The Role of Chemokines in Proangiogenic Action Induced by Human Adipose Tissue-Derived Mesenchymal Stem Cells in the Murine Model of Hindlimb Ischemia

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Key Words

hASCs • GCP2 • MCP1 • Capillary-like structure formation • Proangiogenic action

Abstract

The proangiogenic action of human adipose tissue-derived mesenchymal stem cells (hASCs) transplantation has been shown to be mediated by secretory factors. In this study, we determined if human granulocyte chemotactic protein-2(GCP2) or monocyte chemoattractant protein-1(MCP1) is involved in the proangiogenic action of hASCs transplantation in the hindlimb ischemia model. hASCs secrete GCP2 and MCP1, which leads to increased tubule formation. The downregulation of GCP2 or MCP1 decreased MCP1 and GCP2 secretion, respectively, whereas the external addition of GCP2 or MCP1 increased MCP1 and GCP2, respectively. Additionally, the treatment of GCP2 and MCP1 increased VEGF secretion, while the downregulation of GCP2 and MCP1 showed the opposite effect on VEGF secretion. Downregulation of GCP2 and MCP1 expression also inhibited hASCs-induced proangiogenic action, while the overexpression of GCP2 increased it. Finally, the downregulation of

MCP1 or VEGF inhibited the GCP2 overexpression-induced increase in blood flow recovery. Taken together, these data indicate that the proangiogenic action of hASCs transplantation is mediated by the interaction between GCP2, MCP1 and VEGF, which are secreted from the transplanted cells.

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Introduction

Angiogenesis is an essential biological event encountered in a number of physiological and pathological processes such as embryonic development, wound healing, chronic inflammation and the growth of malignant solid tumors [1-5]. Angiogenesis also plays important roles in tissue regeneration. The early phase of skeletal muscle regeneration is dependent on the invasion of blood vessels into ischemic muscles before regeneration occurs in the hindlimb ischemia model [6].

Chemokines constitute a large family of chemotactic cytokines that regulate cell migration during physiological and inflammatory processes. Chemokines are important for host defense, and also play fundamental roles in the development and homeostasis of the immune system. In addition, chemokines are involved in angiogenesis and

tumorigenesis [7]. CXC chemokines, which act as the regulatory link between inflammation and angiogenesis, provide a fine and coordinated regulation of angiogenesis and inflammation that leads to tissue repair [8].

Mesenchymal stem cells (MSCs) are present in various tissues including bone marrow, peripheral blood and adipose tissues. MSCs are pluripotent and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [9-11]; therefore, MSCs can be used for a wide range of tissue engineering and gene therapy applications [12]. Recently, MSCs were shown to form capillary-like structures in an *in vitro* Matrigel assay [13], and MSCs transplantation was found to induce neovascularization in a hindlimb ischemia model [14]. This proangiogenic action of hMSCs is primarily due to the release of various factors from the transplanted hMSCs [15, 16]. It has also been reported that MSCs derived from adipose tissues have characteristics similar to bone marrow stromal cells (BMSCs) [17, 18]. Moreover, MSCs derived from human adipose tissues have been shown to be differentiated into endothelial cells [19, 20] and to have a proangiogenic effect in the hindlimb ischemia model [19-21]. Additionally, culture expanded human adipose tissue-derived mesenchymal stem cells (hASCs) were found to have a proangiogenic effect in a murine model of hindlimb ischemia, and the differentiation efficiency of the remaining cells that were transplanted into the endothelial or skeletal muscle cells was low [21-24]. Therefore, the proangiogenic action of hASCs is believed to be primarily mediated by the paracrine effects of chemokines or cytokines that are released from the transplanted cells. Data represent the mean $\pm$ SEM of three different experiments [21, 22, 25]. However, the mechanisms by which various factors mediate the paracrine effects have not yet been elucidated.

In this study, we determined if GCP2 or MCP1 is involved in the proangiogenic action induced by hASCs in the hindlimb ischemia model of nude mice.

