VEGFR-1 and -2 Regulate Inflammation, Myocardial Angiogenesis, and Arteriosclerosis in Chronically Rejecting Cardiac Allografts


Objective—Interplay between inflammation and angiogenesis is important in pathological reparative processes such as arteriosclerosis. We investigated how the two vascular endothelial growth factor receptors VEGFR-1 and -2 regulate these events in chronically rejecting cardiac allografts.

Methods and Results—Chronic rejection in mouse cardiac allografts induced primitive myocardial, adventitial, and intimal angiogenesis with endothelial expression of CD31, stem cell marker c-kit, and VEGFR-2. Experiments using marker gene mice or rats as cardiac allograft recipients revealed that replacement of cardiac allograft endothelial cells with recipient bone marrow— or non—bone marrow—derived cells was rare and restricted only to sites with severe injury. Targeting VEGFR-1 with neutralizing antibodies in mice reduced allograft CD11b+ myelomonocyte infiltration and allograft arteriosclerosis. VEGFR-2 inhibition prevented myocardial c-kit+ and CD31+ angiogenesis in the allograft, and decreased allograft inflammation and arteriosclerosis.

Conclusions—These results suggest interplay of inflammation, primitive donor—derived myocardial angiogenesis, and arteriosclerosis in transplanted hearts, and that targeting VEGFR-1 and -2 differentially regulate these pathological reparative processes. (Arterioscler Thromb Vasc Biol. 2007;27:819-825.)

Key Words: angiogenesis ■ inflammation ■ transplantation ■ arteriosclerosis ■ stem cells

Normal adult vasculature is in a quiescent state, but growth of new blood vessels is seen in many physiologic and pathological conditions involving hypoxia and inflammation. A transplanted heart faces many peri- and postoperative nonimmunologic and immunologic stimuli that may be interpreted as a need for new blood vessels. Recent knowledge indicates that angiogenic growth factors are involved in allograft inflammation and the development of cardiac allograft vasculopathy (CAV)—the main reason for poor long-term survival of heart transplant patients.

VEGF functions through 2 tyrosine kinase receptors—VEGFR-1 and VEGFR-2. In addition to endothelial cells (ECs), VEGFR-1 is expressed in bone marrow (BM) progenitor cells, myelomonocytic inflammatory cells, and vascular smooth muscle cells (SMCs). VEGFR-1 in myeloid cells mediates monocyte migration and has a regulatory role in the development of inflammatory diseases such as rheumatoid arthritis and arteriosclerosis. VEGFR-1 expression in SMCs is induced by vascular injury and the receptor is involved in neointimal development. VEGFR-2 elicits the main mitogenic, angiogenic, and permeability effects of VEGF on ECs, and it has a key role in developmental angiogenesis and hematopoiesis. In adults, VEGFR-2 expression is downregulated in nonfetalized vasculature such as in the heart, making these capillaries resistant to VEGF inhibition. In turn, VEGFR-2 is expressed at sites with active angiogenesis eg, after myocardial infarction, increases vascular permeability in ischemic heart, and participates in sepsis—related cardiac dysfunction. VEGF also mobilizes VEGFR-2+ endothelial progenitor cells (EPCs) from BM that either directly differentiate to ECs or participate in angiogenesis by secreting paracrine signals to local ECs. In addition, the recruited EPCs may activate a resident cardiac pool of c-kit+ progenitor cells—cardiac stem cells——that preferably differentiate to ECs in injury.

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We previously showed that VEGF gene transfer enhances cardiac allograft arteriosclerosis, whereas VEGF inhibition with PTK787—which also inhibits other receptor tyrosine kinases—had opposite effects. In this study, we investigated the specific roles of the 2 VEGFR in cardiac allograft inflammation, angiogenesis, and arteriosclerosis, and the contribution of donor and recipient cells in allograft angiogenesis. We found that chronic rejection in transplanted hearts induced donor-derived myocardial, adventitial, and intimal angiogenesis with endothelial expression of CD31, c-kit, and VEGFR-2. VEGFR-1 inhibition reduced allograft CD11b+ myelomonocyte infiltration and allograft arteriosclerosis whereas targeting VEGFR-2 prevented c-kit+ and CD31+ angiogenesis, and decreased allograft inflammation and arteriosclerosis. These results suggest interplay of inflammation, primitive donor-derived angiogenesis, and arteriosclerosis in transplanted hearts, and that VEGFR-1 and -2 differentially regulate these pathological reparative processes.

