Role of FAK phosphorylation in hypoxia-induced hMSCS migration: involvement of VEGF as well as MAPKS and eNOS pathways

Sang Hun Lee,1 Yu Jin Lee,1 Chang Hun Song,2 Young Keun Ahn,3,4,5 and Ho Jae Han1

1Department of Veterinary Physiology, Biotherapy Human Resources Center, College of Veterinary Medicine, Chonnam National University; 2JB Stem Cell Institute, Inc., College of Medicine, Chosun University; and 3The Heart Center, Cardiovascular Research Institute, 4Clinical Trial Center, Chonnam National University Hospital, Gwangju, Korea

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Lee SH, Lee YJ, Song CH, Ahn YK, Han HJ. Role of FAK phosphorylation in hypoxia-induced hMSCS migration: involvement of VEGF as well as MAPKS and eNOS pathways. Am J Physiol Cell Physiol 298: C847–C856, 2010. First published January 20, 2010; doi:10.1152/ajpcell.00418.2009.—Here we show that the effect of hypoxia on human umbilical cord blood mesenchymal stem cell (hMSC) migration is via the modulation of focal adhesion kinase (FAK) and its related signaling pathways. Hypoxia increased hMSC migration and cell viability, whereas lactate dehydrogenase (LDH) release was not affected for up to 48 h (data not shown). In addition, hypoxia increased the level of reactive oxygen species (ROS) generation in a time-dependent manner. Hypoxia-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) was inhibited by the antioxidant (N-acetylcysteine, NAC, 10−6 M) and (taurine, 4×10−6 M). Hypoxia-induced endothelial nitric oxide synthase (eNOS) phosphorylation was regulated by p38 MAPK and SAPK/JNK activation. In addition, hypoxia increased the level of hypoxia inducible factor (HIF)-1α expression, which was blocked by inhibition of eNOS. Also, hypoxia-induced expression of Flk-1, vascular endothelial growth factor (VEGF), and its secreted form were inhibited by HIF-1α small interfering RNA (siRNA). In this hypoxic condition, FAK and Src phosphorylation were increased in a time-dependent manner. Inhibition of Src with specific inhibitor (PP2, 10−8 M) blocked hypoxia-induced FAK activation. Subsequently, hypoxia-induced FAK phosphorylation was blocked by VEGF siRNA. Finally, hypoxia-induced increase of hMSC migration was inhibited by FAK siRNA. The results indicate that hypoxia increases migration of hMSCs via VEGF-mediated FAK phosphorylation and involves the cooperative activity of the ROS, MAPK, eNOS and HIF-1α pathways.

human umbilical cord blood mesenchymal stem cell; focal adhesion kinase; vascular endothelial growth factor

OXYGEN is a potent biochemical signaling molecule and is a major gene regulator during an organism’s development. O2 regulates a broad range of cellular events critical to the growth of multicellular organisms and plays important roles in normal and pathological states (11). The particular response to in vitro O2 deficiency (hypoxia) is cell-type dependent, but O2 affects critical cellular processes such as proliferation (17, 25). The effects of reduced O2 tension have previously been studied to elucidate cellular processes in their natural niches, which have much lower O2 tension than the 21% O2 used in tissue culture systems (10, 14). Exposure of cells to O2 deprivation in vitro reduces proliferation and/or leads to programmed cell death (16, 20). However, there is considerable controversy in the literature regarding cellular responses in a hypoxic state; most of the discrepancies can be explained by differences in O2 concentrations, exposure time, and type of cells. In general, O2 concentrations over 1%, rather than arresting the growth of most kinds of cells, promotes the proliferation of some types of cells. There is increasing evidence that mild hypoxia acts as a potent regulator of various types of stem cells. Therefore, the effects of hypoxia on stem cells are extensive, cell-type specific, and O2 regulated.

