Vascular Endothelial Growth Factor-B–Deficient Mice Display an Atrial Conduction Defect

Karin Aase, PhD; Gabriel von Euler, PhD; Xuri Li, PhD; Annica Pontén, MS; Peter Thorén, MD, PhD; Renhai Cao, PhD; Yihai Cao, MD, PhD; Birgitta Olofsson, PhD; Samuel Gebre-Medhin, PhD; Milos Pekny, MD, PhD; Kari Alitalo, MD, PhD; Christer Betsholtz, MD, PhD; Ulf Eriksson, PhD

**Background**—Vascular endothelial growth factors (VEGFs) and their receptors are essential regulators of vasculogenesis and angiogenesis in both embryos and adults. One of the factors with a still unknown physiological function is VEGF-B, which is expressed in many tissues, including the heart.

**Methods and Results**—Mice carrying a targeted deletion in the VEGF-B gene were developed. In VEGF-B−/− animals, no gross abnormalities were observed in organs that normally show high expression of VEGF-B, such as the heart, muscle, and kidney. Analysis of heart function by ECG showed that adult VEGF-B−/− mice have an atrial conduction abnormality characterized by a prolonged PQ interval. VEGF- or basic fibroblast growth factor–induced corneal angiogenesis was similar in normal and VEGF-B−/− mice.

**Conclusions**—VEGF-B seems to be required for normal heart function in adult animals but is not required for proper development of the cardiovascular system either during development or for angiogenesis in adults. (*Circulation. 2001; 104:358-364.)*

**Key Words:** growth substances ▪ angiogenesis ▪ electrocardiography ▪ atrium ▪ conduction

Vasculogenesis and angiogenesis are complex processes that generate the primary vascular system, which is essential for embryonic development and organ formation. Angiogenesis is also important in adults during reproduction, tissue regeneration, and remodeling and in development and progression of several pathological conditions, such as tumor growth, diabetic retinopathy, and several types of chronic inflammation.1

Several growth factors are believed to be involved in various aspects of vessel growth, remodeling, and physiology. Among them, vascular endothelial growth factor (VEGF) is well characterized and is relatively specific toward vascular endothelial cells (ECs) (reviewed in Reference 2). The biological effects of VEGF include stimulation of EC proliferation, migration, differentiation, tube formation, maintenance of vessel integrity, and regulation of vascular permeability. The distinct actions of VEGF are mediated by receptor tyrosine kinases expressed primarily by ECs, namely VEGF receptors 1 (VEGFR-1, flt-1) and 2 (VEGFR-2, KDR or flk-1).2–4 VEGFR-2 mediates induction of proliferation and migration of ECs, whereas VEGFR-1 appears to be unable to transduce such signals.

Four additional mammalian proteins with high structural homology to VEGF were identified (reviewed in Reference 6), including placenta growth factor (PIGF), VEGF-B, VEGF-C, and VEGF-D. PIGF and VEGF-B bind specifically to VEGFR-1,5–7 and VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 (flt-4).8–10 Specific isoforms of VEGF and PIGF and both isoforms of VEGF-B bind to neuropilin-1 (NP-1).11–13

VEGF-B displays a unique expression pattern with prominent expression in developing heart and in several muscle derivatives during embryonic development.14–16 In the embryonic heart, expression is confined to cardiac myocytes, whereas VEGFR-1 is expressed in the adjacent ECs.14 NP-1 is expressed both in endothelium and in cardiac myocytes during development, suggesting that VEGF-B may also act in an autocrine fashion on cardiac myocytes.15,16

To reveal the functional role(s) of VEGF-B, we generated mice deficient in VEGF-B (VEGF-B−/−) by targeted deletion of the VEGF-B locus. The data suggest that adult VEGF-B−/− mice have a mild cardiac conduction defect, whereas it is not required for proper development of the cardiovascular system.