Materials and Methods

Experimental Design
Mouse chronic rejection heart transplantation model and immunohistochemical stainings were used to identify angiogenesis and progenitor cells in allografts. Marker gene mice and rats, and strain-specific major histocompatibility complex (MHC) class I antibodies, were used to determine whether allograft ECs originate from the donor or from the recipient. Neutralizing antibodies were used to investigate the functional role of VEGFR-1 and -2 on mouse cardiac allograft angiogenesis, inflammation, and arteriosclerosis. For the detailed methods please see the supplemental materials, available online at http://atvb.ahajournals.org.

Mouse Chronic Rejection Heterotopic Heart Transplantation Model
Heterotopic cardiac allografts were transplanted in abdominal position from Balb (B/c, H-2d) to C57 (B6, H-2b) mice (Harlan, Horst, The Netherlands). The recipients received suboptimal FK506 immunosuppression (i.m. formulation, kindly provided by Fujisawa, currently Astellas Pharma, Tokyo, Japan) and the allografts were harvested at 8 weeks.

Origin of Allograft Endothelial Cells
Tiel/LacZ rats were used to as allograft recipients (n=4) or donors (n=14, with or without immunosuppression) to investigate Tiel expression, and the origin of Tiel positive ECs in transplanted hearts. Contribution of BM-derived cells in allograft angiogenesis was investigated using recipient mice with green fluorescent protein-expressing BM cells (GFP-BM, n=3).

VEGFR-1 and VEGFR-2 Inhibition
Cardiac allograft recipients were treated with 800 μg of rat IgG (n=8; Sigma-Aldrich), anti–VEGFR-1 antibody (n=9; MF1, ImClone), anti–VEGFR-2 antibody (n=9; DC101, ImClone), or their combination (n=10) every third day for 10 doses, starting immediately after the transplantation.

Histology and Immunohistochemistry
Arterial occlusion percentage was determined using morphometry. Immunohistochemical stainings were performed using peroxidase ABC method or Alexa Fluor 488 (green) and 568 (red, Promega) secondary antibodies.

Analysis of Immunohistochemical Stainings
Allograft parenchymal inflammatory cells and c-kit+ capillaries were counted from 16 random sections, and are given as the mean density

Figure 1. Primitive myocardial angiogenesis in chronically rejecting cardiac allografts. Only few c-kit+ immunoactive cells were detected in normal mouse hearts (A, arrow) whereas numerous c-kit+ cells were found in myocardium (B, arrow), vein endothelium (B, arrowheads), and arterial adventitia and intima (C) of chronically-rejecting mouse cardiac allografts under FK506 immunosuppression 2 months after transplantation. The c-kit+ cells coexpressed CD31 (D) and VEGFR-2 (E), and some expressed K67 (F, arrows). VEGFR-1 was mainly expressed in the media of arteries (G). The density of myocardial c-kit+ cells in cardiac allografts correlated with CD11b+ myelomonocyte density (H), incidence of arteriosclerotic changes (I) and mean occlusion of allograft arteries (J). A–C, Mayer hemalum staining; no specific immunoreactivity with IgG control (B, inset). Dashed line indicates lamina elastica interna (C). DAPI nuclear staining is shown in blue (D–G). The white box shows the area magnified for the single color images (D–G). Data analyzed by linear regression analysis (H–J). Scale bars=50 μm.

of positive cells or vessels. CD31 and α-SMA immunofluorescence stainings were analyzed with Axioscan 2 microscope and Axiovision 4.2 analysis software (Carl Zeiss) using a semi-automated script.

Real Time RT-PCR
Total RNA was extracted using RNeasy Mini Kit (Qiagen) (n=4 to 6 per group). RT-PCR reactions were carried out using LightCycler (Roche) and the results are given in relation to 18S rRNA molecule numbers.

Statistical Analysis
Data are mean±SEM and analyzed by parametric ANOVA with Dunnett correction to compare the treatment groups to the control group. Linear regression analysis was applied to evaluate relation of c-kit+ cells to CD11b+ cells and to CAV. P<0.05 was regarded as statistically significant.

