Expression of Vascular Endothelial Growth Factor Receptor 1 in Bone Marrow-derived Mesenchymal Cells Is Dependent on Hypoxia-inducible Factor 1

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Bone marrow-derived cells are recruited to sites of ischemia, where they promote tissue vascularization. This response is dependent upon the expression of vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1), which mediates cell migration in response to VEGF or placental growth factor (PLGF). In this study, we found that exposure of cultured mouse bone marrow-derived mesenchymal stromal cells (MSC) to hypoxia or an adenovirus encoding a constitutively active form of hypoxia-inducible factor 1 (HIF-1) induced VEGFR1 mRNA and protein expression and promoted extracellular migration in response to VEGF or PLGF. MSC in which HIF-1 activity was inhibited by a dominant negative or RNA interference approach expressed markedly reduced levels of VEGFR1 and failed to migrate or activate AKT in response to VEGF or PLGF. Thus, loss-of-function and gain-of-function approaches demonstrated that HIF-1 activity is necessary and sufficient for basal and hypoxia-induced VEGFR1 expression in bone marrow-derived MSC.

Bone marrow cells are mobilized into peripheral blood and recruited to sites of ischemia, where they participate in tissue repair and revascularization (1–6). Among the bone marrow cells that have been shown to participate in these responses are endothelial progenitor cells and mesenchymal stromal cells (MSC) (7–14). In mice, mobilization and recruitment of bone marrow cells to sites of ischemia is dependent upon vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) activity (15). VEGFR1, which is encoded by the Flt1 gene in mice, binds VEGF and placental growth factor (PLGF), is expressed by multiple bone marrow-derived cell types, and plays a critical role in cell migration. In contrast, VEGFR2, which is encoded by the Flik gene in mice, binds VEGF but not PLGF, is expressed specifically by endothelial cells and their progenitors, and is required for their survival, proliferation, and differentiation (16, 17). In human umbilical vein endothelial cells (HUVEC), transcription directed by the VEGF1 gene promoter is induced by hypoxia, an effect that is dependent upon a cis-acting hypoxia response element, which contains a putative binding site for hypoxia-inducible factor 1 (HIF-1) (18).

HIF-1 is a transcription factor that consists of an O2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (19–21), which heterodimerize and bind to hypoxia response elements that contain the core nucleotide sequence 5′-RCGTG-3′ (22). HIF-1α is subject to O2-dependent hydroxylation events that block its interaction with coactivators and promote its ubiquitination and degradation (23–28). Under hypoxic conditions, hydroxylation is inhibited because of substrate (O2) deprivation, and HIF-1α accumulates, dimerizes with HIF-1β, and mediates profound changes in gene expression (29). Within each cell type, a specific battery of genes is regulated by HIF-1 (29, 30). The purpose of the present study was to investigate whether HIF-1 regulates VEGFR1 expression in bone marrow-derived cells. Using gain-of-function and loss-of-function approaches, we demonstrate that HIF-1 plays an essential role in the regulation of both basal and hypoxia-induced expression of VEGFR1 in bone marrow-derived MSC.

EXPERIMENTAL PROCEDURES

Reagents—Anti-HIF-1α monoclonal antibody (mAb) H1α67 (31), anti-HIF-1β mAb H1β234 (32), and anti-HIF-2α Ab were from Novus Biologicals (Littleton, CO). Anti-HIF-1α mAb clone 54 was from BD Biosciences (San Diego, CA). Anti-VEGFR1 and VEGFR2 antibodies were from Santa Cruz (Santa Cruz, CA). Phycocerythrin- and fluorescein isothiocyanate-conjugated antibodies were from BD Biosciences. Recombinant human fibroblast growth factor 2 (FGF2), VEGF, and PLGF were from R & D Systems (Minneapolis, MN).

MSC Isolation and Cell Culture—Bone marrow was collected from three 2-month-old C57BL/6 mice (Charles River, Wilmington, MA) by flushing femurs and tibias with phosphate-buffered saline (Invitrogen) with 2 mM EDTA (PBS/EDTA). The cells were washed with PBS/EDTA and plated in a 75-cm2 flask with DMEM (BioWhittaker, Walkersville, MD) and 10% FBS (Gemini Bio-Products, Woodland, CA). After 3 days, nonadherent cells were removed by medium change, and adherent cells were further cultured in DMEM, 10% FBS for an additional 4 days. On day 7, the adherent cells were passaged into a 6-cm dish. The medium was changed two to three times a week, and cell density was maintained at 2–15 × 10^3 cells/cm^2.

