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Time Course of Increased Cellular Proliferation in Collateral Arteries after Administration of Vascular Endothelial Growth Factor in a Rabbit Model of Lower Limb Vascular Insufficiency

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Proliferation of vascular cells has been previously shown to contribute to spontaneous development of coronary collaterals. Recent studies from several laboratories have established that collateral artery growth in both the heart and limb can be enhanced by administration of angiogenic growth factors, or therapeutic angiogenesis. In this study, we sought (1) to define the extent and time course of endothelial cell (EC) and smooth muscle cell (SMC) proliferation accompanying spontaneous collateral development during limb ischemia and (2) to determine the extent to which proliferative activity of ECs and SMCs is augmented during therapeutic angiogenesis with vascular endothelial growth factor (VEGF), a heparin-binding EC-specific mitogen. Ten days after induction of limb ischemia by surgically excising the femoral artery of rabbits, either VEGF (500 to 1000 μg) or saline was administered as a bolus into the iliac artery of the ischemic limb. Cellular proliferation was evaluated by bromodeoxyuridine labeling for 24 hours at day 0 (immediately before VEGF administration) and at days 3, 5, and 7 after VEGF. EC proliferation in the midzone collaterals of VEGF-treated animals increased 2.8-fold at day 5 (P < 0.05 versus control), and returned to baseline levels by day 7. SMC proliferation in midzone collaterals also increased 2.7-fold in response to VEGF (P < 0.05). No significant increase in EC or SMC proliferation was observed in either the stem or re-entry collaterals of VEGF-treated animals compared with untreated ischemic control animals. Reduction of hemodynamic deficit in the ischemic limb measured by lower limb blood pressure was documented at day 7 after VEGF (P < 0.01 versus untreated, ischemic control). These data thus (1) establish the contribution of cellular proliferation to collateral vessel development in limb ischemia and (2) support the concept that augmented cellular proliferation contributes to the enhanced formation of collateral vessels after therapeutic angiogenesis with VEGF. (Am J Pathol 1995, 147:1649–1660)

The paradigm for new blood vessel growth, elegantly outlined by D'Amore and Thompson,[1] suggests that the initiating feature of angiogenesis is the activation of endothelial cells (ECs) within a parent vessel, followed by disruption of the basement membrane and subsequent migration of the ECs into the interstitial space, possibly in the direction of an ischemic stimulus. Concomitant and/or subsequent EC proliferation, intracellular-vacuolar lumen formation, pericyte capping, and production of a basement membrane complete the developmental sequence. This paradigm is typically applied to new capillary formation, or angiogenesis, a process that some have

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suggested may be distinct from the formation of larger blood vessels, or vasculogenesis.[2]

The proliferative component of this paradigm has been investigated in several animal models of naturally occurring collateral vessel development.[3–8] Studies carried out in the canine and swine coronary circulations, as well as the canine and rodent renal arterial circulations, have established that cellular proliferation is indeed a feature of collaterals that develop consequent to arterial occlusion, thereby providing evidence of new blood vessel formation or angiogenesis.

Recent investigations have documented the feasibility of using recombinant formulations of angiogenic growth factors to further augment collateral artery development in similar animal models. This novel strategy for the treatment of vascular insufficiency has been termed therapeutic angiogenesis.[9] The angiogenic growth factors first employed for this purpose comprised members of the fibroblast growth factor (FGF) family.[10–13] More recently, we[9,14,15] and others[16] have shown that the EC-specific mitogen, vascular endothelial growth factor (VEGF),[17,18] is a potent agent for collateral vessel augmentation in the lower extremity and coronary circulations, respectively. Studies performed previously in our laboratory, for example, showed that 500 to 1000 μg of VEGF administered as a single intra-arterial bolus to the internal iliac artery of rabbits in which the ipsilateral femoral artery was excised to induce severe, unilateral hind limb ischemia produced angiographic and histological evidence of enhanced collateral vessel formation within 10 days; consequent amelioration of blood pressure[9] and flow[14] were significantly greater in the ischemic limb of animals treated with VEGF compared with controls.

The extent to which cellular proliferation contributes to collateral vessel development in the setting of lower extremity ischemia has not been previously defined. Accordingly, in the current study we sought to document the contribution to and sequence of cellular proliferation during spontaneous collateral vessel development in the rabbit hind limb model of lower extremity ischemia and, furthermore, determine the extent to which this template is modified by therapeutic angiogenesis.

Materials and Methods

Animal Model

Cellular proliferation of collateral arteries during therapeutic angiogenesis by VEGF was investigated with a rabbit model of hind limb ischemia as previously described.[9,11,14,15] All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 3.5 to 4.0 kg (Pine Acre Rabbitry, Norton, MA) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) after premedication with xylazine (2.5 mg/kg). A vertical incision was performed in one limb, extending inferiorly from the inguinal ligament. The entire femoral artery, including its branches (the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries), was then dissected, ligated, and completely excised from its proximal origin to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external iliac artery extends retrograde to its origin from the common iliac artery. The internal iliac artery, which usually supplies perfusion to the pelvic organs of the rabbit, but not the hind limb, now becomes the sole source of perfusion for the distal hind limb; as such, the blood supply to the distal limb of this animal model becomes almost entirely dependent upon collateral arteries that may originate from the internal iliac artery (Figure 1). Prophylactic antibiotics (enrofloxacin, 2.5 mg/kg; Miles, Shawnee Mission, KS) were administered subcutaneously for a total of 5 days post-operatively. Analgesia (levorphanol tartrate, 60 mg/kg; Roche Laboratories, Nutley, NJ) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. All animals used in this investigation were prepared specifically for this study and were not used for any other separately reported studies.

