Autologous Peripheral Blood-Derived Mononuclear Cells Induced by Erythropoietin Improve Critical Ischemic Limbs

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Purpose: Efficient and secure collection of CD34+ cells are crucial for the angiogenic therapies. We have developed autologous peripheral blood-mononuclear cell (MNC) transplantation induced by erythropoietin (rhEPO) for critical ischemic limbs.

Methods: Seven patients, including five with arteriosclerosis obliterans, one with Buerger’s disease and one with progressive systemic sclerosis, underwent ten cell therapies. The first administration of rhEPO was performed two weeks before apheresis, and the second administration and blood donation were performed one week before apheresis to activate bone marrow. MNCs including CD34+ cells, isolated from peripheral blood by apheresis, were immediately injected intramuscularly into ischemic limbs.

Results: The number of peripheral blood-CD34+ cells had significantly increased from 1.32 ± 0.83/microL, before the rhEPO induction, to 1.86 ± 0.94/microL, before the apheresis. The number of transplanted MNCs ranged between 0.5 × 10⁹ and 16.5 × 10⁹, and that of CD34+ cells, between 0.1 × 10⁶ and 12.7 × 10⁶, accounting for 0.02%–0.1% of MNCs. There were no serious complications. Finger ulcers with Buerger’s disease were significantly improved one month after the transplantations, but the same or other ulcer(s) appeared 2–6 months later. Three patients had an improvement in rest pain, and one patient extended maximum pain-free walking distance.

Conclusions: Erythropoietin-induced autologous peripheral blood-MNC transplantation is a useful and safe alternative for ischemic limbs.

Keywords: erythropoietin, angiogenesis, autologous peripheral blood-derived mononuclear cell transplantation, critical ischemic limbs

INTRODUCTION

Despite the accumulation of clinical results of therapeutic angiogenesis using autologous bone marrow- or peripheral blood-derived mononuclear cells, there are still no absolute perspectives related to the efficacy and mechanism.1–7) Practical, less-invasive and easily applicable therapeutic angiogenesis remains to be established.

Recombinant human granulocyte colony-stimulating factor (G-CSF) has been used clinically to mobilize peripheral blood progenitor cells for hematopoietic cell transplantation and granulocytes for apheresis collection.8) However, angina has frequently occurred after
G-CSF-induced mobilization of peripheral blood progenitor cells in patients with advanced coronary artery disease. Spontaneous splenic rupture secondary to high-dose G-CSF use (20 mcg/kg/day) was also observed in a healthy allogeneic donor of peripheral blood progenitor cells. On the other hand, human recombinant erythropoietin (rhEPO) has been used safely in a clinical setting for renal anemia, and hematopoietic and endothelial cell lineages seem to share common progenitors. Erythropoietin has direct biologic effects on endothelial cells, and the vasculature seems to be an important erythropoietin target. Recently, a significant mobilization of CD34+/CD45+ circulating progenitor cells in peripheral blood was observed by rhEPO administration.

We have newly developed autologous peripheral blood-derived mononuclear cell (MNC) transplantation induced by erythropoietin and blood donation for activating the function of bone marrow, and have been performing this cell transplantation therapy for patients with critical ischemic limbs since 2004. The aim of the present study was to determine the efficacy and safety of the cell transplantation therapy as a therapeutic option for critical ischemic limbs.

**MATERIALS AND METHODS**

**Indication of autologous peripheral blood-derived mononuclear cell transplantation**

Cell therapy was indicated for selected patients whose activities of daily living were extremely disturbed and who did not show any favorable response to medication or conservative and surgical treatments for critical ischemic limbs. Exclusion criteria of the cell therapy were as follows: 1) life expectancy of less than one year, 2) history of alcohol or drug abuse within the past 3 months, 3) malignant tumor or possibility of malignant tumor, 4) history of the malignant tumor within the past 5 years, 5) diabetic proliferative retinopathy, 6) untreated severe ischemic heart disease, and 7) pregnancy or possibility of pregnancy. The institutional review board of Tokushima University Hospital (Clinical Research Number 158) approved the protocol of this study, and written informed consent was obtained from all subjects.

