Molecular Imaging of Vascular Endothelial Growth Factor Receptors in Graft Arteriosclerosis

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Objective—Vascular endothelial growth factor (VEGF) signaling plays a key role in the pathogenesis of vascular remodeling, including graft arteriosclerosis. Graft arteriosclerosis is the major cause of late organ failure in cardiac transplantation. We used molecular near-infrared fluorescent imaging with an engineered Cy5.5-labeled single-chain VEGF tracer (scVEGF/Cy) to detect VEGF receptors and vascular remodeling in human coronary artery grafts by molecular imaging.

Methods and Results—VEGF receptor specificity of probe uptake was shown by flow cytometry in endothelial cells. In severe combined immunodeficiency mice, transplantation of human coronary artery segments into the aorta followed by adoptive transfer of allogeneic human peripheral blood mononuclear cells led to significant neointima formation in the grafts over a period of 4 weeks. Near-infrared fluorescent imaging of transplant recipients at 4 weeks demonstrated focal uptake of scVEGF/Cy in remodeling artery grafts. Uptake specificity was demonstrated using an inactive homolog of scVEGF/Cy. scVEGF/Cy uptake predominantly localized in the neointima of remodeling coronary arteries and correlated with VEGF receptor-1 but not VEGF receptor-2 expression. There was a significant correlation between scVEGF/Cy uptake and transplanted artery neointima area.

Conclusion—Molecular imaging of VEGF receptors may provide a noninvasive tool for detection of graft arteriosclerosis in solid organ transplantation. (Arterioscler Thromb Vasc Biol. 2012;32:1849-1855.)

Key Words: imaging • molecular imaging • transplantation • vascular remodeling

Vascular endothelial growth factor (VEGF) promotes vascular remodeling by enhancing vascular smooth muscle cell (VSMC) migration and promoting vessel wall inflammation and angiogenesis.1-5 The effects of VEGF in the vessel wall are mediated by 2 receptor tyrosine kinase VEGF receptors (VEGFRs). VEGFR-1 is expressed by several cell types in the vessel wall, including endothelial cells (ECs), VSMCs, and monocyte/macrophages.6 VEGFR-2 is predominantly expressed by ECs, although other cells including VSMCs and progenitor cells, are also reported to express VEGFR-2.6,7 Two other coreceptors, neuropilin (NRP)-1 and NRP-2, can bind VEGF165 but not the VEGF121 isoform.8

Graft arteriosclerosis (GA) is the prototypic example of immune-mediated vascular remodeling. GA is the main cause of late organ failure after cardiac transplantation and is characterized by diffuse narrowing of coronary arteries because of concentric neointima formation.9 There is no reliable noninvasive imaging approach for tracking the remodeling process in GA. VEGF is expressed in transplanted hearts, and its expression has been linked to the presence of GA.10 Inhibition of VEGF signaling inhibits vascular remodeling in transplanted arteries.11,12 Thus, alterations in VEGFR prevalence and distribution may provide useful information for tracking the remodeling process in GA. Here, we sought to investigate the use of VEGFR-targeted imaging for assessment of vascular remodeling in human coronary artery grafts. Using a chimeric model of human coronary artery transplantation to immunodeficient mice and an engineered Cy5.5-labeled single-chain VEGF probe (scVEGF/Cy), we established that scVEGF/Cy uptake is significantly increased in GA. Tissue analyses indicate that this enhanced scVEGF/Cy uptake reflects a significant increase in VEGFR-1 expression in the remodeling artery.