**Methods**

**Generation of VEGF-B−/− Mice**

Genomic clones containing the murine VEGF-B gene18 were isolated from a mouse 129/Sw Als1II library (Stratagene). The targeting
vector, pPGK-Neomycin, contained an 8.3-kb NotI/SpeI fragment of homology on the 5' arm and a 1.2-kb KpnI/HindIII fragment on the 3' arm. The targeting construct was electroporated into E14 ES cells. Homologous recombination events were screened with Southern blots. The targeted allele generated a 6.5-kb EcoRI fragment and the wild type a 20-kb fragment by use of a 460-bp probe derived from exon 7 and part of the 3' untranslated region by polymerase chain reaction (PCR) (primers: 5'-GTGAAGCTCCAGCCGAGCA-3' (forward), and 5'-TAGTGTCTTCCATCTTT-3' (reverse)). Targeted ES cells were injected into C57Bl blastocysts as described. The heterozygous F1 mice were crossed and genotyped by PCR. The wild-type allele generated a 316-bp fragment with the primers 5'-GCCCAGCTGTGTGACTGT-3' (forward) and 5'-CCCACCCATGCTACACT-3' (reverse) and the targeted allele a 140-bp fragment with the primers 5'-TGTTCTCCTCTTCCTCATCC-3' (forward) and 5'-ATTGTCTGTTGTGCCAGTC-3' (reverse). The 129/sv/C57Bl hybrid VEGF-B+/2 mice were backcrossed onto a C57Bl background, and animals from the fourth to the sixth backcrosses were used in this work.

Histology
Paraformaldehyde-fixed, paraffin-embedded tissues were cut into 5-μm sections and stained with hematoxylin-eosin by standard procedures. The MEC 13.3 antibody to platelet–endothelial cell adhesion molecule (PECAM, Pharmingen) was used as previously outlined.20 The capillary density and intercapillary distance were estimated by counting the PECAM-positive capillaries in 5 high-power views per section with 5 sections from each animal (VEGF-B-/-, n=3, and VEGF-B-+/-, n=3). To visualize the His-Purkinje system, sections were stained for acetylcholinesterase activity. Frozen unfixed hearts were cut into 10-μm sections and air-dried for 30 minutes. After fixation in 4% paraformaldehyde and subsequent wash, the sections were immersed in staining solution (in mmol/L: sodium acetate 38, sodium citrate 4.8, copper sulfate 3, tetraisopropyl pyrophosphoramide 0.08 [Sigma], potassium ferricyanide 0.5, and acetylthiocholine iodide 0.87, and 0.012% acetic acid) for 3 hours at 4°C. Connexin stainings using antibodies to connexin 40 (Zymed Laboratories) and connexin 43 (Alpha Diagnostic International) were performed as described.14

Northern Blot Analysis and RNase Protection Assays
Total cellular RNA from tissues was analyzed by Northern blotting by standard procedures.21 Full-length cDNA22 or only the deleted segment of the VEGF-B gene (see above) was labeled by random priming (Megaprim kit, Amersham) and used as the probe. For RNase protection analysis, [32P]UTP-labeled riboprobes were synthesized by in vitro transcription (Ambion) of the corresponding mouse cDNA fragments as the templates (see Table 1 for details of the probes used). Thirty to 80 μg of total RNA was analyzed according to the manufacturer’s protocols (Ambion). Protected fragments were separated on polyacrylamide gels, and the signals were quantified (Fuji Bas 1500 phosphorimager). For accurate
quantification, the data were normalized for each sample against the expression of β-actin with a 250-bp β-actin probe (Ambion).

Electrophysiological Measurements
To record the ECGs and other parameters, transmitters were implanted in the abdomen of adult animals (6 to 8 weeks old). Two electrodes were positioned close to the apex of the heart and subcutaneously in the right shoulder, respectively. The animals were allowed to recover for 7 days before data collection. The DATA Science system, the implantable transmitters, telemetry receivers, and a consolidation matrix that relays information from the telemetry receivers, was used. The data acquisition system consisted of a data translation analog-to-digital converter and the program PC-LAB v. 5.0. The data obtained were further analyzed with an Excel macro program and were presented as activity counts per minute, body temperature, heart rate, ECG arrays allowing studies of heart rhythms, and average ECGs for determination of PQ and QRS intervals. The ECGs were recorded 48 times a day for 1 week. Averaged ECGs were from 50 complexes, and the PQ and QRS intervals were calculated from 48 averaged ECGs collected during 24 hours. Eight VEGF-B−/− mice and 8 control littermates were analyzed.

Blood and Serum Composition
Blood (300 µL/mouse) was collected in EDTA tubes (Labdesign) and analyzed at the Institution for Clinical Chemistry, Swedish Agricultural University, Uppsala, Sweden. For serum composition, serum (200 µL/mouse) was collected and analyzed at the Clinical Chemistry Laboratory, Karolinska Hospital, Sweden.

Cornea Pocket Assay
Sustained-release pellets containing recombinant VEGF or basic fibroblast growth factor (bFGF) were implanted in the corneas of VEGF-B−/− mice and in control littermates. The procedures involved and evaluation of the results have been described previously.

