Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors

GENEVIEVE S. MARCHAND, NICOLAS NOISEUX, JEAN-FRANCOIS TANGUAY, AND MARTIN G. SIROIS
Montreal Heart Institute and Department of Pharmacology, University of Montreal, Montreal, Quebec, Canada H1T 1C8

Received 14 March 2001; accepted in final form 4 September 2001

Marchand, Geneviève S., Nicolas Noiseux, Jean-François Tanguay, and Martin G. Sirois. Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors. Am J Physiol Heart Circ Physiol 282: H194–H204, 2002.—Angiogenesis, the formation of new blood vessels from preexisting ones, is a critical component of various pathologies such as tumor progression, rheumatoid arthritis, and retinopathies. Vascular endothelial growth factor (VEGF) is a mitogenic and chemiotactic factor capable of inducing angiogenesis through the activation of its receptors, fetal liver kinase-1 (Flk-1) and fms-like tyrosine kinase-1 (Flt-1), expressed on endothelial cells. The purpose of the present study was to assess if a treatment with antisense (AS) oligonucleotides directed against VEGF receptors Flk-1 or Flt-1 mRNA could prevent VEGF-mediated angiogenesis. With the use of miniosmotic pumps, phosphate-buffered saline, VEGF, or VEGF combined with AS-Flk-1, AS-Flt-1, or AS-scrambled oligonucleotides were released in mouse testis for 14 days. VEGF (1, 2.5, and 5 μg) increased the formation of new capillary blood vessels by 236, 246, and 287%, respectively. The combination of AS-Flk-1 or AS-Flt-1 (200 μg) to VEGF (2.5 μg) reduced by 87 and 85% the formation of new blood vessels, respectively, and the expression of their corresponding proteins. These data demonstrate the therapeutical potential of AS-Flk-1 or AS-Flt-1 to prevent VEGF-mediated angiogenesis in vivo.

Address for reprint requests and other correspondence: M. G. Sirois, Montreal Heart Institute, 5000 Belanger St., Montreal, Quebec, Canada H1T 1C8 (E-mail: mgsirois@cm.umontreal.ca).

Angiogenesis can be defined as the growth of new blood vessels from preexisting vessels and capillaries and is controlled by the balance between proangiogenic and antiangiogenic molecules (11, 43). It differs from vasculogenesis, which consists of an embryonic phenomenon, in which new blood vessels are formed de novo from blood islands composed of committed stem cells (11). Angiogenesis plays an important role in a variety of physiological processes such as wound healing, corpus luteum formation, and embryonic development (19). However, uncontrolled neovascularization can be associated with human diseases, including growth of solid tumors, retinopathies, rheumatoid arthritis, psoriasis, and atherosclerotic plaque growth (19). Angiogenesis is a complex process that includes proliferation and migration of endothelial cells, disruption of the capillary basal membrane, and formation of vascular tubes and networks (15). These events are activated by growth factors binding to endothelial cell surface receptors (9).

Vascular endothelial growth factor (VEGF) is known to be a potent stimulator of proliferation, migration, and differentiation of endothelial cells in vitro (17, 59). It has the ability to trigger the entire sequence of events leading to new vessel growth (18, 26, 69) and can stimulate angiogenesis in vivo in different animal models such as in the rat and rabbit cornea (8, 40), the chorioallantoic membrane (14), and the rabbit bone graft model (8). VEGF binding to endothelial cell receptors also induces the production of nitric oxide (NO), which is considered to be a mediator of VEGF-induced vasodilation and angiogenesis in vivo (29, 35, 49, 61, 64, 66).

The biological activities of VEGF are mediated through two high-affinity receptor tyrosine kinases, which are fms-like-tyrosine kinase-1 (Flt-1) or VEGF receptor 1 (VEGFR-1), and fetal liver kinase-1 (Flk-1) or VEGFR-2 (13), whose expressions are mainly restricted to endothelial cells (54). Flt-1 binds VEGF with high affinity but is weakly expressed by endothelial cells, which makes it difficult to detect. On the other hand, Flk-1 binds VEGF with a lower affinity, although Flk-1 is highly expressed on endothelial cells and easier to detect (4, 62). In addition, Flk-1 appears to be the major transducer of VEGF signals in endothelial cells, which result in cell proliferation, migration, differentiation, tube formation, increase of vascular permeability, and maintenance of vascular integrity (70). As for Flt-1, it seems to have a role in endothelial cell assembly (6).

Inhibition of angiogenesis has emerged as a promising strategy for the treatment of pathological conditions that are dependent on new blood vessel formation (16). Targeting either VEGF and/or its receptors might be potential avenues for the inhibition of angiogenesis because their expression is upregulated in numerous metastatic tumors (5) in the regenerating liver after...
injury (44), in hypoxic vasculature (42, 60), and in the diabetic retina (25).

