**VEGF Blockade Inhibits Lymphocyte Recruitment and Ameliorates Immune-Mediated Vascular Remodeling**

Jiasheng Zhang, Teresa Silva, Timur Yarovinsky, Thomas D. Manes, Sina Tavakoli, Lei Nie, George Tellides, Jordan S. Pober, Jeffrey R. Bender, Mehran M. Sadeghi

**Rationale:** There are conflicting data on the effects of vascular endothelial growth factor (VEGF) in vascular remodeling. Furthermore, there are species-specific differences in leukocyte and vascular cell biology and little is known about the role of VEGF in remodeling of human arteries.

**Objective:** We sought to address the role of VEGF blockade on remodeling of human arteries in vivo.

**Methods and Results:** We used an anti-VEGF antibody, bevacizumab, to study the effect of VEGF blockade on remodeling of human coronary artery transplants in severe combined immunodeficient mice. Bevacizumab ameliorated peripheral blood mononuclear cell–induced but not interferon-γ–induced neointimal formation. This inhibitory effect was associated with a reduction in graft T-cell accumulation without affecting T-cell activation. VEGF enhanced T-cell capture by activated endothelium under flow conditions. The VEGF effect could be recapitulated when a combination of recombinant intercellular adhesion molecule 1 and vascular cell adhesion molecule-1 rather than endothelial cells was used to capture T cells. A subpopulation of CD3+ T cells expressed VEGF receptor (VEGFR)-1 by immunostaining and FACS analysis. VEGFR-1 mRNA was also detectable in purified CD4+ T cells and Jurkat and HSB-2 T-cell lines. Stimulation of HSB-2 and T cells with VEGF triggered downstream ERK phosphorylation, demonstrating the functionality of VEGFR-1 in human T cells.

**Conclusions:** VEGF contributes to vascular remodeling in human arteries through a direct effect on human T cells that enhances their recruitment to the vessel. These findings raise the possibility of novel therapeutic approaches to vascular remodeling based on inhibition of VEGF signaling. 

**Key Words:** vascular endothelial growth factor ■ bevacizumab ■ vascular remodeling ■ transplantation ■ T lymphocytes

**Integrative Physiology**

Vascular endothelial growth factor (VEGF, also referred to as VEGFA) is produced in response to hypoxia, growth factors (eg, epidermal growth factor, platelet-derived growth factor), and proinflammatory cytokines (eg, interleukin-1α and interleukin-6) by endothelial cells (ECs), leukocytes (monocyte/macrophages and T cells), and a number of other cell types. In addition to its well-known role in promoting angiogenesis, VEGF plays an important role in leukemic cell growth and inflammatory disorders. VEGF contributions to the pathogenesis of vascular pathology include promoting angiogenesis, reendothelialization, vascular smooth muscle cell (VSMC) migration, and inflammation in the vessel wall.

Vascular remodeling, as in graft arteriosclerosis and post-angioplasty restenosis, is a common feature of many vascular diseases. There are conflicting data on the role of VEGF in vascular remodeling. As such, VEGF may promote neointima, inhibit neointima, or have opposing effects based on its endogenous or exogenous origin. Given this controversy on the role of VEGF in vascular remodeling and species-specific differences in leukocyte and vascular cell biology between mice and humans, it is difficult to speculate what role, if any, VEGF plays in remodeling of human arteries. In the present study, we sought to address the role of human VEGF in remodeling of human arteries in vivo using chimeric human/mouse models of vascular remodeling and a specific anti-human (but not anti-mouse) VEGF antibody. We demonstrate that specific blocking of human VEGF ameliorates neointima formation in transplanted human coronary arteries and that this effect is, at least in part, through direct effects on T-cell trafficking. Finally, we demonstrate that VEGF enhances human T-cell binding to the endothelium.
under flow and identify a subpopulation of T cells that express potentially functional VEGF receptor (VEGFR)-1.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animal Models

Human coronary artery transplantation in immunodeficient mice was performed as described. In brief, adjacent segments of human coronary artery were implanted into the infrarenal aortae of 8- to 12-week-old C.B-17 severe combined immunodeficient (SCID)/beige mice. A group of animals received 1×10⁶ human peripheral blood mononuclear cells (PBMCs) per mouse (or control buffer), injected intraperitoneally 1 week after transplantation. Other animals were injected with replication incompetent Ad5.CMV-human interferon-γ (IFN-γ) or Ad5.CMV-LacZ through the jugular vein. Bevacizumab or control human immune globulins were administered at a dose of 5 mg/kg on 3 times per week, starting with PBMC transfer or injection of adenovirus. Animals were euthanized at 4 weeks after PBMC transfer or adenovirus injection (5 weeks after coronary artery transplantation), and transplanted arteries were removed and frozen in OCT for further analysis. All experiments were performed under protocols approved by Yale University Institutional Animal Care and Use and Human Investigation Committees.

