Modulation of the angiogenic process is considered as an important therapeutic goal in numerous diseases including diabetic retinopathy, tumor growth, and ischemic diseases. In the setting of ischemia, the formation of new capillary blood vessels is under the control of both hypoxia and inflammation. Macrophages and T lymphocytes promote angiogenesis through the release of proinflammatory cytokines that heighten the production of matrix metalloproteinases (MMPs), leading to matrix degradation, and through the expression of angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2. On the other hand, macrophages and subsets of T cells are also known to produce antiinflammatory cytokines, including interleukin (IL)-10, which we have recently shown to be a major negative regulator of ischemia-induced angiogenesis. Therefore, the inflammatory balance seems to play a critical role in determining the extent of angiogenesis after ischemic injury.

Vascular ischemic diseases are mainly the result of atherosclerotic plaque progression and rupture, in which inflammation plays a major role. The benefit expected from the actors of the inflammatory response that stimulate tissue neovascularization in this setting, including inflammatory cytokines, chemokines, and VEGF, may be outweighed by their potential to promote atherosclerotic plaque progression and complications. Conversely, antiinflammatory cytokines like IL-10, although reducing plaque progression and complications, may greatly limit the angiogenic process with its expected deleterious effects on tissue perfusion and functional recovery. Therefore, identification of factors that may stimulate tissue neovascularization in the setting of ischemic injury without inducing plaque progression is a considerable challenge with major therapeutic consequences.

IL-18 is a major proinflammatory cytokine initially identified as the interferon (IFN)-γ-inducing factor and plays an important role in many inflammatory diseases, including diabetic retinopathy, tumor growth, and ischemic diseases. In the setting of ischemia, the formation of new capillary blood vessels is under the control of both hypoxia and inflammation. Macrophages and T lymphocytes promote angiogenesis through the release of proinflammatory cytokines, leading to matrix degradation, and through the expression of angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2. On the other hand, macrophages and subsets of T cells are also known to produce antiinflammatory cytokines, including interleukin (IL)-10, which we have recently shown to be a major negative regulator of ischemia-induced angiogenesis. Therefore, the inflammatory balance seems to play a critical role in determining the extent of angiogenesis after ischemic injury.

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rheumatoid arthritis,14,15 Crohn’s disease,16 and atherosclerosis. We have recently shown that the expression of IL-18 in human carotid atherosclerotic plaque is highly associated with plaque instability.17 In addition, treatment of apoE knockout mice with IL-18 binding protein (IL-18BP), the endogenous inhibitor of IL-18, inhibits plaque development, limits plaque progression, and induces a switch to a stable plaque phenotype.18 Recent studies in humans confirmed the atherosclerotic destabilizing potential of IL-18 by identifying this cytokine as a strong independent predictor of cardiovascular mortality in patients with stable or unstable angina.19 However, the role of IL-18 in angiogenesis in general is debated, and its specific role in ischemia-induced angiogenesis is unknown. Given the potential for use of antiinflammatory therapy based on inhibition of IL-18 in many inflammatory diseases, including atherosclerosis, we investigated the effect of IL-18BP administration and IL-18 deficiency on ischemia-induced neovascularization in mice hindlimb. Because recent studies have provided compelling evidence that bone marrow–derived endothelial progenitor cells (BM-EPCs) are involved in postnatal vasculogenesis,20 we also determined the effect of IL-18BP administration on the number of bone marrow–derived differentiated BM-EPCs.

