Therapeutical potential of blood-derived progenitor cells in patients with peripheral arterial occlusive disease and critical limb ischaemia

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Aims Despite considerable advances in the therapy of patients with peripheral arterial occlusive disease (PAOD) and critical limb ischaemia (CLI), a substantial number remain, in whom amputation has to be considered the only and final option. Recent evidence from animal models of hind limb ischaemia suggests that neovascularization induced by circulating blood-derived progenitor cells (CPCs) may permit limb salvage. It remains unclear, however, whether an intra-arterial application of autologous CPCs in patients with infrapopliteal PAOD and CLI is safe, feasible, and of potentially beneficial effects.

Methods and results Seven patients with critical PAOD were treated with an intra-arterial infusion of autologous CPCs (39 ± 24 × 10⁶) isolated from peripheral blood. Pre-interventional stimulation with G-CSF and CPC application was well tolerated. Twelve weeks after CPC administration, the pain-free walking distance increased from 6 ± 13 to 195 ± 196 m. A significant increase in the ankle-brachial index, transcutaneous O₂, flow-dependent vasodilation, flow reserve in response to adenosine, and endothelium-dependent vasodilation was observed.

Conclusion These preliminary data in a small series of patients with CLI without surgical or interventional options indicate that CPC application is safe, feasible, and may improve both functional and clinical indices.

KEYWORDS
Circulating progenitor cells; Angiogenesis; Critical limb ischaemia; Neovascularization; Peripheral arterial occlusive disease

Introduction

Despite the technical advances in interventional and surgical revascularization procedures, a substantial number of patients with peripheral arterial occlusive disease (PAOD) and critical limb ischaemia (CLI) remain, in whom amputation has to be considered the only and final option. Recent evidence from animal models suggests that neovascularization induced by circulating blood-derived progenitor cells (CPCs) may permit limb salvage even under conditions of complete vessel occlusion.1,2 It has been shown that ex vivo expanded CPCs transplanted into a mouse model of hind limb ischaemia improve blood-flow recovery and capillary density, and reduce the rate of limb loss. This finding is consistent with the hypothesis that these cells may function as supply side-strategy for therapeutic neovascularization. Phase I clinical studies in patients with acute myocardial infarction indicate that intra-arterial application of in vitro propagated CPCs after interventional revascularization is safe, feasible, and may improve myocardial function.3,4 Therefore, the aim of the present study was to assess the safety, feasibility, and clinical efficacy of intra-arterial application of autologous CPCs in patients with CLI. Moreover, we aimed to determine whether CPC application is associated with an improvement of endothelial function in patients with infrapopliteal PAOD and CLI, not eligible for interventional or surgical revascularization.

Methods

Patients Seven patients <80 years with diffuse infrapopliteal PAOD and CLI1–6 were included (Table 1). Lesions were not accessible for any surgical or interventional treatment. All patients experienced lower limb pain at rest and five out of seven had an ulcer/gangrene (Table 1). Patients with proliferative retinopathy, evidence of malignancies, symptomatic coronary artery disease, Leriche syndrome, terminal renal failure, hepatic dysfunction, history of neoplasm, anaemia, leucopaenia, thrombocytopaenia, other severe diseases were excluded. The protocol of this study was approved by the Ethics Committee of the University of Leipzig, and written informed consent was obtained from all patients before enrolment into the study.
Study protocol

To increase the amount of circulating CPCs, the patients were treated over 4 days with G-CSF (2 × 300 µg s.c./day). After the stimulation, blood (400 mL) was drawn and substituted with 500 mL normal saline. CPCs were isolated and cultured. Cardiac, haematological, infectious, renal, hepatic, metabolic, and clinical parameters were measured before and after cell application to monitor the effect of G-CSF as well as possible side effects due to cell transplantation. Patients were scheduled to receive intra-arterial infusion of CPCs after 4 days of cell culture. Invasive measurement of vasodilatory capacity was performed before cell application and at the end of the study after 12 weeks.

Baseline measurements of the ankle-brachial pressure index (ABI), the transcutaneous oxygen pressure (TcO2), and the subjective pain index [0 (no pain)–10 (most severe pain)] were determined at the beginning. In addition, the pain-free walking distance was assessed. These parameters were reassessed 1 day and 4, 8, and 12 weeks after cell application.

