Critical Roles of Muscle-Secreted Angiogenic Factors in Therapeutic Neovascularization


Abstract—The discovery of bone marrow–derived endothelial progenitors in the peripheral blood has promoted intensive studies on the potential of cell therapy for various human diseases. Accumulating evidence has suggested that implantation of bone marrow mononuclear cells effectively promotes neovascularization in ischemic tissues. It has also been reported that the implanted cells are incorporated not only into the newly formed vessels but also secrete angiogenic factors. However, the mechanism by which cell therapy improves tissue ischemia remains obscure. We enrolled 29 “no-option” patients with critical limb ischemia and treated ischemic limbs by implantation of peripheral mononuclear cells. Cell therapy using peripheral mononuclear cells was very effective for the treatment of limb ischemia, and its efficacy was associated with increases in the plasma levels of angiogenic factors, in particular interleukin-1β (IL-1β). We then examined an experimental model of limb ischemia using IL-1β–deficient mice. Implantation of IL-1β–deficient mononuclear cells improved tissue ischemia as efficiently as that of wild-type cells. Both wild-type and IL-1β–deficient mononuclear cells increased expression of IL-1β and thus induced angiogenic factors in muscle cells of ischemic limbs to a similar extent. In contrast, inability of muscle cells to secrete IL-1β markedly reduces induction of angiogenic factors and impairs neovascularization by cell implantation. Implanted cells do not secrete angiogenic factors sufficient for neovascularization but, instead, stimulate muscle cells to produce angiogenic factors, thereby promoting neovascularization in ischemic tissues. Further studies will allow us to develop more effective treatments for ischemic vascular disease. (Circ Res. 2006;98:1194-1202.)

Key Words: angiogenesis ■ interleukins ■ muscles

Peripheral vascular disease (PVD), mainly caused by atherosclerosis, leads to obstruction of the blood supply to the lower or upper extremities. PVD is known to affect 10% to 15% of the adult population in developed countries and is often associated with coronary artery disease. Arte- riosclerosis obliterans (ASO) is the most common cause of PVD affecting the lower limbs. Peripheral ischemia can also result from various types of vasculitis, including thromboangitis obliterans (TAO) or Buerger’s disease, which affects small- and medium-sized arteries and is related to tobacco use and male sex but not to other coronary risk factors. The 2 cardinal symptoms of limb ischemia are intermittent claudication and rest pain: the latter symptom occurs in patients with critical limb ischemia and coincides with ischemic ulceration and gangrene. The treatments of PVD include pharmacotherapy, percutaneous transluminal angioplasty, and vascular surgery and are chosen depending on the severity of the symptoms and the arteries involved. However, as many as 50% of patients with critical limb ischemia will undergo limb amputation within 1 year because of an insufficient response to the treatments.3,4 Recent progress in understanding the mechanisms under- lying vascular formation in adults as well as during embryogenesis has opened up a therapeutic avenue for patients without any current options.5 Initial therapeutic approaches were aimed at delivering angiogenic factors, such as vascular endothelial growth factors (VEGF) and fibroblast growth factor-2, to ischemic tissues by using recombinant proteins or vectors encoding these factors. A number of preclinical studies reported improvement of perfusion by such methods in animal models of limb ischemia.6,7 Although the initial nonrandomized clinical trials showed beneficial effects, the results of controlled clinical trials have not been consistent.8 More recently, bone marrow–derived circulating endothelial

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progenitors have been identified in the peripheral blood\textsuperscript{8,9} and have been shown to contribute to both physiological and pathological angiogenesis in adults.\textsuperscript{10,11} These findings have led to the development of therapeutic neovascularization techniques using endothelial progenitors. Preclinical studies indicated that implantation of bone marrow mononuclear cells (BM-MNC), which contain endothelial progenitors, into ischemic limbs was very effective.\textsuperscript{12–14} Consequently, this therapeutic strategy was promptly tried for “no-option” patients with critical limb ischemia. The results of the first clinical trial showed that implantation of BM-MNC significantly improved the tissue oxygen concentration and blood flow in ischemic limbs, resulting in a decrease of rest pain and the involution of ischemic ulcers.\textsuperscript{15} Although promising results have been obtained, the mechanism by which cell therapy improves limb ischemia is largely unknown. Because direct incorporation of implanted cells into newly formed vessels is reported to be relatively rare, it has been assumed that angiogenic factors secreted by implanted cells are responsible for the efficacy of cell therapy.\textsuperscript{16,17}

In the present study, we investigated the efficacy of cell therapy using peripheral blood mononuclear cells (PB-MNC) for the treatment of ischemic limbs. The results of our clinical trial showed that increased levels of angiogenic cytokines correlated with the response to treatment. Implantation of mononuclear cells increased the production of the angiogenic cytokines in muscle cells. A deficiency of the angiogenic cytokines in muscle cells blunted the ability of implanted cells to increase vascularization, suggesting that muscle cells but not mononuclear cells were important as a source of the angiogenic cytokines. Accordingly, we propose a novel mechanism whereby implanted cells promotes neovascularization in ischemic tissues.