Recent studies in our laboratory (3) using antisense (AS) oligonucleotides directed against the mRNA of VEGF receptors Flk-1 and Flt-1 demonstrated that in vitro VEGF-induced endothelial cell proliferation, migration, and platelet-activating factor (PAF) synthesis are mediated through the Flk-1 receptor. In contrast, Flt-1 receptor does not seem to be involved in these VEGF-mediated activities in vitro (3). The aim of the present study was to assess if the use of AS oligonucleotides targeting Flk-1 or Flt-1 mRNA could inhibit in vivo VEGF-induced angiogenesis.

MATERIALS AND METHODS

Surgical procedures. The surgical procedures were performed by one trained operator and were performed in accordance to the guidelines set by the Montreal Heart Institute animal care committee and the Canadian Council for Animal Protection. Male C57/Bl6 mice (18–22 g body wt) (Charles River Breeding Laboratories; Saint-Constant, Quebec, Canada) were anesthetized with an injection (100 mg/kg ip) of ketamine HCl (Ketalean; MTC Pharmaceuticals; Cambridge, Ontario, Canada) and xylazine HCl (10 mg/kg) (Rompun; Bayer; Etobicoke, Ontario, Canada). A diagonal incision (2 cm) of the skin was made just above the right groin after the skin was disinfected with 0.5% chlorexidine (Novopharm; Toronto, Ontario, Canada). The rectus abdominis muscle and transversalis fascia were dissected to get access to the peritoneal cavity (Fig. 1A). The right testis was pulled out through the inguinal canal and brought to the skin incision (Fig. 1B). A fine needle (25G5/8) was used to create a micropuncture in the visceral layer of the tunica vaginalis of the testis, near the head of the epididymis where there were no apparent vessels (Fig. 1C). A sterilized polyethylene (PE)-10 catheter (Cole-Parmer; Vernon Hills, IL) was introduced into the testis in a selected area and secured with silk 6-0 thread (Davis and Geck; Wayne, NJ) attached to the tunica vaginalis (Fig. 1, D and E). The testis was repositioned into the scrotum by passing through the inguinal canal, and the rectus sheath was sutured with silk 6-0 (Fig. 1H). The free tip of the catheter inserted in the testis was fixed with silk 6-0 to the rectus sheath to prevent unwanted movements and connected to a larger PE-60 catheter (Becton-Dickinson; Sparks, MD). This latter was adapted to a miniosmotic pump (model 2002, Alza; Palo Alto, CA) with a controlled flow delivery of 0.5 μl/h for 14 days (Fig. 1G). The pump was placed subcutaneously on the abdominal right flank. The wound was then closed with Dexon 5-0 suture (Davis and Geck) and the animals were returned to their cages.

Fig. 1. During surgery, an incision of the skin is made just above the right thigh of the mouse (A), an incision of the rectus sheath is made to access the abdominal cavity, and the right testis is pulled out through the inguinal canal (B). A fine needle (model 25G5/8) is used to create a small hole at the base of the testis where there are no apparent blood vessels (C), a small polyethylene (PE)-10 catheter is inserted in the hole made at the base of the testis (D), and the catheter is fixed at the base of the testis to avoid its movement into the testis (E). At this moment, pictures of 4 different regions of the testis (A1, A2, B1, and B2) at different magnifications are taken with a digital camera (F), the testis is reinserted into the scrotum by passing through the inguinal canal, the rectus sheath is sutured, and a miniosmotic pump (G) prefilled with the substance to be infused into the testis is attached to the free extremity of the catheter. The pump is placed under the skin on the abdominal right flank, and the skin is finally sutured (H).
different concentrations (1, 2.5, and 5 μg/200 μl PBS-0.1% BSA) to obtain a dose-response curve on the induction of blood vessel formation. On the basis of these data (see RESULTS), a group of mice was treated with VEGF (2.5 μg/100 μl PBS-0.1% BSA) combined with AS-Flk-1 (200 μg/100 μl PBS-0.1% BSA), AS-Flt-1 (200 μg/100 μl PBS-0.1% BSA) or AS scrambled (Scr) (200 μg/100 μl PBS-0.1% BSA). Another group of mice was treated with the oligomers (200 μg/200 μl PBS-0.1% BSA) in the absence of VEGF. Finally, in a sham-operated group of animals, the testes were manipulated as above but without the insertion of the catheter and osmotic pump. After 14 days of treatment, the animals were anesthetized and dissected as described above to bring the right testis to the skin incision for image acquisitions. Animals were then euthanized with an overdose of ketamine and xylazine.

Image acquisition and analysis. Pictures of various regions of the testis with inserted catheters were taken at different magnifications (×8.4, ×12, ×24, ×38.4, and ×48) with a color video digital camera (model DKC 5000; Sony) adapted to a binocular (model SZX12; Olympus). To assess the number of new blood vessels on the surface of the testis, the testis was divided into four sections: A1, A2, B1, and B2 (Fig. 1F). For each testis, one picture per section was taken at day 0. A picture of the exact same region was then taken at day 14 after treatment. These pictures were taken at a magnification of ×48, and the pictures at day 0 and day 14 were then compared. The number of new blood vessels present at day 14 but absent at day 0 was counted on each picture for each section. The new blood vessels counted were full-length vessels of at least 150 μm and not the result of sprouting. The surface of the pictures taken at ×48 magnification was 1.288 mm², and the number of new blood vessels was converted as the number of new blood vessels (in mm²) by dividing the number of new blood vessels per field of ×48 by the surface (1.288).