Results

VEGF and VEGFR Expression in Immune-Mediated Vascular Remodeling

Transplantation of segments of human coronary artery to the abdominal aorta of SCID mice followed by adoptive transfer of allogeneic human PBMCs (of which only memory T cells reconstitute the host) led to significant neointima formation and expansive remodeling over a period of 4 weeks, as previously described. The intima and total vessel areas increased from 0.21±0.05 mm² and 0.62±0.12 mm² in control animals that were not given PBMC to 0.67±0.16 mm² and 1.12±0.17 mm² at 4 weeks after PBMC inoculation (n=5, P=0.004 and 0.005, respectively) (Figure 1a and Online Figure I). Both VEGF and its receptor, VEGFR-1, were readily detectable by immunostaining in transplanted coronary arteries, whether in the presence or absence of PBMC transfer, and predominantly localized to the neointima and media. VEGFR-2 expression was less apparent and mainly localized to the luminal endothelium (Figure 1b). There was no significant difference in 18S rRNA-normalized VEGFR-1 mRNA levels detected by quantitative RT-PCR between the 2 groups of animals (n=5 per group). Normalized VEGFR-2 levels were slightly but significantly decreased in transplanted arteries after PBMC transfer (n=5, P<0.05, Figure 1c).

VEGF Inhibition and Modulation of Immune-Mediated Vascular Remodeling

To address the role of VEGF in the pathogenesis of immune-mediated vascular remodeling, we investigated the effect of bevacizumab, a function-blocking anti-human (but not murine) VEGF antibody, on PBMC-induced vascular remodeling in SCID mice transplanted with human coronary arteries. Bevacizumab (5 mg/kg IP, 3 times a week) significantly reduced neointima (0.74±0.07 mm² and 0.52±0.09 mm², respectively, for the control and bevacizumab-treated groups, n=10, P=0.004) and increased lumen areas (0.17±0.06 mm² and 0.26±0.07 mm², respectively, for the control and bevacizumab-treated groups, n=10, P=0.049) at 4 weeks after PBMC reconstitution, demonstrating a nonredundant causal role of human VEGF in this model of immune-mediated human vascular remodeling (Figure 1 and Online Figure 1).

T-cell recruitment, activation, and IFN-γ secretion are key steps in PBMC-induced vascular remodeling. Therefore, to address the mechanism(s) of the modulatory effect of VEGF inhibition, we assessed the effect of bevacizumab on T-cell infiltration, activation, and cytokine production in human coronary artery transplants. There was a significant reduction in the number of intimal human CD45+ cells (leukocytes) in animals treated with bevacizumab (824±152 and 367±121, respectively, for the control and bevacizumab-treated groups, n=7, P=0.02, Figure 3a and 3b), indicating that VEGF blockade inhibits leukocyte accumulation in transplants. This was confirmed by quantitative RT-PCR that demonstrated a significant reduction in GAPDH-normalized human CD3ε transcripts in animals treated with bevacizumab (to 45±15%, n=7, P=0.039, Figure 3c). Similarly, a trend toward reduction of the number of CD69+ cells was detected in animals treated with bevacizumab (Figure 4a and 4b). However, bevacizumab did not affect leukocyte activation beyond its effect on reducing leukocyte infiltration (ratio of infiltrating CD69+ to CD45+ cells: 0.18±0.04 and 0.35±0.12, respectively, for the control and bevacizumab-treated groups, n=7, P=NS; Figure 4c). There was no clear difference in CD31 (EC), α-actin (VSMC), VEGF, or VEGFR expression pattern in arteries from control and bevacizumab-treated animals (data not shown).

Next, we assessed the effect of VEGF inhibition on IFN-γ production in the arterial transplants by quantitative RT-PCR. There was no significant difference in the ratio of IFN-γ to CD3ε transcripts between animals treated with bevacizumab and those treated with the control IgG, indicating that VEGF inhibition does not alter IFN-γ expression by T cells in this model (IFN-γ to CD3ε transcript ratio: 0.21±0.04 and 0.19±0.07, respectively, for the control and bevacizumab-treated groups; n=6, P=NS; Figure 4d).

