Autologous Bone-Marrow Mononuclear Cell Implantation Reduces Long-Term Major Amputation Risk in Patients With Critical Limb Ischemia

A Comparison of Atherosclerotic Peripheral Arterial Disease and Buerger Disease

Naomi Idei, MD; Junko Soga, MD; Takaki Hata, MD; Yuichi Fujii, MD; Noritaka Fujimura, MD; Shinsuke Mikami, MD; Tatsuya Maruhashi, MD; Kenji Nishioka, MD, PhD; Takayuki Hidaka, MD; Yasuki Kihara, MD, PhD; Moniruddin Chowdhury, MD, PhD; Kensuke Noma, MD, PhD; Akira Taguchi, DDS, PhD; Kazuaki Chayama, MD, PhD; Taijirou Sueda, MD, PhD; Moniruddin Chowdhury, MD, PhD, FAHA

Background—Bone-marrow mononuclear cell (BM-MNC) implantation improves ischemic symptoms in patients with critical limb ischemia (CLI). The purpose of this study was to evaluate long-term clinical outcomes after autologous BM-MNC implantation in patients with CLI.

Methods and Results—We assessed long-term clinical outcomes after BM-MNC implantation in 51 patients with CLI, including 25 patients with peripheral arterial disease (PAD) and 26 patients with Buerger disease. Forty-six CLI patients who had no BM-MNC implantation served as control subjects. Median follow-up period was 4.8 years. The 4-year amputation-free rates after BM-MNC implantation were 48% in PAD patients and 95% in Buerger disease, and they were 0% in control PAD patients and 6% in control Buerger disease. The 4-year overall survival rates after BM-MNC implantation were 76% in PAD patients and 100% in Buerger disease, and they were 67% in control PAD patients and 100% in control Buerger disease. Multivariable Cox proportional hazards analysis revealed that BM-MNC implantation correlated with prevention of major amputation and that hemodialysis and diabetes mellitus correlated with major amputation. In Buerger disease, ankle brachial pressure index and transcutaneous oxygen pressure were significantly increased after 1 month and remained high during 3-year follow-up. However, in patients with PAD, ankle brachial pressure index and transcutaneous oxygen pressure significantly increased after 1 month and gradually decreased during 3-year follow-up and returned to baseline levels.

Conclusions—These findings suggest that BM-MNC implantation is safe and effective in patients with CLI, especially in patients with Buerger disease.

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Key Words: autologous bone-marrow mononuclear cell ■ critical limb ischemia ■ peripheral arterial disease ■ Buerger disease

Surgical bypass and percutaneous transluminal angioplasty and combination with pharmacological therapy are options for revascularization and improvement in limb ischemic symptoms in patients with peripheral arterial disease (PAD). PAD patients with no other treatment option must undergo amputation. It has been shown that cell therapy using implantation of bone-marrow or peripheral mononuclear cells as well as endothelial progenitor cells improves clinical symptoms and perfusion parameters in patients with critical limb ischemia (CLI) who have no other treatment option.1-8 We have shown that autologous bone-marrow mononuclear cell (BM-MNC) implantation improves endothelial function in patients with CLI.9 However, the results of studies have not all been equally positive: In some cases, there were no effects or occasionally adverse effects.10 Unfortunately, there has been no

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From the Department of Cardiovascular Medicine (N.I., J.S., Y.F., N.F., S.M., T.M., K.N., T.H., Y.K.), Department of Medicine and Molecular Science (K.C.), Department of Surgery (T.S.), and Department of Cardiovascular Physiology and Medicine (K.N., Y.H.), Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan; the Department of Hematology and Oncology (M.C.), Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; the Division of Regeneration and Medicine (Y.H.), Hiroshima University Hospital, Hiroshima, Japan; and the Department of Oral and Maxillofacial Radiology (A.T.), Matsumoto Dental University, Shiojiri, Japan.

Correspondence to Yukihito Higashi, MD, Department of Cardiovascular Physiology and Medicine, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail yhigashi@hiroshima-u.ac.jp

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BM-MNC implantation study with a randomized, double-blinded, placebo-controlled design.

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PAD is one of the major manifestations of atherosclerosis. PAD is associated with high rates of cardiovascular morbidity and mortality. It is clinically important to assess the effects of cell therapy on long-term outcomes in patients with CLI. Recently, it has been reported that autologous BM-MNC implantation may improve major amputation survival rate in patients with CLI, suggesting that cell therapy using autologous BM-MNC implantation is safe and effective in these patients. However, control patients were not included in that study.

Therefore, in the present study, we evaluated the long-term clinical outcomes in patients with CLI, including patients with atherosclerotic PAD and patients with Buerger disease who underwent BM-MNC implantation and in CLI patients who had no BM-MNC implantation as control subjects.

