Retroinfusion of Embryonic Endothelial Progenitor Cells Attenuates Ischemia-Reperfusion Injury in Pigs
Role of Phosphatidylinositol 3-Kinase/AKT Kinase

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Background—Adult endothelial progenitor cells (EPCs) reduce myocardial infarct size and improve postischemic myocardial function. We have recently shown that clonal embryonic EPCs (eEPCs), derived from 7.5-day-old mice, home specifically to hypoxic areas in tumor metastasis mouse models but spare normal organs and do not form carcinomas. Here, we assessed the potential of eEPCs to limit organ dysfunction after ischemia and reperfusion in a preclinical pig model.

Methods and Results—Pigs were subjected to ischemia (60-minute left anterior descending [LAD] artery occlusion) and reperfusion (7 days). At the end of ischemia, we applied medium with or without 5×10⁶ eEPCs by either pressure-regulated retroinfusion or intravenous transfusion. One hour after reperfusion, ⁹⁹Tc-labeled eEPCs engrafted to a 6-fold higher extent in the ischemic myocardium after retroinfusion than after intravenous application. Regional myocardial function (subendocardial segment shortening [SES] at 150/min, given in percent of nonischemic circumflex region) and infarct size (TTC viability and Methylene-blue exclusion) were determined 24 hours and 7 days later. Compared with medium-treated animals, retroinfusion of eEPCs decreased infarct size (35±4% versus 51±6%) and improved regional myocardial reserve of the apical LAD region (SES 31±4% versus 6±8%), whereas intravenous application displayed a less pronounced effect (infarct size 44±4%; SES 12±3%). Retroinfusion of an equal amount of neonatal coronary endothelial cells (rat) did not affect infarct size (49±5%) nor regional myocardial reserve (16±7%). The eEPC-dependent effect was detected at 24 hours of reperfusion (infarct size 34±7% versus 58±6%) and was sensitive to Wortmannin coapplication (50±5%).

Conclusion—Our findings show that eEPCs reduce ischemia-reperfusion injury in a preclinical pig model. The rapid effect (as early as 24 hours) indicates a role for enzyme-mediated cardioprotection, which involves, at least in part, the phosphatidylinositol 3-kinase/AKT pathway. (Circulation. 2005;112[suppl I]:I-117–I-122.)

Key Words: angiogenesis ■ endothelium-derived factors ■ ischemia ■ infarction

Attention of myocardial ischemia-reperfusion (I/R) injury is experimentally accomplished (ie, by preservation of endothelial function or supplementation of endothelial derived factors). Vasodilation and enhanced perfusion as well as control of inflammation and coagulation are endothelial functions that contribute to postischemic recovery. Besides the central endothelial mediator NO, growth factors protect the heart against I/R injury.1

Application of endothelial progenitor cells of various origins has been put forward as a novel therapeutic concept for treatment of I/R injury in animal models2 and patients.3–5 It is postulated that endothelial progenitor cells (EPCs) promote formation of new blood vessels in the ischemic area,6 a subacute event that requires at least days to become effective. It is thus possible that neovascularization is not the single benefit of cell-based therapy, but in addition, EPCs, providing a rich source of secreted factors, also contribute at early time points to several cardioprotective processes by controlling inflammation and stimulating cell survival.

In support of this notion, many of the pioneering studies were conducted with xenotypic adult EPCs in immunocompromised hosts (eg, human EPCs in rats), for which application was performed before or immediately after the onset of ischemia.6 This protocol fits the most often reported time course of I/R injury (ie, an immediate challenge by overwhelming inflammation).7 In parallel, angiogenic growth

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factors such as vascular endothelial growth factor, signaling via the phosphatidylinositol 3 (PI3)-kinase–AKT pathway, counteract I/R injury as early as 24 hours after ischemia,5 well before functional neovascularization would occur. To further investigate the hypothesis that EPCs exert acute cardioprotective effects, we used a preclinical pig model of ischemia and reperfusion. As a source of EPCs, we used a clonal mouse embryonic embryonic cell line with endothelial progenitor properties because embryonic cells with a stem cell–like phenotype appear to elude acute or subacute host rejection.10,11 The xenogenic application of embryonic EPCs (eEPCs) might be facilitated by their lack of major histocompatibility complex (MHC) class I surface expression and the inertia of resting host natural killer cells against the eEPCs.10 Moreover, eEPCs of this source are recruited to hypoxic tumors10,12 or ischemic myocardium,12 a process that is mediated by selectin adhesion molecules.

