Characterization of bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice: Evaluation of the feasibility of dimethyloxallyl glycine as a mobilization agent

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Abstract. The prolyl hydroxylase inhibitor dimethyloxallyl glycine (DMOG) has been increasingly studied with regards to stem cell therapy. Previous studies have demonstrated that endogenous mesenchymal stem cells (MSCs) may be mobilized into peripheral circulation by pharmaceutical preconditioning. In addition, our previous study confirmed that DMOG, as a novel mobilization agent, could induce mouse/rat MSC migration into peripheral blood circulation. Therefore, the present study conducted studies to characterize bone marrow-derived MSCs (BM-MSCs) collected from mice following DMOG intraperitoneal injection. The surface antigen immune phenotype, differentiation capability, proliferative ability, migratory capacity and paracrine capacity of the BM-MSCs from mice preconditioned with DMOG. DBM-MSCs exhibited slightly distinct characteristics to BM-MSCs from normal saline-treated mice (NBM-MSCs); however, they may have therapeutic potential for future stem cell therapy. In addition, the present study suggested that DMOG may be used as a novel mobilization agent in future clinical trials as no adverse effects were observed.

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Abbreviations: DMOG, dimethyloxallyl glycine; MSCs, mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; DBM-MSCs, bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice; NBM-MSCs, bone marrow-derived mesenchymal stem cells from normal saline-treated mice; CCK-8, Cell Counting kit-8; ELISA, enzyme-linked immunosorbent assay; TGF, transforming growth factor; PDGF, platelet-derived growth factor; HIF-1α, hypoxia-inducible factor-1α; ICR, Male Institute of Cancer Research; NS, normal saline; DMEM/F-12, Dulbecco’s modified Eagle’s medium/Ham’s F-12; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMEM-LG, Dulbecco’s modified Eagle’s medium-low glucose; SDF-1α, stromal cell-derived factor-1α; PPARγ, peroxisome proliferator-activated receptor-γ

Key words: bone marrow-derived mesenchymal stem cells, dimethyloxallyl glycine, immune phenotype, proliferation, multilineage differentiation, migration, paracrine, mobilization agent

Introduction

The prolyl hydroxylase inhibitor dimethyloxallyl glycine (DMOG) is a type of hypoxia-mimetic agent, which has been increasingly studied with regards to stem cell therapy. DMOG, which is a small molecular drug, is a cell-permeable prolyl-4-hydroxylase inhibitor. At normal oxygen tension, DMOG is able to inhibit the effects of hypoxia-inducible factor prolyl hydroxylase, and stabilize the expression of hypoxia-inducible factor-1α (HIF-1α) in cells (1), thus mediating the function of signaling pathways associated with cellular or tissue alterations and repair.

A previous study regarding DMOG application reported that DMOG was able to induce cardioprotective effects via HIF-1α stabilization in rabbits (2). In the past decade, studies on DMOG have gradually increased with regards to its role in various cells and tissues. In recent studies, DMOG has been successfully used to increase bone healing capacity (3), attenuate renal injury in the remnant kidney model (4), induce angiogenesis in ischemic skeletal muscle (5), promote vascularization in the arterovenous loop via HIF-1α upregulation (6),
and provide neuroprotection in a middle cerebral artery occlusion model (7). Furthermore, it has been reported that DMOG may have an important role in mesenchymal stem cell (MSC) transplantation therapy for the functional recovery of ischemic heart disease (8).

