Bone marrow CXCR4 induction by cultivation enhances therapeutic angiogenesis

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Received 25 May 2008; revised 3 September 2008; accepted 11 September 2008; online publish-ahead-of-print 12 September 2008

Aims The chemokine stromal cell-derived factor-1 (SDF-1) and its receptor (CXCR4, CXC chemokine receptor 4) play a critical role in the process of post-natal neovascularization. Here, we investigated the role of CXCR4+ bone marrow cells (BMCs) in neovascularization in a murine hindlimb ischaemia model.

Methods and results We found that the expression of CXCR4 in BMCs was specifically upregulated by cultivation; therefore, we used freshly isolated BMCs and cultivated BMCs, designated as BMCFr and BMCCul, respectively. The increased CXCR4 expression corresponded to the migratory capacity in response to SDF-1α. Real-time reverse transcription–polymerase chain reaction and immunohistochemical analyses revealed that SDF-1α expression was significantly increased in the ischaemic limbs of mice. Blood flow perfusion and capillary density were significantly accelerated in mice implanted with BMCCul as compared with those in mice implanted with BMCFr. The stimulatory effect of BMCCul on neovascularization was significantly impaired when BMCCul derived from CXCR4−/− mice were implanted. The implanted BMCCul showed high retention in the ischaemic limbs. Further, the implantation of BMCCul significantly increased the expression of interleukin (IL)-1β and vascular endothelial growth factor-A in the ischaemic limbs.

Conclusion The upregulation of CXCR4 expression by cultivation may serve as a useful source of BMCs for accelerating therapeutic angiogenesis in ischaemic cardiovascular diseases.

KEYWORDS
Cardiovascular diseases;
Cytokines;
Ischaemia;
Regeneration;
Paracrine effect

1. Introduction

Therapeutic neovascularization has emerged as a novel strategy for the treatment of ischaemic cardiovascular diseases such as peripheral arterial disease and myocardial ischaemia. In particular, implantation of bone marrow-derived stem cells or circulating endothelial progenitor cells (EPCs) successfully promotes therapeutic neovascularization;1–3 however, the mechanism by which EPCs contribute to neovascularization has not been fully understood. The prevailing view is that these cells are recruited to the sites of ischaemia where they directly incorporate into the neovascularure. Indeed, experimental studies using bone marrow transplantation models have established that bone marrow-derived cells can incorporate into the neovascularure following vascular injury.4 In contrast, recent studies have also suggested that bone marrow-derived cells do not incorporate into the vasculature5 and that EPCs may contribute to neovascularization through paracrine mechanisms instead of differentiating into functional endothelial cells.6,7

The chemokine stromal cell-derived factor-1 (SDF-1) and its specific receptor CXC chemokine receptor 4 (CXCR4), which is expressed at high levels on both endothelial and haematopoietic progenitor cells, play a critical role in the development of the heart and blood vessels and the regulation of the motility and differentiation of haematopoietic stem cells.8–10 Experimental deficiency of SDF-1 or CXCR4 in a developing mouse embryo results in multiple defects, which are lethal. In addition, recent evidence indicates an important role of the SDF-1/CXCR4 system in the process of post-natal neovascularization. For instance, SDF-1 and CXCR4 are recently reported to be expressed by or in close proximity to angiogenic vessels in the ischaemic muscle of patients with peripheral artery disease.11 In experimental studies, we and other investigators have demonstrated that bone marrow-derived CXCR4+ cells functioned as smooth muscle progenitors and participated in the neo-intimal formation after vascular injury.12–14 Conversely, Walter et al.15 reported that impaired CXCR4 signalling

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contributes to the reduced neovascularization capacity of EPCs, suggesting that the CXCR4+ cells function as EPCs in ischaemic tissue. Further, a recent study suggested that bone marrow-derived CXCR4+ cells could promote neovascularization in ischaemic tissue by the release of angiogenic factors.16 Thus, the role of CXCR4 cells in neovascularization remains controversial. In the present study, by using a simple cultivation procedure, we found that CXCR4 expression in bone marrow cells (BMCs) is specifically and markedly upregulated. We further investigated the therapeutic efficacy of these CXCR4-upregulated BMCs in a murine model of hindlimb ischaemia. The findings obtained from this study provide new insight into the mechanism underlying BMC-accelerated neovascularization and therapeutic potential of BMCs for the treatment of ischaemic cardiovascular diseases.

