Role of Ischemia and of Hypoxia-Inducible Genes in Arteriogenesis After Femoral Artery Occlusion in the Rabbit

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Abstract—Vascular endothelial growth factor (VEGF) is known to play an important role in angiogenesis. Its place in collateral artery growth (arteriogenesis), however, is still debated. In the present study, we analyzed the expression of VEGF and its receptors (Flk-1 and Flt-1) in a rabbit model of collateral artery growth after femoral artery occlusion. Hypoxia presents the most important stimulus for VEGF expression. We therefore also investigated the expression level of distinct hypoxia-inducible genes (HIF-1α, LDH A) and determined metabolic intermediates indicative for ischemia (ATP, creatine phosphate, and their catabolites). We found that arteriogenesis was not associated with an increased expression of VEGF or the mentioned hypoxia-inducible genes. Furthermore, the high-energy phosphates and their catabolites were entirely within normal limits. Despite the absence of an increased expression of VEGF and its receptors, collateral vessels increased their diameter by a factor of 10. The speed of collateral development could be increased by infusion of the chemoattractant monocyte chemotactic protein-1 but not by infusion of a 30 times higher concentration of VEGF. From these data, we conclude that under nonischemic conditions, arteriogenesis is neither associated with nor inducible by increased levels of VEGF and that VEGF is not a natural agent to induce arteriogenesis in vivo. (Circ Res. 2001;89:779-786.)

Key Words: arteriogenesis ■ vascular endothelial growth factor ■ hypoxia

Coronary artery occlusions often result in fatal consequences for the affected patient. In some rare cases, however, stenosis of one or even occlusion of more coronary arteries were survived without infarction or produced only minimal symptoms. These patients benefited from a slow progression of thrombus formation, permitting a spontaneous development of compensating collateral arteries.1 Knowledge of the molecular mechanisms triggering vessel growth is the basis for the development of efficacious therapies allowing the timely induction of vessel growth in patients at risk.

In the postnatal organism, 2 types of vessel growth exist: angiogenesis, which involves the proliferation of endothelial cells (ECs) and the development of a vascular tree, and arteriogenesis, defined as the development of collateral arteries from preexisting arteriolar connections by growth, requiring the proliferation of ECs and smooth muscle cells (SMCs). Whereas angiogenesis is observed mainly in ischemic territories, arteriogenesis is temporally and spatially dissociated from ischemia in many cases. Only muscular collateral arteries, however, are potentially able to supply enough blood from outside the risk region to prevent the consequences of severe ischemia.

Among several polypeptide growth factors, vascular endothelial growth factor (VEGF) (45-kD, also called VEGF-A; for a review see Neufeld et al2) is viewed as one of the most potent inducers of angiogenesis. The temporal and spatial correlation of VEGF overexpression with angiogenesis during tumor growth, inflammation, and wound healing provides strong evidence for a functional role of VEGF as a key regulator of angiogenesis. The growth factor stimulates vascular endothelial proliferation and macrophage migration and increases microvessel permeability to macromolecules. The effects of VEGF are mediated via 2 distinct high-affinity receptors, Flt-1 and Flk-1. They are expressed almost exclusively on ECs, rendering VEGF an EC-specific mitogen.

Although the role of VEGF in angiogenesis is obvious, it is not clear whether VEGF is also involved in arteriogenesis, and available data are controversial.3–8 To investigate this, we performed studies in a rabbit hindlimb model of collateral artery growth previously developed by Ito et al.9 We analyzed the expression of VEGF on the mRNA and protein levels and performed in situ hybridization studies to localize the site of VEGF expression. Furthermore, we examined the expression of the 2 high-affinity receptors of VEGF, Flt-1 and Flk-1, and we investigated the effect of locally infused VEGF on collateral artery growth. VEGF expression is induced by ischemia and hypoxia, but also other factors can modulate the
expression of VEGF. To define whether arteriogenesis is associated with ischemia, we analyzed the levels of distinct metabolic parameters and the expression of diverse hypoxia-inducible genes (lactate dehydrogenase A, LDH A; hypoxia-inducible factor-1α, HIF-1α).