**Protocol of peripheral blood-derived mononuclear cell transplantation**

RhEPO and blood donation-induced mobilization of peripheral blood-progenitor cells was performed as follows: 1) 6000 IU of rhEPO of was administered subcutaneously two weeks before apheresis, 2) additional subcutaneous administration of 6000 IU of rhEPO and 400 ml of donated blood was performed one week before apheresis, 3) peripheral blood-derived MNCs were collected by apheresis using COBE Spectra™ two weeks after the first rhEPO injection, and 4) the cells were implanted intramuscularly into ischemic limbs (50-100 points) just after the apheresis under epidural or general anesthesia for upper extremities.

**Assessment of critical limb ischemia**

1) **Augmentation of CD34+ mononuclear cells in peripheral blood**

Levels of circulating CD34+, CD34+/CD133+ and CD34+/CD14+ cells in peripheral blood of the enrolled patients were measured by flow cytometry before and after apheresis. CD34 is a marker for hematopoietic progenitor cells, CD133, a marker for hematopoietic or non-hematopoietic progenitor cells, and CD14, a marker for monocytes. Increase in the number of CD34+ cells, in peripheral blood, by the above-mentioned induction was followed before the first and second rhEPO administrations and before apheresis. CD45+ is a marker for leukocyte common antigen. The numbers of peripheral-blood CD34+ cells were calculated by the formula (CD34+/CD45+ cells x the count of white blood cell/microL). The numbers of MNCs and CD34+ cells of the product harvested by apheresis were calculated separately from them.

2) **Assessment of cell therapy for limb ischemia**

The efficacy and safety of cell transplantation therapy for critical ischemic limbs were assessed before and at 1, 2, 3 and 4 weeks and 3 and 6-months after cell transplantation by 1) the rest-pain scale of ischemic limbs, 2) clinical findings of ischemic limbs (ulcer and gangrene), 3) ankle-brachial pressure index, 4) photoplethysmography, 5) thermography, 6) laser doppler perfusion imaging, 7) transcutaneous oxygen and carbon dioxide tensions (TcPO2 and TcPCO2), and 8) digital subtraction angiography.

**Patients’ characteristics**

Seven patients with critical ischemic limbs (male/female = 6/1), including 5 patients with arteriosclerosis obliterans (ASO), one patient with Buerger’s disease and...
one patient with progressive systemic sclerosis (PSS), were enrolled in this study during the period from 2004 to 2010 (Table 1). Only the patient with Buerger’s disease and intractable finger ulcers underwent cell therapy four times, and overall cell therapy was performed ten times. The ages of patients who underwent cell therapy ranged from 45 to 79 years (61 ± 13 years). The patient with Buerger’s disease complained of intractable pain and finger ulcers. The patient with progressive systemic sclerosis and two patients with ASO exhibited toe gangrene, and the remaining three patients with ASO had rest pain. Two of the patients with ASO suffered from chronic renal failure and were on hemodialysis.

Statistical analysis

Changes in the number of peripheral blood-CD34+ cells were expressed as percentages of the number before the 1st rhEPO administration (mean ± SD). Statistical comparisons were performed using the paired Student’s t-test, with a probability value of less than 0.05 taken to indicate significance.

Results

Laboratory examinations showed no polycythemia due to rhEPO administration or anemia due to phlebotomy but showed an increase in reticulocytes indicating activation of bone marrow (Table 2).