Statistical Analyses
The statistical analyses were done by 2-tailed Student’s t test.

Results
VEGF-B−/− Mice Are Viable and Fertile
The inactivation of VEGF-B removes ~330 bp of coding sequence within the VEGF homology domain, including 7 of the 8 invariant cysteine residues involved in intermolecular and intramolecular disulfide bonding and the VEGFR-1 binding epitope, thus producing a null allele (Figure 1A). Intercrossing the heterozygous F1 mice gave rise to offspring with close to expected mendelian ratios of 1:2:1 for VEGF-B−/−, VEGF-B−/+, and VEGF-B+/+ animals. Top, Analysis using full-length VEGF-B cDNA as probe. Bottom, Analysis using a probe covering deleted portion of VEGF-B gene. Results show that −/− mice do not express detectable levels of normal or truncated VEGF-B transcripts and that +/− animals express about half of level of transcripts vs +/+ mice.

Figure 1. Generation of VEGF-B−/− mice. A, Genomic organization of wild-type (wt) and targeted VEGF-B alleles. Neomycin cassette in targeted allele replaced part of exons 3 and 4. Locations of genomic fragments used as probes in Southern blot analysis and restriction endonuclease cleavage sites used in analysis are indicated. B, Southern blot analyses of wt (+/+), heterozygous (+/−), and homozygous VEGF-B−/− animals (−/−) with Sacl- (top) or EcoRI-cleaved DNA (middle and bottom) showing that genomic sequences corresponding to 3′ part of exon 3, intron between exons 3 and 4, and exon 4 are deleted in targeted allele. Probes used were as described in A. C, Ratios of animals with different genotypes are indicated below. D, Northern blot analysis of expression of VEGF-B transcripts in heart and skeletal muscle tissues from +/+, +/−, and −/− animals. Top, Analysis using full-length VEGF-B cDNA as probe. Bottom, Analysis using a probe covering deleted portion of VEGF-B gene. Results show that −/− mice do not express detectable levels of normal or truncated VEGF-B transcripts and that +/− animals express about half of level of transcripts vs +/+ mice.
heart weight, morphology, and histology were investigated in the VEGF-B−/− mice. Visual inspection revealed no major differences (Figure 2A and 2B), and the heart weights did not differ significantly (data not shown). Sectioned VEGF-B−/− hearts showed a normal appearance, including the thickness of walls and trabeculation of the ventricles and development of the atria and the valve region (Figure 2, C through F). Smooth muscle actin staining in the larger vessels was similar in VEGF-B−/− animals and control littermates, suggesting that recruitment and growth of vascular smooth muscle cells along the larger vessels was preserved in the VEGF-B−/− animals (data not shown). The capillary densities and intercapillary distances were examined in PECAM-stained, cross-sectioned myocardium, but no significant differences were obtained between normal and VEGF-B−/− hearts (Figure 2G and 2H and Table 1). These analyses suggest that heart development is largely normal in VEGF-B−/− mice.

Angiogenesis-Associated Gene Expression in Hearts From Normal and VEGF-B−/− Mice

The expression levels of angiogenesis-associated genes were investigated in hearts from normal and VEGF-B−/− animals. Quantitative RNase protection analysis revealed that transcripts encoding VEGF, PI GF, and platelet-derived growth factor (PDGF) A were normal in VEGF-B−/− animals, whereas PDGF-B and VEGF-C were significantly upregulated compared with the normal littermates (Figure 3). The expression levels of several growth factor receptors were also normal. Similarly, the mRNA levels of NP-1, PECAM, urokinase plasminogen activator, and plasminogen activator inhibitor-1 were normal in the mutant animals.

Abnormal ECGs in VEGF-B−/− Mice

The parameters of cardiac function, eg, heart rate and ECG, were investigated in normal and VEGF-B−/− mice. The recordings showed that the VEGF-B−/− mice have normal heart rate and sinus rhythm and display a normal circadian rhythm of the rate fluctuations. Similarly, the body temperature and the spontaneous movements of the VEGF-B−/− animals in the cages were normal (data not shown). Detailed analysis of averaged ECGs showed that the VEGF-B−/− mice had a characteristic prolongation of the PQ interval, whereas the QRS complex appeared normal (Figure 4 and Table 3). The PQ interval, measuring the conduction time from the sinus node through the atrial myocardium and the atroventricular node to the ventricles, was 10% to 15% longer in the VEGF-B−/− mice (P<0.05). Despite the prolonged PQ interval, we did not obtain any evidence of spontaneous arrhythmia in the VEGF-B−/− animals. Serum electrolyte levels (K+, Na+, Ca++) were also similar between normal and mutant mice (Table 2). Visualization of the conduction system by use of antibod-

