Transplantation of Blood-Derived Progenitor Cells After Recanalization of Chronic Coronary Artery Occlusion: First Randomized and Placebo-Controlled Study
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Transplantation of Blood-Derived Progenitor Cells After Recanalization of Chronic Coronary Artery Occlusion
First Randomized and Placebo-Controlled Study

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Abstract—Transplantation of blood-derived circulating progenitor cells (CPCs) has been shown to improve myocardial regeneration after myocardial infarction. It remains unclear whether CPC transplantation exerts beneficial effects also in patients with chronic myocardial ischemia. We initiated a randomized, double-blind, placebo-controlled study evaluating the impact of intracoronary infusion of CPCs on coronary vasomotion and left ventricular (LV) function in patients after recanalization of chronic coronary total occlusion (CTO). After recanalization of CTO, 26 patients (age, 63±2 years; LV ejection fraction, 53±2%) were randomly assigned to the treatment (intracoronary transplantation of CPCs) or control group. Coronary flow reserve in response to adenosine (2.4 mg/min) was measured in the target vessel at the beginning of the study and after 3 months. LV function and infarct size were assessed by MRI and metabolism by 18F deoxyglucose positron emission tomography. CPC application resulted in an increase in coronary flow reserve by 43% from 2.3±0.3 to 3.3±0.5 (P<0.05 versus beginning and control). At 3 months, the number of hibernating segments in the target region (from 2.9±0.6 to 2.0±0.6 segments, P<0.05 versus beginning and control) had declined in the treatment group, whereas no significant changes were observed in the control group. MRI revealed a reduction in infarct size by 16% and an increase in LV ejection fraction by 14% in the treatment group (from 51.7±3.7 to 58.9±3.2%; P<0.05 versus beginning and control) because of an augmented wall motion in the target region. Hence, intracoronary transplantation of CPCs after recanalization of CTO results in an improvement of macro- and microvascular function and contributes to the recruitment of hibernating myocardium. (Circ Res. 2005;97:756-762.)

Key Words: ischemic heart disease ■ endothelial dysfunction ■ progenitor cells ■ hibernating myocardium ■ reperfusion

Recently, bone marrow–derived circulating progenitor cells (CPCs) have been shown to promote formation of entirely new vessels into ischemic tissues by a process termed vasculogenesis.1–3 The CPC-mediated augmentation in myocardial neoangiogenesis in animal experiments prompted researchers to conduct pioneer clinical trials.4–10 In the TOPCARE-AMI (Transplantation Of Progenitor Cells And Regeneration Enhancement in Acute Myocardial Infarction) study, bone marrow–derived and progenitor cells from peripheral blood administered after successful recanalization in acute myocardial infarction were shown to improve myocardial perfusion, increase left ventricular (LV) contractility, and attenuate LV remodeling equally effective.9 Perin et al were able to extend the knowledge with regard to bone marrow–derived stem cell therapy in patients with severe ischemic heart failure: intramyocardial injection of these cells at the site of ischemia was elucidated to increase blood flow and improve regional and global ventricular function.5 Additional evidence is emerging from the first randomized study, BOOST (BOne marOw transfer to enhance ST-elevation infarct regeneration), that indicates an improvement in global LV ejection fraction (LV-EF) of ~7% using bone marrow–derived stem cells in patients with reperfused acute myocardial infarction.10 Even after successful recanalization of chronic total occlusion (CTO) by angioplasty with stent implantation, the myocardium partially remains hibernating, but the underlying mechanisms are poorly understood.11 One might speculate that cardiomyocytes in the border zone of the old infarct are condemned to die by necrosis and apoptosis caused by a persistent impairment of the coronary vasodilatory reserve and, therefore, mutilation of oxygen supply promoting LV remodeling.

Given the therapeutic potential of CPCs, we hypothesized that in patients after successful recanalization of CTO, the additional intracoronary application of CPCs improves coro-
nary vasomotion and enhances global LV function, possibly by the recruitment of hibernating myocardium. In the present study, blood-derived progenitor cells were used because they can be harvested less invasively and seem to be equally effective with respect to the improvement in LV function compared with bone marrow–derived cells in acute myocardial infarction.9

Materials and Methods
An expanded description of the methods is provided in the online data supplement available at http://circres.ahajournals.org.

Selection of Patients
Patients with CTO, clinical signs or objective measures of myocardial ischemia and local wall-motion abnormalities were screened. CTO was defined as an occlusion of a native coronary artery for >30 days with no luminal continuity and with thrombolysis in myocardial infarction flow grade 0 or 1.12 Patients with unstable angina pectoris, indication for coronary artery bypass grafting, any history of malignant disease, and diabetic retinopathy were excluded from study participation.

Study Protocol
This study was approved by the Ethics Committee of the University of Leipzig and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before randomization. After successful recanalization of CTO patients underwent MRI to measure LV function and 18F deoxyglucose positron emission tomography (FDG-PET) to determine myocardial hibernation. Subsequently, all patients were subcutaneously injected twice a day with filgrastin (300 μg of granulocyte colony stimulating factor [G-CSF]) over 4 days to increase the amount of CPCs in the blood. At day 4, 400 mL of venous blood were collected from all patients, mononuclear cells were purified and ex vivo cultured for 4 days in endothelial-specific medium as described in detail below to select for CPCs.1,2,13 Immediately after invasive assessment of coronary vasomotion, patients randomized to the CPC group received intracoronary administration of the selected CPCs, whereas patients in the control group were infused with cell-free serum into the coronary circulation of the target region. The suspension,
containing either CPCs (an average of $69 \pm 14 \times 10^6$ cells) or serum, was prepared by the Department of Transfusion Medicine, University of Leipzig, and provided in a 20 mL syringe ready for intracoronary injection. Neither the interventionalist nor the clinical investigator was aware of whether patients received CPCs or serum. After 3 months, MRI, FDG-PET, and invasive measurement of coronary vasomotion were repeated.