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>SCID</td>
<td>severe combined immunodeficient</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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VEGF Inhibition and IFN-γ–Induced Vascular Remodeling

IFN-γ is a key mediator of PBMC-induced vascular remodeling that can induce remodeling in transplanted arteries in the absence of allogeneic PBMC transfer. Unlike the neointima of PBMC-induced remodeled arteries (which is mainly composed of infiltrating human memory T cells), the neointima in IFN-γ–induced arterial remodeling is rich in VSMCs. To establish whether the observed effect of bevacizumab on reducing vascular remodeling is dependent on...
on its modulatory effect on leukocyte recruitment, we assessed the effect of VEGF blockade on IFN-γ-induced arterial remodeling. As expected, infection with Ad-IFN-γ in SCID mice transplanted with human coronary artery led to a significant increase in the transplant intima and total vessel areas over a period of 4 weeks (intima area: 0.25±0.02 versus 0.12±0.02 mm², and total vessel area: 0.71±0.03 mm² versus 0.43±0.05 mm², respectively, for IFN-γ and control, Lac Z adenovirus-treated animals, n=4 in each group, P=0.037 and 0.02, respectively) (Figure 5a and Online Figure I). A similar pattern of VEGF and VEGFR expression was detected in IFN-γ–induced remodeled arteries (data not shown). However, contrary to its effect on PBMC-induced vascular remodeling, bevacizumab treatment failed to demonstrate any inhibitory effect on IFN-γ–induced vascular remodeling (intima area, 0.38±0.06 mm² versus 0.34±0.07 mm², and total vessel area: 0.93±0.08 mm² versus 0.83±0.10 mm², respectively, for the control and bevacizumab-treated groups, n=5 per group; A indicates adventitia; M, media; and I, intima. Scale bar: 50 μm.)
in each group, \( P=\text{NS}, \) Figure 5b), suggesting that the observed inhibitory effect of bevacizumab on vascular remodeling is dependent on the presence of T cells.

**VEGF and T-Cell Adhesion to Endothelium**

T-cell recruitment to transplanted arteries involves a cascade of events, including chemotaxis, adhesion to ECs, and transmigration, which may be targets of VEGF stimulation. The IFN-\( \gamma \) and VEGF-inducible IP-10 is a readily detectable chemokine in transplanted arteries and is believed to play a role in the recruitment T cells.\(^{15,17}\) We assessed the effect of bevacizumab treatment on IP-10 expression by quantitative RT-PCR (data not shown). Unexpectedly, bevacizumab treatment had no inhibitory effect on IP-10 expression in transplanted arteries. To address whether VEGF has a direct effect on T-cell adhesion to ECs and/or transendothelial migration, we assessed the effect of VEGF on T cells (5 minutes) had no effect on T-cell transmigration through the endothelium under shear stress (data not shown) but significantly increased the number of T cells adhering to the ECs (Figure 6a). The VEGF effect could be recapitulated when recombinant intercellular adhesion molecule 1 (ICAM-1) alone or a combination of recombinant ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), rather than ECs, was used to capture T cells under shear stress (Figure 6b). Pretreatment of T cells with SU5416 or preincubation of ECs with an anti–ICAM-1 antibody inhibited VEGF-induced T-cell adhesion to TNF-treated ECs (Online Figure III).

**VEGF Receptors in T cells**

VEGF-induced enhanced T-cell capture by ECs is highly suggestive of direct effects of VEGF on T cells or at least a subpopulation of T cells. This would imply that T cells express a functional VEGF receptor. In fact, immunostaining of PBMC smear demonstrated the presence of a population of CD3+ T cells that express VEGFR-1 on their surface (Figure 7a). By flow cytometry, 1.2±0.2% of CD3+ cells (\( n=3 \)) were found to express VEGFR-1 (Figure 7b). Quantitative RT-PCR confirmed expression of VEGFR-1 (but failed to convincingly detect expression of VEGFR-2) mRNA by purified CD4+ and CD4+CD45RA− memory T cells (Figure 7c). Jurkat and HSB-2 human transformed T-cell lines
also expressed detectable levels of VEGFR-1 mRNA and protein but lacked VEGFR-2 (Figure 7c and 7d). Using directly conjugated antibody for VEGFR-1 and flow cytometry, we confirmed surface expression of VEGFR-1 by Jurkat and HSB-2 cells (Figure 7e). Stimulation of HSB-2 cells with VEGF triggered detectable but transient phosphorylation of ERK, demonstrating the functionality of VEGFR-1 in this cell line (Figure 7f). Finally, consistent with the presence of a population of VEGFR-1–expressing T cells, VEGF induced ERK phosphorylation in a subset of CD3+ T cells, as detected by intracellular staining and flow cytometry (Figure 7g and Online Figure IV). Similarly, ERK phosphorylation could be induced in a subset of CD3+ T cells on stimulation with placenta growth factor, a VEGFR-1–specific ligand,1 demonstrating the functionality of VEGFR-1 in human T cells (Figure 7g).