Wild-type (WT) and HIF-1α-null (knock-out) mouse embryonic stem cells (ESC) and mouse embryonic fibroblasts (MEF) were maintained in DMEM with 15% FBS, 1% penicillin-streptomycin, nonessen-

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tial amino acids, glutamine, and sodium pyruvate (33, 34). HUVEC (National Cancer Institute, Frederick, MD) were maintained in endothelial basal medium 2 supplemented with endothelial growth medium 2 (Cambrex, Walkersville, MD) and used at passages 3–6. For hypoxic exposure, tissue culture plates were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and flushed at 2 p.s.i. for 3 min with a gas mixture consisting of 1% O₂, 5% CO₂, and balance N₂.

Adenovirus Preparation and Infection—Replication-defective recombinant adenoviruses were constructed as described (29, 30). AdCA5 contains GLUT1, HIF-1β was performed as previously described (29). Nucleotide PCR (qRT-PCR) Analysis

24 h prior to all studies. Detection System (Bio-Rad). For each set of primers, gradient PCR was performed using iQ SYBR Green Supermix and iCycler Real time PCR—Total RNA extraction and cDNA synthesis was based on the threshold cycle (Cₜ) as \( r = 2^{-\Delta(C_{T\text{target}} - C_{T\text{18S}})} \) where \( \Delta C_{T} = C_{T\text{target}} - C_{T\text{18S}} \) and \( \Delta(C_{T\text{target}} - C_{T\text{18S}}} = C_{T\text{experimental}} - C_{T\text{control}} \).

Immunoblot Assays—Whole cell extracts were prepared in radioimmunoprecipitation buffer, fractionated by SDS-PAGE, transferred to a nitrocellulose filter, and subjected to immunoblot assays. 100-μg aliquots of protein were analyzed using primary Ab at 1:1000 dilution. Horseradish peroxidase-conjugated secondary Ab against mouse or rabbit IgG was used at 1:2500 dilution, and the signal was visualized using ECL Plus reagents (Amer sham Biosciences).

Flow Cytometry—MSC were detached with PBS/EDTA, treated with Mouse Fc Block™ for 5 min, incubated with fluorescence-conjugated Ab for 20 min, washed with PBS, and resuspended in PBS for analysis using an LSRII fluorescence activated cell sorter (BD Bioscience). As a control, aliquots of cells were incubated with phycoerythrin- or fluorescein isothiocyanate-conjugated rat IgG₂ Ab.

Immunocytochemistry—The cells were cultured in an 8-well chamber slide, fixed with 4% paraformaldehyde for 30 min at 4 °C, washed with PBS, blocked, and permeabilized with 1% bovine serum albumin, 0.1% Triton X-100, 10% goat serum, PBS for 30 min, incubated with primary Ab (1:300 dilution in PBS, 1% bovine serum albumin), and then incubated with fluorescent isothiocyanate-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (BD Bioscience) for 1 h. After washing, the slides were counterstained with 300 nM DAPI solution (Molecular Probes, Eugene, OR) and analyzed by fluorescence microscopy (BX51, Olympus, Melville, NY).

Migration Assay—MSC were cultured under nonhypoxic (95% air, 5% CO₂) or hypoxic (1% O₂, 5% CO₂/balance N₂) conditions for 24 h, and then 2 × 10⁵ cells were plated per well in the upper chamber of a transwell containing a polycarbonate filter (12-μm pore size; Costar, Cambridge, MA). The lower chamber contained DMEM (Invitrogen) with or without VEGF or PLGF. After a 12-h incubation at 37 °C in 95% air/5% CO₂, the cells in the lower chamber were counted.

Statistics—The data are presented as the means and standard deviations. The differences between subclones or treatment conditions were analyzed by Student’s t test, and p < 0.05 was considered significant.

RESULTS

Characterization of MSC Derived from Bone Marrow of C57BL/6 Mice—Mononuclear cells were isolated from bone marrow of C57BL/6 mice and cultured on uncoated dishes in DMEM with 10% FBS to isolate MSC. By passage 6, a homogenous population of rapidly dividing cells with fibroblastoid morphology was obtained (data not shown). Analysis by flow cytometry at passage 8 revealed that these cells expressed Sca-1, CD44, and CD105 (Fig. 1A), as previously described for human MSC (35, 36). The mouse MSC did not express the myeloid/hematopoietic markers CD11b, CD34, CD45 (Fig. 1A), or c-Kit (data not shown).

