Atherosclerosis obliterans (ASO) is a disease that significantly impacts patients’ quality of life (QOL) because of leg pain, ulceration, and gangrene. In most developed countries, the incidence of severe limb ischemia is estimated to be 50–100 per 100,000 cases every year,1 but the number of patients needing medical treatment for severe limb ischemia will probably increase in the coming years because of the increasing prevalence of diabetes mellitus (DM) and hypertension, aging populations, and tobacco consumption. Current treatment options include medicines, such as anticoagulants and antiplatelet drugs, intravascular treatments, and bypass surgery. However, in patients with diffuse and distal peripheral severe stenosis, amputation of lower extremities is often required, especially in hemodialysis (HD) patients.

Recently, the use of mononuclear cells harvested from the bone marrow or peripheral blood mononuclear cells (PBMCNs) has been reported to improve limb ischemia in such patients, suggesting that this may be a therapeutic strategy for peripheral artery disease.2–4 However, there are no reports of the long-term effects or changes in QOL after PBMCN transplantation for severe ASO in DM patients on HD, who are considered the high-risk group for limb ischemia and amputation. In this study, we examined 7 such patients who underwent autologous PBMCN transplantation for severe intractable ASO and evaluated the effectiveness, duration of effect of this therapy, the QOL, and any complications.

**Background** Severe atherosclerosis obliterans (ASO) can be intractable, especially in diabetic patients on hemodialysis (HD). Recently, the transplantation of autologous peripheral blood mononuclear cells (PBMCNs) has been reported to have beneficial effects, but the long-term effects and impact on quality of life (QOL) have not been studied.

**Methods and Results** Autologous PBMCNs were transplanted into 7 diabetic patients on HD who had severe ASO (5 cases with Fontaine IV and 2 with Fontaine III) after administration of 5μg/kg granulocyte colony stimulating factor; QOL and degree of ischemia was assessed by measuring skin temperature, skin perfusion pressure (SPP), ankle–brachial index (ABI), and ulcer size, and from angiographic findings. At 4 weeks after the procedure, skin temperature was significantly improved, and SPP and ABI also were increased. These beneficial effects persisted for up to 24 weeks. Angiographic findings and ulcer size improved in 3 of 7 and 3 of 4 patients, respectively. SF-36v2 analysis revealed significant improvements in pain scores. No serious complications were detected.

**Conclusion** Transplantation of PBMCNs resulted in improvement in pain and leg ischemia for over 6 months without serious complications. This therapy is safe and effective for severe ASO in diabetic patients on HD. (Circ J 2007; 71: 1193–1198)

**Key Words:** Arteriosclerosis obliterans; Diabetes mellitus; Dialysis; Peripheral blood mononuclear cell transplantation; Quality of life
lants, antiplatelet drugs, and prostaglandins for more than 3 months. We discussed treatment options for every patient by conference with cardiovascular specialists, nephrologists, plastic surgeons, and anesthesiologists. All patients were considered not amenable to surgical, intravascular, or other traditional treatments. Six of the 7 patients had histories of above- or below-knee or toe amputation. Anti-platelet agents, anticoagulants, and prostaglandins were continued during the pretreatment examination and follow-up, except for 1 week before the spinal anesthesia given at the time of transplantation.

**Protocol**

We diagnosed ASO by physical status and angiography, and assessed ischemic conditions by measuring the ankle–brachial index (ABI), the toe–brachial pressure index (form PWV/ABI, Omron Colin, Tokyo), skin perfusion pressure (SPP) (PV2000, Kaneka, Osaka), and skin temperatures using thermal photography (Infraeye 1200A, Nihon Koden, Tokyo) under constant room temperature (25.0±1.0°C) and humidity (50.0±20.0%). The major and minor axes of skin ulcers were measured. These parameters were measured 0, 1, 2, 4, 12, and 24 weeks after treatment, and compared with the pretreatment values.

The patients underwent digital subtraction angiography before and 1 month after treatment. The angiographic score (2) for the formation of new collateral vessels was assessed as +0 (no collateral development), +1 (slight), +2 (moderate), and +3 (rich) at the time at which contrast flow in the main conducting arteries was most clearly visible. To minimize body temperature bias, we performed measurements at a constant room temperature and humidity, after allowing the patient to rest in the room for 15 min. Leg and toe temperatures were adjusted to forehead temperature to correct for differences in other conditions. SPP was measured on the day without HD and the value was adjusted by systemic pressure.