Material and Methods

Animal Model

The present study was performed with the permission of the State of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the German Law for the Protection of Animals. It conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Femoral artery–ligated New Zealand White rabbits were studied as previously described. Tissue samples from control organs, from the musculus (m) quadriceps vastus intermedius (m quadriceps, a muscle that constantly harbors 2 arteriolar connections that grow upon femoral artery ligation) and the m gastrocnemius of control, experimental, and sham-operated animals were collected at distinct intervals after the surgical procedure (n=5 per time point) and snap-frozen in liquid nitrogen.

Application of Solvent, MCP-1, or VEGF

Right femoral arteries of rabbits (18 rabbits; 8 male, 10 female; body weight 3.7±0.5 kg; age 3 to 4 months) were ligated with 2 ligatures ~1.5 cm apart (but not transected), and the animals were randomly assigned to receive either monocyte chemotactic protein (MCP)-1 (3 µg at a rate of 10 µL/h; Peprotech), VEGF (100 µg at a rate of 10 µL/h; Peprotech), or solvent locally via osmotic minipump (2 ML-1, Alza Corp) for 7 days. Surgical procedures were essentially as described before.

Hemodynamic Measurements

Hemodynamic measurements, counting of microspheres, and calculations of collateral conductance were performed as previously described.

Postmortem Angiograms and Isolation of Collaterals

To take postmortem angiograms or to visualize collateral arteries for isolation, rabbit hindlimbs were perfused with contrast medium as described before. Collateral arteries from the m quadriceps of a rabbit. A, After 1 week of sham operation; B, after 1 week of femoral artery occlusion. Collaterals have substantially increased in size and diameter and appear in typical corkscrew formation. C, Excision of collateral arteries.

RNA Isolation and Northern Blot

Total RNA was isolated according to the method of Chomczynski and Sacchi from frozen tissue of organs, skeletal muscle, and collateral arteries from control animals or at distinct time points after operation from sham-operated or experimental animals. Northern hybridization was carried out according to standard procedures.

Reverse Transcription–Polymerase Chain Reaction

Reverse transcription–polymerase chain reaction (RT-PCR) on total RNA from rabbit spleen was done according to standard procedures. The following sense and antisense primers were used: for Flt-1, a 38-mer homologous to mus musculus L07297 position 3773 to 3810, containing an artificial restriction site for SacI: GCT-GGAGTTGTAGTCTGGGGG. For Flk-1, a 36-mer complementary to mus musculus L07297, position 4011 to 3981, in which a KpnI restriction site was introduced: GTACgggATC-CACGGAGTTGTAGTCTGGGG. For Flk-1, a 36-mer homologous to mus musculus position 2519 to 2554 in which a BamH-I restriction site is introduced: GAGCGGAatcCGCTCTTCATAATA-GAAGGTGCCCAGG and a 32-mer, complementary to mus musculus L07297 position 4011 to 3981, in which a KpnI restriction site was introduced: GTACgggATC-CACGGAGTTGTAGTCTGGGG. For Flk-1, a 36-mer homologous to mus musculus position 2519 to 2554 in which a BamH-I restriction site is introduced: GAGCGGAatcCGCTCTTCATAATA-GAAGGTGCCCAGG and a 36-mer, complementary to mus musculus position 2669 to 2638 containing an artificial restriction site for BstXI.

Molecular Probes

For Northern blot analysis, cDNA probes were random-prime labeled with a Rediprime labeling system (Amersham Pharmacia...
Biotech) according to the manufacturer’s instructions. cDNA probes used in our studies were as follows: VEGF, human, 520 bp (provided by Dr K. Weindel, Freiburg, Germany); Flk-1, a 150-bp fragment, and Flt-1, a 238-bp fragment, both synthesized from rabbit spleen via RT-PCR (see above); LDH A, human, 1700 bp (ATCC); and 18S, mouse, 770 bp (provided by Dr I. Oberbäumer, Munich, Germany).

In Situ Hybridization
The techniques used for in situ hybridization were essentially as described before.18 A cDNA clone of human VEGF (see above) was used to generate a 35 S-UTP–labeled single-stranded RNA probe. Slides coated with Kodak NTB-2 emulsion (Eastman Kodak) were developed after 21 days and counterstained with 0.02% toluidine blue.