Materials and Methods

Cell Culture

hASCs were cultured as described by Lee et al. [17]. All protocols involving human subjects were approved by the Institutional Review Board of Pusan National University, and all subjects provided informed consent. Adipose tissues were obtained from 3 different Korean patients (a 50 year-old male patient, a 45 year-old female patient and a 55 year-old female patient) who had undergone abdominoplasty without any

underlying diseases. To isolate hASCs, adipose tissue samples were washed with phosphate-buffered saline (PBS) and then digested at 37°C for 30 minutes with 0.075% type I collagenase. The enzyme activity was then neutralized using α -modified Eagle's medium (α -MEM) containing 10% fetal bovine serum (FBS). Next, the samples were centrifuged at 1200 $\times$ g for 10 minutes, after which the pellet was incubated overnight at 37°C under 5% CO₂ in a control medium (α -MEM, 10% FBS, 100 units/ml of penicillin and 100 μ g/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove any residual nonadherent cells and then maintained at 37°C under 5% CO₂ in the control medium.

Reagents

Recombinant human GCP2/CXCL6, VEGF and MCP1/CCL2 were purchased from R&D systems (Minneapolis, MN, USA).

In vitro tube formation assay

The *in vitro* tube formation assay was conducted using Matrigel (Chemicon, Temecula, CA). Briefly, thawed Matrigel was added to 96-well plates and allowed to solidify at room temperature for 1 hour. The cells were then seeded at a density of 1×10^4 cells per well and subsequently induced to differentiate into endothelium by plating on a 96-well Matrigel-coated plate with EGM-2-MV (EBM2, Endothelial Basal Medium2) which contains growth factors (hydrocortisone, hEGF, FGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and gentamicin) for 6 days. The capillary tube formations were visualized after 12 h. Digital images of 5 random microscopic fields per well were acquired using a Leica DM IRB microscope (Leica Microsystems GmbH, Wetzlar, Germany) and analyzed using the Leica IM50 software. The tube formation was defined by counting the branch points of the formed tubes and the average numbers of branch points were calculated.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplex oligo (on-TARGET plus SMART pool, Thermo Fisher Scientific (Dharmacon), Rockford, IL) of the targeting genes (GCP2, VEGF and MCP1) mRNA or non-targeting duplex oligo (on-TARGET plus siCONTROL, Thermo Fisher Scientific) as a negative control were transfected into hASCs using DharmaFECT Transfection Reagent.

Viral vector construction and transduction

The GCP2 gene on the donor plasmid pCMV $\cdot$ SPORT6-GCP2 (21C Frontier human gene bank) was recombined into the destination vector pLenti6/V5 using rapid BP/LR clonase (Invitrogen, Carlsbad, CA), which resulted in generation of the pLenti6/V5-GCP2 plasmid. The plasmids were sequenced to verify the construct. Replication-defective lentiviruses were produced by transient transfection of 293T cells using Lipofectamine Plus (Invitrogen), lentivirus vectors (pLenti6/V5 and pLenti6/V5-GCP2) and packaging mix (Invitrogen). After transfection, samples of the viruses were harvested at 48 and 72h and filtered through a Millex-HV 0.45 μ m PVDF filter (Millipore, Billerica, MA) and kept at -80°C until use.

Fig. 1. Expression of GCP2, MCP1 and VEGF in hASCs. (A) Total RNAs were isolated from hASCs and cDNA was synthesized with reverse transcriptase. PCR products were separated by agarose gel electrophoresis. GAPDH was amplified as a positive control. (B) GCP-2, MCP1 and VEGF secretion by hASCs. Conditioned media from cell cultures was analyzed by ELISA to determine the GCP2, MCP1 and VEGF concentrations. Data represent the mean $\pm$ SEM of three different experiments. (C) Effects of GCP2 and MCP1 on capillary-like tube formation of hASCs in Matrigel. Capillary-like tube formation of hASCs was induced by culture on EGM-2-MV media in the absence (Cont) or presence of 25ng/ml GCP2 and MCP1 for 12h (Magnification, $\times$ 100). The experiments were performed using hASCs that were isolated from three different donors and the representative photograph was shown. (D) Determination of branching points in tubule formation assay. Data represent the mean $\pm$ SEM of three different experiments. * $p < 0.05$ significantly different from control hASCs (Cont).

Transduction of the hASCs was performed by 6 hours of exposure to dilutions of viral supernatant in the presence of polybrene (5 μ g/ml). The transductants were then selected by culture in blasticidin (10 μ g/ml, Invitrogen).