2. Methods

2.1 Experimental animals

BALB/c-nu/nu mice (male, 8 week old) and C57BL/6 mice (male, 8 week old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The green fluorescent protein (GFP) transgenic mice (GFP mice, background: C57BL/6) were a generous gift from Dr. Masaru Okabe (Osaka University, Osaka, Japan).17 CXCR4 hetero-knockout (CXCR4−/−) mice were generated as described previously.9 The mice were fed a standard diet and water, and were maintained on a 12-h light and dark cycle. All experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals that conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publications No. 85–23, revised 1996).

2.2 Murine hindlimb ischaemia model

Murine hindlimb ischaemia model was produced as described previously.18 Mice were anaesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium. An incision was made in the skin at the mid portion of the right hindlimb overlying the femoral artery. The proximal part of the femoral artery and the distal portion of the saphenous artery were ligated and removed after all the side branches were dissected free. After 24 h, either bone marrow nucleated cells [5 × 10^6 cells in 100 μL phosphate-buffered saline (PBS)] or the same volume of PBS (100 μL) was injected into the ischaemic muscle. The perfusion of ischaemic and non-ischaemic hindlimb was measured using a laser Doppler perfusion imaging system (Moor LDI; Moor Instruments, Wilmington, DE, USA). The perfusion ratio was calculated as the flux ratio between the ischaemic and non-ischaemic limbs. All the measurements were performed in a double-blind manner by two independent researchers.

2.3 Bone marrow cell cultures and other procedures

Whole BMCs were harvested from the wild-type or GFP mice by flushing the femurs with PBS. Red blood cells were lysed with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2) at 4°C for 20 min. The cells (2 × 10⁶) were washed three times with PBS, resuspended in 2 mL PBS, and used as freshly isolated BMCs. The cells were incubated at 95% air, 5% CO₂, in a humidified incubator at 37°C in 100-mm plastic dishes (Falcon; BD Biosciences, San Jose, CA, USA) with 10 mL Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% foetal calf serum (FCS: HyClone, Logan, UT, USA) for the indicated periods, and washed three times with PBS, resuspended in 2 mL PBS, and used as cultivated BMCs. The cell viability as determined by trypan blue exclusion showed a slight loss of cell viability by the Supplementary material online. For cell implantation experiments, we first confirmed the induction of ischaemia 24 h after the surgery by laser Doppler perfusion imaging and then implanted BMCs at the site of hindlimb ischaemia. Neutralizing anti-SDF-1α antibody and interleukin-1 (IL-1) receptor antagonist (IL-1RA) were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). Unless specified otherwise, all other chemicals including AMD3100 were obtained from Sigma.

2.4 Real-time reverse transcription–polymerase chain reaction

RNA extraction and real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis were performed to detect the mRNA expression of IL-1β, vascular endothelial growth factor (VEGF)-A, IL-6, transforming growth factor-β1 (TGF-β1), monocyte chemoattractant protein-1 (MCP-1), and β-actin (for details, see Supplementary material online).

2.5 Immunohistochemistry

Immunohistochemistry for SDF-1α, CD31, VE-cadherin, desmin, VEGF-A, and IL-1β was performed (for details, see Supplementary material online).

2.6 Flow cytometric analysis

Flow cytometric analysis was performed using antibodies against CXCR4, CD3, c-kit, CD31, CD34, Gr-1, CD62L, CCR7, Mac-1, Flk-1, Sca-1, and IgG2a as described previously.1920 Details are described in the Supplementary material online.

2.7 Cell migration assay

To assess the BMC migration activity, transwell migration assay was performed.2122 For details, see Supplementary material online.

2.8 Enzyme-linked immunosorbent assay

The levels of IL-1β and VEGF-A in the culture supernatants were assessed using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s instruction.

2.9 Statistical analysis

Data are expressed as mean ± SEM. Differences with values of P < 0.05 were considered to be statistically significant. For detailed statistical analysis, see Supplementary material online.

3. Results

3.1 Upreregulation of CXCR4 in cultured BMCs

We first examined the effect of cultivation periods on CXCR4 expression in BMCs by flow cytometry and found that CXCR4 expression increased significantly 12 h after cultivation, reached a peak at 24 h and then decreased slightly after 36 h (Figure 1A, P < 0.01). To investigate whether this increased expression is specific for CXCR4, we tested various types of BMC surface markers such as CCR7, L-selectin, Gr-1, CD3, Mac-1, CD34, CD31, Flk-1, Sca-1, and c-kit. Of these markers, only CXCR4 expression was markedly increased after 24 h of cultivation (Figure 1B).