Western Blot
For VEGF detection, protein preparation and Western blot were done according to standard procedures.17 Equal amounts of proteins were separated on a 4% to 12% SDS-PAGE (NOVEX). A single immunoreactive band of ≈45 kDa was visualized in all protein preparations with an enzyme-linked chemiluminescence detection system (Amersham Pharmacia Biotech) and a goat polyclonal anti-human VEGF antibody (1:1000; R&D), which shows cross-reaction with rabbit VEGF.

For HIF-1α, nuclear protein extraction and Western blot were done essentially as described before.19 A single immunoreactive band of ≈120 kDa was visualized in all protein preparations with an enzyme-linked chemiluminescence detection system (Amersham Pharmacia Biotech) and a mouse monoclonal anti-human HIF-1α antibody (1:500; R&D), which shows cross-reaction with rabbit HIF-1α.

Quantification and Statistical Analysis
Signals were quantified with a PhosphorImager (Molecular Dynamics) using ImageQuant software. For all experiments shown in this article, n = 5. Results were expressed as mean±SEM. The ANOVA and subsequent multiple comparisons by Bonferroni test were used for statistical analysis, and results were considered to be statistically significant at a value of P < 0.05.

Results
No animals were lost during or after femoral artery ligation. We also did not observe any gangrene or gross impairment of hindlimb function after femoral artery occlusion.

Presence of Ischemia and Expression of Hypoxia-Inducible Genes
For LDH A, Northern blot results revealed a specific single band of ≈1.7 kb in rabbit- and human-derived tissue (data not shown). Analyzing the mRNA level of LDH A and the protein level of HIF1-α (120 kDa) at distinct intervals (LDH A: 3 hours, 24 hours, 3 days, and 7 days; HIF-1α: 3, 6, 12, and 24 hours) of collateral artery growth in the m quadriceps, we found no change in the levels of either the LDH A mRNA or the HIF-1α protein in experimental compared with sham-operated animals (Figures 2A and 2B). In HUVECs, which were analyzed as a control, hypoxia resulted in a significant 1.2-fold (3 hours) and 1.5-fold (20 hours) induction of the LDH A mRNA compared with normoxia (Figure 2A). For HUVECs and for RASMCs, our Western blot results (positive control) evidenced a minor but significant 1.4-fold increase of the HIF-1α protein after 3 hours of hypoxia compared with normoxia (Figure 2B). The results on the content of distinct purines (ATP, ADP, AMP, adenosine, inosine, hypoxanthine), lactate, and creatine phosphate at 1 and 24 hours and 7 days of collateral artery growth displayed no alteration of the mentioned parameters in the m quadriceps of experimental compared with sham-operated animals (Figure 3, data shown for 24 hours). For comparison, we also...
analyzed the proximal part of the m adductor, the m peroneus, and the m gastrocnemius for the content of the above-mentioned metabolites and the expression of LDH A (the latter was analyzed exclusively in the m gastrocnemius); however, the results again showed no significant changes in either the content of the metabolites or the expression of the mRNA (Figures 2A and 3). Measuring the above-mentioned metabolites in RASMCs (positive control), we found that 3 hours of hypoxia compared with 3 hours of normoxia resulted in minor changes of the ATP level and its breakdown products but a significant increase of the lactate level (Figure 3).

Collateral Artery Growth Does Not Rely on Increased Expression of VEGF

Northern blot results revealed a minor upregulation of the VEGF mRNA (4.6 kb) in the m quadriceps 3 hours after occlusion followed by a significant downregulation to ≈50% of control after 12 and 24 hours (Figure 4A). A comparable downregulation, however, was also found in sham-operated animals. Directly analyzing the expression level of VEGF during the same period in growing collateral arteries, we obtained only weak signals that displayed no difference between the mRNA level of VEGF in experimental compared with sham-operated animals (Northern blot results, data not shown).

