Superoxide induced by a high-glucose concentration attenuates production of angiogenic growth factors in hypoxic mouse mesenchymal stem cells

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Abstract

Previous reports have shown that the paracrine system may be an important mediator in bone-marrow-derived mesenchymal stem cell (MSC) therapy for ischemic diseases. Hyperglycemia and hypoxia have been associated with increased levels of reactive oxygen species; oxidative stress may therefore influence the paracrine effects of MSCs under hypoxic conditions in diabetic patients, although the mechanism underlying this effect remains unknown. Hypoxia-inducible factor 1 α (HIF-1 α) regulates the transcription of hypoxia-inducible genes. We determined the effect of high-glucose concentrations on the production of angiogenic growth factors via HIF-1 α induction in hypoxic MSCs. MSCs were cultured with different glucose concentration (5.6, 11, 20, or 30 mM) for 24 h. The cells were then incubated in a hypoxic chamber (5% O₂) or under normoxia (21% O₂) for 6 or 24 h. Protein levels of HIF-1 α , vascular endothelial growth factor A₁₆₅ (VEGF-A₁₆₅), and

platelet-derived growth factor B (PDGF-B) were attenuated by glucose in hypoxic MSCs in a dose-dependent manner. Treatment with MG132, a specific inhibitor of proteasome activity, significantly reversed the inhibitory effect of high-glucose concentrations in hypoxic MSCs. 4-Hydroxyl-tetramethylpiperidin-oxyl (a cell-permeable superoxide scavenger) or Apocynin (a NADPH oxidase inhibitor) significantly reversed glucose-induced attenuation of VEGF-A₁₆₅, PDGF-B, and HIF-1 α protein levels. Stimulation with a high-glucose concentration (30 mM) significantly increased intracellular superoxide levels in hypoxic MSCs. Our results suggest that in hypoxic MSCs the increase in intracellular superoxide levels induced by high-glucose concentrations may attenuate hypoxia-induced HIF-1 α expression, which in turn attenuates hypoxia-induced VEGF-A₁₆₅ and PDGF-B transcription.

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Introduction

Several clinical trials have shown that the administration of autologous bone-marrow-derived cells induces therapeutic angiogenesis in patients with ischemic heart disease and peripheral arterial disease (Strauer *et al.* 2002, Tateishi-Yuyama *et al.* 2002, Stamm *et al.* 2003). Bone-marrow-derived mesenchymal stem cells (MSCs) are a potent source of critical growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), which protect ischemic tissue (Wang *et al.* 2006). MSCs also protect the mouse myocardium or hindlimb from ischemic injury through elevated release of VEGF (Kinnaired *et al.* 2004a, Tang *et al.* 2004). These findings suggest that the paracrine system may be an important mediator of MSC therapeutic efficacy in ischemic diseases.

Early studies indicated that the ability of transplanted bone marrow cells (BMCs) to promote neovascularization in the ischemic hindlimb is attenuated in diabetic animal models (Hirata *et al.* 2003, Tamarat *et al.* 2004, Sica *et al.* 2006).

Li *et al.* (2006) showed that VEGF production and therapeutic angiogenesis induced by BMCs taken from obese diabetic rats are significantly lower than those of BMCs taken from control rats. Concomitant treatment with antioxidants significantly ameliorated ischemia-induced angiogenesis by i.v. injection of autologous BMCs in the diabetic hindlimb (Sica *et al.* 2006). Injected BMCs isolated from antioxidant-treated diabetic mice increased postischemic neovascularization by 1.5-fold as compared to those from untreated diabetic mice (Ebrahimian *et al.* 2006). The effects of hyperglycemia or hypoxia have been associated with increased levels of reactive oxygen species (ROS; Waypa *et al.* 2001, Brownlee 2005, Gao & Wolin 2008). Oxidative stress may therefore affect the paracrine influence of bone-marrow-derived cells under hypoxic conditions in diabetic patients, but the precise mechanism underlying these effects remains unknown.

Hypoxia caused mouse MSCs to increase the expression and secretion of VEGF (Kinnaired *et al.* 2004b, Wang *et al.* 2007). Hypoxia-inducible gene transcription is regulated by hypoxia-inducible factor-1 (HIF-1), which is essential for

adaptive cellular responses to hypoxia. HIF-1 α , α subunit of HIF-1, is hydroxylated under normoxia but is stabilized under hypoxia. HIF-1 α regulates the transcription of VEGF, platelet-derived growth factor (PDGF), and FGF-2 in response to hypoxia (Nilsson *et al.* 2004, Calvani *et al.* 2006, Copple *et al.* 2009); therefore, HIF-1 α induction may enhance the production of these proteins in mouse MSCs. In previous studies, the effects of high-glucose concentrations on HIF-1 α expression in various mammalian cells were examined. In human dermal microvascular endothelial cells, a high-glucose concentration impaired hypoxia-independent protection of HIF-1 α against proteasomal degradation (Catrina *et al.* 2004). In rat kidney mesangial cells, a high-glucose concentration stimulated HIF-1 α expression and VEGF secretion (Xia *et al.* 2007). Ceradini *et al.* (2008) showed that in hypoxic mouse dermal fibroblasts, intracellular ROS induced by a high-glucose concentration reduced HIF-1 α binding to DNA promoters without affecting HIF-1 α expression. The inconsistencies in these results may be due to the differences in the examined cell types. The effect of a high-glucose concentration on HIF-1 α expression in MSCs has not been previously studied and requires clarification. Earlier studies in acute myocardial infarction models showed that transplantation of bone-marrow-derived cells into the peri-infarction region improved collateral blood flow and cardiac function versus transplantation into the infarction region (Kinnard *et al.* 2004a,b, Yoshioka *et al.* 2005). This finding may be due to the lower reactivity of host vascular endothelial cells to growth factors in severe ischemic regions. The average blood flow ratio in peri-infarction to nonischemic regions of experimental myocardial infarction models or stroke patients is $\sim 25\%$ (Odano *et al.* 1993, Yoshioka *et al.* 2005). Thus, we need to clarify whether oxidative stress induced by a high-glucose concentration under moderate, rather than severe hypoxia (5% O₂; 25% of normal oxygen concentration), affects the production of angiogenic growth factors through HIF-1 α induction in MSCs.