Methods

Subjects

From April 2000 to December 2008, 97 patients with CLI (51 patients with atherosclerotic PAD and 46 patients with Buerger disease) who had severe rest pain and nonhealing ulcers and who were not candidates for angioplasty or surgical revascularization from the Hiroshima University PAD database were enrolled in this study. Buerger disease was diagnosed by the previous criteria, including results of physical examinations, clinical symptoms, and angiographic findings: smoking history, onset before the age of 50 years, infra-arterial arterial occlusive disease, either upper limb involvement or phlebitis migrans, and absence of atherosclerotic risk factors other than smoking. To rule out other vasculitides and hypercoagulable states, rheumatoid factor, lupus anticoagulants, and serological investigations were evaluated. The diagnosis of limb ischemia was confirmed by angiography. CLI was classified according to the guidelines of Trans Atlantic Inter-Societal Consensus II. Fifty-one patients with CLI (25 patients with atherosclerotic PAD and 26 patients with Buerger disease) underwent autologous BM-MNC implantation, and 46 patients with CLI (30 patients with atherosclerotic PAD and 16 patients with Buerger disease) continued to receive conventional therapy after being considered candidates for major amputation. Major amputation is defined as above-the-ankle amputation. This was a nonrandomized trial. Patients stopped smoking before treatment with BM-MNC implantation. The study protocol was approved by the Ethics Committee of Hiroshima University Graduate School of Medicine. Written informed consent for participation in the study was obtained from all subjects.

BM-MNC Implantation

BM-MNCs were isolated and implanted in patients with CLI, as previously described. Briefly, we aspirated ~500 mL of bone marrow from the ileum of each patient under general anesthesia and immediately isolated BM-MNCs using a CS3000-Plus blood-cell separator (Baxter, Deerfield, IL) to obtain a final volume of ~50 mL. We then intramuscularly implanted ~0.75 mL of BM-MNCs into each injection site (total of 40 sites, 1.5 cm in depth) in the gastrocnemius of each ischemic leg, with a 3×3-cm grid using a 22-gauge needle. The numbers of BM-MNCs implanted into ischemic limbs were 1.8×10⁷ ± 0.5×10⁷, and the numbers of CD34⁺ cells included in the implanted BM-MNCs were 3.5×10⁶ ± 1.4×10⁶.

Measurement of Number of Endothelial Progenitor Cells

We measured the number of endothelial progenitor cells (EPCs) in 51 patients with CLI (25 patients with atherosclerotic PAD and 26 patients with Buerger disease) who underwent autologous BM-MNC implantation and 25 healthy subjects (20 men and 5 women; mean age, 51.2±14.1 years). The number of EPCs was analyzed by flow cytometry as previously described. Briefly, samples of BM were placed in tubes containing sodium EDTA (7 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. BM-MNCs were immediately isolated by Ficoll density gradient centrifugation (AXIS-SHIELD, Dundee, Scotland). After thawing, 1×10⁶ mononuclear cells were incubated for 10 minutes with monoclonal antibodies against human fluorescein isothiocyanate–conjugated anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-AC133 (Miltenyi Biotec), and APC-conjugated anti-CD34 monoclonal antibody (Becton Dickinson Biosciences, Franklin Lakes, NJ). To assess background, isotype controls were used as negative controls, based on the species and IgG subclass of each antibody. After incubation, erythrocytes were lysed, and the remaining cells were washed with phosphate-buffered saline, fixed in 2% paraformaldehyde, and analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Biosciences). Each analysis consisted of 500 000 events. To quantify the amount of CD34⁺ ACC133⁺ CD45⁻low cells, the mononuclear cell fraction was gated and analyzed for the expression of AC133 and CD45. Only the AC133⁺ CD45⁻low cells were finally investigated for the count of CD34⁺ cells.

Characterization of Progenitor Cells

Mononuclear cells were isolated by Ficoll density-gradient centrifugation of human blood buffy coats from 50 mL of peripheral blood. Then, 1×10⁶ mononuclear cells were plated on 6-well culture dishes coated with human fibronectin and gelatin and maintained in endothelial cell basal medium-2 (EBM-2, Takara Bio Inc., Otsu, Japan) supplemented with EGM-2 microvascular single aliquots and 5% fetal bovine serum. After 3 days of culture, nonadherent cells were removed. Cytotoxicity analysis of adherent cells was performed on day 4. To detect the uptake of 1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine–labeled acetylated LDL (Di-AcLDL; Molecular Probes, Carlsbad, CA), cultured cells were incubated with Di-AcLDL (10 μg/mL) at 37°C for 1 hour. Thereafter, cells were fixed with 2% paraformaldehyde for 10 minutes and incubated with fluorescein isothiocyanate–labeled Ulex europaeus agglutinin I (lectin, 10 μg/mL; Sigma) for 1 hour. Cells demonstrating double-positive staining lectin and Di-AcLDL were identified to be progenitor cells. Three randomly selected high-power fields per well were counted.