Isolation, characterization, and culture of CPCs

Mononuclear cells (MNCs) were isolated from 400 mL of peripheral blood by Ficoll density-gradient centrifugation. MNCs were resuspended in endothelial cell basal medium supplemented with EGM-2-MV-SingleQuots (final concentration of hydrocortisol 0.2 µg/mL, FGF 4 ng/mL, VEGF 2 ng/mL, IGF 5 ng/mL, EGF 10 ng/mL; all Cambrex, Verviers, Belgium) and 10% patient serum. MNCs were seeded onto gelatin-precoated culture dishes with a density of 1 × 10^6 cells/cm^2 and cultured for 4 days. After thorough rinsing with PBS/EDTA, the adherent cells were detached by trypsin/EDTA and resuspended in 0.9% NaCl solution supplemented with 1% patient serum. After detachment of cells, an aliquot was characterized by flow cytometry using the following antibodies: anti-Ve-cadherin (Bender MedSystems Inc., San Bruno, CA, USA), anti-VEGFR2 (KDR) (R&D Systems, Minneapolis, MN, USA), anti-CD34 (Miltenyi Biotech, Cologne, Germany), anti-CD133 (Miltenyi Biotech), and anti-CD45 (Becton Dickinson, Franklin Lakes, NJ, USA). Human coronary artery endothelial cells (Cell systems, St Katharinen, Germany) were analysed for comparison. CPCs (anti-Ve-cadherin and anti-CD34 double positive cells) were measured before and after stimulation, as well as after application of cultured CPCs using flow cytometry. Cell viability was assessed by Trypan Blue staining.

Clinical assessment

Heart rate, blood pressure, body temperature, haemoglobin, thrombocytes, C-reactive protein, creatinine, urea, uric acid, gamma GT, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and troponin T were measured at the beginning, after G-CSF stimulation, and 1 day, 1 week, and 3 months after cell transplantation. Leucocytes were measured daily from beginning to 3 days after cell application, followed by post-interventional measurements after 1, 2, 3, 4, 6, 8, and 12 weeks. To determine limb status, the criteria of Rutherford et al. were used. Pain-free walking distance was assessed. Doppler-derived arterial segmental pressures on the ankle and brachium were measured using a standard adult cuff. ABI was calculated as the ratio of ankle systolic pressure and brachial systolic pressure (normal range >1.0). TC02 was measured with an oxymonitor (TC02 Monitor VI) (Lawrenz, Bad Soden, Germany). The probe was attached to the skin that was heated to 43.5°C. When a stable steady state was achieved, a pO2 value expressed in mmHg was recorded (normal range > 60). All measurements were made with patients in the supine position, breathing room air. ABI and TcO2 were determined in triplicate.

Invasive measurement of vasodilatory capacity

All cardiovascular medications were withheld for ≥24 h before the measurement of endothelial function as described previously. Saline, acetylsalicylic acid, and adenosine were administered via the infusion catheter in the following order: (i) 0.9% saline (baseline); (ii) acetylsalicylic acid (90 µg/min), (iii) saline (return to baseline), and (iv) adenosine (6 mg/min). Subsequent infusions were administered after 3 min intervals when all variables had returned to prior baseline values. Maximal flow-dependent vasodilatation was measured proximal to the tip of the infusion catheter after the administration of adenosine. Limb blood-flow reserve was calculated as ratio of
mean peak blood-flow velocity during the administration of adenosine to the blood-flow velocity during saline infusion. The agonist-mediated, endothelium-dependent vasodilation was measured distal to the tip of the infusion catheter during administration of acetylcholine. The invasive assessment of endothelium function was repeated 12 weeks after cell application.

**Application of CPCs**

After invasive measurement via crossover sheath (6 French), CPCs were injected using a transfusion catheter. An average of 39 ± 24 × 10⁶ autologous CPCs were slowly administered (within 3 min) by hand injection, without balloon occlusion of the vessel, into the superficial femoral artery of each patient.

