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Dissection of the Biphasic Nature of Hypoxia-Induced Motogenic Action in Bone Marrow-Derived Human Mesenchymal Stem Cells

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Abstract

Hypoxic conditions have been reported to facilitate preservation of undifferentiated mesenchymal stem cell (MSC) phenotype and positively affect their colony-forming potential, proliferation, and migration/mobilization. In this study, designed to dissect mechanisms underlying hypoxia-dependent migration of bone marrow-derived human MSC (hMSC), signal transduction, and molecular mechanisms were evaluated by integrating morphological, molecular, and cell biology techniques, including the wound healing assay (WHA) and modified Boyden's chamber assay (BCA) to monitor migration. Exposure of hMSCs to moderate hypoxia resulted in a significant increase of migration of hMSCs in both WHA (from 6 to 20 hours) and BCA (within 6 hours). Mechanistic experiments outlined the following sequence of hypoxia-dependent events: (a) very early (15 minutes) increased generation of intracellular reactive oxygen species (ROS), which (b) was sufficient to switch on activation of extracellular regulated kinase 1/2 and c-Jun N-terminal protein kinase 1/2, found to be relevant for the early phase of hMSC migration; (c) hypoxia inducible factor-1 (HIF-1)–dependent increased expression of vascular endothelial growth factor (VEGF) (facilitated by ROS) and its progressive release that was responsible for (d) a delayed and sustained migration of hMSCs. These results suggest that hypoxia-dependent migration relies on a previously unrecognized biphasic scenario involving an early phase, requiring generation of ROS, and a delayed phase sustained by HIF-1-dependent expression and release of VEGF. STEM CELLS 2011;29:952–963

INTRODUCTION

Mesenchymal stem cells (MSCs) represent a population of pluripotent cells that can be isolated, purified, and ex vivo expanded by bone marrow samples and other adult tissues [1, 2]. MSCs are stem cells with enormous plasticity [2] being able, when transplanted, to engraft different organ and tissues, including bone [3, 4], cartilage [3, 5], adipose tissue [6], and muscle [7], as well as to differentiate into corresponding specific cell types. Moreover, "in vivo" transplantation of bone marrow-derived MSCs has been reported to represent an effective treatment to repopulate selected tissues after injury, including myocardial infarction [8] and hind-limb ischemia [9, 10]. The mechanisms able to guide the homing of MSCs to the injured tissue have been extensively investigated in the last few years [11–20]. The emerging scenario indicates that MSC migration is stimulated by several polypeptides, including hepatocyte growth factor (HGF) and stromal-derived growth factor-1, through the involvement of their cognate receptors c-met and chemokine (C-X-C motif) receptor 4, respectively [12]. MSCs also migrate following exposure to inflammatory cytokines and engagement of Toll-like receptors [16] as well as in response to a selected number of chemokines [11–14, 16–18] and even erythropoietin [15, 19]. Migration of human MSCs (hMSCs) is favored by expression of matrix metalloproteinases (MMPs), particularly MMP-2 and membrane type 1 MMP [12, 14, 17, 18]. More recently, we have shown that bone marrow-derived hMSCs can migrate in response to additional chemoattractants, including platelet-derived growth factor-BB, vascular endothelial growth factor-A (VEGF-A), angiotensin II, monocyte chemoattractant protein-1, and basic fibroblast growth factor [21, 22]. In particular, we found that all these chemoattractant polypeptides, following interaction with their receptors and activation/recruitment of plasma membrane NADPH-oxidase, required intracellular generation of reactive oxygen species (ROS) and redox-dependent activation of extracellular regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) 1/2 (JNK1/2) isoforms, as critical events for the induction of directional migration of MSCs [22].

In addition to specific polypeptide growth factors and related signaling pathways, there is an increasing evidence indicating that mild hypoxia can act as a potent regulator of various types of stem cells with effects that may differ depending on the specific stem cell type [23, 24]. Along these lines, recent studies have identified hypoxia as a relevant condition able to affect migration of MSCs. Some years ago an "in vivo" experimental study has provided convincing evidence that exposure of rats to chronic hypoxia can result in a dramatic increase in the pool of circulating MSCs in the peripheral blood, suggesting that hypoxia may represent a condition able to selectively mobilize MSCs from bone marrow without affecting significantly the total pool of circulating hematopoietic progenitor cells [25]. More recently, it has been shown that hypoxic preconditioning of cultured MSCs resulted in their increased ability to migrate and enhanced expression of c-met [26]. Moreover, when MSCs submitted to hypoxic preconditioning were transplanted in a mice model of hind-limb ischemia (i.e., a model in which HGF levels were found to be increased in the ischemic muscle), these cells were able to restore more rapid blood flow than in mice transplanted with normoxic MSCs, suggesting that hypoxic preconditioning may improve tissue regenerative potential of MSCs [26]. In a more recent study, evidence was provided confirming that hypoxia per se is a condition able to elicit migration of human umbilical cord blood MSCs and suggesting a mechanism involving VEGF-mediated focal adhesion kinase (FAK) phosphorylation, intracellular generation of ROS, mitogen-activated protein kinases (MAPKs), endothelial nitric oxide synthetase, and hypoxia inducible factor-1α (HIF-1α) pathways [27].

In this study, we have further analyzed the mechanisms involved in hypoxia-induced migration of hMSCs obtained from bone marrow. By using mechanistic approaches, we have dissected and characterized the previously unrecognized biphasic nature of hypoxia-induced motogenic action. Hypoxia-induced migration is switched on by ROS released within minutes by mitochondria that

sustain the activation of Ras/ERK and JNK signaling pathways. This rapid redox-dependent signaling, which is blocked by preventing mitochondrial ROS release and can elicit an early migration in a HIF-1α- and VEGF-independent way, is later on progressively reinforced by the paracrine action of VEGF, which is released in the extracellular medium by MSCs due to HIF-1α-dependent upregulation of VEGF expression.

MATERIALS AND METHODS

Materials

Human recombinant growth factors and cytokines were from PeproTech Inc. (Rocky Hill, NJ, www.peprotech.com). Monoclonal and polyclonal antibodies against phosphorylated and unphosphorylated ERK1/2 or against HIF-1α were from Santa Cruz Biotechnology (Santa Cruz, CA, www.scbt.com), whereas those against phosphorylated and unphosphorylated or JNK1/2 were from Cell Signaling Technology (Beverly, MA, www.cellsignal.com). Monoclonal antibody against β-actin was from Sigma Aldrich SPA (Milan, Italy, www.sigmaaldrich.com/italy.html). SP600125 and PD98095 were from Calbiochem (La Jolla, CA, www.bioscience.org/company/calbio.htm). The enhanced chemiluminescence reagents and nitrocellulose membranes (Hybond-C extra) were from Amersham Pharmacia Biotech (Milan, Italy, www.apbiotech.com). All other reagents were from Sigma Aldrich.

Isolation and Culture of hMSCs

Bone marrow cells were obtained from human donors after informed consent. Aliquots of 2–3 ml of whole bone marrow were seeded in MSC-medium modified Eagle medium (Lonza, Versviers, Belgium, www.lonza.com) at 10% of fetal bovine serum and cultured for 5 days. Adherent cells, when at confluence, were detached by Trypsin/EDTA, seeded at 1000 cm^{-2}, expanded, and used for "in vitro" experiments from passage 3 to passage 7, when displaying a fibroblast-like and α-smooth muscle actin (α-SMA) positive phenotype [21]. Immunophenotypic analysis of hMSCs and their differentiative potential have been described elsewhere [21, 28]. In particular, MSCs used in this study were always more than 90% positive (Fig. 1) for typical antigens like CD90, CD73, CD105, and CD29 but negative for CD34, CD45, and CD14, as showed by cytofluorimetric analysis that was performed as detailed previously [21].