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction Analysis

For RT-PCR assays, the total cellular RNA was isolated from the cells and reverse transcribed using the conventional protocols. PCR amplification was performed using the following primer sets: GAPDH (GeneBank accession No. NM_002046) 5'-TCC ATG ACA ACT TTG GTA TCG-3', 5'-TGT AGC CAA ATT CGT TGT CA-3'; GCP2 (GeneBank accession No. NM_002993), 5'-GTC CTT CGG GCT CCT TGT-3', 5'-AAC TTG CTT CCC GTT CTT CA-3'; MCP1 (GeneBank accession No. NM_002982), 5'-CCC CAG TCA CCT GCT GTT AT-3', 5'-GAG TTT GGG TTT GCT TGT CC-3'; VEGF (GeneBank accession No. NM_001025370), 5'-AAG GAG GAG GGC AGA ATC AT-3', 5'-ATC TGC ATG GTG ATG TTG GA-3'; CXCR1 (GeneBank accession No. NM_000634), 5'-AGT CCA TTG GGC AGG CAG A-3', 5'-ATT CTA CTG CAG GAA CAG CCA ACA-3'; CXCR2 (GeneBank accession No. NM_001557), 5'-CAA TGA ATG AAT GAA TGG CTA AG-3', 5'-AAA GTT TTC AAG GTT CGT CCG TGTT -3'; CCR2 (GeneBank accession No. NM_000647), 5'-CTA CCT TCC AGT TCC TCA TTT TT-3', 5'-ACA TTT ACA AGT TGC AGT TTT CAG C-3'. All of the primer sequences were determined using established GenBank sequences. The designed primers were used to amplify the duplicate PCR reactions, and GAPDH was used as a control.

For real-time -PCR assays, the primer sequences used in the experiment were as follows: β -actin (GeneBank accession No. NM_001101), 5'-CTG GTG CCT GGG GCG-3', 5'-AGC CTC GCC TTT GCC GA-3'; GCP2 (GeneBank accession No. NM_002993), 5'-GTC CTT CGG GCT CCT TGT-3', 5'-AAC TTG CTT CCC GTT CTT CA-3'. Real-time quantitation was done as described [26].

ELISA

To obtain conditioned media, hASCs were incubated with serum-free culture media for 2 days. The incubation of hASCs in serum-free media for 2 days did not affect on cell viability which was determined by trypan blue exclusion test (data not shown). The concentrations of GCP2, MCP1 and VEGF in the conditioned media were then determined using ELISA kits for GCP2, MCP1 and VEGF (R&D Systems) in accordance with the manufacturer's instructions.

Hindlimb ischemia model and cell transplantation

The animal protocol used in this study has been reviewed and approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC) for ethical procedures and scientific care. The neovascularization capacity of the cells was investigated in a murine model of hindlimb ischemia that was made by the ligation of the proximal femoral artery in 8 week-old Balb/C nude mice (Sam: TacN (SD) fbr)[22]. Animals were anesthetized with pentobarbital sodium (0.5 mg/g), and to produce hindlimb ischemia, the proximal portion of the femoral artery, including the superficial and deep branches, was ligated twice using a 7-0 silk suture. After 24 hours, 1×10^6 hASCs (180 μ l of cell suspension in PBS) were injected intramuscularly at three different sites of ischemic legs. The cells were intramuscularly transplanted 24 hours after ligation of the femoral artery. After two weeks, a laser Doppler blood flowmeter (Omegaflow FLO-CI, Omegawave Inc., Osaka, Japan) was used to measure the cutaneous blood flow. The mice were placed on a heating plate at 37°C prior to measurement of blood flow.

Immunohistochemistry

The mice were sacrificed and perfused with 4% (w/v) paraformaldehyde (Sigma, St Louis, MO, USA) after the blood flow was determined. The ischemic hindlimb muscles were then removed and fixed in neutral buffered 4% (w/v) paraformal-