Immunocytochemistry revealed the expression of VEGFR1 in MSC (Fig. 1B and C), as observed in HUVEC (Fig. 1D and E). HUVEC also expressed high levels of VEGFR2 (Fig. 1, F and K), whereas murine MSC did not express VEGFR2 (Fig. 1, H and J), nor did they express the endothelial cell marker CD31 (data not shown).

Effect of Hypoxia or Adenoviral Expression of a Constitutively Active Form of HIF-1α—Injection of nonhypoxic cells with adenovirus AdCA5, which encodes a constitutively active form of HIF-1α, has been shown to result in cell type-specific changes in gene expression that are remarkably similar to those that occur in response to hypoxia (29, 30). When MSC were infected with AdCA5, which also encodes GFP, greater than 95% of the cells were GFP-positive as determined by fluorescence microscopy 24 h after infection (Fig. 2A).
AdCA5-infected MSC constitutively expressed high levels of HIF-1α mRNA as detected by RT-PCR (Fig. 2B). In contrast, cells infected with the control adenovirus AdLacZ expressed levels of HIF-1α mRNA that were comparable with uninfected MSC. qRT-PCR analysis revealed that expressed low levels of HIF-1α mRNA as detected by RT-PCR (Fig. 2C). AdCA5-infected MSC as compared with AdLacZ-infected MSC (data not shown).

Immunoblot assays using mAb H1α67, which was raised against amino acid residues 432–528 of human HIF-1α (31), detected high levels of endogenous HIF-1α protein migrating with an apparent molecular mass of 120 kDa, as previously described (19), in extracts from hypoxic MSC and HeLa cells, whereas nonhypoxic MSC or HeLa cells expressed low levels of HIF-1α (Fig. 2C, top panel). AdCA5 encodes an altered form of human HIF-1α, which includes a deletion (amino acid residues 392–520) and missense mutations (P567T and P658Q) (30), that is not recognized by mAb H1α67. mAb clone 54 was raised against amino acid residues 610–727 of human HIF-1α and recognizes both human HIF-1α and CA5 but does not recognize mouse HIF-1α (Fig. 2C, middle panel). These results demonstrate that AdCA5 infection of MSC results in high levels of CA5 in MSC under nonhypoxic conditions. 91- and 94-kDa proteins corresponding to the 774- and 789-amino acid isoforms of HIF-1β were detected by immunoblot assay using mAb H1β234 (Fig. 2C, bottom panel). HIF-1β expression levels were not affected by hypoxia or adenoviral infection and provide evidence that similar amounts of total protein were analyzed in each lane.

The expression of VEGF, VEGFR1, and VEGFR2 was examined in AdCA5-infected MSC. VEGF is a well-established HIF-1-regulated gene (37) that is transactivated in response to AdCA5 infection in other cell types (29, 30, 38). RT-PCR analysis revealed that both VEGF and VEGFR1 mRNA levels in MSC were increased in response to hypoxia or AdCA5 infection, whereas VEGFR2 mRNA was not detected under any of the treatment conditions (Fig. 2D, left panels). qRT-PCR analysis revealed that compared with uninfected, nonhypoxic MSC, levels of VEGF mRNA increased 6.1 ± 0.2-fold in response to hypoxia, were unchanged after AdLacZ infection, and increased 8.0 ± 0.2-fold after AdCA5 infection (Fig. 2D, right upper panel). VEGF1 mRNA levels increased 2.4 ± 0.1-fold in response to hypoxia, 3.7 ± 0.3-fold in response to AdLacZ infection, and 7.8 ± 0.3-fold in response to AdCA5 infection (Fig. 2D, right lower panel). Thus, compared with their appropriate controls, hypoxia and AdCA5 infection increased VEGF and VEGFR1 mRNA levels in MSC to a similar degree.