### Preparation of Progenitor Cells

Cells were processed according to German law regarding the preparation of pharmaceutical products including hematopoietic stem cells. CPCs were selected as described in the online data supplement and resuspended in a final volume of 20 mL of physiological NaCl supplemented with 10% autologous patient serum. Ninety percent of the selected cells had the typical feature of CPCs to bind lectin and take up acetylated low-density lipoprotein (LDL) (Figure 1A).1,2,10 An aliquot of these cells was further characterized by fluorescence-activated cell sorting (FACS) analysis (Figure 1B).

### Application of Circulating Progenitor Cells

At a mean time of 10±1 days after successful recanalization of CTO, an over-the-wire balloon catheter (Ninja, Cordis, Roden, the Netherlands) was advanced into the stent previously implanted during the reperfusion procedure; the balloon was inflated with low pressure to completely block antegrade blood flow, and a total of 20 mL of progenitor cell suspension (in the CPC group) or cell-free serum (in the control group) was administered distally to the occluding balloon through the central port of the catheter as described in detail in the online data supplement.9

### Invasive Measurement of Coronary Endothelial Function

Saline, acetylcholine, adenosine, and nitroglycerin, respectively, were administered through an infusion catheter into the target vessel to measure coronary vasomotion as described in detail previously.14 Average blood-flow velocity was assessed by Doppler velocimetry (Flow Map, Cardiometrics). Serial coronary angiograms were obtained at the end of each infusion, coronary diameters were assessed, and the vasodilative or vasoconstrictive responses and coronary flow reserve and coronary blood flow were calculated.

### PET, Single-Photon Emission Computed Tomography, and MRI

Myocardial hibernation was determined by FDG-PET and $^{99m}$Tc-tetrofosmine single-photon emission computed tomography according to standard protocols at 8±1 days after recanalization of CTO and at 3 months of follow-up.15–17 LV-EF, end-systolic and end-diastolic volumes, LV mass, and volumes of regions with delayed enhancement (using gadolinium-benzylxoypropionictetraacetate [0.2 mmol/kg body weight]) were measured by cardiac MRI (1.5 T MR scanner; Intera CV, Philips, Best, The Netherlands) according to a previously validated protocol at 8±1 days after recanalization of CTO and at 3 months.15,16

### Follow-Up Studies

Clinical data, medication, and safety laboratory data were recorded. Patients were seen at follow-up visits every week for 3 months.

### Statistical Analysis

Data are expressed as mean±SEM. Comparisons within each group and between the groups were performed using a t test, a Mann–Whitney U test, or a 2-way repeated measures ANOVA, followed by a Tukey post hoc test, where appropriate. A probability value of $P<0.05$ was considered to indicate statistical significance.

### Results

#### Clinical Characteristics at Baseline and Cardiac Medication

After successful recanalization of CTO, 26 patients were randomly assigned to the CPC group (13 patients) or the control group (13 patients). Patients in the control group did not differ significantly from those in the CPC group with respect to the demographic, clinical, angiographic parameters, or medication (supplemental Table I). Cardiac medication did not change during the 4-week period before enrollment or during the 3-month follow-up period.

Of the 26 chronically occluded vessels selected for the study, 11 (42.3%) were left anterior descending coronary artery, 4 (15.4%) the circumflex coronary artery, and 11 (42.3%) the right coronary artery. The distribution of target vessels was comparable between both groups.

#### Clinical Follow-Up

One patient from the control group was excluded from further analysis because the initially successfully reopened chronically occluded vessel was found to be reoccluded at the scheduled time of intracoronary serum application without any chance of successful recanalization. One patient in the CPC group and 1 patient in the control group withdrew consent of study participation because of personal reasons. Therefore, follow-up data for invasive measurement of coronary vasomotor function are available from 12 patients of the CPC group and 11 patients randomized to the control group.

In 1 patient in the CPC group, MRI could not be performed because the patient had an implanted cardioverter defibrillator. Therefore, follow-up data for MRI are available from 11 patients of the CPC group and from 11 patients of the control group.

In 1 patient of the CPC group and 2 patients of the control group, the FDG-PET pictures could not be analyzed because of a superimposition of the heart by intestine. Therefore, FDG-PET data are reported from 11 patients of the CPC group and 9 patients of the control group.

#### Rate of In-Stent Restenosis

Coronary in-stent restenosis occurred in 3 of 12 patients in the CPC group (25%) and in 3 of 11 patients in the control group (27%). However, in-stent restenoses were not flow limiting at rest, and all patients angiographically demonstrated thrombolysis in myocardial infarction flow grade 3. No other clinical events, particularly no recurrences, were evident in either of the groups.

### Stimulation With G-CSF and Serum Inflammatory Markers

One patient in each group reported headache, and 1 patient in the control group developed fever during G-CSF stimulation. However, symptoms were reversible, and body temperature returned to normal immediately after discontinuation of subcutaneous G-CSF injection.