Patients’ health-related QOL (HR-QOL) was evaluated by a questionnaire (SF-36 Health Survey, version 2 [SF-36v2]: SF-36 Medical Outcomes Trust) at 0 and 24 weeks after treatment.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/ gender</th>
<th>Fontaine</th>
<th>HD duration (years)</th>
<th>Amputation history</th>
<th>Total cells (×10⁶/body)</th>
<th>CD34⁺ cells (×10⁶/body)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65/M</td>
<td>III</td>
<td>12</td>
<td>–</td>
<td>×</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>64/F</td>
<td>IV (hand ulcer) &amp; leg gangrene</td>
<td>2</td>
<td>R below-knee &amp; 4 fingers</td>
<td>×</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>66/M</td>
<td>IV (ulcer)</td>
<td>10</td>
<td>L above-knee</td>
<td>×</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>64/F</td>
<td>IV (ulcer)</td>
<td>1.5</td>
<td>L toe</td>
<td>1.19</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>64/M</td>
<td>IV (gangerone)</td>
<td>6</td>
<td>L toe</td>
<td>4.32</td>
<td>12.7</td>
</tr>
<tr>
<td>6</td>
<td>54/M</td>
<td>IV (ulcer)</td>
<td>4</td>
<td>L toe</td>
<td>3.15</td>
<td>8.0</td>
</tr>
<tr>
<td>7</td>
<td>68/F</td>
<td>III</td>
<td>18</td>
<td>L below-knee</td>
<td>1.58</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 1 Patients’ Characteristics**

HD, hemodialysis; Total cells, implanted total mononuclear cells.

![Image of skin temperature improvement](image-url)

Fig 1. Skin temperature improved in 6 of 7 patients. Skin temperature in the lower legs and toes improved at each observational point with significance, even at 1 and 2 weeks after the treatment. The peak elevations of skin temperature were observed at 6 months after the treatment (end of the observation period) in the lower legs (+1.18±1.08°C), and 3 months in the toes (+1.33±0.92°C). *p<0.05; **p<0.01, vs control (before the treatment).
Long-Term Effects of PBSCT for ASO in DM on HD

Circulation Journal Vol.71, August 2007

The SF-36v2 health survey consists of 36 questions that evaluate 8 discrete areas: physical functioning, social functioning, bodily pain, general health perceptions, vitality, role limitations due to emotional problems (role-emotional), role limitations due to physical health problems (role-physical), and mental health. Analysis and scoring of SF-36v2 data were subjected to norm-based scoring to compare with a Japanese standard population according to guidelines developed by Fukuhara et al.5

Therapies
After administration of 50μg·kg⁻¹·day⁻¹ G-CSF (lenogras-

---

**Lower legs**

| Mean±SD | 9.0±10.7 | 8.8±17.4 | 10.8±16.6 | 3.1±22.4 | 18.8±27.5 |
| Δ (mmHg) | 60.0 | 40.0 | 20.0 | 0.0 | 20.0 |

![Graph of Lower legs data](image)

**Toes**

| Mean±SD | -1.1±12.4 | 7.9±5.5 | 0.7±3.5 | -1.4±7.6 | -2.5±16.4 |
| Δ (mmHg) | 60.0 | 40.0 | 20.0 | 0.0 | 20.0 |

![Graph of Toes data](image)

Fig 2. Skin perfusion pressures (SPP) in the lower legs improved more than 10 mmHg in 4 of 7 patients, and less than 10 mmHg in 1 of 7 patients. It also increased early after treatment, but did not show a significant change. SPP in the toes did not change significantly. *p<0.05; **p<0.01, vs control (before the treatment).

**ABI changes**

| Mean±SD | -0.02±0.07 | 0.01±0.09 | 0.04±0.14 | 0.10±0.16 | 0.11±0.15 |
| Δ (mmHg) | 0.4 | 0.2 | 0.1 | 0.0 | 0.0 |

![Graph of ABI changes data](image)

Fig 3. The ankle-brachial index (ABI) improved gradually after treatment. At the end of the observation period, 6 months, the peak elevation of ABI (+0.11±0.15) was seen and ABI improved more than 0.10 in 5 of 7 cases; however, it did not show a significant change.

Table 2 Ulcer Size Changes After the Treatment

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Before</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>50×30</td>
<td>40×25</td>
<td>20×12</td>
<td>12×8</td>
</tr>
<tr>
<td>3</td>
<td>10×8</td>
<td>0×0</td>
<td>0×0</td>
<td>0×0</td>
</tr>
<tr>
<td>4</td>
<td>10×6</td>
<td>10×5</td>
<td>10×5</td>
<td>10×5</td>
</tr>
<tr>
<td>6</td>
<td>20×10</td>
<td>5×5</td>
<td>0×0</td>
<td>0×0</td>
</tr>
</tbody>
</table>

Ulcer size improved in 3 of 4 patients. Patient no. 3 and no. 6 improved completely within 2 weeks and 3 months, respectively.
tim or filgrastim) by subcutaneous injection for 4 days, autologous PBMNCs were harvested using a Spectra cell separator (Cobe, Tokyo, Japan). We checked the number of leukocytes every day during administration of G-CSF, and if leukocyte count before administration of G-CSF was >50,000/μl, administration of G-CSF was reduced to 2.5 μg·kg⁻¹·day⁻¹, and if over 75,000/μl, administration of G-CSF was stopped. After separating PBMNCs and checking the number of CD34+ cells, patients were injected with 0.5 ml of mononuclear fluid in each of 50–100 sites in the muscle of both ischemic limbs, under epidural anesthesia on a single day. Injections were placed 1 cm apart, using a 25 g needle.