MSCs are plastic-adherent, fibroblast-like adult stem cells, which possess the capacity to self-renew and differentiate into numerous types of cells, including adipocytes, osteoblasts, chondroblasts, myoblasts and neuron-like cells (9-11). In addition, MSCs can be obtained from various connective tissue sources, including bone marrow (12), adipose (13), dermal tissue (14), synovial fluid (15), deciduous teeth (16,17) and umbilical cord blood (18). Following adequate stimulation, stem and progenitor cells may leave the cell niche and enter the peripheral blood, which is termed stem cell mobilization (19). Although it has been confirmed that allograft MSCs, following in vitro amplification, can repair numerous types of tissue damage, it has been suggested that in vitro-amplified MSCs may induce the development of tumors (20), thus arousing doubts regarding the safety of allograft MSCs. Therefore, increasing attention has been paid with regards to the repairing effect of autologous MSCs. Unfortunately, the strategy by which endogenous MSCs are used to treat tissue damage is limited by the paucity of circulating MSCs in peripheral blood. Recently, pharmacological preconditioning has been proposed hypothetically and experimentally as an efficient approach for modification and improvement in the function of various organs, tissues and cells. Granulocyte colony-stimulating factor (G-CSF) is a potent stimulator of hematopoietic stem cells mobilization. Numerous studies have attempted to use G-CSF to induce MSC mobilization into the circulation, however, disappointing results have been obtained (21-23). Recent studies have reported that recombinant wingless-related integration site (Wnt)3a (24), insulin-like growth factor-1 + AMD3100 (25) and LiCl (26) mobilize MSCs into the circulation and promote the proliferation and differentiation ability of PB-MSCs. Our previous study confirmed that DMOG was able to mobilize MSCs into peripheral blood circulation (27). Peripheral blood-MSCs were demonstrated to have weaker proliferation and migration ability and similar multilineage differentiation potential when compared with bone marrow-MSCs and the mechanism underlying MSCs mobilization was investigated (28).

MSC mobilization is commonly measured using the fibroblast-colony forming unit assay and flow cytometry. Our previous study demonstrated that compared with normal saline-treated mice, the number of colony forming units and percentage of cluster of differentiation (CD90+/CD45–) cells in the peripheral blood was significantly increased in mice treated with DMOG (27).

Whether DMOG may be feasible as a novel mobilization agent remains to be elucidated. Since bone marrow is one of the most important sources of mobilized peripheral blood MSCs, the present study conducted preliminary studies to characterize BM-MSCs collected from mice following DMOG intraperitoneal injection. The biological properties of BM-MSCs from DMOG preconditioned-mice (DBM-MSCs), including cell morphology, immune phenotype, multilineage differentiation, proliferation, migration and paracrine capacity, were investigated and compared with the properties of BM-MSCs from normal saline-treated mice (NBM-MSCs). The results may provide an experimental basis for the application of DMOG as a novel mobilization agent in future clinical trials.

Materials and methods

Animals and DMOG preconditioning. The present study was approved by the ethics committee of Zhejiang Chinese Medical University (Hangzhou, China). Male Institute of Cancer Research (ICR) mice (age, 8-10 weeks) were purchased from the Zhejiang Chinese Medical University Animal Center (Hangzhou, China; Laboratory Animal Certificate: SCXK 2008-0115). All procedures related to the care of animals were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (29).

ICR mice were randomly assigned into the normal saline control group (NS group) or the DMOG preconditioning group (DMOG group; n=5/group). Mice of the DMOG group received an intraperitoneal injection of DMOG (40 mg/kg in 0.5 ml normal saline; Cayman Chemical Company, Ann Arbor, MI, USA) for 7 consecutive days, whereas mice in the NS group received 0.5 ml normal saline. The DMOG dose was chosen according to our previous study (27).

Isolation and culture of BM-MSCs. Following DMOG or normal saline preconditioning, mice were sacrificed by cervical dislocation and BM-MSCs were obtained from the mice femur and tibia, as previously described (30). Briefly, muscles and adherent tissue were detached, and the epiphyses were removed. The whole bone marrow plugs were flushed using a 25-gauge needle and a 1.0 ml syringe loaded with Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (HyClone; Thermo Fisher Scientific, Inc., Logan, UT, USA). Harvested marrow cells were centrifuged, resuspended, counted, and cultured as described previously (30). All non-adherent cells were removed by media replacement after 24 h. Subsequently, the medium was replaced every 3-4 days. Upon reaching 80-90% confluence, the primary cells were trypsinized (0.25% trypsin-EDTA; Gibco; Thermo Fisher Scientific, Inc.), resuspended in complete culture medium, and subcultured at a 1:2 ratio. BM-MSCs from passages 3-4 were used in the subsequent experiments.

For identification of BM-MSCs, a colony-forming unit fibroblast assay was conducted, and cell surface markers CD90 and CD45 were detected by flow cytometry. The detailed characteristics were confirmed in our previous study (27). CD44+/CD90+/CD45– cells were detected and selected in the present study.