G-CSF application resulted in a comparable increase in serum C-reactive protein levels (supplemental Figure IA) and blood leukocyte count (supplemental Figure IB) in the CPC and the control groups, but both parameters returned to baseline values within 4 days after G-CSF application was stopped. However, neither G-CSF injection nor intracoronary transplantation of CPCs caused any elevation in troponin T levels. In addition, intracoronary application of CPCs/serum was not associated with an increase in body temperature,
leukocyte count, or C-reactive protein levels in the follow-up period of 3 months.

Assessment of Coronary Endothelial Function

Acetylcholine-Induced Pathologic Vasoconstriction and Coronary Blood Flow

At beginning of the study (post-percutaneous coronary intervention [PCI]), patients in the CPC and the control groups had similar responses to intracoronary acetylcholine stimulation, depicted as the percentage change from baseline in the luminal diameter. In the CPC group, the mean vasoconstrictive response after infusions of 0.072, 0.72, and 7.2 μg/min of acetylcholine were -10±5, -21±5, and -34±4%, respectively. In the control group, they were -22±7, -36±12, and -44±15%, respectively (P=NS between the groups).

Three months after CPC application, coronary vasoconstriction was reduced by 88% (from -0.24±0.06 to -0.03±0.04 mm; P<0.01 versus beginning of the study [post-PCI] within the CPC group; P<0.01), by 65% (from -0.51±0.10 to -0.18±0.11 mm; P<0.01 versus post-PCI within the CPC group; P<0.05 for the change versus control group), and by 72% (from -0.87±0.12 to -0.24±0.09 mm; P<0.01 versus post-PCI within the CPC group; P<0.01 versus control group at 3 months), respectively, in response to 0.072, 0.72, and 7.2 μg/min of acetylcholine (Figure 2A). This improved vasodilatory capacity of the epicardial coronary target vessels after CPC application was associated with an increase in coronary blood flow during infusion of acetylcholine (supplemental Table II).

In the control group, the changes in coronary artery diameter and blood flow in response to intracoronary acetylcholine stimulation at 3 months did not differ significantly from those at post-PCI (Figure 2B and supplemental Table II). Moreover, endothelium-independent vasodilatory response did not change from post-PCI to 3 months in either of the groups (supplemental Table II).

Coronary Blood-Flow Reserve

At 3 months, in the CPC group coronary flow reserve was found to be significantly increased by 43% (from 2.3±0.3 at begin to 3.3±0.5 at 3 months, P<0.05 versus post-PCI within the CPC group, P<0.05 versus control group at 3 months) but remained essentially unchanged in the control group (2.7±0.3 at post-PCI; 2.3±0.2 at 3 months) (Figure 3A). In the CPC group, patients with restenosis did not differ from those without restenosis with respect to CFR or endothelium-dependent vasomotion.

Assessment of Myocardial Viability

At beginning of the study (post-PCI), patients in the CPC and the control group were characterized by a similar number of hibernating myocardial segments. At 3 months, the number of myocardial segments with hibernation was significantly reduced by 31% (from 2.9±0.6 segments post-PCI to 2.0±0.6 segments at 3 months; P<0.05 versus post-PCI within the CPC group; P<0.05 versus control group at 3 months), whereas no change was detectable in the control group (from 2.6±0.6 segments post-PCI to 3.6±0.6 segments at 3 months) (Figure 3B).

Assessment of Global LV-Function and Regional Wall Motion by MRI

Post PCI, patients in the CPC and the control group had similar end-diastolic and end-systolic LV volumes. Moreover, global LV function was found to be reduced by a similar degree in both groups. In the CPC group, the global LV-EF was significantly improved by 7.2% at 3 months (P<0.01 versus post-PCI; Figure 4), which was the result of an enhanced local wall motion in the target region. A representative MRI from a patient in the CPC group showing an improvement in local wall motion and global EF 3 months after intracoronary CPC application is shown in the online data supplement (Video Files 1 and 2).

Hence, the end-systolic LV volume decreased by 13% at 3 months (P<0.01 versus beginning), but end-diastolic LV volume remained unchanged. Moreover, the absolute volume of delayed enhancement, indicative of scar tissue, was found to be reduced 3 months after CPC infusion by 16% as compared with post-PCI (supplemental Table II). In the control group, global LV function, end-systolic, and end-diastolic LV volume, as well as the volume of delayed enhancement, remained unchanged (supplemental Table II).

Figure 2. Endothelium-dependent, agonist-mediated coronary vasomotion. A, CPC group: change in coronary target vessel diameter in response to acetylcholine (0.072 μg/min, 0.72 μg/min, 7.2 μg/min) stimulation vs baseline (infusion of 0.9% saline). ●, At beginning of the study (post-PCI); ○, at 3 months. The paradoxic vasoconstriction in response to acetylcholine is significantly blunted 3 months after intracoronary CPC infusion. *P<0.01 vs post-PCI, †P<0.01 vs control group at 3 months, ‡P<0.05 for the change (post-PCI vs 3 months) vs control group. B, Control group: change in coronary target vessel diameter in response to acetylcholine (0.072 μg/min, 0.72 μg/min, 7.2 μg/min) stimulation vs baseline (infusion of 0.9% saline). ○, At beginning of the study (post-PCI); ○, at 3 months. The paradoxic vasoconstriction in response to acetylcholine remains virtually unchanged within the 3 months of the study period.
Figure 3. A, Coronary flow reserve in patients of the CPC (left) and the control group (right). Filled bars represent the beginning of the study (post-PCI); open bars, at 3 months. Coronary flow reserve significantly increases in the CPC-treated patients but remains constant in the control group. *P<0.05 vs post-PCI, #P<0.05 vs control group at 3 months. B, Number of hibernating segments in patients of the CPC (left) and the control group (right). Filled bars represent the beginning of the study (post-PCI); open bars, at 3 months. The number of hibernating segments decreased in the CPC-treated patients and tends to increase in the control group during the study period. *P<0.05 vs post-PCI, #P<0.05 vs control group at 3 months.

