Antiangiogenic Effect of Interleukin-10 in Ischemia-Induced Angiogenesis in Mice Hindlimb

Jean-Sébastien Silvestre, Ziad Mallat, Micheline Duriez, Radia Tamarat, Michel F. Bureau, Daniel Scherman, Nicolas Duverger, Didier Branellec, Alain Tedgui, Bernard I. Levy

Abstract—Ischemia induces both hypoxia and inflammation that trigger angiogenesis. The inflammatory reaction is modulated by production of anti-inflammatory cytokines. This study examined the potential role of a major anti-inflammatory cytokine, interleukin (IL)–10, on angiogenesis in a model of surgically induced hindlimb ischemia. Ischemia was produced by artery femoral occlusion in both C57BL/6J IL-10+/+ and IL-10–/– mice. After 28 days, angiogenesis was quantified by microangiography, capillary, and arteriole density measurement and laser Doppler perfusion imaging. The protein levels of IL-10 and vascular endothelial growth factor (VEGF) were determined by Western blot analysis in hindlimbs. IL-10 was markedly expressed in the ischemic hindlimb of IL-10+/+ mice. Angiogenesis in the ischemic hindlimb was significantly increased in IL-10–/– compared with IL-10+/+ mice. Indeed, angiographic data showed that vessel density in the ischemic leg was 10.2±0.1% and 5.7±0.4% in IL-10–/– and IL-10+/+ mice, respectively (P<0.01). This corresponded to improved ischemic/nonischemic leg perfusion ratio by 1.4-fold in IL-10+/+ mice compared with IL-10–/– mice (0.87±0.05 versus 0.63±0.01, respectively; P<0.01). Revascularization was associated with a 1.8-fold increase in tissue VEGF protein level in IL-10+/+ mice compared with IL-10–/– mice (P<0.01). In vivo electrotransfer of murine IL-10 cDNA in IL-10–/– mice significantly inhibited both the angiogenic process and the rise in VEGF protein level observed in IL-10–/– mice. No changes in vessel density or VEGF content were observed in the nonischemic hindlimb. These findings underscore the antiangiogenic effect of IL-10 associated with the downregulation of VEGF expression and suggest a role for the inflammatory balance in the modulation of ischemia-induced angiogenesis. (Circ Res. 2000;87:448-452.)

Key Words: angiogenesis □ ischemia □ inflammation □ interleukin-10

Angiogenesis is the development of new vessels from preexisting blood vessels. This tightly controlled process is associated with pathological conditions such as tumor growth, diabetic retinopathy, and ischemic diseases. Hence, understanding the mechanism of angiogenesis is of major therapeutic interest.

In ischemic diseases, both hypoxia and inflammation are generally considered to represent fundamental stimuli for angiogenesis.1 The main mechanism of hypoxia-induced angiogenesis involves the rise in hypoxia-inducible factor-1α (HIF-1α) protein resulting in increased expression of vascular endothelial growth factor (VEGF), a specific angiogenic factor.2,3 Neovascularization appears to be also controlled by the inflammatory process that occurs in the ischemic area. Monocytes/macrophages accumulate during vessel growth in ischemic tissues.4 The presence of these inflammatory cells is associated with local secretion of several angiogenic factors, including cytokines such as interleukin (IL)–2 and tumor necrosis factor-α (TNF-α), growth factors such as VEGF and basic fibroblast growth factor (bFGF), and matrix metalloproteinases (MMPs).4,5 Recently, a macrophage-derived peptide, PR39, has been shown to inhibit the degradation of HIF-1α, leading to increased VEGF expression and accelerated formation of vascular structures in vitro.6

During the inflammatory reaction, anti-inflammatory cytokines are also produced and tend to modulate the inflammatory process. However, little information is available regarding the potential role of anti-inflammatory cytokines in ischemia-induced angiogenesis. IL-10, secreted by macrophages and by lymphocytes of the T helper 2 subtype, is an anti-inflammatory cytokine with potent deactivating properties on macrophages. In addition, antitumoral effects of IL-10 have been recently associated with its ability to decrease VEGF, TNF-α, or MMP-9 synthesis and to prevent angiogenesis associated with tumor growth.7,8

We therefore hypothesized that the anti-inflammatory cytokine IL-10 may affect ischemia-induced angiogenesis. We analyzed the angiogenic process in IL-10–deficient (IL-10–/–) mice.
C57BL/6J mice in a model of operatively induced hindlimb ischemia and assessed the effect of in vivo electrotransfer of murine IL-10 cDNA in IL-10−/− mice. We also determined the VEGF protein level in hindlimbs of IL-10+/− mice and IL-10+/+ mice.

