary artery disease, the increases observed have been small and, in several studies of human populations, sE-selectin levels have not emerged as a strong predictor of cardiovascular disease.\textsuperscript{23}

The importance of synergistic interactions between cytokines is becoming increasingly appreciated.\textsuperscript{37} However, this is the first report to our knowledge of enhancement of CAM expression by VEGF/TNF-\( \alpha \) synergy. Furthermore, VEGF also synergized with IL-1\( \beta \) to upregulate E-selectin. Because TNF-\( \alpha \) and IL-1\( \beta \) share many of the same signaling pathways, these findings suggest that VEGF-triggered signal transduction mechanisms can cooperate with a common pathway activated by inflammatory cytokines. VEGF may “prime” endothelial cells so they are capable of responding to lower levels of TNF-\( \alpha \). Consistent with this notion, we observed relatively more synergy with a lower concentration of TNF-\( \alpha \).

The present article is consistent with the broader conclusion that the predominant effect of VEGF alone is not proinflammatory and does not cause endothelial cell activation associated with cardiovascular disease. However, because VEGF can selectively enhance E-selectin expression induced by TNF-\( \alpha \) or IL-1\( \beta \), we propose that the overall impact of VEGF on endothelial function and, by extension, on vascular pathophysiology, may be modified by the local cytokine milieu. A fuller understanding of how VEGF interacts and synergizes with other endothelial cytokines will be key to delineating its role as both a therapeutic and pathogenic factor in cardiovascular disease.

**Sources of Funding**

This work was supported by British Heart Foundation grants RG/02/001 and BS/94001 to I.Z.

**Disclosures**

None.

**References**

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Arterioscler Thromb Vasc Biol. 2007;27:494-502; originally published online December 14, 2006;
doi: 10.1161/01.ATV.0000255309.38699.6c
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

**Materials**—Recombinant human VEGF$_{165}$ and Placental Growth Factor (PlGF) were from R&D Systems, Abingdon, UK. Cyclosporin A (CsA), SU5614 and U0126 were from Calbiochem (Nottingham, UK). Anti-von Willebrand Factor (vWF; clone F8/86), fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies and monoclonal anti-platelet endothelial CAM-1 (PECAM-1 or CD31; clone JC/70A) were from Dako (Ely, UK). Monoclonal antibodies: VCAM-1 (CD106; clone BBIG-V1), ICAM-1 (CD54; clone BBIG-I1) and E-selectin (CD62E; BBIG-E4) were from R&D Systems. Recombinant human TNF-[], IL-1[], and other reagents were from Sigma (Poole, UK), unless otherwise specified.

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were purchased from TCS CellWorks (Buckingham, UK). Cells were cultured in endothelial basal medium (EBM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 12 g/ml bovine brain extract and 50 g/ml gentamycin sulphate (Cambrex Bio Science, Wokingham, UK). For experiments, 1 % (v/v) serum-containing medium, in the absence of other supplements, was used. Plastic culture dishes were precoated with 1 % (w/v) gelatin, and cells were used between passages 1-4.

**Adenoviruses**—Adenoviral constructs (E1,E3-deleted) encoding either VEGF-A$_{165}$ (Ad.VEGF-A$_{165}$) or LacZ (Ad.LacZ) were produced by Ark Therapeutics, Finland, as described. Adenoviruses were desalted using G50 Sephadex columns (Roche Diagnostics Ltd, Lewes, UK) immediately prior to their use in animal studies.

**Collar Placement and Gene Transfer**—All experiments were conducted in accordance with the Animal Care and Ethics Guidelines of University College London, UK. New Zealand White male rabbits (2.5-3.2 kg) were fed a normal diet supplemented with 1.5%
cholesterol for 1 week prior to collar placement and throughout the experiment. In parallel, rabbits were maintained on normal chow without added cholesterol. Placement of a biologically inert, silastic collar (Ark Therapeutics) around the right carotid artery was performed in anesthetized rabbits as described. Five days later, the collared arteries were exposed and 100 µl of each adenoviral vector solution containing 5 x 10⁹ pfu, was placed within the space between collar and artery using a pipette, and the wound sutured.