Focal adhesion kinase (FAK) is a nonreceptor cytoplasmic tyrosine kinase that plays a key role in the regulation of cytoskeletal reorganization, cellular adhesion, growth, survival, and migration (9, 28). Extensive evidence has also conclusively established that FAK is activated in response to both the extracellular matrix and soluble signaling factors. These observations suggest that the FAK family may be at the crossroads of multiple signaling pathways that affect cell and development processes. Recently, it was reported that vascular endothelial growth factor (VEGF) expression is upregulated by hypoxia and stimulates the motility of a range of cell types, including progenitor and stem cells (12, 13, 18). In addition, migration of bone marrow-derived mesenchymal stem cells (MSCs) is induced by growth factor-enriched conditioned media under hypoxic conditions in vitro (3, 4). VEGF-stimulated FAK tyrosine phosphorylation in endothelial cells is associated with increased recruitment of FAK to new focal adhesions and increased endothelial cell migration (34). However, the mechanistic relationship of hypoxia-induced VEGF expression and MSC migration is unclear, although the effect of hypoxia in regulating global gene expression has been reported extensively in human MSCs (hMSCs) (22, 23). Additionally, little is known about the role of VEGF in modulating hypoxia-induced FAK phosphorylation and the related signaling mechanisms have not been defined in hMSCs. Therefore, the aim of this study was to elucidate the effect of FAK hMSC migration and its related pathways.

MATERIALS AND METHODS

Materials. hMSCs were obtained from the JB Stem Cell Institute (Gwangju, Korea). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). 5-(and -6)-Chloromethyl-2-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), N-acetylcysteine (NAC), and taurine were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies to phospho-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, phospho-FAK, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), phospho-SAPK/JNK, endothelial nitric oxide synthase (eNOS), phospho-eNOS, hypoxia-inducible factor-1α...
and 92.3% N2) conditions at a flow rate of 20 l/min. The chamber was incubated in the aforementioned hypoxic conditions. Cells were then

determined with fluorescent antibody staining (HIF-1α, VEGF, and FAK) small interfering RNA (siRNA) specific for eNOS, p38 MAPK, JNK, and OCT4, SOX2, FOXD3, and their expression was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

VEGF collection using trichloroacetic acid precipitation. This protocol was derived from a previously described deoxycholate-trichloroacetic acid protocol (5). Conditioned medium (4 ml) was first cooled on ice in a high-speed centrifuge tube. Sodium deoxycholate or sodium lauroyl sarcosinate was added to a final concentration varying from 0.1%–0.1%. After mixing was completed, trichloroacetic acid was added to a final concentration of 7.5% and precipitate was allowed to form during a 2-h incubation on ice. The mixed protein-detergent precipitate was collected by centrifugation (10,000 g, 10 min, 4°C). The supernatant was carefully removed, 2 ml of tetrahydrofuran precooled in ice was added to the pellet, and vortexing was carried out until the pellet detached from the bottom of the tube and dissolved almost completely. Centrifugation was carried out as described above. The supernatant was removed and the nearly invisible pellet was washed again with 2 ml of tetrahydrofuran. Finally, the pellet was redissolved in 0.4 ml of extraction solution with the help of a sonicator bath for 30 min.

Immunoﬂuorescence staining with p-FAK, p-eNOS, and VEGF. Cells were fixed and treated with a 1:50 dilution of monoclonal antibody against mouse VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit p-FAK and p-eNOS (Santa Cruz Biotechnology) and then incubated for 30 min with a 1:100 dilution of fluorescein isothiocyanate-conjugated secondary antibody raised in rabbit against mouse IgM or in goat against rabbit IgM. Fluorescence images were visualized with a fluorescence microscope (Fluoview 300, Olympus, Japan).

Statistical analyses. The results are expressed as means ± SE. All the experiments were analyzed by ANOVA. In some experiments, this was followed by a comparison of the treatment means with the control using a Bonferroni-Dunn test. A P value < 0.05 was considered significant.

RESULTS

Effect of hypoxia on hMSC migration. The migratory properties of hMSCs cultured under various hypoxic conditions were assessed using scratch (Fig. 1A) and migration assays (Fig. 1B). A significant increase of hMSC migration appeared during 12–24 h of hypoxia. However, hypoxia did not affect the cell viability, lactate dehydrogenase release (which is a marker of structural damage), and Oct-4, SOX-2, FOX-D3, and Rex1 mRNA expression levels under experimental conditions used in this study (data not shown).