Materials and Methods

Experimental Protocol

Male C57BL/6J mice (Ifla Creddo, Lyon, France) underwent surgery to induce unilateral hindlimb ischemia. Animals were anesthetized by isoflurane inhalation. The ligature was performed on the right femoral artery, 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries. Mice were then housed under conventional conditions for 3 or 28 days. To examine the role of IL-18BP in ischemia-induced angiogenesis, a group of mice was injected with the murine IL-18BP expression plasmid, pcDNA3-3IL18BP. Murine IL-18BP isoform d cDNA (accession number No. AF110803), was subcloned into the EcoRI/Ncol sites of mammalian cell expression vector pcDNA3 under the control of the cytomegalovirus promotor (Invitrogen). Mice were injected the day before ligature with 60 μg of pcDNA3-3IL18BP in both tibial cranial muscles, as previously described.18 The control mice were injected with a similar construct devoid of therapeutic cDNA. Transcutaneous electric pulses (8 square wave electric pulses of 200 V/cm, 20 ms duration at 2 Hz) were delivered by a PS-15 electroporator (Genetronics, France) using two stainless steel plate electrodes, placed 4.2 to 5.3 cm apart, at each side of the leg. We have previously shown that this strategy increases IL-18BP plasma levels, decreases plasma IL-18 activity, and inhibits atherosclerotic plaque development and progression.18 In an additional series of experiments, we used male C57BL/6 IL-18 knockout (KO) (kindly provided by Akira et al,21 Hyogo College of Medicine, Nishinomiya, Japan) and C57BL/6 controls in order to examine directly the role of endogenous IL-18 in postischemic angiogenesis. Because all parameters studied were measured at day 3, and day 28 as previously described.4,5 Frozen tissue sections (7 μm) were incubated with rat monoclonal antibody directed against CD31 (20 μg/mL, BD Pharmingen) to identify capillaries. Immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectorstain ABC kit elite, Vector Laboratories). Capillary densities were calculated in randomly chosen fields of a definite area, using Histolab software (Microvision Instrument).

Determination of VEGF, Phosphorylated Akt, Akt, and Endothelial Nitric Oxide Synthase (eNOS) Protein Expression

Tissue samples (gastrocnemius muscle from ischemic and nonischemic legs), obtained at day 3 or 28, were thawed and homogenized in 300 μL of buffer (200 mmol/L sucrose, 20 mmol/L HEPES, pH 7.4) containing protease inhibitors. Protein content was then determined by the method of Bradford. Proteins were separated in denaturing SDS/12% polyacrylamide gels and then blotted onto a nitrocellulose sheet (Hybond ECL, Amersham). Antibodies against VEGF (1:2000, Santa-Cruz-Biotechnology), phosphorylated (phospho-) Akt (1:500, Cell Signaling, Biolabs), Akt (1:1000, Cell Signaling), and eNOS (1:2000, Santa-Cruz-Biotechnology) were then used. As a protein loading control, membranes were stripped, incubated with a goat polyclonal antibody directed against total actin (Santa Cruz-Biotechnology, dilution 1/1000), and specific chemiluminescent signal was detected as previously described.4,5 The proteins were then stained with Ponceau Red (Sigma) for 10 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 to quantification of the transferred total protein bands stained with Ponceau Red (Sigma).

Cellular Localization of Phospho-Akt and VEGF

In an effort to localize phospho-Akt and VEGF-expressing cells, frozen tissue sections from ischemic muscles (7 μm) were incubated with rabbit polyclonal antibody directed against either phospho-Akt (1:50, Cell signaling, Biolabs) or VEGF (1:25, Santa-Cruz-Biotechnology). After incubation with a biotinylated anti-rabbit IgG, immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectorstain ABC kit elite, Vector Laboratories).

Cell Stimulation

HUVECs were obtained from Promocell (Heidelberg, Germany). Cells were used between passages 2 and 4. HUVECs were grown in culture medium (Basal medium, Promocell) supplemented with 10% fetal bovine serum and mitogens. Cells were split to a density of 40,104 cells/cm2 24 hours before start of serum starvation. Cells were washed twice with PBS and 1.5 mL of medium, without serum, complemented with 0.1% bovine serum albumin. After 18 hours of serum starvation, cells were washed with PBS and 1.5 mL of fresh by digital X-ray transducer were assembled in order 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. A quantification zone was delineated by the place of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg.