Materials and Methods

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Sc VEGF/Cy and Oregon green-labeled scVEGF (scVEGF/OG) and the inactivated homolog obtained through multibiotinylation (inVEGF/Cy) were from SibTech, Inc (Brookfield, CT). Labeled scVEGF retains its VEGF activity and binds with high affinity (Kd of 2.8 nmol/L) to VEGFR-2–expressing cells.12 Recombinant human VEGF121 and VEGF165 were purchased from R&D Systems (Minneapolis, MN). VEGF121 lacks the heparin-binding domain of larger VEGFs such as VEGF165, reducing VEGFR-independent binding to heparan sulfates.13

DOI: 10.1161/ATVBAHA.112.252510

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

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Cell Culture
Human umbilical vein ECs were isolated and cultured as described. 11 Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from leukocytes collected by apheresis from healthy adult volunteer donors, under protocols approved by the Yale Human Investigation Committee. 14

DNA Constructs and Transfection
Human embryonic kidney 293 cells (Invitrogen, Eugene, OR) were transiently transfected with human VEGFR-1 or VEGFR-2 expression plasmids (in pcDNA3, kindly provided by Dr William C. Sessa, Yale University) using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Transfected cells were cultured in DMEM media containing 10% fetal bovine serum for 48 hours before further analysis.

Animal Model
All experiments were performed under protocols approved by Yale University and VA Connecticut Institutional Animal Care and Use and Human Investigation Committees. Transplantation of human coronary artery segments to immunodeficient mice was performed as described. 14 Briefly, adjacent segments of human coronary artery were transplanted to the infrarenal aorta of 8- to 12-week-old female C.B-17 severe combined immunodeficiency/beige mice (n=23; imaging, 17; immunostaining, 6). After 1 week, animals were inoculated intraperitoneally with 1x10^6 human PBMCs per mouse (or control buffer), and PBMC reconstitution was verified after 2 weeks by flow cytometry. Animals were used for imaging studies or tissue analysis after 4 weeks as described below.

Imaging
Severe combined immunodeficiency mice transplanted with human coronary artery segments and inoculated with allogeneic PBMCs for 4 weeks and their controls (without PBMC reconstitution) were injected with scVEGF/Cy or inVEGF/cy (10 µg/mouse) through a jugular vein catheter. The animals were euthanized after 24 hours, viscera were removed, and the abdominal aorta was exposed for in situ imaging. Animals were imaged using a Kodak 4000MM imaging system (Carestream Molecular Imaging, New Haven, CT) equipped for in situ imaging. Animals were used for imaging studies or tissue analysis after 4 weeks as described below.

Histology, Morphometry, and Immunostaining
Elastica Van Gieson staining was performed on 5-µm-thick sections according to standard techniques. Morphometric analysis was performed on digitized images of cryostat sections, as described. 15 For immunohistochemistry and immunofluorescent staining, the following antibodies and their isotype-matched nonbinding controls were used: VEGFR-1 (Santa Cruz Biotechnology, Santa Cruz, CA), VEGFR-2 (Cell Signaling Technology, Danvers, MA), CD3 and CD31 (Pharmingen, San Diego, CA). Labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). For immunofluorescent staining, nuclei were stained with 4’,6-diamidino-2-phenylindole (Invitrogen). Images were obtained using a Zeiss LSM 510 microscope.

Real-Time Reverse Transcription Polymerase Chain Reaction
RNA was isolated from frozen tissue sections using Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA) and reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. Real-time polymerase chain reaction was performed on cDNA in triplicates using Taqman gene expression assays (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7500 real-time polymerase chain reaction system. Because of major differences in housekeeping gene expression in the artery without or with PBMC transfer, 16 mRNA expression was normalized to the template and expressed relative to PBMC-treated animals. The following primer sets were used: CD3ε (Hs009999153_m1), VEGFR-1 (Hs01052936_m1), VEGFR-2 (Hs00176677_m1), NRP-1 (Hs00826128_m1), and NRP-2 (Hs00187290_m1).