Intra-Arterial VEGF Administration

The soluble 165-amino-acid isoform of VEGF was purified from transfected Chinese hamster ovary cells as previously described.[19] The purity of the material was assessed by silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and by the presence of a single NH_2-terminal amino acid sequence.

An interval of 10 days between the time of surgery and the administration of VEGF was incorporated to allow for the recovery of rabbits and the stabilization of endogenous development of collateral arteries. At 10 days post-surgery (day 0), a 3 Fr. infusion catheter (Tracker-18, Target Therapeutics, San Jose, CA) was introduced from the carotid artery and advanced into the internal iliac artery of the ischemic limb under fluoroscopic guidance. After the catheter was washed with 3 ml of saline containing 0.1%

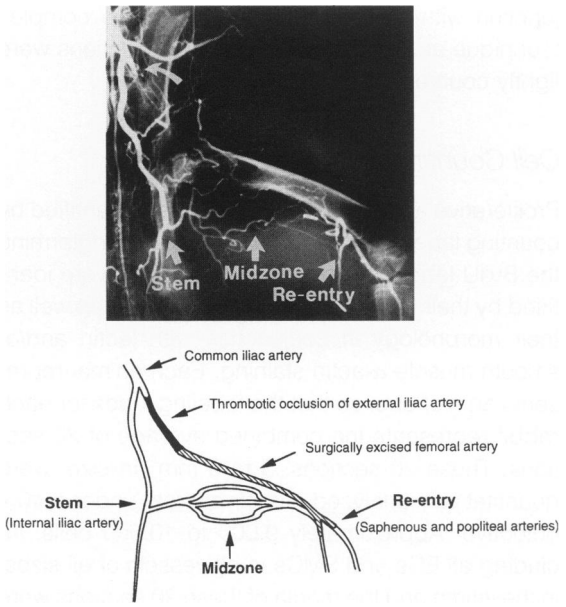

Figure 1. *Classification of collateral vessel anatomy.* A: *Aortic angiography of an ischemic rabbit limb.* Arrows *indicate the stem, midzone, and re-entry collateral arteries.* Curved arrow *indicates the site of thrombotic occlusion of the external iliac artery.* B: *Schematic representation of angiographic findings illustrated in* A.

rabbit serum albumin (Sigma Chemical Co., St. Louis, MO), 500 to 1000 μg of VEGF in 5 ml of saline containing 0.1% albumin was selectively injected into the internal iliac artery as a bolus over a period of 1 minute. The dose of VEGF employed in this study (500 to 1000 μg, or 125 to 285 μg/kg) was previously confirmed to be sufficient to augment collateral artery development in this animal model.[9,14] These same previous studies indicated a lesser therapeutic effect at smaller doses of VEGF (eg, 100 μg), reaching a plateau at 500 μg. Sham-treated animals receiving vehicle alone (an identical volume of saline containing 0.1% albumin) were used as controls to delineate cellular proliferation during natural (ie, spontaneous) collateral artery development.

Lower Limb Calf Blood Pressure Ratio

To evaluate collateral development, the time course of calf blood pressure ratio was assessed in both hind limbs with a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, OR) immediately before administration of VEGF or saline (day 0) and again at the time of sacrifice (days 3, 5, and 7). On each occasion, the hind limbs were shaved and cleaned, the pulse of the posterior tibial artery was identified with a Doppler probe, and the systolic blood pressure of both limbs was determined by standard techniques.[9,14] All measurements were performed by a single observer blinded to the treatment regimen. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Bromodeoxyuridine Labeling

Proliferation of ECs and smooth muscle cells (SMCs) was documented with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU; Sigma). At 24 hours before sacrifice, animals received an intravenous bolus injection of BrdU (25 mg/kg) followed by continuous delivery (25 mg/kg) over a 24-hour period from a subcutaneously implanted osmotic pump (Alzet, Alza, Palo Alto, CA). The animals were euthanized with an overdose of sodium pentobarbital at each of the following time points: day 0 (immediately before VEGF), and days 3, 5, and 7 after VEGF. After animals had been euthanized, a saline flush was immediately performed from a catheter placed in the abdominal aorta to clear the blood. The aorta and the distal vasculature were then perfusion-fixed *in situ* with 100% methanol. Tissue harvesting was performed as follows based in part upon anatomic criteria proposed by Longland[20] (Figure 1). (1) Sections were taken from the principal stem artery, ie, the feeder from which collateral arteries were derived, in this case the distal internal iliac artery. (2) The midzone arteries were removed from the plexus of branching arteries that interconnect the stem and re-entry arteries. Because the midzone collateral arteries were too diminutive to identify macroscopically, tissue harvesting was in this case standardized by removing in a systematic fashion those connective tissues along the mid-portion of the sciatic nerve in the thigh. After tissue processing and immunohistochemical staining, all the arteries in each histological section were identified by light microscopy. Collaterals with a luminal diameter smaller than 20 μm cannot be consistently quantified on standard light microscopic sections;[8] these vessels were therefore excluded from analysis (see below). (3) Sections were taken from the proximal portions of the principal re-entry arteries, ie, those arteries that rejoin the midzone plexus with the distal circulation; in this case the principal re-entry arteries are the saphenous and popliteal arteries. All harvested tissues were further immersion-fixed with 100% methanol for at least 12 hours at 4°C, processed, and embedded in paraffin.

In addition, tissue specimens from the small intestine were harvested from each animal at necropsy. Tissues were fixed in methanol and then embedded in paraffin as described above. These tissues were

used as a positive control for BrdU staining. The brown nuclear staining of epithelial crypt cells was confirmed in all such tissue specimens (Figure 2).