Flow cytometry assay. To detect the effects of DMOG on the immune phenotype of BM-MSCs, flow cytometry was conducted. The fourth generation NBM-MSCs and DBM-MSCs were suspended in phosphate-buffered saline (PBS) at a concentration of 1x10⁶ cells/ml in five tubes. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) isotype controls were added into the two tubes as controls, whereas 2 µl CD44-FITC, 5 µl CD90-PE and 2 µl CD45-FITC...
were added into the three remaining tubes, respectively (all obtained from Abcam (Hong Kong) Ltd., Hong Kong, China). Following a 30 min incubation at room temperature the cells were washed twice with PBS. Subsequently, a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Cell Quest software (version 7.5.3; BD Biosciences) were used to test and analyze the results.

**Multilineage differentiation assay.** The multilineage differentiation potential of the NBM-MSCs and DBM-MSCs was evaluated in detail, as described in the following subsections.

**Adipogenic differentiation.** NBM-MSCs and DBM-MSCs were seeded in 6-well plates at a density of $2 \times 10^4$ cells/cm$^2$. Once the cells reached 90-100% confluence, ICR mouse MSC adipogenic differentiation medium [Cyagen Biosciences (Guangzhou), Inc., Guangzhou, China] was added, and the medium was changed every 3 days. Adipogenic differentiation was assessed in detail after a 21-day incubation period as follows: (a) Oil Red O solution [Cyagen Biosciences (Guangzhou), Inc.] were observed under an inverted fluorescence microscope (ECLIPSE TE2000-S; Nikon Corporation, Tokyo, Japan).

**Osteogenic differentiation.** NBM-MSCs and DBM-MSCs (1 $\times 10^4$ cells/cm$^2$) were seeded in 6-well plates in DMEM-low glucose (DMEM-LG; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 0.1 $\mu$M dexamethasone, 10 $\mu$M β-glycerol phosphate and 50 $\mu$M ascorbic acid (all Sigma-Aldrich, St. Louis, MO, USA), and the medium was replaced every 3 days. Cell morphology was observed under an inverted fluorescence microscope (ECLIPSE TE2000-S). Von Kossa (Sigma-Aldrich) staining was used to reveal mineralized areas.

**Chondrogenic differentiation.** NBM-MSCs and DBM-MSCs were resuspended in serum-free DMEM-high glucose (Gibco; Thermo Fisher Scientific, Inc.) containing insulin-transferrin-selenium-A (Gibco; Thermo Fisher Scientific, Inc.), 0.1 $\mu$M dexamethasone and 200 $\mu$M ascorbic acid, and were plated in 6-well plates at a density of $5 \times 10^4$ cells/ml. Subsequently, 10 ng/ml transforming growth factor (TGF)-β1 and TGF-β3 (PeproTech, Rocky Hill, NJ, USA) were added to the cells. The medium was changed every 3 days, and cell factors were renewed each time. Cells were stained with Toluidine Blue (Sigma-Aldrich) after 21 days and visualized under an inverted fluorescence microscope (ECLIPSE TE2000-S).

**Neuronal differentiation.** The following neuronal differentiation induction media were used in the present study: Pre-induction medium (DMEM-LG containing 1 mM β-mercaptoethanol and 20% FBS), and induction medium (serum-free DMEM-LG containing 5 mM β-mercaptoethanol). NBM-MSCs and DBM-MSCs were plated at a density of $1 \times 10^5$ cells/well on 18x18 mm slides on 6-well plates. Once the cells reached ~70% confluence, differentiation was successively induced by incubation with pre-induction medium for 24 h, followed by incubation with induction medium for 3-6 h.