**Discussion**

In the present study, we provide new information on the role of VEGF in vascular remodeling, demonstrating that human VEGF inhibition with bevacizumab ameliorates immune-mediated arteriosclerosis. Although early studies suggested that VEGF may prevent vascular remodeling by enhancing reendothelialization,3 other studies pointed to a more nuanced picture where, depending on the species and source of VEGF, it may enhance or inhibit neointima formation.5–8 The VEGF effect on vascular remodeling is probably multifaceted and may include modulation of VSMC migration,20,21 angiogenesis, and immune and inflammatory responses.4,17,22–25 The immune-modulatory effects of VEGF have been linked to modulation of monocytes2,22,25,26 and/or lymphocyte recruitment17,25 via regulation of angiogenesis,25 chemokine production (IP-10, monocyte chemotactic protein-1),17,22,25 EC activation,27 and dendritic cell maturation.28 In parallel, through induction of nitric oxide production by ECs, VEGF can function as an antiinflammatory agent and limit VSMC proliferation.29,30 An extra layer of complexity in interpreting these observations is raised by established differences in VEGF biology between mice and humans. For example, it is reported that the VEGF effect on chemokine production by ECs is species-specific. VEGF alone can induce IP-10 in murine but not human ECs, where it can potentiate the IFN-γ effect.17

The chimeric human/mouse models of immune-mediated vascular injury provided us with a unique opportunity to investigate the effect of human VEGF blockade on vascular remodeling in human coronary arteries. Because of the species-specificity of the bevacizumab effect,10 one can be reasonably confident that human VEGF, presumably produced by human vascular cells and/or adoptively transferred human PBMCs, is involved in the pathogenesis of remodeling in this model. Although we did not directly investigate the source of human VEGF in this model, ECs, VSMCs,31 and leukocytes (monocyte/macrophages and activated T lymphocytes)32–34 have been shown to produce VEGF under specific experimental conditions. VEGF was detectable by immunostaining in transplanted arteries. Although it is possible that VEGF is produced elsewhere and deposited in the vessel wall,3 its nonuniform distribution suggests that it is locally produced by vascular cells exposed to ischemia/reperfusion, cytokines, or growth factors.35 Interestingly, VEGF protein is detected in transplanted arteries 5 weeks after transplantation, presumably long after the initial exposure to ischemia, even in the absence of PBMC transfer. The molecular mechanisms of this sustained VEGF expression in the transplanted arteries remains to be determined.

Bevacizumab ameliorated PBMC-induced but not the closely related IFN-γ–induced vascular remodeling. In addition, VEGF blockade had no apparent effect on T-cell activation and polarization. Memory CD3+ T lymphocytes expressing the CD45RO marker constitute the bulk of transplant-infiltrating human leukocytes in this model, where very few CD68+ macrophages and dendritic cells are detectable,15 whereas VSMCs constitute the major cells of neointima in IFN-γ–induced vascular remodeling.13,16 This led us to identify leukocyte recruitment as the critical step in the observed modulatory effect of bevacizumab on GA. Although we did not directly address this possibility here, VEGF might facilitate lymphocyte recruitment through induction of endothelial adhesion molecules, ICAM-1, and VCAM-1.27 The direct effects of VEGF on mononuclear cells6,37 are well recognized. However, much less is known about the direct VEGF effect on T-cell responses. It is reported that VEGF is involved in T-cell recruitment in alloimmunity.17,38 Whereas IP-10 production plays a part in this VEGF effect, other yet to be discovered mechanisms appear to play a major role in the VEGF effect in alloimmunity.17 Similar to our own findings, previous studies have failed to demonstrate an effect of VEGF on lymphocyte activation.17 It is reported that VEGF modifies rat T-cell cytokine secretion profile toward a Th1 phenotype.10 However, we did not detect any effect of VEGF in vitro (data not shown) or its blockade in vivo on IFN-γ...
Figure 7. VEGF receptor expression and signaling by T cells. a, Immunofluorescent staining of PBMC smear, demonstrating VEGFR-1 expression (stained in red) on a subset of CD3+ T lymphocytes stained in green (arrow). Nuclei are stained by DAPI in blue. Insets show a higher magnification of a CD3 positive VEGFR-1–positive cell. The figure is representative of 3 independent experiments. b, Flow cytometry of lymphocytes demonstrating surface expression of VEGFR-1 in a subset of CD3+ T cells. The figure is representative of experiments with blood from 3 different donors. c, VEGFR-1 expression in T-cell subsets. Total RNA from purified CD4+ CD45RA− Jurkat, and HSB-2 cells was isolated and used to detect VEGFR-1 mRNA by quantitative RT-PCR. The levels of VEGFR-1 mRNA expression were normalized to the levels of GAPDH. The figure is representative of 2 independent experiments. d, Lysates from Jurkat and HSB-2 cells (50 μg/lane) and MVEC (30 μg/lane) were analyzed for expression of VEGFR-1 and VEGFR-2 by immunoblotting. HSP90 was used as a loading control. The figure is representative of 3 independent experiments. e, Surface expression of VEGFR-1 in Jurkat and HSB-2 cells is shown after staining with PE-conjugated VEGFR-1 or isotype control antibody and by flow cytometry. The figure is representative of 4 experiments. f, Phosphorylation of ERK in HSB-2 cells that were incubated with 50 ng/mL VEGF for the indicated time was detected by immunoblotting. Bar graph represents the data from 3 independent experiments. *P<0.05 compared with time 0. g, Phosphorylation of ERK detected by intracellular staining and flow cytometry in a subset of CD3+ PBMCs incubated for 10 minutes with VEGF (50 ng/mL) or placenta growth factor (100 ng/mL). Phorbol 12-myristate 13-acetate (PMA, 50 nmol/L) was used as positive control for ERK phosphorylation. The figure represents 1 of 3 experiments with similar results.
production. Searching for potential additional mechanisms, and in the absence of a demonstrable inhibitory effect of bevacizumab on IP-10 production, we hypothesized that VEGF may directly act on T lymphocytes and modulate their recruitment to the vessel wall. Therefore, to investigate whether VEGF can affect other critical steps in T-cell recruitment to the artery, namely, T-cell adhesion to the endothelium and transendothelial migration, we studied the effect of VEGF under both static and flow conditions. CD4+ effector memory cells expressing integrin receptors for endothelial adhesion molecules readily migrate to the sites of inflammation and mediate allogeneic responses in reconstituted SCID/beige mice. To dissociate the VEGF effects on ECs from its potential effects on T cells, VEGF signaling in TNF-activated ECs was inhibited with SU5416. VEGF had little effect on T-cell binding to ECs under static conditions (data not shown). However, it significantly enhanced T-cell capture by activated ECs under flow conditions. This effect was recapitulated when a combination of recombinant ICAM-1 and VCAM-1 replaced activated ECs.