To confirm that the new blood vessels observed at day 14 were not preexisting blood vessels that have been vasodilated by a VEGF treatment, we did another set of experiments in which pictures were taken at day 0 and day 14 (in VEGF-treated groups). The Alzet miniosmotic pump was then removed and a new set of pictures were taken 3 days later (day 17); in such, the VEGF effect was no longer involved because VEGF has a plasmatic half-life of 3 min (15).

The images taken before (day 0) and after treatment (day 14 and in some cases at day 17) were then compared and different parameters were determined: the number of new blood vessels, the length and diameter of the new vessels, change in the diameter of preexisting vessels, and immunohistochemistry analysis. The number of new blood vessels was determined by counting directly on the pictures the number of new vessels created by various treatments. The length and diameter of the vessels were calculated by computerized digital planimetry with the use of a dedicated video binocular and customized software (NIH Image version 1.6).

Selection of AS oligomers. The AS oligomer sequences were selected in function of previous studies (3, 10, 58) and had specific characteristics [no more than three consecutive guanosines, the incapacity to form hairpins without or minimal capacity to dimerize together, and had between 15 and 20 bases length (18 bases in the present study)]. The murine Flt-1 and Flk-1 cDNA, respectively, were obtained from GenBank (Accession Numbers D28498 and X70842). Four different AS oligonucleotide phosphorothioate backbone sequences have been selected at this time, two targeting mice Flt-1 mRNA (AS1, AS1-Flt: 5’-AgAG CAg ACA CCC gAg CAg-3’; AS 2, AS2-Flt: 5’-CCC TGa gCC ATA TCC TGt-3’) and two targeting mice Flk-1 mRNA (AS 1, AS1-Flk: 5’-AgAG ACC ACA gAg CAg CAg-3’; AS 2, AS2-Flk: 5’-AgAT ATg TCT TTT gTg-3’). Two Scr phosphorothioate sequences (Scr Flt, Scr-Flt: 5’-ACT gTC CAC TGc gAg TTC-3’; Scr Flk, SCR-Flk: 5’-TTt Ctg gTA TGc ATT gTg-3’) were also selected as negative controls. The oligomers were used under sterile conditions.

Immunohistochemistry of Flk-1, Flt-1, and endothelial cell NO synthase expression. After the animals were euthanized, the testes were isolated, fixed in 10% formalin PBS-buffered solution, and processed for standard histological procedures. Testes sections were cut into 6-μm-long sections and deparaffinized in xylene and ethanol baths, and endogenous peroxidase activity was quenched in a solution of methanol (200 ml) plus hydrogen peroxide (30%, 50 ml). Nonspecific binding of primary antibodies was prevented by preincubating the tissues with 5% serum from the species used to raise the secondary antibodies. Testes sections were then exposed to primary antibodies for 1 h in endothelial NO synthase (ecNOS) or 2 h in Flk-1 and Flt-1. The primary antibodies used were monoclonal anti-mouse Flk-1 IgG (Santa Cruz Biotechnology; Santa Cruz, CA) diluted 1:500, 1,000, and 2,500, rabbit polyclonal anti-human Flt-1 IgG (Santa Cruz Biotechnology) diluted 1:100, 250, and 500, and monoclonal anti-human ecNOS IgG (Transduction Laboratories; Mississauga, Ontario, Canada) diluted 1:2,500, 5,000, and 10,000. Purified nonspecific mouse IgG (for Flk-1 and ecNOS detection) or rabbit IgG (for Flt-1) were used as primary negative control antibodies. On incubation, the primary antibodies were washed with PBS, and the slides were incubated for 60 min either with a biotinylated goat anti-rabbit (for Flt-1 detection) or a goat anti-mouse IgG (for Flk-1 and ecNOS detection) (1:400) (Vector Laboratories; Burlingame, CA). Peroxidase labeling was achieved with incubation using an avidin/peroxidase complex (ABC kit; Vector), and antibody visualization was established after a 5-min exposure to 3,3’-diaminobenzidine solution (DAB kit; Vector). Testes were counterstained with Gill’s hematoxylin no. 3 solution, rinsed in tap and distilled water, and then mounted with a permount solution.

Statistical analysis. Data are means ± SE. Statistical comparisons were determined by analysis of variance, followed by a paired or unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. Data were considered significantly different if a value of P < 0.05 was observed.

RESULTS

Angiogenesis assessment. The infusion of PBS (200 μl) during a 14-day period with a miniosmotic pump adapted to a catheter inserted in the testis induced the formation of 1.86 ± 0.37 new blood vessels/mm (Figs. 2A and 3A). This formation of new blood vessels was not different from the one observed in control sham-operated animals: 1.58 ± 0.27 new blood vessels/mm (Fig. 3A). Treatment with VEGF at different concentrations (1, 2.5, and 5 μg/200 μl) delivered on a 14-day period increased significantly the number of new blood vessels by 236% (P < 0.01), 246% (P < 0.01), and 287% (P < 0.01), respectively, compared with sham control groups (Figs. 2B and 3A).