Characterization of bone marrow-derived human mesenchymal stem cells (hMSCs) used in this study. hMSCs expanded ex vivo and used in this study were characterized by cytofluorimetric analysis as detailed previously [21]. Briefly, before cytofluorimetric analysis 2×10^5 to 5×10^5 cells were immunolabeled for 20 minutes using mouse anti-human antibodies conjugated with either fluorescein isothiocyanate or phycoerythrin (Becton Dickinson, San Jose, CA) raised against human CD antigens. MSCs were typically more than 90% positive for antigens expressed by MSCs including CD90, CD105, CD29, CD29, and CD73 and mostly negative for CD34, CD45, and CD14.

Cell Migration

Cell migration of hMSCs was evaluated under either standard normoxic conditions (37°C, 5% CO_2, control cells) or directly under hypoxic conditions (3% oxygen) in a dedicated incubator equipped with a Clark electrode by using the wound healing assay (WHA), to monitor nonoriented migration or chemokinesis (migration being evaluated after 20 hours), or modified Boyden's chamber assay (BCA), to monitor oriented migration (migration evaluated after 6 hours of incubation), as described previously [21, 29, 30]. For both assays, cells were first plated in either collagenated 24-

well dishes (for WHA) or normal Petri dishes (for BCA) under standard normoxic conditions and in normal culture medium for 24 hours; the complete medium was then replaced with a serum-free medium for additional 24 hours. Migration was then evaluated as follows:

WHA (Nonoriented Migration).

Monolayers in 24 wells were disrupted to generate a linear wound with a sterile cell scraper of 1 mm, washed to remove debris, and then incubated under either normoxic (control cells) or hypoxic conditions in serum-free medium for 20 hours. Proliferation was abolished by the use of mitomycin, a mitogenic inhibitor, immediately before placing cells under hypoxia. For the evaluation of "wound closure" in the different experimental conditions, 10 randomly selected areas along each wound were photographed under phase contrast microscopy to count cells invading the artificial wound.

BCA (Oriented Migration).

MSCs were detached from Petri dishes and cells (2×10^4) were suspended in serum-free medium and then placed in the upper compartment of the chamber. The chamber was then placed under normoxic or hypoxic conditions, and after 6 hours, collagenated filters were collected and cells migrated within the filter were stained with crystal violet and then counted in 10 randomly selected microscopic fields (×25) for each chamber.

Molecular Biology Procedures

Total cell extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% or 7.5% acrylamide gels. The blots were incubated with desired primary antibodies and then with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline-Tween containing 2% (w/v) nonfat dry milk [29, 31] and developed with the enhanced chemiluminescence reagents according to manufacturer's instructions.

Target short interference RNA (siRNA) sequences for the downregulation of human JNK isoforms are: (a) 5′ GAAAGAATGTCCTACCTTCT 3′, found in both JNK1 mRNA (nucleotide 393–412) and JNK2 mRNA (nucleotide 425–444) [32]; (b) 5′ GTGGAAAGAATTGA TATATAA 3′, found in JNK1 mRNA; and (c) 5′ AAGAGAGCT TATCGTGAACTT 3′, found in JNK2 mRNA. Target siRNA sequence for downregulation of human HIF-1α is: 5′ AGGAAG AACTATGAACATAAA 3′.

siRNAs and related nonsilencing controls were synthesized by Qiagen-Xeragon (Germantown, MD, www.qiagen.com). For transfection, the Amaxa nucleofection technology (Amaxa, Koln, Germany, www.amaxa.com) was used [33]. JNK1/2 and HIF-1α protein levels were analyzed by Western blot analysis at 96 and 120 hours after transfection, respectively. VEGF-A protein levels in culture medium obtained from either MSCs cultured under normoxic conditions or exposed to hypoxia for the desired time were evaluated by an immunoprecipitation procedure, as described previously [34].

Detection of Intracellular and In Vivo Levels of ROS

Intracellular levels of ROS were detected by means of the semiquantitative 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence technique, as detailed previously [34], in cells exposed to the desired stimulus for 15 minutes or to 50 μM hydrogen peroxide (H_2O_2, positive control).

Morphological Analysis

Indirect immunofluorescence was performed on hMSCs exactly as described previously [21]. Final dilution of primary antibodies was 1:250 (α-SMA), 1:50 (phospho-JNK 1/2), and 1:50 (HIF-1α). Immunopositivity was revealed by the appropriate secondary Cy3-conjugated (1:1000 dilution) or Cy2-conjugated (1:200 dilution) antibodies (Amersham Pharmacia Biotech, Milano, Italy). Nuclei were stained using 4,6-diamino-2-phenyilindole (DAPI), and slides were examined with an Olympus Fluoview 300 confocal laser scanning microscope.

Evaluation of Necrotic Cell Death and Apoptosis

hMSCs exposed to hypoxia up to 48 hours were evaluated for necrotic cell death (release in culture medium of lactate dehydrogenase), morphology, and DAPI fluorescence staining to detect apoptosis as reported elsewhere [30, 33].

Statistical Analysis

Data in bar graphs represent means ± SEM, and were obtained from average data of at least three independent experiments. Luminograms and morphological images are representative of at least three experiments with similar results. Statistical analysis was performed by Student's *t* test or analysis of variance, when appropriate ($p < .05$ was considered significant).

RESULTS

Conditions of Moderate Hypoxia Induce Migration of hMSCs Involving Generation of ROS and Activation of ERK1/2 and JNK1/2

As a first experimental approach, we decided to use the standard WHA to investigate whether moderate hypoxia (i.e., 3% O_2, nonextreme hypoxic conditions that, being compatible with those reported in several tissues undergoing a scenario of damage, inflammation and repair, are able to induce classic HIF-related responses but unable to significantly affect MSCs viability) was able to stimulate nonoriented migration of hMSCs. We found that exposure of MSCs to hypoxia induced a significant increase in the number of cells invading the artificial lesion. Such an increase in migration reached a maximum at the standard time point for this assay (exposure to hypoxia for 20 hours), as already shown by others on MSCs [27] and by us on other cells [29], but interestingly it was already detectable starting from exposure to hypoxia for 6 hours (Fig. 2A). To check the real significance of this early migration of MSCs, cells were also placed in the classic Boyden's chambers and again exposure to controlled conditions of hypoxia resulted in an increased number of MSCs migrated into the collagen coated filters after 6 hours (Fig. 2B). It should be noted that these conditions of moderate controlled hypoxia did not affect viability of hMSCs neither in terms of necrotic cell death nor in terms of apoptosis induction, as evaluated by morphology, lactate dehydrogenase release, and DAPI staining (data not shown).