To determine the types of BMCs in which CXCR4 expression was increased by cultivation, double staining with CXCR4 and Gr-1 (granulocytes), CD3 (T cells), or Mac-1 (monocytes/
granulocytes) was performed and analysed by flow cytometry. The increased expression of CXCR4 after 24 h of cultivation was detected in Gr-1$^+$, CD3$^+$, and Mac-1$^+$ cells (Figure 1C and D). Further, there was no significant difference in the percentage of Gr-1$^+$, CD3$^+$, and Mac-1$^+$ cells between freshly isolated cells and cultivated cells (see Supplementary material online, Figure S2). The subsequent experiments were carried out using the control (freshly isolated) and cultivated BMCs (24 h of cultivation), designated BMCFr and BMCCul, respectively.

Since the culture medium contained 10% FCS, we examined the effect of FCS on CXCR4 expression. No difference was observed in the CXCR4 expression between 2 and 10% FCS-containing medium (Figure 2A(a)). However, CXCR4 expression in the medium containing murine serum was lower than that in the medium containing 10% FCS (Figure 2A(b)). Murine serum inhibited the cultivation-induced CXCR4 expression in a dose-dependent manner (Figure 2A(c)), suggesting the presence of inhibitory factors in the murine serum. Since internalization of CXCR4 by SDF-1 treatment was previously reported, we added the neutralizing SDF-1 antibody (10 μg/mL) to the culture medium. However, neutralization of SDF-1α had no effect on CXCR4 expression (Figure 2A(d)). The neutralizing capacity of anti-SDF-1 antibody was confirmed (see Supplementary material online, Figure S3).

To investigate the mechanisms of upregulated CXCR4 by the cultivation, CXCR4 mRNA expression and protein localization were determined before and after the cultivation. Real-time RT-PCR analysis showed no significant increase of CXCR4 mRNA expression by the incubation (Figure 2B). Further, confocal microscopic analysis revealed that CXCR4 was translocated to the cell surface after the cultivation (Figure 2C). To investigate whether CXCR4 upregulation in BMCCul could be decreased in vivo situation, we examined CXCR4 expression at 1 and 6 h following the intravenous injection of GFP$^+/BMCCul$ (isolated from GFP mice). The CXCR4 expression in the injected cells at 6 h decreased significantly as compared with that at 1 h (Figure 2D, P < 0.05).

To determine whether the observed upregulation of CXCR4 expression by cultivation was functionally relevant to the migratory capacity in response to SDF-1α, we used BMCs isolated from GFP mice and performed transwell migration assay. The number of migrated GFP$^+/BMCCul$ was significantly higher than that of migrated GFP$^+/BMCFr$ under the baseline conditions (Figure 2E and F). The addition of SDF-1α (100 ng/mL) to the lower chamber induced the migration of GFP$^+/BMCCul$, but not GFP$^+/BMCFr$. Further, pretreatment of GFP$^+/BMCCul$ with CXCR4 antagonist AMD3100 (30 μM) completely prevented its migration in response to SDF-1α (P < 0.05).
3.2 SDF-1α expression in hindlimb ischaemia

Recent reports demonstrated that the SDF-1/CXCR4 system contributes to the recruitment of bone marrow-derived vascular progenitor cells and participates in the pathophysiology of cardiovascular diseases.13–15,19 Therefore, we investigated whether SDF-1α is upregulated in a murine model of hindlimb ischaemia. Real-time RT–PCR analysis revealed that SDF-1α mRNA expression was significantly increased in the ischaemic limbs as compared with that in the sham-operated limbs (Figure 3A, P, 0.05). Immuno-histochemical staining demonstrated that the expression of SDF-1α protein was also increased at the site of hindlimb ischaemia (Figure 3B). To identify the cells expressing SDF-1α, we performed double immunofluorescent staining with SDF-1α and desmin (skeletal muscles) or CD31 (endothelial cells), and found that SDF-1α was co-localized with endothelial cells (Figure 3C).