Fig. 2. Interaction between GCP2, MCP1 and VEGF secretion in hASCs. (A) GCP2, MCP1 and VEGF expression in GCP2, MCP1 and VEGF siRNA or non-target siRNA-transfected hASCs was evaluated by RT-PCR. (B) The effects of GCP2, MCP1 and VEGF downregulation on the secretion of various factors by hASCs. hASCs were transfected with GCP2, MCP1 and VEGF siRNA or non-target siRNA oligos. (C) The effects of the addition of GCP2, MCP1 and VEGF on cytokines secretion by hASCs. Confluent monolayers of hASCs were treated for 48 h with 25ng/ml GCP2, MCP1 and VEGF. Data represent the mean $\pm$ SEM of three different experiments. * p <0.05 significantly different from control hASCs. (D) Effect of MCP1 and VEGF downregulation on cytokine secretion by GCP2-overexpressing hASCs. GCP2-overexpressing hASCs were transfected with MCP1 and VEGF siRNA or non-target siRNA oligos. Conditioned media from cultured cells was analyzed by ELISA to determine the concentrations GCP2, MCP1 and VEGF. Data represent the mean $\pm$ SEM of three different experiments. * p <0.05 significantly different from control lentivirus-transduced cells (LV). # p <0.05 significantly different from GCP2-overexpressing cells (LV-GCP2).

dehydrate at 4°C for 24 hours prior to embedding in paraffin and sectioning. Transverse muscular sections (5 μ m) were deparaffinized and an antigen retrieval was then performed. To accomplish this, the samples were autoclaved for 10 min at 121°C in 10mM citrate buffer (pH 6.0). The sections were then incubated in blocking buffer (0.3% Triton X-100 and 10% goat or horse serum in PBS) for 1 h, and subsequently incubated overnight with human and mouse-reactive primary antibody (vWF; 1:500, Chemicon, Temecula, CA, USA) in incubation buffer (0.1% Triton X-100, 1% goat or horse serum and 1% BSA in PBS) at 4°C. The next day, the samples were washed with PBS containing 0.1% Tween-20, after which biotinylated secondary antibodies were added for 30 min at room temperature. Detection was then conducted using the Vectastain ABC Elite Kit (Vector Laboratories). After extensive rinsing and incubation with an avidin-biotin immunoperoxidase complex, staining was visualized with the DAB system (Nichirei, Tokyo, Japan), and the sections were counter-stained with Mayer's hematoxylin.

Statistical analysis

All results are presented as the means $\pm$ SEM. Differences between mean values of multiple groups were evaluated by ANOVA followed by Turkey's procedure. Comparisons between groups were made using an unpaired Student's t-test. A P <0.05 was considered to be statistically significant.

Results

Expression of GCP2, MCP1 and VEGF in hASCs

We determined if hASCs express GCP2, MCP1, VEGF and their receptors. RT-PCR and ELISA analysis showed that hASCs express and secrete GCP2, MCP1 and VEGF (Fig. 1A and B), that they express CCR2, CXCR1 and CXCR2 and the receptors of GCP2 and MCP1 (Fig. 1A). We also evaluated tube formation of

Fig. 3. Effect of GCP2 overexpression on tubule formation and blood flow recovery in a hindlimb ischemia model. (A) Overexpression of GCP2 in hASCs by lentivirus. GCP2 mRNA levels were determined in pLenti-vector transduced (LV) or GCP2-overexpressing hASCs (LV-GCP2) by RT-PCR and real time PCR. (B) Proangiogenic action of GCP2-overexpressing hASCs in a hindlimb ischemia model. PBS, vector-transduced (LV) or GCP2-overexpressing (LV-GCP2) hASCs were injected intramuscularly 24 hours after ligation. Quantitative analysis using a Doppler laser was conducted, and the data are presented as the ratio of perfusion in an ischemic leg compared with that of a nonischemic leg over time on day 14. (C) Determination of microvascular density. Ten fields from each section were selected, and the vWF positive cells were counted (Magnification, x 200). Data represent the mean $\pm$ SEM of five different experiments. * $p < 0.05$, significantly different from the control PBS group. # $p < 0.05$, significantly different from lentivirus-transduced cells (LV).

MCP1 and GCP2 using a Matrigel assay in EGM-MV media to determine if GCP2 or MCP1 plays a functional role in hASCs. The results revealed that MCP1 and GCP2 at concentrations of 25nM induced increased tubule formation in hASCs (Fig. 1C and D).

