Granulocyte Colony-Stimulating Factor
—— A Noninvasive Regeneration Therapy for Treating Atherosclerotic Peripheral Artery Disease ——

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Background  The purpose of this study was to determine whether treatment with granulocyte colony-stimulating factor (G-CSF), which mobilizes endothelial progenitor cells from bone marrow, can safely improve the clinical outcomes of patients with atherosclerotic peripheral artery disease (PAD).

Methods and Results  Thirty-nine patients with intractable PAD were randomly assigned to 3 groups: a negative control group (n=12) treated with conventional drug therapy; a positive control group (n=13) treated with conventional drug therapy plus bone marrow transplantation (BMT); and a G-CSF group (n=14) treated with conventional therapy plus subcutaneous injection of 2–5μg/kg of recombinant human G-CSF once daily for 10 days. One month after treatment, subjective symptoms improved significantly in the G-CSF and BMT groups. Ankle-brachial pressure index and transcutaneous oxygen pressure increased significantly in the BMT and G-CSF groups, but no such improvements were seen in the group receiving conventional therapy alone.

Conclusions  G-CSF improves the clinical signs and symptoms of patients with intractable PAD to the same degree as BMT does. This noninvasive treatment may thus represent a useful new approach to managing the disease.  (Circ J 2006; 70: 1093–1098)

Key Words: Angiogenesis; Bone marrow cells; Granulocyte colony-stimulating factor; Limb ischemia

Endothelial progenitor cells (EPC) derived from bone marrow have the potential to mediate vasculogenesis. Moreover, the therapeutic potential of bone marrow cell transplantation (BMT) has been confirmed in animal models, in which direct injection into ischemic hindlimbs induces vasculogenesis and improves deteriorated exercise capacity. It was also recently reported that direct autologous BMT has a beneficial effect in patients with atherosclerotic peripheral artery disease (PAD); however, the treatment method is complex, requiring general anesthesia, aspiration of a large amount of bone marrow, separation of the mononuclear cells, and multiple injections into the ischemic lower limbs.

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that mobilizes CD34+ EPC from the bone marrow into the peripheral blood. Clinically, G-CSF has been used for the collection of stem cells used in allogeneic or syngeneic peripheral blood stem cell (PBSC) transplantation. Notably, G-CSF appears to induce vascular and myocardial tissue regeneration and accelerate the healing process in animal infarcted myocardium. A related molecule, granulocyte-macrophage colony-stimulating factor (GM-CSF), improves coronary arterial collateral flow in humans with extensive coronary artery disease. In the present study, we tested whether subcutaneous injection of G-CSF would improve the signs and symptoms of lower limb ischemia in patients with intractable atherosclerotic PAD.

Methods

Patient Population  We obtained written informed consent from all subjects after the university ethics committee approved this study. The subjects were patients with critical limb ischemia with rest pain, non-healing ulcers, or both (Fontaine III or IV), and who were ineligible for percutaneous transarterial angioplasty and/or surgical reconstruction. Patients with proliferative retinopathy, neoplasia or critical coronary and/or cerebral vascular complications were excluded, as were patients who had undergone percutaneous coronary intervention within 3 months prior to the study.

Procedure  The primary outcomes of interest for this study were the safety and feasibility of treating critical limb ischemia using G-CSF. A total of 39 individuals were randomly assigned to 3 groups. A conventional therapy group (n=12), which served as a negative control, were treated with conventional drug therapies, including antiplatelet agents, anticoagulants and vasodilators (eg, prostaglandins). A BMT group (n=13), which served as a positive control for regeneration therapy, received conventional drug therapy plus direct injection of mononuclear cells derived from the
bone marrow. As previously described, the BMT entailed aspiration of marrow cells under general anesthesia, after which mononuclear cells were isolated using a CS3000-Plus blood-cell separator (Baxter, Deerfield, USA) and were concentrated in a final volume of 50 ml. We then divided the mononuclear cells (1×10⁹ to 3×10⁹ cells) into 100 aliquots (0.5 ml each) and injected them into the gastrocnemius of patients during the study.