VEGFs, the prototypic member of the VEGF family of glycoproteins, interacts with several receptor tyrosine kinase VEGFRs. VEGFR-2 is the main mediator of mitogenic effects of VEGF in ECs, where VEGFR-1 may serve as a decoy receptor to fine-tune VEGF responses. VEGF binding to VEGF-2 triggers a cascade of signaling events, including p44/42 MAPK and AKT phosphorylation, which regulate EC proliferation and migration. VEGFR-1 is the predominant VEGF receptor in a number of nonendothelial cells, including VSMCs and monocytes. Compared with VEGFR-2, less is known about signaling events triggered by VEGFR-1 activation. Our data demonstrate that at least a subset of human T cells (as well as 2 human T-cell lines), express VEGFR-1. Although we are yet to fully characterize this population, another group of investigators recently reported that activated murine T cells express VEGFR-1 and can migrate in response to VEGF. VEGF- and placenta growth factor–induced ERK phosphorylation demonstrated the functionality of VEGFR-1 in human T cells in our study.

VEGF-induced T-cell capture by recombinant ICAM-1 alone or to a greater degree to a combination of ICAM-1 and VCAM-1 (which, unlike ICAM-1, supports rolling) suggests that VEGF may alter integrin expression and/or activation of T cells. VEGF signaling through VEGFR-2 can activate β1, β3, and β5 integrins on ECs. It is reasonable to speculate that a similar mechanism may exist for VEGFR-1 in T cells. The flow requirement in the VEGF-induced augmented adhesion suggests an additive or synergistic effect on leukocyte integrin activation and/or expression. ECs express mechanosensory complexes that contain VEGFR-2, PECAM-1, VE-cadherin, and αvβ3. Flow-induced VEGF-2 activation (in the absence of ligand) has been demonstrated, as has an altered cellular pattern of αvβ3 in its active conformation. Although these responses have not been demonstrated in leukocytes exposed to flow, it is likely that an analogous mechanosensory complex exists in T cells, linking VEGF responses and integrin activation. We are currently studying the effects of VEGF and flow on T-cell β1 and β2 integrin function. Together, our data strongly support a role for VEGF in regulating T-cell recruitment in immune-mediated vascular remodeling, possibly through regulation of VEGFR-1 signaling. A similar phenomenon may exist in other forms of vascular remodeling. Although we have not directly assessed the effect of T-cell VEGFR-1 blockade on neointima formation in our model, given the challenges of T-cell–specific growth factor receptor targeting in vivo, it is interesting to note that VEGFR-1 tyrosine kinase–deficient mice display reduced neointima formation after cuff placement. This effect may also be, at least in part, mediated by T cells. These VEGF effects raise the possibility of novel therapeutic approaches to vascular remodeling based on inhibition of VEGF signaling. Given the important role of T cells in many aspects of immunity, the implications of our findings go beyond vascular diseases and may extend to other immune and inflammatory disorders.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis, leukemic cell growth, and inflammation.
- Vascular remodeling is a common feature of many vascular diseases, including graft arteriosclerosis and postangioplasty restenosis.
- Interferon-γ plays a nonredundant role in the development of graft arteriosclerosis.