Tissue Staining

Proliferating cells were identified with a mouse monoclonal antibody against BrdU (Dako, Carpinteria, CA). Paraffin-embedded sections (6 μm) were cut on to poly-L-lysine-coated slides and air dried overnight. Sections were incubated with 2 N HCl for 10 minutes at 37°C to denature DNA. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide in phosphate-buffered saline. Sections were then incubated with anti-BrdU antibody (1:50 dilution) for 45 minutes. Bound primary antibody was detected by an avidin-biotin-immunoperoxidase method according to the supplier's guidelines (Elite Avidin-Biotin Detection System, Signet Laboratories, Dedham, MA). Sections were lightly counterstained with hematoxylin. Additional staining for *Bandeiraea simplicifolia* lectin B4 (BSLB4)[21] and smooth muscle α-actin was performed on adjacent sections to identify ECs and SMCs. For lectin staining, sections pretreated with 0.3% hydrogen peroxide were incubated with 5 μg/ml biotinylated lectin (Vector Laboratories, Burlingame, CA) overnight at 4°C. After washing, slides were incubated with peroxidase-conjugated streptavidin (BioGenex Laboratories, San Ramon, CA) for 1 hour; 3-amino-9-ethyl-carbazole (Signet Laboratories) was then applied as a substrate for the enzyme, resulting in a brown reaction product. Sections were lightly counterstained with hematoxylin. Monoclonal antibody against smooth muscle α-actin (HHF-35, Enzo Diagnostics, New York, NY) was used for SMC staining in conjunction with the avidin-biotin peroxidase complex technique as described previously.[22] Sections were lightly counterstained with hematoxylin.

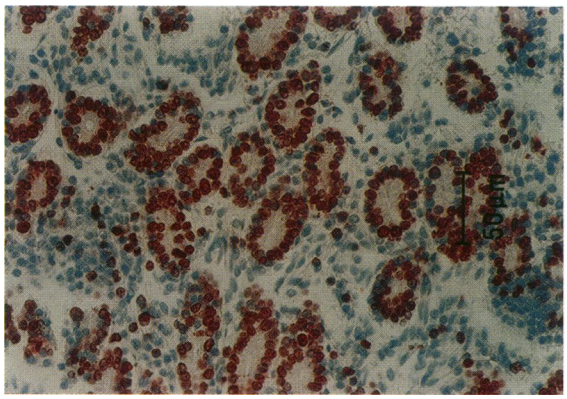

Figure 2. *Immunostaining for BrdU. Tissue specimens from the small intestine were harvested from each animal at necropsy. These tissues were used as a positive control for BrdU staining. The brown nuclear staining of epithelial crypt cells was confirmed in all such tissue specimens.*

Cell Counting

Proliferative activity of limb arteries was quantified by counting labeled *versus* unlabeled cells to determine the BrdU labeling index. ECs and SMCs were identified by their position within the vessel wall as well as their morphology in conjunction with lectin and/or smooth muscle α-actin staining. Each animal represents an n of one, so that the labeling index for each rabbit represents the combined average of 20 sections. These 20 sections, 3 to 5 mm^2 in size, were quantitatively analyzed in their entirety under a 40× objective. Approximately 9,000 to 10,000 cells, including all ECs and SMCs in all vessels of all sizes, in the intima and the media of these 20 sections were individually counted, following which the BrdU labeling index, ie, the fraction (percentage) of labeled nuclei, was determined for each rabbit by averaging the indices of all sections examined. BrdU-positive cells were identified principally in vessels 20 to 100 μg in diameter. The EC labeling index (percentage) was defined as the number of BrdU-positive ECs divided by the total number of ECs in the intima. The SMC labeling index (percentage) was defined as the number of BrdU-positive SMCs divided by the total number of SMCs in the media. Previous work from our laboratory has indicated that >95% of the cells in the intima of this model are ECs, and >95% of the cells in the media are SMCs (S. Takeshita, unpublished data). Nevertheless, we cannot exclude with certainty the possibility that some small fraction of the 9,000 to 10,000 denominator cells counted individually for each of the 41 rabbits could be non-ECs or non-SMCs, including pericytes or fibroblasts. All measurements were performed by a single observer blinded to the treatment regimen.

Statistics

Results were expressed as mean ± SE. Statistical significance was evaluated by unpaired Student's *t*-test for comparisons between two means and analysis of variance followed by Scheffé's procedure for more than two means. A value of $P < 0.05$ was interpreted to denote statistical significance.

Results

Proliferative activity of collateral artery ECs and SMCs was studied as a function of anatomic collat-

eral zone and time course in 41 rabbits with surgically induced lower limb ischemia; 5 rabbits were sacrificed at day 0 (immediately before administration of VEGF or saline); 19 rabbits at days 3 (n = 5), 5 (n = 8), and 7 (n = 6) after VEGF administration; and 17 rabbits at days 3 (n = 5), 5 (n = 8), and 7 (n = 4) after saline administration. All 41 rabbits were labeled with BrdU for 24 hours before sacrifice as described above.

Time Course of Lower Limb Blood Pressure Ratio (Figure 3)

The time course of lower limb blood pressure ratio was measured to document physiological evidence of collateral artery development.[9,14] The blood pressure ratio at day 0 was 0.25 ± 0.04 in control group and 0.29 ± 0.04 in VEGF-treated group (*P* value not significant). In the ischemic, untreated control animals, no significant increase in blood pressure ratio was recorded between days 0 and 7; specifically, blood pressure ratio in the control group measured 0.23 ± 0.13 at day 3; 0.33 ± 0.09 at day 5; and 0.23 ± 0.11 at day 7. In contrast, among the VEGF-treated group, a statistically significant increase in the blood pressure ratio was observed by day 7 ($P < 0.01$ *versus* day 0); specifically, blood pressure ratio in the VEGF group measured 0.40 ± 0.06 at day 3; 0.32 ± 0.09 at day 5; and 0.57 ± 0.04 at day 7. The day 7 blood pressure ratio for VEGF-treated animals was also significantly increased when compared with that recorded for the control group at the same time point ($P < 0.05$).

