Transfer of endothelial progenitor cells improves myocardial performance in rats with dilated cardiomyopathy induced following experimental myocarditis

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Abstract

Endothelial progenitor cells (EPCs) home to sites of tissue injury and differentiate into mature endothelial cells. Their transfer feasibility has been proven in models of hindlimb ischemia and myocardial infarction. We investigated, the effect of delivery of spleen-derived EPC in a rat model of inflammatory-mediated myocardial damage. Male Lewis rats (N = 25) were immunized against myosin. Healthy donor Lewis rats were sacrificed, their spleens harvested, separated on Ficoll gradient centrifugation, and grown on fibronectin coated plates with endothelial cell medium for 5 days. Ten days after myosin immunization, spleen cell derived EPC were collected, and labeled 2 × 10⁷ cells per rat were injected into the femoral vein of diseased rats. Cell transplantation was repeated twice, 2 and 4 weeks after initial cell transfer. Rats with inflammatory-mediated cardiomyopathy exhibited a significant mobilization of EPC from the bone marrow to the periphery and their ability to adhere to fibronectin, mature endothelial cells and cultured cardiomyocytes was significantly reduced when compared to healthy rats. Transfer of EPC resulted in a functional improvement in cardiac performance evident by higher fractional shortening by echocardiography (a 15% increase). Histological studies exhibited reduced scar tissue and thickened ventricular walls in rats receiving EPC as compared with untreated animals. EPC transfer is effective in attenuating myocardial damage in a model of non-ischemic dilated cardiomyopathy.

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1. Introduction

Dilated cardiomyopathy (DCM) is associated with significant mortality in humans and is most frequently secondary to ischemic heart disease [1]. However, some patients develop DCM as a result of prior autoimmune myocarditis [2]. Supporting this contention is the rat model of myosin-induced myocarditis in which a robust inflammatory response in the myocardium is followed by extensive scar replacement and consequent DCM [3,4].

Endothelial progenitor cells (EPC) are present in the bone marrow and the peripheral blood and exhibit phenotypic markers of mature endothelial cells [5,6]. Although no consensus has yet been accomplished as to their precise characterization, EPC can generally be defined as cells with the capacity to differentiate into mature endothelial cells when grown under appropriate conditions in vitro. Considerable interest has centered on EPC in recent years for two reasons. The first is that their circulating numbers have been shown to be altered in several vascular disorders [7–12] and they appear to respond to a set of well-characterized stimuli such as growth factors and cytokines. Patients with atherosclerotic risk factors appear to have reduced number of circulating EPC [7,8], whereas patients with myocardial infarction and active ischemic heart disease [11,12] exhibit a transient increase in their number. The second line of studies emphasizing the importance of EPC, report on the feasibility of cell transfer as means of facilitating angiogenesis thereby attenuating organ damage induced by ischemia. Thus, it has been shown that transfer of EPC ameliorates myocardial remodeling following coronary artery ligation and increases vascular perfusion in the hindlimb.
ischemia model [13,14]. The encouraging results of these experiments prompted initial studies in humans that appear successful in attenuating remodeling following acute myocardial function [15–17].

In the current study, we hypothesized that a dysregulated EPC pool is present in a model of DCM induced after autoimmune myocarditis and could contribute to myocardial dysfunction. The latter assumption was tested by administration of ex vivo expanded EPC and testing their effect on myocardial function.

2. Methods

2.1. Induction of experimental autoimmune myocarditis (EAM) and DCM

Cardiac porcine myosin (Sigma) was dissolved in a solution of potassium chloride, 0.3 mol/l, and phosphate-buffered saline (PBS), 0.2 mol/l, at a concentration of 10.0 mg/ml [18]. Male Lewis rats were immunized with 1.0 mg of cardiac myosin in an equal volume of Freund’s complete adjuvant containing 10.0 mg/ml of heat-killed Mycobacterium tuberculosis. The same protocol was repeated 1 week later. Rats were studied for EPC number and function, 6 weeks after a booster injection.

2.2. EPC transfer protocol

Spleen cell derived EPCs were collected from healthy, male Lewis rats and 2 × 10^7 1,1′-dioctadecyl-3,3,3′,3′-tetracyanomethyindocarbocyanine (DiI, Molecular Probes) labeled cells per rat or PBS were injected intravenously into femoral vein of two groups of recipient rats after induction of myocarditis, 12 and 13 in number, respectively. Transplantation was repeated for 2 consecutive weeks. Functional myocardial assessment by echocardiography, and sacrifice of the rats was carried out at the time when scar formation is evident, 6 weeks after booster immunization.

2.3. Preparation of EPC for intravenous transfusion

Spleens from Lewis rats were explanted and mechanically minced, and mononuclear cells (MNC) were isolated using a Ficoll gradient (Sigma). MNCs were stained with the fluorescent dye DiI for incorporation in the cell membrane according to the manufacturer’s instructions. Labeled cells were counted, and 2 × 10^7 cells were resuspended in 500 µl PBS for intravenous injection.