Capillary Density

Microangiographic analysis was completed by assessment of capillary densities in ischemic and nonischemic muscles at day 3 and day 28, as previously described.4,5 Frozen tissue sections (7 μm) were incubated with rat monoclonal antibody directed against CD31 (20 μg/mL, BD Pharmingen) to identify capillaries. Immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectorstain ABC kit elite, Vector Laboratories). Capillary densities were calculated in randomly chosen fields of a definite area, using Histolab software (Microvision Instrument).
starvation medium containing human recombinant protein IL-18 (50 ng/mL, Serono) or Wortmannin (30 nmol/L, Sigma) was added where indicated. Two hours later, human recombinant protein VEGF165 (3 ng/mL, Sigma) was added for 30 minutes, where indicated. Protein extracts were obtained by lysis in 200 μL of buffer (SDS 20%, Na-vanadate 100 mmol/L, Tris 0.5 mol/L, pH 7.4) containing protease inhibitors. Western blot analysis was then performed as described above.

Flow Cytometry Analysis

EPCs are thought to derive from Sca-1-positive bone marrow-derived mononuclear cells.20 The percentage of mononuclear cells expressing the EPC marker protein Sca-1 was then determined by flow cytometry (Calibur). Seven days after ischemia, mononuclear cells were isolated from peripheral blood (300 μL) and from bone marrow of mice treated with either the empty pcDNA3 plasmid or the pcDNA3-mIL18BP plasmid (n = 5 per group). Bone marrow cells were obtained by flushing the tibias and femurs. Low-density mononuclear cells were isolated by density-gradient centrifugation with Ficoll. Mononuclear cells were then incubated with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against Sca-1 (D7, BD Pharmingen). Isotype-identical antibodies served as controls.

EPC Differentiation Assay

Immediately after isolation, 5 × 10² bone marrow–derived mononuclear cells were also plated on 35-mm cell culture dishes coated with rat plasma vitronectin (Sigma) and gelatin (0.1%) and maintained in endothelial basal medium (EBM2, Bio Whittaker). After 4 days in culture, nonadherent cells were removed and adherent cells underwent immunochemicals analysis.

To detect the uptake of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (AcLDL-Dil), cells were incubated with AcLDL-Dil (Tebu) at 37°C for 1 hour. Cells were then fixed with 2% paraformaldehyde, incubated with a primary polyclonal rabbit antibody directed against von Willebrand factor (vWF) (DAKO) for 1 hour, and with FITC-labeled monoclonal anti-rabbit IgG (H+L) for 30 minutes (Coulter). Dual-stained cells positive for both AcLDL-Dil and vWF were judged to be EPCs, and they were counted per well. Three independent investigators evaluated the number of EPCs per well by counting 3 randomly selected high-power fields. Results are then expressed as percentage of total number of mononuclear cells.

Statistical Analysis

Results are expressed as mean ± SEM. One-way analysis of variance ANOVA was used to compare each parameter. Post hoc Bonferroni’s t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. A value of P < 0.05 was considered as statistically significant.

Results

Effects of IL18-BP and IL-18 Deficiency on Ischemia-Induced Angiogenesis

Microangiography

At day 3, ischemic/nonischemic leg angiographic score ratios were comparable between the 3 groups (n = 4 per group) (Figure 1). In contrast, at day 28, angiographic score showed 1.6-fold increase in mice treated with IL-18BP (n = 7) compared with controls (n = 12) (P < 0.01). Similarly, vessel density increased by 1.9-fold in IL-18 KO mice (n = 7) compared with controls (n = 12) (P < 0.01).

Capillary Density

Microangiographic data were confirmed by capillary density analysis after CD31 staining. At day 3, no difference in capillary number was found between the 3 groups (data not shown). At day 28, capillary density of the ischemic leg of control mice (n = 7) was lower than that of the nonischemic leg (415 ± 31 vessels versus 688 ± 42 vessels/mm²; P < 0.01). However, capillary density of the ischemic leg of IL-18BP–treated mice (n = 7) was significantly higher (1.4-fold increase) than that of control mice (n = 7) (588 ± 48 versus 415 ± 31 vessels/
mm², respectively; \( P=0.01 \) and did not differ from the level observed in the nonischemic leg.