Statistical Analysis

Clinical data were analyzed with unpaired 2 group t-tests and nonparametric Mann-Whitney tests as appropriate. Results are presented as means±SD. Analyses were performed with JMP 5.0.1J software (SAS Institute, Cary, NC, USA). P-values below 0.05 were considered to be statistically significant.

Results

In most patients, skin temperature in the lower legs and toes improved at each observational point, even at 1 and 2 weeks after the treatment (Fig 1). The peak elevations of skin temperature were observed at 6 months after the treatment (+1.18±1.08°C) and at 3 months in the toes (+1.33±0.92°C). The SPP in the ankles also increased early after treatment (Fig 2); however, the SPP in the toes did not show a significant change. ABI also improved gradually after the treatment (Fig 3). The peak elevation of ABI was seen at the end of the observation period (ie, 6 months: +0.11±0.15) and ABI improved more than 0.10 in 5 of 7 cases; however ABI did not show a significant change.

Ulcer size improved in 3 of 4 patients (Table 2). Ischemic leg pain improved in 6 of 7 patients within 2 weeks (Table 3), and continued to the end of the observation period. Angiographic improvements were seen in 3 of 7 patients at 1 month after the treatment (Fig 4). After treatment, bodily pain on the HR-QOL improved with significance (p<0.01) and no HR-QOL parameter got worse after the treatment (Fig 5). We could not find any relationship between the number of CD34+ cells injected and clinical outcomes. There was no patient who had worsened ASO symptoms or ulcer size, new cardiovascular events, or other serious complications. One patient with moderate proliferative diabetic retinopathy had minor retinal bleeding 1 week after the treatment, which recovered within 1 month without functional loss. One patient with progressive toe gangrene (patient No. 5) before transplantation required toe amputation despite treatment.
Discussion

The number of patients with ischemic limbs is increasing, especially those with DM on HD. These patients represent one of the highest risk groups for peripheral arterial disease and their treatment options are often limited to amputation. The poor outcomes of these patients may be due to vascular calcification and poor physical status. Many new angiogenic therapeutic methods using mononuclear cells and recombinant gene therapy for ischemic limbs have been recently reported and shown to have beneficial effects. Treatment with bone marrow mononuclear cells (BMMNCs) was reported to promote angiogenesis in the diabetic foot, and Rehman and Tateno reported that PBMNCs produce more angiogenic factors and cytokines than BMMNCs, which may also make them a more useful source. Our results support this hypothesis. CD34– cells might also contribute to endothelium when CD34 cells were used alone. Rehman et al. reported that there is a positive relationship between these factors, but Kinnaird et al. did not find a relationship between the number of CD34+ cells injected and potential improved angiogenic effects. In our study, we did not find a relationship between the number of CD34+ cells injected and potential improved angiogenic effects. In our study, we did not find a relationship between the number of CD34+ cells injected and potential improved angiogenic effects.

It is still unknown whether there is a relationship between the number of CD34+ cells injected and potential improved angiogenic effects. In our study, we did not find a relationship between outcome and the number of CD34+ cells injected. For example, patient No. 2, who received 100 fewer CD34+ cells than patient No. 5, had a much bigger improvement in blood flow and ulcer condition than patient No. 5. Saigawa et al. and Ishida et al. reported that there is a positive relationship between these factors, but Kinnaird et al. and Rehman et al. reported that this benefit was related to the level of angiogenic factors produced not merely the number of cells. By examining human microvascular endothelial cells, Rookmaaker et al. reported that CD34− cells can induce migration of CD34+ cells to the endothelium because they observed little new angiogenesis and no recruitment to endothelium when CD34 cells were used alone. Our results support this hypothesis. CD34− cells might also influence angiogenesis and have therapeutic effects.

In the present study, most patients showed peripheral blood flow improvement within 1 or 2 weeks, with continued effects over 6 months. This early improvement suggests the...
effects of cytokines after the procedure, because it is too early for significant angiogenesis to have occurred. The later, persistent effects might be because of angiogenesis, as 6 months is likely too long to sustain an acute inflammatory response. Our results suggest the hypothesis that most of the angiogenic effects are related to such angiogenic factors as interleukin (IL)-1, PDGF, and IL-8.12–15

Therapeutic angiogenesis is an important future therapeutic target for cardiovascular and peripheral ischemic diseases. Our findings suggest long-term effects of autologous PBMNCs transplantation, not only on clinical data but also on QOL, for severe ischemic limb disease, even in diabetic patients on HD who comprise one of the highest risk groups, without serious complications.

Acknowledgments

We thank our colleagues Drs Shigemoto Nakanishi, Yoshihiro Naruse, Akiko Maehara (Cardiovascular center, Toranomon Hospital), and Mayumi Okada (Department of anesthesiology) for their helpful advice.

References