Migration Assay

We measured cell migration response to vascular endothelial growth factor (VEGF) in 51 patients with CLI (25 patients with atherosclerotic PAD and 26 patients with Buerger disease) who underwent autologous BM-MNC implantation and 25 healthy subjects (20 men and 5 women; mean age, 51.2±14.1 years). Cell migration was evaluated using a modified Boyden chamber assay as previously described. Briefly, isolated progenitor cells were detached mechanically by using a cell scraper, harvested by means of centrifugation, resuspended in 300 μL of EBM, and counted. The 2×10⁴ progenitor cells were placed in the upper chamber of a modified Boyden chamber (FluroBlock, Becton Dickinson Biosciences). The chamber was placed in a 24-well culture dish containing EBMs and culture medium for control and human recombinant VEGF (50 ng/mL; Sigma). After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification of cells that had migrated, cell nuclei were stained with 4′,6-diamino-phenylindole (DAPI, Sigma). Migrated cells in the lower chamber were counted manually in the 3 random high-power fields. Each experiment was performed in triplicate.

Statistical Analysis

Results are presented as mean± SD. All reported probability values were 2-tailed. Values of P<0.05 were considered significant. Continuous variables were compared by using ANOVA for multiple
groups and the t test between 2 groups. Categorical variables were compared by means of the Fisher exact test. Time-to-event end-point analyses were performed by using the Kaplan-Meier method. A log-rank test was used to compare survival in the groups. Multivariable Cox proportional hazard regression analysis was performed to identify independent predictors for major amputation in addition to univariate analysis. First, variables were selected by univariate analysis adjusting for age, using the level of \( P < 0.05 \) as an indicator of significance. Next, a search for the best model was conducted in stages. Initially, all factors selected in the univariate analysis were included in the model. Nonsignificant variables were successively eliminated until all remaining variables except age were significant (\( P < 0.05 \)). Medians and interquartile ranges were determined for the follow-up time. Comparisons of variables before and after BM-MNC implantation were performed with adjusted means by analysis of covariance with the use of baseline data as covariates. Comparisons of time course curves of ankle brachial pressure index (ABPI), transcutaneous oxygen pressure (TcO2), and visual analog pain score (VSA) with 10 levels after BM-MNC implantation were performed using 2-way ANOVA for repeated measures on 1 factor followed by Bonferroni correction for multiple paired comparisons. Missing ABPI, TcO2, and VSA values were imputed using the last observation carried forward method. Data were processed using the software package Statview (SAS Institute, Cary, NC) or Super ANOVA (Abacus Concepts, Berkeley, CA).

Results

Clinical Characteristics

Baseline clinical characteristics in the BM-MNC implantation group and no–BM-MNC implantation group (control group) are summarized in Table 1. There were notable differences in baseline clinical characteristics, including age, risk factors, complications, and drugs used, between patients with atherosclerotic PAD and patients with Buerger disease. These were no significant differences between parameters for patients with atherosclerotic PAD in the BM-MNC implantation group and those in the control group or between parameters for patients with Buerger disease in the BM-MNC implantation group and those in the control group (Table 1).