**Laser Doppler Perfusion Imaging**

Microangiographic and capillary density measurements were associated with changes in blood perfusion. Hindlimb blood flow recovery occurred in either group. However, in IL-18BP treated mice (\( n=7 \)), a greater increase in blood flow was evident by day 28 compared with control animals (\( n=12 \)) (1.5-fold, \( P<0.01 \), Figure 2). Hindlimb blood flow recovery was also higher in IL-18 KO mice (\( n=7 \)) in reference to control mice (\( n=12 \)) (\( P<0.05 \) and \( P<0.001 \) at day 21 and 28, respectively).

**Regulation of VEGF, Phospho-Akt, and eNOS Protein Level**

**VEGF**

At day 3, no changes in VEGF protein level were observed between the ischemic and nonischemic legs, in either group. At day 28, in control mice, VEGF protein content tended to increase in the ischemic leg when compared with the nonischemic one, but this did not reach statistical significance. In contrast, VEGF protein level of the ischemic leg was markedly upregulated by 128% and 137% in IL-18BP–treated mice and IL-18 KO mice, respectively, compared with controls (\( P<0.05 \)) (Figure 3).

**Phospho-Akt**

At day 3, phospho-Akt protein level was unchanged in ischemic and nonischemic hindlimbs regardless of the treatment. At day 28, in control mice, phospho-Akt protein content was increased by 65% in ischemic hindlimb over that of nonischemic one (\( P<0.05 \)). This increase in phospho-Akt content of the ischemic leg doubled in mice treated with IL-18BP (110% increase; \( P<0.01 \) as compared with the increase in the ischemic leg of control mice). Similarly, phospho-Akt content of the ischemic leg was further enhanced in IL-18 KO mice (110% increase, \( P<0.05 \) as compared with the increase in the ischemic leg of control mice). In addition, no significant changes in Akt protein level were observed in either group (Figure 4). In an effort to localize VEGF and phospho-Akt expressing cells, we used immunohistochemical techniques and demonstrated a positive phospho-Akt staining mainly in capillaries of ischemic legs, whereas positive VEGF staining was mainly detected in myocytes (Figure 5).

**eNOS**

At day 3, eNOS protein content was unaffected in ischemic (112±15%, 103±24% versus 108±25%) and nonischemic legs (100±20%, 94±21% versus 109±23%) for control, IL-18BP–treated animals, and IL-18 KO mice, respectively. At day 28, in control mice, eNOS levels were raised by 55% in the ischemic leg in reference to the nonischemic one (159±12% versus 100±15%, respectively; \( P<0.05 \)). Such an increase was affected neither by IL-18BP treatment (160±12%; \( P=0.61 \) versus ischemic control) nor by IL-18 deficiency (158±20%; \( P=0.75 \) versus ischemic control).

**Effect of IL-18 on VEGF-Induced Akt Phosphorylation**

To test whether IL-18 may modulate VEGF/Akt pathway, we analyzed phospho-Akt protein content in whole-cell lysates of HUVECs treated with VEGF. VEGF raised the phospho-Akt protein level by 200% compared with untreated cells (\( P<0.05 \); Figure 6). In addition, the PI3-kinase inhibitor

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**Figure 2.** A, Ischemia-induced changes in hindlimb blood flow monitored in vivo by laser Doppler perfusion imaging performed at day 28 after ischemia in mice treated with pcDNA3-mIL18BP (IL-18 BP), mice deficient for IL-18 (IL-18KO), and controls. In color-coded images, normal perfusion is depicted in red; marked reduction in blood flow of ischemic hindlimb is in blue. B, Quantitative evaluation of blood flow expressed as a ratio of blood flow in ischemic limb to that in nonischemic one. Values are mean±SEM, \( n=12 \) for controls, \( n=7 \) for IL-18BP and IL-18KO groups. *\( P<0.05 \), **\( P<0.01 \), and ***\( P<0.001 \) vs control mice.
wortmannin completely abolished VEGF-induced Akt phosphorylation, which fell below unstimulated background, as previously described.22 Interestingly, VEGF-induced Akt phosphorylation was prevented by addition of IL-18, suggesting an important role of IL-18 pathway in Akt phosphorylation by VEGF (Figure 6).