Materials and Methods

Animals

Male, 5-week-old C57Bl/6 mice were used (Japan SLC, Inc., Hamamatsu, Japan) in accordance with the guidelines of the Japanese Association for Laboratory Animal Science, which comply with international rules and policies.

Preparation of mouse bone marrow mesenchymal stem cells

Isolation of mouse MSCs has previously been described (Peister *et al.* 2004). In brief, BMCs were harvested from 16 male C57Bl/6 mice (5-week-old) by flushing out the femoral and tibial cavities with a complete culture medium that included the following: α -minimal essential medium,

10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all obtained from Invitrogen). Cells were passed through a 30 μ m nylon mesh to remove any remaining clumps of bone marrow. Cells were washed with complete medium and centrifuged for 5 min at 50 g. The cell pellets were re-suspended and cultured in 100 mm dishes with complete medium at 37 °C and 5% CO₂. MSCs selectively adhere to the polystyrene surface, and non-adherent cells were removed 2 days after plating. Adherent cells were further propagated through 4–5 passages.

To identify the cells as MSCs, the cells were analyzed by flow cytometry using FITC-conjugated rat anti-mouse cluster of differentiation 44 (CD44), rabbit anti-mouse CD105, rat anti-mouse CD34, and rat anti-mouse CD45 (all obtained from AbD Serotec, Oxford, UK). The cells were analyzed using a flow cytometer FACS Calibur (Becton-Dickinson, Rutherford, NJ, USA) equipped with a 488 nm argon ion laser and Cell Quest software (Becton-Dickinson). Signals were obtained using a 530 nm bandpass FITC filter. The determination was based on the mean fluorescence intensity of 5000 cells.

To evaluate the differentiation potential of the MSCs toward osteoblasts, subconfluent MSCs were treated with bone morphogenetic protein 2 (3 nM), ascorbic acid (50 μ g/ml), and β -glycerol phosphate (10 mM) for 7 days. The cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, and stained for alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitroblue tetrazolium chloride (0.1 mg/ml; Abcam, Cambridge, MA, USA) for 2 h at room temperature. To evaluate the differentiation potential of MSCs toward adipocytes, subconfluent MSCs were treated with insulin (100 nM), indomethacin (50 μ M), and dexamethasone (100 nM) for 7 days. The cells were washed twice with PBS, fixed with 10% formaldehyde for 20 min, and stained with 0.18% Oil Red O solution (60% isopropyl alcohol) for 20 min at room temperature.

When cultures became confluent, the cells were harvested and re-plated at a density of 1×10^6 cells/dish. After 24 h incubation, the culture medium was discarded and the cells were treated with normal glucose concentration (5.6 mM), a high-glucose concentration (11, 20, or 30 mM), or 24.4 mM mannitol plus 5.6 mM glucose. After 24 h, cells were incubated in an Invivo₂ 300 Hypoxia Chamber (Ruskin Technology, Pencoed, UK) at 37 °C, 5% CO₂, and 5% O₂ for 6 or 24 h. To avoid re-oxygenation of the hypoxia-treated cells, we collected total RNA, the cell lysates, and the culture supernatants in an Invivo₂ 300 Hypoxia Workstation (Ruskin Technology) at 37 °C, 5% CO₂, and 5% O₂. All other cell cultures were incubated under normoxia (37 °C, 5% CO₂, and 21% O₂) for 6 or 24 h. To assess cell viability, the total number of surviving cells was counted after staining with 0.4% trypan blue solution (Sigma) and the cell survival rate was calculated as the percentage of the total re-plated cells.