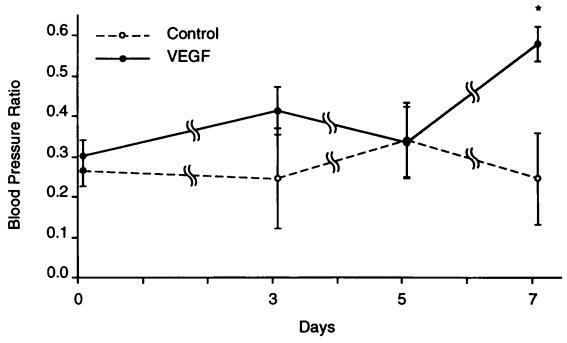

Figure 3. *Time course of lower limb blood pressure ratio measurements. The time course of lower limb blood pressure ratio (ischemic/normal limb) was measured to document physiological evidence of collateral artery development. In ischemic, untreated control animals (- - - -), no statistically significant increase in blood pressure ratio was observed from day 0 to day 7. In contrast, among the VEGF-treated group (——), a statistically significant increase in the blood pressure ratio was observed by day 7 (*P < 0.01, versus day 0). The day 7 blood pressure ratio of VEGF-treated animals was also significantly higher than that of the controls at the same time point (*P < 0.05, versus control). Interruption of both dashed and solid lines indicates that blood pressure measurement was not continuously performed on a daily basis but was limited to days 0, 3, 5, and 7.*

Time Course of Cellular Proliferation Associated with Spontaneous Collateral Development (Tables 1 and 2; Figure 4)

Stem Artery

In the stem artery of ischemic, untreated control animals, no significant change in proliferative activity of either ECs or SMCs was observed during the 7-day period after administration of saline (Figure 4, A and B). Although the percentage of labeled ECs appeared to increase progressively from day 0 (0.21 ± 0.13%) to day 3 (0.46 ± 0.29%), day 5 (0.89 ± 0.24%), and day 7 (1.35 ± 0.99%), these increments in the labeling index did not achieve statistical significance. Likewise, a similar frequency of labeled SMCs was observed at day 0 (0.40 ± 0.21%), day 3 (0.52 ± 0.33%), day 5 (0.38 ± 0.08%), and day 7 (0.47 ± 0.38%).

Midzone Artery

Proliferative activity of both ECs and SMCs was also stable in the midzone collateral arteries over the 7-day study period (Figure 4, C and D). For ECs, proliferative activity was 1.78 ± 1.53% at day 0, 0.47 ± 0.38% at day 3, 1.51 ± 0.39% at day 5, and 1.71 ± 0.95% at day 7. For SMCs, the labeling index was 1.87 ± 1.05% at day 0, 0.59 ± 0.34% at day 3, 2.08 ± 0.48% at day 5, and 1.88 ± 1.11% at day 7.

Re-Entry Artery

In re-entry arteries, proliferative activity of both ECs and SMCs of control arteries was again statistically unchanged from 0 (0.49 ± 0.35% and 0.23 ± 0.10%, respectively) through 7 days (0.85 ± 0.85% and 0.03 ± 0.03%, respectively) after saline administration (Figure 4, E and F).

Time Course of Cellular Proliferation after VEGF (Tables 1 and 2; Figure 4)

Stem Artery

For the VEGF group, proliferative activity of both ECs and SMCs in the stem artery failed to show a statistically significant deviation (peak or nadir) at any single time point over the 7-day period. Moreover, proliferative activity of stem ECs in the VEGF-treated animals at

Table 1. *Proliferative Activity of Endothelial Cells in Collateral Arteries of Untreated (Control) and VEGF-Treated Animals with Hind Limb Ischemia*

	Baseline, day 0	VEGF	EC proliferative activity (%)			
			Day 3	Day 5	Day 7	Days 3–7
Stem	0.21 ± 0.13	0	0.46 ± 0.29	0.89 ± 0.24	1.35 ± 0.99	0.84 ± 0.23
		+	4.00 ± 2.25	1.65 ± 0.59	0.56 ± 0.35	1.93 ± 0.68
Midzone	1.78 ± 1.53	0	0.47 ± 0.38	1.51 ± 0.39	1.71 ± 0.95	1.25 ± 0.31
		+	1.56 ± 0.87	4.25 ± 1.19*	2.81 ± 1.27	3.09 ± 0.70†
Re-entry	0.49 ± 0.35	0	0.21 ± 0.11	0.69 ± 0.43	0.85 ± 0.85	0.64 ± 0.31
		+	1.06 ± 0.57	0.92 ± 0.38	0.17 ± 0.11	0.69 ± 0.22
All zones	0.82 ± 0.52	0	0.40 ± 0.17	1.03 ± 0.21	1.30 ± 0.49	0.94 ± 0.17
		+	2.29 ± 0.89	2.33 ± 0.55‡	1.18 ± 0.50	1.94 ± 0.36§

Values are mean ± SEM. +, animals that received VEGF; 0, animals that did not receive VEGF.
*P = < 0.05 *versus* midzone ECs of the controls at day 5.
†P < 0.05 *versus* re-entry ECs of the VEGF-treated group at days 3 to 7; P < 0.05 *versus* midzone ECs of the controls at days 3 to 7.
‡P < 0.05 *versus* all zones ECs of the controls at day 5.
§P < 0.05 *versus* all zones ECs of the controls at days 3 to 7.