Materials and Methods

Animals

Generation and genotyping of interleukin (IL)-1β-deficient mice have been previously described.\textsuperscript{18} C57BL/6 mice were purchased from SLC Japan. All mice used in this study were 8 to 12 weeks old. All experimental procedures were performed according to the guidelines of Chiba University for animal experiments and the protocols were approved by our institutional review board.

Collection of Mouse PB-MNC and BM-MNC

Donor mice were euthanized with a lethal dose of anesthetic, after which whole blood or bone marrow were harvested. Mononuclear cells were subsequently separated using Histopaque 1083 (Sigma). To deplete putative endothelial progenitors, the mononuclear cells were further incubated with a rat anti-mouse VEGF receptor-2 antibody (eBiosciences) at a concentration of 0.5 μg per 1 million cells. Then the population of cells negative for VEGF receptor-2 was prepared using a magnetic cell sorter (Miltenyi Biotec) according to the instructions of the manufacturer. The control cell population was prepared using an isotype control anti-rat IgG2a antibody. Sorted cells were washed twice with PBS and resuspended in PBS at a concentration of 1 million cells per 100 μL for the subsequent experiments. In all experiments, the viability of the mononuclear cells was more than 96.0%, as judged by Trypan blue dye exclusion.

Hindlimb Ischemia Model

After the mice were anesthetized, the proximal part of the femoral artery and the distal portion of the saphenous artery were ligated and stripped out after all side branches were dissected free. After 24 hours (designated as day 1), either mononuclear cells (1.0×10\textsuperscript{6} cells in 100 μL of PBS) or PBS (100 μL) was injected into the ischemic muscle. As a rescue model, some of the IL-1β-deficient mice were treated with PB-MNC on day 1 and given an intramuscular injection of 0.5 ng of mouse recombinant IL-1β (IBL) on days 3, 5, and 7 after the operation. The mice were euthanized at the indicated times. Before death, hindlimb perfusion was measured with a laser Doppler perfusion analyzer (Moor Instruments).

Bone Marrow Transplantation Model

Before bone marrow transplantation, 8- to 10-week-old male wild-type or IL-1β-deficient mice were exposed to total body irradiation (9 mGy). Bone marrow cell suspensions were isolated by flushing the femurs and tibias harvested from wild-type or IL-1β-deficient mice. Bone marrow cell suspensions (1.5×10\textsuperscript{7} cells) were injected intravenously via the tail vein within 6 hours of irradiation. The chimeric rate was more than 95%, as determined by fluorescence-activated cell sorting analysis of chimeric mice transplanted with bone marrow cells from the green fluorescent protein (GFP) transgenic mice.

Histology

Vastus and rectus femoris muscle tissues were removed from the ischemic limbs after systemic perfusion with PBS and immediately embedded in OCT compound (Sakura Finetechanical). Then each specimen was snap frozen in liquid nitrogen and cut into 6-μm sections. The sections were stained with antibodies for myosin (MF-20), CD31 (Pharmingen), or IL-1β (Santa Cruz Biotechnology) and counterstained with hematoxylin or 4’,6-diamidino-2-phenylindole (DAPI). Two transverse sections of the entire muscle were photographed digitally at a magnification of ×100 (12 to 16 photographs per mouse), and these photographs were reviewed in a blinded manner. Capillary endothelial cells were identified by immunoreactivity for CD31 and quantified as the number per square millimeter.

Cell Culture

C2C12 myoblasts were cultured as described previously.\textsuperscript{19} Briefly, cells were maintained in DMEM supplemented with 20% FBS (growth medium) until use. In all experiments, the cells were seeded into 60-mm or 100-mm plastic culture dishes at a concentration of 5×10\textsuperscript{4} cells/cm\textsuperscript{2} (day 0) and were incubated at 37°C in a mixture of 95% air and 5% CO\textsubscript{2}. After 24 hours (day 1), the growth medium was changed to DMEM supplemented with 2% horse serum (differentiation medium). On day 4, the medium was replaced by fresh differentiation medium with or without PB-MNC. On day 5, cells were harvested after the mononuclear cells were completely removed by washing 5 times with PBS.