Serum samples were taken for assay of total cholesterol, Low Density Lipoprotein- and High Density Lipoprotein-cholesterol (Roche Diagnostics Ltd), 7 days before initiation of the high-cholesterol diet, 1 day after initiating the diet and 9 days after gene transfer. Nine days after gene transfer, animals were euthanized; collared and contralateral control arteries were then excised. Arteries were flushed with ice-cold saline and divided into two segments. The proximal part was immersion-fixed in 1 % paraformaldehyde/phosphate buffered saline (PBS) pH 7.4, for 6 h, rinsed in 70 % ethanol, and embedded in paraffin. The distal part was either immersion-fixed in 4 % paraformaldehyde/PBS pH 7.4, for 30 min, rinsed in PBS for 15 min, cryoprotected in sucrose, embedded in OCT compound (Miles), and stored at −80˚C or snap frozen in liquid nitrogen and stored at −80˚C for total RNA extraction.

**RNA Isolation and Reverse-transcription (RT) PCR**—Total RNA was isolated from 30-50 mg frozen tissue (pooled from 2 carotids or non-targeted organs) using an adapted RNeasy spin-column method, as described (RNeasy Fibrous Tissue Mini Kit, QIAGEN, Crawley, UK). Total RNA (500ng) was reverse transcribed using Superscript III RT (Invitrogen, Paisley, UK) and random hexamers according to the manufacturer’s instructions. PCR was performed using Platinum® Taq DNA Polymerase (Invitrogen) using β-actin as a reference gene. Nested PCR was performed for the amplification of
VEGF-A165, using transgene specific primers (5’ primers selected from the CMV promoter and 3’ primers from the coding region). Primers for PCR of β-actin and VEGF-A165 were as described. PCR products were run on a 2 % agarose gel, alongside 50kb DNA markers (Invitrogen).

LacZ Staining—Detection of β-galactosidase activity was performed by overnight incubation of whole arterial segments or 6 μm sections in the dark at 37°C in β-gal staining solution as described.

Immunohistochemistry—The following antibodies were used: mouse IgG1 to rabbit VCAM-1 (Rb1/9; gift of MI Cybulsky) at a 1:100 dilution in frozen sections; E-selectin (10 μg/ml; R & D Systems), macrophage-specific RAM11 (1:50, Dako); mouse anti-human CD31 (1:500; Dako); smooth muscle cell-specific β-actin antibody (1:150; Dako); anti-VEGF mouse mAb sc-7269 (1: 200; Santa Cruz Biotechnology Inc, CA). Primary antibodies were diluted in Tris-buffered saline, pH 7.2. Staining was performed on deparaffinized or frozen sections as described and visualised using a Vectastain Elite ABC Kit (Vector Laboratories, Peterborough, UK). The 5 μm frozen sections were fixed for 10 min in acetone at −20°C and then air-dried. Negative controls for all immunostainings were performed by omitting the primary antibody.

Morphometry and Image Analysis—Images of sections at x5 and x40 were acquired with a high resolution colour camera (Zeiss microscope, Jenoptik Camera) and analyzed using automated image analysis software (Image J, National Institute of Health). Intimas were defined as the regions between luminal endothelium and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminas, and analysed blindly. Intima/media ratios (I/M) were determined in serial sections cut at 500 μm intervals and stained with haematoxylin and eosin; I/M values were averaged and
expressed as means ± SE Neovascularisation was quantified by counting the numbers of CD31-positive vessels. CD31 staining was regarded as positive if a single cell or a vessel with a lumen was present and numbers of CD31-positive cells and vessels were expressed per mm² total adventitial area. Total RAM-11 positive macrophages were counted in the intima and expressed per mm². VCAM-1 and E-selectin immunostaining was quantified using Image J on high resolution (1300 x 1030 pixel) images captured using OpenLab 3.14 software (Improvision Ltd), and for VCAM-1 is expressed as the number of pixels representing endothelial immunostaining as a percentage of the total endothelial pixel count, and for E-selectin is expressed as the positively stained area as a percentage of total tissue area in the sections.