Table 1 Patients’ characteristics at first cell therapy

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Status of Limb ischemia</th>
<th>Previous treatment</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ASO</td>
<td>rest pain</td>
<td>Femoro-popliteal bypass, medication</td>
<td>lumbar spinal canal stenosis</td>
</tr>
<tr>
<td>2</td>
<td>ASO</td>
<td>rest pain</td>
<td>Femoro-popliteal bypass, medication</td>
<td>DM, HL, S/P CABG, CRF on HD</td>
</tr>
<tr>
<td>3</td>
<td>ASO</td>
<td>rest pain</td>
<td>medication</td>
<td>DM, HL</td>
</tr>
<tr>
<td>4</td>
<td>ASO</td>
<td>toe gangrene rest pain</td>
<td>Aorto-femoral bypass, medication</td>
<td>smoking</td>
</tr>
<tr>
<td>5</td>
<td>ASO</td>
<td>toe gangrene rest pain</td>
<td>medication</td>
<td>S/P CABG, CRF on HD, cerebral hemorrhage</td>
</tr>
<tr>
<td>6</td>
<td>Buerger</td>
<td>finger ulcers rest pain</td>
<td>medication</td>
<td>DM, HL</td>
</tr>
<tr>
<td>7</td>
<td>PSS</td>
<td>toe gangrene rest pain</td>
<td>medication</td>
<td>DM, HL, cerebral infarction</td>
</tr>
</tbody>
</table>

ASO: arteriosclerosis obliterans; PSS: progressive systemic sclerosis; DM: diabetes mellitus; HL: hyperlipidemia; S/P CABG: postoperative state of coronary artery bypass grafting; CRF on HD: chronic renal failure on hemodialysis

1) Augmentation and collection of CD34+ mononuclear cells in peripheral blood by erythropoietin administration and blood donation

Fluorescence–activated cell storing analysis of peripheral blood using CD34, CD133 and CD14 cells before and after apheresis demonstrated effective collection of peripheral blood-derived MNCs including CD34+ cells (Fig. 1A). The change of the number of peripheral blood-CD34+ cells in each patient, not on hemodialysis, is shown from before the 1st rhEPO administration to before apheresis in Fig. 1B. The number of peripheral blood-CD34+ cells had increased from 1.32 ± 0.83/μL before the rhEPO induction to 1.92 ± 1.29/μL before the 2nd rhEPO administration and to 1.86 ± 0.94/μL before apheresis (Fig. 1B). There was a significant difference between the number of peripheral blood CD34+ cells before the 1st rhEPO administration and that before apheresis. The number of MNCs harvested by apheresis ranged between 0.5 × 10^6 and 16.5 × 10^6 (mean ± SD, 10.0 × 10^6 ± 4.7 × 10^6). Consequently, the number of CD34+ cells in the product harvested by apheresis ranged between 0.1 × 10^6 and 12.7 × 10^6 (6.5 × 10^6 ± 4.5 × 10^6) and accounted for 0.06 ± 0.03% (0.02%–0.1%) of the MNCs (Table 3). Induction of peripheral blood CD34+ cells was performed without any serious complications; however, patients over 65 years of age or with chronic renal failure patients on hemodialysis had small numbers of peripheral blood CD34+ cells in the harvested MNCs.
Table 2  Laboratory examination around rhEPO and blood donation-induced mobilization of peripheral blood-derived progenitor cells and apheresis in a patient with Buerger’s disease

<table>
<thead>
<tr>
<th></th>
<th>1st Cell Therapy</th>
<th>2nd Cell Therapy</th>
<th>3rd Cell Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base line</td>
<td>2nd EPO</td>
<td>pre Aphe</td>
</tr>
<tr>
<td></td>
<td>5/10/’05</td>
<td>5/18</td>
<td>5/23</td>
</tr>
<tr>
<td>WBC (*10³/uL)</td>
<td>6.8</td>
<td>8.1</td>
<td>8.8</td>
</tr>
<tr>
<td>RBC (*10⁶/uL)</td>
<td>4.26</td>
<td>4.31</td>
<td>4.31</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.2</td>
<td>14.2</td>
<td>14.3</td>
</tr>
<tr>
<td>Platelet (*10³/uL)</td>
<td>343</td>
<td>309</td>
<td>319</td>
</tr>
<tr>
<td>Reticulocyte (%)</td>
<td>17</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>60.2</td>
<td>62.5</td>
<td>60.9</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>29.1</td>
<td>27.3</td>
<td>27.7</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>6.1</td>
<td>5.9</td>
<td>6.1</td>
</tr>
</tbody>
</table>