Interaction between MAPKs and eNOS in hypoxia-induced FAK phosphorylation. To ensure that the hypoxia used in this study induces typical cell responses to hypoxic stress, the reactive oxygen species (ROS) generation was first observed. Significant increases in ROS appeared from 15 min of hypoxic exposure (Fig. 2, A and B). Next, we determined whether ROS was involved in hypoxia-induced MAPK phosphorylation. As shown in Fig. 2C, hypoxia initially increased and then decreased p38 MAPK and SAPK/JNK phosphorylation in a time-dependent manner. Each signal pathway was blocked by antioxidant NAC (10−6 M) and taurine (4×10−6 M) (Fig. 2D). In addition, p38 MAPK siRNA and SAPK/JNK siRNA inhibited the hypoxia-induced increase of phosphorylated FAK protein in Western blot analysis (Fig. 2E), and inhibitors of p38
MAPK (SB 203580, 10⁻⁷ M) and SAPK/JNK (SP 600125, 10⁻⁶ M) abrogated the hypoxia-induced increase of FAK phosphorylation as detected by immunofluorescence staining (Fig. 2F). In the next step, we carried out an experiment to ascertain the involvement of hypoxia-induced eNOS phosphorylation with FAK phosphorylation. As shown in Fig. 3, A and B, hypoxia increased eNOS phosphorylation in a time-dependent manner; the signal pathway was blocked by NAC and taurine (Fig. 3C). Hypoxia-induced eNOS phosphorylation was obviated by the SB-203580 and SP-600125 inhibitors (Fig. 3D). Furthermore, eNOS siRNA blocked the hypoxia-induced phosphorylation of FAK (Fig. 3, E and F). These observations were consistent with the suggestion of hypoxia-induced increase of FAK phosphorylation via ROS generation and the MAPKs and eNOS signal pathways.

**Interaction between HIF-1α and VEGF in hypoxia-induced FAK phosphorylation.** HIF-1α expression was examined to determine whether the activation of HIF-1α was involved in hypoxia-induced FAK phosphorylation. The increase of HIF-1α expression was observed 6–12 h after exposure to hypoxic conditions (Fig. 4A). Increased HIF-1α was blocked by the eNOS siRNA (Fig. 4B) in experiments to examine the associations between the signaling molecules in the cells. HIF-1α-specific siRNA inhibited the hypoxia-induced increase in HIF-1α expression (Fig. 4C). Hypoxia-induced increase of cell migration was attenuated by HIF-1α-specific siRNAs (Fig. 4, D and E). In addition, hypoxia-induced FAK phosphorylation was inhibited by HIF-1α-specific siRNAs in Western blot analysis (Fig. 4F) and immunofluorescence staining (Fig. 4G) experiments. VEGF expression was examined to determine whether the activation of VEGF was involved in hypoxia-induced FAK phosphorylation. The hypoxia-induced increase of Fak-1 (VEGF receptor 2), secreted VEGF, and VEGF expression was observed 12–48 h after exposure to hypoxic conditions (Fig. 5A). To examine the relationship among the signaling molecules, cells were transfected with a pool of HIF-1α-specific siRNAs or nontargeting siRNA. In this experiment, VEGF expression was blocked by HIF-1α siRNAs (Fig. 5, B and C), which was evidence of the contribution of HIF-1α to the upregulation of VEGF in hypoxic hMSCs. VEGF-specific siRNA inhibited the hypoxia-induced increase of Flk-1, secreted VEGF, and VEGF expression (Fig. 5D). As shown in Fig. 5, E and F, hypoxia-induced FAK phosphorylation was attenuated by VEGF siRNA. Collectively, the results supported the idea of a hypoxia-induced increase of FAK phosphorylation via HIF-1α and VEGF signaling pathways.

**Effect of FAK/src on hypoxia-induced cell migration.** The level of FAK phosphorylation was examined to determine whether FAK and Src could be involved in hypoxia-induced cell migration. As shown in Fig. 6A, hypoxia increased FAK and Src phosphorylation for 1–24 h before a decrease. The hypoxia-induced increase of FAK phosphorylation level was blocked by the 10⁻⁸ M of the Src inhibitor (PP2) (Fig. 6, B and C). FAK-specific siRNA inhibited the hypoxia-induced increase in FAK phosphorylation level (see Fig. 6, D, E, and F). The results are consistent with hypoxia-mediated increase in cell migration via FAK phosphorylation.

