Effects of Aging and Hypoxia-Inducible Factor-1 Activity on Angiogenic Cell Mobilization and Recovery of Perfusion After Limb Ischemia

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Abstract—Ischemia is a stimulus for production of angiogenic cytokines that activate local vascular cells and mobilize angiogenic cells to the circulation. These responses are impaired in elderly patients with peripheral arterial disease. Hypoxia-inducible factor (HIF)-1 mediates adaptive responses to ischemia, including production of angiogenic cytokines. In this study, we demonstrate that aging and HIF-1 loss-of-function impair the expression of multiple angiogenic cytokines, mobilization of angiogenic cells, maintenance of tissue viability, and recovery of limb perfusion following femoral artery ligation. We show that HIF-1 directly activates transcription of the gene encoding stem cell factor and that mice lacking the cognate receptor C-KIT have impaired recovery from ischemia. Administration of AdCA5, an adenovirus encoding a constitutively active form of HIF-1α, improved the recovery of perfusion in older mice to levels similar to those in young mice. Injection of AdCA5 into nonischemic limb was sufficient to increase the number of circulating angiogenic cells. These results indicate that HIF-1 activity is necessary and sufficient for the mobilization of angiogenic cells and that HIF-1α gene therapy can counteract the pathological effects of aging in a mouse model of limb ischemia. (Circ Res. 2007;101:1310-1318.)

Key Words: angiogenesis ▪ hypoxia ▪ vascularization

Atherosclerotic stenosis of vessels responsible for perfusion of the heart or lower limbs results in ischemia, which may progress to myocardial infarction or critical limb ischemia (CLI) requiring amputation, respectively. Despite advances in the medical and surgical treatment of these conditions, ischemic tissues produce angiogenic cytokines that induce blood vessel formation and remodeling of existing vessels.

In mice, limb ischemia resulting from femoral artery ligation induces mobilization into peripheral blood (PB) of endothelial progenitor cells and other angiogenic cells from bone marrow (BM), blood vessels, or other tissues, and recruitment of these cells to the ischemic limb, where they promote recovery of tissue perfusion by stimulating angiogenesis, arteriogenesis, and vasculogenesis.

Here, we use the term circulating angiogenic cells (CACs) to denote a heterogeneous population that includes endothelial progenitor cells, which incorporate into the endothelium of new or remodeling vessels, as well as myeloid, mesenchymal, and hematopoietic stem cells, which promote vascular growth and remodeling through production of angiogenic cytokines.

CACs are enumerated by culturing PB mononuclear cells (MNCs) under endothelial growth conditions or by flow cytometry using antibodies against cell surface receptors characteristic of endothelial and progenitor cells. Among inbred mouse strains, the number of CD45+/CD13−/Flk1+ cells in PB is correlated with the magnitude of the response to an angiogenic stimulus such as administration of vascular endothelial growth factor (VEGF).

In experimental animals, recovery of blood flow to ischemic tissue is increased by administration of angiogenic cytokines, but clinical trials have not demonstrated efficacy. Animal studies suggest that involvement of multiple angiogenic growth factors is required for production of stable, functional vessels. Administration of CACs or BM-MNCs promotes tissue vascularization and repair in mice. However, randomized clinical trials involving administration of autologous cells to patients with CLI or myocardial infarction have demonstrated modest or no improvement. The contrast...
between dramatic responses in animals and disappointing clinical outcomes may reflect the fact that young, healthy animals are used in most laboratory studies, whereas ischemic cardiovascular disease in patients is the result of a chronic process in which aging, genetic modifiers, and environmental factors impair physiological responses to tissue hypoxia and ischemia.17 Understanding these processes may lead to development of efficacious therapies.

Hypoxia-inducible factor (HIF)-1 is a transcription factor that mediates adaptive responses to hypoxia and ischemia. HIF-1 regulates the expression of hundreds of genes, including those encoding angiogenic cytokines such as VEGF, placental growth factor (PLGF), angiopoietin 1 (ANGPT1), ANGPT2, and stromal-derived factor (SDF)-1.18–21 HIF-1 also mediates cell-autonomous responses to hypoxia in endothelial cells.21–23 AdCA5, an adenovirus encoding a constitutively active form of the HIF-1α subunit, has been shown to promote angiogenesis and arteriogenesis in animal models.19,24 as have other modified forms of HIF-1α.25 A phase I trial involving administration of adenovirus encoding a HIF-1α/VP-16 fusion protein to no-option patients with CLI has been reported.26 In this study, we investigated the effects of aging and HIF-1 gain- or loss-of-function on ischemia-induced angiogenic cytokine expression, CAC mobilization and recruitment, and recovery of tissue perfusion in mice with limb ischemia.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Limb Ischemia Model
Unilateral femoral artery ligation was performed as described.27 Serial measurements of limb perfusion were obtained by laser Doppler perfusion imaging (LDPI).