No polycythemia due to rhEPO administrations or anemia due to blood donation were shown. Reticulocytes were increased from 17–21‰ to 25–30‰ by human recombinant erythropoietin administrations and blood donation indicating activation of bone marrow. EPO: human recombinant erythropoietin; Aphe: apheresis; Base line: before the 1st rhEPO administration; 2nd EPO: after the 2nd rhEPO administration; preAphe: before apheresis; postAphe: after apheresis

Fig. 1  
A: Fluorescence-activated cell storing analysis of peripheral blood using CD34, CD133 and CD14 cells before and after apheresis. The results of peripheral blood-cell sorting analysis of patient 1 are shown. CD34+/ CD133+ mononuclear cells were effectively collected from peripheral blood by apheresis. 
B: Changes in the number of CD34+ cells in peripheral blood of patients not on hemodialysis from before the 1st rhEPO administration to before apheresis. Peripheral blood CD34+ cells increased to 159 ± 64% (82%–245%) before the 2nd rhEPO administration and to 137 ± 35% (88%–175%) before apheresis. There was a significant difference between the percentage of peripheral blood CD34+ cells before the 1st rhEPO administration and that before apheresis. 
rhEPO: human recombinant erythropoietin
2) Outcomes of critical limb ischemia after cell transplantation

There were no adverse complications caused by the trials (Table 4). Three patients showed subjective improvement in the rest-pain scale of ischemic limbs and exhibited objective appearance of pulse waves in a toe photoplethysmogram. Only one patient showed notable improvement of maximum pain-free walking distance from 160m before cell transplantation to 915m after cell transplantation. The two patients with ASO and the patient with PSS associated with advanced toe/foot gangrene required amputation of the lower limb within 3 months after cell transplantation.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Status of limb ischemia before cell therapy</th>
<th>Outcome of limb ischemia rest-pain scale (before→after)</th>
<th>Maximum walking distance after cell therapy (before→after)</th>
<th>Adverse complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ASO</td>
<td>rest pain</td>
<td>rest-pain scale (7→7)</td>
<td>wheelchair</td>
<td>none</td>
</tr>
<tr>
<td>2.</td>
<td>ASO (on HD)</td>
<td>rest pain</td>
<td>rest-pain scale (10→5)</td>
<td>48m→100m</td>
<td>none</td>
</tr>
<tr>
<td>3.</td>
<td>ASO</td>
<td>rest pain</td>
<td>rest-pain scale (2→0)</td>
<td>160m→915m</td>
<td>none</td>
</tr>
<tr>
<td>4.</td>
<td>ASO</td>
<td>toe gangrene rest pain</td>
<td>amputation</td>
<td>/</td>
<td>none</td>
</tr>
<tr>
<td>5.</td>
<td>ASO</td>
<td>toe gangrene rest pain</td>
<td>amputation</td>
<td>/</td>
<td>none</td>
</tr>
<tr>
<td>6. -1st.</td>
<td>Buerger</td>
<td>finger ulcer rest pain</td>
<td>temporarily improved rest-pain scale (6→3)</td>
<td>/</td>
<td>none</td>
</tr>
<tr>
<td>-2nd.</td>
<td>finger ulcer rest pain</td>
<td>temporarily improved rest-pain scale (3→1)</td>
<td>/</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>-3rd.</td>
<td>finger ulcer rest pain</td>
<td>temporarily improved</td>
<td>/</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>-4th.</td>
<td>finger ulcer rest pain</td>
<td>temporarily improved</td>
<td>/</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>PSS</td>
<td>toe gangrene rest pain</td>
<td>amputation</td>
<td>/</td>
<td>none</td>
</tr>
</tbody>
</table>