**What New Information Does This Article Contribute?**

- VEGF blockade inhibits neointima formation in immune-mediated vascular remodeling, at least in part, through a reduction in T-cell accumulation in the neointima.
- A subset of T cells expresses functional VEGF receptor-1.
- VEGF enhances T-cell capture by the activated endothelium under flow conditions.

There are conflicting data on the role of VEGF in vascular remodeling, with some data indicating that VEGF promotes neointima formation whereas others point to an opposite effect.

Transplantation of human coronary artery segments to infrarenal aorta of severe combined immunodeficient mice followed by adoptive transfer of allogeneic human peripheral blood mononuclear cells or treatment with interferon-γ leads to significant remodeling of the vessel graft. We show that bevacizumab, a blocking anti-human VEGF antibody, inhibits peripheral blood mononuclear cell–induced but not interferon-γ–induced neointima formation. Human VEGF blockade leads to a reduction in T-cell accumulation in the neointima, without changes in T-cell activation or polarization. This led us to identify a novel subset of T cells that express functional VEGF receptor-1 and demonstrate that human T-cell capture by activated endothelium under flow conditions is enhanced by VEGF. Our findings establish a novel role for VEGF in inflammation and immunity and raise the possibility of novel therapeutic approaches to vascular remodeling based on inhibition of VEGF signaling in leukocytes.
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Material and Methods

Reagents
All reagents were from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Bevacizumab (Avastin) was from Genentech, Inc. (South San Francisco, CA). Human immune globulins were purchased from Talecris Biotherapeutics, Inc. (Research Triangle Park, NC). Recombinant human TNFα, VEGF, PIGF, ICAM-1/Fc, VCAM-1/Fc chimera proteins and control IgG1Fc were purchased from R&D Systems (Minneapolis, MN).

Cell Culture
Human umbilical vein endothelial cells (HUVECs) and dermal microvascular endothelial cells (MVECs) were isolated and cultured as described 1, 2. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood or leukapheresis product by density centrifugation from adult volunteer donors, under protocols approved by the Yale Human Investigation Committee 3. CD4+ cells were isolated using Dynal CD4 Positive Cell Isolation Kit (Invitrogen, Carlsbad, CA) according to manufacturer’s recommendation, and were found to be >99% pure. Memory (CD45 RA+) T cells were isolated from CD4+ cells by depletion of CD45RA+ cells using anti-CD45RA mAb and pan-mouse IgG beads. Jurkat and HSB-2 T cell lines were from ATCC (Manassas, VA). They were cultured in RPMI-1640 supplemented with 10 % FBS (Lonza, Walkersville, MD), 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin.

Adenoviral Preparations
Sub-confluent 293A cells (Invitrogen) were infected with replication incompetent adenovirus encoding human IFN-γ (Ad5.CMV-human IFN-γ, from Qbiogene, Carlsbad, CA) or LacZ (Ad5.CMV-LacZ, from Qbiogene) and cultured according to manufacturer’s recommendations. After two days, infected cells were harvested and lysed by freeze-thawing to release viral particles. Cell debris was then removed by centrifugation and the supernatant containing the viral particle transferred to a fresh tube. Viral particles were purified using Adenopack-100 purification kit (Sartorius Stedim North America Inc., Bohemia, NY) following the manufacturer’s instructions. Viral titer was determined by plaque assay using Thiazolyl Blue Tetrazolium Bromide (MTT) to visualize the plaques. The purified virus was stored at -80 °C until further use.

Animal Models
Human coronary artery transplantation in immunodeficient mice was performed as described 3. Briefly, adjacent segments of human coronary artery were implanted into the infra-renal aortae of 8-12 week old C.B-17 SCID/beige mice (n=48). A group of animals received 1 x 10⁸ human PBMCs per mouse (or control buffer), injected intra-peritoneally one week after transplantation. Reconstitution was verified by flow cytometry. Other animals were injected with replication incompetent Ad5.CMV-human IFN-γ or Ad5.CMV-LacZ (5 x 10⁶PFU) through the jugular vein. Bevacizumab or control human immune globulins were diluted in saline and were administered at a dose of 5 mg/kg, ip, three times per week, starting with PBMC transfer or injection of adenovirus. Animals were sacrificed at 4 weeks after PBMC transfer or adenovirus injection (5 weeks after coronary artery transplantation), and transplanted arteries were removed, and frozen in OCT for further analysis. All experiments were performed under protocols approved by Yale University Institutional Animal Care and Use and Human Investigation Committees.