2.4. Assessment of colony forming units and EPC phenotyping

Spleen-derived MNCs or bone marrow derived cells were seeded on 24-well plates coated with fibronectin in 0.5 ml endothelial basal medium (CellSystems) supplemented with 1 µg/ml hydrocortisone, 3 µg/ml bovine brain extract, 30 µg/ml gentamicin, 50 µg/ml amphotericin B, 10 µg/ml human endothelial growth factor, and 20% fetal calf serum (FCS). After 5 days in culture, cells were washed with PBS and resuspended. Adherent cells were incubated with 2.4 µg/ml DiI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL, Molecular Probes) for 1 h. For CFU cultures, spleen, and BM derived MNC were cultured for 7 days in endothelial basal medium with change of medium every third day. The number of CFU was counted by two independent observers.

For further confirmation of endothelial phenotype of CFU, the following antibodies were used for immunofluorescent and flow-cytometric analysis: Rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-flk-1 (A-3) and goat polyclonal anti-CD31 (PECAM-1, M-20); all from Santa Cruz as described previously [19].

2.5. Functional assessment of EPC (adherence assays) in DCM and healthy rats

EPCs (day 7) from DCM and healthy Lewis rat were washed with PBS and gently detached with 0.5 mmol/l EDTA in PBS. After centrifugation and resuspension in basal complete medium supplemented with 5% FCS, identical cell numbers were placed onto culture dishes in which immobilized fibronectin, rat cardiomyocytes or bovine aortic endothelial cells (BAEC) were grown to near complete confluence. DiI-labeled EPC were incubated for 30 min at 37 °C. Adherent cells were counted by two blinded investigators.

2.6. Assessment of myocardial performance by echocardiography

Echocardiographic studies were performed 4 weeks after surgery using a 7.5-MHz phased-array transducer (Acuson, USA). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt.) as a supplement to maintain mild anesthesia. M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular end-diastolic diameter (EDD) and end-systolic diameter (ESD), and LV fractional shortening (FS) were measured from three consecutive cardiac cycles by the American Society for Echocardiography leading-edge method. FS was calculated by the formula:

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\text{LVFS} (\%) = \frac{(E_D - E_S)}{E_D} \times 100.
\]

2.7. Enzyme linked immunosorbent assay (ELISA) for anti-myosin antibodies

Sera obtained from all rats at sacrificed was studied for anti-myosin IgG antibody levels by ELISA as previously described [18].

2.8. Assessment of vascular density, fibrous area and LV thickness

Assessment of vascular density was performed by staining slides from all rats with anti-CD31 antibody (see above).
Positive CD31 cells were counted in 30 fields of each section at ×400 magnification. Scar formation was studied after staining with Masson’s trichrome by performing morphological assessment of the positively stained area. Area of fibrosis was measured by image processing in ×40 magnification (image-pro-plus). Maximal left wall thickness was measured in three different levels for each case and an average value was calculated for each of the rats from all study groups.

2.9. Statistical analysis

Comparisons between healthy and DCM rats and between EPC and PBS transfused animals were performed by the Student’s t-test. Level of significance was set at 0.05.

3. Results

To test the hypothesis that EPC pool is deranged in rats with DCM, we assessed the number and function of EPC in Lewis rats with DCM as compared to healthy animals. Rats with DCM had significantly increased numbers of CFU (30.7 ± 4.5 per well) recovered from the spleen, as compared with control animals 10.1 ± 1.4 per well, \( P < 0.01 \) (Fig. 1A).

As opposed to the spleen pool, CFU exhibiting endothelial phenotype from bone marrow of DCM rats were significantly reduced as compared to healthy age matched animals (Fig. 1B).

We validated the endothelial characteristics of these colonies by demonstrating their staining for Tie-2, CD31 and VEGF-R2. Next, we hypothesized that the functional properties of EPC from DCM rats are compromised. To prove this hypothesis, several sets of adhesion studies were done. We assumed that a functional EPC is one that is capable of adhering to matrix, mature endothelial cell and intact cardiomyocytes, and tested for these adhesion properties, in vitro. The ability of EPC obtained from the spleen of DCM rats to adhere to immobilized fibronectin was reduced as compared with healthy rats (13.7 ± 7.7 cells per high power field [HPF] vs. 25.1 ± 9.8 cells per HPF, respectively; \( P < 0.001 \)). A similar effect was evident when the adhesive properties of spleen (Fig. 2A) and bone marrow (Fig. 2B) derived EPC from DCM rats to adhere to BAEC were compared with those of healthy rats.

Spleen cell derived EPC from DCM rats adhered less potently to neonatal rat cardiomyocytes in comparison with EPC from healthy rats (33.4 ± 7.2 cells per HPF vs. 53.0 ± 15.5 cells per HPF, respectively; \( P < 0.01 \) ) (Fig. 2C).