Results

Chronic Rejection Induces Primitive Myocardial Angiogenesis in Cardiac Allografts
We detected only occasional stem cell marker c-kit immuno-reactive cells in cross sections of nontransplanted mouse hearts (Figure 1A, arrow). In contrast, numerous myocardial capillary-like c-kit+ cells (Figure 1B, arrows) and c-kit+ vein ECs (Figure 1B, arrowheads) were seen in chronically-rejecting cardiac allografts harvested 2 months after the
operation. In allografts with severe arteriosclerotic changes, c-kit+ cells were also found in the adventitia and intima of coronary arteries (Figure 1C). Allograft myocardial c-kit+ cells were nearly all positive for endothelial marker CD31 (Figure 1D), and coexpressed VEGFR-2 (Figure 1E). The majority of c-kit+ capillaries did not express proliferation marker Ki67 but some c-kit+ cells with nuclear Ki67 immunoreactivity were also detected (Figure 1F, arrows). In contrast to the preferential expression of VEGFR-2 in the endothelium, VEGFR-1 was mainly expressed in allograft α-SMA+ SMCs (Figure 1G). In peripheral blood, over 50% of VEGFR-1+ cells coexpressed the myelomonocyte marker CD11b (data not shown).14

A positive correlation was verified between the density of c-kit+ capillaries in the myocardium and the number of allograft-infiltrating CD11b+ myelomonocytic inflammatory cells (Figure 1H), as well as with the incidence of arteriosclerotic changes (Figure 1I) and the mean occlusion of allograft arteries (Figure 1J). These results indicate that chronic rejection in transplanted hearts induces myocardial, adventitial, and intimal angiogenesis with endothelial expression of primitive markers c-kit and VEGFR-2.

**Endothelial Replacement With Recipient-Derived Cells Is Rare in Cardiac Allografts**

As recipient-derived circulating EPCs could differentiate to ECs in the transplanted heart, we next determined the origin of cardiac allograft ECs by using marker gene rats (Tie1/LacZ) or mice (GFP-BM) as allograft recipients. When Tie1/LacZ allografts were transplanted to wild-type (WT) recipients, areas with abundant X-gal reactivity in allograft endothelium was detected (Figure 2A and 2B) indicating Tie1 expression in the donor ECs. Next, WT cardiac allografts were transplanted to Tie1/LacZ recipients to detect recipient-derived ECs in the transplanted hearts. Only few donor-derived X-gal+ ECs (Figure 2C and 2D, arrows) localizing to severely fibrotic areas were seen in cross sections in a total of 14 WT cardiac allografts.

Additionally, GFP-BM mice were used as cardiac allograft recipients allowing the detection of BM-derived cells in the allografts. The majority of allograft-infiltrating CD11b+ myelomonocytic cells expressed GFP (data not shown). Although GFP+ cells often surrounded allograft blood vessels, no colocalization with allograft CD31+ (Figure 2E) or c-kit+ capillaries (Figure 2E) was detected.

Furthermore, donor- and recipient-specific MHC class I antibodies were used to identify the source of ECs in allograft arteriosclerotic arteries. Numerous recipient MHC class I+ cells were found around occluded arteries (Figure 2G, arrows) whereas only few positive cells were detected in the intima (Figure 2G). In contrast, abundant donor MHC Class I immunoreactivity was found in the neointima (Figure 2H, arrows). The contribution of recipient-derived SMCs to neointimal formation34 was not assessed as MHC Class I expression was low in SMCs34 (Figure 2G and 2H).

**VEGFR-2 Inhibition Normalizes C-Kit+ and CD31+ Capillary Density in Chronically Rejecting Cardiac Allografts**

To investigate the functional role of VEGFR-1 and -2, mouse cardiac allograft recipients were treated with rat IgG, or antibodies against VEGFR-1, VEGFR-2, or both. Two months after heart transplantation, targeting VEGFR-2 reduced the density of myocardial c-kit+ capillaries (Figure 3A) and CD31+ capillaries (Figure 3B) in the allograft to the level found in nontransplanted mouse hearts (Figure 3A and 3B, dashed lines). VEGFR-1 inhibition also resulted in a smaller decrease in c-kit+ capillary density (P=NS with Dunnett correction, P<0.05 with LSD correction). VEGFR-1 or -2 inhibition did not change the density of SMC coated vessels (Figure 3C) indicating that VEGFR-2 inhibition specifically regulated angiogenesis at microvascular level.