Histology, Morphometry and Immunostaining
Hematoxylin and eosin (H&E), and Elastica-van Gieson (EVG) staining were performed using standard techniques on 5-μm-thick, fixed cryostat sections. For morphometric analysis, microscopic measurements were performed on digitized images of cryostat sections. The area inside the endothelium, internal elastic lamina (IEL), and external elastic lamina (EEL) were measured and
averaged from 5 sections at 100 µm intervals using computer-assisted image analysis and ImageJ software program (ImageJ, NIH). For immunofluorescent staining, samples were incubated in the presence of nonbinding controls or anti-human VEGF, VEGFR-1, CD69, Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA), VEGFR-2 (Cell Signaling Technology, Danvers, MA), CD3 and CD31 (Pharmingen, San Diego, CA) and CD45 (GAP8.3, ATCC), and followed by labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). These anti-VEGFR antibodies cross-react with corresponding murine receptors. Nuclei were stained with DAPI. To quantify leukocyte infiltration and activation in transplants, CD45 and CD69 positive cells were counted on high power photomicrographs of a representative section for each animal and the results were averaged for several animals.

Reverse Transcription Quantitative Real Time Polymerase Chain Reaction (Q-RTPCR)
Frozen tissue sections were air-dried, and then scraped off slides into RNA lysis buffer (Stratagene, La Jolla, CA). Total RNA was isolated from transplanted coronary arteries using Absolutely RNA® Nanoprep Kit (Stratagene, La Jolla, CA) and reverse transcribed using QuanTitect® Reverse Transcription Kit (QIAGEN, Valencia, CA) following manufacturers’ instructions. Quantitative real time PCR was performed on cDNA in triplicates (except for a few samples where only one replicate was available) using Taqman® gene expression assays (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7500 Real-Time PCR System. Expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S rRNA. The following primer sets were used: human CD3ε (Hs99999153_m1), human IFN-γ (Hs00174143_m1), human IP-10 (Hs00171042_m1), human VEGFR-1 (Hs01052936-m1), human VEGFR-2 (Hs0017667-m1), human 18S rRNA (4352930E) and human GAPDH (Hs99999905_m1). For analyses of VEGFR mRNA expression in T cells, total RNA was isolated from 10^6 purified CD4+, CD4+CD45RA-, Jurkat and HSB-2 cells. Quantitative PCR was performed on cDNA in triplicates was performed using QuantiTect SYBR Green PCR kit (QIAGEN) using the following primer sets: VEGFR-1: 5'-ATCATCCGAAAGCAAGGTGTG-3' and 5'- AAACCCATTGGCAGACATCTGT-3', VEGFR-2: 5'-AGGCAGCTCACAGTCCTAGAGC-3', and 5'-GTCTTTTCTGGGCACCTTCTA-3', GAPDH: 5'-AGTCAACGGATTTGGTCGTAT-3', and 5'-GGGATCTCGCTCCTGGAAGA-3'.

T cell transmigration and adhesion assays
HUVEC grown to confluence on 35 mm human plasma fibronectin coated coverglasses were stimulated with TNF-α (1 ng/ml, 20 h) and incubated with 5 µM SU5416 for 2h before the flow assay. In some experiments, ECs were also incubated with anti-ICAM-1 mAb or control IgG (10µg/ml, 30 minutes). CD4+ T cells (1 million in 500 µl) were also treated with vehicle or 100 ng/ml VEGF for 5 minutes, then applied to the EC monolayer under flow (1 dyne/cm^2) for 2 minutes, followed by flowing medium for 8 minutes, on a 37° C heated surface. In some experiments, T cells were treated with SU5416 (5 µM, 2h) or vehicle control. Samples were fixed and stained with anti-CD45 mAb, followed by Alexafluor 488 or 647 conjugated goat anti-mouse IgG, mounted on slides using mounting medium containing DAPI (Molecular Probes), and examined by microscopy. A FITC filter was used to detect Alexafluor 488-stained cells, a DAPI filter used to detect DAPI-stained nuclei, and a Cy5 filter was used to detect Alexafluor 647 stained cells. Using a 40X/0.60 korr Ph2 objective, phase contrast optics were used to determine whether CD4+ T cells were either on top or underneath the EC monolayer for transmigration assays. (T cells that were captured but had not spread or transmigrated were round and bright when viewed under phase contrast. CD4+ T cells that were spread, but still on top of the EC monolayer were surrounded by a bright corona of light in contrast to those that had transmigrated.) The percentage of transmigrated CD4+ T cells were calculated for 200 cells per sample by analyzing ten groups of 20 cells each, calculating the percentage for each group, and calculating the mean and SEM for the ten groups. For cell capture experiments, the number of CD45+ cells in ten fields (1000 x 1000 pixels) per sample of triplicate
samples viewed with a 10X objective were counted to determine cells/field. For the recombinant protein adhesion assays, 35 mm Petri dishes were coated with goat anti-human IgG, blocked with BSA, then coated with human IgG, rhICAM-1 Ig fusion protein (fused to Fc fragment of human IgG1), rhVCAM Ig fusion protein, or both rhICAM-1 Ig fusion protein and rhVCAM Ig fusion protein. T cells were treated with VEGF and flowed across the samples (0.5 dyne/cm²), fixed, stained with DAPI, and quantitated as above.