Interaction between GCP2, MCP1 and VEGF secretion in hASCs

To evaluate the interaction between GCP2, MCP1 and VEGF secretion in hASCs, we measured the GCP2, MCP1 and VEGF levels in conditioned media that was used to culture GCP2, MCP1 and VEGF siRNA-transfected cells. RT-PCR analysis showed efficient downregulation of the expression of the target genes in siRNA transfected cells (Fig. 2A). In addition, ELISA revealed that the downregulation of GCP2 inhibited the secretion of MCP1 and VEGF, and that the downregulation of MCP1 inhibited GCP2 and VEGF secretion. However, downregulation of VEGF did not affect the secretion of GCP2 and MCP1 (Fig. 2B). We then measured the effect of the external addition of GCP2, MCP1 and VEGF on the cytokine levels. The addition of 25ng/ml GCP2 to hASCs led to a significant increase in MCP1 and VEGF secretion. Moreover, the addition of 25ng/ml MCP1 led to increased GCP2 and VEGF secretion, but the addition of 25ng/ml VEGF did not affect the levels of GCP2 or MCP1 present in the conditioned media (Fig. 2C). The overexpression of GCP2 in hASCs led to increased secretion of MCP1 and VEGF, similar to the external addition of GCP2. Finally, the downregulation of MCP1 expression by siRNA reduced the GCP2 levels

in the GCP2-overexpressing cell line, whereas the downregulation of VEGF did not affect the levels of GCP2 and MCP1 (Fig. 2D).

Role of the interactions among GCP2, MCP1 and VEGF in the proangiogenic actions of hASCs in vivo

We evaluated the interaction between GCP2 and MCP1 secretion to determine if it affected the *in vivo* proangiogenic action. To accomplish this, we first determined the effects of GCP2 overexpression on the proangiogenic action of hASCs. GCP2 was overexpressed in hASCs using the lentiviral gene transfer system, and this overexpression was confirmed by RT-PCR and real-time PCR (Fig. 3A). When GCP2-overexpressing hASCs were transplanted into the hindlimb ischemia model, there was higher blood flow recovery and vascular density than when control cells were transplanted (Fig. 3B and 3C).

We also examined the proangiogenic action of hASCs transfected with siGCP2, siMCP1 and siVEGF in the hindlimb ischemia model. There was lower blood flow recovery and microvascular density in hASCs transfected with siGCP2, siMCP1 or siVEGF than in hASCs transfected with the control siRNA (Fig. 4). Additionally, the downregulation of VEGF expression showed a stronger inhibitory effect on blood flow recovery than the downregulation of GCP2 and MCP1 expression (Fig. 4). Furthermore, the increased blood flow recovery action by GCP2 overexpression was partly inhibited by the transfection of MCP1 or VEGF siRNA (Fig. 5). Finally, the VEGF siRNA transfection had a stronger

Fig. 4. Effects of GCP2, MCP1 or VEGF siRNAs on the proangiogenic action of hASCs in a hindlimb ischemia model. (A) Proangiogenic action of GCP2, MCP1 and VEGF siRNAs in a hindlimb ischemia model. Quantitative analysis was conducted as in Fig. 3 B. (B) Determination of microvascular density. Ten fields from each section were selected and the vWF positive cells were counted (Magnification, x 200). Data represent the mean $\pm$ SEM of five different experiments. * $p < 0.05$, significantly different from control siRNA transfected cells (siCont). # $p < 0.05$, significantly different from GCP2 and MCP1 siRNA-transfected hASCs.

inhibitory effect on the blood flow recovery that was induced by GCP2 overexpression than MCP1 siRNA transfection (Fig. 5).

Discussion

Vascularization occurs via three distinct processes, vasculogenesis, angiogenesis and arteriogenesis. Angiogenesis is a process by which new capillary blood vessels sprout from a preexisting blood vessel [27], while arteriogenesis describes the remodelling of pre-existing arterio-arteriolar anastomoses into completely developed and functional arteries [28]. Chemokines have been shown to play an important role in angiogenesis [29], and the paracrine action of chemokines or cytokines released from transplanted hASCs has been shown to be an important mechanism for their proangiogenic action in a hindlimb ischemia model [21, 22, 24, 25].

Recently, roles of specific factors in the proangiogenic actions of hASCs have been reported in hindlimb ischemia models. Kondo et al [30] reported that SDF-1 play a role in the ASCs-mediated angiogenesis in part by facilitating mobilization of endothelial progenitor cells, and Cai et al [25] showed that the downregulation of HGF in human ASCs significantly impaired in their ability to promote reperfusion in a murine hindlimb ischemia model. The combined treatment of FGF-2 and hASCs significantly increased blood perfusion, as well as survival of transplanted cells and secretion of human angiogenic factors (VEGF, HGF and FGF2). However, the roles of GCP2 or MCP-1 and the interaction between chemokines in hindlimb ischemia models have not been reported.