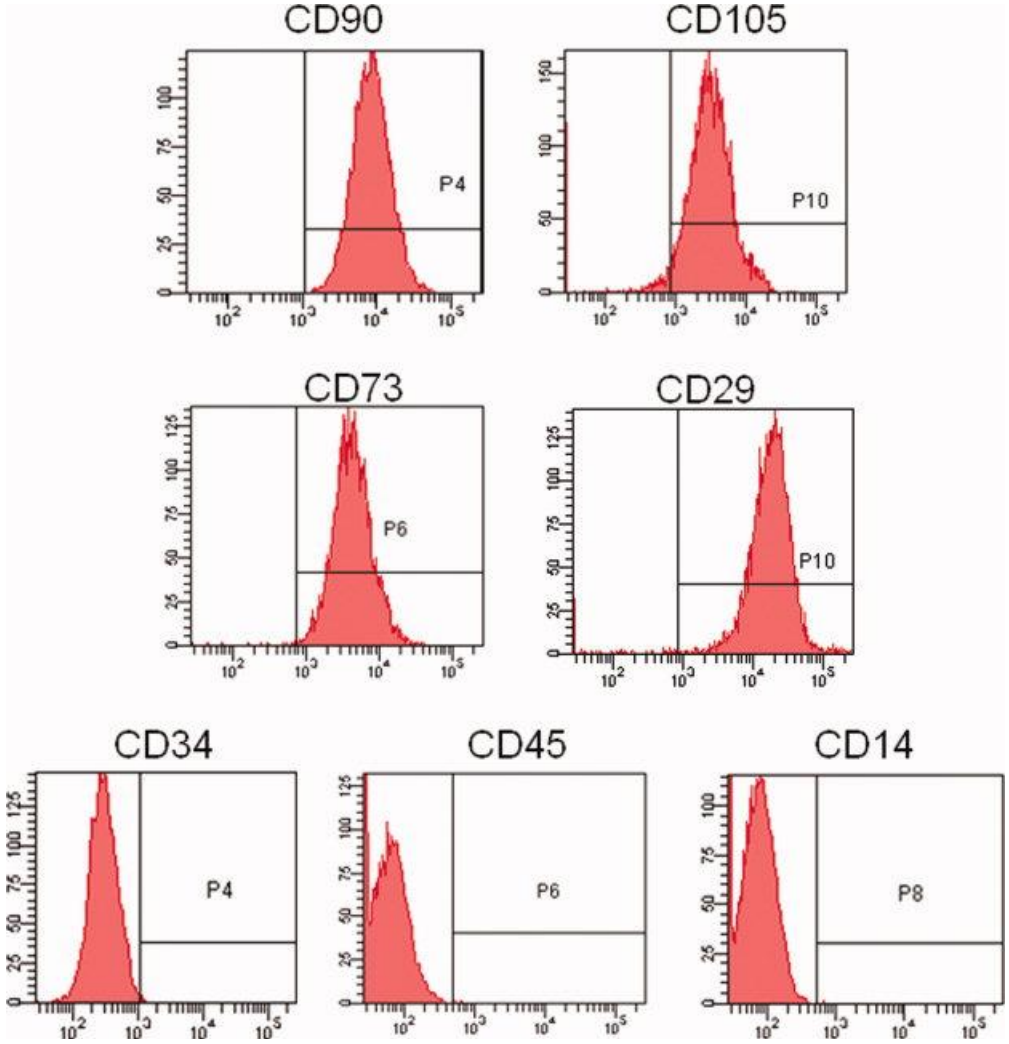

Figure 1. Characterization of bone marrow-derived human mesenchymal stem cells (hMSCs) used in this study. hMSCs expanded ex vivo and used in this study were characterized by cytofluorimetric analysis as detailed previously [21]. Briefly, before cytofluorimetric analysis 2 × 105 to 5 × 105 cells were immunolabeled for 20 minutes using mouse anti-human antibodies conjugated with either fluorescein isothiocyanate or phycoerythrin (Becton Dickinson, San Jose, CA) raised against human CD antigens. MSCs were typically more than 90% positive for antigens expressed by MSCs including CD90, CD105, CD29, CD29, and CD73 and mostly negative for CD34, CD45, and CD14.

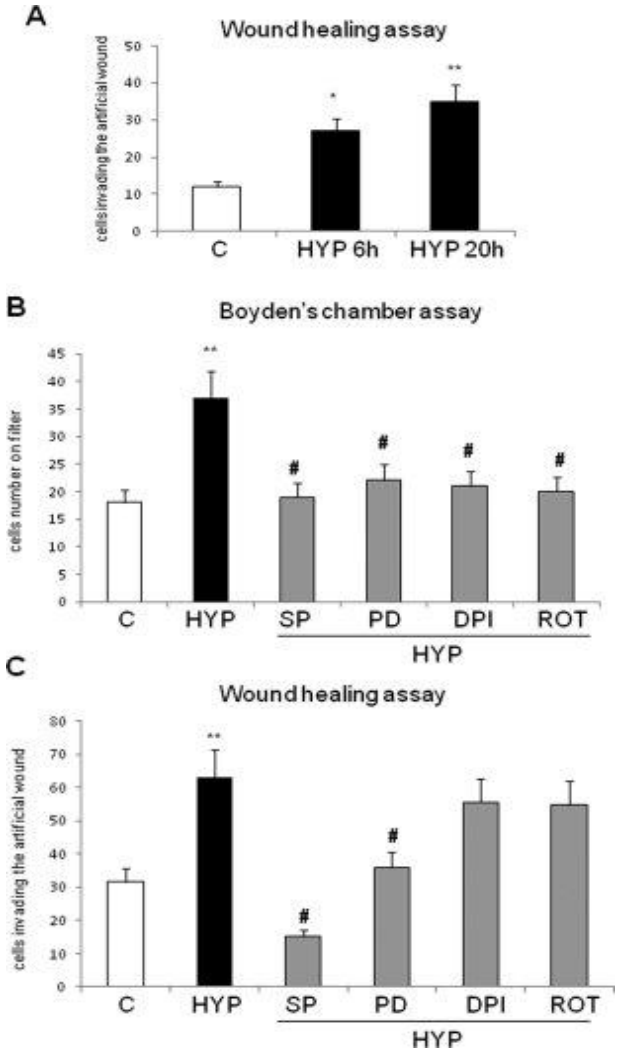

Figure 2.

Migration of human mesenchymal stem cells (hMSCs) in response to hypoxia. Wound healing assay (WHA) **(A, C)** and Boyden's chamber assay **(B)** were performed on hMSCs. Cells were either maintained in normoxic conditions (control), exposed to moderate hypoxia (3% O_2), or pretreated with the pharmacological inhibitor of c-Jun N-terminal kinase 1/2 (SP600125, 20 μM) (30′), with the pharmacological inhibitor of MAP kinase/ERK kinase 1 (PD98095, 30 μM) (30′), or with the inhibitors of mitochondrial electron transfer (complex I) of diphenylene iodonium (1 μM) (1 hour) or rotenone (2.5 μM) (immediately before treatment) before exposure to hypoxic conditions for 6 hours (Boyden's chamber) or 20 hours (WHA). Data in bar graphs represent mean ± SEM (n = 4, in triplicate) and are expressed as number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. *, $p < .05$ and **, $p < .01$ versus control values. #, $p < .01$ versus values in cells exposed to hypoxia alone. Abbreviations: C, control; DPI, diphenylene iodonium; HYP, hypoxia; ROT, rotenone; SP, SP600125; PD, PD98095.

As a second approach, on the basis of previously obtained data supporting a major promigratory role for ERK1/2, JNK1/2, and intracellular generation of ROS [21, 22], MSCs before exposure to hypoxic conditions were pretreated for 30 minutes with either pharmacological inhibitors of MEK1 (PD98095), the ERK1/2 upstream kinase, or JNK1/2 (SP600125) as well as with either rotenone (immediately before treatment) or diphenylene iodonium (DPI) (1 hour before treatment) to block mitochondrial release of ROS, which is known to occur in target cells early after exposure to hypoxic conditions [34–36]. As shown in Figure 2B, 2C, hypoxia-induced migration in either

Boyden's chambers or WHA was drastically inhibited by both pharmacological inhibitors of ERK and JNK signaling pathways. Indeed, Western blot analysis revealed that hypoxia resulted in an early (i.e., within 15 minutes) activation/phosphorylation of both ERK1/2 and JNK1/2 (Fig. 3A). Moreover, the absolute relevance of JNK1/2 activation for MSC migration in both WHA and Boyden's chambers under hypoxic conditions was unequivocally documented by experiments (Fig. 3B, 3C) in which MSCs were transfected with a specific siRNA able to significantly silence the expression of both isoforms (Fig. 3D) [13]. However, rotenone or DPI, which were effective in reducing the early hypoxia-dependent increase in ROS (Fig. 3E), led to abrogation of MSC migration in Boyden's chamber (Fig. 2B) but failed in inhibiting migration in the WHA (Figs. 2C), suggesting that ROS may be more relevant in sustaining the initial phase of MSC migration.