Table 1. Clinical Characteristics in the BM-MNC Implantation Group and Control Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Atherosclerotic PAD (n=30)</th>
<th>Buerger (n=16)</th>
<th>Atherosclerotic PAD (n=25)</th>
<th>Buerger (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>69.3±9.5*</td>
<td>42.9±9.8</td>
<td>68.1±8.2†</td>
<td>43.2±10.0</td>
</tr>
<tr>
<td>Sex, men/women</td>
<td>22/8</td>
<td>14/2</td>
<td>16/7</td>
<td>23/3</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.2±3.7</td>
<td>22.4±4.0</td>
<td>23.1±3.9</td>
<td>22.5±4.1</td>
</tr>
<tr>
<td>Rutherford category, n (％)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8 (27)</td>
<td>3 (19)</td>
<td>7 (28)</td>
<td>8 (31)</td>
</tr>
<tr>
<td>5</td>
<td>16 (53)</td>
<td>9 (56)</td>
<td>13 (52)</td>
<td>14 (54)</td>
</tr>
<tr>
<td>6</td>
<td>6 (20)</td>
<td>4 (25)</td>
<td>5 (20)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Previous treatment, n (％)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bypass graft</td>
<td>9 (30)*</td>
<td>2 (13)</td>
<td>5 (20)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>PTA</td>
<td>10 (33)*</td>
<td>1 (6)</td>
<td>5 (20)*†</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Complications, n (％)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>21 (70)*</td>
<td>1 (6)</td>
<td>17 (68)*†</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>22 (73)*</td>
<td>1 (6)</td>
<td>18 (72)*†</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>15 (50)*</td>
<td>1 (6)</td>
<td>12 (48)*†</td>
<td>2 (8)</td>
</tr>
<tr>
<td>CRF (hemodialysis)</td>
<td>10 (33)*</td>
<td>0 (0)</td>
<td>8 (30)*†</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>8 (27)</td>
<td>0 (0)</td>
<td>6 (24)*†</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>8 (27)*</td>
<td>0 (0)</td>
<td>8 (30)*†</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Medication, n (％)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiplatelet</td>
<td>29 (97)</td>
<td>16 (100)</td>
<td>24 (96)</td>
<td>24 (92)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>8 (27)*</td>
<td>1 (6)</td>
<td>6 (24)*†</td>
<td>2 (8)</td>
</tr>
<tr>
<td>ARBs</td>
<td>3 (10)*</td>
<td>0 (0)</td>
<td>4 (16)*†</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>13 (43)*</td>
<td>0 (0)</td>
<td>14 (56)*†</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Statins</td>
<td>14 (47)*</td>
<td>1 (6)</td>
<td>9 (36)*†</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Sulfonylurea and/or metformin</td>
<td>17 (57)*</td>
<td>1 (6)</td>
<td>14 (56)*†</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Insulin</td>
<td>7 (23)</td>
<td>0 (0)</td>
<td>5 (20)*†</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Smoker, n (％)</td>
<td>21 (70)</td>
<td>16 (100)</td>
<td>15 (60)</td>
<td>25 (96)</td>
</tr>
</tbody>
</table>

PTA indicates percutaneous transluminal angioplasty; CRF, chronic renal failure; ACE, angiotensin-converting enzyme; and ARB, angiotensin type 1 receptor blocker.

All results are presented as mean±SD.

* \( P < 0.05 \) versus before (0 days) in the same group.

† \( P < 0.05 \) versus Buerger control.
Major Amputation-Free Survival Rate After Autologous BM-MNC Implantation

The follow-up periods are summarized in Table 2. Kaplan-Meier analysis showed that the 55 atherosclerotic PAD patients with and without BM-MNC implantation had a significantly worse long-term outcome of major amputation than did the 42 patients with Buerger disease with and without BM-MNC implantation (Figure 1). The major amputation-free survival rate for patients with Buerger disease who underwent BM-MNC implantation was significantly different than the rates for patients with atherosclerotic PAD who underwent BM-MNC implantation, patients with Buerger disease who did not undergo BM-MNC implantation, and patients with atherosclerotic PAD who did not undergo BM-MNC implantation ($P=0.0029$, $P<0.0001$, and $P<0.0001$, respectively), and the major amputation-free survival rate for patients with atherosclerotic PAD who underwent BM-MNC implantation was higher than that for patients who did not undergo BM-MNC implantation ($P<0.0001$ and $P<0.0001$, respectively), but there was no significant difference between major amputation-free survival rates for patients with Buerger disease who did not undergo BM-MNC implantation and patients with atherosclerotic PAD who did not undergo BM-MNC implantation ($P=0.0981$). The 4-year amputation-free rates after BM-MNC implantation were 48% in patients with atherosclerotic PAD and 95% in patients with Buerger disease, and they were 0% in control atherosclerotic PAD patients and 6% in control Buerger disease patients (Figure 2). Multivariable Cox proportional hazards analysis revealed that BM-MNC implantation correlated with prevention of major amputation and that hemodialysis and diabetes mellitus correlated with major amputation (Table 3).

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Table 2. Follow-Up Period of Major Amputation-Free Survival

<table>
<thead>
<tr>
<th>Patients</th>
<th>Median (Q25, Q75), Month</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (n=97)</td>
<td>4.6 (1.4, 30.2)</td>
<td>19 d to 6.4 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD (n=55)</td>
<td>2.0 (1.2, 24.2)</td>
<td>21 d to 6.4 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD with BM-MNC implantation (n=25)</td>
<td>28.5 (4.8, 44.4)</td>
<td>1.1 mo to 6.4 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD without BM-MNC implantation (n=30)</td>
<td>1.3 (1.1, 1.7)</td>
<td>21 d to 8.4 mo</td>
</tr>
<tr>
<td>Buerger disease (n=42)</td>
<td>20.7 (2.4, 35.9)</td>
<td>19 d to 6.4 y</td>
</tr>
<tr>
<td>Buerger disease with BM-MNC implantation (n=26)</td>
<td>26.7 (21.1, 42.7)</td>
<td>4.4 mo to 6.1 y</td>
</tr>
<tr>
<td>Buerger disease without BM-MNC implantation (n=16)</td>
<td>1.5 (1.0, 3.75)</td>
<td>19 d to 6.4 y</td>
</tr>
</tbody>
</table>

Q25, Q75 indicate lower, upper quartiles.