Effect of AS oligonucleotides on the formation of new blood vessels. On the basis of our data in Fig. 3A, we used VEGF at a dose of 2.5 μg for the following experiments. As mentioned above, the infusion of VEGF (2.5 μg/200 μl) for a period of 14 days induced the formation of 5.48 ± 0.96 new blood vessels/mm² (P < 0.01 com-
pared with sham control group) (Fig. 3B). The combination of AS1-Flk-1 (200 μg), AS2-Flk-1 (200 μg), AS1-Flt-1 (200 μg), or AS2-Flt-1 (200 μg) with VEGF (2.5 μg) into 200 μl (final volume) decreased the formation of new blood vessels by 85, 87, 85, and 71%, respectively, compared with the VEGF-treated group (P < 0.01) (Figs. 2, C and D, and 3B). The combination of AS-Scr (200 μg) with VEGF (2.5 μg) into 200 μl (final volume) led to the formation of 5.34 ± 0.64 new blood vessels/mm², which was statistically not different from the group treated with VEGF alone (Fig. 3B). In another group, we tested the effect of the AS and Scr oligomers in PBS-treated mice, and these oligomers did not alter significantly the number of preexisting blood vessels and the basal formation of new blood vessels mediated by PBS (data not shown).

The length and the diameter of the new blood vessels were also determined. The average length of the new blood vessels in all studied groups fluctuated from 245 to 324 μm. The average length of new blood vessels under VEGF treatment (2.5 μg/200 μl) was 284 ± 10 μm (Table 1). The diameter of the new blood vessels has been measured as well, and all had a capillary-like diameter with an average diameter fluctuating from 6.30 to 9.04 μm, including an average diameter of 8.52 ± 0.40 μm under VEGF treatment (2.5 μg/200 μl) (Table 1).

Vasodilatory effect of VEGF on preexisting blood vessels. VEGF is a vasodilatory mediator; consequently, we wanted to assess if the new blood vessels observed on a sustained infusion of VEGF were due to the dilation of preexisting capillaries or due to its angiogenic potential. First, we looked at the vasodilatory effect of VEGF on preexisting blood vessels with a diameter <20 μm and on vessels with a diameter between 20 and 100 μm. Pictures of the testes to be treated were taken at day 0 before treatment and at day 14, the miniosmotic pump was then removed, and another set of pictures was taken 3 days later at day 17. In the control sham-operated group, there was no change in the diameter of preexisting vessels at days 14 and 17 compared with the diameter observed at day 0 (Fig. 4A). A treatment with VEGF (2.5 μg/200 μl) delivered on a period of 14 days did not mediate a vasodilation of preexisting vessels with a diameter <20 μm. The diameter of these vessels increased by 6% at day 14 compared with day 0 and decreased by 3% at day 17 compared with day 14 (P = not significant) (Fig. 4A). However, the infusion of VEGF (2.5 μg/200 μl) over a period of 14 days increased by 40% the dilation of preexisting blood vessels having a diameter between 20 and 100 μm compared with untreated arteries (day 0) (P < 0.01). However, this vasodilatory effect mediated by
Vessel density, length, and diameter of new blood vessels according to treatment

VEGF infusion was abrogated within a period of 3 days after the arrest of VEGF infusion (day 17) and was no longer significant compared with day 0 (Fig. 4A).

Effect of AS oligonucleotides on VEGF-mediated vasodilation of preexisting blood vessels. In sham-operated mice, the diameter of preexisting blood vessels (20–100 μm in diameter) did not fluctuate significantly from day 0 to day 14, and the mean diameter was set as the 100% baseline diameter. A treatment with VEGF (2.5 μg/200 μl) delivered on a 14-day period induced the vasodilation of preexisting blood vessels (20–100 μm diameter) by 48% (P < 0.01 compared with control sham-operated mice) (Fig. 4B). The combination of AS1-Flk-1 (200 μg), AS2-Flk-1 (200 μg), AS1-Flt-1 (200 μg), or AS2-Flt-1 (200 μg) with VEGF (2.5 μg) into 200 μl (final volume) abrogated the VEGF vasodilatory effect of preexisting blood vessels (20–100 μm diameter) (P < 0.01) (Fig. 4B). Treatment with a Scr oligomer did not reduce the VEGF-mediated vasodilatory effect. In fact, it even increased the vasodilation of preexisting blood vessels (Fig. 4B). The combination of AS or Scr

![Graph A](image1)

![Graph B](image2)

Fig. 3. VEGF angiogenic effect and its inhibition by AS oligonucleotide gene therapy. A: effect of a sustained infusion of VEGF (1, 2.5, and 5 μg) on a 14-day period on the formation of new blood vessels in mouse testes compared with control sham-operated and PBS-treated groups. B: combination of AS oligomers targeting either Flk-1 or Flt-1 mRNA (AS-Flk-1 or AS-Flt-1; 200 μg) to VEGF (2.5 μg) abrogated the formation of new blood vessels, whereas AS scrambled (Scr) oligomers (200 μg) did not prevent VEGF-angiogenic activity. n = 5–11 animals per treatment. **P < 0.01 and ***P < 0.001 vs. sham; ††P < 0.01 vs. VEGF.