**Immunofluorescence.** Immunofluorescence assay was used to identify the expression of the nerve cell-specific marker Nestin, and the astrocyte-specific marker glial fibrillary acidic protein (GFAP). Briefly, slides were gently washed twice with PBS, and were fixed with immune dyeing fixative (Hangzhou Dawen Biotec Co., Ltd., Hangzhou, China) for 10 min. Subsequently, immune dyeing wash buffer (Hangzhou Dawen Biotec Co., Ltd.) was used to wash the slides, and the slides were blocked with immune dyeing block buffers (Hangzhou Dawen Biotec Co., Ltd.) for 60 min. The following liquid was aspirated, and the slides were then incubated with polyclonal rabbit anti-mouse Nestin (1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-20978) and GFAP (1:100; Santa Cruz Biotechnology, Inc.; cat. no. sc-9065) primary antibodies for 90 min at room temperature. Following three washes, FITC-conjugated goat polyclonal anti-rabbit immunoglobulin G-H&L secondary antibody [1:100; Abcam (Hong Kong) Ltd.; cat. no. ab97050] was added to the slides and incubated for 30 min. The slides were rinsed three times, and were incubated with 4,6-diamidino-2-phenylindole for 5 min. Following a further three washes with PBS, the specimens were sealed with glycerin and observed under an inverted fluorescence microscope (ECLIPSE TE2000-S).

**Cell Counting kit (CCK)-8 cell proliferation assay.** In order to determine the influence of DMOG on BM-MSC proliferation, NBM-MSCs and DBM-MSCs were trypsinized, neutralized, and centrifuged at 100 x g for 6 min. Resuspended in fresh complete medium, a 200 $\mu$l cell suspension was added to 96-well plates at a density of $2 \times 10^4$ cells/well. After 24 h, the cells were washed twice with PBS, and were fixed with immune dyeing fixative (Hangzhou Dawen Biotec Co., Ltd., Hangzhou, China) for 10 min. Subsequently, immune dyeing wash buffer (Hangzhou Dawen Biotec Co., Ltd.) was used to wash the slides, and the slides were blocked with immune dyeing block buffers (Hangzhou Dawen Biotec Co., Ltd.) for 60 min. The following liquid was aspirated, and the slides were then incubated with polyclonal rabbit anti-mouse Nestin (1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-20978) and GFAP (1:100; Santa Cruz Biotechnology, Inc.; cat. no. sc-9065) primary antibodies for 90 min at room temperature. Following three washes, FITC-conjugated goat polyclonal anti-rabbit immunoglobulin G-H&L secondary antibody [1:100; Abcam (Hong Kong) Ltd.; cat. no. ab97050] was added to the slides and incubated for 30 min. The slides were rinsed three times, and were incubated with 4,6-diamidino-2-phenylindole for 5 min. Following a further three washes with PBS, the specimens were sealed with glycerin and observed under an inverted fluorescence microscope (ECLIPSE TE2000-S).

**Cell migration assay.** In order to determine the effects of DMOG on BM-MSCs migration capacity, a Transwell assay was conducted. NBM-MSCs and DBM-MSCs were resuspended in DMEM containing 2% FBS. Briefly, 600 $\mu$l culture medium containing 150 ng/ml stromal cell-derived factor-1α (SDF-1α; PeproTech) was placed into the lower chamber of the 24-well plate, and 150 $\mu$l cell suspension ($1 \times 10^4$ cells/ml) was plated into the upper chamber of the 24-well plate that contained Transwell inserts (diameter, 6.5 mm; pore size, 8 $\mu$m; Corning, Inc., Corning, NY, USA). Following a 15 h incubation, the inside compartments were removed and a cotton swab was used to remove the cells. Following fixation with 4% paraformaldehyde, crystal violet staining and flushing with double distilled water, the cells that had migrated to the lower chamber were observed under a microscope. The migrated cells were counted in five random microscope fields (ECLIPSE TE2000-S).

**Enzyme-linked immunosorbent assay (ELISA).** An ELISA was performed to analyze the paracrine capacity of the cells. NBM-MSCs and DBM-MSCs were plated in 12-well plates at a density of $5 \times 10^4$ cells/well. The medium was discarded after 24 h and the cells were washed twice with PBS. Subsequently,
500 µl serum-free DMEM-LG was added and the supernatants were collected after 48 h. TGF and platelet-derived growth factor (PDGF) concentration was detected using corresponding ELISA kits. Mouse TGF (β IG-H3) and PDGF ELISA kits (Wuhan Boster Biological Co., Ltd., Wuhan, China) were used, according to the manufacturer’s protocol.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 statistical software (SPSS IBM, Armonk, NY, USA). Experiments were repeated at least three times and data are presented as the mean ± standard deviation. Student’s two-tailed t-test was used to compare the two independent experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology, immune phenotype and multilineage differentiation ability is similar in DBM-MSCs and NBM-MSCs. To detect the immune phenotype and differentiation potential of the cells, NBM-MSCs and DBM-MSCs were cultured in parallel. The homogeneous layer of fibroblast-like BM-MSCs obtained from DMOG-preconditioned mice was similar to that obtained from NS-treated mice (Fig. 1A).