Figure 1. Results of Kaplan-Meier analysis of major amputation-free rate in atherosclerotic PAD patients with and without BM-MNC implantation and in Buerger disease patients with and without BM-MNC implantation.

Table 3. Multivariable Cox Regression Analysis of Factors for Major Amputation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MNC implantation</td>
<td>20.93</td>
<td>9.64–45.45</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Age $&gt;65$ y</td>
<td>0.85</td>
<td>0.44–1.63</td>
<td>0.619</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.52</td>
<td>0.28–0.97</td>
<td>0.039</td>
</tr>
<tr>
<td>CRF (hemodialysis)</td>
<td>0.41</td>
<td>0.20–0.81</td>
<td>0.011</td>
</tr>
</tbody>
</table>

CRF indicates chronic renal failure.
Survival Rate After Autologous BM-MNC Implantation

The follow-up periods were summarized in Table 4. Kaplan-Meier analysis showed that the 55 atherosclerotic PAD patients with and without BM-MNC implantation had significantly worse long-term mortality than did the 42 Buerger disease patients with and without BM-MNC implantation (Figure 3). The 4-year overall survival rates after BM-MNC implantation were 76% in patients with atherosclerotic PAD and 100% in patients with Buerger disease, and they were 67% in control atherosclerotic PAD patients and 100% in control Buerger disease (Figure 4). Overall survival rate after BM-MNC implantation was markedly lower in patients with atherosclerotic PAD than in patients with Buerger disease. BM-MNC implantation did not alter the overall survival rate of patients with atherosclerotic PAD or patients with Buerger disease. Table 5 shows the etiology of death in patients with atherosclerotic PAD who underwent BM-MNC implantation and in control subjects.

ABPI, Tco₂, and VSA After Autologous BM-MNC Implantation

Figure 5 shows the changes in ABPI, Tco₂, and VSA during a 3-year follow-up period after BM-MNC implantation. ABPI was significantly lower in patients with atherosclerotic PAD than in patients with Buerger disease at any time point. In patients with Buerger disease, ABPI was significantly increased after 1 month and remained high during the 3-year

Table 4. Follow-Up Period of Survival

<table>
<thead>
<tr>
<th>Patients</th>
<th>Median (Q25, Q75), Month</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (n=97)</td>
<td>32.5 (20.4, 43.7)</td>
<td>2 wk to 6.8 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD (n=55)</td>
<td>31.5 (16.5, 44.8)</td>
<td>2 wk to 6.3 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD with BM-MNC implantation (n=25)</td>
<td>43.3 (24.9, 53.2)</td>
<td>1.8 mo to 6.4 y</td>
</tr>
<tr>
<td>Atherosclerotic PAD without BM-MNC implantation (n=30)</td>
<td>24.8 (12.6, 35.4)</td>
<td>2 wk to 6.3 y</td>
</tr>
<tr>
<td>Buerger disease (n=42)</td>
<td>30.5 (23.1, 42.1)</td>
<td>7 mo to 6.8 y</td>
</tr>
<tr>
<td>Buerger disease with BM-MNC implantation (n=26)</td>
<td>28.3 (23.1, 42.4)</td>
<td>9.6 mo to 6.1 y</td>
</tr>
<tr>
<td>Buerger disease without BM-MNC implantation (n=16)</td>
<td>33.3 (18.4, 39.6)</td>
<td>7 mo to 6.8 y</td>
</tr>
</tbody>
</table>

Q25, Q75 indicate lower, upper quartiles.
follow-up period. However, in patients with atherosclerotic PAD, ABPI was significantly increased after 1 month and gradually decreased during the 3-year follow-up period and returned to baseline levels. Tco₂ was significantly lower in patients with atherosclerotic PAD than in patients with Buerger disease at 12, 24, and 36 months after BM-MNC implantation. In patients with Buerger disease, VSA was significantly decreased after 1 month and remained low during the 3-year follow-up period. In patients with atherosclerotic PAD, VSA was significantly decreased after 1 month and gradually increased during the 3-year follow-up period and returned to baseline levels. VSA was significantly higher in patients with atherosclerotic PAD than in patients with Buerger disease at 24 and 36 months after BM-MNC implantation.

Acute Adverse Effects of Autologous BM-MNC Implantation
No severe acute adverse effects were observed in any of the patients who underwent autologous BM-MNC implantation. Four of the 51 patients had slight fever elevation within 3 days, 3 patients had worsened pain at lesions in which cells were implanted within 2 days, and 1 patient had vertigo within 1 day after autologous BM-MNC implantation.