**VEGFR-1 and -2 Inhibition Reduces Inflammation in Chronically Rejecting Cardiac Allografts**

Immunohistochemical analysis showed that targeting VEGFR-1, VEGFR-2, or both profoundly reduced the density of allograft-infiltrating CD11b+ myelomonocytic cells (supplemental Figure IA, available online at http://atvb.ahajournals.org). VEGFR-2 inhibition also resulted in a similar reduction in CD8+ (Figure IB) and CD4+ (supplemental Figure IC) lymphocyte density in the allograft (for the combination group: P=NS with Dunnett correction and P<0.05 with LSD correction).
Angiogenesis is a prominent feature in the intima\(^6\)–\(^8\) and adventitia\(^9\) of cardiac allograft coronary arteries and it may be a driving force for the development of CAV.\(^5\),\(^40\) Here we show that in addition to intimal and adventitial angiogenesis, chronic rejection induces the expression of primitive markers c-kit and VEGFR-2 in allograft myocardial capillaries. As the density of myocardial c-kit\(^+\) capillaries correlated with the severity of cardiac allograft inflammation and arteriosclerosis, alloimmune and ischemic stimuli may be important regulators of the myocardial angiogenesis we observed. This primitive c-kit\(^+\) angiogenic response probably represents a repair process that, interestingly, in light of the present VEGF intervention results, may in fact aggravate inflammation and arteriosclerosis in transplanted hearts. Importantly, there may be a balance between early capillary formation and later destruction of allograft capillaries as seen in skin transplants.\(^41\)

In experiments using marker gene animals and donor or recipient specific antibodies, we found only few recipient-derived ECs in the transplanted hearts and they were restricted to severely fibrotic areas. These observations—together with earlier findings using heterotopic heart transplantation models\(^14\),\(^42\)—suggest that recipient-derived circulating cells do not differentiate into allograft EC unless the injury to the allograft extensive. Although this argues against direct involvement of recipient-derived EPCs in allograft angiogenesis, these circulating cells may have important paracrine effects as seen in nontransplantation situations.\(^26\)–\(^28\) Our results on the origin and c-kit\(^+\) phenotype of allograft EC further indicates that donor-derived progenitor cells—such as resident cardiac stem cells\(^29\),\(^30\),\(^43\) or adventitial stem cells\(^44\)–\(^46\)—directly participate in allograft angiogenesis. Alternatively, the hypoxic and inflammatory signals related to the transplantation may have induced dedifferentiation of allograft ECs to a more primitive phenotype. Interestingly, EPC-derived soluble factors such as VEGF, VEGF-B, stromal cell derived factor-1, and insulin-like growth factor-1,\(^27\) and also hepatocyte growth factor\(^47\) may regulate the functions of c-kit\(^+\) cardiac progenitor cells, and the present results suggest important role for VEGF-2.

VEGF is perhaps the most important angiogenic cytokine and it has also many proinflammatory properties.\(^3\) The present findings support the regulatory role of VEGF in the pathogenesis of alloimmune responses\(^8\),\(^9\) and CAV\(^5\),\(^6\) in transplanted hearts, and shed light to the mechanisms, and the 2 VEGFR involved. In transplanted hearts VEGFR-2 inhibition reduced myocardial angiogenesis to the level seen in normal hearts consistent with the important angiogenic role for VEGFR-2.\(^3\) In addition, targeting VEGF-2 decreased inflammatory cell infiltration, and production of IP-10 and MCP-1 in the allograft similarly to previous reports with anti-VEGF therapies.\(^8\) Our results thus suggest that VEGF-2 in cardiac allografts functions mainly at the endothelial level and regulates both pathological capillary angiogenesis and inflammation. Involvement of VEGFR-2 in cardiac inflammation may be a more general phenomenon as the receptor participates in cardiac angiogenesis.

**Effect of VEGF-1 and -2 Inhibition on Allograft Cytokine mRNA Levels**

Finally, we used real time RT-PCR to determine the mRNA levels of inflammatory cytokines IFN-inducible protein-10 (IP-10) and monocyte chemotactic protein-1 (MCP-1) that are potentially regulated by VEGF in cardiac allografts,\(^8\),\(^35\) and the mRNA levels of stem cell factor (SCF) that is the ligand for c-kit. VEGFR-2 inhibition decreased allograft IP-10 mRNA by approximately 50% alone and by 75% in combination with VEGFR-1 inhibition (Figure 5A), and MCP-1 mRNA by 50% (Figure 5B). In contrast, allograft tumor necrosis factor (TNF)-\(\alpha\) (Figure 5C) and SCF (Figure 5D) mRNA levels were similar in the control and treatment groups. These results indicate that the VEGFR-2 inhibition regulated at least in part the T cell and monocyte recruitment by decreasing IP-10 and MCP-1 production,\(^8\),\(^35\) respectively. Also, the effect of VEGFR-2-inhibition on c-kit\(^+\) capillaries was not associated with changes in SCF production.