Statistical Analysis

Data are shown as mean±SEM unless otherwise noted. Changes of the rest pain scale and walking distance over time were analyzed by the rank sum test. Differences of patient background factors between responders and nonresponders were analyzed with the χ\textsuperscript{2} test or Fisher’s exact test. Comparison between multiple groups was performed by 1-way ANOVA followed by the Bonferroni procedure for comparison of mean values. Comparisons between two groups were done with the two-tailed Student’s t test or 2-way ANOVA. For these analyses, of P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Therapeutic Neovascularization Using PB-MNC for Critical Limb Ischemia

Because most patients with critical limb ischemia have concomitant coronary artery disease and cerebrovascular disease, the collection of bone marrow cells, which requires
general anesthesia, could cause fatal events in such patients. Given that angiogenic factors secreted from BM-MNC play a major role in cell therapy, PB-MNC may be more beneficial because collection of PB-MNC is much safer and less expensive. To examine the effects implanting PB-MNC, we produced a model of hindlimb ischemia in C57BL/6 mice and implanted PB-MNC into the ischemic limbs of these animals. Implantation of PB-MNC significantly increased perfusion compared with that in the control group (PBS) as assessed by laser Doppler analysis (Figure 1A). The capillary density in ischemic limbs was significantly greater in the PB-MNC group than in the PBS group (Figure 1B). Although BM-MNC has been reported to contain much more (100-fold) endothelial progenitors than PB-MNC, there was no significant difference of neovascularization between the PB-MNC group and the BM-MNC group (Figure 1A and 1B), indicating that implantation of PB-MNC is as efficient as BM-MNC for the treatment of limb ischemia and suggesting that endothelial progenitors do not play a pivotal role in the treatment of limb ischemia at least by PB-MNC.

After obtaining approval from the ethical committee of Chiba University Graduate School of Medicine, we started a pilot clinical trial to investigate whether therapeutic neovascularization by implantation of PB-MNC is feasible and effective for ischemic limbs. We enrolled 29 patients with critical limb ischemia caused by ASO or TAO who had no treatment options. More than 80% of the patients were recommended to undergo major amputation by their doctors before enrollment because of unhealed ischemic ulcers or gangrene despite conventional therapy (Tables 1 and 2). Approximately half of the patients had chronic renal failure and were on dialysis 3 times weekly (Table 1). Apart from 5 TAO subjects, most of the patients (82.7%) had 1 or more complications (Table 1). We implanted PB-MNC into the ischemic limbs of these patients twice within a 1-month period and estimated their response at 2 months, 6 months, and 1 year after treatment (as described in the online data supplement). Rest pain was significantly decreased and was nearly normalized by 1 year after treatment (Figure 1C). The maximum walking distance also improved significantly, and this improvement was preserved for a year (Figure 1D). Three patients (11.1%) underwent major amputation, which was much less frequent compared with the reported annual rate of amputation in patients with critical limb ischemia (50%).

Figure 1. Effect of implanting PB-MNC. A, Limb perfusion measured by a laser Doppler analyzer at 2 weeks after treatment (photographs). Scale bar=100 μm. The graph shows the ratio of ischemic limb (left) to nonischemic limb (right) blood flow. Data are shown as mean±SEM. *P<0.05, **P<0.01 vs PBS-treated group (n=8). B, Immunohistochemistry for CD31 (brown) in ischemic limbs at 2 weeks after treatment with PBS, PB-MNC, or BM-MNC. Scale bar=100 μm. The number of CD31-positive cells per square millimeter is shown. Data are shown as mean±SEM. *P<0.05, **P<0.01 vs PBS-treated group (n=8). C, Changes of the rest pain scale after implantation of PB-MNC. D, Improvement of the maximum walking distance after implantation. Data are shown as mean±SEM. *P<0.001 vs 0 month; #P<0.05, ##P<0.001 vs 2 months (n=29). E, Nonhealing ischemic ulcer (left) is completely healed by 6 months after implantation (right).
TABLE 1. Patient Background

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<td>Any complications</td>
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Data are shown as mean±SD or the no. of patients (%). PTA indicates percutaneous transluminal angioplasty; CRF, chronic renal failure; HD, hemodialysis; CAD, coronary artery disease; CVD, cerebrovascular disease.

Improvement of ischemic ulcers was observed 16 of 24 patients (66.7%) (Figure 1E), whereas only 31% of the patients showed substantial improvement of the ankle–brachial pressure index (ABPI), presumably because of underestimation of improvement in this index of hemodialysis patients. There were no major complications related to the treatment, such as death or coronary events. Three patients died of unrelated causes, including pneumonia and chronic renal failure (11.1%), which was lower than the reported average mortality rate of patients with critical limb ischemia (≈25%).1 These results indicate that implantation of PB-MNC was a safe and effective treatment for critical limb ischemia.