Cell Cultures
PB-MNCs were isolated, cultured, and stained with fluorescein isothiocyanate–lectin and/or 1,1′,3,3′,3′,3′-tetramethylindocarbocyanine (DiI)-labeled, acetylated low-density lipoprotein as described.12 Mouse embryonic stem cells (ESCs) were cultured as described.18

Flow Cytometry
PB-MNCs were isolated and CD34+/Flk1+ cells,13 and CD45+/CD13/Flk1+ cells14 were analyzed as described. CXCR4+/Sca1+ cells were analyzed by flow cytometric analysis of whole blood.

Real-Time Quantitative RT-PCR
Total RNA extraction, cDNA synthesis, and real-time PCR were performed as described.19,24

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation assay was performed using the KItg primers shown in Table I in the online data supplement.

Statistical Analysis
Comparison of recovery over time and mRNA expression in wild-type (WT) versus heterozygous (HET) mice at 2 or 3 different ages was performed by 2- or 3-way ANOVA, respectively, with Bonferroni post hoc comparisons. CACs and stem cell factor (SCF) mRNA were analyzed by Student’s t test.

Results
Effects of Aging and Partial HIF-1α Deficiency on Recovery of Limb Perfusion
To induce limb ischemia, femoral artery ligation was performed on male littermate mice that were either WT or HET for a null (knockout [KO]) allele at the Hif1α locus encoding HIF-1α. The HET mice develop normally and are indistinguishable from their WT littermates but have impaired responses to hypoxia and ischemia, whereas homozygosity for the KO allele results in failed vascularization and embryonic lethality.18,28 We compared recovery of perfusion in young adult (2 months), middle-aged (8 months), and aged (20 months) HET and WT ischemic mice. LDPI was performed for serial, noninvasive determination of perfusion in the ischemic as compared with nonischemic limb in the immediate pre- and postoperative periods and up to 35 days later. Compared with 2-month-old mice, 8-month-old mice showed impaired recovery of perfusion (Figure 1A). Twenty-month-old mice showed an even more severe impairment. At each age, recovery of perfusion in HET mice was less than in their WT littermates. Three-way ANOVA revealed significant effects on the limb perfusion ratio of: time after ligation (P<0.001); age (P<0.001); and genotype (P<0.01).

Genotype and aging also had significant effects (P<0.001 for each) on the degree of neurological impairment of the ischemic limb (Figure 1B). The ultimate effect of inadequate recovery of perfusion is tissue death, leading to autoamputation of toes or the entire foot (Figure 1C). The effect of aging on limb salvage was highly significant (P<0.0005, Fisher’s exact test). Partial HIF-1α deficiency was associated with increased frequency and severity of amputation in each age group, although the differences did not reach statistical significance. Taken together, these results demonstrate that aging and Hif1α genotype have additive effects on vascular and tissue recovery in CLI.

Angiogenic Cell Mobilization Is Impaired by Aging and HIF-1α Deficiency
We next tested the hypothesis that the observed differences in recovery of perfusion were associated with differences in mobilization of angiogenic cells in response to limb ischemia. MNCs were isolated from PB of 2- and 8-month-old C57BL/6J mice before or 3 days after limb ischemia and cultured in the presence of endothelial cell growth factors, and the number of CACs was determined. In response to limb ischemia, the number of CACs in PB on day 3 increased significantly in 2-month-old, but not in 8-month-old, mice (Figure 2A). Similar studies were performed comparing HET and WT mice. The number of CACs in PB was similar in nonischemic 2-month-old WT and HET littermates, but the ischemia-induced increase in CACs was observed only in WT mice (Figure 2B). Flow cytometric analysis of PB-MNCs on day 3 after femoral artery ligation revealed a 2-fold increase in cells that coexpressed VEGF receptor (VEGFR2) (also known as Flk1) and the progenitor marker CD34 (VEGFR2+/CD34+) in ischemic, as compared with nonischemic, WT mice (Figure 2C). The number of VEGFR2+/CD34+ cells in PB of ischemic, compared with nonischemic, HET mice was

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not increased and was 4-fold lower than in ischemic WT mice (Figure 2C). CXCR4$^+/Sca1^+$ cells were also mobilized into the PB of WT, but not HET, mice 3 days after femoral artery ligation (Figure 2D). The number of CXCR4$^+/Sca1^+$ CACs was not increased on day 2 in WT mice, thus establishing day 3 as the onset of mobilization. Taken together, the results presented in Figure 2 suggest that the effects of aging and Hif1a genotype on recovery of perfusion are, in part, attributable to impaired ischemia-induced mobilization of CACs.