**Discussion**

Figure 3. The effect of VEGFR inhibition on capillary angiogenesis and arteriosclerosis in chronically rejecting mouse cardiac allografts. Chronically rejecting mouse cardiac allograft recipients with suboptimal FK506 immunosuppression received IgG (n=8), or monoclonal antibodies against VEGFR-1 (MF1, n=9), VEGFR-2 (DC101, n=9), or both (n=10) for 30 days. At 8 weeks, VEGFR-2 inhibition decreased allograft myocardial c-kit\(^+\) (A) and CD31\(^+\) capillary density (B). VEGFR inhibition did not change \(\alpha\)-SMA\(^+\) vessel density (C). A, Mayer hemalum staining. The dashed red line indicates nontransplanted mouse heart values (A and B). Data are given as mean±SEM, by ANOVA with Dunnett correction, comparing the treatment groups with the control group. Scale bars=50 \(\mu\)m.

A similar result was also obtained on the mean occlusion of allograft arteries (Figure 4A). Morphometric analysis of allograft arteries revealed that targeting VEGFR-1, VEGFR-2, or both decreased the incidence of allograft arteries with intimal changes (Figure 4A). This effect was not associated with changes in SCF production. The effect of VEGFR inhibition on capillary angiogenesis in the allograft myocardium is shown in Figure 4B. VEGFR inhibition did not change the vessel density of myocardial c-kit\(^+\) capillaries correlated with the primitive phenotype of the myocardial angiogenesis we observed. This primitive c-kit\(^+\) angiogenic response probably represents a repair process that, interestingly, in light of the present VEGF intervention results, may in fact aggravate inflammation and arteriosclerosis in transplanted hearts. Importantly, there may be a balance between early capillary formation and later destruction of allograft capillaries as seen in skin transplants.\(^41\)

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dysfunction during sepsis, and also in vascular permeability following myocardial infarction.

In contrast to VEGFR-2, VEGFR-1 was primarily found in allograft SMCs and in peripheral blood myelomonocytic cells. As VEGFR-1 directly regulates SMCs during arterial injury, VEGFR-1 inhibition in the current study may have directly decreased SMC recruitment to the intima. VEGFR-1 inhibition also profoundly reduced myelomonocytic recruitment to the allograft consistent with its role in monocytes and inflammatory diseases. Although VEGFR-2 inhibition prominently decreased the density of myocardial c-kit+ cells, also VEGFR-1 inhibition had a similar but more subtle effect. This indicates that also VEGFR-1 may in part regulate the capillary angiogenesis, and possibly involves cross-talk with VEGFR-2 or indirect inflammation-mediated effects. The reason why combined VEGFR-1 and -2 inhibition did not have a beneficial additive effect may be explained by the moderate injury in the current experimental setting. Supporting this, our unpublished trachea transplantation findings (Krebs et al, 2007) show additive beneficial effect after severe but not after moderate tracheal injury.

In conclusion, we found that chronic rejection in cardiac allografts induced donor-derived capillary angiogenesis. Also, selective VEGFR-inhibition prevented allograft angiogenesis and had beneficial effects on inflammation and arteriosclerosis. These results suggest therapeutic applications for anti-VEGF strategies during pathological angiogenesis and inflammation in transplanted hearts.
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Disclosure

D.J. Hicklin and Y. Wu are employees of ImmClone Systems Inc.

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*Equal contribution

Expanded Materials and Methods

Experimental design

Mouse chronic rejection heart transplantation model and immunohistochemical stainings were used to identify angiogenesis and progenitor cells in allografts. Marker gene mice and rats, and strain-specific major histocompatibility complex (MHC) class I antibodies, were used to determine whether allograft EC originate from the donor, or from the recipient. Neutralizing antibodies were used to investigate the functional role of VEGFR-1 and -2 on mouse cardiac allograft angiogenesis, inflammation and arteriosclerosis.