After evaluating various routes of application, we established that pressure-regulated retroinfusion leads to enhanced eEPC recruitment. After this application strategy, we analyzed infarct size and regional recovery of myocardial function 7 days after ischemia (1-hour left anterior descending [LAD] artery occlusion) and reperfusion. To further elucidate the molecular basis underlying the early progenitor cell–mediated cardioprotective mechanisms, we assessed the level of PI3-kinase/AKT signal cascade activation in cardiomyocytes 24 hours after eEPC application in vitro and in vivo.

Methods

Animals

German pigs were purchased from a local farm (Oberschleissheim, Germany). Animal care and all experimental procedures were performed in strict accordance with the German and National Institutes of Health animal legislation guidelines and were approved by the local animal care and use committees.

Reagents

All chemicals were purchased from Sigma. Contrast agent Solutrast (both Santa Cruz Biotechnology),15 myeloperoxidase assay, 14 and TUNEL-positive cells.

Cardiomyocyte Cell Culture

Rat neonatal ventricular cardiomyocytes were prepared as described previously.13 After plating on 6-well plates, hypoxia was induced for 4 hours, followed by reoxygenation for 1 hour. Where indicated, eEPCs were placed in a permeable insert (Bioscop; Becton Dickenson) in the well before hypoxia. The AKT inhibitor SH-5 (AG Scientific) and the PI3-kinase inhibitor Wortmannin (Sigma) were added to the myocyte compartment before hypoxia. At the end of the experiment, Trypan blue was added for 2 minutes, and 5 microscopic fields were photographed and quantified (Zeiss AxioVision).

Pig I/R Model

All pig experiments were conducted in the Institute for Surgical Research at the University of Munich. Pigs (n=6 per experimental group unless otherwise indicated) were anesthetized and instrumented as described previously.13 Briefly, a balloon was placed in the LAD distal to the bifurcation of the first diagonal branch and inflated with 4 atm (0.41 MPa). Correct localization of the coronary occlusion and patency of the first diagonal branch was ensured by injection of contrast agent.

eEPC Application

The infusion of eEPCs (5×10⁶ cells per animal) was done either systemically or into the anterior interventricular vein draining the parenchyma perfused by the LAD. Every regimen started at 55 minutes of ischemia and lasted until 5 minutes of reperfusion. Systemic infusion was performed via the external jugular vein. In retrogradely treated groups (including saline-treated control experiments), we used continuous pressure-regulated retroinfusion of isothermic Tyrode solution (25 mL/min) with or without 5×10⁶ eEPC or endothelial cells (ECs) for 10 minutes. In all groups, the percutaneous transendoluminal coronary angioplasty balloon was deflated at 60 minutes of ischemia, and the onset of reperfusion was documented angiographically.

In a subset of experiments (n=4 per group), reperfusion was maintained for 24 hours. Where indicated, Wortmannin (20 μg/kg) was retroinfused with or without eEPCs (5×10⁶ cells). At this dosage, no difference to control experiments was observed with Wortmannin retroinfusion alone.

At the end of the experiment (24 hours or 7 days after ischemia), sternotomy was performed, and ultrasonic crystals were placed in the noninfarcted and infarcted area at risk (AAR) as well as in theCx perfusion area in a standardized manner. Subendocardial segment shortening (SES) was performed under resting heart rate as well as at 120/min and 150/min atrial pacing (for 1 minute each). After assessing infarct size (TTC viability and Methylene-blue exclusion),14 tissue samples were analyzed for the presence of EFGP–labeled eEPCs by immunocytochemistry as described above. Immunoblotting for AKT-1 and p-AKT (both Santa Cruz Biotechnology),13 myeloperoxidase assay,14 and TUNEL staining14 were performed as described previously. Tissue of 2 slices of each region of the heart (nonischemic, noninfarcted AAR, infarct) was analyzed for 15 fields each for TUNEL–positive cells.

Radioactive Labeling of eEPCs

The ⁹⁹Tc-HMPAO (Ceretec; Amersham Healthcare) kit was reconstituted with 20 mCi (740 MBq) fresh ⁹⁹Tc-eluante in a total volume of 5.0 mL. For stabilization of the complex, 2.0 mL of a cobalt(II) chloride solution (0.1 μg/mL) was added after 2 minutes. Freshly prepared ⁹⁹Tc-HMPAO (2 to 5 mCi; 74 to 185 MBq) was slowly added to the EPC suspension and incubated for 10 to 30 minutes at 37°C, providing a labeling efficiency of 45% to 80% and cell viability of >80%, decreasing to 60% after 3 hours. A cell suspension with ~5×10⁶ cells containing 1 mCi (37 MBq) was used for each experiment.