Expression of Angiogenic Cytokines Is Impaired by Partial HIF-1α Deficiency and Aging

The signal for mobilization of CACs arises in the ischemic tissue with the hypoxia-induced expression of genes encoding angiogenic cytokines, including PLGF, SDF-1, and VEGF. Based on the kinetic analysis of CAC mobilization described above, RNA was isolated from calf muscle 3 days after femoral artery ligation for analysis by quantitative RT-PCR. In 2-month-old mice, the ischemia-induced expression of ANGPT2, HIF-1α, PLGF, SCF, and SDF-1 mRNA was significantly reduced in HET as compared with WT mice. Mean mRNA levels were not significantly different in the nonischemic limbs of HET versus WT mice (data not shown). Thus, partial HIF-1α deficiency does not significantly impair basal production of angiogenic cytokines but has a major effect on the expression of angiogenic cytokines in response to limb ischemia. A significant effect of aging across both genotypes was demonstrated for ANGPT1, ANGPT2, HIF-1α, MCP-1, PLGF, SCF, and SDF-1 mRNA. Effects of genotype and/or aging were also observed for ANGPT1 and VEGF but were not significant because of large variation within groups. Two-way ANOVA also revealed a significant interaction (P<0.01) between genotype and age on the expression of ANGPT1, ANGPT2, HIF-1α, PLGF, SCF, and SDF-1 mRNA.

To confirm that the observed changes in mRNA levels were associated with changes in protein expression, we analyzed lysates from calf muscles of 3 pairs of WT and HET littermate mice 3 days after femoral artery ligation. VEGF protein expression was induced by ischemia in 3 of 3 WT and 2 of 3 HET mice. Although there was great variation among the mice, ischemia-induced expression was greater in the WT mouse of each littermate pair (Figure 3B). The ischemia-induced HIF-1α- and age-dependent expression of angiogenic cytokines provides a molecular basis for the ischemia-induced HIF-1α- and age-dependent mobilization of CACs (Figure 2); eg, cells bearing VEGFR2 or CXCR4 are mobilized in response to VEGF or SDF-1, respectively. We hypothesized that ischemia-induced cytokine expression promotes recruitment of mobilized CACs to the ischemic limb, but detecting the relatively small numbers of such cells presents a technical challenge. To overcome this problem, mRNAs encoding receptors for angiogenic cytokines were quantified by quantitative RT-PCR (Figure 3C) using the same samples that were analyzed for angiogenic cytokine mRNAs (Figure 3A). A significant difference in
cytokine receptor mRNA levels was demonstrated in the ischemic limbs of 2-month-old HET versus WT mice by 2-way ANOVA (P<0.05). In particular, C-KIT mRNA levels showed the greatest increase in ischemic compared with nonischemic limbs and the most significant difference between WT and HET mice (Figure 3C).

Effects of Aging and Genotype on Ischemia-Induced HIF-1α Expression
To further investigate the basis for the marked reduction in ischemia-induced expression of mRNAs encoding angiogenic cytokines in aged and HET mice, HIF-1α protein levels were analyzed by immunoblot assay. In 2-month-old WT mice, HIF-1α protein levels in the nonischemic limb were below the level of detection, whereas in the ischemic limb, HIF-1α was easily detected (Figure 4). In contrast, there was no detectable increase in HIF-1α protein levels in the ischemic limbs of HET mice. In 8-month-old WT mice, ischemia-induced HIF-1α levels were lower than in 2-month-old WT mice. In 20-month-old WT mice, ischemia-induced HIF-1α levels were below the limits of detection, similar to HET mice at all ages. The observed effects of aging and genotype on HIF-1α protein levels (Figure 4) are qualitatively similar to their effects on the expression of mRNAs encoding angiogenic cytokines (Figure 3A). The effect of aging on HIF-1α expression provides a molecular basis for the interaction between genotype and age on cytokine mRNA expression that was reported above (Figure 3A).

HIF-1 Mediates Kitlg Gene Expression in Response to Hypoxia
The finding of increased SCF mRNA expression in ischemic limb muscle is of great interest because SCF is the ligand for the C-KIT receptor, which is expressed by many CACs. Administration of C-KIT+ BM cells has been shown to promote angiogenesis and repair in ischemic tissue. C-KIT mRNA levels were significantly increased in the ischemic limb of 2-month-old WT mice, consistent with recruitment of C-KIT+ cells. Previous studies have reported that SCF expression is induced by hypoxia, and the impairment of ischemia-induced SCF mRNA in HET mice suggested that HIF-1 may activate transcription of the Kitlg gene encoding SCF. Alternatively, HIF-1–dependent expression of another cytokine might recruit SCF–expressing cells to the ischemic tissue. To test whether hypoxia induces cell-autonomous SCF mRNA expression, mouse ESCs that were WT or homozygous for the Hif1a KO allele were analyzed. Incubation of WT ESCs for 24 hours under hypoxic conditions induced significantly increased SCF mRNA relative to cells incubated under nonhypoxic conditions (Figure 5A). In contrast, SCF mRNA expression was not induced by hypoxia in KO ESCs.