3.3 Neovascularization by BMCFr or BMCCul

To investigate the therapeutic potential of CXCR4+–BMCs, we injected PBS (control), BMCFr or BMCCul into the site of hindlimb ischaemia 24 h after the surgery and assessed the tissue blood flow perfusion using a laser Doppler imaging system (Figure 4A). The perfusion was significantly increased in mice implanted with BMCCul as compared with that in the control mice (Figure 4B, P, 0.05) and mice implanted with BMCFr. The density of endothelial cells that was determined by CD31 and VE-cadherin expression on Day 21 after the surgery increased significantly in the BMCCul-implanted limbs as compared with that in the limbs of the control mice (Figure 4C and D). To further investigate the role of CXCR4, we isolated BMCs from the wild-type and CXCR4+/− mice, and compared the effect of wild-type BMCCul and CXCR4+/− BMCCul on blood flow perfusion in the ischaemic limbs. The perfusion was significantly
impaired in mice implanted with CXCR4<sup>−/−</sup> BMC<sup>Cul</sup> as compared with that in mice implanted with the wild-type BMC<sup>Cut</sup> (Figure 4E).

3.4 Fate of BMC<sup>Fr</sup> or BMC<sup>Cul</sup> implanted into the ischaemic hindlimb

The CXCR4<sup>−/−</sup>-BMCs have been shown to differentiate into vascular cells such as endothelial cells or smooth muscle cells. 14,15 To investigate the fate of implanted BMCs in the ischaemic limbs as well as in other tissues, BMC<sup>Fr</sup> and BMC<sup>Cul</sup> obtained from GFP mice were implanted into the ischaemic limbs, and the cells isolated from the ischaemic limbs, bone marrow, peripheral blood, and the spleen were then collected and analysed on Days 1, 3, and 21 after implantation by flow cytometry and histology. On Day 1 after implantation, the percentage of GFP<sup>+</sup> cells was considerably higher in the GFP<sup>+</sup>/BMC<sup>Cul</sup>-implanted limbs than in the GFP<sup>+</sup>/BMC<sup>Fr</sup>-implanted limbs (Figure 5A and B, P < 0.05). However, the GFP<sup>+</sup> cells in the GFP<sup>+</sup>/BMC<sup>Cul</sup>-implanted limbs disappeared from Day 3 to Day 21 (Figure 5B). No GFP<sup>+</sup> cells were detected in the bone marrow, peripheral blood, and the spleen of mice implanted with GFP<sup>+</sup>/BMC<sup>Cul</sup> (Figure 5A).

3.5 Interleukin-1β and vascular endothelial growth factor mRNA expression in vivo and in vitro

Since several cytokines and angiogenic factors have been shown to be involved in the BMC-accelerated neovascularization,6,7 we examined the expression of IL-1β, VEGF-A, IL-6, TGF-β, and MCP-1 in the BMC-implanted ischaemic limbs by real-time RT–PCR analysis. The mRNA expression of IL-1β, VEGF-A, IL-6, and MCP-1, but not TGF-β1, was significantly higher in the ischaemic limbs (control; PBS) than in the sham-operated limbs (Figure 6A, see Supplementary material online, Figure S4A, P < 0.05). The implantation of CXCR4<sup>−/−</sup>-BMCs tended to increase further the IL-1β mRNA levels. In comparison with the control or that of BMC<sup>Fr</sup>, the implantation of BMC<sup>Cul</sup> remarkably increased mRNA expression of VEGF-A and IL-6 (P < 0.05). We next tested whether the skeletal muscle cells express angiogenic factors. Immunohistochemistry revealed that the expression of VEGF and IL-1β was clearly visible in the skeletal muscle.
cells of ischaemic limbs at 2 weeks after BMC<sup>cul</sup> implantation; in particular, the regenerating myocytes expressed these factors (see Supplementary material online, Figure S5).

The expression of the angiogenic factors in isolated BMCs was investigated in vitro. The IL-1β mRNA expression markedly increased after 3 h of cultivation and then decreased, whereas the VEGF-A mRNA expression gradually increased and reached a peak at 24–48 h after cultivation (Figure 6B, see Supplementary material online, Figure S4B). The mRNA expression of IL-6 and MCP-1, but not TGF-β1, also increased during the cultivation. Further, the mRNA levels of IL-1β and VEGF-A, but not IL-6, TGF-β1, and MCP-1, were significantly lowered in CXCR4<sup>−/−</sup> BMCs. The protein levels of IL-1β and VEGF-A in the culture supernatants were also lowered in CXCR4<sup>−/−</sup> BMCs (Figure 6C). Treatment of BMCs with IL-1 receptor antagonist (IL-1RA: 1 μg/mL) showed a partial but significant inhibition of VEGF-A mRNA expression induced by the 24 h incubation period (Figure 6D).