Investigating the expression level of VEGF after 3 and 7 days and 3 weeks in the m quadriceps and after 3 days in growing collateral arteries proper, we found a comparable abundance in experimental and sham-operated animals; however, the total level of the VEGF mRNA was much higher in the skeletal muscle (m quadriceps) than in the arteries themselves (Figure 4B, data not shown for 3 weeks). For a further control, we analyzed the mRNA level of VEGF in different organs. The transcript was expressed at very high levels in the heart and slightly less in the uterus of a pregnant rabbit. In the central nervous system, we detected an abundance comparable to that in arteries; in the bone marrow, however, it was just above the detection limit (Figure 4B). For VEGF-B (1.6 kb), which showed cross-hybridization with our cDNA probe, we could not detect a change in experimental compared with sham-operated animals in any of the tissue samples analyzed. With the exception of the bone marrow, the level of VEGF-B mRNA expression in all tissue samples was lower than that of VEGF (Figure 4B).

Exclusively in HUVECs, which were analyzed as a control, we observed that 20 hours of hypoxia resulted in a significant 1.5-fold induction of the VEGF mRNA compared with 20 hours of normoxia. The expression level of VEGF-B was not changed (Figure 4C).

Expression Level of the VEGF Transcript in Skeletal Muscle Cells

For in situ hybridization studies, tissue samples gained from the m quadriceps of experimental and sham-operated animals at 3, 6, 12, and 24 hours after femoral occlusion were hybridized with a radiolabeled probe for VEGF. Results displayed neither quantitative differences in the mRNA abundance of VEGF nor differences in the cell types expressing VEGF. The results revealed, however, that VEGF is expressed primarily in skeletal muscle cells, as demonstrated by the density of the labeled antisense probe in the nuclei of that cell type but not in larger vessels or connective tissue (Figure 5; results are shown for the m quadriceps at 3 hours of collateral artery growth).

Comparable Protein Abundance of VEGF in Skeletal Muscle and Collateral Arteries

Western blot results revealed no alteration of the protein level of VEGF in experimental compared with sham-operated animals either during the first 24 hours (m quadriceps) or after 3 days (collaterals) of collateral artery growth. It is notable, however, that the abundance of the VEGF protein in collateral arteries was about as high as in the m quadriceps (Figure 6).

Expression Level of Flt-1 and Flk-1 During Arteriogenesis

Investigating the mRNA level of Flt-1 and Flk-1 via Northern blot, we found a very low abundance of both transcripts in all tissue samples analyzed. For Flt-1, we obtained a weak signal of 7.5 kb in the central nervous system and the heart. The uterus of pregnant rabbits, however, did not show demonstrable quantities of Flt-1 mRNA. The expression pattern of Flk-1...
(≈6.0 kb) in the control organs corresponded to that of Flt-1 (Figure 7).

Analyzing the mRNA level of Flt-1 and Flk-1 during the first 24 hours and at days 3 and 7 of collateral artery growth in the m quadriceps and at days 3 and 21 in true collateral arteries, we found no difference in the expression level of the 2 transcripts in experimental compared with control or sham-operated animals (Figure 7).

Influence of Exogenous VEGF on Collateral Artery Growth

We tested whether infusion of VEGF (for 7 days) in rabbit hindlimb after occlusion of the femoral artery might result in an enhanced collateral artery growth. To obtain a positive control, a second group of rabbits received MCP-1 and a third solvent. Figure 8A shows a postmortem angiogram of a normal rabbit hindlimb; Figure 8B to 8D shows angiograms of rabbit hindlimbs after treatment. In all animals tested, occlusion of the femoral artery resulted in the formation of collateral vessels displaying a corkscrew formation, a typical sign of growing collateral arteries. This type of collateral was found primarily in the adductor longus, adductor magnus, and vastus intermedius muscles connecting the perfusion bed of the arteria femoralis profunda to that of the arteria saphena parva in the adductor muscles and the perfusion bed of the arteria circumflexa femoris lateralis to that of the arteriae genuales in the quadriceps muscle. Angiograms taken from hindlimbs of animals treated with solvent (number of collaterals [NOC] 15.4 ± 2.2) and VEGF (NOC 15.6 ± 3.6) showed comparable diameters of collateral arteries (Figure 8B and 8C). The most prominent morphological effect was...
seen in animals treated with MCP-1 (NOC 30.1±3.3) (Figure 8D).