### TABLE 2. Summary of Investigated Physiological Parameters of Normal and VEGF-B−/− Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type</th>
<th>VEGF-B−/−</th>
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<tbody>
<tr>
<td>Weight (1 mo), g*</td>
<td>20.48±1.99</td>
<td>19.48±1.36</td>
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<tr>
<td>Males</td>
<td>15.16±1.39</td>
<td>15.35±1.39</td>
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<tr>
<td>Heart†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capillary density, counts/field</td>
<td>52.3±8.0</td>
<td>51.7±8.7</td>
</tr>
<tr>
<td>Intercapillary distance, μm</td>
<td>1.3±2.7</td>
<td>1.3±2.1</td>
</tr>
<tr>
<td>Blood composition‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes, 10⁹/L</td>
<td>0.11±0.04</td>
<td>0.22±0.17</td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>161.6±6.85</td>
<td>159.4±11.1</td>
</tr>
<tr>
<td>B-TPK, 10⁹/L</td>
<td>710.4±92.3</td>
<td>680.5±151.3</td>
</tr>
<tr>
<td>B-EPK, 10⁹/L</td>
<td>10.04±0.64</td>
<td>9.88±0.91</td>
</tr>
<tr>
<td>B-LPK, 10⁹/L</td>
<td>6.17±1.40</td>
<td>5.49±1.59</td>
</tr>
<tr>
<td>Serum electrolytes, mmol/L†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>152.7±2.1</td>
<td>149.0±6.8</td>
</tr>
<tr>
<td>K</td>
<td>6.8±0.5</td>
<td>6.8±0.4</td>
</tr>
<tr>
<td>Ca</td>
<td>2.6±0.1</td>
<td>2.6±0.03</td>
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Two-tailed Student’s t test showed no significant differences in any of the tests. Hb indicates hemoglobin; B-TPK, total number of thrombocytes; B-EPK, total number of erythrocytes; and B-LPK, total number of leukocytes.

* n=13 wild-type (wt) males, 15 wt females, 11 VEGF-B−/− males, and females; †n=3 wt/VEGF-B−/−; ‡n=7 wt and 11 VEGF-B−/−.
ties to connexin 40 and 43 and acetylcholinesterase stainings indicated that the gap junctions between the atrial myocytes were intact and that the His-Purkinje system appeared normal (data not shown).

VEGF-B<sup>−/−</sup> Mice Have Normal VEGF- and bFGF-Induced Angiogenic Responses

The normal development of VEGF-B<sup>−/−</sup> mice suggested that embryonic and postnatal angiogenesis do not require VEGF-B. VEGF-B offspring produced by VEGF-B<sup>−/−</sup> females appear normal, ruling out any rescue of embryonic development by maternal VEGF-B. To analyze the angiogenic response in adult animals, sustained-release pellets containing recombinant VEGF or bFGF were implanted into the corneas of VEGF-B<sup>−/−</sup> mice and control littermates. The stimulation of blood vessel growth from the limbus region was recorded after 5 days. This analysis revealed no significant difference between mutant animals and control littermates in the ability to generate corneal vessels in response to VEGF or bFGF (Figure 5).

**Discussion**

In this study, functional alteration was observed in VEGF-B<sup>−/−</sup> mice that displayed a significant increase in the PQ interval in the ECG. This 10% to 15% increase in time required for atrial conduction did not generate arrhythmia or any other known malfunction of the heart. An increased PQ time occurs in first-degree atrioventricular block in humans, a condition caused primarily by atrioventricular nodal dysfunction. There could be several causes for the conduction delay, eg, enhanced vagal tone, defects in the conduction system, electrolyte disturbances, or ischemia. Tissue ischemia seems unlikely, because we found no defects in the cardiac vasculature. Also, the conduction system and serum electrolytes were normal, and the basal heart rate was not affected, suggesting that there is no enhanced vagal tone. Thus, the mechanisms underlying the altered conduction properties in the VEGF-B<sup>−/−</sup> animals remain unknown.