**DISCUSSION**

This study demonstrates that hypoxia increases the migration of hMSCs via VEGF-mediated FAK phosphorylation through the ROS, MAPK, eNOS, and HIF-1α pathways. Growth in hypoxic conditions (1%–3% O₂) may be beneficial for hMSCs, as this O₂ tension is reminiscent of the physiological conditions encountered by hMSCs in the bone marrow (2%–7% O₂). In the present study, hypoxia-induced ROS generation initiated signaling via phosphorylation events, leading to a transient and early increase in MAPKs such as p38 MAPK and SAPK/JNK. MAP kinases have been shown to be activated by hypoxia and ischemia and may play an important role in the adaptive response to hypoxia (9, 31, 32). We observed a transient and early increase in the p38 MAPK and SAPK/JNK levels under hypoxic conditions. Activation of MAPKs was inhibited by NAC and taurine. Several lines of evidence have shown that eNOS activation is upregulated by hypoxia (20). However, it has remained unclear how MAPK signaling transduction pathways elicited their actions on the...
Fig. 2. Effect of reactive oxygen species (ROS), p38 mitogen-activated protein kinase (MAPK), and stress-activated protein kinase/c-Jun NH2 terminal kinase (SAPK/JNK) hypoxia-induced focal adhesion kinase (FAK) phosphorylation. A: cells were incubated in hypoxic condition for 0–30 min, and then dichlorofluorescein (DCF)-sensitive cellular ROS was observed by confocal microscopy. The example shown is a representative of three independent experiments. B: cellular level of H2O2 was measured after the cells were incubated in the hypoxic condition for 0–30 min. The values are reported as means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. 0 time, #P < 0.05 vs. normoxia alone. C: cells were exposed to hypoxic conditions for 0–180 min, and phosphorylated p38 MAPK and SAPK/JNK were detected. D: cells were pretreated with N-acetylcysteine (NAC) and taurine for 30 min before 1 h of hypoxic exposure and analyzed by Western blot analysis (p38 MAPK and SAPK/JNK). E: cells were transfected for 48 h with either SMARTpool of p38 MAPK and SAPK/JNK small interfering RNAs (siRNAs, 100 nmol/l) or a nontargeting (NT) control siRNA (100 nmol/l) before hypoxia exposure for 12 h. The total lysates were subjected to SDS-PAGE and blotted with p-FAK antibodies. Bottom panels depict means ± SE of four independent experiments as determined from densitometry relative to β-actin. *P < 0.05 vs. control, **P < 0.05 vs. hypoxia alone. F: cells were pretreated with SB-203580 (p38 MAPK inhibitor, 10−7 M) or SP-600125 (SAPK/JNK inhibitor 10−6 M) for 30 min before hypoxia exposure for 12 h. Cells were fixed and permeabilized, and then phosphorylated FAK was detected by immunofluorescence staining. The example shown is representative of four independent experiments.
upregulation of eNOS induced by hypoxia. We examined the link between other signaling molecules and the eNOS phosphorylation confirmed downstream of MAPKs, even if this pathway is not the only pathway capable of converging to this subfamily of eNOS. Presently, inhibition of MAPKs blocked eNOS phosphorylation during hypoxia and inhibition of the MAPKs/eNOS pathway consequently decreased the level of HIF-1α expression, suggesting that HIF-1α is downstream of the MAPKs-eNOS pathway, consistent with previous results (17, 27, 33). The HIF-1-mediated transcriptional response
facilitates adaptation to hypoxia through activation of VEGF (33). The present study also demonstrated that hypoxia promotes VEGF expression in hMSCs. The induction of VEGF by hypoxia was also partly dependent on HIF-1α activation, consistent with previous results (27).