![Graph C](image3)

Fig. 4. VEGF vasodilatory effect on preexisting blood vessels and its inhibition by AS oligonucleotide gene therapy. A: in the sham-operated control group, there was no change in the diameter of preexisting blood vessels at days 14 and 17 postprocedure. VEGF (2.5 μg) infusion on a 14-day period did not modulate the vascular tone of preexisting blood vessels with a diameter <20 μm. However, VEGF increased significantly the diameter of preexisting blood vessels with a diameter from 20 to 100 μm compared with untreated arteries (day 0). The arrest of VEGF infusion abrogated its vasodilatory effect within 3 days (day 17). B: combination of AS oligomers targeting either Flk-1 or Flt-1 mRNA (AS-Flk-1 or AS-Flt-1; 200 μg) to VEGF (2.5 μg) inhibited the vasodilatory effect of VEGF on preexisting vessels with a diameter from 20 to 100 μm. Addition of a Scr oligomer to VEGF did not inhibit VEGF vasodilatory effect. n = 4–11 animals per treatment. **P < 0.01 vs. day 0; ††P < 0.05 vs. day 14; *P < 0.01 and ***P < 0.001 vs. sham; ††P < 0.01 vs. VEGF.

Table 1. Vessel density, length, and diameter of new blood vessels according to treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>New Blood Vessels, mm²</th>
<th>Length, μm</th>
<th>Diameter, μm</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.58 ± 0.27</td>
<td>251.2 ± 15.5</td>
<td>7.12 ± 0.26</td>
<td>6</td>
</tr>
<tr>
<td>PBS</td>
<td>1.86 ± 0.37</td>
<td>324.1 ± 17.0</td>
<td>6.62 ± 0.66</td>
<td>11</td>
</tr>
<tr>
<td>VEGF (2.5 μg)</td>
<td>5.48 ± 0.96</td>
<td>284.3 ± 10.0</td>
<td>8.52 ± 0.40</td>
<td>8</td>
</tr>
<tr>
<td>AS1-Flk-1 (200 μg)</td>
<td>2.17 ± 0.36</td>
<td>278.7 ± 13.1</td>
<td>7.74 ± 0.42</td>
<td>7</td>
</tr>
<tr>
<td>AS2-Flk-1 (200 μg)</td>
<td>2.08 ± 0.40</td>
<td>278.9 ± 13.0</td>
<td>6.30 ± 0.59</td>
<td>7</td>
</tr>
<tr>
<td>AS1-Flt-1 (200 μg)</td>
<td>2.15 ± 0.40</td>
<td>285.2 ± 13.3</td>
<td>9.04 ± 0.44</td>
<td>8</td>
</tr>
<tr>
<td>AS2-Flt-1 (200 μg)</td>
<td>2.71 ± 0.23</td>
<td>267.4 ± 13.5</td>
<td>8.45 ± 0.48</td>
<td>7</td>
</tr>
<tr>
<td>AS scrambled (200 μg)</td>
<td>5.34 ± 0.64</td>
<td>245.1 ± 6.4</td>
<td>7.94 ± 0.28</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals treated per group. PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor; AS, antisense; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase-1.
oligomers to PBS did not alter the basal diameter of the preexisting blood vessels (20–100 μm diameter) (data not shown).

**Effect of VEGF on the number and diameter of new blood vessels.** In previous figures, we showed under sustained VEGF infusion the presence of new blood vessels within 14 days and stated that they were <10 μm in diameter. In addition, we observed that VEGF infusion had no vasodilatory effect on preexisting blood vessels with a diameter <20 μm, but induced the vasodilation of preexisting blood vessels with a diameter >20 μm, and that this effect was abrogated on the arrest of VEGF infusion (Fig. 4A). These results suggest that the new blood vessels observed in our study cannot be the result of a vasodilation of preexisting blood vessels with a diameter <20 μm because VEGF does not induce vasodilation of these small blood vessels.

Nevertheless, to ensure that the new blood vessels observed under VEGF infusion were not the result of an unexpected vasodilation of small preexisting capillaries (<10 μm diameter), we performed an additional study, in which we quantified the number and the diameter of new blood vessels at days 14 and 17 in control sham-operated and VEGF-treated mice (Fig. 5). In control sham-operated mice, we observed the formation of 1.71 ± 0.40 new blood vessels/mm² with a mean diameter of 8.01 ± 0.40 μm at day 14; these parameters were not significantly different at day 17 (Fig. 5). A treatment with VEGF (2.5 μg/200 μl) infused on a 14-day period induced in the present study the formation of new blood vessels (4.40 ± 0.40 vessels/mm²) (P < 0.001) compared with the control group, and these new vessels had a diameter of 7.92 ± 0.47 μm, which is not statistically different from the diameter of new blood vessels formed in the control sham-operated group (Fig. 5). The infusion of VEGF was terminated by the removal of the miniosmotic pump at day 14, and the same parameters were evaluated 3 days later (day 17) (Fig. 5). The number and diameter of new blood vessels 3 days after the removal of VEGF were not statistically different from those obtained at day 14 under VEGF treatment (Fig. 5).