**Statistical analysis**

Data are expressed as mean ± SEM. Data were tested for normal distribution by the Kolmogorow-Smirnow test and for homogeneity of variances using Levene’s test. In case of a normal distribution, values obtained before and after the intervention were compared applying a paired t-test. When data were distributed in a non-normal fashion, comparisons (beginning vs. 12 weeks) were performed by a Wilcoxon signed rank test on an intra-individual basis using SPSS 11.5 for windows (SPSS Inc., Illinois, CA, USA). A Bonferroni-Holm correction was applied to account for the inflation of the type I error due to multiple testing. The Bonferroni correction was applied to the ABI, the TcO₂, the subjective pain index, the count, which returned to physiological levels after cytokine treatment (Figure 1). Additionally, the cells were characterized by markers of stem/progenitor cells (CD34: 32 ± 6%; CD133: 23 ± 3%; Figure 1). A total of 66 ± 11% of attached cells were positive for the panleucocyte marker CD45, a monocyte antigen. The viability of the applied cells was 91 ± 1% (data not shown).

At the beginning, CPC concentration was 130 ± 50 cells/mL rising to 510 ± 100 cells/mL after G-CSF stimulation. Subsequent to intra-arterial application of cultured CPCs, the concentration of CPCs in the circulatory blood was 105 ± 35 cells/mL.

**Clinical follow-up**

Stimulation with G-CSF led to an increase in leucocyte count, which returned to physiological levels after cytokine treatment (Figure 2). Typical muscle or joint pain, fever, chills, or flu-like symptoms after C-CSF application were not reported. Application of CPCs was well tolerated, and procedure-related side effects like vascular damage, thrombotic complications, infections, bleedings, aneurysms,
In one patient, a toe that was already necrotic at study entry had to be amputated after 3 weeks; however, wound healing was prompt and remarkably uncomplicated. In other patients, a regression in ulcer size and healing of previously necrotic areas were observed.

Twelve weeks after CPC administration, a 30-fold increase in the pain-free walking distance from $6.4 \pm 12.5$ to $195 \pm 196$ m ($P = 0.016$; Figure 3A) was observed. In addition, a decrease of the subjective pain index (beginning: $8 \pm 1$ vs. 12 weeks: $2 \pm 2$; $P < 0.001$; Figure 3B), a significant augmentation in ABI (beginning: $0.48 \pm 0.09$ vs. 12 weeks: $0.64 \pm 0.11$; $P < 0.001$; Figure 3C), and TcO$_2$ (beginning: $15 \pm 10$ vs. 12 weeks: $35 \pm 9$ mmHg; $P < 0.001$; Figure 3D) were documented.

Owing to prior thigh amputation of the contra-lateral leg in one patient, ABI and TcO$_2$ could only be measured in six of seven patients. ABI (beginning: $0.84 \pm 0.12$ vs. 12 weeks: $0.85 \pm 0.11$; n.s.) and TcO$_2$ (beginning: $27 \pm 3$ vs. 12 weeks: $36.6 \pm 6$; n.s.) of the contra-lateral leg did not change significantly 12 weeks after intervention.

**Invasive flow measurement**

Twelve weeks after transplantation of CPCs, a five-fold increase in flow-dependent vasodilation (from $0.9 \pm 0.3$ at beginning to $5.0 \pm 1.4$% at 12 weeks; $P = 0.016$; Figure 4A) and a 140% increase in the adenosine-dependent flow reserve (from $3.6 \pm 1.3$ at beginning to $4.9 \pm 1.7$ at 12 weeks; $P = 0.004$; Figure 4B) in the superficial femoral artery could be detected. The endothelium-dependent vasodilation in response to acetylcholine, expressed as the percent change from baseline (saline infusion) in the luminal diameter after $90 \mu$g/min acetylcholine administration, improved significantly (from $-2.6 \pm 1.9$ at beginning to $4.3 \pm 1.7$% at 12 weeks; $P = 0.021$; Figure 4C).

Interestingly, of three patients who initially exhibited a paradoxical vasoconstriction to acetylcholine infusion, two had a normal vasodilatory response to acetylcholine 12 weeks after cell application.

**Discussion**

The present phase I trial in patients with severe PAOD and CLI, who were stimulated with G-CSF and treated with intra-arterial transplantation of autologous CPCs, showed excellent periprocedural safety results. No major cardiovascular events including death, stroke, or aggravation of PAOD or CAD occurred during intervention or follow-up. Procedure-related side effects like worsening of PAOD, infections, bleedings, aneurysms, or renal failure have not been observed.