Number of EPCs
The number of EPCs was smaller in patients with atherosclerotic PAD than in patients with Buerger disease and normal control subjects (561 ± 307/mL versus 947 ± 471/mL and 1012 ± 418/mL, P < 0.0001, respectively), whereas there was no significant difference between the number of EPCs in the control group and that in the Buerger disease group (Figure 6).

Cell Migration Response to VEGF
Cell migration response to VEGF was smaller in patients with atherosclerotic PAD than in patients with Buerger disease and normal control subjects (32 ± 15/high-power field versus 65 ± 21/high-power field and 69 ± 21/high-power field, P < 0.0001, respectively) (Figure 7), whereas there was no significant difference between the cell migration response to VEGF in the control group and that in the Buerger disease group (Figure 7).

Discussion
The present study is the first study showing long-term safety and efficacy of BM-MNC implantation in patients with CLI with inclusion of a nontreated reference group using a nonrandomized study design. BM-MNC implantation decreased the rate of major amputation in patients with CLI, both patients with atherosclerotic PAD and patients with Buerger disease, compared with that in control patients. However, clinical symptoms and perfusion parameters improved at the early phase after BM-MNC implantation in both groups but remained improved a long period only in the Buerger disease group, suggesting that beneficial effect of cell therapy is remarkable in patients with Buerger disease.

Indeed, some pilot studies have shown that cell therapy failed to improve clinical symptoms and perfusion parameters in patients with CLI.10,21 However, growing evidence has
shown that BM-MNCs, peripheral blood MNCs, and peripheral blood MNCs mobilized by granulocyte colony-stimulating factor improve limb ischemia and that there is no severe transplantation-related complications during a relatively short period of less than 24 weeks.1–3,5–9,22,23 Unfortunately, there have been only a few studies on long-term follow-up of clinical symptoms and outcomes, including major amputation, for more than 2 years after cell therapy.4,15,24,25 These studies also clearly showed that cell therapy reduces the rate of major amputation, one of the primary end points. In addition, there has been no information on predictors of major amputation with BM-MNC implantation. In the present study, multivariable Cox proportional hazards analysis showed that BM-MNC implantation correlated with prevention of major amputation.

Figure 6. A, Representative measurements of the number of EPCs by flow cytometry in a normal control subject, a patient with Buerger disease, and a patient with atherosclerotic PAD. B, Comparisons of numbers of EPCs in normal control subjects, patients with Buerger disease, and patients with atherosclerotic PAD.
amputation. Diabetes mellitus and hemodialysis correlated with major amputation in patients with CLI. These findings suggest that CLI patients with diabetes mellitus who are undergoing hemodialysis may not be eligible for cell therapy.

Although BM-MNC implantation decreased the major amputation rate in patients with atherosclerotic PAD, perfusion parameters such as ABPI and TcO₂ gradually decreased during the 3-year follow-up period and returned to baseline levels. In patients with Buerger disease, BM-MNC implantation markedly decreased the major amputation rate, and ABPI was increased after 1 month and remained high during the long-term follow-up period. Recently, Matoba et al reported that BM-MNC implantation did not alter ABPI and TcO₂ in patients with atherosclerotic PAD or patients with Buerger disease for 3 years in a series of Therapeutic Angiogenesis by Cell Transplantation (TACT) trial, whereas BM-MNC implantation led to extension of the amputation-free interval and improvement in ischemic pain. The reason for this discrepancy is unclear. In a previous TACT trial, ABPI and TcO₂ were significantly improved in patients with atherosclerotic PAD at 4 and 24 weeks after BM-MNC implantation. ABPI and TcO₂ in patients with atherosclerotic PAD were lower in the present study and in the previous TACT trial than in the TACT follow-up study (0.39±0.26 and 0.35±0.14 versus approximately mean 0.6 and 13±13 mm Hg and 28±10 mm Hg versus approximately mean 30 mm Hg). In patients with Buerger disease also, ABPI and TcO₂ were lower in the present study than in the TACT follow-up study (0.54±0.33 versus approximately mean 0.8 and 10±10 mm Hg versus approximately mean 37 mm Hg). The differences in the severity of PAD may partially explain why there are differences in changes in perfusion parameters between the studies.