For in vivo pressure/flow relations, infusion of VEGF for 1 week led to only a minor increase in collateral conductance, and the values did not show statistical significance compared with solvent (Figure 8E). Collateral conductance was significantly increased, however, in animals treated with MCP-1 (Figure 8E).

Temperature Changes After Occlusion
Measuring the temperature of the muscles at 1 hour, 24 hours, and 7 days of collateral artery growth, we consistently found a minor reduction in the upper leg (m quadriceps 0.60±0.12°C; m adductor proximal 0.25±0.10°C) and a strong reduction in the lower leg (m peroneus 1.22±0.16°C; m gastrocnemius 1.43±0.17°C) of experimental compared with sham-operated animals (P<0.05).

Discussion
VEGF has been reported to be a potent inducer of angiogenesis during development, in the adult during the female reproductive cycle, and under pathological conditions. Recent studies suggested that VEGF might also play a role in collateral artery growth, but the results were controversial.3–8

In our study, we defined the involvement of VEGF in arteriogenesis in a rabbit hindlimb model of vessel growth, in which occlusion of the femoral artery has been shown to result in the growth of collateral arteries in the thigh within 3 days.9 The cell proliferation of these vessels could already be demonstrated 24 hours after femoral artery occlusion.20

To characterize whether adaptive collateral artery growth is associated with an increased expression of VEGF, we analyzed the abundance of the transcript in the rabbit directly in growing collateral arteries and as a function of time in the m quadriceps, a skeletal muscle of the upper leg that consistently harbors collaterals. For the m quadriceps, Northern blot results displayed a significant downregulation of the VEGF mRNA in the experimental tissue. However, a similar downregulation was also seen in sham-operated animals, indicating that the altered mRNA level of VEGF in experimental animals was not due to arteriogenesis but rather to the experimental procedure. In growing collaterals, we found no change of the mRNA level of VEGF compared with control or sham-operated animals; however, the mRNA abundance of VEGF in collateral arteries was very low compared with the surrounding muscle tissue. Our Western blot results showed an almost comparable level of the VEGF protein in skeletal muscle tissue and in collateral arteries but did not show an increased protein level of VEGF during arteriogenesis. These data clearly demonstrate that collateral artery growth is not associated with an increased expression of VEGF.

Although other growth factors and cytokines can modulate the expression of VEGF, hypoxia is the most potent stimulus.
The hypoxia-induced transcription of VEGF is mediated via the binding of HIF-1 (α/β heterodimer) to a HIF-1 binding site located in the VEGF promoter. Furthermore, hypoxia promotes the stabilization of the VEGF mRNA by proteins that bind to sequences located in the 3′ untranslated region of VEGF. In addition, under unfavorable stress conditions, such as hypoxia, an efficient cap-independent translation of VEGF is maintained via the use of an internal ribosomal entry site at the 5′ UTR. Our analysis of VEGF during collateral artery growth, however, displayed no increased expression of VEGF on either the mRNA or the protein level, indicating that arteriogenesis is not associated with hypoxia. To test these results, we analyzed the expression level of LDH A, known to be induced by hypoxia and containing a HIF-1 binding site. We furthermore analyzed the protein level of HIF-1α. Our results showed that none of the mentioned genes was upregulated during arteriogenesis, representing additional evidence that in our model, collateral artery growth is not associated with hypoxia.

Hypoxia is a condition that occurs mainly by exposure to high altitude. Hypoxia is similar to ischemia in that hypoxia-inducible genes are also upregulated in ischemia. It differs from ischemia in that only the latter leads to a catabolism of the high-energy phosphates, ie, from ATP to ADP, AMP, adenosine, inosine, and hypoxanthine. It causes a decrease of creatine phosphate and, as a consequence of anaerobic glycolysis, an increased concentration of lactate. Analyzing these markers of ischemia, we found no change of any of the described parameters in our rabbit model in either the upper leg (m quadriceps, m adductor proximal) or the lower leg (m gastrocnemius, m peroneus). This was surprising, because one would expect that at least the lower leg would suffer from femoral artery occlusion, particularly because this surgical procedure was described to cause profound perfusion deficits in the lower leg of rabbits. Furthermore, our own measurements on the gastrocnemius of experimental animals displayed a significant reduction of the temperature, suggesting underperfusion. The degree of underperfusion, although marked, was not enough to cause any structural changes in terms of infarction or focal necrosis. The observation that ischemia could not be detected in the lower leg argues strongly against a role of ischemia as a cause for collateral growth, which was marked and resulted in a 10-fold increase in the diameter and a 20-fold increase in conductance. Why femoral artery ligation is not associated with ischemia in the lower leg, however, cannot be explained on the basis of our present results and needs further investigation.