Immunoblot assays revealed that MSC expressed VEGFR1 but not VEGFR2 protein (Fig. 2E, left panels), results that were consistent with mRNA analyses (Fig. 2D) and immunocytochemistry (Fig. 1). By densitometric analysis, levels of VEGFR1 protein increased 1.8-fold in response to hypoxia, 1.3-fold in response to AdLacZ infection, and 3.0-fold in response to AdCA5 infection, relative to the levels of VEGFR1 protein in uninfected, nonhypoxic MSC (Fig. 2E, right panel). Thus, VEGFR1 mRNA and protein levels in MSC increased in response to hypoxia or expression of a constitutively active form of HIF-1α under nonhypoxic conditions.

Inhibition of HIF-1 in MSC by Expression of a Dominant Negative Form of HIF-1α—MSC were infected with a retrovirus expressing a dominant negative form of HIF-1α (rVQC-DN), and a stably transduced subclone was selected. As a control, a subclone of MSC stably transduced with a retrovirus expressing GFP (rVQC-GFP) was also selected. After selection, 100% of MSC were stably transduced with rVQC-GFP and expressed GFP as determined by fluorescence microscopy (Fig. 3A).

Immunoblot assays revealed that HIF-1α protein expression was induced in hypoxic MSC transduced with rVQC-GFP, whereas levels of HIF-1α protein were reduced under nonhypoxic and hypoxic conditions in MSC transduced with rVQC-DN (Fig. 3B). HIF-1αDN competes with endogenous HIF-1α for dimerization with HIF-1β. In contrast to HIF-1α–HIF-1β heterodimers, HIF-1αDN–HIF-1β heterodimers cannot bind to DNA or activate transcription (21). HIF-1α protein that fails to form heterodimers is degraded at an increased rate, thus accounting for the modestly reduced levels of HIF-1α in hypoxic cells expressing HIF-1αDN (39). HIF-1αDN contains two deletions (amino acid residues 4–27
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The expression of VEGF, GLUT1, and VEGFR1 mRNA was examined in MSC transduced with rvQC-DN or rvQC-GFP. As in the case of the VEGF gene, GLUT1 is a well-established HIF-1 target gene (33, 40). qRT-PCR analysis revealed that VEGF, GLUT1 and VEGFR1 mRNA levels were all increased in response to hypoxia in nontransduced MSC (data not shown) and MSC transduced with rvQC-GFP, but not in MSC transduced with rvQC-DN (Fig. 3C). Compared with cells transduced with rvQC-GFP, levels of VEGFR1 mRNA expression in MSC transduced with rvQC-DN were decreased 5.0-fold under nonhypoxic conditions (Fig. 3C) and indicate that reduced HIF-1 activity results in reduced VEGFR1 mRNA and protein levels.

Effect of Inhibiting HIF-1α Expression by RNA Interference — A second approach to induce HIF-1 loss-of-function was also utilized. MSC were transduced with a retrovirus encoding an shRNA that targeted HIF-1α mRNA for degradation (rvShRNAHIF-1α) or a retrovirus encoding a scrambled negative control shRNA (rvShRNASHC), and subclones of stably transduced MSC were selected. HIF-1α mRNA levels in MSC transduced with rvShRNAHIF-1α were decreased by 60% relative to the levels of HIF-1α mRNA in MSC transduced with rvShRNASHC (Fig. 4A). Immunoblot assays revealed reduced HIF-1α protein levels in hypoxic MSC transduced with rvShRNAHIF-1α compared with MSC transduced with rvShRNASHC under both nonhypoxic and hypoxic conditions (Fig. 4B).

VEGFR1 mRNA levels were decreased under both nonhypoxic and hypoxic conditions in MSC transduced with rvShRNAHIF-1α compared with MSC transduced with rvShRNASHC (Fig. 4C). VEGFR1 protein expression was also strikingly reduced in both hypoxic and nonhypoxic MSC transduced with rvShRNAHIF-1α compared with MSC transduced with rvShRNASHC (Fig. 4D). Thus, both gain-of-function studies (Fig. 2) and loss-of-function studies using two independent experimental approaches (Figs. 3 and 4) demonstrate that HIF-1 is an essential posi-
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HIF-1 Activity Is Required for VEGF- or PLGF-stimulated Migration of MSC—VEGFR1 plays a critical role in ischemia-induced angiogenesis (15) and in the recruitment of bone marrow-derived progenitor cells to sites of ischemia in response to production of VEGF and PLGF, both of which bind to VEGFR1 (41). VEGF-induced migration of monocytes is also mediated via VEGFR1 (42, 43). VEGF and PLGF were recently shown to stimulate the chemotactic migration of human MSC (44). The role of HIF-1 in promoting cytokine-induced migration of MSC was tested in an ex vivo transwell migration assay. VEGF-induced migration of nonhypoxic MSC dose-dependently (Fig. 5A, subgroup N). Hypoxic pretreatment potentiated VEGF-induced migration (Fig. 5A, subgroup H).