Real-time reverse transcription-PCR

After 6 h exposure to normoxic or hypoxic conditions, total RNA from treated cells was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. After spectrophotometric quantification, reverse transcription was carried out using random primers and MultiScribe reverse transcriptase (Applied Biosystems, Foster, CA, USA). The cDNA was quantified by real-time PCR using TaqMan Universal PCR master mix and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Based on published reports, the following primers and TaqMan probes were used: β -actin, forward: 5'-ATGCTCCCCGGGCTG-TAT-3'; reverse: 5'-TCACCCACATAGGAGTCCTT-CTG-3'; TaqMan probe: 5'-(FAM)-ATCACACCC-TGGTGCCTAGGGCG-(TAMRA)-3'; HIF-1 α , forward: 5'-AGACAGACAAAGCTCATCCAAGG-3'; reverse: 5'-GCGAAGCTATTGTCTTTGGGTTTAA-3'; TaqMan probe: 5'-(FAM)-CTGCCACTTTGAATCAAAGAAATA-CTGTTCTGAG-(TAMRA)-3'; VEGF-A₁₆₅, forward: 5'-GCACTGGACCCTGGCTTTACT-3'; reverse: 5'-ACT-TGATCACTTCATGGGACTTCTG-3'; TaqMan probe: 5'-(FAM)-CCATGCCAGTGGTCCCAGGCTG-(TA-MRA)-3'; PDGF-B, forward: 5'-CATCCGCTCCTTTG-ATGATCTT-3'; reverse: 5'-ATGAGCTTTCCAACTCG-ACTCC-3'; TaqMan probe: 5'-(FAM)-CCTGCTGCAC-AGAGAGACTCCGTAGATGAA-(TAMRA)-3'. The PCR amplification profile was as follows: 50 °C, 2 min; 95 °C, 10 min; and 40 cycles of denaturing at 95 °C for 15 s, and annealing/extension for 1 min at 60 °C. The threshold cycle (C_t), the cycle at which emission rises above the baseline, was determined for both the target gene and β -actin for each sample, and the relative quantity of target gene transcript was determined by using a comparative C_t method as described in the ABI Prism sequence detection system manual (Applied Biosystems). For each sample, ΔC_t was calculated by subtracting each target C_t value from the mean β -actin C_t value. Relative quantification of the target transcript levels was determined by evaluating the expression $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ represents the subtraction of the ΔC_t determined for the control from that determined for each treatment group.

Western blot analysis

After 6 or 24 h exposure to normoxic or hypoxic conditions, the cells were lysed in cold buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 mM Na₄P₂O₇, 20 mM NaF, 1 mM Na₃VO₄, 10 mg/ml aprotinin); and centrifuged at 10 000 *g* for 15 min. The protein extracts (15 μ g) were electrophoresed on a 7.5–15% SDS-polyacrylamide gel and blotted on a polyvinylidene difluoride membrane with a semidry blotting apparatus. After blocking, primary antibodies were applied at the following concentrations: rabbit anti-mouse VEGF-A₁₆₅ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-mouse PDGF-B (Santa Cruz), rabbit anti-mouse FGF-2

(Santa Cruz), goat anti-mouse gp91^{phox} (Santa Cruz), goat anti-mouse xanthine oxidase (XO; Santa Cruz), rabbit anti-mouse p22^{phox} (Santa Cruz), and rabbit anti-mouse β -actin (Acris Antibodies GmbH, Hiddenhausen, Germany) at 1 μ g/ml; rabbit anti-mouse HIF-1 α (Affinity Bioreagents, Golden, CO, USA) at 0.25 μ g/ml. Secondary peroxidase-conjugated antibodies were added and immunoreactive proteins were visualized by the ECL Western Blotting detection kit (Amersham Biosciences). Densitometry of the immunoreactive bands was performed by a luminescent image analyzer (LAS 3000; Fuji Film, Tokyo, Japan) and software for image analysis (Multi Gauge version 3.1; Fuji Film). The density of the bands was normalized to β -actin as the internal standard.

ELISA

After 24 h exposure to normoxic or hypoxic conditions, MSC supernatants were collected. VEGF-A₁₆₅ and PDGF-B were quantified by ELISA using a commercial ELISA kit (Invitrogen), according to the manufacturer's instructions. All samples and standards were measured in duplicate. The protein content was determined by the Bradford method (Bradford 1976), with BSA as the standard. The VEGF-A₁₆₅ or PDGF-B content in the supernatant was normalized to the protein content of the cell layer.

Figure 1 Expression of CD44, CD105, CD34, and CD45 on mouse bone-marrow-derived cells. Representative histograms show the expression of CD44 (A), CD105 (B), CD34 (C), and CD45 (D) in mouse bone-marrow-derived cells evaluated by single-color flow cytometry as described in section 'Materials and Methods'. Log fluorescence (horizontal axis) for the antibodies of CD44, CD105, CD34, or CD45 is demonstrated in the white histograms, and the isotype control peak is black. Values represent the percentage of cells expressing CD44, CD105, CD34, or CD45 (mean $\pm$ S.E.M.; $n=4$).

Flow cytometric determination of intracellular superoxide levels

Intracellular superoxide levels were measured by flow cytometry as the fluorescence of ethidium, which represents the superoxide-sensitive oxidation products of dihydroethidium (Sharikabad *et al.* 2001). Cells were incubated for 10 min with a dihydroethidium probe (2 mM, 0.15% dimethylsulfoxide) at 37 °C. Cells were fixed with 1% paraformaldehyde and protected from light at 4 °C. A flow cytometer FACS Calibur (Becton-Dickinson), equipped with a 488 nm argon ion laser and Cell Quest software (Becton-Dickinson), was used to measure superoxide levels. Signals were obtained by using a 575 nm bandpass filter for ethidium. Determinations were based on the mean fluorescence intensity of 5000 cells.