**Materials and Methods**

**Experimental Protocol**

Male C57BL/6J IL-10−/− mice and IL-10+/− mice (Transgenic Alliance) underwent surgery to induce unilateral hindlimb ischemia. Animals were anesthetized by inhalation of isoflurane. The ligature was performed on the right femoral artery, 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries. Mice (7 animals per group) were then housed under specific pathogen-free conditions for 28 days. Animals were cared for in accordance with guidelines published by the National Institutes of Health (NIH publication No. 85-23, revised 1985), and the study protocol was approved by the local ethics committee.

**Quantification of Angiogenesis**

**Microangiography**

Vessel density was evaluated by high-definition microangiography (Trophy system) at the end of the 28-day treatment period. Mice were anesthetized (isoflurane inhalation), and a longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta and to inject a contrast medium (barium sulfate, 1 g/mL). Angiography of hindlimbs was then assessed and images (3 per animal) were acquired by a digital x-ray transducer. Images were then assembled to obtain a complete view of the hindlimbs. The vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area. Quantification area was limited by the place of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg. The time between artery femoral ligature and angiography (28 days) was determined as being optimal for vascularization after ischemia (data not shown).

**Capillary and Arteriole Densities**

Microangiographic analysis was completed by assessment of capillary and arteriole densities. Ischemic and nonischemic muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. Sections (7 μm) were first incubated for 30 minutes in PBS containing 5% BSA at room temperature and then 1 hour with either mouse monoclonal antibody directed against human smooth muscle actin α1 (dilution 1:50) to identify arterioles or with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50) to identify capillaries. Arteriole immunohistochemistry was achieved by treating sections with H2O2 3% and with a biotinylated secondary antibody with a horseradish peroxidase–streptavidin conjugate (dilution 1:50). Capillaries were revealed with a fluorescent FITC anti-rabbit antibody (dilution 1:10). Capillary and arteriole densities were then calculated in randomly chosen fields of a definite area using Optilab/Pro software.

**Laser Doppler Perfusion Imaging**

To provide functional evidence for ischemia-induced changes in vascularization, laser Doppler perfusion imaging experiments were performed in IL-10−/− and IL-10+/+ mice (n=3) as previously described.9

**Intramuscular Electrotransfer of Expression Plasmid IL-10 cDNA**

IL-10+/− mice were injected at day 1 after femoral artery occlusion with the IL-10 expression plasmid, pCor–IL-10, and with the control empty plasmid, pCor, into tibial cranial muscles of ischemic and nonischemic legs of the mouse (7 animals per group), as previously described.10 We previously showed that IL-10 cDNA transfection results in a marked increase in IL-10 plasma level up to 21 days.10

**Determination of IL-10 and VEGF**

**Protein Expression**

Tissue samples were thawed and homogenized in 300 μL of buffer (200 mmol/L sucrose and 20 mmol/L HEPES [pH 7.4]) containing protease inhibitors. Protein content was then determined by the method of Bradford.11 Proteins were separated in denaturing SDS/12% polyacrylamide gels and then blotted onto a nitrocellulose sheet (Hybond enhanced chemiluminescence [ECL], Amersham). Antibodies against IL-10 (Pharmingen) and VEGF (Santa Cruz Biotechnology) were used at a dilution of 1:2000. Specific protein was detected by chemiluminescent reaction (ECL+ kit, Amersham) followed by exposure of the membranes to Hyperfilm ECL (Amer-sham). The proteins were then stained with Ponceau Red (Sigma) for 5 minutes. Quantifications were performed by densitometric analysis after scanning using the Bio-Rad gel Doc 1000. Results are expressed as a ratio of quantification of the specific band on autoradiogram to quantification of the transferred total protein bands stained with Ponceau Red.

**Statistical Analysis**

Results are expressed as mean±SEM. One-way ANOVA was used to compare each parameter. Post hoc Bonferroni t-test comparisons were then performed to identify which group differences accounted for the significant overall ANOVA. A value of P<0.05 was considered significant.