Table 2. *Proliferative Activity of Smooth Muscle Cells in Collateral Arteries of Untreated (Control) and VEGF-Treated Animals with Hind Limb Ischemia*

	Baseline, day 0	VEGF	SMC proliferative activity (%)			
			Day 3	Day 5	Day 7	Days 3–7
Stem	0.40 ± 0.21	0	0.52 ± 0.33	0.38 ± 0.08	0.47 ± 0.38	0.44 ± 0.12
		+	0.11 ± 0.04	0.84 ± 0.27	0.26 ± 0.14	0.44 ± 0.14
Midzone	1.87 ± 1.05	0	1.59 ± 0.34	2.08 ± 0.48	1.88 ± 1.11	1.59 ± 0.37*
		+	0.54 ± 0.26	5.57 ± 1.45†	2.24 ± 1.14	3.19 ± 0.84‡
Re-entry	0.23 ± 0.10	0	0.01 ± 0.01	0.17 ± 0.08	0.03 ± 0.03	0.10 ± 0.05
		+	0.11 ± 0.10	0.09 ± 0.06	0.06 ± 0.04	0.08 ± 0.03
All zones	0.83 ± 0.39	0	0.43 ± 0.18	0.88 ± 0.24	0.82 ± 0.46	0.74 ± 0.16
		+	0.26 ± 0.11	2.26 ± 0.71	0.86 ± 0.43	1.29 ± 0.35

Values are mean ± SEM. +, animals that received VEGF; 0, animals that did not receive VEGF.
*P < 0.05 *versus* stem artery SMCs of the controls at days 3 to 7; P < 0.005 *versus* re-entry artery SMCs of the controls at days 3 to 7.
†P < 0.05 *versus* midzone SMCs of the controls at day 5.
‡P < 0.005 *versus* stem and re-entry SMCs of the VEGF-treated group at days 3 to 7.

days 3, 5, and 7 did not differ significantly from findings in ischemic, untreated control animals at the same time points (Figure 4, A and B). It was nevertheless intriguing to note that certain of the VEGF-treated animals showed very high proliferative activity of the stem ECs at day 3 after VEGF. The highest EC-BrdU index observed in this group of animals was 11.4%. No such exceptions were observed among the ischemic, untreated controls.

Midzone Artery

In contrast to observations made in the stem and re-entry (see below) arteries, a significant increase in proliferative activity as a function of time was observed in midzone arteries after VEGF (Figures 4, C and D, and 5). In VEGF-treated animals, proliferative activity of ECs increased from day 3 (1.56 ± 0.87%) to day 5 (4.25 ± 1.19%) and then subsequently decreased by day 7 (2.81 ± 1.27%). Proliferative activity of SMCs also increased in parallel with ECs between day 3 (0.54 ± 0.26%) and day 5 (5.57 ± 1.45%) and then returned to control levels by day 7 (2.24 ± 1.14%). The degree of proliferative activity noted for midzone ECs in the VEGF-treated group at day 5 was 2.8 times higher than in the day 5 control group (4.25 ± 1.19% *versus* 1.51 ± 0.39%, $P < 0.05$) and that of SMCs was 2.7 times higher than control (5.57 ± 1.45% *versus* 2.08 ± 0.48%, $P < 0.05$).

Re-Entry Artery

In re-entry arteries, proliferative activity of both ECs and SMCs of VEGF-treated animals was stable over 7 days after treatment. Furthermore, proliferative activity in the re-entry arteries of the VEGF-treated animals at days 3, 5, and 7 was not statistically greater than that of the ischemic, untreated controls at any of these same time points (Figure 4, E and F).

Discussion

Ambiguity persists regarding the relative contribution of proliferation *versus* migration of ECs to the