Mouse Chronic Rejection Heterotopic Heart Transplantation Model

Male Balb/c (B/c, H-2^d) and C57BL/6J (B6, H-2^b) mice weighing 25-30 g (Harlan, Horst, The Netherlands) were used. Heterotopic cardiac allografts were transplanted in abdominal position from Balb to C57 mice. The donor heart was perfused with 1 ml of +4°C 0.9% NaCl with 500 IU heparin. The recipients received FK506 (i.m.)
VEGFR in cardiac allograft angiogenesis

formulation, kindly provided by Fujisawa, currently Astellas Pharma, Tokyo, Japan) s.c. 3.0 mg/kg/d for the first week and 1.5 mg/kg/d thereafter. Allografts were harvested at 8 weeks. This immunosuppression was chosen after preliminary studies with different FK506 dosing, as the administration resulted in good allograft survival and development of cardiac allograft vasculopathy. Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. Animals received care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377-3, revised 1996).

Origin of Allograft Endothelial Cells

Wistar rats that express LacZ under the promoter of EC receptor Tie1 (Tie1/LacZ)\(^1\) were used. To investigate donor-derived Tie1 expression in transplanted hearts, Tie1/LacZ hearts were transplanted to non-immunosuppressed wild type recipients that were sacrificed at 5 days (n=4). To investigate the replacement of EC with recipient-derived Tie1-expressing cells in transplanted hearts, DA allografts (AG-B4, RT1\(^a\), Harlan) were transplanted to Tie1/LacZ recipients. Allograft recipients were either left non-immunosuppressed (n=4) and the allografts were harvested at day 8, or the recipients received cyclosporine A (CsA, Novartis, Basel, Switzerland) (n=10), and the allografts were harvested at 4 months or if the function of the allograft deteriorated. CsA was diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany) and administered subcutaneously 1.5 mg/kg/d for the first week and 1.0 mg/kg/d thereafter. After harvesting, samples were flushed in phosphate buffer, fixed in 0.2% glutaraldehyde, stained with X-gal solution for 3 days, washed overnight, fixed in 4% PFA, embedded in paraffin and counterstained with Nuclear Fast Red.
Replacement of allograft cells with BM-derived cells was investigated using C57 mice that had received a BM transplant from green fluorescent protein-expressing syngenic mice (GFP-BM) as Balb cardiac allograft recipients (n=3). The mouse chronic rejection heterotopic heart transplantation model was used. Samples were first incubated in 2% paraformaldehyde for 30 min, then in 20% sucrose overnight, embedded in TissueTek and snap frozen in liquid nitrogen.

**Inhibition of VEGFR-1 and VEGFR-2 Signaling**

The mouse chronic rejection model was used. Cardiac allograft recipients were treated with 800 µg of rat IgG (n=8; Sigma-Aldrich, St. Louis, MO), anti-VEGFR-1 antibody (n=9; MF1, ImClone, New York, NY), anti-VEGFR-2 antibody (n=9; DC101, ImClone) or their combination (n=10). Antibodies were administered i.p. every third day for 10 doses, starting immediately after the transplantation.

**Histology**

Cardiac allograft vasculopathy was determined in a blinded manner from paraformaldehyde-fixed paraffin sections stained with hematoxylin-eosin and Resorcin fuchsin for internal elastic lamina using computer-assisted image processing (NIH Image version 1.62, US National Institutes of Health; http://rsb.info.nih.gov/nih-image/) and measuring the area surrounded by the internal elastic lamina and vessel lumen. Arterial occlusion percentage was determined as the ratio of neointimal area and internal elastic lamina area. The incidence of arteriosclerotic changes was determined by the percentage of arteries with intimal changes.
VEGFR in cardiac allograft angiogenesis