The subjective symptoms and blood flow parameters, including pain-free walking distance, ankle–brachial pressure index (ABPI) and transcutaneous oxygen pressure (TcO₂) were evaluated before and 1 month after the treatments. ABPI was measured 3 times at each time point. In some cases, such as hemodialysis patients, ABPI becomes elevated when total area of ulcer became half or less. Rest pain scale: +4, severe pain unresolved with non-steroidal anti-inflammatory drugs (NSAIDs); +3, moderate pain NSAIDs necessary; +2, slight pain NSAIDs unnecessary; +1, very slight pain; 0, completely resolved. All data were obtained by investigators who were unaware of the treatment allocation. Anti-hrombotic drugs such as aspirin and warfarin were administered to all of the subjects during the study.

**Statistical Analysis**

Values are expressed as means±SE. Differences in patient characteristics among the 3 groups were evaluated using the χ² test. Baseline and follow-up rest pain scale values were compared using the Wilcoxon signed rank test. Changes in ABPI and TcO₂ from baseline to 1 month after treatment were analyzed with 2-tailed paired t-test.

**Results**

**Clinical Characteristics of Patients**

Table 1 shows the clinical characteristics of the patients in the 3 therapy groups. No significant differences were seen with respect to age, gender, the severity of the limb ischemia, or such complications as diabetes mellitus, hemodialysis and ischemic cardiac/cerebral disease.

**Effects After 1 Month of Treatment**

As shown in Table 1 and Fig 1, the pretreatment values for rest pain, ABPI and TcO₂, which reflect the severity of lower-limb ischemia, were similar among the 3 therapy groups. One month after treatment, however, rest pain scale values had decreased significantly in the G-CSF and BMT groups, but not in the conventional therapy group (Table 1). ABPI in the G-CSF and BMT groups increased significantly from 0.47±0.03 and 0.43±0.05 respectively, at baseline to 0.56±0.04 and 0.53±0.06 respectively, at 1 month after treatment. At the same time, TcO₂ (mmHg) increased 1 month after treatment in both the G-CSF group (27±4 vs 37±3, p<0.05) and the BMT group (24±6 vs 32±8, p<0.05). Indeed, there was no significant difference between the results in the BMT and G-CSF groups (Fig 1), indicating equivalent improvements in blood flow. In contrast, no such improvements were seen in the control group (ABPI:

Table 1 Characteristics and Changes in the Symptoms and Blood Flow Parameters of the 3 Groups of Patients With Peripheral Artery Disease

<table>
<thead>
<tr>
<th></th>
<th>G-CSF group (n=14)</th>
<th>BMT group (n=13)</th>
<th>Control group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69±2</td>
<td>62±3</td>
<td>68±2</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>7/7</td>
<td>11/2</td>
<td>7/5</td>
</tr>
<tr>
<td>Fontaine grade (III/IV)</td>
<td>7/7</td>
<td>5/8</td>
<td>7/5</td>
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<tr>
<td>Complications</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7/14</td>
<td>5/13</td>
<td>5/12</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>4/14</td>
<td>4/13</td>
<td>1/12</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>3/14</td>
<td>4/13</td>
<td>3/12</td>
</tr>
<tr>
<td>Old myocardial infarction</td>
<td>6/15</td>
<td>4/13</td>
<td>5/12</td>
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<tr>
<td>Effect of treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improvement in pain-free walking distance 1 month after treatment</td>
<td>2/4</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Improvement in ischemic ulcers 1 month after treatment</td>
<td>3/7</td>
<td>3/8</td>
<td>1/6</td>
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<tr>
<td>Rest pain scale</td>
<td></td>
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<tr>
<td>Pre-treatment</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 month after treatment</td>
<td>2.5±0.3</td>
<td>2.8±0.3</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>Improvement of ABPI by &gt;0.10 1 month after treatment</td>
<td>1.9±0.3*</td>
<td>1.9±0.4*</td>
<td>2.1±0.3</td>
</tr>
</tbody>
</table>

*p<0.05 compared with the pre-treatment values in each group.