Effect of IL-18BP on EPCs
EPCs are thought to derive from Sca-1–positive mononuclear cells.20 The percentage of Sca-1–positive mononuclear cells in the peripheral blood remained unchanged in IL-18BP–treated mice compared with control animals (31.5±13% versus 28.5±13%, respectively). Similarly, the overall number of Sca-1–positive mononuclear cells isolated from bone marrow did not differ among IL-18BP–treated mice and control animals (4.35±0.95% versus 5.87±0.22%, respectively). Furthermore, IL-18BP treatment did not affect the total number of peripheral blood or bone marrow mononuclear cells (data not shown).

EPCs were isolated and cultivated from bone marrow mononuclear cells and characterized as dual-stained cells positive for AcLDL-Dil and vWF. The percentage of cells with double positive staining was almost undetectable in nonischemic animals (<5%, n=4). Ischemia induced a marked increase in the percentage of cells with double positive staining for AcLDL-Dil and vWF (48±3%; P<0.001 versus nonischemic animals). Such an effect was further expanded by IL-18BP treatment (85±2%; P<0.001) (Figure 7). Thus, IL-18BP treatment seems to stimulate the differentiation of mononuclear cells into EPCs rather than increase the number of circulating progenitor cells.
Discussion
In the present study, we show that endogenous IL-18 pathway is a critical modulator of ischemia-induced neovascularization. In vivo intramuscular electroporation of a murine IL-18BP expression plasmid significantly enhanced new capillary development in the ischemic hindlimb. This was associated with increased local expression of VEGF and phosphorylation of Akt, and increased number of cultured differentiated BM-EPCs. Similar proangiogenic effects were observed using IL-18 KO mice, strongly suggesting a major antiangiogenic role for endogenous IL-18 in postischemic injury.

Classically, proinflammatory cytokines have been shown to promote neovascularization through various mechanisms, including increased recruitment of inflammatory cells, production of angiogenic factors, and increased matrix degradation. However, not all proinflammatory cytokines have proangiogenic properties. IL-12 and IFN-γ, for example, are major proinflammatory cytokines of the T-helper cell type 1 response (Th1), and exert significant antiangiogenic properties in defined models of angiogenesis, although their precise role in ischemia-induced angiogenesis is unknown. IL-18, which is also a major Th1 cytokine and synergizes with IL-12 to induce the production of IFN-γ, has been reported to exert IFN-γ-dependent antiangiogenic properties in tumor models, when administered alone or in combination with IL-12. However, in a recent study, IL-18 was reported to act as a mediator of angiogenesis in inflamed synovia of patients with rheumatoid arthritis, stimulating the migration of microvascular endothelial cells. In addition to the controversial role of IL-18 in angiogenesis, these previous

Figure 6. A, Representative Western blot of phospho-Akt and Akt protein content in HUVEC extracts treated with IL-18, VEGF, IL-18+VEGF, or VEGF + wortmannin in the absence of serum. Baseline levels of phospho-Akt are those found in cells grown in the absence of serum. Cells grown in 10% serum were used as a positive control for Akt phosphorylation. B, Quantitative evaluation of phospho-Akt protein levels. Values are mean ± SEM; n=5 per group. *P<0.05 vs control (0% serum); †P<0.05 vs VEGF-treated cells.

Figure 7. A, Representative images of EPCs isolated from bone marrow of mice without femoral artery ligation (Sham) and of mice treated with pcDNA3-mIL18BP (IL-18BP) or empty plasmid (Cont). EPCs were characterized as adherent cells with double-positive staining for AcLDL-Dil and von Willebrand factor (vWF). B, Quantification of double-positive cells in mice treated with pcDNA3-mIL18BP or empty plasmid. Values are mean ± SEM, n=5 per group. **P<0.001, vs control mice; †††P<0.001, vs mice without femoral artery ligation (Sham).
studies used exogenous administration of IL-18 to examine its role in angiogenesis. Therefore, their conclusions may not be extrapolated to the understanding of the role of endogenously produced IL-18 in the particular setting of ischemic injury. In our study, treatment of mice with IL-18BP, the endogenous inhibitor of IL-18, resulted in a significant increase in ischemia-induced angiogenesis without affecting vessel density of the nonischemic leg. These results were confirmed in IL-18 KO mice, which showed enhanced postischemic neovascularization. Our data strongly suggest that in vivo inhibition of IL-18 signaling in ischemic tissues promotes new capillary vessel formation and should be considered as a potential therapeutic strategy to enhance tissue perfusion in this setting. It is important to point out that such IL-18–targeted strategy, although promoting postschismic neovascularization, will also lead, as previously shown, to atherosclerotic plaque stabilization, therefore reducing the risk of recurrent ischemic events.