**Effect of AS oligonucleotides and VEGF on Flk-1 and Flt-1 protein expression.** By immunohistochemistry analysis, we performed a semiquantitative analysis of Flk-1 and Flt-1 receptors present on vascular endothelial cells of mouse testes under normal condition (nontreated, sham operated, or PBS infused) (Figs. 6A and 7A). Under VEGF sustained infusion (2.5 μg/200 μl; 14-day period), we observed that the protein expression of Flk-1 and Flt-1 remained similar to the control sham-operated group compared with the control sham-operated group (Figs. 6B and 7B). We then investigated the effect of AS1-Flk-1 (200 μg), AS2-Flk-1 (200 μg), AS1-Flt-1 (200 μg), or AS2-Flt-1 (200 μg) combined with VEGF (2.5 μg) into 200 μl (final volume). A treatment with either AS1-Flk-1 or AS2-Flk-1 blocked Flk-1 protein expression (Fig. 6C) without affecting Flt-1 protein expression (Fig. 7C), whereas a treatment with either AS1-Flt-1 or AS2-Flt-1 blocked Flt-1 protein expression (Fig. 7D) without affecting Flk-1 protein expression (Fig. 6D). Treatment with Scr oligomers did not alter the expression of Flk-1 and Flt-1 (Figs. 6E and 7E). Purified nonspecific mouse and rabbit IgG were used as primary negative control antibodies, and in each case we could not detect any positive staining (data not shown).

**EcNOS protein expression.** VEGF mediated a vasodilation of preexisting blood vessels with a diameter between 20 and 100 μm, and such vasodilation was abrogated by AS oligomers targeting either Flk-1 or Flt-1 mRNA but not with Scr oligomers (Fig. 4B). Consequently, we wanted to confirm that the inhibition of VEGF-mediated vasodilation by the AS oligomers was not due to a nonselective downregulation of ecNOS protein expression. By immunohistochemistry analysis, we confirmed ecNOS protein expression on vascular endothelial cells of the mouse testis under normal conditions (nontreated, sham operated, or PBS infused) and in the VEGF-treated group (Fig. 8, A and B). The combination of AS oligomers either against Flk-1 or Flt-1 mRNA as well as Scr oligomers with VEGF did not alter the ecNOS protein expression on vascular endothelial cells of the mouse testis (Fig. 8, C–E). Purified nonspecific mouse IgG was used as primary negative control antibody, and we could not detect any positive staining (data not shown).

**DISCUSSION**

In the present study, we developed a new model of angiogenesis in which we were able to 1) investigate the VEGF angiogenic activity, 2) downregulate by AS oligonucleotide gene therapy the protein expression of Flk-1 and Flt-1, 3) prevent VEGF-mediated angiogenes-
Fig. 6. Positive Flk-1 protein expression on vascular endothelial cells was detected by immunohistochemistry (cells stained in brown; arrow). Basal expression was maintained in control sham-operated mice (A), VEGF infusion maintained the level of Flk-1 protein expression (B), and treatment with AS-Flk-1 prevented Flk-1 protein expression (C), whereas treatment with either AS-Flt-1 (D) or AS-Scr oligomer (E) did not alter the vascular Flk-1 protein expression (magnification ×1,000).

Fig. 7. Positive Flt-1 protein expression on vascular endothelial cells was detected by immunohistochemistry (cells stained in brown; arrow). Basal expression was maintained in control sham-operated mice (A), VEGF infusion maintained the level of Flt-1 protein expression (B), a treatment with AS-Flk-1 did not alter Flt-1 protein expression (C), and a treatment with AS-Flt-1 prevented Flt-1 protein expression (D), whereas an AS-Scr oligomer did not alter the vascular Flt-1 protein expression (E) (magnification ×1,000).
4) demonstrate that Flt-1 and Flk-1 are required to mediate VEGF vasodilatory effect. It is possible to mimic under in vitro conditions different activities related to angiogenesis, namely, endothelial cell proliferation, migration, formation of tubulelike structures, and the release of angiogenic mediators (33). However, it remains that these in vitro experiments cannot entirely reproduce the angiogenic process obtained under in vivo conditions.