The cell surface antigen expression of NBM-MSCs and DBM-MSCs was analyzed by flow cytometry for three samples. The two types of cells were positive for CD44 (93.8 and 97.9%, respectively) and CD90 (93.5 and 96.6%, respectively), but not CD45 (2.59 and 1.70%, respectively). Experiments were performed in triplicate, and there was no significant difference between them (P>0.05). NBM-MSCs, bone marrow-derived mesenchymal stem cells from normal saline-treated mice; DBM-MSCs, bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice; CD, cluster of differentiation.

Figure 1. Morphology and immune phenotype of NBM-MSCs and DBM-MSCs. (A) NBM-MSCs and DBM-MSCs from passage 3 were observed under an inverted fluorescence microscope. Scale bar, 500 µm. (B) Surface antigen markers on passage 3 NBM-MSCs and DBM-MSCs were detected by flow cytometry. NBM-MSCs and DBM-MSCs expressed CD44 (93.8 and 97.9%, respectively) and CD90 (93.5 and 96.6%, respectively), but not CD45 (2.59 and 1.70%, respectively). Experiments were performed in triplicate, and there was no significant difference between them (P>0.05). NBM-MSCs, bone marrow-derived mesenchymal stem cells from normal saline-treated mice; DBM-MSCs, bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice; CD, cluster of differentiation.
for 14-21 days. Subsequently, the presence of cytoplasmic olesomes, the formation of calcium precipitation, or the secretion of acid glycosaminoglycan was examined by specific staining. NBM-MSCs and DBM-MSCs were able to be differentiated into adipocytes that contained secreting orange-red lipid droplets, osteoblasts that are surrounded by black calcium precipitation and chondrocytes that have cell matrix that dyes purple and nuclei that stain blue (Fig. 2A). In addition, DBM-MSCs induced by β-mercaptoethanol for 5 h were immunocytochemically shown to display typical neuron-like characteristics, and express nerve cell-specific marker Nestin, but not astrocyte-specific marker GFAP (Fig. 2B; data not shown). These results suggest that the multilineage differentiation capacity of DBM-MSCs was similar to that of NBM-MSCs.

Cell proliferation is slightly reduced in DBM-MSCs compared with NBM-MSCs. To determine the influence of DMOG on MSCs proliferation, the number of MSCs derived from the two

Figure 2. Multilineage differentiation capacity of NBM-MSCs and DBM-MSCs. (A) NBM-MSCs and DBM-MSCs were cultured in Ad., Os., or Ch. differentiation medium. Adipogenic differentiation was stained with Oil Red O. Osteogenic differentiation was stained with Von Kossa. Chondrogenic differentiation was stained with Toluidine Blue. (Magnification, x100). (B) NBM-MSCs and DBM-MSCs were induced into neuron-like cells. Immunofluorescence staining: Nuclei were dyed blue with DAPI and Nestin expression was dyed green. (Magnification, x200). NBM-MSCs, bone marrow-derived mesenchymal stem cells from normal saline-treated mice; DBM-MSCs, bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice; Ad., adipogenic; Os., osteogenic; Ch., chondrogenic; DAPI, 4',6-diamidino-2-phenylindole.