Discussion
In this first randomized, placebo-controlled, and double-blinded study using intracoronary infusion of CPC, we are showing that intracoronary infusion of CPC after successful recanalization of CTO improves coronary endothelial function and augments metabolism in the target region in patients with symptomatic coronary atherosclerosis. These profound effects were coupled with an improvement in wall-motion abnormalities in the target area, a significant decrease in end-systolic volume and scar size resulting in an increase in global LV-EF. In conclusion, these data support the concept that intracoronary application of CPC after successful recanalization of CTO contributes to a recruitment of hibernating myocardium.

From these results, an important question arises: Is the primary mode of CPC action the improvement in endothelial function and vasculogenesis that leads to the recruitment of hibernating myocardium, or does endothelial function improve secondary to the CPC-mediated enhancement in metabolism and function? Clearly, both mechanisms are possible. The attenuated vasoconstriction of epicardial vessels in response to acetylcholine after CPC therapy might originate from the homing of locally administered CPC into gaps within the endothelial cell layer, thereby preventing the direct vasoconstrictive action of acetylcholine on the smooth muscle. This hypothesis is in accordance with data from animal and human studies proposing that a CPC-mediated reconstitution of damaged endothelium might result in an improvement of endothelium-dependent vasodilatation.19,20 Because CPCs have also been shown to release survival factors, it is alternatively conceivable that those cytokines promote the survival and proliferation of resident endothelial cells within the epicardial coronary arteries.21 Additionally, the increase in the coronary flow reserve might be the consequence of an enhanced CPC-mediated vasculogenesis. The latter theory is supported by numerous, elegant animal studies proving the contribution of CPC and bone marrow–derived stem cells to neovascularization and linking them to the improvement in perfusion in ischemic tissues.1–7 Even more evidence is currently being derived from human studies, in which the application of progenitor cells in reperfused acute myocardial infarction and the intramyocardial injection of bone marrow stem cells in patients with ischemic heart failure has been considered suitable for achievement of therapeutic angiogenesis that might contribute to an improvement in local and global LV function.5,8–10

Nevertheless, the situation faced by CPCs that had been administered into the coronary circulation after successful recanalization of CTO is different: in case of prior myocardial infarction, the dead myocardium has already been replaced by scar tissue and remodeling of the left ventricle has started, which is possibly limiting the therapeutic benefits of an additional CPC application. Even in this hostile environment CPCs prevail: they did contribute to an improvement in metabolism, a reduction in infarct size, and an augmentation in EF. However, the question whether bone marrow–derived progenitor cells, including CPCs, and the progeny are competent to replace scar and rejuvenate damaged myocardium and whether this occurs by fusion or transdifferentiation is unanswered so far. Whereas earlier animal studies have suggested a regeneration of significant amounts of contractile myocardium consisting of newly formed endothelium, smooth muscle cells, and cardiomyocytes by intramyocardial injection of bone marrow stem cells,22 recent experiments question these results and the proposed mechanisms.23–25 Nevertheless, because CPCs were recently shown to retrain the capability to differentiate into functional cardiomyocytes, endothelial cells and smooth muscle cells, scar replacement by de novo generation of myocardium leading to an improvement in cardiac function cannot be excluded at this time.26 Additionally, early tissue-committed stem cells, expressing cardiac and endothelial-lineage markers, which were recently recognized in the bone marrow and the circulating blood, might contribute to the reconstitution of functional myocardium in the present study.27 There was a considerable variation in the percentage of CD34+ and CD133+ cells per patient. However, we did not detect any association between the number or percentage of stem cells or CPCs and the
outcome with respect to endothelial function or hibernation. In our understanding, this finding is not surprising because the therapeutic effects of stem cells, of CPCs in particular, depend not only on their number but also on their functional capacities, which are known to be deteriorated by risk factors.28

More recently, a population of so-called cardiac progenitor cells was identified in the heart of different mammals, including humans.29,30 Those progenitors with stem cell properties were proven to contribute to the regeneration of functional myocardium after infarction as well.28,29,31 It has been anticipated that intramyocardial or intracoronary administration of BMCs or CPCs might activate cardiac progenitor cells in a paracrine manner, thereby promoting cardiac repair. Therefore, it is conceivable that CPCs, which might represent a progeny of early-tissue committed stem cells, or paracrine-activated cardiac progenitor cells contribute to the replacement of scar tissue by functional myocardium. Moreover, it must be kept in mind that cytokines released from CPCs might contribute to the recruitment of immune cells, such as monocytes, macrophages, and lymphocytes.28 These immune cells, which have a high activity of matrix metalloproteinases, will possibly replace surrounding scar tissue, thereby reducing the myocardial area with an hyperenhancement in MRI.