Short interfering RNA treatment

Subconfluent cells were transfected with short interfering RNAs (siRNAs) using siPORT NeoFX Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Two kinds of siRNA targeting HIF-1 α (sense, 5'-GCUUCUGUUAUGAGGCUCATT-3'; antisense,

5'-UGAGCCUCA-UAACAGAAGCTT-3', GenBank accession number, NM_010431 exon 2), (sense, 5'-GGAUACAAG-CUGCCUUUUU-TT-3'; antisense, 5'-AAAAAGGCA-GCUUGUAUCCTC-3', GenBank accession number, NM_010431 exon 9), and nontargeting siRNA (Silencer Negative Control siRNA) were purchased from Ambion. Mock control cells were transfected without oligonucleotides under the same conditions. After transfection, the cells were grown for 24 h and then exposed to normoxic or hypoxic conditions with or without MG132 (5 μ M; a specific inhibitor of proteasome activity). The cells were lysed and whole-cell lysates were extracted as described above.

Preparation of mitochondria-enriched fractions and measurement of mitochondrial complexes I and III activity

After 24 h exposure to normoxic or hypoxic conditions, cells were disrupted mechanically by a glass/Teflon homogenizer and centrifuged at 4 500 *g* for 10 min. The mitochondria-enriched pellets were frozen in 10 mM Tris, pH 7.6, and kept at -80 °C.

Figure 2 Differentiation potential of mouse bone-marrow-derived cells. (A and B) To evaluate the differentiation potential of mouse bone-marrow-derived cells toward osteoblasts, subconfluent cells were treated with bone morphogenetic protein 2 (3 nM), ascorbic acid (50 μ g/ml), and β -glycerol phosphate (10 mM) for 7 days. Representative photomicrographs reveal positive staining of cuboidal cells for alkaline phosphatase. (C and D) To evaluate the differentiation potential of the cells toward adipocytes, subconfluent cells were treated with insulin (100 nM), indomethacin (50 μ M), and dexamethasone (100 nM) for 7 days. Representative photomicrographs reveal positive staining with Oil Red O. Bars represent 100 μ m (A and C) and 50 μ m (B and D). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0305>.

Complexes I and III activity were assayed by previously established methods (Jarreta *et al.* 2000, Janssen *et al.* 2007). Complex I activity (NADH-decylubiquinone oxidoreductase) was measured at 340 nm (U-3010 Spectrophotometer, Hitachi) using the acceptor 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (80 mM) and 200 μ M NADH as donor, in 10 mM Tris (pH 8.0)

buffer containing 1 mg/ml BSA, 0.24 mM KCN, and 0.4 μ M antimycin A for 5 min. To permeabilize the mitochondrial internal membrane to NADH, the sample was incubated in distilled water for 3 min at 37 °C. Complex III activity (ubiquinol cytochrome *c* reductase) was measured at 550 nm using 40 μ M oxidized cytochrome *c* as the acceptor and 80 μ M decylubiquinol as the donor in 10 mM KH_2PO_4

Figure 3 Effects of a high-glucose concentration on the expression of VEGF-A₁₆₅, PDGF-B, FGF-2, and HIF-1 α in mouse mesenchymal stem cells under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM), high glucose (11, 20, or 30 mM), or 5.6 mM glucose + 24.4 mM mannitol. After 24 h, the cells were incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 24 h. VEGF-A₁₆₅, PDGF-B, FGF-2, and HIF-1 α protein levels were determined by western blot analysis as described in section 'Materials and Methods'. (A) Representative ECL gel documents show the protein expression in MSCs under normoxic or hypoxic conditions for 24 h. The bands of β -actin in the lower panel are shown for internal standards. (B and C) Bar graphs show quantitative analysis of the bands by densitometry. Values were normalized to the optical density values of β -actin bands. Data represent means $\pm$ S.E.M. ($n=4$). * $P<0.01$ versus MSCs treated with 5.6 mM glucose at normoxia. # $P<0.05$ versus hypoxic MSCs treated with 5.6 mM glucose.

Figure 4 Effects of a high-glucose concentration on the production of VEGF-A₁₆₅ or PDGF-B in mouse mesenchymal stem cells under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM), high glucose (11, 20, or 30 mM), or 5.6 mM glucose + 24.4 mM mannitol. After 24 h, the cells were incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 24 h. Culture supernatants were collected. The production of VEGF-A₁₆₅ or PDGF-B in the culture supernatants was determined by ELISA. Data represent means ± S.E.M. (n=4). *P<0.01 versus MSCs treated with 5.6 mM glucose at normoxia. #P<0.05; ##P<0.01 versus hypoxic MSCs treated with 5.6 mM glucose.

(pH 7.8), 1 mg/ml BSA, and 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, and 0.2 mM ATP for 2 min. The addition of 1 μM antimycin A allowed us to distinguish between the reduction of cytochrome *c* catalyzed by complex III and the nonenzymatic reduction of cytochrome *c* by reduced quinone.

Proteasome activity assay

After 6 h exposure to normoxic or hypoxic conditions, cells were lysed in cold buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 mM Na₄P₂O₇, 20 mM NaF, 1 mM Na₃VO₄) and centrifuged at 10 000 *g* for 15 min. Proteasome activity in the cell lysates was evaluated by the Proteasome Activity Assay kit (Chemicon International, Inc., Billerica, MA, USA). The assay is based on detection of fluorophore 7-amino-4-methylcoumarin (AMC) after the cleavage from the labeled substrate Leu-Leu-Val-Tyr-AMC by the proteasome (Meng *et al.* 1999). Free AMC fluorescence is quantified using a fluorometer (PerkinElmer, Waltham, MA, USA). All samples and standards were measured in duplicate. The data in the cell lysates were normalized to the protein contents.