Flow Cytometry
Expression of VEGFR-1 and VEGFR-2 on human umbilical vein ECs and human embryonic kidney 293 cells was detected by staining live cells with PE-conjugated anti-VEGFR-1, VEGFR-2, or the corresponding isotype control antibody (R&D Systems). scVEGF uptake was assessed in cells exposed with scVEGF/O (10 nmol/L) for 1 hour at 37°C in the absence or presence of VEGF_165 (added 10 minutes before scVEGF/O). At least 5000 cells that satisfied a gate on forward and side scatter to eliminate aggregates and debris were acquired using a FACSci Kalibur flow cytometer (Becton Dickinson, Mountain View, CA). 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 nonparametric values) or 1-way ANOVA with the Tukey post hoc analysis was used to assess the significance of differences. Two-tailed Spearman correlation was used to test the association between 2 variables. P<0.05 was considered significant.

Results
Cellular Uptake of scVEGF
scVEGF is a fusion protein containing 2 amino acid 3 to 112 fragments of human VEGF_121 cloned head to tail and an N-terminal Cys-tag for site-specific conjugation of imaging and therapeutic moieties. 12 To investigate whether scVEGF, similar to VEGF, is internalized upon binding to scVEGF/O, human ECs were exposed to scVEGF/O. Using flow cytometry, we detected considerable scVEGF/O uptake by ECs. This uptake was inhibited in the presence of excess VEGF_165 or VEGF_121 in a concentration-dependent manner, indicating binding specificity (Figure 1A). Microscopic analysis of ECs exposed to scVEGF/Cy confirmed scVEGF uptake and its localization in intracellular compartments (Figure 1B). Transient overexpression of either VEGFR-1 or VEGFR-2 (but not a control plasmid) in human embryonic kidney 293 cells promoted scVEGF/O uptake by transfected cells, indicating that both receptors can mediate scVEGF uptake (Figure 1C).

Near-Infrared Fluorescent Imaging of VEGFRs in GA
To detect VEGFRs in GA, we used an established model of immune-mediated vascular remodeling. 14 In this model, transplantation of segments of human coronary artery
which often express some degree of native atherosclerosis or diffuse intimal thickening) to severe combined immunodeficiency mice followed by adoptive transfer of allogeneic human PBMCs leads to considerable vascular remodeling of the transplanted arteries over a period of 4 weeks. scVEGF/Cy was intravenously injected to transplant recipients at 4 weeks after PBMC inoculation. Transplant recipients without PBMC transfer were used as control. In situ near-infrared fluorescent (NIRF) imaging at 24 hours after scVEGF/Cy injection demonstrated high focal uptake of scVEGF/Cy in artery grafts after PBMC transfer (Figure 2A and Figure IA in the online-only Data Supplement). In contrast, human artery autofluorescence was readily detectable, independent of the presence of PBMCs. The specificity of scVEGF/Cy uptake in coronary transplants was addressed in an additional group of 3 animals at 4 weeks after PBMC transfer. NIRF imaging after intravenous injection of an inactivated homolog of scVEGF/Cy (inVEGF/Cy) demonstrated no visually detectable uptake in artery grafts (Figure 2A and Figure IB in the online-only Data Supplement). Imaging-derived quantitative analysis of tracer uptake demonstrated significantly higher background-corrected mean fluorescence intensity in PBMC-reconstituted compared with control animals (192.9±70.3 versus 16.2±4.7 arbitrary units, respectively; n=7 in each group; P<0.001; Figure 2B). Tracer uptake specificity was confirmed by quantitative analysis of the mean fluorescence intensity of inVEGF/Cy, which was as low as the level seen in control animals (19.5±13.2; n=3; P=0.05 compared with PBMC-reconstituted animals injected with scVEGF/Cy; Figure 2B). There was no difference in autofluorescence between the 2 groups of animals (17.7±4.2 versus 19.0±2.6 arbitrary units, respectively; n=7 in each group; P=0.36; Figure 2C).