The data from ESCs indicated that SCF mRNA expression is induced by hypoxia in a cell-autonomous and HIF-1–dependent manner. HIF-1 regulates gene expression by binding to the DNA sequence 5’-(A/G)CGTG-3’, which was identified within the proximal promoters of the mouse and human Kitlg genes encoding SCF (Figure 5B). Chromatin immunoprecipitation assay assays were performed to analyze the binding of HIF-1 to the Kitlg promoter. In WT cells, HIF-1 binding was demonstrated under nonhypoxic conditions (20% O2) and increased binding was observed under hypoxic conditions (1% O2), whereas in KO cells, HIF-1 binding was not detected under hypoxic or nonhypoxic conditions (Figure 5C). HIF-1–binding data showed remarkable agreement with SCF mRNA expression data (compare Figure 5A and 5C).

Impaired Recovery of Perfusion in C-KIT Mutant W/W’ Mice
To further investigate the role of SCF→C-KIT signaling in the vascular response to limb ischemia, we performed femoral artery ligation in 2-month-old W/W’ mice, which contain a point mutation that results in loss of C-KIT receptor tyrosine kinase activity. Compared with WT littermates,
Acute Inhibition of HIF-1 Increases Ischemic Tissue Damage and Functional Impairment

2-Methoxyestradiol (2ME2) is an endogenous antiangiogenic metabolite of estradiol that has low affinity for estrogen receptors but has been shown to inhibit hypoxia-induced HIF-1α protein expression by a mechanism that involves microtubule disruption.33 As a means of inducing acute HIF-1 loss-of-function in vivo, we administered 2ME2 (200 mg/kg IP) or vehicle to 2-month-old WT mice immediately following femoral artery ligation and each day thereafter. Immunoblot assay revealed that 2ME2 treatment effectively inhibited ischemia-induced HIF-1α protein expression (Figure 7A).

Taken together, the ischemia-induced expression of mRNAs encoding HIF-1α and angiogenic factors was significantly different in 2ME2-treated versus vehicle-treated mice (Figure 7B) as demonstrated by 2-way ANOVA ($P<0.05$). The recovery of perfusion in mice receiving 2ME2 was significantly impaired over the 21-day time course ($P<0.01$) and at the end point ($P<0.001$) as compared with vehicle-treated littermates (Figure 7C). Neurological defects (Figure 7D) and tissue damage (Figure 7E) were observed in the ischemic limbs of all mice treated with 2ME2 compared with none of the mice treated with vehicle.

AdCA5 Promotes Recovery of Limb Perfusion in Young and Old Mice

The studies of HET and 2ME2-treated mice demonstrated by genetic and pharmacological approaches that HIF-1 loss-of-function impairs the angiogenic response to limb ischemia. To analyze the effect of HIF-1 gain-of-function, we compared the effect of administering AdCA5, which encodes a constitutively active form of HIF-1α,19,21,24 and AdLacZ, which encodes Escherichia coli β-galactosidase. Immediately following surgery, AdLacZ or AdCA5 was injected along the former course of the excised femoral artery. In 2-month-old C57BL/6J mice, AdCA5 administration significantly improved recovery of blood flow (Figure 8A). These results are consistent with those previously obtained in a limb ischemia

Figure 3. Analysis of angiogenic cytokines and cognate receptors. A and C, RNA was isolated from calf muscles of 2- and 20-month-old WT and HET mice (n=3 to 5 each) 3 days after femoral artery ligation and analyzed by quantitative RT-PCR. The ratio of mRNA expression in ischemic/nonischemic limbs was determined, and mean values ($\pm$SEM) are plotted and analyzed by 2-way ANOVA with Bonferroni correction. A, HIF-1α and angiogenic cytokine mRNA expression. *$P<0.01$ compared with WT; #$P<0.05$ for 2 month vs 20 month. B, VEGF protein expression as determined by immunoblot assay. Total protein on the membrane was stained with Ponceau S to evaluate loading. C, Angiogenic cytokine receptor mRNA expression. **$P<0.01$. 

W/Wv mice had significantly reduced recovery of perfusion as well as increased motor dysfunction and tissue damage (Figure 6). 