Additionally, it is conceivable that CPCs, which are rich in proteases, transmigrate through the vessel wall and remove scar tissue by digestion.32 These latter 2 mechanisms could potentially explain the reduction in infarct size in the absence of significant cardiac regeneration. However, independent of whether a de novo generation of myocardium, rejuvenation of damaged myocardium (eg, by cell fusion), or improvement of endothelial function is a key mechanism of the CPC therapy, the majority clinical studies, including the present study, had an improvement in cardiac function in common, which is, from the point of view of the clinician, the most important finding.22,23 Additionally, these findings in conjunction with the observed augmentation of myocardial perfusion and metabolism in the CPC-treated patients argues against concerns that CPC application might promote the growth of noncardiac tissue within the heart.33

Lately, G-CSF stimulation to augment the number of CPCs has been discussed controversially; 1 study had been stopped prematurely because of an increased rate of in-stent restenosis in an inconsistent population of patients who had experienced myocardial infarction within the last 4 to 280 days.34 In our present study, the binary rate of in-stent restenosis in the carefully selected control group compares favorable with results from previous trials assessing the effects of PCI and bare-metal stent implantation in patients with CTO, eg, GISSOC, STOP, and TOSCA, respectively.35–37 Most importantly, after successful recanalization of CTO, G-CSF stimulation for 4 days followed by intracoronary CPCs application was not associated with an increased risk of in-stent restenosis or progression of coronary artery disease in our study. Notably, microcirculatory response was also improved in CPC-treated patients who demonstrated in-stent restenosis, and none of our patients experienced a worsening of local wall-motion or an expansion of the LV end-systolic volume after intracoronary CPC application, independent of whether an in-stent restenosis was present at follow-up or not.

The combined therapy with intracoronary transplantation of blood-derived progenitor cells after successful recanalization of chronic coronary occlusion was associated with a blunting of paradoxic vasoconstriction in the target region and an enhanced myocardial perfusion. The CPC-mediated rescue of hibernating myocardium resulted in an improvement in local wall motion and global LV function in the absence of any harmful short-term side effects. These data suggest that intracoronary transplantation of progenitor cells, in addition to standard therapy, not only has the potential to improve recovery after acute myocardial infarction but might also partially retrieve the deleterious effects of chronic coronary occlusion on LV remodeling.

Acknowledgments
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References


Online Supplement

Expanded Methods

Selection of Patients

Patients with CTO, clinical signs or objective measures of myocardial ischemia – e.g. angina pectoris or ST-segment depression in the ECG during exercise - and local wall motion abnormalities (hypokinetic, akinetic, or dyskinetic segments, respectively) in left ventricular angiography were screened. CTO was defined as an obstruction of a native coronary artery for more than 30 days with no luminal continuity and with Thrombolysis In Myocardial Infarction (TIMI) flow grade 0 or 1. Patients with unstable angina pectoris, indication for coronary artery bypass grafting, any history of malignant disease and diabetic retinopathy were excluded from study participation. Twenty six patients (age <75 years), who matched the above-mentioned criteria were eligible for the study after successful recanalization of CTO by angioplasty and stent implantation. A restoration of TIMI flow grade 3 with a residual stenosis < 30% assessed by quantitative coronary angiography was considered a technical success.

Study protocol

This study was approved by the Ethics Committee of the University of Leipzig and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before randomization. Additionally, the study was registered at and approved by the “Paul-Ehrlich-Institute”, which is responsible for the monitoring of an adequate processing and use of blood products including stem cells in humans in Germany. After successful recanalization of CTO patients underwent magnetic resonance imaging (MRI) to measure left ventricular function and F-18 deoxyglucose positron emission tomography (FDG-PET) as well as ⁹⁹ᵐTc-tetrofosmine SPECT to determine myocardial hibernation. Subsequently, all patients
were subcutaneously injected twice a day with filgrastin (300 µg, G-CSF: granulocyte colony stimulating factor) over 4 days in order to increase the amount of CPCs in the blood. At day 4, 400 mL of venous blood were collected from all patients, mononuclear cells were purified and ex vivo cultured for 4 days in endothelial specific medium as described in detail below to select CPCs. Immediately after invasive assessment of coronary vasomotion, patients randomized to the CPC group received intracoronary administration of the selected CPC’s, whereas patients in the control group were infused with cell-free serum into the coronary circulation of the target region. The suspension –either containing CPCs or serum- was prepared by the Department of Transfusion Medicine and provided in a 20 mL syringe ready for intracoronary injection. Neither the interventionalist nor the clinical investigator was aware of whether patients received CPCs or serum. After 3 months, MRI, FDG-PET, SPECT and invasive measurement of coronary vasomotion were repeated.

**Preparation of Progenitor Cells**

Cells were processed according to the German law in adherence to GMP/GLP practices. Mononuclear cells (MNC) were isolated from 400 ml of venous blood by density gradient centrifugation with Histopaque-1077 (Sigma, Deissenhofen, Germany). Immediately after isolation, total MNC were plated on gelatine coated cell culture flasks (1 % gelatine dissolved in PBS, Biochrom KG, Berlin, Germany) with a cell density of $1 \times 10^6$ cells/cm$^2$. The cells were maintained for 4 days in endothelial basal medium (Cambrex, Verviers, Belgium) supplemented with EGM SingleQuots (Cambrex, Verviers, Belgium; resulting in a final concentration of fibroblast growth factor: 4 ng/ml, of vascular endothelial growth factor: 2 ng/mL, of insulin-like growth factor I: 5 ng/mL, of epidermal growth factor: 10 ng/mL in the cell culture medium) and 10% human serum, collected from each individual patient. Additionally, the cell
culture medium was supplemented with ascorbic acid (final concentration, 75 ng/mL) and hydrocortisone (0.2 µg/mL). After 4 days of culture nonadherent cells were removed by a thorough washing with phosphate buffered saline (PBS), and the adherent cells were detached with trypsin/EDTA (Biochrom KG, Berlin, Germany). The collected cells were washed twice with PBS containing 2 mmol/L EDTA and resuspended in a final volume of 20 ml physiological NaCl supplemented with 10% autologous patient serum. 90% of the selected cells had the typical feature of CPCs to bind lectin and take up acetylated LDL (Figure 1A). An aliquot of these cells was further characterized by FACS analysis (FACS-Calibur (Becton-Dickinson) using the following antibodies: anti-CD-3 and anti-CXCR4 (Becton-Dickinson, Heidelberg, Germany), anti-KDR (R&D Systems, Wiesbaden, Germany), anti-CD-34 and anti-CD-133 (Miltenyi, Bergisch-Gladbach, Germany), anti VE-cadherin (Bender MedSystems, San Bruno, CA, USA) (Figure 1B). Cell viability was tested by trypan blue exclusion. Contamination of the cultured CPCs by mycoplasma was ruled out applying a PCR based method (EuroClone, Wetherby, UK). The number of CPCs recovered from 400 mL of blood varied between 22x10^6 and 200x10^6 cells.