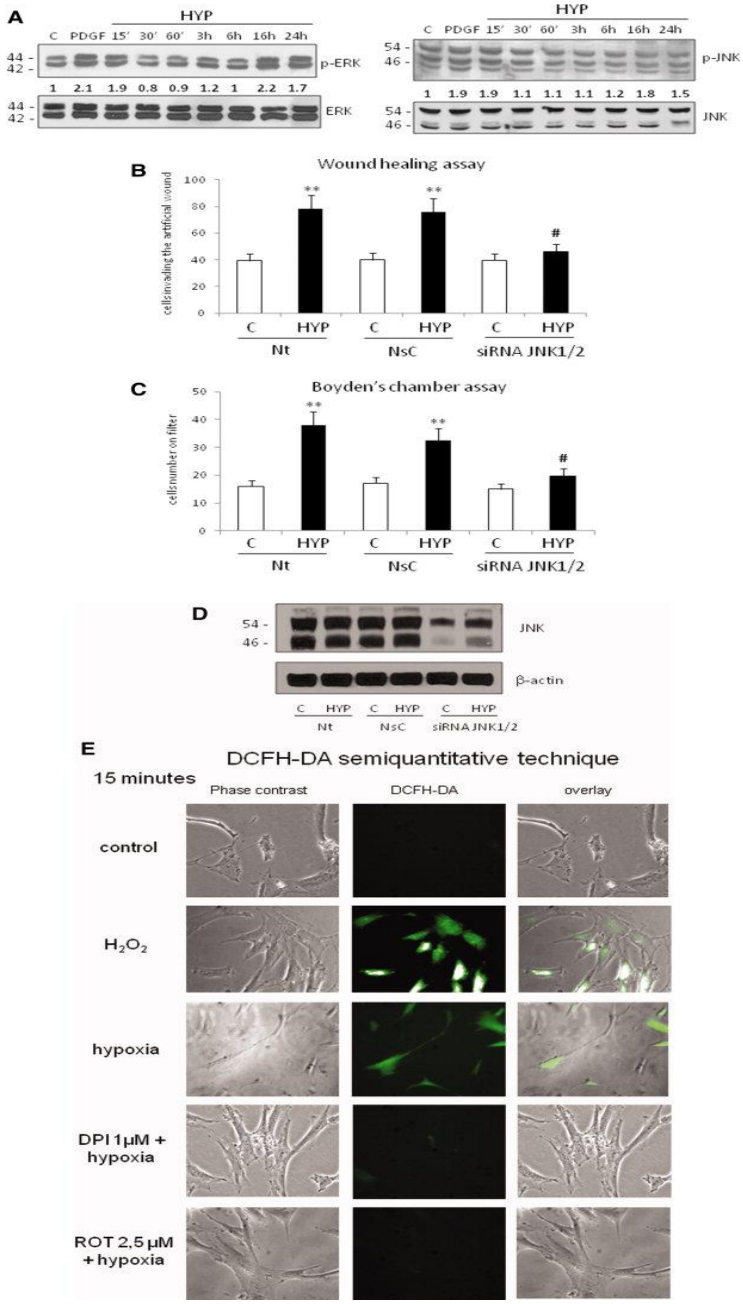

Figure 3.

Conditions of moderate hypoxia-induce migration of human mesenchymal stem cells (hMSCs) by involving reactive oxygen species (ROS) generation and activation of ERK1/2 and c-Jun N-terminal

kinase 1/2 (JNK1/2). Confluent and 24 hours starved hMSCs were incubated for 15 minutes under normoxic conditions in the absence (control) or in the presence of platelet-derived growth factor-BB (10 ng/ml), used here as positive control, or exposed to hypoxia (3% O_2) for the indicated time points. **(A):** Levels of phosphorylated and unphosphorylated ERK1/2 (p44 and p42) and JNK1/2 (p46 and p54) were detected by Western blot analysis on total lysates by using specific antibodies. Phospho-ERK (p-ERK) and phospho-JNK (p-JNK) bands were submitted to scanning laser densitometry and then normalized to respective unphosphorylated proteins; data were then numerically expressed as fold increase versus the respective control bands. **(B, C):** hMSCs were then silenced for JNK1/2 as described in Materials and Methods section. Migration in the wound healing assay (WHA) **(B)** and in Boyden's chamber **(C)** was assessed 96 hours after transfection in cells that were either not transfected, transfected with a nonsilencing control siRNA, or transfected with the desired siRNA. Cells were then either maintained in normoxic conditions (control cells) or exposed to moderate hypoxia (3% O_2) for 6 hours (Boyden's chambers) or for 20 hours (WHA). Data in bar graphs represent mean ± SEM (n = 4, in triplicate) and are expressed as number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. **, $p < .01$ versus control values. #, $p < .01$ versus values in cells exposed to hypoxia and not treated with JNK1/2 siRNA. **(D):** JNK1/2 protein levels were also analyzed by Western blotting 96 hours after transfection to check silencing efficiency. Sample loading was evaluated by reblotting the same membranes with antibodies raised against β-actin. Generation of intracellular ROS (green fluorescence) was detected by the semiquantitative technique based on the use of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) **(E)** at 15 minutes in cells maintained in normoxic conditions (control), in cells exposed to hypoxia alone (3% O_2) or pretreated with the inhibitors of mitochondrial electron transfer (complex I) diphenylene iodonium (1 μM) (1 hour) or rotenone (2.5 μM) (immediately before treatment) before exposure to hypoxic conditions, or in cells treated with hydrogen peroxide (50 μM), the latter used as positive control. For any condition, three images are offered of the same field, representing phase contrast image (left column), DCFH-DA positive fluorescence (middle column), and their digital overlay (right column). Abbreviations: C, control; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DPI, diphenylene iodonium; ERK, extracellular regulated kinase; H_2O_2, hydrogen peroxide; HYP, hypoxia; JNK, c-Jun N-terminal kinase; Nt, not transfected; NsC, nonsilencing control; PDGF, platelet-derived growth factor; p-ERK, phosphor-ERK; p-JNK, phospho-c-Jun N-terminal kinase; ROT, rotenone; siRNA, short interference RNA.

It should be remarked, in this connection, that, as already mechanistically shown for MSCs and other cells able to migrate [22, 30], the simple increase in intracellular levels of ROS is a signal sufficient to stimulate migration of hMSCs. This concept has been reconfirmed in this study by treating, under conditions of normoxia, MSCs with very low (0.1 μM) and not cytotoxic doses of menadione (MEN) and 2,3-dimethoxy-1,4-naphtoquinone (DMNQ), two compounds able to induce an increase in intracellular levels of superoxide or hydrogen peroxide, respectively (Supporting Information Fig. 1A). MEN and DMNQ were able to significantly stimulate migration of hMSCs in both WHA and Boyden's chamber, as documented by data included in Supporting Information Figure 1B, 1C.

