

# Autologous transplantation of peripheral blood endothelial progenitor cells (CD34<sup>+</sup>) for therapeutic angiogenesis in patients with critical limb ischemia

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**Aim.** Intramuscular injection of endothelial progenitor cells (EPCs) may constitute an alternative treatment strategy for patients with critical limb ischemia (CLI). We performed transplantations of EPCs (CD34<sup>+</sup>) extracted from peripheral blood in patients with CLI. The objective of this report is to present the method and early results of intramuscular autologous peripheral blood CD34<sup>+</sup> cell transplantation in the ischemic limb.

**Methods.** CD34<sup>+</sup> cell transplantation was performed in 2 limbs of 2 patients with CLI, in cases in which it was not possible to perform surgical or percutaneous revascularization. The patients received a granulocyte colony-stimulating factor (G-CSF) prior to the treatment. CD34<sup>+</sup> cells were retrieved from peripheral blood and injected directly into the muscle of the ischemic limb.

**Results.** CD34<sup>+</sup> cells retrieved in patient 1 were  $1 \times 10^5$ /ml and in patient 2 were  $1.6 \times 10^5$ /ml. Transcutaneous oxygen pressure in the foot increased and clinical symptoms improved. Newly visible collateral blood vessels were directly documented by angiography.

**Conclusion.** Satisfactory clinical improvement was achieved by using peripheral blood EPCs (CD34<sup>+</sup>) in the patients with CLI. No complications arose following the intramuscular administration of peripheral blood CD34<sup>+</sup> cells.

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Intramuscular transplantation of endothelial progenitor cells (EPCs) may constitute an alternative treatment strategy for patients with critical limb ischemia (CLI). This strategy is designed to promote the development of supplemental collateral blood vessels that can constitute endoge-

nous bypass conduits around occluded native arteries, a concept called therapeutic angiogenesis.<sup>1</sup> Preclinical studies in animal models and early results of clinical trials have demonstrated that intramuscular injection of EPCs derived from bone marrow can promote the neovascularization of ischemic tissues.<sup>2-4</sup> Previous studies have also established that bone marrow-derived EPCs are present in normal systemic circulation.<sup>5-7</sup>

This study describes the initial clinical experience of circulating CD34 antigen-positive EPC (CD34<sup>+</sup>) transplantation for patients with CLI. Two patients with CLI that could not be treated with surgical or percutaneous revascularization underwent direct CD34<sup>+</sup> cell transplantation in their ischemic limbs. There were no complications, and the patients experienced marked symptomatic improvement and/or objective evidence of improved limb perfusion. In this report we explain the method of intramuscular autologous peripheral blood CD34<sup>+</sup> cell transplantation in the ischemic limb and show results.

## Materials and methods

### Patients

The inclusion criteria for intramuscular CD34<sup>+</sup> cell transplantation were drafted to restrict treatment to those patients with chronic CLI who did not show improvement in response to conventional therapies and who were not optimal candidates

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for surgical or percutaneous revascularization. Subjects were excluded if they had cancer or diabetes. The patients gave written informed consent before participation.

#### *Granulocyte colony-stimulating factor (G-CSF) administration*

Five to 6 days before apheresis, the 2 patients were treated with G-CSF (Filgrastin; Gran®, San-kyo, Co. Ltd. Japan) administered by subcutaneous injection at approximately 2~3 µg/kg/day. Since G-CSF has been reported to increase platelet aggregation and platelet activity,<sup>8</sup> oral antiplatelet drug and intravenous heparin (10 000-15 000 µ/day) were administered concomitantly.

#### *Peripheral blood CD34<sup>+</sup> cell retrieval and intramuscular CD34<sup>+</sup> cell transplantation*

Apheresis was initiated 5 to 6 days after the start of G-CSF administration. The basilic, cephalic or median antecubital vein was punctured with an 18-G needle to conduct apheresis. CD34<sup>+</sup> cells were retrieved from peripheral blood using Haemonetics Multi (Haemonetics, Multi Component System TM, USA). Cell suspensions of 60 ml and 80 ml were obtained by apheresis. The number of CD34<sup>+</sup> cells retrieved was 1×10<sup>5</sup>/ml and 1.6×10<sup>5</sup>/ml, respectively.

The patients were anesthetized with spiral anesthesia. The cell suspension was injected directly into the muscle of the ischemic thigh and calf using a 26-G needle in a total of 50 points (1 ml/point) spaced 1 cm apart.

#### *Transcutaneous oxygen pressure (TcPO<sub>2</sub>), ankle brachial pressure index (ABI), and angiography*

TcPO<sub>2</sub> examination was performed at rest, in a supine position before CD34<sup>+</sup> cell transplantation as well as 7 days and 14 days post-transplantation. A transcutaneous oxygen sensor (TCM3 Radiometer, Copenhagen, Denmark) was placed at 3 regions: the dorsum of the foot, the anteromedial calf at about 10 cm below the patella, and the thigh at about 10 cm above the patella.

ABI was measured with an apparatus (ABI Form, AT Company, Japan) that simultaneously measures the upper and lower extremity pressure.