Factors Associated With the Response to Treatment

We next investigated the factors related to the response to treatment. We divided the patients into a group of responders (n=21) and a group of nonresponders (n=8) according to a response score estimated from the improvement of rest pain, ischemic ulcers, walking distance, and ABPI. Then we compared their background factors and laboratory data. There were no significant differences of patient background factors, including age, gender, and coronary risk factors, between responders and nonresponders (Table 2). The serum level of C-reactive protein, a marker of inflammation induced by cytokines, was significantly higher in the responders after implantation (Table 2). Therefore, we examined the plasma levels of various angiogenic cytokines in the patients at days 1, 3, 7, and 14 after implantation. We found that the peak levels of IL-1β, IL-6, and VEGF, but not tumor necrosis factor-α or granulocyte colony-stimulating factor, were markedly higher in responders than in nonresponders (Figure 2A; Figure I in the online data supplement). In contrast, there were no significant differences of the number of implanted mononuclear cells and CD34-positive cells between the 2 groups (Figure 2A). Consistent with these results, deprivation of putative endothelial progenitors (VEGF receptor-2–positive cells) did not affect the ability of implanted mononuclear cells to promote neovascularization in a mouse model of hindlimb ischemia (Figure 2B). Thus, we speculated that the

TABLE 2. Clinical Response and Patient Background

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<th>Nonresponders (n=8)</th>
<th>Responders (n=21)</th>
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<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
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<td>13 (61.9%)</td>
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<td>TAO</td>
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<td>8 (38.1%)</td>
<td></td>
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<td>Ischemic status</td>
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</tr>
<tr>
<td>Fontaine 3</td>
<td>1 (12.5%)</td>
<td>3 (14.3%)</td>
<td>NS</td>
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<td>Fontaine 4</td>
<td>7 (87.5%)</td>
<td>18 (85.7%)</td>
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<td>Previous revascularization</td>
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<td>Duration of illness (month)</td>
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<td>CRF on HD</td>
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<td>CAD</td>
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<td>CVD</td>
<td>3 (37.5%)</td>
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<tr>
<td>Diabetes</td>
<td>6 (75.0%)</td>
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Data are shown as mean±SD or the no. of patients (%). CRF indicates chronic renal failure; HD, hemodialysis; CAD, coronary artery disease; LVEF, left ventricular ejection fraction; CVD, cerebrovascular disease; MBP, mean blood pressure; FBS, fasting blood sugar; T-Cho, total cholesterol; Cho, cholesterol; CRP, C-reactive protein. Patients with osteomyelitis were excluded for analysis of CRP data.
increase of angiogenic factors secreted by implanted mononuclear cells might influence the efficacy of this therapy.

**Increased Expression of IL-1β in Ischemic Limbs Is Crucial for Neovascularization**

Because IL-1β is known to be a potent angiogenic cytokine and induces a number of angiogenic factors including VEGF,20,21 we examined the role of IL-1β on neovascularization induced by mononuclear cell implantation in a model of hindlimb ischemia. When PB-MNC from wild-type mice were implanted into the ischemic limbs of wild-type mice, blood flow and capillary density were significantly increased compared with those in a PBS-treated group (Figure 3A and 3B). Unexpectedly, PB-MNC derived from IL-1β-deficient mice18 increased the blood flow of the ischemic limbs in wild-type mice as efficiently as wild-type PB-MNC (Figure 3A). Likewise, implantation of IL-1β-deficient PB-MNC significantly increased capillary density compared with the PBS-treated group (Figure 3B). In contrast, implantation of wild-type PB-MNC into ischemic limbs of IL-1β-deficient mice had no effect on neovascularization (Figure 3A and 3B). This suggests that implantation of PB-MNC induces IL-1β expression in ischemic limbs and promotes neovascularization by the induction of angiogenic factors such as VEGF.

However, implantation of wild-type PB-MNC failed to induce VEGF expression in the ischemic limbs of IL-1β-deficient mice (Figure 3C). The pattern of IL-1β expression in ischemic limbs was very similar to that of VEGF (Figure 3D). A significant increase of IL-1β expression was observed in the ischemic limbs of wild-type mice treated with wild-type or IL-1β-deficient PB-MNC, whereas there was no induction of IL-1β in the ischemic limbs of IL-1β-deficient mice after implantation of wild-type PB-MNC (Figure 3D). These results suggest that implantation of PB-MNC induces IL-1β expression in ischemic limbs and promotes neovascularization by the induction of angiogenic factors such as VEGF.