Dissection of Early and Late Mechanisms Able to Sustain Hypoxia-Induced Migration of MSCs

According to current knowledge, we next designed experiments to evaluate the hypoxia-dependent involvement of HIF-1α stabilization/translocation and VEGF expression in MSC migration. Time course analysis revealed that exposure of hMSCs to hypoxia resulted, as expected, in an increased HIF-1α stabilization/involvement, as appreciated by either Western blot (Fig. 4A) or immunofluorescence (Fig. 4B); HIF-1α stabilization was already evident early (i.e., from 15 minutes to 2 hours) after exposure of MSCs to hypoxic conditions. HIF-1α stabilization reached an

apparent peak at 2 hours, but was significantly elevated through the entire experimental period (i.e., up to 24 hours; Fig. 4A). When MSCs were pretreated with rotenone (immediately before treatment) and DPI (1 hour before treatment) before exposure to hypoxia for 2 hours, this resulted in a significant inhibition of HIF-1α positive nuclear staining (Fig. 4B), indicating that, as already suggested [34–36], ROS of mitochondrial origin may offer a major contribution to HIF-1α stabilization. This specific concept has been confirmed (Fig. 4C, 4D) once again by a strategy, already mentioned in the previous paragraph and adopted in a previous study [22], by which ROS-related stabilization of HIF-1α was reproduced in MSCs by simply eliciting intracellular generation of ROS under normoxic conditions following treatment of cells with MEN or DMNQ for 2 hours, as revealed by Western blot analysis and immunofluorescence.

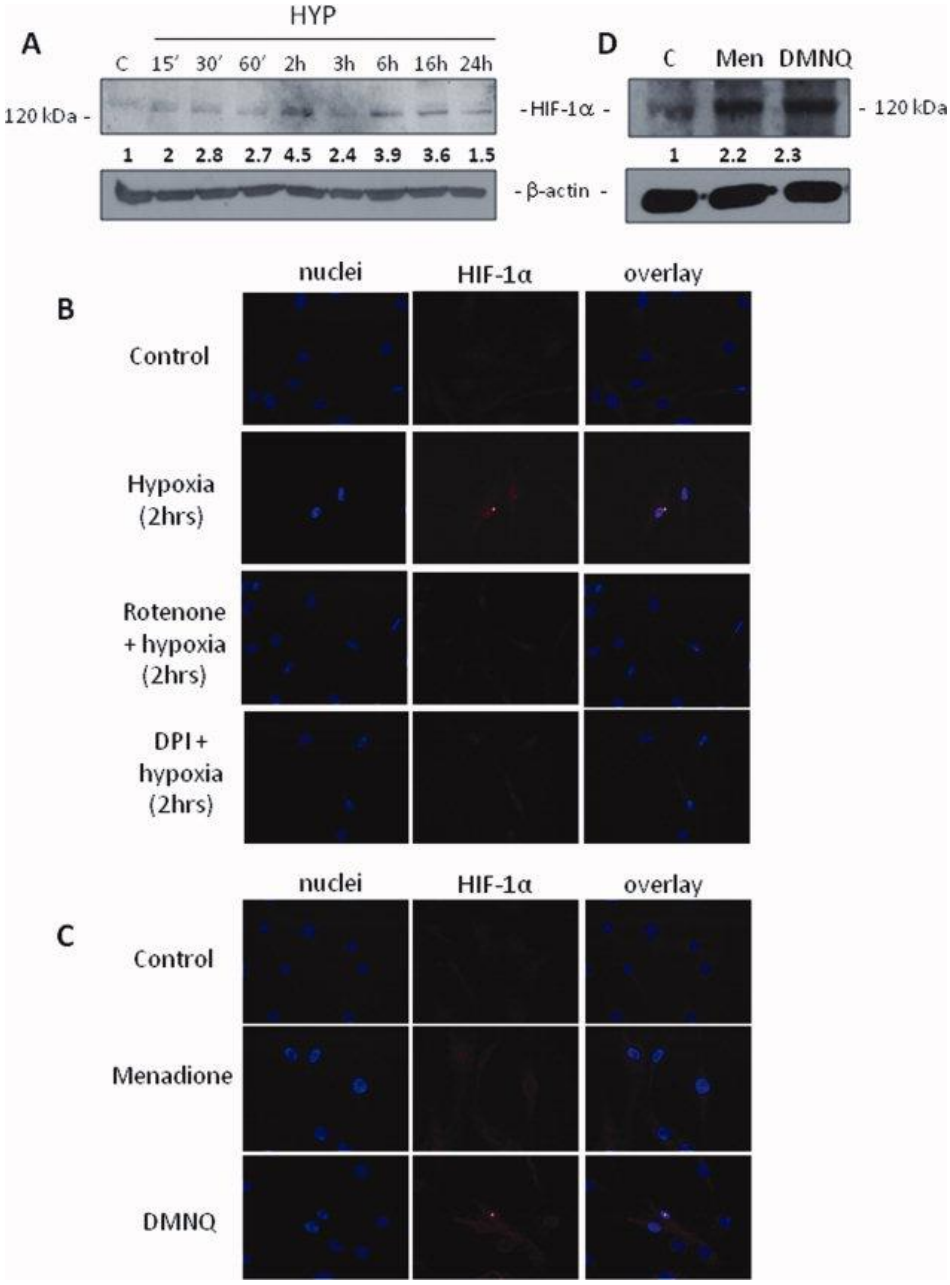

Figure 4.

Reactive oxygen species contribute significantlty to HIF-1α stabilization under hypoxia. Confluent and 24 hours serum starved human mesenchymal stem cells (hMSCs) were exposed to hypoxic conditions (3% O_2) for the indicated time points. **(A):** Levels of HIF-1α (120 kDa) were detected in

Western blot analysis on total lysates by using a specific antibody. Sample loading was evaluated by reblotting the same membranes with antibodies raised against β-actin. **(B):** Levels of HIF-1α were also evaluated by means of indirect immunofluorescence in hMSCs maintained for 2 hours (i.e., the peak time for HIF-1α protein levels) either in normoxic conditions (control), exposed to hypoxia (3% O_2), or pretreated with the inhibitors of mitochondrial electron transfer (complex I) diphenylene iodonium (1 μM) (1 hour) or rotenone (2.5 μM) (immediately before treatment) before exposure to hypoxia. **(C):** Positive immunofluorescence staining for HIF1-α was also evaluated in cells exposed for 2 hours to normoxic conditions alone (control) or treated with menadione (0.1 μM) or DMNQ (0.1 μM). For any condition, three images are offered of the same field representing nuclear 4,6-diamino-2-phenyilindole fluorescence (left column), immunopositivity for HIF-1α (middle column) and the result of their electronic merging (right column). **(D):** Levels of HIF-1α (120 kDa) were finally detected in Western blot analysis on total lysates obtained from confluent and 24 hours serum starved hMSCs exposed for 2 hours to normoxic conditions alone (control) or treated with menadione (0.1 μM) or DMNQ (0.1 μM). Sample loading was evaluated by reblotting the same membranes with antibodies raised against β-actin. HIF-1α bands (Fig. 4A, 4D) were submitted to scanning laser densitometry and then normalized to β-actin levels; data were then numerically expressed as fold increase versus the respective control bands. Abbreviations: C, control; DMNQ, 2,3-dimethoxy-1,4-naphtoquinone; DPI, diphenylene iodonium; HIF-1α, hypoxia inducible factor-1α; HYP, hypoxia; MEN, menadione.