GCP2, which was originally isolated from conditioned medium of cytokine-stimulated osteosarcoma cells [31],

Fig. 5. Effects of MCP1 or VEGF siRNA on proangiogenic action of GCP2-overexpressing hASCs in a hindlimb ischemia model. PBS, vector-transduced (LV) or GCP2-overexpressing (LV-GCP2) hASCs were injected intramuscularly 24 hours after ligation. Quantitative analysis was performed as in Fig. 3 B. Data represent the mean $\pm$ SEM of three different experiments. * $p < 0.05$ significantly different from PBS. # $p < 0.05$ significantly different from GCP2-overexpressing cells (LV-GCP2).

is also produced by mesenchymal cells such as fibroblasts and macrovascular endothelial cells [32]. The angiogenic properties of GCP2 have been demonstrated in the rat corneal micropocket model of neovascularization [33] and in human tumor cell allografts [34]. MCP1 is a member of the C-C family of chemokines that promotes the recruitment and activation of monocytes/macrophages that play critical roles in the process of inflammation in various diseases [35, 36]. MCP-1 has been shown to play an important role in arteriogenesis as well as angiogenesis [37]. For example, Schaper et al. demonstrated that MCP1 is a potent enhancer of arteriogenesis in ischemic tissue [38, 39]. Additionally, Heil et al. [40] demonstrated that mice deficient in C-C chemokine receptor-2 (CCR2), a major functional

receptor for MCP1, exhibited impaired arteriogenesis.

In this study, we first demonstrated that GCP2 affected MCP1 secretion and vice versa in hASCs, and that their interaction modulates the recovery of blood flow induced by hASC transplantation in a hindlimb ischemia model of nude mice. These results indicate that the interaction between GCP-2 and MCP-1 secretion in hASCs can contribute to efficient vascularization by simultaneous promotion of angiogenesis and arteriogenesis. We previously reported that TLR ligands, which can be released from injured tissues, increased GCP2 and MCP1 expression in hASCs [41]. Therefore, the proangiogenic action of GCP2 and MCP1 could be further intensified *in vivo* because hASCs can be stimulated by endogenous TLR ligands that are released from injured muscles.

The results of this study showed that GCP-2 and MCP-1 increased VEGF secretion (Fig. 2C) and that, despite the finding that downregulation of VEGF did not affect GCP2 or MCP1 levels in hASCs (Fig. 2B), VEGF downregulation by siRNA transfection showed a stronger inhibitory effect on hASCs-induced blood flow recovery in a hindlimb ischemia model than MCP1 downregulation (Fig. 4A). These findings suggest that the proangiogenic action of GCP2 or MCP1 is primarily due to the induction of VEGF secretion, not the direct action of GCP2 or MCP1. Although there have been no reports of GCP-2 inducing VEGF secretion, Hong et al. [42] showed that MCP-1 induced VEGF secretion in human aortic endothelial cells, and that MCP-1-induced angiogenesis in chick chorioallantoic membrane was mediated by

VEGF secretion, which is consistent with the results of the present study.

The roles of VEGF or MCP1 in angiogenic action have been well established [42]; however, long term administration of single factor induces deleterious effects *in vivo* in addition to leading to blood flow recovery. For example, implantation of fibroblasts continuously expressing VEGF via a retroviral vector induced tissue edema, inflammation and hemangiomas [43]. Moreover, prolonged expression of MCP1 induced atherosclerosis in an experimental model [44], even though bolus administration of MCP1 is beneficial for collateral development in limb ischemia [38]. The interaction of chemokines secreted by hASCs observed in this study suggests that the synergistic action of multiple angiogenic factors on ischemic tissues secreted from hASCs leads to efficient blood flow recovery without inducing the adverse side effects associated with high concentrations of single factors.

In conclusion, our study demonstrated that the interactions of secreted GCP2, MCP1 and VEGF play an important role in the proangiogenic action of hASCs transplantation in a mouse hindlimb ischemia model. These findings provide new insights regarding the therapeutic efficacies of hASCs transplantation in ischemic diseases.

Acknowledgments

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