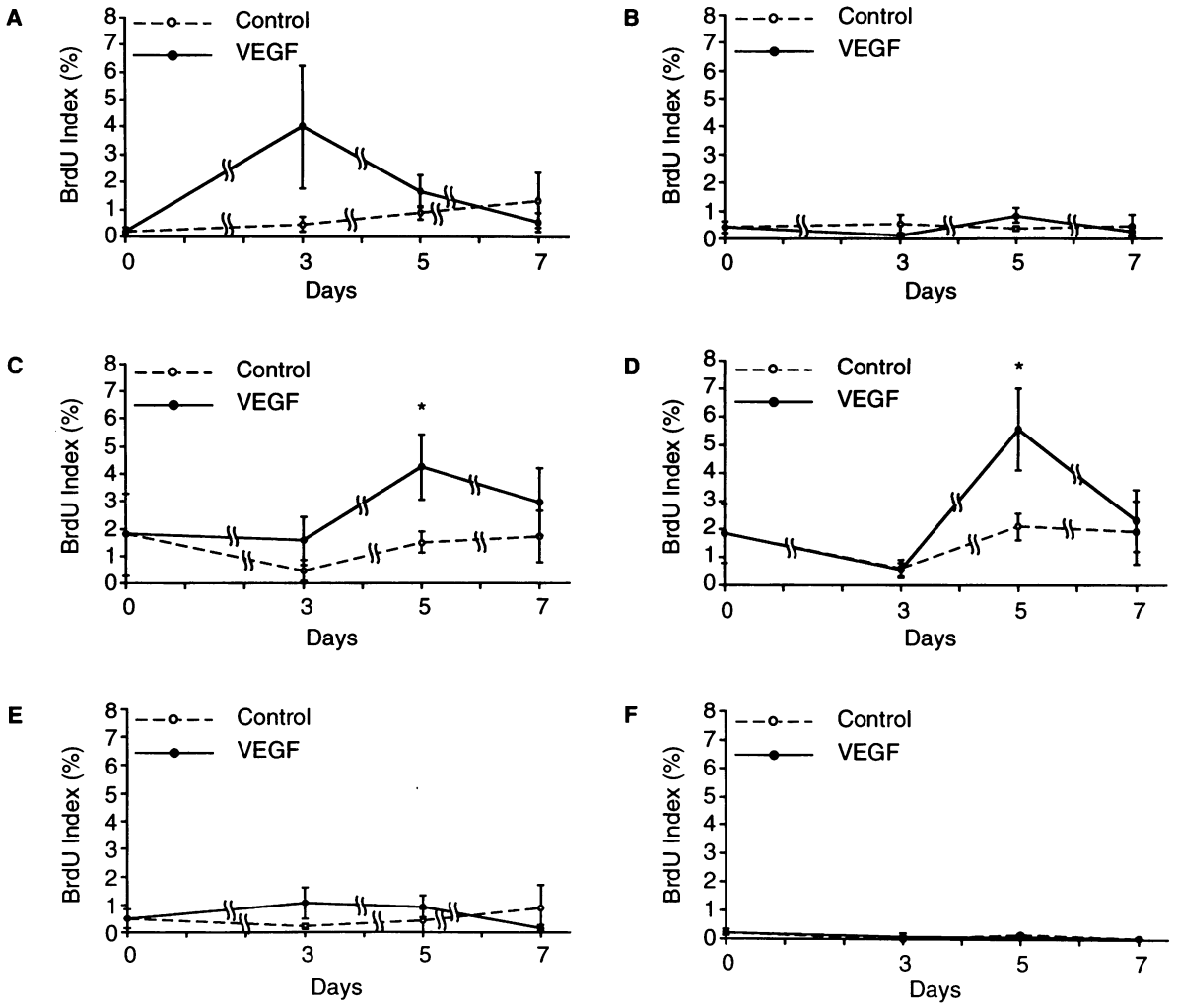

Figure 4. *Time course of proliferative activity of collateral arteries. BrdU labeling index (%) of stem ECs (A) and SMCs (B); midzone ECs (C) and SMCs (D); and re-entry ECs (E) and SMCs (F). Midzone EC and SMC proliferation of VEGF-treated animals (——) was significantly higher than that of ischemic, untreated controls (- - - -) at day 5 after treatment (*P < 0.05). Interruption of solid and dashed lines indicates that tissue retrieval was not continuously performed on a daily basis but was limited to days 0, 3, 5, and 7.*

development of new blood vessels, or angiogenesis. Sholley et al,[23] using a model of inflammation-induced angiogenesis of the rat cornea, demonstrated that initial vascular sprouting does not require EC proliferation. EC proliferation in this model was suppressed by X-irradiation with 2000 or 8000 rads before application of the inflammatory stimulus. In irradiated corneas displaying no cellular proliferation, vascular sprouting at 2 days was similar to that seen in contralateral shielded corneas. Although neovascular growth was subsequently blunted and ultimately ceased by 4 to 7 days, these experiments documented the critical if not exclusive roles of migration and redistribution of pre-existing ECs in the commencement of neovascularization. Similar implications resulted from work by Nicosia et al[24]; fibronectin was shown to promote, in a dose-dependent fashion, the elongation of microvessels that sprout from explants of rat aorta placed in serum-free collagen gel, despite the fact that neither DNA synthesis nor mitotic activity was increased in comparison with fibronectin-negative gels. Fibronectin was therefore inferred to promote angiogenesis *in vitro* by migratory recruitment of pre-existing ECs.

In contrast to these *in vivo* inflammatory and *in vitro* organ culture models, angiogenesis that develops in response to experimental vascular obstruction, ie, collateral vessel development, has been shown by several previous investigators to involve proliferation of not only ECs but SMCs as well (Table 3). Several important principles were elucidated by these studies.

First, evidence of EC proliferation is nearly absent in normal arteries,[4,5] a finding that is consistent with