The effect of infecting cells with AdLacZ or AdCA5 was analyzed next. The numbers of migrating AdLacZ-infected MSC in the presence of 10 and 100 ng/ml VEGF were not significantly different from those obtained using uninfected, nonhypoxic MSC (Fig. 5A, AdLacZ). In contrast, infection of MSC with AdCA5 potentiated VEGF-induced migration (Fig. 5A, AdCA5). Thus, AdCA5-infected cells showed an augmented migration response to VEGF under nonhypoxic conditions that was comparable with the effect of hypoxia on uninfected cells.

The effect of HIF-1 loss-of-function on VEGF-induced cell migration was analyzed in MSC transduced with rvQC-GFP or rvQC-DN. Whereas VEGF dose-dependently induced migration of MSC transduced with rvQC-GFP, MSC transduced with rvQC-DN showed no stimulation of migration in response to 10 and 100 ng/ml VEGF (Fig. 5B). In addition, hypoxic pretreatment did not promote VEGF-stimulated migration in MSC transduced with rvQC-DN.

The migration of MSC subclones transduced with rvShRNAHIF-1α or rvShRNA_{SNC} was also analyzed. In MSC stably transduced with rvShRNA_{SNC}, the number of migrating MSC was increased by VEGF in a dose-dependent manner (Fig. 5C). Hypoxic pretreatment potentiated VEGF-induced migration of MSC transduced with rvShRNA_{SNC}. In contrast, in MSC transduced with rvShRNAHIF-1α, the number of migrating MSC was not increased in the presence of 10 or 100 ng/ml VEGF (Fig. 5C). Even hypoxic pretreatment could not promote significant VEGF-stimulated migration of MSC transduced with rvShRNA-
Thus, loss-of-function (Fig. 5, B and C) and gain-of-function (Fig. 5A) approaches demonstrate that HIF-1 activity is necessary and sufficient to promote migration of MSC in response to VEGF.

PLGF also dose-dependently induced migration of MSC transduced with rvShRNA_{SNC} and this effect was potentiated by hypoxic pretreatment (Fig. 5D). In contrast, the migration of MSC transduced with rvShRNA_{HIF-1α} was not stimulated by 10 or 100 ng/ml PLGF (Fig. 5D). Even hypoxic pretreatment could not promote PLGF-stimulated migration of these cells.

Bromodeoxyuridine incorporation assays revealed that treatment of MSC with VEGF or PLGF did not stimulate cell proliferation (data not shown), which was an expected result because proliferative signals are transduced only by VEGF binding to VEGFR2, which the MSC do not express. Thus, inhibition of HIF-1α expression specifically blocks the migration of MSC in response to VEGF or PLGF, the ligands for VEGFR1.

HIF-1 Activity Is Required for VEGF- or PLGF-stimulated Akt Phosphorylation—To analyze signal transduction downstream of VEGFR1, phosphorylation of the serine-threonine kinase Akt was analyzed.
VEGF induced significantly increased Akt phosphorylation in MSC transduced with rvQC-GFP, whereas in MSC transduced with rvQC-DN, there was no significant increase in Akt phosphorylation in response to VEGF (Fig. 6A). VEGF treatment also significantly increased Akt phosphorylation in MSC transduced with rvShRNASNC but not in MSC transduced with rvShRNAHIF-1α/H9251 (Fig. 6B). PLGF treatment significantly increased Akt phosphorylation in MSC transduced with rvShRNASNC but not in MSC transduced with rvShRNAHIF-1α (Fig. 6C). To demonstrate that the effects of HIF-1 loss-of-function were specific to Akt phosphorylation mediated by VEGFR1 signaling, MSC were stimulated with FGF2. HIF-1α RNA interference had no significant effect on Akt phosphorylation induced by FGF2 (Fig. 6D). Thus, HIF-1 activity is specifically required for signal transduction from VEGFR1, but not from FGF receptors, to Akt in MSC.