Several in vivo models of angiogenesis have been elaborated throughout the years. These models include avian chorioallantoic membrane, neovascularization in the cornea (19), matrix implants, inflammatory granuloma response (30), window models (41), cancer models (68), and wound healing (8). Each model has different advantages, disadvantages, and limitations. For instance, the chick chorioallantoic membrane model is based on the application of a disk containing a growth factor placed on the chorioallantoic membrane of a fertilized egg. This system is nonmammalian, and, for certain, it is not truly considered as an in vivo model because it involves embryonic angiogenesis, which is not necessarily applicable to disease-driven angiogenesis (1, 34). Another in vivo model commonly used is the corneal neovascularization model, in which a device (pellet) releasing an angiogenic factor is implanted into the stroma of the cornea of the animals. The surgical technique is difficult and can produce undesirable inflammation by itself (2, 21). In fact, there is probably no perfect angiogenic model, and the decision about which animal model is the most suitable to be used should be based on different variables, including the objectives, the angiogenic stimulus to be studied, the quantitative analysis, the animal species, and the practical and economical aspects.

We believe that our angiogenic model in the mouse testis offers several advantages. It provides the possibility to work with mammalian animal. The testis has a moderate vascular network at the surface of the tunica vaginalis, which is very easy to locate and to measure. Consequently, it is also very easy to see the formation of new capillary-like blood vessels under angiogenic conditions, and the results are highly reproducible. The surgical procedure is relatively easy, and produces no inflammatory response at the expected angiogenic site. Furthermore, while the testis is pulled out through the natural inguinal canal for the surgical procedure and for image acquisitions, there is consequently no scar tissue or fibrosis formation on the testis. In this model, drugs and mediators of interest can be delivered locally in the testis through a catheter adapted to a miniosmotic pump placed distally. This latter approach provides a significant advantage as it allows (if desired) to modify the treatment by the removal/replacement of the delivering miniosmotic pump on a simple skin incision at the level of the abdominal flank, and thus without having to handle the treated testis. If applicable, the angiogenic inhibitors can be given by other routes (i.e., orally and intravenously).

In our study, we observed that a sustained infusion for 14 days of VEGF in the mouse testis induced the formation of new capillary-like blood vessels with a

Fig. 8. Positive endothelial nitric oxide synthase (ecNOS) protein expression on vascular endothelial cells was detected by immunohistochemistry (cells stained in brown; arrow). Basal expression in control sham-operated mice (A) and VEGF infusion maintained the level of ecNOS protein expression (B). Treatment with AS-Flk-1 (C), AS-Flt-1 (D), or an Scr oligomer (E) did not alter the vascular ecNOS protein expression (magnification ×1,000).
normal blood flow. In addition, we assessed that a treatment with AS directed against the mRNA of VEGF receptors Flk-1 or Flt-1 for a period of 14 days abrogated almost completely the VEGF-mediated angiogenesis. These results suggest that both VEGF receptors Flk-1 and Flt-1 are essential for VEGF in vivo angiogenic activity. Our results are in agreement with previous reports, in which the importance of VEGF receptors in vascular development has been illustrated using gene-targeting approaches. Indeed, targeted disruption of either Flt-1 or Flk-1 gene expression in mice prevents normal vascularization and embryonic development leading to embryonic lethality, but the two knockouts have distinctive phenotypes (62). For instance, Flk-1-deficient mice produce neither differentiated endothelial cells nor organized blood vessels and also possess no hematopoietic precursors, suggesting that this receptor is essential for development of both endothelial and hematopoietic precursors (56). In contrast, the Flt-1 knockout mice possess mature differentiated endothelial cells but have large, disorganized vessels (20). Stimulation of either receptor by VEGF triggers their phosphorylation, although the tyrosine kinase activity of Flt-1 is usually weak, about 10 times less than Flk-1 kinase, and in the absence of serum starvation, VEGF-dependent Flt-1-phosphorylation is often difficult to detect (3, 55, 62). Transfection of human Flt-1 or Flk-1 receptor cDNA in cells that do not normally express VEGFR demonstrated that the ability of VEGF to stimulate proliferation, migration, and actin reorganization is mediated by Flk-1 but not Flt-1 (55, 62). In vivo VEGF-mediated vascular permeability increases are also Flk-1 dependent (32, 63). The role of Flt-1 in angiogenesis is less clear. It has recently been shown that homozygote mice without the kinase domain of Flt-1 developed almost normal blood vessels (28). These results indicate that early in embryogenesis, the extracellular domain of Flt-1 is sufficient to rescue the lethal abnormality in Flt-1 null mutant mice (20). It is proposed (57) that the major role of Flt-1 is to negatively regulate the levels of endogenous VEGF by absorbing the ligand with the extracellular domain of Flt-1. Thus the lethality of Flt-1-deficient mice would not be due to the lack of some special signaling from Flt-1 but due to the lack of negatively regulation in the growth and differentiation of hemangioblasts (20, 57).

In mature animals, Flt-1 is localized to the endothelium in adult tissues (12, 42, 50, 53) and overexpressed in the vasculature of hypoxic tissues (24, 42, 60). Flt-1 activation by VEGF induces tissue factor expression and chemotaxis in monocytes; Flt-1 is also implicated in VEGF stimulation of urokinase and plasminogen activator inhibitor-1 and of metalloproteinase expression in smooth muscle cells (70). Stimulation of Flt-1 plays also a role in contact inhibition of endothelial cell growth and in endothelial cell assembly (6). All of these events support the hypothesis that Flt-1 activation of Flt-1 is also essential to VEGF-mediated angiogenesis in mature animals.