**Application of Circulating Progenitor Cells**

At a mean time of 10±1 days after successful recanalization of CTO, an over-the-wire balloon catheter (Ninja, Cordis, Roden, Netherlands) was advanced into the stent previously implanted during the reperfusion procedure. To promote adhesion and potential transmigration of the infused cells through the endothelium, the balloon was inflated with low pressure to completely block antegrad blood flow for a period of two minutes. During this time, 5 mL of progenitor cell suspension (in the CPC group) or cell-free serum (in the control group) were administered distally to the occluding balloon through the central port of the catheter. This manoeuvre was repeated 4
times to accommodate infusion of 20 mL progenitor cell suspension containing an average of $69\pm14\times10^6$ CPCs (range from $22\times10^6$ to $200\times10^6$ of CPCs administered per patient) interrupted by 2 minutes of reflow by deflating the balloon to minimize extensive ischemia. In patients of the control group, the same procedure was performed, except that 20 mL of cell-free serum were infused into the coronary target circulation. Afterwards, coronary angiography was repeated to ensure vessel patency and unimpaired blood flow in all patients.

**Invasive Measurement of Coronary Endothelial Function**

Endothelial function was measured as previously described. Briefly, a 6-French catheter was used to cannulate the left or right coronary artery. A 2.5-French infusion catheter (Transit Infusion Catheter, Cordis, Miami, Fl, USA) was than advanced over a guide wire containing a 12-MHz, pulsed Doppler ultrasound velocimeter (FlowMAP, Cardiometrics, Endosonics, Rancho Cordova, CA, USA) into a non-branching segment of the reopened target vessel. The tip of the guide wire was positioned 1 cm distal to the end of the infusion catheter, close to an anatomical landmark. The maximal and mean blood-flow velocity measured by the Doppler velocimeter was continuously registered throughout the test protocol and drug infusion. To determine coronary blood flow, the mean peak velocity was multiplied by the cross sectional area of the vessel segment of interest and is expressed in milliliters per minute.

**Drug Infusion and Quantitative Angiography**

Saline (0.9 percent in order to determined baseline diameter), acetylcholine (to assess endothelium-dependent vasomotion; 10 mg per milliliter, Dispersa, Germering, Germany), adenosine (to measure flow-dependent vasodilatation; 3 mg per milliliter, Sanofi, Winthrop, Munich, Germany), and nitroglycerin (to elucidate
endothelium independent vasomotion; 1 mg per milliliter, Schwarz Pharma, Monheim, Germany) were administered through the infusion catheter as previously described in detail. Serial coronary angiograms were obtained in the same projection at the end of each infusion, coronary diameters were assessed and the vasodilatative or vasoconstrictive responses as well as coronary flow reserve were calculated.

**PET and SPECT**

FDG-PET was performed using a whole-ring ECAT EXACT HR+ PET scanner (Siemens/CTI, Knoxville, TN, USA). After an overnight fast, non-diabetics received a single dose of 250 mg acipimox (Olbemox, Pharmacia Upjohn, Erlangen) followed by 50 g of glucose 60 minutes later. After one hour, a mean dosage of 370 MBq of $^{18}$F-FDG was injected and PET images were acquired for 30 min, including a 10 min transmission scan. Diabetic patients underwent hyperinsulinemic euglycemic clamping and $^{18}$F-FDG was administered when glucose plasma levels started to decrease. The images were reconstructed with 2D filtered back projection employing a Hann filter FWHM 7.9 mm.

For perfusion scans, 400 MBq $^{99m}$Tc-tetrofosmine were injected after an overnight fast and image acquisition was started one hour later. For acquisition a dual-head camera with detectors positioned at an angle of 90° (ADAC Lab. Vertex) equipped with ultra-high resolution collimators and two 153Gd-line sources for attenuation correction was used. Sixteen views per detector were recorded for a period of 80 s each from left posterior oblique 45° to right anterior oblique 45° position. Image reconstruction was performed using an iterative algorithm with attenuation and scatter correction.
According to the nomenclature, the left ventricle was divided into 17 segments corresponding to the territories of the 3 major coronary arteries. Mean signal intensities of the segments in the target region corresponding to the re-opened CTO and the reference area were calculated Post-PCI and after 3 months of follow up by FDG-PET. The segment of the reference area with the highest mean signal intensity (MSI) in the perfusion study was considered normal (100 %), all other segments were adjusted applying a computerized automatic procedure. Segments with reduced perfusion (<80% of reference) with relatively to the perfusion increased glucose metabolism (>10%) were classified as hibernating segments.