**Results**

**Expression of IL-10 in Ischemic Hindlimb of IL-10+/+ Mice**

We first verified whether ischemia-induced angiogenesis was associated with a rise in IL-10 expression in the ischemic tissue. In IL-10+/+ mice, a marked expression of IL-10 was detected in the ischemic leg compared with the nonischemic leg. As expected, in IL-10−/− mice, IL-10 protein level was undetectable in both ischemic and nonischemic hindlimbs (Figure 1).

**Vessel Density in IL-10+/− Mice**

**Microangiography**

In IL-10−/− mice, vessel density was reduced in the ischemic (right) leg compared with the nonischemic (left) leg...
In the ischemic hindlimb, vessel density was increased by 1.8-fold in IL-10−/− mice compared with IL-10+/+ mice (10.2 ± 0.5% versus 5.7 ± 0.4%, respectively, P < 0.01) and was similar to that observed in the nonischemic leg (10.2 ± 0.5% versus 9.8 ± 0.4%, respectively, NS). In the nonischemic hindlimb, vessel density was not different in IL-10−/− mice and IL-10+/+ mice (9.8 ± 0.4% versus 9.3 ± 0.7%, respectively, NS) (Figure 2).

Capillary and Arteriole Densities
Microangiographic data were confirmed by capillary and arteriole density analysis. In IL-10+/+ mice, capillary and arteriole density was decreased in the ischemic leg compared with the nonischemic one (434 ± 15 and 5.3 ± 0.3 vessels/mm² versus 864 ± 34 and 12 ± 3 vessels/mm², respectively, P < 0.01) and was similar to that observed in the nonischemic leg (10.2 ± 0.5% versus 9.8 ± 0.4%, respectively, NS). In the nonischemic hindlimb, vessel density was not different in IL-10+/+ mice and IL-10−/− mice (9.8 ± 0.4% versus 9.3 ± 0.7%, respectively, NS) (Figure 3).

Laser Doppler Perfusion Imaging
Microangiographic and capillary density measurements corresponded to improved perfusion in ischemic hindlimb of IL-10−/− mice. Indeed, ischemic/nonischemic leg perfusion ratio increased by 1.4-fold in IL-10−/− mice compared with IL-10+/+ mice (0.87 ± 0.05 versus 0.63 ± 0.01, respectively, P < 0.01).

Effect of Electrotransfer of pCor–IL-10 in IL-10−/− Mice
To demonstrate that enhanced ischemia-induced angiogenesis in IL-10−/− mice was due to the IL-10 deficiency, in vivo intramuscular electrotransfer of pCor IL-10 was performed in IL-10−/− mice.

Microangiography
In the ischemic hindlimb of transfected IL-10−/− mice, vessel density was significantly reduced (7.4 ± 0.5% versus 10.2 ± 0.5% in nontransfected IL-10−/− mice, P < 0.05) in such a way that it was no longer significantly different from that in IL-10−/− mice (7.4 ± 0.5% versus 5.7 ± 0.4%, NS). In contrast, electrotransfer of the control expression plasmid (pCor) did not affect the angiogenic process in IL-10−/− mice (9.7 ± 0.9% in mice transfected with the control pCor plasmid versus 10.2 ± 0.5% in nontransfected mice).

In the nonischemic hindlimb of IL-10−/− mice, intramuscular administration of IL-10 or empty pCor did not affect vessel density (Figure 2).
**Capillary and Arteriole Densities**

In the ischemic hindlimb of transfected IL-10⁻/⁻ mice, capillary and arteriole densities were decreased by 1.3-fold compared with nontransferred IL-10⁻/⁻ mice (P<0.05). The number of capillaries and arterioles became not significantly different from that of IL-10⁻/⁻ mice (Figure 3).

In the nonischemic hindlimb of IL-10⁻/⁻ mice, injection of IL-10 did not change capillary and arteriole densities (data not shown).