G-CSF, granulocyte colony-stimulating factor; BMT, bone marrow transplantation; ABPI, ankle-brachial pressure index.
0.48±0.03 vs 0.47±0.03, TcO2: 26±5 vs 26±5). An increase in ABPI of more than 0.1 was noted in 7 of 13 patients in the G-CSF group and in 7 of 12 patients in the BMT group (Table 1). These incidences were significantly higher than that seen in the conventional therapy group (1 of 10 patients). Significant improvement of ischemic ulcers was seen in 3 of 7 and 3 of 8 patients, in the G-CSF and BMT groups, respectively, but was seen only 1 of the 6 patients with ulcers in the conventional therapy group (Fig 2, Table 1).

**Adverse Effects**

Although 5 patients receiving G-CSF complained of bone pain, it was not clinically serious. No major complications (ie, death, liver or renal dysfunction, spleen hypertrophy, or myocardial or cerebral infarct) occurred in either the BMT or G-CSF group. However, 1 day after completing the 10-day G-CSF treatment, ventricular fibrillation followed by complete recovery after immediate external electrical cardioversion occurred in 1 patient in the G-CSF group. This patient had an old myocardial infarction and showed frequent ventricular premature beats. A rapid fall in serum potassium (2.7 mmol/L at the onset of ventricular fibrillation) due to severe diarrhea of unknown etiology was thought to be related to the arrhythmia. There was no clinical evidence of acute myocardial or cerebral infarction. Subsequent coronary angiography revealed no significant coronary stenosis.

**Peripheral Blood Cell Counts**

In the G-CSF group before treatment, the average numbers of white blood cells, granulocytes, monocytes and CD34-positive cells in the peripheral blood were 6,082±478/µL, 4,000±479/µL, 396±52/µL and 0.72±0.07/µL, respectively (Fig 3). During the 10-day treatment protocol, as shown in Fig 3, those numbers peaked respectively at 32,770±1,069/µL, 29,763±1,024/µL, 1,415±290/µL and 4.74±0.90/µL (p<0.05 for each parameter), but then declined to the pretreatment levels within 4 days after completing the treatment. There were no significant changes in lymphocyte, red blood cell or thrombocyte counts throughout the experiment. The BMT and conventional therapy groups showed no significant changes in peripheral blood cell counts. The cumulative dose of G-CSF administered to patients in the G-CSF group ranged from 0.85 mg/10 days to 2.10 mg/10 days (average=1.59±0.91 mg/10 days).
Therapeutic Effect of G-CSF

Although conventional therapy provided patients participating in this study with no relief from their PAD, subcutaneous administration of G-CSF led to significant improvements in inflow blood pressure and oxygenation, as reflected by ABPI and TcO2, respectively, which was associated with a concomitant reduction in rest pain scale values. Standard assessment criteria for interventional therapy for PAD indicate that increases in ABPI of more than 0.1 are desirable. The incidence of such increases seemed higher in the G-CSF group than the conventional therapy group.

It is well known that the placebo effect is very powerful in end-stage patients and can induce even physiological changes. Nevertheless, this trial was not designed as a placebo-controlled study because the frequency occurrence of bone pain makes G-CSF a potential unblinding agent.11 In fact, approximately one-third of patients receiving G-CSF in the present study experienced bone pain, even though we used only a low dose of the drug. To overcome this situation, in addition to a negative control (conventional therapy group), the therapeutic effect of G-CSF injection was compared with that of BMT, which has been shown to exert a real clinical effect4 and therefore served as a positive control. We found that G-CSF showed a favorable clinical effect equivalent to that afforded by BMT. Moreover, because placebo effects are especially strongly associated with invasive and complicated procedures12 the simplicity of subcutaneous injection of G-CSF, as compared to the much more complex and invasive BMT therapy, encourages us to believe that the G-CSF-mediated improvement is not explained by a placebo effect.