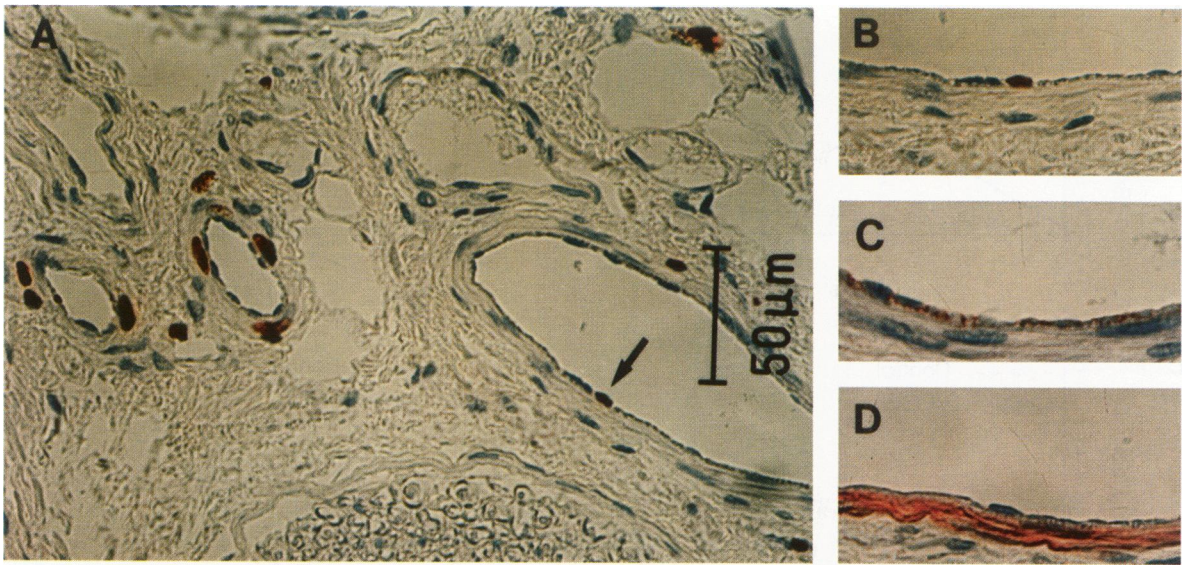

Figure 5. *Representative histological section of midzone collateral arteries at day 5 after VEGF.* **A:** *Immunostaining for BrdU. Brown nuclear staining indicates BrdU positivity.* **B:** *Higher magnification of BrdU-positive EC indicated by* **arrow** *in* **A**. *Identification of the cell type as EC was confirmed by positive staining for lectin* (**C**) *and negative staining for smooth muscle α-actin* (**D**) *in adjacent sections. Hematoxylin counterstain.*

an estimated EC turnover time of "thousands of days" in quiescent microvasculature.[25] Even a relatively low percentage of EC proliferation observed in response to arterial occlusion or exogenous growth factors may therefore represent considerable enhancement of EC proliferative activity and, when considered in relation to a denominator of thousands of ECs, is clearly sufficient to provide the basis for new blood vessel formation. Second, peak EC proliferation, which contributes to naturally occurring collateral development in the setting of vascular occlusion, varies from 2.6 to 3.5% in the canine coronary circulation,[4,7] from 5 to 6% in the rodent renal vasculature,[5,6] and <1% in swine coronaries.[8] The contrasting rates of EC proliferation between the canine and swine coronary circulations are indeed representative of the contrasting propensity for natural collateral artery development in these two species. Third, proliferation of SMCs, the additional requisite cell type for the formation of larger blood vessels (vasculogenesis), is an implicit component of angiogenesis, regardless of animal species or circulatory site. Schaper et al[3] in fact speculated nearly 25 years ago that "it is tempting to assume that EC proliferation not only serves the purpose of forming the endothelium of a finally larger artery but rather actively participates in the development of the tunica media." Fourth, proliferative activity, for SMCs as well as ECs, is highest at the level of the smallest diameter collateral vessels, the so-called midzone collateral segments.[3,4,7,8] Fifth, although evidence of EC and SMC proliferation alone does not necessarily distinguish new vessel development from an increase in the size of pre-existing vessels, adjunctive data regarding increased capillary density[9,26] support the notion that proliferative activity does in fact reflect true angiogenesis.

Table 3. *Evidence for Vascular Cell Proliferation during Spontaneous Angiogenesis* in Vivo

First author (year)	Species (n)	Circulation	Index of proliferation	Time course	Proliferative activity	
					ECs	SMCs
Schaper (1969)	Dog (6)	Coronary	Mitoses	Peak = 2–3 weeks*	60 cells	53 cells
Schaper (1971)	Dog (11)	Coronary	[^{3}H]Thymidine	Peak = 3 weeks*	3.5% of all ECs and SMCs	
Cowan (1978)	Dog (3)	Renal	[^{3}H]Thymidine	18–39 days	5.5%	Increased
Ilich (1979)	Rat (38)	Renal	[^{3}H]Thymidine	Peak = 1–4 days	5–6%	ND
Pasyk (1982)	Dog (10)	Coronary	[^{3}H]Thymidine	Peak = 4 days	2.6%	1.6%
White (1992)	Pig (17)	Coronary	[^{3}H]Thymidine	Peak = 2–3 weeks*	0.7%	0.5%

ND, not done.
*In these studies, peak refers to time after placement of an ameroid constrictor; occlusion in this case typically develops at approximately 17 days post-operatively.

In contrast to those studies shown in Table 1, all of which concern spontaneous collateral development, limited investigation has been performed regarding the extent to which EC and/or SMC proliferation are altered by interventions designed to augment collateral vessel development, ie, therapeutic angiogenesis.[9] Graham et al[26] established arteriovenous (AV) fistulae at the popliteal level in a canine model of hind limb ischemia, following which the vein proximal to the AV anastomosis was ligated; this so-called "arteriovenous reversal (AVR)" was designed to augment perfusion to the distal limb in a retrograde manner. A consistent feature of this procedure (in humans[27] as well as animals) was the development of an extensive network of new vessels in the proximity of the AV anastomosis. A significant increase in uptake of tritiated thymidine, administered 3 hours before death, was observed in hind limbs treated by AVR *versus* controls and, when coupled with histological evidence of increased capillary density, was interpreted as evidence for therapeutically augmented EC proliferation. More recently, Unger et al[13] administered BrdU to dogs in which collateral artery development had been provoked by application of an ameroid constrictor around the left circumflex coronary artery; after administration of basic FGF (bFGF) directly into the circumflex distal to the constrictor, cellular proliferation was found to be increased in collateral-dependent viable myocardium as well as sites of myocardial infarction. Based on morphology and location, cells immunopositive for BrdU were judged to consist predominantly of ECs.