ASO: arteriosclerosis obliterans; PSS: progressive systemic sclerosis; rest-pain scale of ischemic limbs (before cell implantation→6 months after cell implantation)
A 48-year-old man with Buerger’s disease and intractable finger ulcers underwent successful 1st cell transplantation. Clinical symptoms with finger ulcers had significantly improved one month after MNC transplantation; however, an intractable ulcer on the same finger appeared two months later (Fig. 2A). Intermittent MNC transplantations were performed four times. Each cell transplantation resulted in an improvement, one month later, and the appearance of an ulcer(s) on the same/other finger ulcer(s) 2–6 months later. Laser Doppler perfusion imaging (LDPI) and TcPO₂ data showed an increase in peripheral tissue blood flow step by step with increase in the number of cell therapies (Fig. 2B and 2C).

**Fig. 2**

A: Intractable finger ulcers in a 48-year-old man with Buerger’s disease before and after the first cell therapy. Fourth finger ulcer of the right hand and fifth finger ulcer of the left hand were significantly improved one month after cell transplantation.

B: Changes in transcutaneous oxygen tension of both third fingers of the patient before and after 1st–3rd cell therapies. The values of TcPO₂ were increased one month after every cell therapy, but slightly decreased within 3 months. Overall, a slight increase in TcPO₂ was observed after cell therapies.

TcPO₂: transcutaneous oxygen tension

C: Changes in Laser Doppler perfusion imaging of both hands in the patient before and after cell therapies. Perfusion imaging improved after every cell transplantation. Local tissue perfusion seems to increase step by step with an increase in the number of cell therapies. A: right hand, B: left hand.

ROI: region of interest

ROI 1 indicates that of the great toe, ROI 2 indicates that of the second toe, ROI 3 indicates that of the third toe, and ROI 4 indicates that of the fourth toe. Y-axis shows the value (voltage) of mean perfusion in each ROI.

**DISCUSSION**

Some angiogenesis therapies, using endothelial progenitor cells or extended modifications for peripheral arterial disease, were exploited, according to its basic rule, since the Japan Trial for Therapeutic Angiogenesis Using Cell Transplantation and have been in clinical use. In particular view of safety concerns, clinical use of the several therapeutic angiogenesis using autologous bone marrow- or peripheral blood-derived MNCs have been accumulated, however, the superior efficacy of those cell therapies has not yet been achieved. Practical, less-invasive and easily applicable therapeutic angiogenesis remains to be established. Peripheral blood MNC
transplantation is expected to be developed as an efficient and safe optional treatment for critical ischemic limbs.

**Mechanistic explanation of how transplanted mononuclear cells work in critical ischemic limbs**

The patient who had symptoms that were most suggestive of Buerger’s disease and intractable finger ulcers underwent four successful cell transplantations that resulted in an improvement, one month later, though the patients had a relapse of the same/other finger ulcer(s), 2–6 months later. Clinical improvement of the finger ulcers coincided with the timing of peak values of local LDPI and TcPO₂, after each cell therapy. Those facts imply that the cell therapy itself plays some role in healing ischemic ulcers and that its working mechanism was through the upregulation of some tissue cytokines such as classic angiogenic growth factors consequent to the transplanted MNCs.¹⁷–²⁰ There was a recent report that human CD34+ exosomes may represent a significant component of the paracrine effect of progenitor cell transplantation for therapeutic angiogenesis.²² Not only the long survival and the increase of transplanted MNCs, but also the potential of paracrine effects provided by the local secretion of cytokines, may underline the longevity of improvement of ischemic limbs. In particular, the anti-apoptosis effect of augmented cytokines may result in the improvement. The resident population of apoptotic cells that are competent to respond to cytokines may also constitute a potentially limiting factor in those cell therapies. Since there was a long period (up to 6 months) until disappearance of finger ulcers, a more progressed cell therapy, with fine conservative management, will provide complete remission of finger ulcers in the near future. It is necessary to further investigate how rhEPO-induced MNCs participate in the restoration of damaged tissue circulation.