**Magnetic Resonance Imaging**

Cardiac MRI (1.5 T MR scanner, Intera CV, Philips, Best, The Netherlands) was performed 8±1 days after recanalization of CTO and 3 months after intracoronary progenitor cell infusion. All images were acquired in the supine position using a phased-array body surface coil during breath holds of 4-8 seconds. Vectorcardiographic triggering was performed. Cine images were acquired in the standard views (4- and 2-chamber view, short axis throughout the entire left ventricle) using a steady-state free precession sequence with retrospective gating (20-25 phases per cardiac cycle; repetition time (TR) 3.2 ms, echo time (TE) 1.2 ms, flip angle 60°. Typical in-plane spatial resolution was 1.8x1.8 mm with a slice thickness of 8 mm.

In addition, delayed enhancement images were recorded 10-15 minutes after injection of gadolinium-BOPTA (0.2 mmol/kg body weight) using a 3D inversion recovery sequence (TR 2.8 ms, TE 1.1 ms, flip angle 15°) The inversion time was individually adapted to null normal myocardium. Typical in-plane spatial resolution was 2.0x2.0 mm with a slice thickness of 5 mm.
Offline image analysis was performed by two independent observers blinded to patient identity and assignment. Left ventricular ejection fraction (LV-EF), endsystolic and enddiastolic volumes, LV mass, and volumes of regions with delayed enhancement were calculated as described previously.\textsuperscript{13} Additionally, images were analyzed applying a standard 17 segment model. Segmental wall thickening was assessed semiquantitatively and evaluated by a score to be either normal (5), mildly hypokinetic (4), moderately hypokinetic (3), severely hypokinetic (2), akinetic (1), or dyskinetic (0), resulting in a maximum score of 85.\textsuperscript{9} An improvement in contraction of individual segments was considered to represent a functional recovery of local wall motion. Moreover, the segmental delayed enhancement was determined according to the following classification: 0\%, >0 to \(\leq\) 25\%, >25\% to \(\leq\) 50\%, >50\% to \(\leq\) 75\%, and \(>75\%\) of either volume extent or transmural extent.

**Follow-up Studies**

Clinical data, medication, and safety laboratory data were recorded. Patients were seen at follow-up visits every week for 3 months.

**Statistical Analysis**

Data are expressed as mean\(\pm\)SEM. Based on the TOP-CARE AMI trial, sample size was calculated assuming that CFR - as the primary end point - improves by 1.0\(\pm\)0.9 in the CPC group whereas no change in CFR was expected in the control group.\textsuperscript{5} A sample size of 11 patients in each group provided the study with 85\% power to detect the above-mentioned difference in CFR. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variance with Levene’s test. Comparisons within
each group and between the groups were performed with the use of two-way repeated measures ANOVA, followed by a Tukey post hoc test. In case of a non-normal distribution of the data, a Mann Whitney U test or a Wilcoxon signed rank test was applied for the inter- and intragroup comparison, respectively.
A t-test was used to compare the absolute and the percentage changes (from the initial study to the follow-up assessment at 3 months) between the two groups in case of a normal distribution of the data. Otherwise a Mann-Whitney U test was applied. Categorical variables were tested using the chi-square test. A p value of less than 0.05 (by two-sided testing) was considered to indicate statistical significance.

References:


9. Cerqueira MD, Weissman NJ, Dilsizian V, Jacobs AK, Kaul S, Laskey WK, Pennell DJ, Rumberger JA, Ryan T, Verani MS. Standardized myocardial segmentation and nomenclature for tomographic imaging of the heart: a statement for healthcare professionals from the Cardiac Imaging Committee of


Online Table 1: Clinical Characteristics and Cardiac Medication

<table>
<thead>
<tr>
<th></th>
<th>CPC (n=13)</th>
<th>Control (n=13)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gender (female/ male)</td>
<td>4/9</td>
<td>2/11</td>
<td>0.645</td>
</tr>
<tr>
<td>age (years)</td>
<td>64±2</td>
<td>62±3</td>
<td>0.611</td>
</tr>
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<td>1/2/3 vessel disease</td>
<td>8/4/1</td>
<td>6/4/3</td>
<td>0.291</td>
</tr>
<tr>
<td>time of CTO (months)</td>
<td>7.5±2.9</td>
<td>8.8±2.9</td>
<td>0.766</td>
</tr>
<tr>
<td>number of implanted stents</td>
<td>1.9±0.4</td>
<td>1.8±0.2</td>
<td>0.900</td>
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<td>body height (cm)</td>
<td>170±2</td>
<td>171±2</td>
<td>0.863</td>
</tr>
<tr>
<td>body weight (kg)</td>
<td>81±3</td>
<td>81±3</td>
<td>0.959</td>
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<td>systolic blood pressure (mmHg)</td>
<td>131±5</td>
<td>130±6</td>
<td>0.895</td>
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<tr>
<td>diastolic blood pressure (mmHg)</td>
<td>77±3</td>
<td>78±3</td>
<td>0.748</td>
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<td>serum glucose (mmol/L)</td>
<td>5.3±0.2</td>
<td>5.7±0.6</td>
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<tr>
<td>serum LDL cholesterol (mmol/L)</td>
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<td>3.2±0.3</td>
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<td>history of diabetes mellitus (n)</td>
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<td>4</td>
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<tr>
<td>history of arterial hypertension (n)</td>
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<td>12</td>
<td>1.000</td>
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<td>history of hypercholesterolemia (n)</td>
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<td>9</td>
<td>0.521</td>
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<tr>
<td>history of smoking (n)</td>
<td>0</td>
<td>3</td>
<td>0.214</td>
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<tr>
<td>family history of CAD (n)</td>
<td>2</td>
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<td>0.425</td>
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<tr>
<td>Cardiac Medication:</td>
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<td></td>
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<tr>
<td>Aspirin &amp; Clopidogrel (n)</td>
<td>13</td>
<td>13</td>
<td>1.000</td>
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<tr>
<td>Beta-blocker (n)</td>
<td>9</td>
<td>12</td>
<td>0.322</td>
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<tr>
<td>ACE inhibitor (n)</td>
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<td>8</td>
<td>0.603</td>
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<tr>
<td>Statin (n)</td>
<td>9</td>
<td>9</td>
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<tr>
<td>Nitrate (n)</td>
<td>1</td>
<td>4</td>
<td>0.322</td>
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<tr>
<td>Calcium antagonist (n)</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
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Clinical characteristics and cardiac medication. CTO: chronic total occlusion.
# Online Table 2: Coronary Blood Flow and MRI Data