In high-risk patients with CLI, CPC infusion was not associated with an increased risk of infections/inflammatory activation and was well tolerated. Cardiac, haematological, infectious, renal, hepatic, metabolic, and clinical parameters like body temperature, heart rate, and blood pressure remained within normal limits. The short-term safety, absence of adverse events, and feasibility of CPC application in this phase I study are encouraging to further pursue the strategy of CPC application in ischaemic syndromes. This is reassuring for the design and execution of larger-scale randomized studies using in vitro cultures of CPCs. Furthermore, significant improvements in both clinical parameters (relief of resting pain and increase in

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**Figure 1** FACS-analysis of one patient showing isolated MNCs, attached (AT$^{pos}$) or non-attached cells (AT$^{neg}$) after 4 days of culture as well as human coronary artery endothelial cells. The shaded area of each box denotes negative antigen gate and the white area denotes positive gate. CD34 and CD133 are characterizing haematopoetic stem cells. KDR and VEcad are markers for endothelial cells.

**Figure 2** Leucocyte counts before and after CPC application for each individual patient. (Pat-1 open square), Pat-2 (open triangle), Pat-3 (reverse open triangle), Pat-4 (open diamond), Pat-5 (open circle), Pat-6 (cross), Pat-7 (plus). S denotes the time of stimulation and A refers to the date of application of CPCs.
pain-free walking distance) and endothelial function could be observed.

As this is a safety and feasibility study and because of the high degree of intervention, the study population was limited similar to comparable studies. The study design does not permit the attribution of the positive effects to the CPC application.

**Rationale for CPC application in CLI**

Conventionally, revascularization by either interventional recanalization or bypass surgery is considered the therapy of choice in patients with CLI. However, in the presence of long-distance vascular occlusions with peripheral vessels too small to provide sufficient run-off for successful revascularization strategies, only conservative options are left. In these patients, various pharmacological interventions have been tried in the past (pentoxiphyllin etc.) with little long-lasting effects.

Recently, progenitor cell infusions were tested in animal experiments of chronic hind limb ischaemia with complete occlusion of the femoral artery by surgical ligation. Even under the conditions of complete vascular occlusion, cell-infusion was associated with limb salvage and improved

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**Figure 3** Pain-free walking distance (A), the subjective pain-index [0 (no pain) to (10) most severe pain] (B), ABI (C) and TcO2 (D) is depicted for each individual patient [Pat-1 (open square), Pat-2 (open triangle), Pat-3 (reverse open triangle), Pat-4 (open diamond), Pat-5 (open circle), Pat-6 (cross), Pat-7 (plus)] after application of the cells. The *P*-value refers to a paired t-test comparing the results at 12 week vs. baseline.

**Figure 4** Flow-dependent vasodilation, representing the adenosine-induced change in the diameter of the proximal target vessel segment before and after cell application is illustrated in (A). The adenosine-dependent flow reserve which is defined as mean peak flow velocity divided by the velocity at rest is displayed in (B). The agonist-mediated endothelium-dependent vasodilation (response to acetylcholine), expressed as the per cent change from baseline in the luminal diameter after 90 μg/min acetylcholine administration is shown in (C). Each line represents an individual patient [Pat-1 (open square), Pat-2 (open triangle), Pat-3 (reverse open triangle), Pat-4 (open diamond), Pat-5 (open circle), Pat-6 (cross), Pat-7 (plus)].
resting perfusion in treated animals. These findings are consistent with the hypothesis that CPCs home in ischaemic tissue areas and form new collaterals to improve peripheral perfusion. On the basis of these findings, we initiated the present study investigating a strategy of CPC harvesting, extra-corporal cultivation, and interventional intra-arterial application in selected patients with CLI.

The current literature does not describe a definite number of cells to be applied to obtain a certain benefit. Until now, no correlation between applied cell number and clinical outcome is known. Therefore, we aimed to apply the maximal number of isolated CPCs. To generate a high number of CPCs, patients were stimulated with G-CSF.

Clinical effects of G-CSF stimulation
In the present study, G-CSF was used to increase the number of CPCs prior to harvesting. Contrary to the concern of Tateishi-Yuyama et al., G-CSF treatment did not aggravate symptoms of angina pectoris or precipitate arterial thrombosis because of leucocytosis and hypercoagulability, we did not observe any adverse events in our patients associated with G-CSF application. Patients did not complain about muscle or joint pain, fever chills, or flu-like symptoms possibly because of their pain-relief medication.