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model in young rabbits, in which AdCA5 administration increased blood pressure and blood flow by increasing the luminal area of collateral arteries in the ischemic limb as demonstrated by arteriography and immunohistochemistry.24

Two limitations of the experiment described above with regard to its translational relevance were that it was performed with young mice that recover without treatment and that it involved large doses of adenovirus that might be difficult to scale up for clinical use. However, a 3-fold lower dose of AdCA5 also significantly improved recovery of perfusion in 8-month-old mice (Figure 7B). Notably, recovery of 8-month-old mice treated with AdCA5 was similar to that of control (AdLacZ-treated) 2-month-old mice (compare Figure 8A and 8B), indicating that HIF-1 gain-of-function overcame the effect of aging on recovery of blood flow in 8-month-old mice.

We hypothesized that improved recovery of blood flow in ischemic tissue of AdCA5-treated mice may be attributable in part to increased mobilization of CACs. To test whether HIF-1 gain-of-function was sufficient to mobilize CACs in the absence of femoral artery ligation, AdLacZ or AdCA5 was injected into nonischemic hind limb adductor muscle of 2-month-old C57BL/6J mice and 3 days later PB was analyzed by flow cytometry. The number of CD45^+CD13^+Flk1^+ CACs was significantly increased in AdCA5-treated mice (Figure 8C). Thus, gain-of-function (Figure 8C) and loss-of-function (Figure 2B–D) studies demonstrate that HIF-1 is necessary and sufficient, respectively, for mobilization of CACs.
Discussion

CLI is the end-stage outcome of multiple types of peripheral arterial disease, including atherosclerosis and thromboangiitis obliterans (Buerger disease). In this study, we have attempted to integrate the effects of aging, genetic variation, and environmental factors such as drug treatment on tissue perfusion, viability, and function in response to CLI. We demonstrate that aging and Hif1a genotype have significant effects on these processes and that adenoviral or pharmaco-
logical intervention that increases or decreases HIF-1α expression can also have a major impact on the adaptive response to ischemia. It is striking that variation in only 2 factors (age and Hif1α genotype) was sufficient to generate a broad range of outcomes (Figure 1).

Based on the data presented above and in previous studies,19–25 HIF-1 promotes tissue vascular responses to ischemia by inducing the expression of genes encoding multiple angiogenic growth factors, including ANGPT1, ANGPT2, PLGF, VEGF, SDF1, and, as demonstrated for this first time in this report, SCF. These factors induce chemotaxis of cells bearing their cognate receptors (TIE2, VEGFR1, VEGFR2, CXCR4, and C-KIT, respectively), leading to the mobilization of CACs from BM and/or other tissues to PB and subsequent homing from PB to the ischemic limb. We demonstrate that HIF-1 mediates transcriptional activation of the Kitlg gene encoding SCF and that C-KIT signaling is required for recovery of perfusion following femoral artery ligation.

The impaired recovery of perfusion in HET mice demonstrates the effect of genetic variation on vascular pathophysiology. The loss of ischemia-induced HIF-1α protein expression in the HET ischemic limbs was striking and resulted in decreased expression of multiple angiogenic cytokine mRNAs. HIF-1α mRNA expression was also induced by ischemia in a HIF-1–dependent manner, which may provide a mechanism for signal amplification as increased levels of HIF-1α mRNA result in increased synthesis of HIF-1α protein, which accumulates rapidly in ischemic tissue because of decreased O2-dependent degradation.

A priori, the observed impairment of ischemic recovery in HET mice could reflect subtle developmental defects rather than a defect in physiological adaptation. We therefore chose to administer 2ME2 to WT mice as a means of acutely inhibiting HIF-1 and as a model for an environmental influence, such as pharmacotherapy for a coexisting condition (2ME2 [Panzem], is currently in clinical trials as an anticancer agent). An additional clinically relevant basis for choosing 2ME2 was the recent proposal that 2ME2 might represent “new and improved hormone replacement therapy for athero-sclerosis” that “could be used clinically to prevent or treat cardiovascular disease.”34 However, 2ME2 does not bind to estrogen receptors but instead inhibits HIF-1α expression by destabilizing microtubules.33 Most importantly, our data indicate that 2ME2 has the potential to exacerbate rather than to prevent ischemic cardiovascular disease.

Aging was associated with impaired induction of HIF-1α protein and angiogenic cytokine mRNA expression in the ischemic limb, impaired CAC mobilization in PB, impaired recovery of perfusion, and impaired limb salvage. Aging effects were observed as early as 8 months of age and progressed significantly at 20 months. These aging effects may be attributable in part to the progressive age-dependent impairment of HIF-1α protein expression that was observed in the ischemic limbs of WT mice. However, recovery of perfusion was impaired to a greater extent in 8-month-old WT mice than in 2-month-old HET mice, whereas ischemia-induced HIF-1α protein expression was impaired to a greater extent in 2-month-old HET mice than in 8-month-old WT mice, which indicates that aging exerts effects that are independent of impaired HIF-1α expression. Nevertheless, AdCA5 administration stimulated recovery of perfusion in 8-month-old C57BL/6 mice to levels similar to those observed in 2-month-old control mice.