Previous studies demonstrated that infiltration of macrophages plays a critical role in IL-1β-induced neovascularization.22,23 To test this, we injected PB-MNC from GFP transgenic mice into the ischemic limbs of wild-type or IL-1β-deficient mice and performed histological analyses at 5 days after treatment. The number of infiltrating host-derived (GFP-negative) macrophages was significantly fewer in the ischemic limbs of IL-1β-deficient mice than in those of wild-type mice (supplemental Figure II). PB-MNC implantation significantly induced macrophage infiltration compared with PBS-treated mice (supplemental Figure II). There was no difference in infiltration of macrophages into nonischemic limbs between wild-type and IL-1β-deficient mice.
Thus, infiltration of macrophages may be also involved in the mechanisms underlying IL-1β/H9252-induced neovascularization.

To investigate whether host-derived hematopoietic cells contribute to induction of IL-1β by mononuclear cell implantation, we produced chimeric mice. When PB-MNC from IL-1β-deficient mice were implanted into the ischemic limbs of wild-type mice treated with PBS (PBS→Wild), wild-type PB-MNC (Wild→Wild), or IL-1β-deficient PB-MNC (KO→Wild) and the limbs of IL-1β-deficient mice treated with PBS (PBS→KO) or wild-type PB-MNC (Wild→KO). Immuno-CD31+ cells in ischemic limbs. C, Expression of VEGF in ischemic limbs of the same mice at 1 week after treatment was analyzed by the ribonuclease protection assay (RPA). Relative expression to that of L32 (the internal control) is shown. D, Expression of IL-1β in ischemic limbs of the same mice at 1 week after implantation was analyzed by RPA and relative expression to that of L32 is shown. ND indicates not detected. Data are shown as mean±SEM. *P<0.05, **P<0.01 vs wild-type mice treated with PBS (n=5). E, F, and G, The graph shows blood flow (E), capillary density (F), and expression of IL-1β (G) in the ischemic limbs of wild-type mice treated with PBS (PBS→Wild), wild-type PB-MNC (Wild→Wild), or IL-1β-deficient PB-MNC (KO→Wild) and the limbs of IL-1β-deficient mice treated with wild-type bone marrow treated with IL-1β-deficient PB-MNC (KO→Wild→KO-BM). The values are shown as described in the legend of Figure 1A for blood flow and capillary density. Data are shown as mean±SEM. *P<0.05, **P<0.01 vs PBS→Wild (n=5).

To determine the type of cells in which IL-1β expression is induced by implantation of PB-MNC, we performed immuno-histological analysis of ischemic limbs after implantation. Expression of IL-1β was localized to the skeletal muscle cells, as identified by staining for sarcomeric myosin, and was particularly detected in the regenerating tissues (Figure 4A). Likewise, in situ hybridization revealed that IL-1β was predominantly expressed by muscle cells and, to a lesser extent, by other types of cells such as vascular cells and infiltrating leukocytes (Figure 4B and data not shown). To test whether PB-MNC could increase the expression of wild-type bone marrow (Figure 3G), which suggests a minor role of host-derived hematopoietic cells in the production of IL-1β after treatment.

**Critical Roles of Muscle-Secreted Angiogenic Factors**

To determine the type of cells in which IL-1β expression is induced by implantation of PB-MNC, we performed immuno-histological analysis of ischemic limbs after implantation. Expression of IL-1β was localized to the skeletal muscle cells, as identified by staining for sarcomeric myosin, and was particularly detected in the regenerating tissues (Figure 4A). Likewise, in situ hybridization revealed that IL-1β was predominantly expressed by muscle cells and, to a lesser extent, by other types of cells such as vascular cells and infiltrating leukocytes (Figure 4B and data not shown). To test whether PB-MNC could increase the expression of wild-type bone marrow (Figure 3G), which suggests a minor role of host-derived hematopoietic cells in the production of IL-1β after treatment.
angiogenic cytokines by muscle cells, we coincubated mouse embryonic muscle cells (C2C12) with PB-MNC from wild-type mice for 24 hours under differentiating conditions and harvested muscle cells after intensive wash with PBS to eliminate PB-MNC from the samples. Coincubation with PB-MNC increased the expression of various angiogenic cytokines such as IL-1β, IL-6, and VEGF by C2C12 cells (Figure 4C). C2C12 cells treated with PB-MNC as well as nontreated cells expressed myosin (Figure 4D), whereas expression of CD31 was not detected in these cells (Figure 4C), indicating that contamination of PB-MNC was negligible. Coincubation of C2C12 cells with human PB-MNC also induced expression of mouse cytokines as determined by RPA using a mouse-specific probe (supplemental Figure III). We, therefore, concluded that implantation of mononuclear cells promotes neovascularization by stimulating muscle cells to increase the production of angiogenic cytokines such as IL-1β.