The relevance of the role of HIF-1α for MSC migration was further investigated by using a specific siRNA able to efficiently silence this subunit [34]. Under these experimental conditions, we found that hypoxia-induced migration of HIF-1α silenced MSCs (efficiency of silencing is proven in Fig. 5C) in the WHA was almost completely prevented (Fig. 5A). However, migration of MSCs in Boyden's chambers exposed to hypoxia for 6 hours was not affected by HIF-1α silencing (Fig. 5B), suggesting that hypoxia-induced MSC migration may indeed rely on the existence of two distinct phases and/or mechanisms.

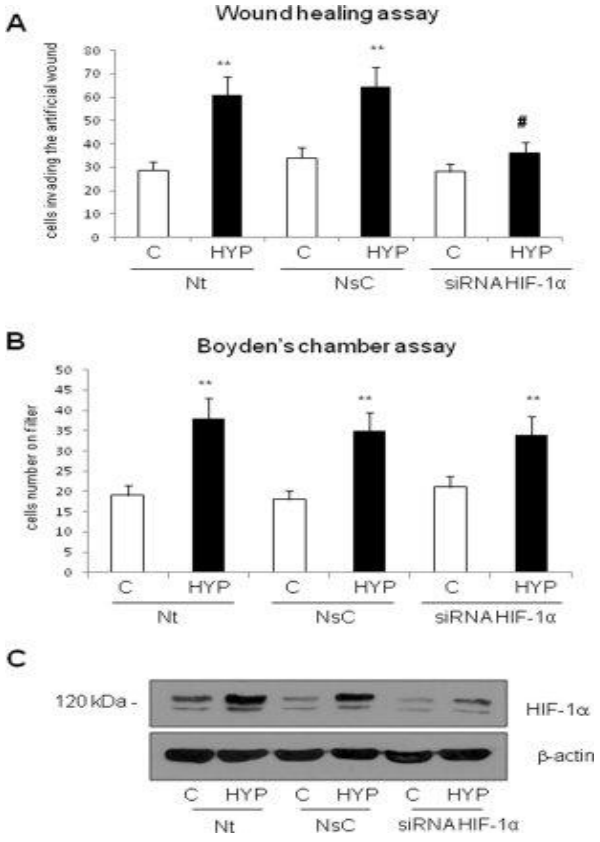

Figure 5.

Hypoxia-induced human mesenchymal stem cells (hMSCs) late migration requires HIF-1α-dependent mechanisms. hMSCs were efficiently silenced for HIF-1α as detailed in Materials and Methods section. Migration of MSCs in both wound healing assay (WHA) **(A)** and Boyden's chamber **(B)** was assessed 120 hours after initial transfection in cells either not transfected, transfected with a nonsilencing control siRNA, or transfected with the desired HIF-1α siRNA. Cells were then either exposed to normoxic conditions (control cells) or to hypoxia (3% O_2). Data are expressed as numbers of cells migrated in the artificial lesion or in the filter of Boyden's chambers. Data in bar graphs represent mean ± SEM (n = 4, in triplicate) and are expressed as number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. **, $p < .01$ versus control values. #, $p < .01$ versus values in cells exposed to hypoxia and not treated with HIF-1α siRNA. HIF-1α protein levels were analyzed by Western blot analysis 120 hours after transfection to check silencing efficiency. Sample loading was evaluated by reblotting the same membranes with antibodies raised against β-actin. Abbreviations: C, control; HIF-1α, hypoxia inducible factor-1α; HYP, hypoxia; Nt, not transfected; NsC, nonsilencing control; siRNA, short interference RNA.

We next performed experiments designed to investigate the role of VEGF, a classic target gene for HIF-1 and a growth factor that has been shown to stimulate both nonoriented migration and chemotaxis in hMSCs [22]. In our experiments, hypoxia-induced migration in the WHA, which was almost abolished by HIF-1α silencing, was found to be significantly inhibited (Fig. 6A) also by pretreating cells (30 minutes) with either a neutralizing antibody directed against VEGF receptor type 2 (VEGF-R2) or a pharmacological inhibitor of VEGF-R2 tyrosine kinase (SU1498). As this was suggesting a hypoxia- and HIF-1α-related release of VEGF-A in the extracellular medium, we next performed experiments in normoxic conditions in which hMSCs were exposed in Boyden's chambers to hypoxic-conditioned medium (i.e., HCM, medium collected at desired time points from MSC cultured under hypoxia). Only HCM media collected at 16 hour and 24 hour time points were found to exert a very significant chemotactic effect on hMSCs (Fig. 6B). Accordingly, by evaluating protein levels in HCM by an immunoprecipitation procedure (Fig. 6C), we noted that VEGF-A levels were significantly increased in HCM collected at 16 hours but not at earlier time points, suggesting that VEGF-A is unlikely to be responsible for early migration of MSCs under hypoxic conditions.

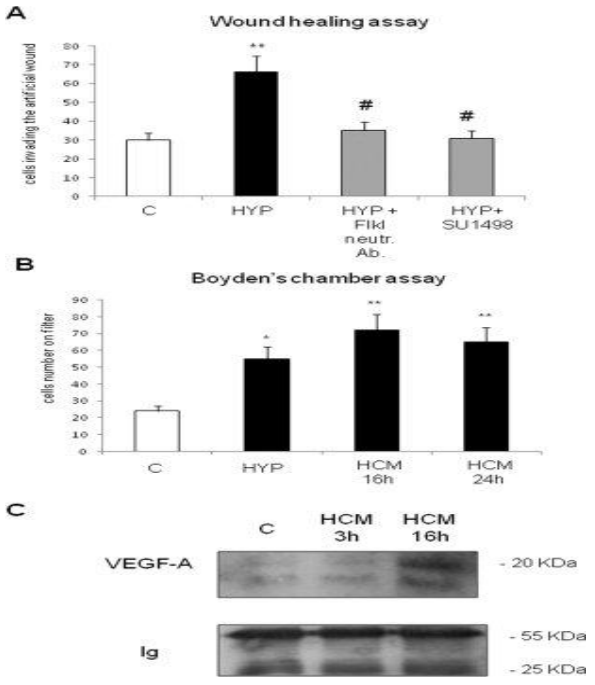

Figure 6.

Late HIF-1-dependent mesenchymal stem cells (MSCs) migration acts through the extracellular release of vascular endothelial growth factor (VEGF). Wound healing assay (20 hours) was performed on human MSCs (hMSCs) **(A)** exposed to either normoxic conditions (control), to moderate hypoxia alone (3% O_2), or to hypoxia after pretreatment for 30 minutes with either a specific neutralizing antibody directed against VEGF receptor type 2 (VEGF-R2 or Flk1) or a pharmacological inhibitor of VEGF-R2 tyrosine kinase (SU1498 0.045 mg/ml). MSC migration in Boyden's chamber **(B)** was evaluated in cells exposed to normoxic medium (control, i.e., a medium obtained after 16 hours of incubation of MSCs under normoxic conditions), hypoxia (3% O_2), or hypoxic-conditioned medium (i.e., medium collected after 16 or 24 hours from hMSCs cultured under hypoxia). Data are expressed as number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. Data in bar graphs represent mean ± SEM (n = 4, in triplicate) and are expressed as number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. *, $p < .05$ and **, $p < .01$ versus control values; #, $p < .01$ versus values obtained in cells exposed to hypoxia alone. VEGF-A (20 kDa) protein levels were analyzed following specific immunoprecipitation performed in either normoxic medium (control) and in hypoxic medium collected after 3 or 16 hours of hypoxia **(C)**. VEGF-A bands were submitted to scanning laser densitometry and then normalized to Ig levels; data were then expressed as fold increase versus the respective control bands. Abbreviations: C, control; Flk1 neutr. ab., VEGF receptor type 2 neutralizing antibody; HCM, hypoxic-conditioned medium; HYP, hypoxia; VEGF-A, vascular endothelial growth factor-A.