A major challenge of contemporary biomedical research is to develop appropriate models of chronic human diseases. Our studies illustrate the effects of aging, genotype, and environment on the presentation and progression of limb ischemia. Unlike young WT mice, which recover spontaneously without permanent tissue damage and therefore are not a model of CLI, aging and partial HIF-1α deficiency impair recovery of blood flow and result in permanent tissue damage. The evaluation of novel therapies in animal models with multifactorial impairment of vascularization may represent a more effective strategy for identifying candidates for clinical trials in patients with ischemic cardiovascular disease.

Acknowledgments

We thank Andrea Gambotto and Susan Schoonover for large-scale adenovirus preparations and Nick Dordai for advice on flow cytometry.

Sources of Funding

This work was supported by NIH grant R01-HL55338, American Diabetes Association grant 1-06-RA-121, and the Johns Hopkins University Institute for Cell Engineering.

Disclosures

Under a licensing agreement between Genzyme Corporation and Johns Hopkins University, G.L.S. is entitled to a share of fees received by the University from sales of technology described in this study. Terms of this arrangement are managed by the University in accordance with its conflict of interest policies.

References


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_Circ Res._ 2007;101:1310-1318; originally published online October 11, 2007; doi: 10.1161/CIRCRESAHA.107.153346

_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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Supplemental Materials and Methods

Mouse Strains
The \textit{Hif1a}^\text{tm1jhu} mouse strain was generated by homologous recombination to replace exon 2 of the \textit{Hif1a} gene with a \textit{neo}^R expression cassette in the J1 line of embryonic stem cells, which was originally derived from 129/Sv mice, followed by injection of the genetically modified cells into C57BL/6J blastocysts.\textsuperscript{1} The strain has been maintained by HET x WT intercrosses for the last 10 years and the mice therefore segregate the \textit{Hif1a}^\text{tm1jhu} allele on an outbred 129 x B6 genetic background. \textit{W/Wv} mice and colony controls were purchased from The Jackson Laboratory.

Limb Ischemia Model
Complete femoral artery excision from the left hind limb of male mice was performed using an operative procedure developed in the laboratory of the late Dr. Jeffrey Isner,\textsuperscript{2} which was modified to include disruption of the subcutaneous branch in the medial thigh. Perfusion of the hind limbs was measured by laser Doppler perfusion imaging (LDPI; Moor Instruments) which utilizes a near-infrared laser diode to measure subcutaneous blood flow as a function of light scattering by moving red blood cells (Doppler shift). This technique allows for repeated, non-invasive, quantitative measurements of tissue perfusion that are expressed as the ratio of signal in the ischemic and contralateral non-ischemic hind limb. Perioperative LDPI provides a method of demonstrating successful surgical outcome (\textit{i.e.} presence of critical limb ischemia). The serial changes in perfusion that are observed following femoral artery excision and revascularization have been shown to correlate with changes in vessel density as determined by histological analysis.\textsuperscript{2} In addition to the quantitative analysis of perfusion in the foot by LDPI, the animals
were clinically evaluated and scored for physical evidence of ischemic tissue damage and impairment of motor function.\textsuperscript{3}

**Mononuclear Cell (MNC) Cultures**

Peripheral blood was obtained by cardiac puncture. MNCs were isolated by density gradient centrifugation in Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). Remaining red blood cells were lysed by addition of Ammonium Chloride Solution (Stem Cell Technologies, Vancouver, Canada). MNCs were suspended in EBM-2 supplemented with EGM-2MV (Cambrex, Walkersville, MD) and plated on rat vitronectin (Sigma-Aldrich) coated dishes at 1.5x10\textsuperscript{6} cells/cm\textsuperscript{2}.\textsuperscript{4} After incubation for 4 days, the cells were incubated with 2 µg/ml of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL; Molecular Probes) for 2 h, rinsed in PBS, fixed in 2% paraformaldehyde for 5 min at room temperature, incubated in PBS containing 20 µg/ml of FITC-labeled Bandeira simplificifolia agglutinin-1 (BS-1 lectin; Sigma-Aldrich) for 30 min at 4\textdegree C, rinsed with PBS, and incubated in PBS containing 300 nM DAPI (Molecular Probes, Eugene, OR). Five fields were photographed under fluorescence microscopy at 200x magnification from each of 2 wells and the mean (± SEM) number of cells was determined. In our hands, all of the cultured cells that were positive for AcLDL uptake were also positive for lectin binding. Because the lectin-binding assay did not provide any additional specificity, we discontinued its use in later studies.