Figure 5 Effects of a high-glucose concentration on the mRNA expression of VEGF-A₁₆₅, PDGF-B, and HIF-1α in mouse mesenchymal stem cells under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM) or high glucose (30 mM). After 24 h, the cells were incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 6 h. After 6 h exposure to normoxic or hypoxic conditions, total RNA was extracted. VEGF-A₁₆₅, PDGF-B, and HIF-1α transcript levels were determined using real-time RT-PCR and normalized to a control value (5.6 mM, 21% O₂) as described in section 'Materials and Methods'. Data represent means ± S.E.M. (n=4). *P<0.01 versus MSCs treated with 5.6 mM glucose at normoxia. †P<0.01 versus MSCs treated with 30 mM glucose at normoxia. ‡P<0.05; ##P<0.01 versus hypoxic MSCs treated with 5.6 mM glucose.

Statistical analysis

All results were expressed as means $\pm$ S.E.M. Comparisons between the means of multiple groups were analyzed by one-way ANOVA and Scheffe's multiple comparison test. The number of MSCs from different animals is shown as *n*. In all tests, differences were considered statistically significant at $P < 0.05$.

Results

Identification of bone-marrow-derived cells with MSCs

Flow cytometry showed that the primary bone-marrow-derived cells were CD34[−], CD44⁺, CD45[−], and CD105⁺ (Fig. 1). To evaluate the differentiation potential toward osteoblasts, subconfluent cells were treated with bone morphogenetic protein 2 (3 nM), ascorbic acid (50 μ g/ml), and β -glycerol phosphate (10 mM) for 7 days. Photomicrographs revealed positive staining of cuboidal cells for alkaline phosphatase (Fig. 2A and B). To evaluate the differentiation potential toward adipocytes, subconfluent cells were treated with insulin (100 nM), indomethacin (50 μ M), and dexamethasone (100 nM) for 7 days. Photomicrographs revealed positive staining with Oil Red O (Fig. 2C and D). These findings suggest that the bone-marrow-derived cells have the same properties as mouse MSCs.

Effect of a high-glucose concentration on VEGF-*A*₁₆₅, PDGF-B, and FGF-2 expression in MSCs under normoxic or hypoxic conditions

The MSC survival rate under normoxic or hypoxic conditions was unaffected by high-glucose concentrations (data not shown). Exposure to a high-glucose level under normoxia did not affect cellular protein levels of VEGF-*A*₁₆₅, PDGF-B, or FGF-2 (Fig. 3A and B). In contrast, stimulation with a high-glucose concentration (30 mM) under hypoxic conditions significantly attenuated VEGF-*A*₁₆₅ and PDGF-B protein levels. This was not due to an osmotic effect because 24.4 mM mannitol plus 5.6 mM glucose did not attenuate protein levels of VEGF-*A*₁₆₅ or PDGF-B. In contrast, FGF-2 protein levels were unaffected by high-glucose concentrations under hypoxic conditions.

Exposure to high-glucose concentrations under normoxia did not affect the cellular production of VEGF-*A*₁₆₅ or PDGF-B in the MSCs (Fig. 4), but under hypoxic conditions stimulation with high-glucose concentrations (30 mM) significantly attenuated VEGF-*A*₁₆₅ and PDGF-B production. VEGF-*A*₁₆₅ and PDGF-B production were not attenuated by 24.4 mM mannitol plus 5.6 mM glucose.

Exposure to high-glucose concentrations under normoxia did not affect the mRNA level of VEGF-*A*₁₆₅ or PDGF-B in the MSCs (Fig. 5), but under hypoxic conditions a high-glucose concentration (30 mM) significantly attenuated the mRNA expression of VEGF-*A*₁₆₅ and PDGF-B.

Effect of a high-glucose concentration on HIF-1 α expression in MSCs under hypoxic conditions

Under normoxia, HIF-1 α protein was scarcely detected by western blot analysis of MSC extracts and exposure to high-glucose concentrations had no effect on HIF-1 α protein levels (Fig. 3A and C). In contrast, stimulation with a high-glucose concentration (30 mM) significantly inhibited hypoxia-induced accumulation of HIF-1 α protein.

To study the processes by which stimulation with a high-glucose concentration interferes with HIF-1 α upregulation in hypoxia, the effect of a high-glucose concentration on HIF-1 α mRNA levels under normoxia and hypoxia was investigated. Exposure to a high-glucose concentration did not affect HIF-1 α mRNA content under either normoxia or hypoxia, indicating that stimulation with a high-glucose concentration does not regulate HIF-1 α transcription (Fig. 5).

Previous studies have suggested that proteasome-mediated degradation may regulate HIF-1 α expression and function (Salceda & Caro 1997, Kallio *et al.* 1999). Thus, the inhibitory effect of high-glucose concentrations on HIF-1 α protein levels in hypoxic MSCs in the presence or absence of MG132, a specific inhibitor of proteasome activity, was studied. Treatment with 5 μ M MG132 significantly reversed the inhibitory effect of a high concentration of glucose on HIF-1 α protein levels in hypoxic MSCs (Fig. 6), but

Figure 6 Effect of MG132, an inhibitor of proteasome activity, on the protein expression of HIF-1 α in mouse mesenchymal stem cells under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM) or high glucose (30 mM). After 24 h, the cells were treated with or without 5 μ M MG132 and incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 6 h. HIF-1 α protein level was determined by western blot analysis. Representative ECL gel documents show the protein expression in MSCs. Bar graphs show quantitative analysis of the bands by densitometry. Values were normalized by the optical density values of β -actin bands. Data represent means $\pm$ S.E.M. (*n* = 4). * $P < 0.01$ versus MSCs treated with 5.6 mM glucose at normoxia. # $P < 0.01$ versus hypoxic MSCs treated with 5.6 mM glucose. † $P < 0.01$ versus hypoxic MSCs treated with 30 mM glucose.