4. Discussion

The major findings of this study are as follows: (i) the CXCR4 expression in BMCs was specifically increased after cultivation in vitro; the increased CXCR4 expression corresponded to the migratory capacity in response to SDF-1α; (ii) SDF-1α expression was significantly increased in the ischaemic limbs of mice; (iii) blood flow perfusion and capillary density were significantly improved in mice implanted with BMC<sup>cul</sup>. The perfusion was significantly impaired in mice implanted with CXCR4<sup>−/−</sup> BMCs; (iv) BMC<sup>cul</sup> showed high retention in the implanted ischaemic limbs on Day 1 after implantation; however, these cells disappeared after Day 3; (v) implantation of BMC<sup>cul</sup> increased the expression of angiogenic factors including IL-1β and VEGF-A in the ischaemic limbs. These findings extend previously published data, thereby indicating that CXCR4 expression essentially modulates the migratory and angiogenic capacities of cultured BMCS. Our data provide new insights into the mechanism underlying CXCR4<sup>+</sup>–BMC-accelerated neovascularization. Further, the bone marrow-derived CXCR4<sup>+</sup>–BMCs might be a promising source for therapeutic neovascularization for ischaemic cardiovascular diseases.

We demonstrated that the simple cultivation of BMCs markedly increased the expression of CXCR4 but not that of CCR7, l-selectin, Gr-1, CD3, Mac-1, CD34, CD31, Flk-1, Sca-1, and c-kit; this suggests that CXCR4 may specifically respond to the culture conditions used for the cultivation of BMCs. Consistent with our finding, Yamaguchi et al.<sup>25</sup>

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Figure 4 Neovascularization by BMC<sup>fr</sup> or BMC<sup>cul</sup>. BMC<sup>fr</sup> (5 × 10<sup>6</sup> cells), BMC<sup>cul</sup>, or PBS (control) was injected into the site of hindlimb ischaemia 24 h after the surgery. Blood flow perfusion was measured using a laser Doppler perfusion imaging system. (A) Representative photographs of blood flow imaging on Day 21 after the surgery are shown. (B) Quantitative analysis of the blood flow ratio was performed. Data are expressed as means ± SEM (n = 9 for each). *P < 0.05 vs. control. (C) The adductor muscles were excised 21 days after the surgery and immunohistochemically stained with antibodies against CD31 and VE-cadherin. Representative photographs are shown (n = 3). Scale bar represents 100 μm. (D) Quantitative analysis of CD31<sup>+</sup> endothelial cells was performed. Data are expressed as means ± SEM (n = 3 for each). *P < 0.05 vs. wild-type BMC<sup>cul</sup>. (E) Wild-type BMC<sup>cul</sup> (5 × 10<sup>6</sup> cells) or CXCR4<sup>−/−</sup> BMC<sup>cul</sup> was injected into the site of hindlimb ischaemia 24 h after the surgery. Blood flow perfusion was measured using a laser Doppler perfusion imaging system. Quantitative analysis of the blood flow ratio was performed. Data are expressed as means ± SD (n = 6 for each). *P < 0.05 vs. wild-type BMC<sup>cul</sup>.
previously demonstrated that ex vivo expanded EPCs showed an increased expression of CXCR4 and that locally delivered SDF-1 augments vasculogenesis and subsequently promotes ischaemic neovascularization in vivo by augmenting EPC recruitment in ischaemic tissues. In the present study, we particularly examined the role of paracrine effect by bone marrow-derived cells in neovascularization of ischaemic tissues. Since freshly isolated peripheral blood cells from mice showed low expression of CXCR4, we hypothesized that murine serum could influence the expression of CXCR4 in BMCs. Interestingly, the culture medium supplemented with murine serum prevented the cultivation-induced CXCR4 expression, at least in part, in a dose-dependent manner, suggesting that murine serum may contain certain inhibitory factor(s) that affect CXCR4 expression. Internalization of CXCR4 by leucocytes after exposure to SDF-1α is well documented. Therefore, we tested this possibility; however, neutralization of SDF-1α had no effect on the cultivation-induced CXCR4 expression. Internalization of CXCR4 by leucocytes after exposure to SDF-1α is well documented. Therefore, we tested this possibility; however, neutralization of SDF-1α had no effect on the cultivation-induced CXCR4 expression. In addition, although the mRNA expression of CXCR4 was not affected by the cultivation, endogenous CXCR4 protein translocated to the cell surface after the cultivation. These findings suggest that upregulation of CXCR4 might be due to translocation but not by de novo synthesis in the cultivated BMCs.