FAK serves as a cellular signaling switch in that FAK participates in a variety of cellular processes and binding interactions, although cell survival and mobility may be mutually complementary programs (29). The present results strongly implicated FAK in signal transduction linked to cell migration and correlate their activities with activation of MAPKs and eNOS, HIF-1α, or VEGF. Likewise, inhibition of the MAPK and eNOS signal pathways blocked hypoxia-induced FAK phosphorylation, also suggesting the importance of
hypoxia-induced increase of FAK phosphorylation. As far as we know, FAK is positioned at a crossroad for multiple signaling pathways and mediates bidirectional signaling. The “outside-in” signaling directs rearrangements in cytoskeletal organization and nuclear gene expression in response to changing conditions in the circulation, and an “inside-out” signaling component stimulates cells to change their extracellular matrix and the behavior of neighboring cells and tissues (29). The present direct demonstration of the attenuation of FAK phosphorylation by HIF-1α or VEGF siRNA suggests that focal adhesion is altered in response to HIF-1α and VEGF expression. Recently, FAK silencing has been linked with inhibition of hypoxia-induced HIF-1α and VEGF, indicating an involvement of FAK-dependent signaling pathway in the evolution of hypoxia-induced HIF-1 stabilization and VEGF expression in retinal pigment epithelial cells (35). So, FAK might lie both upstream and downstream of HIF-1, which is dependent on the cell types, maturity, and environmental conditions. Moreover, our finding in the present result is that VEGF-induced FAK phosphorylation is blocked by the specific Src family kinase inhibitor PP2. Our findings are consistent with previous studies showing that Src induces FAK phosphorylation in vitro and vivo (6, 7). Because FAK and Src are strongly implicated in signal transduction linked to cell migration, the marked inhibitory effect of PP2 on VEGF-induced cell migration is consistent with a role for Src-mediated FAK phosphorylation in the migratory response to VEGF (2, 21), although it does not in itself provide direct evidence for such a role. Elucidation of the connections between the Src-dependent phosphorylation pathway and downstream and proximal molecules are likely to yield novel insights into the signal transduction mechanisms mediating the biological actions of VEGF (2, 21). Although we do not exclude the possibility that the inhibition of Src might have other effects independent of the inhibition of FAK phosphorylation, the fact that FAK is both a major target for Src and is strongly implicated in the mechanisms underlying cell migration and survival supports the argument that VEGF-induced FAK phosphorylation mediated via Src contributes to the promotion of responses to VEGF.

Finally, we observed that hypoxic conditions better maintained the stem cell properties of undifferentiated hMSCs, while preserving their multilineage differentiation ability. These findings indicate that O2 may be an important feature in the evolution of hMSCs, and exogenous regulation of the O2 concentration may be used to guide the developmental patterns of hMSC. In addition, understanding the O2-sensitive adaptive pathways in hMSCs may help develop therapeutic strategies to treat various hypoxia-related diseases. In conclusion, hypoxia increases migration of hMSCs via VEGF-mediated FAK phosphorylation through the ROS, MAPKs, eNOS, and HIF-1α pathways.

**REFERENCES**


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Fig. 6. Effects of Src/FAK on hypoxia-induced hMSC migration. A: cells were exposed to hypoxic conditions for 0–48 h, and phosphorylated FAK/Src was detected. B and C: cells were pretreated with PP2 (Src inhibitor, 10^{-8} M) pretreated for 30 min before being incubated in hypoxic conditions for 12 h. FAK was determined by Western blot analysis and immunofluorescence staining. The example shown is representative of four independent experiments. D: cells were transfected for 48 h with either a SMARTpool of FAK siRNAs (100 nmol/l) or a NT control siRNA using LipofectAMINE 2000 before hypoxia exposure for 12 h, and then phosphorylated FAK were detected by Western blot analysis. Bottom panels depict means ± SE of four independent experiments for each condition as determined from densitometry relative to β-actin. *P < 0.05 vs. control, **P < 0.05 vs. hypoxia alone. E: cells were transfected for 48 h with either a SMARTpool of FAK siRNAs or a NT control siRNA before hypoxia exposure for 24 h, and then cells were stained with 5 μM calcein AM. Fluorescence was quantified with a plate reader. The values are reported as means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. control, **P < 0.05 vs. hypoxia alone.