Figure 7 Effect of HIF-1α siRNA on the expression of VEGF-A₁₆₅ or PDGF-B in MSCs under normoxic or hypoxic conditions. Subconfluent MSCs were transfected with nontargeting siRNAs (negative), siRNA targeting HIF-1α exon 2 or HIF-1α exon 9. Mock control cells (mock) were subjected to transfection without oligonucleotides under the same conditions. After the transfection, cells were grown for 24 h and then exposed to normoxic or hypoxic conditions for 24 h. (A) VEGF-A₁₆₅, PDGF-B, and HIF-1α transcript levels were determined using real-time RT-PCR and normalized to a control value (mock, 21% O₂). (B and D) Twenty-four hours after transfection, MSCs were treated with normal glucose (5.6 mM) or high glucose (30 mM). After 24 h, the cells were treated with or without 5 μM MG132 and incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 6 h. VEGF-A₁₆₅, PDGF-B, FGF-2, or HIF-1α protein levels were determined by western blot analysis. Representative ECL gel documents show the protein expression in MSCs. (C and E) Bar graphs show quantitative analysis of the bands by densitometry. Values were normalized to the optical density values of β-actin bands. Data represent means ± S.E.M. (n=4). *P<0.05; **P<0.01 versus normoxic MSCs transfected with nontargeting siRNA. #P<0.05; ##P<0.01 versus hypoxic MSCs treated with 30 mM glucose. *P<0.05; **P<0.01 versus hypoxic MSCs treated with 5.6 mM glucose.

treatment with MG132 did not affect HIF-1 α protein levels in normoxic MSCs.

As HIF-1 α may regulate the transcription of VEGF and PDGF in response to hypoxia, the attenuation of VEGF-A₁₆₅ and PDGF-B by a high-glucose concentration in hypoxic MSCs may be due to the decreased HIF-1 α protein level. To confirm this hypothesis, the role of HIF-1 α in the regulation of VEGF-A₁₆₅ or PDGF-B expression in MSCs by suppression of HIF-1 α using siRNA was studied. siRNA directed against HIF-1 α significantly inhibited the induction of HIF-1 α under hypoxic conditions (Fig. 7A, D, and E), but not under normoxia (Fig. 7B and C). Suppression of HIF-1 α using siRNA significantly attenuated the expression of VEGF-A₁₆₅ and PDGF-B at mRNA and protein levels under hypoxic conditions (Fig. 7A, D, and E), whereas siRNA slightly attenuated the mRNA levels of VEGF-A₁₆₅ and PDGF-B under normoxia. In addition, HIF-1 α siRNA diminished the inhibition of HIF-1 α , VEGF-A₁₆₅, and

PDGF-B proteins by high-glucose concentrations in hypoxic MSCs. Although MG132 treatment reversed the inhibitory effect of high-glucose concentrations in MSCs transfected with the nontarget control siRNA, HIF-1 α siRNA diminished the preventive effect of MG132 (Fig. 7D and E).

Effect of 4-hydroxyl-tetramethylpiperidin-oxyl or Apocynin on VEGF-A₁₆₅, PDGF-B, or HIF-1 α expression in MSCs under hypoxic conditions

The effects of high concentrations of glucose or hypoxia have been associated with increased levels of ROS (Waypa *et al.* 2001, Brownlee 2005, Gao & Wolin 2008). Therefore, we studied whether the inhibitory effect of a high-glucose concentration on HIF-1 α protein levels in hypoxic MSCs is due to increased ROS levels. A cell-permeable superoxide scavenger, 4-hydroxyl-tetramethylpiperidin-oxyl (Tempol; 100 μ M), significantly reversed the glucose-induced attenuation of

Figure 8 Effect of Tempol or Apocynin on VEGF-A₁₆₅, PDGF-B, or HIF-1 α expression in MSCs stimulated by a high-glucose concentration under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM) or high glucose (30 mM). Tempol (100 μ M) or Apocynin (1 mM) was added to the cells simultaneously. After 24 h, the cells were incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 24 h. VEGF-A₁₆₅, PDGF-B, or HIF-1 α protein levels were determined by western blot analysis. (A) Representative ECL gels show protein expression in MSCs. (B) Bar graphs show quantitative analysis of the bands by densitometry. Values were normalized to the optical density values of β -actin bands. (C) The production of VEGF-A₁₆₅ or PDGF-B was determined by ELISA as the concentration of VEGF-A₁₆₅ or PDGF-B in the culture supernatants. Data represent means $\pm$ S.E.M. ($n=4$). * $P<0.01$ versus MSCs treated with 5.6 mM glucose at normoxia. # $P<0.05$; ## $P<0.01$ versus hypoxic MSCs treated with 5.6 mM glucose. * $P<0.05$ versus hypoxic MSCs treated with 30 mM glucose.