Western blotting
Cells were lysed with ice-cold lysis buffer (0.05M Tris pH 7.4, 0.15M NaCl, 1% Igepal) supplemented with 1x complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and 1x phosphatase inhibitors (Calbiochem, La Jolla, CA). Cleared lysates (30 μg/lane for HDMVEC and 50 μg/lane for Jurkat or HSB-2 cells) were separated in 10% (for phospho- and total ERK) or in 7.5% (for VEGFR-1 and -2) gels by SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked with the Blocking Buffer for Near Infra Red Fluorescent Western Blotting (Rockland Immunochemicals, Gilbertsville, PA) for 1 h. Primary antibodies for ERK, phospho-ERK, VEGFR-2 (all from Cell Signaling), VEGFR-1 (Santra Cruz), HSP90 (BD Biosciences) and the corresponding Alexa-Fluor-680 or IR800-labeled secondary antibodies were diluted in the blocking buffer supplemented with 0.1% Tween-20. Immunoreactive bands were acquired and quantified using LI-COR’s Odyssey Infrared Imaging System (Lincoln, NE).

Flow Cytometry
Expression of VEGFR-1 on peripheral blood lymphocytes and Jurkat and HSB-2 cells was analyzed by staining live cells with PE-conjugated anti-VEGFR-1 antibody or the corresponding isotype control antibody (R&D Systems). Phospho-ERK was detected by intracellular staining of paraformaldehyde-fixed cells (Phosflow, Becton Dickinson, Mountain View, CA) according to manufacturer's instructions. A total of 50,000 cells that satisfied a gate on forward and side scatter to eliminate aggregates and debris were acquired using a FACS Calibur flow cytometer (Becton Dickinson,) at Yale University Cell Sorter Facility. Data analysis was performed using FlowJo software (Tree Star Inc, Ashland, OR).

Statistical analysis
All values are expressed as mean±SEM. Two-tailed ratio t-test (for paired non-parametric values) or one-way ANOVA with the Tukey post-hoc analysis were used to assess the significance of differences. p <0.05 was considered as significant.

References:


Supplement Material

Online Figure I: Representative low-magnification examples of Elastica-van Gieson staining of adjacent segments of human coronary artery from 4 different donors transplanted to SCID/beige mice in the absence of, or 4 weeks after human PBMC transfer (first row), reconstituted with human PBMCs, and treated with either control human IgG or bevacizumab for 4 weeks (second row), 4 weeks after intravenous injection of either AD-LacZ or AD-IFN-γ (third row), or injected with AD-IFN-γ, and treated with either human IgG or bevacizumab for 4 weeks (forth row). Scale bar: 100 μm.
Online Figure II: Inhibition of VEGF signaling by SU5416. ECs were pretreated with 5 μM SU5416 for 30 min prior to exposure to VEGF (100 ng/ml) for 10 min. VEGF-induced phosphorylation of ERK and its inhibition by SU5416 were analyzed by immunoblotting. Bar graphs represent the data from three experiments.
Online Figure III: Inhibition of VEGF-induced T cell adhesion to ECs by SU5416 and anti-ICAM-1 antibody. CD4+ T cells were treated with vehicle (veh) or SU5416 for 2 hours followed by treatment with 100 ng/ml VEGF for 5 minutes before flowing over HUVEC treated with TNF (1 ng/ml, 20 h), SU5416 (5 µM, 2 h), and anti-ICAM-1 mAb or control IgG (30 minutes) for 2 minutes, followed by 8 minutes of medium only in triplicates. Graph represents mean and SEM of the number of T cells/field from 10 fields per sample. *: p<0.001 between VEGF and the others, except anti-ICAM (p<0.01).
Online Figure IV: Phosphorylation of ERK detected by intracellular staining and flow cytometry in a subset of CD3+ PBMCs incubated for the indicated times with VEGF (50 ng/ml). Phorbol 12-myristate 13-acetate (PMA, 50 nM) was used as positive control for ERK phosphorylation. The figure represents one of three experiments with similar results.