VEGFRs in Remodeling Human Coronary Arteries

Histological analysis of coronary transplants showed that, as expected, adoptive transfer of allogeneic human PBMCs to transplanted animals had led to significant neointima formation and expansive remodeling over a period of 4 weeks, with the intimal area increasing from 0.16±0.05 mm² in control animals to 0.71±0.09 mm² at 4 weeks after PBMC inoculation (n=7; P<0.001; Figure 3). Similarly, the total vessel area significantly increased (from 0.60±0.10 to 1.10±0.12 mm²; P<0.001) and the lumen area significantly decreased (from 0.17±0.02 to 0.03±0.01 mm²; P<0.05) after PBMC reconstitution. Immunostaining of transplanted human coronary arteries, in the presence or absence of PBMCs, demonstrated that VEGFR-1 expression predominantly localizes in the tunica media (Figure 3C). After adoptive transfer of PBMCs, VEGFR-1 was also expressed in the neointima (Figure 3C). VEGFR-2 expression predominantly localized to the luminal endothelium in both groups of animals (Figure II in the online-only Data Supplement). NIRF imaging of coronary grafts after in vivo scVEGF/Cy administration showed that the Cy5.5 signal localized in VEGFR-1 positive areas of the neointima in PBMC-reconstituted animals (Figure 3C). The colocalization of the Cy5.5 signal with neointimal CD3-positive cells implicated T lymphocytes in scVEGF/Cy uptake in vivo (Figure III in the online-only Data Supplement). Little Cy5.5 signal could be detected in the absence of PBMC transfer or after inVEGF/Cy administration (Figure 3C).
Biological Correlates of scVEGF/Cy Uptake in Transplanted Arteries

VEGFR expression in artery grafts was quantified by real-time polymerase chain reaction. There was no significant difference in VEGFR-2 expression between control and PBMC-reconstituted animals. However, VEGFR-1 was significantly higher in human coronary artery transplants 4 weeks after adoptive transfer of human PBMCs (Figure 4). Expression of VEGF coreceptors, NRP-1 and NRP-2, was also significantly higher in human coronary artery grafts after PBMC transfer (Figure 4).

Enhanced uptake of scVEGF/Cy in remodeling coronary arteries in conjunction with VEGFR-1 upregulation suggests that VEGFR-1 may be the primary target for scVEGF/Cy in this model. Indeed, there was a significant correlation between scVEGF/Cy uptake and VEGFR-1 (as well as NRP-1 and NRP-2, but not VEGFR-2) expression in GA samples (Table 1). Morphological assessment of transplanted coronary arteries demonstrated a significant correlation between scVEGF/Cy uptake in vivo and the neointima, lumen, and total vessel areas, but not the media area after PBMC transfer (Table 2), suggesting that VEGFR imaging can detect vascular remodeling in GA.

Discussion

In this study, we demonstrate that a tracer with specificity for VEGFRs, scVEGF/Cy, specifically accumulates in remodeling human coronary arteries in GA, and its uptake in vivo correlates with indices of vascular remodeling. VEGF, the prototypic growth factor for ECs, plays an important role in normal development, homeostatic response to ischemia, cancer, inflammation, and immune response. VEGF effects are mediated by its binding to VEGFRs, including VEGFR-1 and VEGFR-2. VEGFR-2 is expressed by ECs and mediates the proangiogenic effects of VEGF on ECs. More recently, VEGFR-2 expression has been described in other cells, including VSMCs and T cells. Less is known about VEGFR-1, which is expressed by a wide variety of primary cells, including ECs, VSMCs, monocytes, and lymphocytes. The role of VEGF and VEGFRs in vascular remodeling remains controversial. Although several studies point to a causal role of VEGF in vascular remodeling, other studies have reached an opposite conclusion. Recently, using a VEGF-blocking antibody, we demonstrated that VEGF plays a key role in the pathogenesis of vascular remodeling in GA and linked this effect to VEGFR-1 on T cells. These findings suggest that VEGFR-targeted imaging may provide important information on the development of vascular remodeling in GA.