Cell Type-specific Regulation of VEGFR1 Expression by HIF-1—To investigate whether VEGFR1 expression is induced by hypoxia in a HIF-1-dependent manner in other cell types, VEGFR1 mRNA levels were determined in MEF and ESC that were either WT or homozygous for a null allele at the locus encoding HIF-1α (33, 34). qRT-PCR analysis...
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revealed that, compared with nonhypoxic cells, VEGFR1 mRNA levels were increased 2.0 ± 0.1- and 4.5 ± 0.2-fold in hypoxic WT MEF and ESC, respectively (Fig. 7). However, hypoxia did not significantly increase VEGFR1 mRNA levels in HIF-1α-null MEF or HIF-1α-null ESC. These results indicate that HIF-1 activity is required for hypoxia-induced VEGFR1 gene expression in MEF and ESC.

When HUVEC were infected with AdCA5, which also encodes GFP, greater than 95% of the cells were GFP-positive as determined by fluorescence microscopy 24 h after infection (Fig. 8A). In HUVEC, VEGF mRNA expression was induced 15.6 ± 0.2- and 8.0 ± 0.1-fold by hypoxia or AdCA5 infection, respectively (Fig. 8B). GLUT1 mRNA expression was also induced in HUVEC exposed to hypoxia or AdCA5 (Fig. 8C). The expression of VEGFR1 mRNA was induced 3.6 ± 0.1-fold by hypoxia but was not induced by AdCA5 infection in HUVEC (Fig. 8D). Immunoblot assays revealed that VEGFR1 protein expression was increased in hypoxic as compared with nonhypoxic HUVEC but was not increased in AdCA5-infected as compared with AdLacZ-infected HUVEC (Fig. 8E). Taken together, the data in Fig. 8 (A–E) indicate that AdCA5 infection of HUVEC is efficient and induces the expression of the HIF-1 target genes VEGF and GLUT1 but not VEGFR1.

HIF-2α can also dimerize with HIF-1β and regulate the expression of an overlapping group of HIF-1 target genes (45–48) in some cell types (49, 50). HIF-1α mRNA was detected in MSC and HUVEC by RT-PCR (Fig. 8F). HIF-2α mRNA in MSC was barely detectable at passage 3 and undetectable at passage 10, whereas HUVEC strongly expressed HIF-2α mRNA. Immunoblot assays revealed that compared with MSC, lower levels of HIF-1α were induced by hypoxia in HUVEC (Fig. 8G). The 91- and 94-kDa isoforms of HIF-1α were detected in HUVEC, whereas predominantly the 91-kDa isoform was expressed in MSC. HIF-2α protein was not detected in lysates of nonhypoxic or hypoxic MSC, whereas HUVEC expressed detectable levels of HIF-2α protein, which increased in response to hypoxia (Fig. 8H). Because the antibody used in the immunoblot assay was raised against human HIF-2α protein, it was possible that the failure to detect HIF-2α protein in murine MSC was due to reduced cross-species reactivity of the antibody. However, in the same immunoblot assay, hypoxia-inducible expression of HIF-2α protein was demonstrated in mouse peripheral blood mononuclear cells (Fig. 8H, mPBMNC).

DISCUSSION

HIF-1 Is Required for VEGFR1 Expression in Bone Marrow-derived MSC—Given the important role of VEGFR1 in the recruitment of bone marrow-derived cells to sites of ischemia, we investigated whether HIF-1 played a role in the transcriptional regulation of VEGFR1 expression in mouse MSC. In a previous study, the expression of VEGFR1 mRNA in HUVEC and the activity of the Flt1 gene promoter were induced ~2-fold in response to hypoxia, and this response was localized to a 40-bp fragment located 1 kb 5′ to the transcription start site containing the core HIF-1-binding site sequence 5′-ACGTG-3′, mutation of which abrogated its ability to function as a hypoxia response element (18). Although this study implicated HIF-1 in the control of VEGFR1 transcription in HUVEC, the recognition that HIF-1 regulates many genes in a cell type-restricted manner (30) necessitated the direct testing of this hypothesis. Indeed, our study suggested that in HUVEC, HIF-1 (defined here as the HIF-1α-HIF-1β heterodimer) does not regulate VEGFR1 expression. Our results suggest that VEGFR1 expression in HUVEC is mediated by HIF-2α-HIF-1β heterodimers. The same conclusion was obtained in another study (51) by investigators who demonstrated HIF-2α-mediated VEGFR1 expression in HUVEC using gain-of-function and loss-of-function strategies that were similar to the rigorous approach we have taken to demonstrate HIF-1α-mediated VEGFR1 expression in MSC. In mouse endothelial progenitor cells, chromatin immunoprecipitation studies have demonstrated the binding of HIF-1α-HIF-1β heterodimers to the Flt1 promoter (52). The demonstration of cell type-specific effects of HIF-1α and HIF-2α is important because of the interest in overexpressing each of these factors as a means of stimulating tissue vascularization in patients with ischemic cardiovascular disease. These data suggest that the consequences of overexpressing HIF-1α or HIF-2α protein will depend upon the target cell population.