Figure 3. Cell proliferation was detected using the CCK-8 assay. There were significant differences from day 3 to day 7 between the NBM-MSCs and DBM-MSCs. Data are presented as the mean ± standard deviation. *P<0.05. CCK-8, Cell Counting kit-8; BM-MSCs, bone marrow-derived mesenchymal stem cells from normal saline-treated mice; DBM-MSCs, bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice; OD, optical density.
groups was measured using the CCK-8 test. DBM-MSCs were in a latent period of growth between days 1 and 3, exhibited logarithmic growth between days 4 and 6, and reached a plateau after day 6. Compared with NBM-MSCs, the proliferation rate of DBM-MSCs was reduced, and starting from day 3 the total number of cells was significantly decreased compared with the number of NBM-MSCs at the same time (P=0.003, P=0.001, P=0.004, P=0.007 and P=0.003 for day 3 to day 7, respectively). The results also indicated that DBM-MSCs had a similar latent period, logarithmic phase and plateau to the NBM-MSCs (Fig. 3). Both cell growth curves were ‘S’-shaped; however, the proliferative ability of DBM-MSCs was slightly reduced compared with NBM-MSCs.

Migratory ability is slightly reduced in DBM-MSCs compared with NBM-MSCs. The Transwell assay was used to compare differences in migratory ability between DBM-MSCs and NBM-MSCs. As shown in Fig. 4A, the migrated cells from the two groups were detected (Fig. 4A). Results indicated that following 15 h of SDF-1α as a chemoattractant, the number of DBM-MSCs that had migrated to the lower compartment was reduced compared with the number of NBM-MSCs (92.00±4.85 vs. 101.40±5.18; n=5; P=0.018; Fig. 4B). These results suggest that the migratory ability of DBM-MSCs was inferior to that of NBM-MSCs.

DBM-MSCs exhibit a similar TGF and PDGF secretory capacity compared with NBM-MSCs. In order to determine whether cytokine production was affected by DMOG, the TGF and PDGF cytokine concentrations were detected in DBM-MSCs and NBM-MSCs by ELISA. Compared with NBM-MSCs, TGF secretion of DBM-MSCs was decreased; however, there was no significant difference between the groups (689.9±40.2 vs. 746.4±43.8 ng/ml; P=0.066; Fig. 5A). Conversely, PDGF secretion of DBM-MSCs was increased; however, this difference was also not significant (1323.5±110.3 vs. 1207.9±98.7 ng/ml; P=0.119; Fig. 5B). These results indicate that DBM-MSCs have a similar secretory capacity with regards to TGF and PDGF, as compared with NBM-MSCs.

**Discussion**

Our previous study revealed that MSCs could be mobilized into peripheral blood circulation by hypoxia induction, and the transcription factor HIF-1α had a pivotal role in hypoxia-induced MSCs mobilization (31). Considering clinical and ethical safety, hypoxia mobilization is not feasible in clinical therapy. However, it has been reported that similar effects may be obtained using the prolyl hydroxylase inhibitor DMOG (32), which is a type of hypoxia-mimetic agent that stabilizes and upregulates HIF-1 signaling under normoxic conditions.

The present study investigated the biological properties of BM-MSCs obtained from ICR mice preconditioned with DMOG. Previous studies have mainly investigated the effects of DMOG by directly preconditioning stem cells in vitro, in order to assess the benefits (3,8). However, in the present study,
MSCs constantly update

The most common method used to identify whether analyzed cell populations are capable of multilineage differentiation. Wnt and Rho are the main signaling pathways associated with regulation of adipogenic differentiation of MSCs (37). Adipogenic stimuli induce terminal differentiation of committed preadipocytes via the epigenomic activation of peroxisome proliferator-activated receptor-γ (PPARγ). The coordination of PPARγ with CCAAT/enhancer-binding protein transcription factors is able to maintain adipocyte gene expression (38). In addition, Wnt, Notch and bone morphogenetic protein signaling has an important role in the regulation of MSCs osteogenic differentiation (39). DMOG has been reported to increase the bone healing capacity of adipose-derived MSCs by promoting osteogenic differentiation and angiogenic potential in rat critical-sized calvarial defects (3). In addition, a previous study suggested that TGF-β signals have a pivotal role in chondrogenic differentiation (40). Hypoxia-enhanced chondrogenesis of BM-MSCs has also been reported to occur via activation of the mitogen-activated protein kinase P38 pathway (41). Furthermore, sirotn 1 activation may be essential for the induction of neuronal differentiation, due to its effects on mammalian target of rapamycin downregulation and neurite outgrowth stimulation (42). In the present study, compared with NBM-MSCs, DBM-MSCs exhibited similar adipogenic, osteogenic, chondrogenic and neuronal differentiation abilities, thus suggesting that DMOG had no obvious stimulatory or inhibitory effects on BM-MSCs multilineage differentiation. While previous studies have predominantly investigated the effects of DMOG by directly preconditioning stem cells in vitro (3,8), the present study investigated the biological properties of BM-MSCs obtained from ICR mice preconditioned with DMOG. By contrast to in vitro treatment, the current study hypothesizes the in vivo treatment would be influenced by complex metabolic reactions in the animal body. No notable change in the differentiation ability of BM-MSCs was observed in the present in vitro culture and our previous study also demonstrated DMOG could mobilize MSCs to the peripheral blood with no effect on differentiation in pretreated mice (28). Thus, DMOG appears to be feasible as a stem cell mobilization agent.