The SDF-1/CXCR4 system plays a critical role in the process of post-natal neovascularization. To explore the therapeutic potential of BMCs that express high levels of CXCR4 by cultivation in ischaemic cardiovascular diseases, we produced a murine model of hindlimb ischaemia. Consistent with previous reports, we showed that SDF-1α expression was clearly upregulated at the endothelium of ischaemic site. In the present study, the implantation of BMCs accelerated capillary formation and improved blood flow, and this improved blood flow was significantly impaired in mice implanted with CXCR4+/− BMCs. SDF-1 expression in endothelial cells is shown to be regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1), which induces SDF-1 expression in the ischaemic tissues. In particular, the recruitment of CXCR4+ cells into the ischaemic tissues is mediated by the hypoxic gradients through HIF-1-induced SDF-1 expression. Recently, it was reported that bone marrow-derived circulating cells are retained in close proximity to angiogenic vessels by SDF-1. Therefore, we hypothesized that BMCs might exert a potent activity for neovascularization and retention in the ischaemic tissues. Indeed, our data revealed an increased retention of BMCs implanted in the ischaemic limbs, suggesting that SDF-1 functions as an entrapment signal for CXCR4+ cells in the ischaemic tissues. However, the implanted cells disappeared at the early phase after implantation. Further, no GFP+ cells were detectable in the ischaemic tissues of the mice implanted with BMCs on Day 21 after the surgery. Thus, it is unlikely that CXCR4+BMCs differentiate into endothelial cells or smooth muscle cells in the ischaemic limbs.
The precise mechanism by which CXCR4<sup>+</sup> cells could accelerate neovascularization has not been fully understood; however, our data strongly suggest that a paracrine mechanism is responsible for this acceleration. Recently, Tateno et al.<sup>7</sup> reported that implantation of peripheral mononuclear cells promoted the skeletal muscle regeneration in the ischaemic limbs and that the regenerated muscle-secreted angiogenic factor IL-1β induces neovascularization, indicating a critical role of IL-1β-mediated paracrine. In addition, Grunewald et al.<sup>27</sup> demonstrated that VEGF plays an important role in neovascularization induced by the SDF-1/CXCR4 system. Consistent with these reports, we observed that the expression of IL-1β and VEGF was markedly upregulated in the ischaemic limbs implanted with BMCCul. The cultivated BMC<sup>cul</sup> expressed IL-1β at the early phase and VEGF at the later phase in vitro, and this VEGF induction was partially prevented by treatment with IL-1RA, indicating that VEGF was induced at least partially through IL-1β induction. Moreover, the levels of IL-1β and VEGF-A expression were significantly lowered in CXCR4<sup>+/−</sup> BMCs. Taken together, the implanted CXCR4<sup>+</sup>-BMCs might secrete IL-1β in the ischaemic limbs, and this secreted IL-1β induces subsequent VEGF production, thereby resulting in acceleration of neovascularization.

There are several limitations in our study. First, other angiogenic factors such as IL-6 and MCP-1 were upregulated in the ischaemic limbs implanted with BMC<sup>cul</sup> in vivo as well as in the cultivated BMCs in vitro, suggesting that other angiogenic factors may participate in its neovascularization. Secondly, the role of angiogenic factors induced by the regenerated skeletal muscles also remains unclear. Thus, further investigations are required to elucidate the precise mechanism underlying CXCR4<sup>+</sup>-BMCs-accelerated neovascularization.

In conclusion, we demonstrated that cultivation specifically stimulates CXCR4 expression in BMCs, and implantation of these cultivated CXCR4<sup>+</sup>-BMCs enhanced therapeutic neovascularization and restored blood flow of the ischaemic hindlimbs in mice. Since the SDF-1/CXCR4 system is profoundly demonstrated to modulate the angiogenic activity and homing capacity of vascular stem cells, the upregulation of CXCR4 expression by cultivation may be useful for accelerating therapeutic neovascularization by BMCs in ischaemic cardiovascular diseases.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Funding

This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology (to M.T.), the Ministry of Health, Labor and Welfare (to M.T. and U.I.), and Takeda Science Foundation (to M.T.).
Acknowledgements

We thank Junko Nakayama, Tomoko Hamaji, and Kazuko Misawa for excellent technical assistance and Masaru Okabe (Osaka University) for providing GFP mice.

Conflict of interest: none declared.

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