DISCUSSION

This study, as previously shown by others [25–27] and by us in a preliminary report [37], confirms that exposure to hypoxic conditions can result in an increased migration of bone marrow-derived hMSCs and offers for the first time a mechanistic dissection of the previously unrecognized biphasic nature of hypoxia-dependent motogenic action on hMSCs. In particular, this study (Fig. 7) envisages a biphasic, time-dependent, and interrelated sequence of events, suggesting that MSC migration induced by hypoxia represents the result of an early phase, mainly related to rapid redox changes and related activation of ERK and JNK signaling, as well as of a late and sustained phase that relies mainly on increased stabilization of HIF-1α and related, HIF-1 mediated, upregulation of VEGF-A expression.

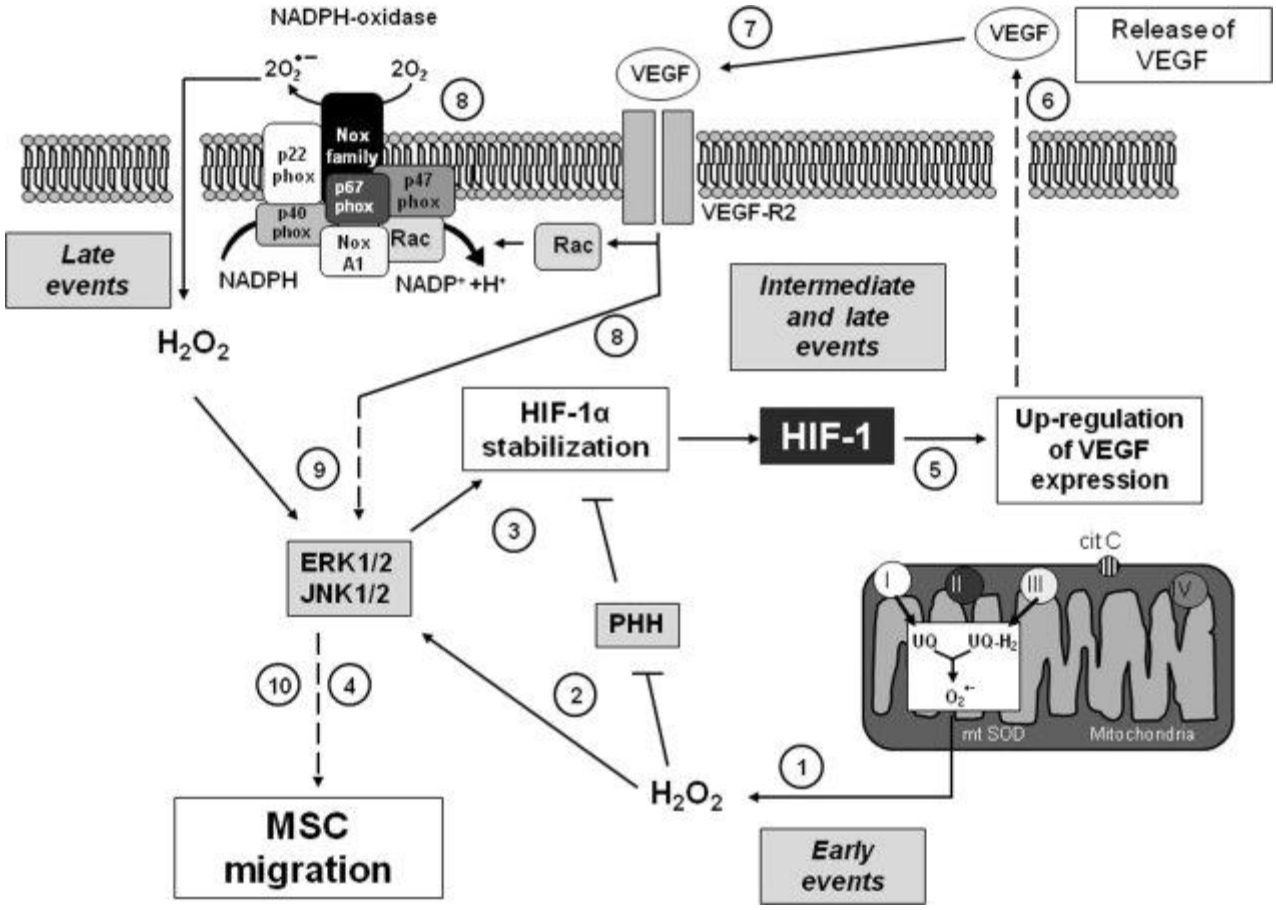

Figure 7.

Biphasic nature of hypoxia-induced migration in bone marrow-derived human mesenchymal stem cells. Hypoxia can induce migration by a biphasic scenario involving early and late events. Early migration relies on hypoxia-dependent release of reactive oxygen species (ROS) by mitochondria which operates within minutes from exposure of MSCs to hypoxic conditions [1]. Intracellular ROS, mainly hydrogen peroxide, lead to HIF-1α stabilization (i.e., the prerequisite for the transcriptional activity of the heterodimer HIF-1) through a mechanism that, according to literature, should involve either inhibition of PHH or, possibly, activation of mitogen-activated protein kinases like ERK1/2 and c-Jun N-terminal kinase 1/2 (JNK1/2) [2, 3]. In any case, hypoxia-dependent activation of ERK1/2 and JNK1/2 is sufficient to initiate and sustain early migration of MSCs [4]. On the other hand, early stabilization/recruitment of HIF-1α leads to HIF-1 transcriptional activity on vascular endothelial growth factor (VEGF) gene expression [5] that, in turn, leads to a progressive release of the growth factor in the extracellular medium that reach significance within 16 hours [6]. VEGF is then primarily responsible for late migration by acting in a paracrine/autocrine way through interaction with its cognate receptor VEGF-R2 [7]. This ligand–receptor interaction, as shown previously, leads in parallel to Rac-related activation of plasma membrane NADPH-oxidase [8] and ROS generation [9], the latter event being able to significantly contribute to promigratory activation of ERK1/2 and JNK1/2 [10]. Abbreviations: ERK1/2, extracellular regulated kinase 1/2; HIF-1α, hypoxia inducible factor-1α; H_2O_2, hydrogen peroxide; JNK1/2, c-Jun N-terminal kinase 1/2; MSC, mesenchymal stem cell; mt SOD, mitochondrial superoxide dismutase; PHH, prolyl hydroxylase; VEGF, vascular endothelial growth factor; VEGF-R2, VEGF receptor type 2.