Our in situ analysis displayed a strong staining for VEGF in the skeletal muscle cells (m quadriceps) of experimental and sham-operated animals, but not in cells of larger vessels or their closer vicinity. Our Western blot results, however, showed an almost equal abundance of the protein in the m quadriceps and in (growing and nongrowing) collateral arteries. This suggests that VEGF exerts a biological function both during collateral artery growth and under normal physiological conditions in maintenance of the vascular system via action on the ECs of a vessel in a paracrine manner.

To characterize whether the action of VEGF during collateral artery growth is mediated via the availability of its receptor, we characterized the mRNA level of Flt-1 and Flk-1; however, we could not find an increased expression of either of the 2 receptors during arteriogenesis. These data are in keeping with the unchanged expression level of VEGF, which was shown to have the capability to potentiate the expression of both its receptors. These data confirm that in our in vivo system, collateral artery growth occurs independently of ischemia (hypoxia), because it was demonstrated that Flt-1, which, like VEGF, contains a HIF-1 consensus binding site, is upregulated under hypoxic conditions on the transcriptional level in vivo.

To analyze whether we could stimulate collateral artery growth in our animal model system via application of VEGF (its activity was tested and confirmed in advance by analyzing tissue factor expression in monocytes after stimulation with VEGF, data not shown), we infused the growth factor via osmotic minipump in a concentration >30-fold higher than the active concentration of MCP-1. The results showed, however, that application of VEGF had only minor effects on the diameter of collateral arteries and on collateral conductance compared with solvent or MCP-1, which has already been demonstrated to be a potent inducer of collateral artery growth. VEGF has been shown not to be a direct mitogen for arterial endothelium in vivo. Collateral artery growth involves the proliferation not only of ECs but also of SMCs; however, the receptors for VEGF are expressed only on ECs. It is therefore likely that the effect of VEGF, although minor, was due to an indirect mechanism. It is reasonable that circulating blood cells, in particular monocytes, are involved, because they are the only cells besides ECs that express a VEGF receptor (Flt-1). Furthermore, monocytes have previously been described to play an important role in arteriogenesis. VEGF activates monocytes, induces integrin expression on monocytes, and promotes monocyte adhesion and migration. However, if VEGF has an indirect positive effect on collateral artery growth via activation of monocytes, this might be counterbalanced by a feature of VEGF itself, since this growth factor has been shown to reduce SMC proliferation in arteries.

If not VEGF and ischemia, one might then ask what the driving forces are for arteriogenesis. In our model, collateral artery growth occurs as an adaptive response to femoral artery occlusion, ie, the experimental procedure redirects flow through preexisting arteriolar connections, which then in turn enlarge by growth. This strongly implies that such hemodynamic forces as pressure and shear stress are the stimuli for this type of vessel growth. Recent results from our own laboratory showed that MCP-1 strongly promotes arteriogenesis, indicating that monocytes, specifically growth factors supplied by monocytes, play an important role. Our own results (unpublished data, 2001) as well as other reports show evidence that fibroblast growth factors have a prominent function in arteriogenesis.

In summary, our data clearly show that collateral artery growth under nonischemic conditions, in contrast to ischemic conditions, is neither associated with an increased expression of VEGF nor significantly inducible by an increased availability of the growth factor in vivo.
Acknowledgments
This work was supported by Max-Planck-Gesellschaft funding. We want to thank E. Neubauer, S. Thomas, M. von Bruehl, and M. Heil from our laboratory and R. Heidenreich and C. Wild from G. Breier's laboratory for all their support.

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Circ Res. 2001;89:779-786; originally published online September 13, 2001;
doi: 10.1161/hh2101.098613

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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