Currently, it is believed that VEGFR-1 is expressed almost exclusively by the vasculature<sup>27,28</sup> and not by myocytes, favoring the view that the conduction defect might be indirect. Both VEGF-B and NP-1 are expressed in myocardium, however, at least during development<sup>13,14,17</sup> and it cannot be excluded that the observed effect is mediated via NP-1 expressed in cardiac myocytes.

The present study suggests that VEGF-B is dispensable for normal development and for maintenance of the adult vasculature. This may be partly because VEGF-B specifically binds VEGFR-1 but not VEGFR-2, which is critically required for proliferation of EC precursors. The physiological role of VEGFR-1 is unclear, because deletion of its intracellular tyrosine kinase domain is not necessary for normal vessel development<sup>29</sup> whereas the extracellular domain is critical for its function.<sup>30</sup>

Redundant activities of PlGF and VEGF, the 2 other known VEGFR-1 ligands, may contribute to the weak phe-

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**Table 3. Data From ECG Measurements of Normal and VEGF-B<sup>−/−</sup> Mice**

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>VEGF-B&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>538±58</td>
<td>526±36</td>
<td>NS</td>
</tr>
<tr>
<td>PQ interval, ms</td>
<td>35.7±2.0</td>
<td>39.7±2.5</td>
<td>0.014</td>
</tr>
<tr>
<td>ORS interval, ms</td>
<td>11.6±1.0</td>
<td>12.2±0.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

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**Figure 3.** Analysis of angiogenesis-associated transcripts in hearts from wild-type (+/+) and VEGF-B<sup>−/−</sup> mice. mRNA levels for several growth factors, their receptors, genes involved in proteolysis in angiogenesis, and PECAM were quantified by RNase protection assays. Expression levels were normalized in each experiment with β-actin as an internal control. Expression levels in +/+ mice were arbitrarily set to 1. PDGF-B and VEGF-C were significantly upregulated in −/− animals (P<0.05). RNA samples from 5 different animals were analyzed for each group. ND indicates not detectable; uPA, urokinase plasminogen activator; and PAI, plasminogen activator inhibitor.

**Figure 4.** ECGs from control (+/+) and VEGF-B<sup>−/−</sup> mice. A, Representative primary recordings from +/+ and VEGF-B<sup>−/−</sup> mice. Bar=100 ms. B, Averaged ECGs from +/+ and VEGF-B<sup>−/−</sup> mice. Data show that no signs of arrhythmia can be detected in VEGF-B<sup>−/−</sup> mice and that averaged ECG is normal, except for 10% to 15% prolongation of PQ interval.
VEGFR-2, which has a lower affinity for VEGF. Thereby, VEGF would be competed off VEGF-B and PlGF, might act by occupying available binding sites on VEGFR-1. Several previous studies, both in vitro and in vivo, have suggested that VEGFR-1 specific ligands, like VEGF-B and PlGF, might act by occupying available binding sites on VEGFR-1. Thereby, VEGF would be competed off from this receptor and it would be made better available to VEGFR-2, which has a lower affinity for VEGF. Although this hypothesis is attractive, and more VEGF could bind to VEGFR-1 in VEGF-B−/− embryos, we find no evidence for decreased activity of VEGF via VEGFR-2 in cardiac tissue, such as retarded tissue growth or decreased vessel density. Similarly, we obtained no evidence for a compensatory upregulation of the 2 known VEGFR-1 ligands in the VEGF-B−− mice. In fact, significant alterations in expression levels of several genes involved in angiogenesis were found only for PDGF-B and VEGF-C. Whether the upregulations of these 2 factors in the VEGF-B−− hearts are connected or independent events remains to be established. It is known, however, that VEGF stimulation of ECs in vitro (presumably via VEGFR-2) induces expression of PDGF-B (U.E., unpublished observation, 2000), suggesting that the increased expression of PDGF-B and VEGF-C may be connected.

During the preparation of this article, Bellomo et al. published the results from their VEGF-B knockout model. They report phenotypes different from ours, e.g., a reduction in heart size, a decrease in the thickness of the walls of the left ventricle, and impaired ability to repay coronary flow after transient coronary occlusion. It would be interesting to know the molecular mechanisms underlying these phenotypes, because we could not detect a similar reduction in heart size or a reduced thickness of the walls of the left ventricle in our VEGF-B−− animals. In summary, the VEGF-B−− mice generated in this study developed normally but displayed a characteristic defect in the atrial conduction system reminiscent of certain abnormal conditions found in humans. Further studies will be necessary to reveal the molecular mechanisms underlying these defects.

Acknowledgments

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