Lower limb intra-arterial digital subtraction angiography (IA-DSA) was performed in the pre-

transplantation period and in the 2<sup>nd</sup> week of post-transplantation to evaluate the neovascularization in the ischemic limbs.

## **Results**

Two patients with CLI who were unsuitable for surgical or percutaneous revascularization underwent direct CD34<sup>+</sup> cell transplantation in the ischemic limb.

Patient 1 was a 78-year-old non-diabetic man with chronic CLI Fontaine class III (rest pain) who was treated daily with analgesics. IA-DSA revealed complete occlusion of the left superficial femoral artery, proximal popliteal artery, anterior tibial, posterior tibial and peroneal arteries and no suitable site for a conventional bypass.

Patient 2 was a 74-year-old non-diabetic man with chronic CLI Fontaine class III who was also treated daily with analgesics. This patient was referred to our institution after 3 previous bypass revascularizations (femoro-femoral bypass, left femoro-popliteal bypass with auto-saphenous vein graft, left femoro-popliteal bypass with prosthetic Dacron grafts), 2 surgical revisions, and iliac percutaneous transluminal angioplasty with stent placement.

#### *Clinical status*

Clinical improvement was achieved in the acute postoperative course (approximately 7 days post-transplantation) in both patients, and treatment with analgesics was eliminated or reduced to sporadic use.

#### *White blood cells (WBC) and platelet count variations*

Figure 1A shows the variation in WBC counts. Before the G-CSF administration, the WBC counts were normal in both patients. On the day of apheresis (pre-apheresis), the WBCs increased to approximately 30 000/ml. On the post-transplantation day 1, counts were around 20 000/ml and continued to decrease progressively to a normal range: post-transplantation day 7 for patient 1 and post-transplantation day 14 for patient 2.

Figure 1B shows the variations in platelet counts. G-CSF administration did not significantly change the platelet count in either patient. However, leu-

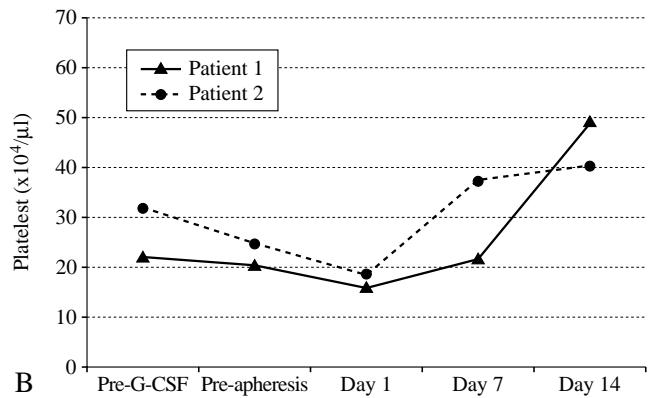
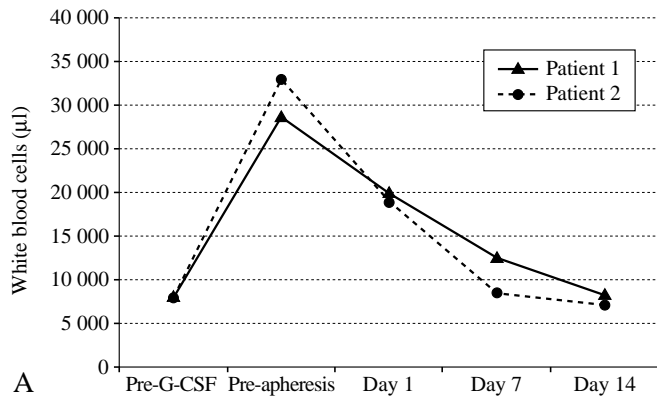


Figure 1.—Variations in white blood cell counts (A) and platelet counts (B) prior to G-CSF administration, on the day of apheresis, and on post-transplantation day 1, day 7, and day 14.

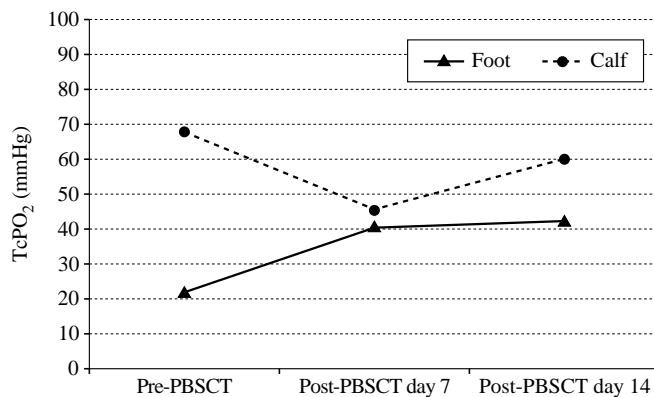


Figure 2.—Variations in TcPO<sub>2</sub> prior to peripheral blood EPC transplantation, on day 7, and on day 14 post-PBSCT (patient 1).

kapheresis decreased the platelet count by about 25% in both patients but the count returned to normal up to 1 week later. No hemorrhagic complication occurred in either patient.