We then examined whether treatment with IL-1β restores the efficacy of PB-MNC implantation in the ischemic limbs of IL-1β–deficient mice. We observed that neovascularization occurred in the ischemic tissues at 1 week and that the appearance of the skeletal muscles became normal by 3 weeks after implantation (Figure 4E). Implantation of wild-type PB-MNC did not induce neovascularization in the ischemic limbs of IL-1β–deficient mice at 1 week, and, thus, necrosis occurred by 3 weeks after implantation (Figure 4E). In contrast, administration of IL-1β in conjunction with implantation of wild-type PB-MNC significantly increased neovascularization (P<0.01 versus IL-1β–deficient mice treated with wild-type PB-MNC; n=3) and prevented necrosis of the ischemic limbs in IL-1β–deficient mice at 3 weeks (Figure 4E). Consistent with the results of histological analyses, improvement of blood flow by PB-MNC implantation in wild-type mice was significantly better than in IL-1β–deficient mice (Figure 3A and supplemental Figure IV).
Moreover, administration of IL-1β in conjunction with implantation of wild-type PB-MNC partially improved the effect of PB-MNC implantation on blood flow in IL-1β-deficient mice (supplemental Figure IV). However, administration of IL-1β without PB-MNC implantation did not improve limb ischemia in wild-type mice (supplemental Figure V). Thus, it is conceivable that induction of IL-1β is prerequisite for the efficacy of PB-MNC implantation, but an IL-1β only treatment is insufficient for limb salvage.

Discussion

The present study provided a possible mechanism for the process of neovascularization during cell therapy for limb ischemia. The results of our clinical trial showed that angiogenic cytokines, especially IL-1β, were associated with the response to treatment. Many previous studies have suggested that angiogenic factors secreted by implanted cells play a critical role in therapeutic neovascularization. In contrast, our in vitro and in vivo studies demonstrated that the implanted mononuclear cells did not secrete cytokines sufficient for neovascularization but, instead, stimulated muscle cells to produce IL-1β. This is consistent with our observation that most of the implanted cells disappeared from the ischemic tissues as early as 3 days after implantation, which was before reconstruction of the vascular system started (K.T., T.M., I.K., unpublished data, 2006). Thus, it is likely that muscle cells but not implanted cells are a major source of angiogenic cytokines in ischemic limbs.

We noted that IL-1β was predominantly expressed by myocytes with central nuclei in ischemic limbs, indicating that these cells are regenerating myocytes (Figure 4A). We also observed that implantation of mononuclear cells increased the number of central nucleated myocytes in ischemic limbs (K.T., T.M., I.K., unpublished data, 2006). We therefore propose a novel model of neovascularization, in which implanted mononuclear cells enhance muscle regeneration and the increased expression of IL-1β by regenerating myocytes promotes neovascularization via induction of angiogenic factors, thereby contributing to limb salvage. Implantation of wild-type mononuclear cells increased the number of regenerating myocytes in the ischemic limbs of IL-1β-deficient mice compared with PBS-treated IL-1β-deficient mice (Figure 4E and supplemental Figure VI). However, the regeneration of myocytes was not accompanied by neovascularization at 1 week, and, thus, necrosis occurred by 3 weeks after implantation (Figure 4E). These results suggest that implantation of mononuclear cells promotes muscle regeneration and that the regenerated muscle-secreted IL-1β induces neovascularization, leading to suitable conditions for further muscle regeneration. Thus, although expression of IL-1β by muscle cells is required for mononuclear cells promoting neovascularization, administration of IL-1β could not obviate the need for injection of mononuclear cells.

We recently reported a similar finding that granulocyte colony-stimulating factor acts directly on cardiomyocytes and promotes neovascularization in ischemic myocardium by inducing the production of angiogenic factors. Our results also coincide with recent evidence suggesting an important role for the interaction between macrophages and skeletal muscle in the process of muscle regeneration after injury. During this process, muscle cells modulate macrophage invasion by releasing cytokines such as monocyte chemoattractant protein-1 and VEGF. On the other hand, macrophages release factors in vitro that promote the proliferation of muscle cells. Macrophages are also present in vivo at sites where muscle regeneration occurs, and macrophage depletion significantly impairs muscle regeneration after transplantation of myogenic cells, suggesting a critical role of macrophages in muscle repair. However, there are no definitive studies to show which factors (if any) are released by macrophages in vivo to influence the process of muscle repair. In our study, it is unclear how the implanted mononuclear cells stimulated muscle cells to proliferate and induce the expression of angiogenic factors. It would be also interesting to determine whether the similar mechanisms are involved in other modes of therapeutic vascularization such as cytokine therapy. Further studies on the roles of cellular communications will allow us to develop more effective treatments for ischemic vascular disease.