VEGF imaging was introduced as a promising approach for detection of cancer and angiogenesis, and many VEGF-based tracers have been developed and evaluated in animal models for these applications. One example is scVEGF labeled with Cy5.5, 99mTc, 64Cu, or 68Ga, which can detect tumor angiogenesis by NIRF, single-photon emission computed tomography, or positron emission tomography imaging. The scVEGF/Cy signal has been linked to receptor-mediated endocytosis and subsequent intracellular retention of the highly charged Cy5.5 moiety. Although much of the focus of VEGF imaging
Figure 3. Histological analysis of human coronary artery grafts. **A**, Examples of elastic Van Gieson staining of human coronary artery grafts without or with peripheral blood mononuclear cell (PBMC) transfer. Scale bar, 200 μm. **B**, Morphometric analysis of the grafts. n=7 in each group. *P<0.05, **P<0.001. **C**, Representative vascular endothelial growth factor receptor (VEGFR)-1 immunohistochemistry (IHC, top) and near-infrared fluorescent imaging of Cy5.5-labeled single-chain VEGF tracer (scVEGF/Cy) and inactivated homolog of scVEGF/Cy (inVEGF/Cy) uptake in coronary artery grafts without or 4 weeks after human PBMC transfer. Arrowheads point to Cy5.5-positive areas. Elastic laminae are visualized through their autofluorescence. L indicates lumen; I, intima; M, media; A, adventitia. Scale bar, 50 μm.
has been on targeting VEGFR-2 expressed by ECs, here we demonstrated that both VEGFR-1 and VEGFR-2 can mediate scVEGF/Cy uptake by target cells. This allowed us to investigate the expression and functionality of these receptors as targets for imaging vascular remodeling in GA.

Normal arteries express both VEGF receptors. Although VEGFR-2 predominantly localizes to luminal endothelium, VEGFR-1 is mostly expressed in the media where VSMCs constitute the majority of cells. Immune-mediated vascular remodeling is associated with expansion of the intima, which in this model of GA primarily consists of CD3+ T cells and matrix components.14,26 There is little, if any, change in the area of the media in GA. Here, we showed that in addition to its expression in the media, VEGFR-1 is also expressed in the neointima of remodeling arteries in GA. Quantitative assessment of VEGFR expression demonstrated a significant increase in VEGFR-1 (but not VEGFR-2) mRNA transcripts in GA. Interestingly, the development of GA was also associated with significant upregulation of NRP-1 and NRP-2, 2 VEGFR coreceptors.

NIRF imaging of transplanted arteries was remarkably consistent with histological findings. Because inherent depth limitation of NIRF imaging precluded in vivo imaging of transplanted coronary arteries in the mouse, we relied on in situ imaging to investigate scVEGF/Cy uptake in artery grafts. There was only a minimal probe uptake in control animals in the absence of vascular remodeling. After allogeneic PBMC transfer and in conjunction with the development of GA, scVEGF/Cy uptake significantly increased in artery grafts, with the uptake correlating directly with neointimal and total vessel areas and inversely with luminal area. The lack of inVEGF/Cy uptake in PBMC-reconstituted animals excluded the possibility that the scVEGF/Cy signal is merely because of neointima mass as opposed to VEGFR expression in the neointima. Tracer uptake in the transplanted arteries localized to VEGFR-1 and CD3-positive areas of the neointima, implicating T lymphocytes in the uptake of scVEGF/Cy in this model. Recently, we identified a population of VEGFR-1–expressing CD3+ T cells and demonstrated that VEGFRs play a role in T-cell endothelial adhesion.11 Therefore, it is likely that intimal inflammatory cells mediate much of the enhanced scVEGF/Cy uptake in GA. Interestingly, despite considerable VEGFR-1 expression in the media, scVEGF/Cy uptake in the media is minimal. This may be explained by the probe’s limited access to the media (eg, because of the barrier function of elastic laminae). Alternatively, it is possible that there are differences in VEGFR functionality (eg, because of NRP expression)8 in the neointima, which lead to enhanced scVEGF/Cy uptake in GA in vivo.

