Therapeutic Potential of Ex Vivo Expanded Endothelial Progenitor Cells for Myocardial Ischemia

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Background—We investigated the therapeutic potential of ex vivo expanded endothelial progenitor cells (EPCs) for myocardial neovascularization.

Methods and Results—Peripheral blood mononuclear cells obtained from healthy human adults were cultured in EPC medium and harvested 7 days later. Myocardial ischemia was induced by ligating the left anterior descending coronary artery in male Hsd:RH-rnu (athymic nude) rats. A total of 10⁶ EPCs labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate were injected intravenously 3 hours after the induction of myocardial ischemia. Seven days later, fluorescence-conjugated Bandeiraea simplicifolia lectin I was administered intravenously, and the rats were immediately killed. Fluorescence microscopy revealed that transplanted EPCs accumulated in the ischemic area and incorporated into foci of myocardial neovascularization. To determine the impact on left ventricular function, 5 rats (EPC group) were injected intravenously with 10⁶ EPCs 3 hours after ischemia; 5 other rats (control group) received culture media. Echocardiography, performed just before and 28 days after ischemia, disclosed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the EPC group than in the control group by day 28. Regional wall motion was better preserved in the EPC group. After euthanization on day 28, necropsy examination disclosed that capillary density was significantly greater in the EPC group than in the control group. Moreover, the extent of left ventricular scarring was significantly less in rats receiving EPCs than in controls. Immunohistochemistry revealed capillaries that were positive for human-specific endothelial cells.

Conclusions—Ex vivo expanded EPCs incorporate into foci of myocardial neovascularization and have a favorable impact on the preservation of left ventricular function. (Circulation. 2001;103:634-637.)

Key Words: endothelium ■ stem cells ■ ischemia ■ regeneration ■ neovascularization

C ollateral circulation attenuates myocardial ischemia in coronary artery disease. Recently, novel approaches that may augment collateral circulation in ischemic heart disease have been tested in preclinical and clinical studies. Gene transfer of angiogenic growth factors, for example, reportedly attenuates tissue ischemia through stimulating angiogenesis at sites of neovascularization.1–3 Circulating CD34 antigen–positive endothelial progenitor cells (EPCs), recently isolated from the peripheral blood of adult species,4–6 represent an alternative approach. Indeed, there is now evidence to suggest that part of the favorable impact of angiogenic growth factor therapy involves the mobilization of bone marrow–derived EPCs.7–9 Accordingly, we transplanted ex vivo expanded EPCs in a model of rat myocardial infarction and investigated the incorporation of EPCs into sites of neovascularization, physiological indices of left ventricular (LV) function, and histological findings 4 weeks after EPC transplantation.

Methods

Cell Culture

Total human peripheral blood mononuclear cells were isolated from healthy volunteers by density-gradient centrifugation, and they were cultured in endothelial cell (EC) basal medium-2 (EBM-2, Clonetics) for 7 days.4 The vast majority of these ex vivo expanded cells are of endothelial lineage and, as such, they constitute the ex vivo expanded EPC-enriched fraction.10

Animal Model of Myocardial Ischemia

All procedures were performed in accordance with St Elizabeth’s Institutional Animal Care and Use Committee. Male athymic nude rats (Hsd:RH-rnu rats, Harlan Sprague Dawley, Indianapolis, Ind) aged 6 to 8 weeks were anesthetized with sodium pentobarbital (50 mg/kg IP). Myocardial ischemia was induced by ligating the left anterior descending (LAD) coronary artery.11 Immediately before euthanization, rats were injected with an overdose of pentobarbital.
Transplantation of Ex Vivo Expanded EPCs

Three hours after inducing myocardial ischemia, rats received intravenous injections of $10^6$ culture-expanded human EPCs resuspended with 200 µL of EBM-2 ($n=5$) or the same volume of EBM-2 without cells ($n=5$). These rats were killed 28 days after myocardial ischemia.

To evaluate the incorporation of EPCs into ischemic myocardium, cells were labeled with fluorescent carbocyanine $1,1'-9$-dioctadecyl-1-to-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes). 10 Athymic nude rats ($n=2$) received DiI-labeled EPCs ($10^6$ cells) by intravenous injection 3 hours after myocardial ischemia. This subgroup of rats was killed on day 7 after myocardial ischemia. Thirty minutes before euthanization, 500 µg of Bandeiraea simplicifolia lectin I (Vector Laboratories), the murine-specific EC marker, was administered intravenously.

Histological Assessment of Transplanted Animals

At necropsy, hearts were sliced in a bread-loaf fashion into 8 transverse sections from apex to base and fixed with 4% paraformaldehyde. In 2 rats injected with DiI-labeled EPCs, fixed tissues were embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen for fluorescence microscopy. In the remaining 10 rats, paraffin-embedded tissues were used to measure the average ratio of fibrosis area to LV area. Immunohistochemical staining was performed using antibodies prepared against the murine-specific EC marker isolectin B4 (Vector Laboratories), as well as antibodies against the human-specific EC markers CD31 (DAKO) and ulex europaeus lectin type 1 (Vector Laboratories).10 Capillary density was evaluated morphometrically by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium, subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4. All morphometric studies were performed by 2 examiners (H.M., H.I.) who were blinded to treatment.

Physiological Assessment of LV Function

Transthoracic echocardiography (SONOS 5500, Hewlett Packard) was performed just before (baseline) and 28 days after myocardial ischemia. LV diastolic (LVDd) and systolic (LVDs) dimensions and fractional shortening were measured at the midpapillary muscle level. Regional wall motion score was examined per published criteria.12 All procedures and analyses were performed by an experienced researcher (H.-C.G.) who was blinded to treatment.