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References


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Critical Roles of Muscle-Secreted Angiogenic Factors in Therapeutic Neovascularization

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Materials and Methods

Patients

We enrolled patients aged 20 years or older who suffered from ischemic rest pain and/or non-healing ischemic ulceration due to ASO or TAO despite conventional therapy, including nonsurgical and surgical revascularization. Before entry into this study, we performed physical examination, laboratory tests, X-rays, electrocardiography, echocardiography, carotid artery ultrasonography, cardiac scintigraphy, and coronary angiography in all candidates. They also underwent arteriography of the lower extremities, measurement of ABPI, a laser Doppler study, and thermography to confirm the existence of PVD unsuitable for non-surgical or surgical revascularization. We excluded patients with proliferative diabetic retinopathy or those with malignancy during the previous 5 years. We also excluded patients with limb ischemia due to causes other than ASO or TAO, such as collagen vascular disease. Patient enrolment was completed by May 2004, and a total of 29 patients underwent therapeutic neovascularization by implantation of PB-MNC. We obtained written informed consent from all of these patients. The ethical committee of Chiba University Graduate School of Medicine reviewed and approved the study protocol.

Study design

The primary outcome was the safety of the treatment based on the occurrence of adverse events related to the treatment. Secondary outcomes were the incidence of major amputation of treated limbs, improvement of rest pain, walking distance, ischemic ulcers (more than 25% reduction in size), and ABPI (an increase of more than 0.1). Rest pain scale was determined as follows: +4, severe pain unsolved with morphine, pentazocine, or non-steroidal anti-inflammatory drugs (NSAID); +3, moderate pain NSAID necessary; +2, slight pain NSAID unnecessary; +1, very slight
pain; 0, complete resolved. Non-responder was defined as a patient who revealed no improvement of rest pain, walking distance, ischemic ulcers, or ABPI. We performed follow-up examinations at 2 months, 6 months, and 12 months after treatment. We also monitored adverse events, including the progression of atherosclerosis, by the examinations described above.

**Procedure for clinical implantation of PB-MNC**

PB-MNC were harvested by peripheral blood apheresis with a COBE Spectra Apheresis System (Gambro). The system was operated in the manual mode with the target haematosis being set at 2.0–3.0%. Patient underwent apheresis for 240 min while awake and resting quietly. When the patient had severe anaemia (hemoglobin<7.4 g/dl) or left ventricular dysfunction (ejection fraction<45%), the apheresis time was reduced to 180 min. The total leukocyte count and mononuclear cell count in the apheresis product was $1.75 \times 10^{10} \pm 0.73$ (mean±SD) and $1.09 \times 10^{10} \pm 0.39$ (mean±SD), respectively. Part of the product was subjected to FACS analysis to determine the number of CD34-positive cells. The product was concentrated to 20 ml by centrifugation at 2,000×g for 10 min and then was implanted on the same day.

PB-MNC were injected intramuscularly into the ischemic lower extremities at sites 1 to 3 cm apart, with each injection being 0.1 to 0.3 ml in volume. We examined the plasma levels of VEGF, interleukin (IL)-1β, IL-6, tumor necrosis factorα (TNFα), and granulocyte colony-stimulating factor (G-CSF) in the patients by using human-specific ELISA (R & D systems). We measured these cytokines at 1, 3, 7, and 14 days after treatment and used the peak levels of cytokines for a comparison between responder and non-responder because the time point at which each cytokine reached its maximum varied.
Western blot analysis

Samples were prepared in lysis buffer (10 mM Tris-HCL, pH 8, 140 mM NaCl, 5 mM EDTA, 0.025% NaN₃, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 µg/ml leupetin, 2 µg/ml aprotinin, 50 mM NaF, and 1 mM Na₂VO₃). The lysates (30 µg) were resolved by SDS polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with the primary antibody followed by a horseradish anti-mouse immunoglobulin G antibody (Jackson). The primary antibodies used for Western blotting were anti-myosin antibody (MF-20) and anti-actin antibody (Sigma).

Extraction of RNA and ribonuclease protection assay (RPA)

Total RNA was extracted from hindlimb muscle tissue or C2C12 cells by the guanidinium thiocyanate-phenol chloroform method. The levels of IL-1β, IL-6, and VEGF mRNA were examined with a multi-probe ribonuclease protection assay system (BD Biosciences) according to the manufacturer’s instructions. The level of expression of each of these genes was quantified by the Bio Imaging Analyzer System (Fuji Photo Film).