The involvement of HIF-1α and VEGF-A is strongly supported by the following evidence: (a) hypoxia-related significant increase in HIF-1α stabilization is rapidly detected in MSCs (apparent

peak at 2 hours) and persists throughout the entire experimental protocol; (b) VEGF-A, which is known to efficiently stimulate both nonoriented migration and chemotaxis of hMSC ([22, 27], and this study), is progressively released in the extracellular medium starting from 16 hours; (c) HCM obtained at 16 and 24 hours from cells cultured under hypoxia, which contain significantly increased levels of VEGF-A, efficiently stimulate chemotaxis of MSCs; moreover, hypoxia-induced migration of MSCs in the WHA is significantly prevented by pretreating cells with either a neutralizing antibody against VEGF-R2 or with SU1498, a pharmacological inhibitor of VEGF-R2-related tyrosine kinase, two approaches that we have previously used with other VEGF-A responding cells [29]; (d) finally, efficient silencing of HIF-1α resulted in a significant prevention of hypoxia-related migration of MSCs in the standard WHA. All these results are conceptually analogous to those very recently published by Lee et al. [27]. These authors on the basis of their data proposed, as a main message of their study, that MSC migration under hypoxia was the result of HIF-1α-mediated upregulation of VEGF-A expression, with VEGF-A released by hypoxic MSCs being the real "promigratory" factor acting by involving FAK phosphorylation. These authors, who described migration of MSCs in the "scratch test" (an assay that should be considered as homologous to the WHA assay used in this study), detected first signs of VEGF-A-related migration at 12 hours with best results (including inhibition of migration by using siRNA against HIF-1α) described after 24 hours. Although these authors described, as we did in our study, early generation of intracellular ROS and activation of JNK isoforms, ROS were mainly considered as mediators able to contribute to increased HIF-1α stabilization, as already proposed for different cells by several groups (reviewed in [35, 36]). Our results, particularly those obtained under normoxia by inducing generation of intracellular ROS by means of treating MSCs with MEN and DMNQ (i.e., a procedure resulting in early migration of target cells, including MSCs, [22]), not only confirm this role of ROS but also propose a further additional role for ROS. Whether HIF-1α stabilization by mitochondrial ROS under hypoxic conditions is concerned, this event has been mainly ascribed to a ROS-related inhibition of prolyl hydroxylases (PHH, the enzymes that in normoxic conditions, by hydroxylating HIF-1α, allow its binding to von Hippel–Lindau tumor suppressor protein and subsequent ubiquitination and proteasomal degradation) [35, 36]. However, ROS-related HIF-1α stabilization and upregulation of expression of target genes may also rely on redox-dependent activation of MAPKs, with HIF-1α being recognized then as a putative downstream signaling target for MAPKs-mediated phosphorylation [27, 35, 36]. Data in this study and those obtained in a previous one [22] seem to suggest that both ROS-dependent events may indeed contribute to HIF-1α stabilization under hypoxia.

Here, on the basis of this study and previous data obtained on MSCs as well as on other migrating cells [22, 34], we propose a relevant and critical additional role for ROS in the genesis of MSC migration. In particular, in our analysis the crucial result in dissecting migration in an early and late phase as well as the starting point for proposing a role for ROS-related mechanisms has been the lack of effect of HIF-1α silencing on early migration of MSCs that we observed by using Boyden's chambers. This data unequivocally rules out the early involvement of HIF-1α-related VEGF-A expression and is further confirmed by the fact that MSC migrate (Boyden's chamber) only in response to hypoxia conditioned media collected quite late (not before 16 hours of exposure to hypoxia).

According to our interpretation, the early phase of hypoxia-induced migration is likely to be first switched on by a rapid increase in intracellular levels of ROS that, in agreement with current literature [27, 34–39], are released within minutes by mitochondria in hypoxia-exposed MSCs, as shown here by using the DCFH-DA technique in the absence or in the presence of the inhibitors of mitochondrial electron transport chain rotenone and DPI. Both inhibitors, by acting as blockers of mitochondrial complex I, not only almost completely abolished the rise in intracellular ROS due to their release from mitochondria but also were very effective in preventing early MSC migration in

Boyden's chamber, being perhaps much less efficient in inhibiting late migration (i.e., as detected at 20 hours in the WHA), which in turn seems an additional proof that late migration is more dependent on HIF-1α and VEGF.

Another critical step is then represented by ROS-dependent activation of ERK1/2 and JNK1/2 in the induction of MSC migration. Along these lines, a number of considerations should be recalled. First, the direct link between the rise in intracellular levels of ROS and redox-related activation (i.e., phosphorylation) of both ERK1/2 and JNK1/2 is a well-established issue in the scenario of redox signaling and has been suggested to result from either direct activation of defined signaling components (redox mediated rearrangement of the three-dimensional structure either in monomeric isoforms or heterodimeric complexes) or from inhibition of phosphatases that should be responsible for the negative feed-back control of activated signaling pathways (such as protein tyrosine phosphatases, JNK specific phosphatases, etc) [40–42]. Moreover, it is well known that activation of MAPKs, in particular JNK isoforms, has a mechanistic relationship with migration of human cells (reviewed in [43]).

Even more pertinent for MSC migration, in a recent study [22], we have shown that both hMSCs and human activated, myofibroblast-like, hepatic stellate cells (HSC/MFs, a cell type able to migrate and having a major role in liver fibrogenesis, see [44, 45]) to migrate in response to polypeptide chemoattractant factors require, as a common critical step, NADPH-oxidase-related intracellular generation of ROS that, in turn, can trigger nonoriented migration (chemokinesis) and chemotaxis through a mechanism involving redox-sensitive activation of ERK1/2 and JNK1/2. In the same study, we also provided mechanistic evidence that migration of MSCs and HSC/MFs (in both WHA and Boyden's chamber) could be stimulated in a polypeptide-independent way by simply eliciting an increased intracellular level of ROS by treating cells with MEN or DMNQ, two chemicals leading to a DCFH-DA-detectable intracellular rise of superoxide anion and hydrogen peroxide, respectively. All the results obtained in this study, including particularly those indicating that JNK1/2 silencing can prevent hypoxia-induced migration of MSCs in both experimental systems (WHA and Boyden's chamber), are then consistent with the emerging issue that a simple increase in intracellular levels of ROS, whatever the specific event involved (i.e., mitochondrial release, ligand-receptor-mediated activation of plasma membrane NADPH-oxidase, entry of hydrogen peroxide from the extracellular necroinflammatory environment, etc.), can be sufficient to induce migration in motile cells.

Our result also provide a realistic and mechanistic interpretation of two previously published relevant studies, the first one describing hypoxia-dependent mobilization of multipotent MSCs into peripheral blood [25] and the second reporting that hypoxic preconditioning of MSCs prior to “in vivo” transplantation can result in a significant improvement of their tissue regenerative potential [26]. Finally, the general message coming from this study reinforces the concept that hypoxia, which is easily detectable in several relevant human clinical conditions of tissue injury (particularly in chronic inflammatory and fibroproliferative diseases and in malignant tumors) may favor homing/engraftment of MSCs in damaged tissues.

CONCLUSION

This study shows that hypoxia-dependent induction of hMSC migration proceeds through a biphasic scenario involving distinct but interrelated early and late events (Fig. 7). Hypoxia-induced migration is switched on by ROS released within minutes by mitochondria that sustain the activation of Ras/ERK and JNK signaling pathways. This rapid redox-dependent signaling can be blocked by preventing mitochondrial ROS release and can elicit an early migration in a HIF-1α- and VEGF-A-independent way. Intracellular rise of ROS can also reinforce the late phase of hypoxia-

induced MSC migration by contributing significantly to HIF-1α stabilization that, in turn, is responsible for upregulation of VEGF-A expression and the consequent paracrine promigratory action of VEGF-A released extracellularly by hypoxic MSCs. The scenario also includes the notion, already established in previous work, that induction of migration by VEGF-A released extracellularly from hypoxic MSCs operates not only through the standard signal transduction pathway but also is reinforced through the VEGF-A/VEGF-R2-mediated activation of NADPH-oxidase, leading to further ROS-dependent activation of ERK1/2 and JNK1/2.

The overall message emerging from this study is that hypoxia-dependent induction of MSC migration is characterized by an additional level of complexity when compared with what previously envisaged. The biphasic scenario and related mechanisms here outlined suggest that not only the switching of migration of bone marrow-derived MSCs reaching an injured tissue may be potentially very rapid (i.e., a matter of few minutes to few hours) but also allow to reasonably speculate that such a rapid redox-mediated response to microenvironment signals (then hypoxia and ROS in addition to growth factors and chemokines, which in turn may lead to further ROS generation) may contribute to the effective engraftment of MSCs.

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