#### TcPO<sub>2</sub>, ABI, and angiography

TcPO<sub>2</sub> and ABI measurements were performed on day 7 and day 14 post-transplantation. A slight increase in the TcPO<sub>2</sub> value was observed in the foot (Figure 2). However, no increase in ABI value was observed. IA-DSA performed 2 weeks post-transplantation showed an increase in collateral vessels in the ischemic limb at the calf (Figure 3).

### Discussion

Therapeutic angiogenesis is a strategy designed to promote the development of supplemental col-

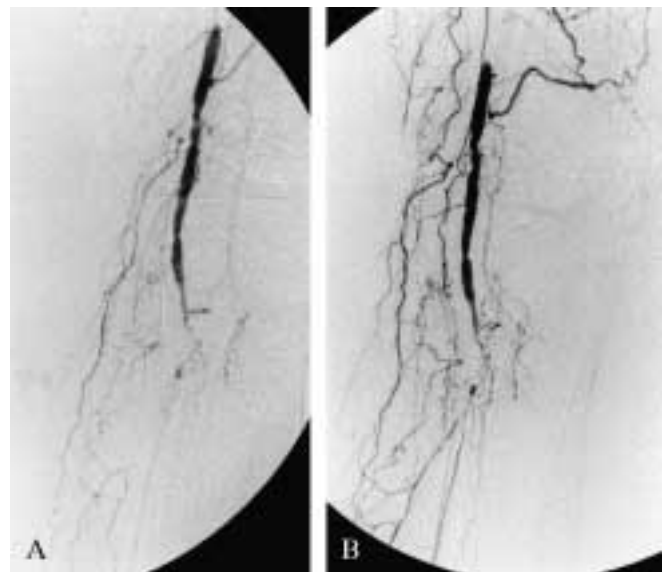


Figure 3.—Patient 1: digital subtraction angiography before (A) and 2 weeks after (B) peripheral blood EPC transplantation.

lateral vessels that can constitute an endogenous bypass conduit around occluded native arteries.<sup>1</sup> Angiogenesis is based on 3 components of the angiogenic process. One is enzymatic degradation of the basement membrane of the parent venule. Another is endothelial cell migration. The migrating endothelial cells elongate and align with one another to create a solid sprout. A lumen forms by a curvature that occurs within each endothelial cell. The 3<sup>rd</sup> is endothelial cell proliferation. Endothelial cell proliferation further increases the length of a sprout. Two hollow sprouts join at their tips to form a loop, after which blood flow begins. Pericytes position themselves along the base of the loop and new sprouts

grow from the apex of the loop to continue the angiogenic process.<sup>9</sup>

Experimental studies have established that numerous angiogenic factors, including vascular endothelial growth factor (VEGF),<sup>10,11</sup> basic fibroblast growth factor (bFGF),<sup>12,13</sup> and hepatocyte growth factor (HGF),<sup>14,15</sup> promotes collateral artery development in animal models of limb and myocardial ischemia.

Recent study have reported that bone marrow cells (BMCs) induce angiogenesis and improve the damaged ischemic heat function in patients<sup>2</sup> and in an ischemic hind limb animal model.<sup>3,4</sup> Another recent study has also demonstrated that BMCs and blood-derived EPCs, such as CD34<sup>+</sup> cells, stimulates vascularization.<sup>5,7</sup> Previous studies have established that bone marrow-derived EPCs are present in the normal systemic circulation.<sup>5,16</sup> However, EPCs should be mobilized from the bone marrow to peripheral blood for collection. We administered G-CSF to mobilize and increase the progenitor population before retrieval from peripheral blood. Thus, the number of CD34<sup>+</sup> cells removed by apheresis was equivalent to that from the treatment using BMC transplantation.

In addition, a previous study on G-CSF activity demonstrated that after G-CSF is administered, endothelial cells express an activation/differentiation cycle (including proliferation and migration) related to angiogenesis.<sup>17</sup> A family of glycoprotein molecules, termed colony-stimulating factors (CSF), control the proliferation, maturation, and functional activities of granulocytes, macrophages, and their precursors.<sup>18-20</sup> The idea that hematopoietic growth factors are angiogenic growth factors and *vice versa* is supported by 2 observations: 1) endothelial cells and hematopoietic cells have a common origin in embryogenesis, and 2) angiogenic factors of the FGF-type induce mesoderm and stimulate the formation of blood cells.<sup>21</sup> In a comparison of G-CSF to bFGF, G-CSF resulted in a lower maximal proliferation of endothelial cells, whereas migration was on the same order of magnitude with both factors.<sup>17</sup>

## Conclusion

In conclusion, satisfactory clinical improvement was achieved using peripheral blood EPCs (CD34<sup>+</sup>) in patients with CLI, and no complications due

to the use of G-CSF or the intramuscular administration of peripheral blood CD34<sup>+</sup> cells were noted. Our clinical data support the use of peripheral blood cells as a possible source of stem cells for therapeutic angiogenesis in CLI.

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