Dosages of Injected G-CSF

We strictly controlled the daily dosage of G-CSF (2–5 µg/kg) to maintain the peripheral blood leukocyte count at approximately 30,000/µl. The occurrence of life-threatening complications from G-CSF injection, including myocardial and cerebral infarctions13 and spontaneous rupture of the spleen14 has been reported in the setting of PBSC transplantation in healthy humans, where the daily injected dose of G-CSF is usually 5–10 µg/kg, and peripheral leukocyte counts frequently exceed 50,000/µl. With G-CSF, as with most other drugs, the incidence of adverse effects is a direct function of the dosage.11 For PBSC mobilization in healthy donors, Harada et al reported that a G-CSF dose of less than 10 µg/kg per day is acceptable11 whereas Murata et al recommended 8.8 µg/kg per day or less.11 Harada et al also demonstrated that G-CSF facilitates platelet aggregation and recommended that PBSC transplantation donors take aspirin.15 Our subjects were not healthy volunteers, but were patients with atherosclerotic PAD in which thrombosis is almost always combined with atherosclerotic lesions not only in peripheral arteries but also in coronary and cerebral arteries and aorta. In earlier studies, moreover, the response to G-CSF varied considerably from patient to patient,16 and the basal numbers of granulocytes, monocytes and CD34-positive cells in peripheral blood generally correlated with the increases in peripheral white blood cell counts seen following G-CSF injection.17 By contrast, the strict control of the daily dosage enabled us to keep the increases in the peripheral blood of granulocytes, monocytes and CD34-positive cells, as well as white blood cells, relatively constant among patients, as evidenced by the small standard deviations. Thus, careful injection of low, controlled doses of G-CSF, combined with antiplatelet agents and anticoagulants, produced no serious complications related to G-CSF injection. Recently, serious concerns were raised about the safety of G-CSF injection aimed at vascular regeneration in patients with ischemic coronary artery disease. The use of G-CSF after the onset of acute myocardial infarction led to unexpectedly high restenosis rate.18 In addition, other investigators reported that G-CSF injection was associated with new onset of myocardial infarction in 2 of 12 cases with intractable coronary artery disease.19 The subcutaneous injection doses of G-CSF were 10µg/kg per day in those 2 studies, which were 2-fold higher than the dosage used in the present study. Moreover, we performed coronary angiography in all patients and excluded cases of significant coronary stenosis. We also excluded patients who had undergone coronary intervention.

Discussion
therapy within the previous 3 months. These facts might explain the different findings concerning adverse effects between the current study and the others.18,19

Mechanisms of G-CSF Effect

In the G-CSF group, CD34+ cells, which are a possible marker of EPC mobilized from bone marrow into peripheral blood, were increased approximately 7-fold over pretreatment levels. Such mobilization of EPC has been previously shown to induce angiogenesis followed by improved inflow blood pressure and TcO2+4 Thus, 1 possible mechanism of the beneficial effects of G-CSF is the development of new vasculature mediated by CD34+ cells in the peripheral blood. This favorable role of CD34+ cells is supported by the fact that cyclophosphamide, which also mobilizes CD34+ cells, exerts beneficial effects on post-infarct myocardial function.20 Moreover, the involvement of endothelial precursor cells in the mechanism of restoring blood flow was confirmed by our previous clinical study, which demonstrated that G-CSF injection also improved blood flow in ischemic myocardium.21 It is well known that G-CSF contributes to the healing of myocardial infarction,22 diabetic ulcers of the skin,23 and ischemic necrosis resulting from doxorubicin extravasation.24 A recent study also demonstrated that intramuscular injection of G-CSF induced angiogenesis through mechanisms independent of EPC mobilization.25 These findings suggest that other possible mechanisms of G-CSF, such as direct angiogenesis and wound healing, may play a role.

Study Limitations

In the current study, the BMT group served as a positive control on the basis of a recently published report. The saline group also served as a negative control. However, this study was a small trial with no placebo control. Therefore, it may not be appropriate to conclude that G-CSF is effective. In addition, the G-CSF-dose dependency of therapeutic effects was not examined in the protocol of the present study of low-dose injection of G-CSF. Also, the association between the beneficial effect and the increase of white blood cells is unclear. Thus, larger trials with high and low doses of G-CSF are needed to substantiate our findings.

Conclusion

G-CSF therapy was associated with an improvement in the clinical signs and symptoms of atherosclerotic PAD. This noninvasive treatment may represent a useful new approach to managing PAD and therefore warrants further study.

Acknowledgments

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