Some patients with Buerger disease had a normal range of ABPI because of the location of sites of stenosis or occlusion in arteries below the ankle. Therefore, in the present study, before BM-MNC implantation, there was a significant difference in ABPI between patients with Buerger disease and patients with atherosclerotic PAD. We do not know the precise mechanisms by which perfusion parameters, ABPI and TcO₂, and clinical symptoms were increased and maintained in patients with Buerger disease, but perfusion parameters and symptoms temporally increased and gradually decreased in patients with atherosclerotic PAD. Because patients with Buerger disease had few cardiovascular risk factors related to development of atherosclerosis, improve-
mment of perfusion parameters and symptoms with BM-MNC implantation may be maintained. On the other hand, advanced age and existence of cardiovascular risk factors in patients with atherosclerotic PAD may contribute to the failure of maintenance of improved perfusion parameters and symptoms.

It is well known that there are no significant differences in mortality and morbidity of cardiovascular outcomes between Buerger disease patients and healthy subjects and that Buerger disease patients have a lower mortality rate than that of atherosclerotic PAD patients, because Buerger disease is not associated with cardiovascular risk factors. Indeed, in the present study, overall mortality rate was markedly higher in atherosclerotic PAD patients than in patients with Buerger disease. No Buerger disease patients with or without BM-MNC implantation died during the follow-up period. There was no significant difference between overall survival rates of atherosclerotic PAD patients who underwent BM-MNC implantation and those who did not undergo BM-MNC implantation or between overall survival rates of patients with Buerger disease who underwent BM-MNC implantation and those who did not undergo BM-MNC implantation. There was no difference in etiology of death concerning cardiovascular complications between atherosclerotic PAD patients with and without BM-MNC implantation. The survival rate of atherosclerotic PAD patients with CLI who did not undergo BM-MNC implantation is comparable to the survival rates in previous published studies. A recent clinical trial has also shown a similar survival rate of patients with CLI who underwent autologous BM-MNC implantation. These findings suggest that BM-MNC implantation does not alter mortality in patients with CLI.

On the other hand, Miyamoto et al reported that in an unblended and uncontrolled pilot study, long-term adverse effects after BM-MNC implantation were observed in 4 of 8 patients with Buerger disease and that 1 patient suddenly died 20 months after implantation at the age of 30 years. In the present study, none of patients including Buerger disease patients who underwent BM-MNC implantation and control patients who had no BM-MNC implantation died during follow-up periods (mean, 2.8 ± 1.4 years; range, 7 months to 6.8 years). In the TACT follow-up study also, 3-year overall survival rate after BM-MNC implantation was 100% in 41 patients with Buerger disease. Most studies have shown that cell therapy is promising for angiogenesis and has no severe adverse effects in patients with Buerger disease.

In the present study, the number of EPCs and cell migration response to VEGF were significantly decreased in patients with atherosclerotic PAD compared with those in patients with Buerger disease. Interestingly, the number of and migration of EPCs were similar in the Buerger disease group and control group. Yamamoto et al reported that EPCs from Buerger disease patients displayed high expression of endothelial lineage molecules compared with their counterparts obtained from PAD patients. These findings suggest that the number of and function of EPCs and expression of EPC marker molecules may contribute to differences in perfusion parameters and major amputation rate after BM-MNC implantation between patients with atherosclerotic PAD and patients with Buerger disease.

Gene therapy using VEGF, fibroblast growth factor, and hepatocyte growth factor plasmid and adenoviral vectors encoding their genes may also improve clinical symptoms and limb perfusion in patients with CLI. Recently, Haro et al have reported comparison of the efficacy and safety of gene and cell therapy trials in patients with PAD by a meta-analysis. Indeed, although some studies failed to improve clinical symptoms, the efficacy, safety, and feasibility of both gene therapy and cell therapy have been established, suggesting that these therapies are promising new strategies for CLI. Future large-scale studies with a randomized, double-blinded, and placebo-controlled design are needed to confirm the effects of gene therapy and the effects of cell therapy alone and the effects of combined gene therapy and cell therapy on clinical symptoms and cardiovascular outcomes in patients with CLI.

**Study Limitations**

Although we selected patients with CLI who had no other treatment option as a reference, the study design was not a prospective randomized trial. In addition, the number of subjects in the present study was relatively small. However, major amputation after autologous BM-MNC implantation was prevented in many patients with atherosclerotic PAD and patients with Buerger disease who had no other treatment option. Although no severe adverse effects were observed after BM-MNC implantation in the present study, further studies are needed to evaluate the adverse effects and outcomes, including not only cardiovascular outcomes but also onset of malignancy, during a much longer follow-up period using a prospective, randomized, controlled study design.

It is clinically important to know the viability of injected cells. Unfortunately, there is no information on the viability of injected cells in humans. Some experimental studies have demonstrated the possibility that adult BM cells can differentiate into vascular endothelial cells or endothelial progenitor cells in a hind limb ischemic model and in a heart ischemic model. However, Ziegelhoeffer et al have recently shown that although implantation of BM-derived cells increases collateral vessel formation in an ischemic limb model, these cells are not incorporated into the growing vasculature, suggesting that angiogenesis with adult cells is due to several angiogenic growth factors and cytokines released by implanted cells. In addition, it has been shown that mobilization of BM-derived cells contributes to angiogenesis in response to hypoxia without transdifferentiation into endothelial cells. Results of several studies support the concept that angiogenic growth factors and cytokines released by implanted cells predominantly promote angiogenesis.