G-CSF stimulation led to an increase of CPCs, whereas the concentration of CPCs after intra-arterial application of cultured CPCs did not differ from the levels measured before G-CSF stimulation. This is most likely attributed to the fact that CPCs which are not homing in the target region are removed from the circulation by liver and spleen.

However, it is conceivable that the CPC peak could already affect neovascularization by mobilization of endogenous progenitor cell populations as evidenced in patients with coronary artery disease. G-CSF has only been tested for treatment of infected diabetic foot ulcers. However, peripheral perfusion was not an endpoint in this study, and the effect on ulcer healing remains under discussion. It should be emphasized that because of the nature of our study, the effectiveness of G-CSF stimulation vs. CPC application cannot be readily assessed. Prospective trials comparing G-CSF alone vs. G-CSF plus harvesting and re-injection of CPCs are needed to resolve this controversy.

Clinical effects of progenitor cell application
So far, most studies of progenitor cell application are descriptive in the sense that they do not elucidate which cell type is involved in the process of neovascularization. As reported by other groups, the cell suspension used contained a heterogeneous population of CPCs. Approximately 50% of the cells showed endothelial characteristics as demonstrated by the expression of KDR and Vcadherin. Additionally, the cells expressed cell surface markers of stem/progenitor cells and the panleucocyte marker CD45, which is consistent with the literature.

Owing to the clinical nature of the study, little is known about the fate of the injected cells and one can only speculate about the amount of endothelial differentiation, cell fusion, or migration into other tissue layers.

Therapeutic angiogenesis induced by autologous transplantation of stem cells was previously studied in patients with chronic limb ischaemia. However, the method used for stem-cell harvesting was more invasive than the technique used in this study. With regard to the clinical effects, our results (ABI, TcO2) are well in line with the data reported by Tateishi-Yuyama and reinforce the strategy of therapeutic neovascularization.

Effects of CPC application on vascular endothelial function
Endothelial function and flow velocity in the superficial femoral artery of patients with CLI were invasively measured for two reasons: (i) to accurately determine regional perfusion after maximal vasodilation of collateral circulation by adenosine; and (ii) to assess possible direct effects of CPC attachment/integration into the vascular wall at the site of CPC infusion.

Evidence is accumulating that the vascular endothelium does not only regenerate by cell division of locally sessile endothelial cells but that CPCs may also integrate into areas of damaged endothelial cell layer and improve a previously pathological endothelium-dependent vasodilation. This argument is supported by an improved endothelial function in the femoral artery at the site of CPC injection in our study.

Alternatively, the improved peripheral perfusion may have contributed to augmented shear stress and consecutive shear stress-related upregulation of local NO-generation by increased ecNOS expression and/or activity. Due to the CLI, the application of L-NMMA to determine whether basal NO-production was improved was not possible.

Potential factors involved in clinical improvement
As there is no control group, the observed changes cannot be unambiguously attributed to the CPC administration. Other contributing factors might be spontaneous improvement, placebo effect, and medical therapy.

Although there is no control group, it is rather rare that PAOD improves without intervention or exercise training. A significant change of ABI and TcO2 in the contra-lateral leg could not be detected.

In many patients, placebo effects play a very important role regarding subjective evaluation of pain. In contrast, ABI, TcO2, and endothelial function are objective parameters difficult to influence.

Cardiovascular medication did not change in any of the patients during the study period. Therefore, the observed clinical effects are unlikely to be caused by drug therapy. We are aware that in the typical PAOD patient, beta blockers should be used with extreme caution as soon as limb salvage is at stake. However, in the context of the present study with close weekly clinical follow-up, we felt that the risk-benefit ratio was acceptable. Recently, it has been demonstrated that statins might improve the mobilization and quality of CPCs. However, because of the small population in this study, it is not possible to draw any conclusion about effects of statins on clinical outcome or endothelial function.

Conclusion
The present study documents for the first time that not only the injection of bone marrow cells but also the intra-arterial
application of CPCs after G-CSF stimulation is safe and feasible in patients with PAOD and CLI. The results of this study indicate that it might be as well an effective therapeutic approach, however, larger-scale randomized studies are needed to prove this promising option.

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