In situ hybridization

In situ hybridization was carried out as previously described.¹ ² A 570 bp Pst-I fragment of the IL-1β cDNA (provided by Dr Yoichiro Iwakura)³ ⁴ was subcloned into pBluescript II SK (Stratagene), linearized by restriction enzymes and transcribed by RNA polymerases (Roche), whereby the digoxigenin labeled the antisense and sense probes. Muscle samples were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), then in sucrose and embedded with OCT compound. Sections were cut, airdried and then fixed in 4% PFA for 15 minutes at room
temperature. After washing with PBS, sections were treated with proteinase K (5 µg/ml for 15 minutes), refixed with PFA, washed with PBS, treated with 0.2 M hydrochloride for 10 minutes and washed again with PBS. Samples were acetylated for 10 minutes, washed with PBS and dehydrated with ethanol. Hybridization mixture (50% formamide, 10 mM Tris-HCl pH 7.6, 200 µg/ml tRNA, 1×Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and the probe) was then applied on top of the section, overlaid with parafilm, and incubated overnight at 50°C in a humidified chamber. Sections were washed for 30 minutes with 2×SSC/50% formamide at 50°C, treated with RNase for 30 minutes at 37°C, washed with 2×SSC for 20 minutes at 50°C and washed twice with 0.2xSSC for 20 minutes each at 50°C. Sections were rinsed with buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl), blocked with 10% sheep serum in buffer 1 for 2 hours and incubated with alkaline phosphatase-conjugated sheep polyclonal antidigoxigenin antibody (Roche) diluted 1:4000 in buffer 1 for 30 minutes. After washing twice with buffer 1, alkaline phosphatase activity was detected in the presence of NBT/BCIP (Roche).

References


3. Gray PW, Glaister D, Chen E, Goeddel DV, Pennica D. Two interleukin 1 genes
in the mouse: cloning and expression of the cDNA for murine interleukin 1 beta. 


Figure legends

**Supplementary Figure 1**  Factors associated with the response to treatment. Results are shown as box plots representing median, 25th and 75th percentiles as boxes and the range of data as bars. IL-6, Interleukin 6; TNFα, tumor necrosis factorα; G-CSF, granulocyte colony-stimulating factor. *P=0.08 versus non-responders (responders, n=21; non-responders, n=8).

**Supplementary Figure 2**  Immunohistochemistry for macrophages. Wild-type PB-MNC was implanted into the ischemic limbs of wild-type (Wild→Wild) or IL-1β-deficient mice (Wild→KO). The ischemic limbs of wild-type mice were also treated with PBS (PBS→Wild) and served as a control. Five days after PB-MNC implantation, the sections of the ischemic or non-ischemic limbs were stained with anti-Mac3 antibody. The graph indicates the number of infiltrating macrophages into the ischemic or non-ischemic limbs (Non-ischemic). *P<0.05 versus PBS→Wild, #P<0.01 versus Wild→Wild (n=5).

**Supplementary Figure 3**  Mononuclear cell implantation increases the production of angiogenic factors by muscle cells. C2C12 cells were cultured under differentiating conditions with or without human PB-MNC (2.5×10^5 cells/ml) for 24 hr and expression of mouse angiogenic cytokines was examined by RPA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control.

**Supplementary Figure 4**  Laser Doppler analysis. The graph shows relative blood flow in the ischemic limbs of wild-type mice treated with PBS (PBS→Wild), wild-type PB-MNC (Wild→Wild) and the limbs of IL-1β-deficient mice treated with PBS (PBS→KO), wild-type PB-MNC (Wild→KO) or wild-type PB-MNC plus IL-1β (Wild+IL-1β→KO). Data are shown as mean±SEM. *P<0.05 versus PBS-treated group (n=5).
Supplementary Figure 5  Effect of IL-1β treatment on limb ischemia. The graphs show relative blood flow (A) and capillary density (B) in the ischemic limbs of wild-type mice treated with PBS (PBS→Wild), IL-1β (IL-1β→Wild) or wild-type PB-MNC (Wild→Wild). Data are shown as mean±SEM. *P<0.05, **P<0.01 versus PBS-treated group (n=3-5).

Supplementary Figure 6  Immunohistochemistry for CD31 in the ischemic limbs of IL-1β-deficient mice treated with wild-type PB-MNC. Scale bar: 100 μm.
Supplementary Figure 1

A. TNF-α (ng/dL)

B. G-CSF (ng/dL)

C. IL-6 (ng/dL)

Clinical response

*P=0.08
Supplementary Figure 2

![Graph showing Mac-3 positive cells/mm² for different groups: Non-ischemic Wild, Non-ischemic KO, PBS, Wild, Non-ischemic KO.](image-url)
Supplementary Figure 3
Supplementary Figure 4

![Graph showing blood flow for different groups: PBS, Wild, KO, Wild + IL-1β, KO.](image)
Supplementary Figure 5

A

B

PBS → Wild
IL-1β → Wild
PIM → Wild

Blood flow

CD31-positive cells/mm²

PBS → Wild
IL-1β → Wild
PIM → Wild

* **
Supplementary Figure 6