The 4-year overall survival rates after BM-MNC implantation were 76% in patients with atherosclerotic PAD and 100% in patients with Buerger disease in this study. The relatively large dropout rate (>20%) in patients with atherosclerotic PAD may produce bias in the analyses regarding differences in ABI, Tco2, and VSA after BM-MNC implantation between patients with PAD and patients with Buerger disease.
disease because the patients who dropped out may be different from those who completed the study. We used the last observation carried forward approach, one of the single imputation methods for missing data, instead of the complete-case method because it is considered good practice not to ignore dropout regardless of whether data were informatively or noninformatively missing. Comparison of the reason for death between atherosclerotic PAD patients with and without BM-MNC implantation suggests that missing data caused by dropout occurred at random. Dropout rate was indeed more than 20% during the whole observation period in patients with atherosclerotic PAD, but it was less than 10% at 12 months. Significant differences in ABPI, TcO2, and VSA after BM-MNC implantation between patients with PAD and patients with Buerger disease were mainly observed between baseline and 12 months, suggesting that the influence of last observation carried forward is small during this period. Conversely, the findings after 24 months should be carefully interpreted because dropout rate was more than 20%. Furthermore, because the small sample size may influence the results, further study using a large number of patients with a small proportion of missing data are necessary to clarify our findings.

In the present study, we used multivariable Cox proportional hazard regression analysis to identify independent predictors for major amputation in addition to univariate analysis. We finally could select diabetes mellitus, hemodialysis, and BM-MNC implantation as independent predictors for major amputation. Diabetes mellitus and hemodialysis are well established as important predictors for major amputation. Diabetes mellitus and hemodialysis are well established as important predictors for major amputation. However, further study including a large number of patients with chronic critical limb ischemia is necessary to clarify our findings.

In conclusion, BM-MNC implantation reduces major amputation rate and is safe and effective in patients with CLI, especially in patients with Buerger disease.

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Disclosures
None.

References
Preclinical studies and clinical trials have shown that cell therapy, including autologous bone-marrow mononuclear cell (BM-MNC) implantation, improves clinical symptoms and increases collateral vessel formation in patients with peripheral arterial disease (PAD). Unfortunately, there have been few studies on the long-term follow-up of clinical symptoms and events such as major amputation and mortality, and there has been no information on predictors of major amputation with BM-MNC implantation. We found that autologous BM-MNC implantation decreases the rate of major amputation in patients with critical limb ischemia (CLI), both patients with atherosclerotic PAD and patients with Buerger disease. The results of this study showed that BM-MNC implantation, improves clinical symptoms and increases collateral vessel formation in patients with peripheral arterial disease. 

**CLINICAL PERSPECTIVE**

Preclinical studies and clinical trials have shown that cell therapy, including autologous bone-marrow mononuclear cell (BM-MNC) implantation, improves clinical symptoms and increases collateral vessel formation in patients with peripheral arterial disease (PAD). Unfortunately, there have been few studies on the long-term follow-up of clinical symptoms and events such as major amputation and mortality, and there has been no information on predictors of major amputation with BM-MNC implantation. We found that autologous BM-MNC implantation decreases the rate of major amputation in patients with critical limb ischemia (CLI), both patients with atherosclerotic PAD and patients with Buerger disease, compared with that in control patients. After BM-MNC implantation, the amputation-free rate was markedly worse in patients with atherosclerotic PAD than in patients with Buerger disease. Overall, the survival rate was also markedly worse in patients with atherosclerotic PAD than in patients with Buerger disease. The results of this study showed that BM-MNC implantation was an independent predictor of prevention of major amputation and that hemodialysis and diabetes mellitus were independent predictors of major amputation. In the present study, we confirmed that BM-MNC implantation is safe and effective in patients with CLI during long follow-up periods. Patients with Buerger disease, but not PAD patients who have diabetes mellitus and are undergoing hemodialysis, are eligible for treatment with BM-MNC implantation. Future large-scale studies with a randomized, double-blinded, and placebo-controlled design are needed to confirm the effects of cell therapy on the clinical symptoms and cardiovascular outcomes in patients with CLI.
Autologous Bone-Marrow Mononuclear Cell Implantation Reduces Long-Term Major Amputation Risk in Patients With Critical Limb Ischemia: A Comparison of Atherosclerotic Peripheral Arterial Disease and Buerger Disease

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