Figure 9 Effect of a high-glucose concentration on the expression of NADPH oxidase or xanthine oxidase, the activity of mitochondrial complexes I and III, and the intracellular superoxide level in MSCs under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM) or high glucose (11 or 30 mM). After 24 h, the cells were incubated under normoxic (21% O₂) or hypoxic (5% O₂) conditions for 24 h. (A and B) The protein levels of p22^{phox}, gp91^{phox}, or xanthine oxidase were determined by western blot analysis. (A) Representative ECL gel documents show the protein expression in MSCs. (B) Bar graphs show quantitative analysis of the bands by densitometry. Values were normalized to the optical density values of β-actin bands. (C) Mitochondrial complexes I and III activity were measured in mitochondria-enriched fractions spectrophotometrically as described in section 'Materials and Methods'. (D) The intracellular superoxide levels were determined by flow cytometry as the fluorescence of ethidium using the dihydroethidium probe. Data were based on the mean fluorescence intensity of 5000 cells. Data represent means ± S.E.M. (n=4). *P<0.05 versus normoxic MSCs treated with 5.6 mM glucose. †P<0.05 versus normoxic MSCs treated with 30 mM glucose. #P<0.05; ##P<0.01 versus hypoxic MSCs treated with 5.6 mM glucose. *P<0.05 versus hypoxic MSCs treated with 30 mM glucose.

VEGF-A₁₆₅, PDGF-B, and HIF-1α protein levels under hypoxic conditions (Fig. 8A and B). Apocynin (1 mM; an NADPH oxidase inhibitor) also significantly reversed the attenuation of VEGF-A₁₆₅, PDGF-B, and HIF-1α proteins levels induced by high-glucose concentrations. At normoxia, neither Tempol nor Apocynin affected the expression of VEGF-A₁₆₅, PDGF-B, or HIF-1α protein levels. Tempol or Apocynin significantly reversed the glucose-induced attenuation of VEGF-A₁₆₅ and PDGF-B production (Fig. 8C).

Effect of a high-glucose concentration on the intracellular superoxide level in MSCs under hypoxic conditions

XO, NADPH oxidase, lipoxygenases, mitochondria, or the uncoupling of nitric oxide synthase can produce intracellular ROS. The expression and activity of NADPH oxidase were enhanced by a high-glucose concentration or hypoxia

(Muzaffar *et al.* 2005, Ebrahimiyan *et al.* 2006, Taye *et al.* 2010). In this study, we showed that stimulation with a high-glucose concentration (30 mM) significantly increased gp91^{phox} expression in MSCs under hypoxic conditions (Fig. 9A and B), whereas a high-glucose concentration did not affect p22^{phox} and gp91^{phox} expression in MSCs at normoxia. A high-glucose concentration did not affect XO expression in MSCs under normoxic or hypoxic conditions (Fig. 9A and B). Mitochondrial superoxide was released from the electron respiratory chain via complexes I and III (Kudin *et al.* 2004), which were the main targets of hyperglycemia-induced injury (Rosca *et al.* 2005). This study showed that stimulation with a high-glucose concentration (30 mM) slightly increased the activity of complexes I and III in MSCs under hypoxic conditions (Fig. 9C). In contrast, we could not detect lipoxygenases, nitric oxide synthases, or tetrahydrobiopterin in the cell lysates of MSCs

Figure 10 Effects of a high-glucose concentration on proteasome activity in mouse mesenchymal stem cells under normoxic or hypoxic conditions. MSCs were treated with normal glucose (5.6 mM) or high glucose (11, 20, or 30 mM). After 24 h, the cells were incubated at normoxia (21% O₂) or in a hypoxic chamber (5% O₂) for 24 h. Proteasome activity in the cell lysates was evaluated by the Proteasome Activity Assay kit as described in section 'Materials and Methods'. Bar graphs show quantitative analysis of the cell lysates by the fluorometer. Data represent means $\pm$ S.E.M. ($n=4$). * $P<0.01$ versus MSCs treated with 5.6 mM glucose at normoxia. # $P<0.05$; ## $P<0.01$ versus hypoxic MSCs treated with 5.6 mM glucose.

stimulated by a high-glucose concentration under normoxic or hypoxic conditions (data not shown).

Flow cytometry analysis using superoxide-sensitive dihydroethidium showed that stimulation with a high-glucose concentration (30 mM) significantly increased intracellular superoxide level in MSCs at normoxia (Fig. 9D). Under hypoxic conditions, the effect of a high-glucose concentration (30 mM) was significantly enhanced. Tempol significantly suppressed superoxide production when stimulated by a high-glucose level in hypoxic MSCs.

Effect of a high-glucose concentration on proteasome activity in MSCs under hypoxic conditions

We tested the hypothesis that the inhibitory effect of high-glucose concentrations on the HIF-1 α protein levels in hypoxic MSCs was due to the enhancement of proteasome-mediated degradation. Proteasome activity at a normal glucose concentration (5.6 mM) under hypoxic conditions was significantly lower than that under normoxic conditions (Fig. 10). Stimulation with a high-glucose concentration recovered the decreased proteasome activity in hypoxic MSCs in a dose-dependent manner.