Statistical Methods

The results are given as mean values±SEM. Statistical analysis was done using 1-way ANOVA. Whenever a significant effect was obtained with ANOVA, we used multiple comparison tests between the groups with the Student-Newman–Keuls procedure (SPSS statistical program). Differences between groups were considered significant for P<0.05.

Results

eEPC Recruitment After Systemic or Regional Application in Pigs

A prerequisite of cell-based therapeutic approaches is an efficient congregation to the desired site. Here, we infused
99Tc-labeled eEPCs systemically or directed them to the ischemic area by selective retroinfusion of a cardiac vein (see Methods). One hour later, we observed a robust recruitment of eEPCs to the ischemic myocardial tissue, an effect further enhanced by retroinfusion (Figure 1A). The ischemic area contained 0.5% of total applied radioactivity for systemic application and 2.7% for retroinfusion. Fucoidin, a selectin antagonist, abrogated the effect of retroinfusion entirely. In addition, the lung sequestered considerable amounts of eEPCs, unlike all other tissues investigated (muscle, liver, spleen, skin). Because hydrophilic 99Tc released from unviable cells is eliminated renally, we also detected substantial radioactivity in the kidney (Figure 1B). After 24 hours, numerous eEPCs were found in the ischemic area (Figure 2; supplemental Figure I, available online at http://circ.ahajournals.org) in smaller and larger microcirculatory vessels, whereas the cell recruitment in the nonischemic area was limited. At day 7, the number of cells decreased by 42% (Figure 2C), with eEPCs found either adjacent to or integrated in the vessel wall (Figure 2B).

### eEPCs Attenuate I/R Injury in Pigs

We examined the impact of eEPCs on infarct size, assessed as the ratio of nonviable myocardium to the AAR. We found that this ratio was reduced 7 days after retroinfusion of eEPCs (39±5% versus 51±5%; Figure 3A). Interestingly, systemic infusion of eEPCs did not suffice to reduce infarct size significantly (44±6%), similar to retroinfusion of mature ECs (49±5%). However, the extension of the infarct (AAR/LV area) did not differ between the different groups (Figure 3B).

We also documented a significant improvement to the SES in the apical LAD region (Figure 3C), an area that most likely is experiencing infarction in our model. Unlike controls, in which 23% of the nonischemic shortening level was present at rest, further declining at 120/min and 150/min atrial pacing, eEPC retroinfusion preserved regional myocardial contraction reserve (at 150/min), an effect not found after systemic eEPC infusion or EC retroinfusion (Figure 3C).

### Mechanisms of eEPC-Mediated Cardioprotection

Because postischemic inflammation plays a detrimental role after myocardial infarction, we analyzed the activity of myeloperoxidase, a characteristic enzyme of polymorphonuclear (PMN), in postischemic heart tissues. Interestingly, at day 7, only eEPC retroinfusion limited the increase in myeloperoxidase activity that is otherwise specifically found...
in the infarct zone (Figure 4). Myeloperoxidase levels of the lung did not differ between controls or eEPC-treated groups (1509±110 and 1562±151 U/g, respectively).

As a next step, we investigated whether eEPCs may protect cardiomyocytes directly during hypoxia/reoxygenation in vitro. Indeed, eEPCs, physically separated by semipermeable inserts, significantly increased survival of neonatal cardiomyocytes after 4 hours of hypoxia and 1 hour of reoxygenation (Figure 5). The cardiomyocyte protection occurred rapidly (after 1 hour of reoxygenation), raising the possibility of post-translational activation of survival signals, such as the PI3-kinase/AKT pathway. Indeed, we found that inactivation of PI3-kinase by Wortmannin or AKT by SH-5 blunted the cardioprotection of eEPCs (Figure 5), even if Wortmannin was applied only onto the cardiomyocytes, whereas selective inhibition of eEPCs with Wortmannin revealed no significant effect. Of note, in this in vitro system lacking immune cells, mature neonatal ECs also increased cardiomyocyte survival.