**ELISA**—Analysis of total (cell-surface and intracellular) VCAM-1 expression was performed using confluent HUVECs in 96 well plates (2 × 10⁴ cells/well in quadruplicate) as described.¹⁹ E-selectin, ICAM-1 and vWF were analysed by substituting anti-VCAM-1 with anti-E-selectin (5 μg IgG₁/ml), anti-ICAM-1 (1 μg IgG₁/ml) or anti-vWF (1.2 μg IgG₁/ml). Cells were incubated with 25 ng/ml VEGF in 1 % serum-containing medium for times indicated. In some experiments TNF-α was added directly into the media for the last 6 h to a final concentration of 100 U/ml (5 ng/ml) (or other concentrations as indicated) except for basal (unstimulated) controls. Primary antibody binding was detected by StreptABComplex/HRP Duet kit and 0-phenylenediamine chromogenic substrate (Dako). Absorbances were measured at 492 nm by a microtitre plate spectrophotometer (SpectroMax 250). Cell protein per well was measured using the Bradford reagent (Bio-Rad Laboratories, Hemel Hempstead, UK).

**Flow Cytometry**—Cell surface VCAM-1, E-selectin, ICAM-1 and PECAM-1 was measured in confluent HUVECs (3 × 10⁵ cells/well in triplicate) as described.¹⁹ Cells were
incubated with factors as described for ELISA and in results. After washing with PBS, plates were placed on ice and the cells gently removed using a cell lifter. Primary antibody binding and negative control antibody binding (at equivalent IgG1 concentrations) were detected using goat anti-mouse FITC-conjugated antibodies. Intact cells were gated on forward scatter versus side scatter linear amplifications and 5 \( \times \) \( 10^3 \) cells were analysed for FITC fluorescence per well by FACScan (BD Biosciences, Cowley, UK) using Cellquest software.

**Reverse Transcription and Quantitative Real-time PCR**—After treatment of confluent HUVECs with factors as described in results, total RNA was extracted by using RNeasy Mini Kit (QIAGEN), and quantitative real-time PCR was performed as described\(^\text{17}\) using primers for CAMs (MWG-BIOTECH Ltd, Milton Keynes, UK) to sequences in the 3’ untranslated region of the genes to ensure the specificity of the fragment, and ensuring that amplified fragments were 200-300 base pairs in size (see supplementary Table I). Amplification of fragments of the predicted size was verified by conventional RT-PCR methodology. Real-time PCR was performed by using the LightCycler-FastStart DNA Master SYBR Green I Kit and Lightcycler System with LightCycler3 Software (Roche Diagnostics). Data were normalised to the reference gene, GAPDH, and are presented as the mean fold changes compared with control.

**Soluble E-selectin (sE-selectin) Immunoassay**—Confluent HUVECs (3 \( \times \) \( 10^5 \) cells/well in triplicate) were preincubated with factors as described. Media was then replaced with fresh 1 % serum-containing medium and cells incubated for the times indicated. Cell culture supernatants were centrifuged (15 000 g, 10 min) to remove particulates, diluted 1:1 with sample diluent and assayed by commercial immunoassay for human sE-selectin (R&D Systems). The optical density was read at 450 nm with the wavelength correction set at
650 nm for increased accuracy. The lower limit of detection of sE-selection was typically 
\(~0.5\) ng/ml. The intra-assay and inter-assay coefficients of variation were 4.6 % and 13.2 
%, respectively. After removal of media supernatants, HUVEC monolayers were lysed in 
lysis buffer [50 mM Tris-HCL, 150 mM NaCl, 10 mM EDTA, 1 % (v/v) Triton X-100, 0.5 
% (v/v) NP40 and protease inhibitor cocktail tablets (Complete\textsuperscript{TM}; Roche Diagnostics), 
pH7.6] and total cellular protein assayed using the Bradford assay.

*Statistical Analysis*—Differences in VCAM-1 expression and morphometric differences 
between different treatment groups in rabbits were evaluated by ANOVA and Bon 
Ferroni Correction (SPSS). Data were considered statistically significant at \( p<0.05 \). The 
statistical analysis of independent cell culture experiments was performed using Student’s \( t- \) 
test, or, alternatively, one-way or two-way ANOVA with *post hoc* analysis by Fisher’s 
PLSD, where appropriate. Results are shown as means ± SE or ± SD, and \( p<0.05 \) was 
considered significant.
FIGURE LEGENDS FOR SUPPLEMENTARY DATA