Table 1. Correlation Between scVEGF/Cy Uptake and VEGFR Expression

<table>
<thead>
<tr>
<th>Variable</th>
<th>R Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-1</td>
<td>0.56</td>
<td>0.046</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>−0.19</td>
<td>0.51</td>
</tr>
<tr>
<td>Neuropilin-1</td>
<td>0.55</td>
<td>0.043</td>
</tr>
<tr>
<td>Neuropilin-2</td>
<td>0.71</td>
<td>0.006</td>
</tr>
</tbody>
</table>

scVEGF/Cy indicates Cy5.5-labeled single-chain VEGF tracer; VEGFR, vascular endothelial growth factor receptor.

Table 2. Correlation Between scVEGF/Cy Uptake and Morphometric Indices

<table>
<thead>
<tr>
<th>Variable</th>
<th>R Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intima area</td>
<td>0.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Media area</td>
<td>0.42</td>
<td>0.14</td>
</tr>
<tr>
<td>Lumen area</td>
<td>−0.85</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total vessel area</td>
<td>0.75</td>
<td>0.002</td>
</tr>
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scVEGF/Cy indicates Cy5.5-labeled single-chain VEGF tracer.
Imaging VEGFRs appear to be a promising approach for detection of vascular remodeling in GA. In addition to its potential as an investigational tool for vascular biology research, our findings suggest that scVEGF-based imaging can serve as a clinical tool for tracking the development of vascular remodeling and response to therapy in cardiac transplantation. Although the clinical application of fluorescent probes in clinical cardiovascular medicine is probably limited to invasive approaches (such as intravascular imaging), radiolabeled homologs of scVEGF may be used for noninvasive imaging. Although imaging coronary arteries in humans is complicated by the small size of coronary arteries and cardiac motion, the diffuse nature of GA would facilitate cardiac imaging and the clinical application VEGFR imaging in GA. Finally, the development of VEGFR-1– and VEGFR-2–specific probes based on characterized mutations in VEGF can further enhance the specificity of imaging information.

Sources of Funding
This work was supported by National Institutes of Health Program Project HL70295, R01 HL085093, R01 HL043331, and a Department of Veterans Affairs Merit Award to M.S.

Disclosures
J.M.B. has equity in SibTech, Inc. The other authors have no conflicts to report.

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Arterioscler Thromb Vasc Biol, 2012;32:1849-1855; originally published online June 21, 2012; doi: 10.1161/ATVBAHA.112.252510

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

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Supplemental Figure I: VEGFR imaging in GA. a) NDIR imaging of human coronary arteries transplanted in SCID mice without or with adoptive transfer of human PBMCs showing considerable graft probe uptake in PBMC reconstituted animals following injection with scVEGF/Cy but not with inVEGF/Cy, the non-binding probe. Arrows point to human coronary arteries transplanted end to end into murine abdominal aorta. b) Autofluorescence imaging demonstrating no difference in the graft signal between different animals.
Supplemental Figure II: VEGFR-2 expression in coronary grafts. Representative immunofluorescent staining of VEGFR-2 expression (in red) in coronary artery grafts in the absence or 4 weeks after human PBMC transfer. Nuclei are stained with DAPI in blue. L: lumen, I: intima. Scale bar: 20 µm.
Supplemental Figure III: scVEGF/Cy uptake in GA. High magnification Cy5.5 imaging (in purple) and CD3 staining (in green) of the coronary artery graft from a mouse injected with scVEGF/Cy four weeks after PBMC reconstitution, demonstrating uptake of the probe in lymphocyte rich areas of neointima. Nuclei are stained with DAPI in blue. I: intima, M: media, A: adventitia. Scale bar: 20 µm.