**Regulation of VEGF Protein Level**

In the ischemic hindlimb of IL-10⁻/⁻ mice, VEGF level was increased by 179±21% (P<0.01) compared with IL-10⁻/⁻ mice. Interestingly, intramuscular electrotransfer of pCor-IL-10 in IL-10⁻/⁻ mice prevented this increase; VEGF level in IL-10⁻/⁻ mice transfected with pCor-IL-10 was similar to that in IL-10⁻/⁻ mice (125±18% versus 100±15%, NS) (Figure 4). In the nonischemic hindlimb, VEGF protein level was much lower than in the ischemic hindlimb and was similar in IL-10⁻/⁻ and IL-10⁻/⁻ mice injected with pCor IL-10 or empty pCor (Figure 4).

**Discussion**

The main results of this study are that IL-10 negatively modulates ischemia-induced angiogenesis and that this effect is associated with the downregulation of VEGF expression.

Signs of inflammation are present in the ischemic hindlimb. However, although evidence is accumulating on the activating role of proinflammatory cytokines in angiogenesis, little is known about the role of the anti-inflammatory component of the reaction. Among the anti-inflammatory cytokines, we considered IL-10 as a cytokine with a putative antiangiogenic effect. IL-10 is produced by macrophages and could therefore be produced locally within the angiogenic area. Indeed, in the present study, we detected marked levels of IL-10 in the ischemic tissue of IL-10⁻/⁻ mice.

Our study demonstrates that in IL-10⁻/⁻ mice, vessel growth was specifically increased in the ischemic hindlimb with no effect in the nonischemic contralateral hindlimb. This suggests that hypoxia was the primary event leading to a local inflammatory reaction and angiogenesis. Ischemic tissue may produce chemoattractant proteins favoring monocyte migration. Monocytes that are activated to remove necrotic tissue may also contribute to angiogenesis through the production of proinflammatory cytokines with angiogenic properties. In addition, it has been shown that cytokines, such as IL-1β, strongly increase HIF-1α activity in human hepatoma cells in culture, emphasizing a possible role of HIF-1α as a trans-acting factor in the angiogenic process as well. Interestingly, in vivo LPS administration increases capillary density, as well as the number of macrophages in nonoccluded hindlimb. It is therefore likely that recruitment and activation of resident macrophages are sufficient to activate the angiogenic process.

Our finding of enhanced angiogenesis in ischemic hindlimb of IL-10⁻/⁻ mice underscores the importance of the inflammatory balance in the control of the angiogenic process. The marked angiogenesis associated with tissue ischemia in IL-10⁻/⁻ mice was due to the deficiency in IL-10.

Indeed, in vivo electrotransfer of murine IL-10 cDNA in IL-10⁻/⁻ mice was able to prevent the increased angiogenesis observed in the ischemic hindlimb of these animals.

IL-10 may act by deactivating macrophages with decreased production of angiogenic factors, including bFGF and TNF-α, as previously shown in ischemic cardiac tissue. Interestingly, antibodies to proinflammatory cytokines blunt the angiogenic response to VEGF and bFGF in a model of ocular angiogenesis, which suggests that proinflammatory cytokines are instrumental in triggering the angiogenic process. IL-10 may also directly modulate several cellular pathways that play an important role in the regulation of angiogenesis. Regulation of VEGF protein level has been shown to be a key event in the angiogenic process associated with hindlimb ischemia. Interestingly, VEGF protein level was markedly enhanced in the ischemic hindlimb of IL-10⁻/⁻ mice and returned to baseline level in IL-10⁻/⁻ mice trans-
fected with pCor-IL-10. This finding indicates that the antiangiogenic effect of IL-10 was likely due to a downregulation of VEGF expression. In addition, IL-10 might inhibit cyclooxygenase-2, which has been reported to affect the angiogenic process. An important effector mechanism in angiogenesis involves MMP production and activation. Inhibition of MMP activity is sufficient to block the angiogenic response to bFGF in rat cornea. IL-10 could therefore exert its angiogenic activity by inhibiting MMP synthesis and/or stimulating tissue inhibitors of MMPs, as previously described in human mononuclear phagocytes.

In conclusion, the present study demonstrates the antiangiogenic effect of IL-10 in mice with operatively induced hindlimb ischemia. This antiangiogenic effect was associated with the downregulation of VEGF content. The present work underscores the major role of the inflammatory balance in the modulation of ischemia-induced angiogenesis. Further studies are necessary to determine the exact mechanism of the antiangiogenic effect of IL-10. Our results open the way for therapeutic strategies aimed at decreasing IL-10 production to activate the angiogenic process in ischemic tissues.

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