In an attempt to investigate the potential mechanisms involved in the proangiogenic effects related to IL-18 inhibition, we first examined the effect of IL-18 inhibition on the VEGF pathway. The increase in the revascularization process observed in IL-18BP–treated or IL-18 KO mice was associated with local upregulation of VEGF expression and Akt phosphorylation. Conversely, eNOS protein level was not affected. VEGF expression in ischemic hindlimb was localized to nonendothelial cells, mostly muscle cells, whereas phosphorylation of Akt occurred preferentially in endothelial cells, suggesting that the potential interplay between at least muscle cells and endothelial cells may modulate the angiogenic process. In vitro, VEGF–induced Akt phosphorylation in HUVECs was completely abrogated by addition of IL-18, pointing to a potent inhibitory role of IL-18 on Akt phosphorylation in endothelial cells. Taken together, our findings suggest that there exist at least 2 levels of regulation of neovascularization by endogenous IL-18: inhibition of VEGF expression by nonendothelial cells present in the ischemic hindlimb and inhibition of Akt phosphorylation in endothelial cells after stimulation with VEGF and presumably other angiogenic stimuli. The increase in both VEGF expression and Akt phosphorylation is likely to promote angiogenesis through various mechanisms as previously reported in models of ischemic injury. However, the precise molecular pathways responsible for the inhibitory effect of IL-18 on VEGF expression and Akt phosphorylation remain to be elucidated.

Recent studies have provided strong evidence for an important role of BM–EPCs in postnatal physiological and pathological neovascularization. In the setting of ischemic injury, Takahashi et al have shown that the development of limb ischemia induced BM–EPC mobilization that homed to sites of injury and significantly contributed to vasculogenic neovascularization. Moreover, it has been shown that mobilization and differentiation of EPCs were highly dependent on activation of phosphatidylinositol (PI) 3-kinase/Akt pathway. Mobilization of EPCs could also be modulated by granulocyte macrophage–colony stimulating factor (GM-CSF), which also stimulates hematopoietic progenitor and myeloid lineage cells and may act as a proinflammatory cytokine. However, the potential role of other cytokines in EPC mobilization associated with postischemic inflammatory responses is currently unknown. Because endothelial cells may express receptors for IL-18, we examined the effect of IL-18BP administration on EPC mobilization and differentiation. In vivo electrotransfer of IL-18BP cDNA constantly resulted in a significant increase in differentiated EPC number, whereas treatment with the empty plasmid did not affect EPC differentiation. Other blood or bone marrow cell counts were not affected by IL-18BP administration, suggesting a specific effect on EPC differentiation, which could be related to the potential of IL-18 inhibitors to promote Akt phosphorylation. This is the first study to show that treatment with an antiinflammatory cytokine may increase the number of bone marrow–derived differentiated EPCs and promote postschismic neovascularization.

In conclusion, we show that in vivo inhibition of IL-18 activity in mice stimulates tissue neovascularization in response to ischemic injury. Promotion of postischemic neovascularization is associated with increased expression of VEGF, increased phosphorylation of Akt, and increased number of cultured differentiated BM–EPCs. The proangiogenic properties of the endogenous inhibitor of IL-18, IL-18BP, associated with its known antiinflammatory and antiatherosclerotic effects, make IL-18BP, and other IL-18 inhibitors, ideal candidates for the treatment of ischemic diseases.

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