To test whether the eEPC-mediated cardiomyocyte protection requires the PI3-kinase/AKT pathway in vivo, we retroinfused Wortmannin concomittantly with eEPCs. Tissue obtained after 24 hours of reperfusion indicated an increase in phosphorylated AKT in the infarct region treated with eEPC retroinfusion, an effect blunted by Wortmannin (Figure 6A and 6B). Vice versa, eEPC retroinfusion blocked leukocyte influx and an increase in TUNEL-positive cells. These beneficial effects were abolished in the presence of Wortmannin (Figure 6C and 6D). Importantly, infarct reduction by eEPC retroinfusion at this early time point (24 hours) was inhibited by Wortmannin (Figure 7A), indicating that a signaling mechanism through PI3-kinase/AKT is provided by the eEPCs. The AAR/LV ratio did not differ significantly between the different groups (Figure 7B).

**Discussion**

In the present study, we investigated the fate and function of eEPCs applied to postischemic myocardium in pigs. We
observed an enhanced proportion of recruited eEPCs in the ischemic region after selective, pressure-regulated retroinfusion compared with systemic intravenous application or antegrade intracoronary infusion (Figure 1A). Retroinfusion of a limited number of eEPCs (5 × 10^6 cells), but not systemic infusion, sufficed to exert cardioprotection with respect to infarct size and regional myocardial function of the ischemic area (Figure 3). The progenitor status of the transfused cell population was essential for cardioprotection because neonatal coronary ECs did not have a significant beneficial effect in vivo (Figure 3). Early protection, rather than neovascularization, appeared to improve myocyte survival, involving activation of the PI3-kinase/AKT pathway and a decrease in postischemic inflammation (Figures 5 through 7). Of note, the pigs benefited from xenotransplantation of mouse eEPCs but not rat mature neonatal coronary ECs. Our previous data indicated that murine eEPCs are immunoprivileged in allogeneic settings because of a lack of MHC I expression and resistance to nonactivated natural killer cells.

The initial process of cell recruitment to the postischemic heart has been described for adult progenitor cells, essentially relying on specific adhesion molecules like selectins and β2-integrins, and chemoattractants like stromal cell-derived factor 1. Consistently, myocardial ischemia in the pig model induced eEPC recruitment in the ischemic region, although systemic application was not sufficient to confer cardioprotection, most likely because of the relatively small number of eEPCs. Thus, 5 × 10^6 eEPCs per experiment would translate to ∼2000 cells/mL, a number that is 100-fold lower than the cell concentration we found was needed for cardioprotection after systemic eEPC application in mice. This mass of infused cells might carry various risks related to lung sequestration (Figure 1B).

For eEPCs, homing to tumor tissue has been described, involving ligands for P-selectin and E-selectin expressed on the eEPC surface. Similar to our mouse model, fucoidin, an unspecific selectin antagonist, blocked eEPC recruitment to the postischemic myocardium (Figure 1A). Selectin mediation indicates a striking similarity of eEPC recruitment to the homing of leukocytes, which preferentially occurs at the venular site of the vascular tree, as targeted via retroinfusion. However, regardless of the application, the lung rapidly attracts a substantial number of cells, which might, in turn, re-enter circulation and travel to the ischemic region, as shown previously. In contrast, the substantial amount of tracer found in the kidney rather reflects elimination of 99Tc,
equally released from eEPCs in all groups. No other organ revealed significant amounts of eEPCs at this time point.

Using the retroinfusion approach, we found attenuation of I/R injury 7 days after ischemia (Figure 3). Searching for potential mechanisms, we found an increase of capillary density in the ischemic area (supplemental Figure II). In parallel, eEPCs were also capable of providing rapid protection against myocyte cell death in vitro via humoral mediators (Figure 5), suggesting early, post-translational effectors. One candidate for enhancing myocyte survival,20,21 the PI3-kinase/AKT pathway, was of particular interest because humoral activators such as insulin-like growth factor-1,22 leukemia inhibitory factor,23 and thymosin-β423 are highly expressed in eEPCs (3.3-fold, 4.0-fold, and 7.1-fold the average expression; Affymetrix analysis).12a Using inhibitors like Wortmannin and SH-5 in vitro, we found that PI3-kinase/AKT increased cardiomyocyte survival (Figure 5). In vivo, this survival signal was fully active (Figure 6A) and effective (Figure 7) at 24 hours of reperfusion, decreasing postischemic inflammation24 and cardiomyocyte apoptosis25 (Figure 6C and 6D), both known to contribute to I/R injury.

In summary, we found that eEPCs provide cardioprotection after I/R when healing is enhanced by selective retroinfusion. This early cardioprotective effect can be attributed to eEPC-mediated stimulation of the PI3-kinase/AKT pathway in host cells and attenuation of the inflammatory response and apoptosis. The abundance of cultured eEPCs and their specific cardioprotective features described here might offer a novel therapeutic approach of acute ischemic syndromes of the heart.

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References

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