<table>
<thead>
<tr>
<th></th>
<th>CPC Group</th>
<th>Control Group</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Post PCI</td>
<td>3 Months</td>
<td>#</td>
<td>p value</td>
<td>Post PCI</td>
<td>3 Months</td>
<td>$</td>
<td>p value</td>
<td>†</td>
<td>p value</td>
</tr>
<tr>
<td><strong>Change in CBF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ach [7.2 µg/min]</td>
<td>-5.5 ± 37.6</td>
<td>124.0 ± 28.1</td>
<td>&lt;0.05</td>
<td></td>
<td>4.4 ± 25.0</td>
<td>14.0 ± 24.3</td>
<td>0.686</td>
<td></td>
<td>&lt;0.05</td>
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<tr>
<td>NTG [0.2 mg bolus]</td>
<td>219.7 ± 32.4</td>
<td>198.2 ± 38.2</td>
<td>0.594</td>
<td></td>
<td>184.1 ± 32.6</td>
<td>181.6 ± 32.1</td>
<td>0.953</td>
<td></td>
<td>0.973</td>
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<tr>
<td><strong>MRI Data</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV-EF global [%]</td>
<td>51.7 ± 3.7</td>
<td>58.9 ± 3.2</td>
<td>&lt;0.001</td>
<td></td>
<td>55.8 ± 2.8</td>
<td>55.8 ± 2.6</td>
<td>0.987</td>
<td></td>
<td>&lt;0.01</td>
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<tr>
<td>ESV [mL]</td>
<td>72.2 ± 9.1</td>
<td>62.6 ± 8.9</td>
<td>&lt;0.01</td>
<td></td>
<td>70.8 ± 8.2</td>
<td>66.6 ± 6.3</td>
<td>0.196</td>
<td></td>
<td>0.240</td>
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<tr>
<td>EDV [mL]</td>
<td>148.3 ± 11.9</td>
<td>148.1 ± 10.6</td>
<td>0.962</td>
<td></td>
<td>156.1 ± 9.7</td>
<td>148.5 ± 8.0</td>
<td>0.179</td>
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<td>0.271</td>
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<tr>
<td>Wall motion score</td>
<td>71.3 ± 4.2</td>
<td>77.2 ± 3.5</td>
<td>&lt;0.001</td>
<td></td>
<td>76.7 ± 2.1</td>
<td>78.4 ± 2.1</td>
<td>0.262</td>
<td></td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Myocardial mass [g]</td>
<td>135.8 ± 12.9</td>
<td>131.3 ± 11.5</td>
<td>0.087</td>
<td></td>
<td>124.0 ± 9.2</td>
<td>125.5 ± 8.7</td>
<td>0.658</td>
<td></td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>Infarct size [mL]</td>
<td>16.8 ± 4.1</td>
<td>13.8 ± 3.7</td>
<td>&lt;0.001</td>
<td></td>
<td>10.5 ± 3.7</td>
<td>9.4 ± 3.4</td>
<td>0.133</td>
<td></td>
<td>0.063</td>
<td></td>
</tr>
</tbody>
</table>

# p value for the comparison within the CPC group (Beginning of the study (post PCI) vs. 3 Months).

$ p value for the comparison within the Control group (Post PCI vs. 3 Months).

† p value for the comparison of the change (Post PCI vs. 3 Months) between the CPC group and the control group.
Figure Legends

Online Figure 1:

CRP level

A: The increases in CRP and the resolution of it are show patient by patient for the CPC group (left panel) and the control group (right panel).

Blood leukocyte count

B: The increases in blood leukocyte count and the resolution of it are show patient by patient for the CPC group (left panel) and the control group (right panel).

From day –8 (S) to day –4 patients of both groups were injected with G-CSF twice a day. At day –4 (B) 400 mL of venous blood were collected to isolated CPC, and G-CSF application was discontinued. At day 0 (A) patients in the CPC group were treated by intracoronary transplantation of CPC, whereas patients in the control group received a intracoronary injection of cell-free serum.
**Video Legends**

**Video Files:** Representative MRI examples from a patient of the CPC group at beginning of the study (post PCI) and 3 months after intracoronary CPC application

**SA-beginning:** Short axis view of the left ventricle from a patient of the CPC group 8 days after successful recanalization of a chronic total occlusion of the left anterior descending coronary artery. The hypokinetic segments (the septum bordering the anterior wall and the anterior wall itself) can be clearly recognized.

**SA-3months:** Short axis view of the left ventricle from the same patient of the CPC group 3 months after intracoronary CPC administration. The septum and the anterior wall are showing an improved wall motion as compared to post PCI and are now almost normally contracting.