In the present study, both gain-of-function and loss-of-function studies definitively established that HIF-1 is an essential regulator of VEGFR1 mRNA and protein expression in bone marrow-derived MSC. Surprisingly, even the basal expression of VEGFR1 was dependent upon HIF-1 activity, indicating that the HIF-1α protein levels detected in nonhypoxic MSC (Figs. 3B and 4B) were sufficient to drive VEGFR1 expression. Functional expression of HIF-1α under nonhypoxic culture conditions has been reported for several cell types, including ESC (33), macrophages (53, 54), and pulmonary artery smooth muscle cells (55). Inhibition of HIF-1 activity by either dominant negative or RNA interference approaches markedly reduced VEGFR1 mRNA and protein levels and eliminated VEGFR1 signal transduction, as measured by cell migration or Akt activation, in response to VEGF or PLGF stimulation. Whereas hypoxia potentiated MSC migration in response to VEGF or PLGF, this effect was lost when HIF-1 activity was inhibited. The ability of hypoxia to stimulate the angiogenic properties of bone marrow-derived cells (56, 57) may be due in part to the HIF-1-mediated induction of VEGFR1 expression that we have demonstrated. However, in vivo studies are required to investigate this hypothesis, especially given the heterogeneous nature of bone marrow cells, which is in contrast to the homogeneous population of MSC that were the focus of this study.
Expanding Role of HIF-1 in the Control of Tissue Vascularization—

Three principal mechanisms have been identified by which tissue vascularization can be modulated (reviewed in Ref. 58): angiogenesis is the process in which new blood vessels branch off of existing vasculature; arteriogenesis is the process in which pre-existing collateral blood vessels are remodeled to permit increased blood flow following occlusion of a large conduit vessel; and vasculogenesis is the process by which bone marrow-derived cells are recruited to participate in the de novo formation of new blood vessels. Accumulating data indicate that HIF-1 plays multiple important roles in all three of these processes.

Initial studies demonstrated the critical role of HIF-1 in the hypoxia-induced expression of VEGF and other angiogenic cytokines during angiogenesis (30, 33, 37, 59, 60). More recently, AdCA5 injection into skeletal muscle was shown to promote arteriogenesis in a model of critical limb ischemia, an effect that was shown to involve increased production of angiogenic cytokines (38). In addition, a polymorphism in the human HIF1A gene has been associated with absence of coronary collaterals in patients with ischemic coronary artery disease (61).

In addition to its role in promoting the expression of cytokines that signal to vascular endothelial cells and/or smooth muscle/pericytes, HIF-1 has also been shown to mediate cell autonomous effects in endothelial cells subjected to hypoxia (29, 62, 63). In the present study, we have implicated HIF-1 in the process of vasculogenesis through its control of VEGFR1 expression. Increased HIF-1 activity may promote recruitment of MSC by increasing expression of VEGFR1 and may also stimulate angiogenesis in ischemic tissue by increasing expression of VEGF. Production of arteriogenic cytokines has been proposed as a mechanism by which MSC may stimulate the remodeling of collateral vessels (11, 12). HIF-1-dependent autocrine VEGF signaling appears to play a critical functional role in endothelial cells (62). In ESC, HIF-1-dependent autocrine VEGF signaling through VEGFR1 promotes cell survival under hypoxic conditions (64). These studies underscore the multiple roles played by HIF-1 in mediating responses to ischemic cardiovascular disease. Further studies are needed to determine whether HIF-1 gain-of-function may improve the ability of bone marrow-derived cells to modulate tissue vascularization.

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