We observed that VEGF can induce a vasodilation of blood vessels with a diameter of 20–100 μm; such vasodilation was not observed in microvessels with a diameter <20 μm, and this latter effect can be explained by the fact that these vessels (<20 μm diameter) are composed mainly of a monolayer of endothelial cells with no or sparse smooth muscle cells surrounding them, which would provide the capacity to modulate the vascular tone. What is even more interesting is that a treatment with AS oligonucleotides against the mRNA of VEGF receptors Flk-1 or Flt-1 inhibited the vasodilation of preexisting blood vessels (20–100 μm diameter) mediated by VEGF, whereas Scr oligomers had no such effect. This result shows that both VEGF receptors Flk-1 and Flt-1 are essential for the VEGF vasodilatory effect. Previous studies (27, 61) have shown that VEGF induces a vasodilation of coronary arteries via the release of endothelial NO. Other reports have shown that NO may play a significant role in VEGF-mediated effects. NO release could mediate VEGF vascular permeability increase (45–47, 49, 64). In addition, NO may be directly involved in angiogenesis (31, 45, 49). On the other hand, other reports (23, 36–38, 48) have shown that the blockade of endogenous NO synthesis in endothelial cells is proinflammatory and can promote neutrophil-endothelial adhesion, vascular permeability, and endothelial injury. Others have shown that NO has antiangiogenic activity (51, 52) and that VEGF inflammatory and angiogenic effects are NO independent (7, 22, 39). These differences may depend on the level of NO production (48, 51). However, to the best of our knowledge, our study is the first one to establish under in vivo conditions the contribution of Flk-1 and Flt-1 on VEGF vasodilatory activity.

By immunohistochemistry analysis on testes sections, we confirmed that AS oligonucleotides against the mRNA of Flk-1 and Flt-1 reduced selectively their corresponding protein expression, whereas the Scr oligomers did not alter the Flk-1 and Flt-1 protein expression. Nevertheless, because the use of AS oligomers targeting either Flk-1 or Flt-1 mRNA abrogated the VEGF vasodilatory effect, and that VEGF may mediate the release of NO, we performed an ecNOS immunohistochemistry analysis to ensure that the AS oligomers did not directly or indirectly alter ecNOS protein expression. Our study confirmed the ecNOS protein expression on testes vasculature of control and VEGF-treated mice and that neither the AS oligomers targeting Flt-1 or Flk-1 mRNA nor Scr oligomers altered the ecNOS protein expression. These data confirm that the inhibition of VEGF-induced vasodilation and angiogenesis is not caused by the inhibition of vascular ecNOS protein expression.

Despite the fact that VEGF induced solely a vasodilation of preexisting blood vessels with a diameter >20 μm and that the diameter of the new blood vessels observed was <10 μm, we nevertheless wanted to confirm that the new blood vessels observed on a 14-day VEGF infusion was not due to the vasodilation of preexisting capillaries that could not be seen at day 0 before VEGF treatment. To discard such a possibility, we infused VEGF for 14 days, then removed the
miniosmotic pump, and collected additional images 3 days later (day 17). At day 14, we observed new blood vessels with a diameter <10 μm and preexisting blood vessels with a diameter between 20 and 100 μm that were vasodilated compared with the diameter observed at day 0. Three days after the arrest of VEGF infusion (day 17), the vasodilatory effect of VEGF on preexisting blood vessels (20–100 μm diameter) could no longer be observed, but the new blood vessels observed at day 14 were still present at day 17 and their diameter was maintained (<10 μm). These data confirm that the new blood vessels observed at day 14 were the result of VEGF angiogenic activity in the mouse testis.

In summary, the present study introduces a convenient and reproducible model that allows investigation of in vivo angiogenesis. By using AS oligonucleotides gene therapy, we have been able to downregulate the protein expression of Flk-1 and Flt-1, and in both cases we abrogated VEGF angiogenic activity. Therefore, our results suggest that the blockade of VEGF receptors expression by AS gene therapy may provide a new therapeutic approach to prevent diseases associated with pathological angiogenesis.

We thank Caroline Boucher and Véronique Philibert for technical assistance.

This study was supported by Medical Research Council of Canada Grant MT-14378, Canadian Institutes of Health Research Grant MOP-43919, and by a Heart and Stroke Foundation of Quebec assistance. (to M. G. Sirois). M. G. Sirois is a recipient of a scholarship from the Heart and Stroke Foundation of Canada. G. S. Marchand is a recipient of a studentship from the Fonds pour la Formation de Chercheurs et d’Aide à la Recherche-Fonds de la Recherche en Santé du Québec foundation.

REFERENCES

23. Kitajima I, Unoki K, and Maruyama I. Phosphorothioate oligodeoxynucleotides inhibit basic fibroblast growth factor-in...


AJP-Heart Circ Physiol • VOL 282 • JANUARY 2002 • www.ajpheart.org