Immunohistochemical and Immunofluorescence Stainings

Immunohistochemistry was performed using cryostat sections, peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories) and 3- amino-9- ethylcarbazole (Vectastain). MHC Class I stainings were performed with tyramine signal amplification kit (TSA, Perkin Elmer, Wellesley, MA). Immunofluorescence double stainings were performed using Alexa Fluor 488 (green) and 568 (red, Promega, Madison, WI) secondary antibodies. Antibodies and dilutions were CD4 (5 \( \mu \)g/ml, 553043), CD8 (5 \( \mu \)g/ml, 553027), CD11b (5 \( \mu \)g/ml, 553308), biotinylated anti-Balb MHC class I (0.5 \( \mu \)g/ml with TSA, 553564) and biotinylated anti-C57 MHC class I (6.25 \( \mu \)g/ml with TSA, 550550) from BD Pharmingen, San Diego, CA; VEGFR-1 (2 \( \mu \)g/ml, sc-316-G) and VEGFR-2 (2 \( \mu \)g/ml, sc-315-G) from Santa Cruz, Santa Cruz, CA; CD31 (0.5 \( \mu \)g/ml, ab7388) from Abcam, Cambridge, UK; c-kit (1 \( \mu \)g/ml, AF1356) from R&D Systems, Minneapolis, MN; \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, 1:5000, A2547) from Sigma-Aldrich; and Ki67 (1:1000, NCL-Ki67p) from Novocastra Laboratories Ltd, New Castle, UK.

Analysis of Immunohistochemical Stainings

Allograft parenchymal inflammatory cells and c-kit+ capillaries were counted from four random fields from each quadrant of the graft section using 400x magnification and given as the mean density of positive cells or vessels. CD31 and \( \alpha \)-SMA immunofluorescence stainings were analysed using Axioplan 2 microscope and Axiovision 4.2 analysis software (Carl Zeiss, Oberkochen, Germany). CD31+ capillaries were analyzed from four random fields with cross-sectional capillaries using 400x magnification. \( \alpha \)-SMA+ coated vessels were analyzed from allograft cross section using 100x magnification. A semi-automated script was used to quantify
CD31+ capillaries and α-SMA+ coated vessels, and the results are given as the mean density of positive vessels.

**RNA isolation and reverse transcription**

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) \(n=4-6\) per group. Reverse transcription of mRNA was performed using M-MLV reverse transcriptase (Sigma-Aldrich), recombinant RNasin ribonuclease inhibitor (Promega), and random nonamers (Sigma-Aldrich).

**Real-time RT-PCR**

Real-time RT-PCR reactions were carried out in a LightCycler using LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche, Basel, Switzerland). Measurement of the PCR product was performed at the end of each extension period. The number of copies of the gene of interest was calculated from the corresponding standard curve using LightCycler software (Roche) and given in relation to 18S rRNA molecule numbers. The following primers for mouse IP-10 (Gene Bank accession no. M33266), SCF (acc. No. NM_013598), MCP-1 (acc. No. NM_011333), TNF-\(\alpha\) (acc. No. NM_013693), were used: IP-10 fwd 5'-TTACCCAGTGATGGCTAGTCCTA-3', bwd 5'-CCCTTGGGAAGATGGTGGTT-3', SCF fwd 5'-GTCATTGTTGGCTACGAGATA-3', bwd 5'-AACACGAGGTCATCCACTGA-3', MCP-1 fwd 5'-AGCACCAGCCAACTCTCACTGA-3', bwd 5'-CGGGTCAACTTCAATCTCGGTCAAGG-3', TNF-\(\alpha\) fwd 5'-GGGCCACCACCGCTTTGTCT-3', bwd 5'-GCCACTCCAGCTGCTCTCCAC-3'.

5
**Statistical Analysis**

Data are mean ± SEM, and analyzed by parametric ANOVA with Dunnett’s correction to compare the treatment groups to the control group. Linear regression analysis was applied to evaluate relation c-kit+ cells to CD11b+ cells and to CAV. *P*<0.05 was regarded as statistically significant.

**References**


**Figure legends**

**Figure I.** The effect of VEGFR inhibition on inflammation in chronically rejecting mouse cardiac allografts. VEGFR-1 or -2 inhibition decreased of CD11b+ myelomonocytic cell density in cardiac allografts (A). VEGFR-2 inhibition decreased the number of CD8+ (B) and CD4+ lymphocytes (C). Mayer’s hemalum staining. Data as in Figure 3. Scale bars 50 μm.
Figure I

A

CD11b+ cells/mm²

p<0.001

IgG
VEGFR1 Ab
VEGFR2 Ab
VEGFR1+2 Ab

B

CD8+ lymphocytes/mm²

p<0.05

IgG
VEGFR1 Ab
VEGFR2 Ab
VEGFR1+2 Ab

C

CD4+ lymphocytes/mm²

p<0.05

IgG
VEGFR1 Ab
VEGFR2 Ab
VEGFR1+2 Ab