**Flow Cytometry**

The number of CD34\textsuperscript{+}/VEGFR2\textsuperscript{+} cells in MNCs isolated from peripheral blood were determined in control mice and in mice 3 days status post femoral artery ligation by fluorescence activated cell sorting (FACS). 2 x 10\textsuperscript{5} mononuclear cells isolated from peripheral blood were incubated
for 20 minutes in the dark with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against CD34 (eBioscience), followed by phycoerythrin (PE)-conjugated monoclonal antibody against mouse Flk-1 (BD Biosciences). After incubation, cells were washed with staining buffer (0.5% BSA/2mM EDTA in PBS), and 500,000 total events were analyzed. The mononuclear cell population was gated and positive cells were expressed as percentage of gated cells.

On day 3 following adenoviral injection, the number of CD45−CD13+/Flk1+ cells was determined as previously described. The antibodies used were PerCP-CD45, FITC-CD13, and PE-Flk1 (BD Biosciences). 5-6 µl of each antibody was added to 100 µl of whole blood and incubated for 25 min at 4°C in the dark. 1 ml of ammonium chloride solution (Stem Cell Technologies) was added and incubated for 10 min at 4°C in the dark to lyse red blood cells. Mononuclear cells were pelleted, resuspended in staining buffer, and FACS was performed with an acquisition of 500,000 events.

CXCR4+/Sca1+ cells were analyzed by FACS using 100-µl aliquots of whole blood to which was serially added: 1 µl of Fc block (CD16/CD32; BD Pharmingen); 2 µl each of PE-CXCR4 and FITC-Sca1 antibody (BD Pharmingen); and 1 ml of ammonium chloride solution. After incubation and centrifugation, the pellet was resuspended in 500 µl of staining buffer. 150,000 events were acquired by FACS analysis (LSRII, Becton Dickinson), the live cell population in the FSC vs SSC window was gated, and data were recorded from the dot plot of FITC vs PE.

Real-time Quantitative Reverse-Transcriptase PCR

RNA isolation and analysis was performed as previously described. Total RNA was extracted from cells or calf muscle using RNeasy Mini kits (Qiagen) and treated with DNase I (Ambion). Two µg of total RNA were used for first-strand synthesis with iScript cDNA Synthesis system.
Real-time PCR was performed using iQ SYBR Green Supermix and iCycler Real-time PCR Detection System (BioRad). For each set of primers, gradient PCR was performed for determination of the optimal annealing temperature. Serial dilutions of cDNA samples were analyzed to determine efficiency and dynamic range of the PCR. An assay requirement was that the standard deviation for the cycle threshold ($C_T$) among 3-5 replicate samples was $< 0.3$. $C_T$ was plotted vs log (ng input RNA) and the best-fit line was constructed. An assay requirement was that the correlation coefficient of the line was $> 0.99$. The slope (m) of the line was used to determine PCR efficiency (E) based on the formula $E = (10^{l/m})^{-1}$. In order for samples to be compared, efficiencies could vary by no more than 5%. Thus, stringent criteria were imposed to assure linearity of the assays. The relative expression, $R$, of each target mRNA was calculated based on the threshold cycle (Ct) according to the formula $R = 2^{-\Delta Ct}$, where $\Delta Ct = C_{t\text{target}} - C_{t\text{control}}$; the target was HIF-1$\alpha$, ANGPT1, ANGPT2, MCP-1, PLGF, SCF, SDF-1, or VEGF mRNA; and the control was 18S rRNA or RPL13A mRNA. Results were normalized to the mean value for the non-ischemic wild-type samples. The fold increased mRNA expression in the ischemic limb, $F$, was calculated according to the formula $F = 2^{\Delta(\Delta Ct)}$, where $\Delta(\Delta Ct) = \Delta Ct_{\text{ischemic}} - \Delta Ct_{\text{non-ischemic}}$. For embryonic stem (ES) cells, results were normalized to those obtained from wild-type cells at 20% O$_2$.

**Immunoblot Assays**

Tissue lysates were prepared from ischemic and non-ischemic calf muscle as previously described.$^7$ Anti-HIF-1$\alpha$ and anti-β–actin rabbit polyclonal antibodies were obtained from R&D Systems and Santa Cruz Biotechnology, respectively. Anti-VEGF mouse monoclonal antibody was obtained from Santa Cruz Biotechnology.
ES Cell Cultures

Mouse ES cells were maintained in DMEM supplemented with 15% fetal bovine serum, non-essential amino acids, glutamine, pyruvate, and 1% penicillin-streptomycin as previously described.¹

Hypoxic Cell Cultures

For hypoxic exposures, cells were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed for 3 min at 2 psi with a gas mixture containing 1% O₂, 5% CO₂, and balance N₂, the chamber was sealed, and incubated at 37°C. Non-hypoxic cultures (20% O₂) were performed in a standard tissue culture incubator containing 95% air and 5% CO₂.