Statistical Analysis

All values were expressed as mean±SE. Unpaired $t$ tests were performed to compare values between treated and control rats. Paired $t$ tests were used to compare echocardiographic parameters between baseline and day 28. $P<0.05$ was considered statistically significant.

Results

Ex Vivo Expanded EPCs Incorporate into Foci of Myocardial Neovascularization

DiI-labeled human EPCs were found principally in the ischemic area, contributing to a vascular network that included rat ECs (Figure 1a through 1c). In contrast, exogenous human EPCs were rarely distributed to nonischemic myocardium outside the risk area defined by the LAD occlusion (Figure 1a). Thus, ex vivo expanded EPCs, administered
intravenously, incorporated into foci of neovascularization in segments of that portion of myocardium subserved by the occluded LAD.

**Exogenous Human EPCs Differentiate to Mature ECs in the Rat Heart**

Human CD31-positive and ulex europaeus lectin type 1–positive mature ECs were identified in the vasculature of that portion of myocardium subserved by the occluded LAD (Figure 1d and 1e). Thus, ex vivo expanded and intravenously administered human EPCs differentiated to mature ECs in rat ischemic myocardium.

**Morphometric Evaluation of Capillary Density and Infarct Size**

Capillary density 28 days after the development of myocardial ischemia was significantly greater in rats receiving human EPCs than in control rats ($290.1 \pm 21.5$ versus $191.1 \pm 17.8$/mm$^2$, $P=0.0009$; Figure 2a through 2c). The ratio of percent fibrosis area/entire LV area was significantly lower in rats receiving EPCs than in rats in the control group ($8.9 \pm 0.9$ versus $17.8 \pm 1.4\%$, $P=0.0007$; Figure 2d through 2f).

**Ex Vivo Expanded EPCs Preserve LV Function After Myocardial Ischemia**

LVDD, LVDS, fractional shortening, and regional wall motion scores at baseline were not significantly different between rats receiving EPCs versus control rats. In rats receiving EPCs and in control rats, LVDD and LVDS increased significantly during the 28 days after myocardial ischemia ($P<0.01$ in both groups), whereas fractional shortening significantly decreased and regional wall motion scores significantly worsened ($P<0.01$ in both groups).

By day 28, however, LVDD and LVDS were significantly lower in rats receiving EPCs than in control rats (LVDD: $0.87 \pm 0.03$ versus $0.93 \pm 0.01$ cm, $P=0.032$; LVDS: $0.68 \pm 0.03$ versus $0.79 \pm 0.02$ cm, $P=0.005$). Fractional shortening on day 28 was significantly higher in rats receiving EPCs than in controls ($21.3 \pm 0.6\%$ versus $15.3 \pm 2.2\%$, $P=0.0004$). Regional wall motion was also better preserved in rats receiving EPCs than in those in the control group ($25.3 \pm 0.8$ versus $30.6 \pm 1.0$, $P=0.0021$; Figure 2g).

Thus, echocardiographic examination performed before and after myocardial ischemia suggests that the intravenous.
administration of ex vivo expanded EPCs had a favorable impact on the preservation of LV function in rats with myocardial ischemia.

**Discussion**

In the present study, fluorescence microscopy was used to document the incorporation of ex vivo expanded and intravenously administered EPCs into foci of myocardial neovascularization in ischemic hearts. Exogenous EPCs were rarely observed in nonischemic areas. Immunohistochemical staining also revealed that transplanted EPCs (recognized by staining with an antibody specific for human as opposed to rat ECs) complete differentiation into mature ECs.

The recent demonstration that the bone marrow–derived EC precursors present in peripheral blood may home to and incorporate into sites of neovascularization has prompted an investigation of cell-based approaches. In certain cases, this has involved the use of total bone marrow cells that were transplanted intramyocardially and were shown to enhance neovascularization at sites of myocardial injury. These approaches not only merit but require further investigation. Parenteral harvest and administration of cultured EPCs is clearly less invasive than bone marrow aspiration with direct myocardial transplantation. However, the safety of ex vivo culture expansion of the cells for clinical applications remains to be demonstrated. Also implicit in the use of the total bone marrow mononuclear cell populations is the potential for differentiation into nonendothelial lineage cells, including osteoblasts, chondroblasts, and fibroblasts. To avoid this potential problem, we transplanted ex vivo cultured EPCs that were expanded and enriched in a prespecified culture system.

The strategy of therapeutic neovascularization used here contrasts with those approaches in which genes, growth factors, or cells are delivered by local administration to optimize activity in the target region. Previous studies performed in our laboratory indicated that intravenously injected EPCs may specifically home to the sites of nascent neovascularization and differentiate into mature ECs. Indeed, this finding was the basis for studies that established proof of the concept that exogenously administered EPCs could accelerate revascularization and promote limb salvage in mice with hindlimb ischemia. The current study extends these previous findings by documenting that exogenously administered EPCs home to foci of myocardial neovascularization, augment vascularity, and exert a favorable impact on the preservation of LV function.

Capillary density, a direct anatomic reflection of neovascularization, was significantly greater in rats transplanted with EPCs than in control rats in this study. Enhanced neovascularization after the administration of ex vivo expanded EPCs led to a reduction in LV dilatation and a preservation of contractile performance after myocardial ischemia. The precise mechanism of these favorable effects of EPCs on cardiac function requires further elucidation.

Rescue of hibernating myocardium, recently documented in human subjects after therapeutic angiogenesis, may have contributed to this improved physiology. The statistically significant reduction in the extent of myocardial fibrosis may also be a factor; previous clinical and experimental studies demonstrated that late reperfusion of the infarct vascular bed attenuates left ventricular remodeling, including infarct expansion. EPC transplantation may thus inhibit LV remodeling after myocardial infarction via an improvement in myocardial blood flow.

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