The current study aimed to document the contribution to and sequence of cellular proliferation during spontaneous collateral vessel development in an animal model of lower extremity ischemia and determine the extent to which this is modified by therapeutic angiogenesis. EC proliferative activity (BrdU index) among collateral arteries developing in the untreated, ischemic limbs of control animals varied from 0 to 3.3% in stem segments, from 0 to 4.1% in midzone segments, and from 0 to 3.6% in the re-entry segments. Thus, regardless of anatomic site, EC proliferation varied between 0 and 4.1% per day, similar to the range reported previously by Pasyk et al[7] and White et al[8] for canine and swine coronary artery collaterals, respectively. EC proliferation among the ischemic untreated controls was not disproportionately greater for any individual collateral zone or individual time point. For SMCs, proliferative activity among the control animals was lower than that of ECs in the stem and re-entry zones; the degree of SMC proliferative activity in the midzone was similar to that of ECs and thereby significantly greater than that of the stem and re-entry zones. With regard to time course, however, the proliferative activity of SMCs during spontaneous collateral artery development did not vary significantly as a function of time.

The apparently modest extent of EC proliferation documented during spontaneous collateral development in this model is in reality quite substantial considering that (1) EC proliferation is nearly absent in normal arteries[4,5]; (2) the estimated EC turnover time for ECs in quiescent microvasculature is "thousands of days"[25]; and (3) the denominator in the current analyses represented thousands of cells.

A single intra-arterial bolus of recombinant human $VEGF_{165}$ was used to accomplish therapeutic angiogenesis in this study. The 165-amino-acid isoform of VEGF employed is the most prevalent of the four VEGF isoforms in humans and is intermediate between the 121 and 189 VEGF isoforms with regard to heparin affinity. Previous studies performed in our laboratory have demonstrated that the dose and route of administration employed in the current study produce statistically significant augmentation of collateral vessel development[9]; moreover, amelioration of hemodynamic deficit in the ischemic limb, assessed both by measurements of blood pressure and more recently blood flow,[14] have been shown to be significantly greater in animals receiving VEGF than in untreated controls. It has been previously proposed[12] that the heparin-binding feature of angiogenic growth factors such as VEGF may explain in part their protracted efficacy as a result of binding of the growth factor by heparan sulfate proteoglycans present on the luminal surface of the vascular endothelium and/or within the extracellular matrix. Such heparan sulfate binding of $VEGF_{165}$ may indeed contribute to the observation made in the present study that peak EC proliferative activity was delayed to day 5 after VEGF administration.

Administration of VEGF in the current study was shown to augment EC proliferation in the midzone collateral vessels by roughly threefold compared with control animals; this difference was most profound, and statistically significant, at day 5 after VEGF. The increase in EC proliferation observed at day 5 after VEGF was followed by reduction of the hemodynamic deficit in the ischemic limb by day 7. The improved hemodynamic measurements documented in the treatment group in the current study are unlikely to be the result of vasodilatory effects of VEGF; in our experience such effects are quite transient and do not persist beyond 2 hours after administration of VEGF.[28] Previous studies from our group have clearly documented that the increase in lower

limb blood pressure ratio correlates with an increase in the number of angiographically visible collateral arteries as well as an increase in the limb blood flow measured by Doppler flow guidewire,[9,14] ie, improvement of collateral artery formation in the ischemic limb.

In the stem arteries, EC proliferation among VEGF-treated animals was quite high (up to 11.4%) in selected cases but did not reach statistical significance for the treated group as a whole when compared with controls. At the level of the re-entry arteries, EC proliferation among VEGF-treated animals was in no case >2.6%. Thus, a statistically significant increase in EC proliferation in response to VEGF versus controls was limited to the midzone collaterals. These findings suggest that the locus of augmented EC proliferation consequent to therapeutic angiogenesis is similar to that previously noted during naturally occurring collateral development,[4] namely, the smallest diameter, or midzone, collateral vessels.

Despite the fact that the mitogenic effects of VEGF have been previously shown to be limited to ECs,[17] the proliferative activity of SMCs in the midzone collaterals also increased by approximately threefold. The augmentation in midzone SMC proliferation at day 5 was statistically significant, compared with that observed in naturally occurring collateral artery development in the present study and exceeds that reported in previous studies of natural collateral development[4,7,8] as well. Although VEGF has been shown to interact with lower affinity binding sites to induce mononuclear phagocyte chemotaxis,[29,30] higher affinity binding sites presumed to mediate the mitogenic effects of VEGF are limited to ECs.[17,28] Increased SMC proliferation observed in the present study is therefore not likely to represent a direct effect of VEGF. Two indirect effects are possible. The ability to induce vascular permeability is a well known feature of VEGF, responsible in fact for its alternate designation as vascular permeability factor.[31–33] It is possible that extravasation of certain angiogenic growth factors from circulating blood might result in activation of SMC proliferation.

Alternatively, ECs stimulated by VEGF may secrete factor(s) that promote SMC proliferation. It has been previously shown, for example, that VEGF induces expression of tissue-type plasminogen activators, potent mitogens for cultured human SMCs,[34] in cultured bovine microvascular ECs.[35] Platelet-derived growth factor, a mitogen and chemoattractant for vascular SMCs,[36] which is expressed by ECs[37–39] in a polarized manner[40] sufficient to provide an organizing gradient for SMCs, has been previously shown to be induced in human umbilical vein ECs by the addition of another angiogenic growth factor, aFGF;[41] the possibility that VEGF may similarly up-regulate EC production of growth factors mitogenic for SMCs,[42] has to our knowledge not yet been studied.

In summary, spontaneous collateral artery development observed in response to limb ischemia, as well as that seen after administration of VEGF, is characterized by active cellular proliferation of ECs and SMCs in the midzone arteries, maximal at day 5 after VEGF and followed by hemodynamic evidence of improvement in limb perfusion by day 7. These data thus support the concept that augmented cellular proliferation plays an important role in the process of enhanced collateral artery development after therapeutic angiogenesis and, furthermore, provide a basis for rational selection and/or timing of adjunctive therapies designed to optimize collateral development via effects on cellular proliferation, migration, and/or associated lysis of the extracellular matrix.

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