A cell growth curve was generated using the CCK-8 assay, and the proliferative ability of DBM-MSCs was slightly reduced, compared with that of NBM-MSCs. These results suggested that DMOG slightly inhibited BM-MSCs proliferation. However, DMOG treatment maintained a normal growth curve, thus suggesting that DMOG had no obvious cytotoxic effects on BM-MSCs as only a slightly reduced proliferation was observed. This result is similar to the findings of a previous study on the effects of DMOG on adipose-derived MSCs (3). In the in vivo microenvironment, MSCs constantly update themselves, with the majority of cells maintained in the latent period of growth. There are few studies that have reported the effects of DMOG on MSCs proliferation. A previous study demonstrated that DMOG was able to inhibit the proliferation of vascular smooth muscle cells in vitro (43). Furthermore, it has been reported that DMOG may significantly reduce the apoptosis of MSCs, stabilize the expression of HIF-1α to induce glucose transport protein synthesis, and reduce the release of mitochondrial cytochrome c, thus promoting protein kinase phosphorylation (44). Therefore, the reduction in the proliferative ability of DBM-MSCs may be associated with the varying expression levels of proteins involved in cell cycle regulation.
The present study detected the migratory capacity of DBM-MSCs, and SDF-1α was used as a chemotaxon. The results demonstrated that DBM-MSCs possessed weaker migratory ability compared with NBM-MSCs. Our research group and others have revealed that the SDF-1α/CXC chemokine receptor (CXCR) axis has an important role in mediating MSCs migration (45,46). In addition, Wang et al. (47) indicated that CXCR4- and CXCR7 receptors were co-expressed in BM-MSCs and synergistically promoted BM-MSC migration. However, the in vitro migration assay employed in the present study may not directly mimic the in vivo conditions necessary for BM-MSCs migration. Hu et al. (48) demonstrated that pretreatment of the BM-MSCs with the CXCR4 antagonist AMD3100 significantly inhibited the mobilization of BM-MSCs in vitro and in vivo. Therefore, the decreased migratory capacity of DBM-MSCs may be associated with reduced CXCR expression.

Paracrine capacity is one of the main mechanisms by which MSCs exert their functions on damage repair, blood vessel formation and blood supply (49). In the present study, TGF and PDGF concentrations were detected in cell culture supernatants. DBM-MSCs exhibited reduced TGF secretion and increased PDGF secretion compared with NBM-MSCs. Ng et al. (50) reported that paracrine TGF-β and PDGF signaling was essential for MSCs differentiation and proliferation. TGF-β is significantly associated with chondrogenesis, whereas PDGF is significantly associated with adipogenesis and chondrogenesis. Although there were differences in cytokine secretion between DBM-MSCs and NBM-MSCs, no statistical significance was detected.

In conclusion, the results of the present study provide a novel insight into the biological changes of BM-MSCs obtained from mice preconditioned with DMOG. DBM-MSCs were similar in aspects of cell morphology, immune phenotype, multilineage differentiation, and TGF and PDGF secretion, but were slightly distinct with regards to proliferative and migratory capacity compared with NBM-MSCs; however, they may have therapeutic potential for future stem cell therapy. In addition, the present study suggested that DMOG may be used as a novel mobilization agent in future clinical trials as no adverse effects were observed with the mobilization of MSCs to the peripheral blood.

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