**Role of erythropoietin induction in the cell therapy**

Erythropoietin can induce endothelial cell proliferation and differentiation and accelerate the migration of endothelial progenitor cells to peripheral blood.¹¹ Erythropoietin levels were significantly increased on the day of peripheral blood stem cell collection, 2 weeks after chemotherapy, and G-CSF administration in comparison with levels of other cytokines.²³ Exposure to moderate hypoxia leads to an increase in progenitor cells in tissue and circulation, the effect of which is dependent on erythropoietin.²⁴ Serum levels of erythropoietin are significantly associated with the number and function of circulating endothelial progenitor cells as well as with the number of progenitor cells in bone marrow and may help in identifying patients with impaired endothelial progenitor cell recruitment capacity. Strategies that increase serum erythropoietin concentrations may be promising for augmenting the regenerative properties of peripheral blood progenitor cells.

Although therapeutic angiogenesis, using many CD34+ cells from bone marrow, has been introduced in patients with peripheral artery disease, the invasiveness limits clinical application for cell therapy.¹ On the other hand, therapeutic angiogenesis using peripheral blood-derived MNCs is less invasive, and it also has the disadvantage of a small number of MNCs harvested by apheresis. Endothelial progenitor cells consist of about 0.01% of bone marrow-derived MNCs, and those of peripheral blood-derived MNCs is also anticipated being extremely low.

**Pathophysiological factors influencing the efficacy and safety of the cell therapy**

Other investigators have demonstrated that the number of peripheral blood-derived MNCs induced by G-CSF for cell therapy to ischemic limbs was 2.3-11.6 × 10¹⁰ and that the number of CD34+ cells was 1.37-14.9 × 10⁷ (mean, 0.13%; 0.07%-0.29%).²⁵,²⁶ Pretreatment with rhEPO caused significant mobilization of CD34+ circulating progenitor cells in peripheral blood and increased the number of functionally active endothelial progenitor cells in patients (week 2, 312 ± 31%; week 8, 308 ± 40%) as well as in healthy subjects (week 8, 194 ± 15%).¹¹ Our previous study also revealed that the combination of rhEPO administration and blood donation resulted in a significantly greater increase in the number of CD34+ and CD34+/CD133+ MNCs than that in the case of blood donation only and that rhEPO administration was a significant independent factor for the magnitude of increase in CD34+/CD133+ MNCs.²⁷ The results of the present study suggest that the optimal timing of apheresis for cell therapy is one week after 2nd rhEPO administration and blood donation as our protocol.

Although the mechanisms by which stem cells might contribute to angiogenesis and the origin of neovascular endothelial cells are controversial,²²,²⁸ our results seem to imply that the efficacy of induction of autologous peripheral blood-derived MNCs by rhEPO and blood donation is dependent on the number of transplanted CD34+ cells.
and the patient’s pathogenesis. Among patients less than 55 years of age or without chronic renal failure requiring hemodialysis, CD34+ cells were increased to 0.07%–0.1% of MNCs by our scheduled induction, and 9.2-16.1 \times 10^6 MNCs and 7.4-12.7 \times 10^6 CD34+ cells were transplanted to ischemic limbs. Although the augmentation by rhEPO was inferior to that by G-CSF, peripheral blood MNCs induced by rhEPO showed subjective and objective improvements to some degree without serious adverse effects such as angina and myocardial infarction. The acuity of ischemic limbs seems to be a limiting factor, and recipients with Buerger’s disease may be more favorable than those with ASO. In any case, early cell transplantation therapy is recommended as an optional treatment for selected patients with peripheral arterial disease.

**CONCLUSIONS**

The findings suggest that autologous peripheral blood-mononuclear cell transplantation induced by rhEPO is a useful and safe alternative for patients with peripheral arterial disease, particularly for patients with Buerger’s disease and intractable finger ulcers.

**REFERENCES**

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