Discussion

This study demonstrates that in mouse MSCs, a high-glucose concentration significantly attenuated hypoxia-induced expression of HIF-1 α , VEGF-A₁₆₅, and

PDGF-B (Figs 3, 5, and 6). Treatment with Tempol, a superoxide scavenger, significantly reversed the attenuation of HIF-1 α , VEGF-A₁₆₅, and PDGF-B by a high-glucose concentration (Fig. 8). This study also found that stimulation with a high-glucose concentration significantly increases intracellular superoxide levels and proteasome activity in hypoxic MSCs (Figs 9D and 10). Yang *et al.* (2003) showed that in rat renal medullary interstitial cells, superoxide generators significantly inhibited HIF-1 α levels induced by hypoxia or proteasome inhibitors and Tempol significantly increased HIF-1 α levels. In addition, treatment with MG132, a specific inhibitor of proteasome activity, significantly reversed the attenuation of HIF-1 α at the protein level by a high-glucose concentration (Fig. 6). Thus, in hypoxic MSCs, the increase in the intracellular superoxide level by stimulation with a high-glucose concentration may cause HIF-1 α degradation. HIF-1 α regulates the transcription of VEGF-A and PDGF-B under hypoxic conditions in cultured mouse cells (Nilsson *et al.* 2004, Copple *et al.* 2009). We found that the suppression of HIF-1 α using directed siRNA significantly attenuated the expression of VEGF-A₁₆₅ and PDGF-B under hypoxic conditions (Fig. 8A–C). Although the inhibition of proteasome-mediated degradation by MG132 treatment reversed the attenuation of HIF-1 α , VEGF-A₁₆₅, and PDGF-B protein levels by a high-glucose concentration in hypoxic MSCs (Fig. 7D and E), HIF-1 α siRNA diminished the preventive effect of MG132. Thus, attenuation of hypoxia-induced VEGF-A₁₆₅ and PDGF-B expression may be due to the downregulation of hypoxia-induced HIF-1 α accumulation by glucose in high concentrations.

Stimulation with a high-glucose concentration (30 mM) significantly increased intracellular levels of ROS in endothelial cells at normoxia (Brownlee 2005). We also showed that stimulation with a high-glucose concentration (30 mM) significantly increased the activity of mitochondrial complex III and the intracellular superoxide level in MSCs under normoxic conditions (Figs 9C and 10). In contrast, severe hypoxia (2% O₂ or 0% O₂) alone increased the intracellular levels of ROS (Waypa *et al.* 2001, Gao & Wolin 2008), whereas we showed that moderate hypoxia (5% O₂) did not affect the expression of NADPH oxidase and XO, mitochondrial activity, or superoxide production (Figs 9 and 10). These disparate results may be due to the differences in the tested hypoxic conditions.

HIF-1 α mediates hypoxic induction of FGF-2 mRNA and protein accumulation in human umbilical vein endothelial cells (Calvani *et al.* 2006). In hypoxic human breast carcinoma MCF-7/ADR cells, FGF-2 gene expression is mediated through the c-Jun N-terminal kinase signal transduction pathway (Le & Corry 1999). In this study, FGF-2 protein levels were not affected by high-glucose concentrations in hypoxic MSCs (Fig. 3A and B). Thus, FGF-2 expression in hypoxic MSCs may be HIF-1 α independent.

Recently, Weil *et al.* (2009) showed that severe hypoxia (1% O₂) enhanced the production of VEGF in human

MSCs (hMSCs) by seven- to eight-fold, and that a high-glucose concentration (20 or 30 mM) did not affect VEGF production in severe hypoxia-treated hMSCs. In contrast, we showed that moderate hypoxia (5% O₂) in MSCs yielded a 30% increase in VEGF production compared with MSCs at normoxia, and that a high-glucose concentration (30 mM) yielded a 30% decrease in VEGF production in MSCs under moderate hypoxic conditions. We speculate that, under severe hypoxia, the induction of VEGF may be greater than the attenuation of VEGF by high-glucose concentrations. In fact, no significant differences in intracellular superoxide level in MSCs treated with a high-glucose concentration (30 mM) were noted between the 5% O₂ and 1% O₂ concentrations (unpublished data). Thus, HIF-1 α degradation by stimulation with high-glucose concentrations in 1% O₂ may not be greater than that under 5% O₂.

We analyzed the effect of a high-glucose concentration in MSCs under hypoxic conditions for a short time (6 or 24 h). *In vivo* studies by several investigators demonstrated that therapeutic angiogenesis was achieved 24 h after the implantation of bone-marrow-derived cells into ischemic regions (Kamihata *et al.* 2001, Onda *et al.* 2008). The therapeutic benefit of the cell therapy persists for several weeks. Thus, further studies will be required to reveal whether the effect of a high-glucose concentration in MSCs persists for longer periods under hypoxic conditions.

In summary, the current studies suggest that, in hypoxic MSCs, intracellular superoxide levels induced by a high-glucose concentration may attenuate hypoxia-induced expression of HIF-1 α , VEGF-A, and PDGF-B. The mechanism involved may serve to explain the fact that the paracrine effects of bone-marrow-derived cells under hypoxic conditions were impaired in diabetic patients. A high proportion of patients suffering acute stress such as stroke or myocardial infarction develop hyperglycemia, even in the absence of a preexisting diabetes diagnosis (Capes *et al.* 2001). Thus, acute hyperglycemia in patients with stroke or myocardial infarction may inhibit therapeutic angiogenesis by autologous bone-marrow-derived cells through induction of superoxide production. Cotreatment with antioxidants may ameliorate the impaired therapeutic efficacy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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