Chromatin Immunoprecipitation Assay (ChIP)

Wild type and HIF-1α knockout mouse embryo fibroblast cultures were incubated at 20% or 1% O₂ for 24 hours as described.⁸ ChIP was performed using the ChIP Assay Kit (Upstate-Cell Signaling Solutions), rabbit polyclonal antibodies against HIF-1α (R&D Systems), and oligonucleotide primers that were designed based on the DNA sequence of the mouse Kitlg gene flanking the putative HIF-1 binding site (Table S1). Mouse Kitlg and human KITLG gene nucleotide sequences were obtained from GenBank (accession numbers NT_039500.6 and NT_019546.15, respectively).

2-Methoxyestradiol Treatment

Immediately following femoral artery ligation and daily thereafter, mice received an intraperitoneal injection of 2-methoxyestradiol (200 mg/kg; 2ME2) or vehicle alone. The 2ME2
administered was formulated by or on behalf of EntreMed, Inc. (Rockville, MD), which provided both the 2ME2 suspension (Panzem®) and vehicle.

**Adenoviral Treatment**

AdCA5 encodes a modified form of HIF-1α that contains a deletion of amino acids 392 to 520 and two missense mutations (Pro567Thr and Pro658Gln) that inhibit O₂-dependent degradation of the protein.⁶ AdLacZ encodes *E. coli* β-galactosidase. Large-scale adenoviral production was performed at the NHLBI PEGT Vector Core Facility, University of Pittsburgh. Immediately after femoral artery ligation, AdlacZ or AdCA5 was directly injected at 4 sites in the thigh muscle and 4 sites in the calf muscle of the ischemic limb. A total of 6 x 10⁸ or 2 x 10⁸ plaque-forming units (pfu) were injected into the left thigh muscle of 2- and 8-month-old mice, respectively. For non-ischemic mice, percutaneous intramuscular adenoviral injection was performed at 8 sites in the left leg.
References


Table S1. Sequences of oligonucleotide primers

1. Sequences of primers for qrt-PCR analysis of HIF-1α and angiogenic cytokines:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Fwd Sequence</th>
<th>Rev Sequence</th>
</tr>
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<tbody>
<tr>
<td>RPL13A</td>
<td>GAGGCCCTACCATTTCCGA</td>
<td>GGCTTCAGCCGAACACCTT</td>
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<tr>
<td>HIF-1α</td>
<td>CCACAGGACAGTACAGGATG</td>
<td>TCAAGTCTGAATAATACC</td>
</tr>
<tr>
<td>PLGF</td>
<td>GGATGTGCTCTGTGAATGC</td>
<td>CCTCTGAGTGGCTGGTTAC</td>
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<td>VEGF</td>
<td>GGCTGCTGTAACGATGAAG</td>
<td>CTCTCTATGTGCTGGCTTTG</td>
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<td>SDF1</td>
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<td>TTCGGGTCAATGCACACTTG</td>
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<td>ANGPT1</td>
<td>CTACCAACAACAAACAGATCC</td>
<td>CTCCCTTTAGCAAAACACCTTC</td>
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<td>SCF</td>
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<td>AGCCAATTACAAGGAAATGAG</td>
</tr>
<tr>
<td>18S</td>
<td>GAATCGAACCCTGATTCCCCGTC</td>
<td>CGGCGACGACCCATTCGAAC</td>
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</table>

2. Sequences of primers for Kitlg gene ChIP assay:

Fwd: GGGGTACCGGTCAACCTCTCTTCCAGGATC 
Rev: GAAGATCTCTTGTTTCAGGAAGATC
3. Sequences of primers for qrt-PCR analysis of angiogenic cytokine receptors:

**TIE-2**
Fwd  5’ GTG TAG TGG ACC AGA AGG 3’
Rev  5’ CTT GAG AGC AGA GGC ATC 3’

**CKIT**
Fwd  5’ ATC CCT GTT GTG TCT GTG 3’
Rev  5’ CTC CTG GCG TTC ATA ATT G 3’

**CXCR4**
Fwd  5’ AGC ATG AC G GAC AAG TAC C 3’
Rev  5’ GAT GAT ATG GAC AGC CTT ACA C 3’

**VEGFR1**
Fwd  5’ ACA TTG GTG GTG GCT GAC TCT C 3’
Rev  5’ CCT CTC CTT CGG CTG GCA TC 3’

**VEGFR2**
Fwd  5’ GCG GGC TCC TGA CTA CAC 3’
Rev  5’ CCA AAT GCT CCA CCA ACT CTG 3’