FIG. 1. Effects of Ad.VEGF delivery on intimal thickening in hypercholesterolaemic rabbits. Panel A - periadventitial Ad.lacZ delivery to collared carotid arteries resulted in efficient β-galactosidase expression predominantly in the adventitia. Panel B - RNA was extracted from collared transfected carotid arteries in cholesterol-fed rabbits, and VEGF-A165 transgene expression was determined by RT-PCR in the presence (+) or absence (-) of template using β-actin as a reference gene. A predicted PCR product of 547 bps corresponding to VEGF-A165 was detected only in arteries transduced with the appropriate adenovirus and was absent from Ad.lacZ-transduced arteries (lac), or the adenoviral backbone (Ad), or in uninfected control contralateral arteries from the same rabbit (CA). A product of 90 bps corresponding to β-actin was present at a similar level in all samples. RT-PCR of Ad.PIGF2 transduced in parallel into rabbit carotid arteries was used as a negative control, and Ad.VEGF-A165 expressed in HUVECs as a positive control (+ve). Panel C - arterial VEGF-A expression was detected by immunostaining with a specific antibody for VEGF-A. Ad.lacZ-infected and uninfected sham-operated (control) arteries stained with VEGF-A antibody, showed little detectable expression. VEGF-A immunostaining in Ad.VEGF-A165-transduced arteries was detected mainly in the adventitia, with some neointimal staining. Panel D - collar placement and gene transfer were performed in rabbits on a normal diet and I/M ratios were determined in collared carotid arteries infected with Ad.lacZ (n = 10), or Ad.VEGF-A165 (n=10) as described in Experimental Procedures. Intimal thickening was significantly increased in Ad.VEGF-A165-transduced rabbit carotid arteries. Representative H&E sections indicating the position of the internal elastic lamina (IEL) are shown.
FIG. II. Angiogenic response to Ad.VEGF in collared arteries

A. Sections of collared carotid arteries in rabbits on a high cholesterol diet infected with either no adenovirus (control), or Ad.lacZ, or Ad.VEGF-A<sub>165</sub> were immunostained with CD31 antibody. CD31 staining is shown at x10 (upper and lower left photomicrographs) and x40 magnification (lower right, magnification of boxed area in lower left photomicrograph). An increase in CD31-positive microvessels occurred in the adventitia of arteries transduced with Ad.VEGF-A<sub>165</sub>. 

B. The numbers of adventitial CD31-positive microvessels and cells in rabbits fed high cholesterol or normal diets were quantified and results are presented as CD31-positive vessels/mm<sup>2</sup>; *p < 0.05 for Ad.VEGF-A<sub>165</sub> versus Ad.lacZ.

FIG III. VEGF and TNF-α synergistically increase TF mRNA levels. HUVECs were pre-treated for 24 hours either with (VEGF, VEGF+TNF-α) or without (basal, TNF-α) 25 ng/ml VEGF, and then incubated with 100 U/ml TNF-α for a further 6 hours. Expression of mRNA for TF (A) and COX-2 (B) was then determined by quantitative real time RT-PCR. TF was increased by VEGF/TNF-α (grey bar, p<0.05) compared with TNF-α alone. COX-2 showed no synergistic upregulation with VEGF and TNF-α (C). Bars show means ± SE from 3 independent experiments using different cell batches.
Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’ to 3’</th>
<th>Reverse 5’ to 3’</th>
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<tr>
<td>GAPDH</td>
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<td>COX-2</td>
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<td>CTGAGGCACTGAAACATTCG</td>
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Fig 1

A

B

PIGF2 VEGFA
M CAAd lac + + +ve M
VEGFA
β-actin
547bp
90bp

C

Ad.LacZ
Ad.VEGFA
Ad.VEGFA x40

D

Normalized

LacZ VEGFA

Ad.LacZ IEL
Ad.VEGFA
Fig II

A

Control
Ad.LacZ
Ad.VEGF

B

High cholesterol
Normal

CD31/mm²

Ad.LacZ  Ad.VEGF
0  5  10  15  20  25  30  35

*
Fig III

A

TF/GAPDH fold change

basal  VEGF  TNF-α  VEGF + TNF-α

B

COX-2/GAPDH fold change

basal  VEGF  TNF-α  VEGF + TNF-α