In order to confirm the endothelial nature of the CFU and cells employed for adhesion, two sets of validation studies were carried out. MNC grown for 5 days on fibronectin coated plates in the presence of endothelial cell medium were stained for the coexpression of Dil-AcLDL and BS1 and double stained cells were always more than 90% of the adherent population (Fig. 3A). CFU were stained separately for three markers of endothelial cells including CD31, Tie-2 and VEGF receptor 2 (Fig. 3B).

Having established that spleen cell derived EPC from DCM rats are increased in numbers and exhibit compromised functional properties, we speculated that these derangements could contribute to myocardial dysfunction. EPC obtained by culturing Ficoll-eluted splenocytes in endothelial cell medium on plates coated with fibronectin, were injected intravenously twice to rats following myosin immunization. Phenotypic characterization of the transferred cells was performed confirming that ~90% of the cells exhibited uptake of Dil-acLDL and the lectin BS-1 (Fig. 3A).

We confirmed homing of the transferred EPC to the diseased myocardium, by demonstrating the presence of Dil-labeled cells in the hearts of the rats (data not shown). Myocardial performance of DCM rats transfused with EPC assessed by echocardiography was improved as compared with non-treated DCM rats. This was evident by the improved FS (approximately 15% increase) in EPC transferred rats (Fig. 4).

Next, we investigated whether functional improvement in EPC transfused rats was also associated with histopathological evidence of attenuated damage. By H&E staining, we
observed that myocardial thickness was significantly reduced in PBS- as compared with treated rats (2.4 ± 0.4 mm vs. 3.7 ± 0.8 mm, respectively, \(P < 0.05\)) (Fig. 5A). We have also found that scar formation as assessed by Masson’s trichrome staining was significantly attenuated in EPC treated rats (positively stained area of 16 ± 2.9%) as compared with non-treated animals (26 ± 9.1%; \(P < 0.05\), providing an explanation for the improved performance observed in the echocardiographic studies (Fig. 5B).

Aiming to determine whether functional improvement conferred by EPC delivery, was mediated by enhanced vascularization, we evaluated vascular density by staining with anti-CD31 antibodies. Vascular density did not differ significantly between DCM rats treated with EPC (37.3 ± 7.6 CD31 cells per field) and PBS treated (42.6 ± 10.9 CD31 cells per field) (Fig. 5C). To rule out that differences in functional myocardial performance was a result of attenuated systemic autoimmunity to myosin, we employed ELISA for assessment of IgG anti-myosin antibodies and found similar levels between EPC and PBS treated rats (Fig. 5D).

4. Discussion

The principal finding of the current study is that transfer of spleen cell derived EPCs partially attenuated myocardial damage induced by experimental myocarditis. The beneficial effects were evident both by functional assays employing transthoracic echocardiography and also by assessing left ventricular wall thickness by histopathology. These findings were associated with a reduction of fibrous tissue content in the hearts of EPC treated rats, an observation that is consistent with the improved functional performance noted by echocardiographic studies.

There is now growing number of reports that support the role of spleen cell and bone marrow derived EPCs in recovering myocardial function in experimental disorders associated with ischemia. Indeed, EPC have been shown to improve blood flow in the hindlimb ischemia model as well as in models of myocardial infarction induced by ligation of the left anterior descending artery in rats [13,14]. EPC transfusion studies have also been successful in models in which endothelial denudation was performed [20] and are in line with the contention that these cells are capable of promoting angiogenesis and vasculogenesis. These experimental studies were soon followed by small scale, yet promising clinical trials in humans, essentially proving that cell transfer is capable of accelerating myocardial recovery after myocardial infarction [15–17].

Herein, we demonstrated for the first time, that the benefit brought by EPC transfer extends not only to experimental models induced by tissue ischemia, but also to DCM induced by experimental myocarditis. In this model, myocardial dysfunction results from replacement of cardiomyocytes by a fibrous scar formed by migrating fibroblasts that follow mas-
sive infiltration of antigen presenting cells as well as autoim-
mune lymphocytes [21].

Several mechanisms have been proposed to account for the beneficial effects observed in EPC transfer studies [5,6]. Whereas intuitively, transformation to resident mature endo-
thelial cells appears most important, other mechanisms are perhaps of at least equal significance. Thus, it has been sug-
gested that secretion of angiogenic cytokines produced by EPC could act by directly improving myocardial performance and vascular repair and growth. We have attempted to

explore several mechanisms by which the beneficial effects of EPC transfer were mediated. Initially, we compared rats with DCM to healthy, age matched control animals. We found that the numbers of spleen cell derived EPC in rats with DCM was significantly increased as compared with healthy ani-
mals whereas bone marrow derived EPC were reduced in the former in comparison with the latter group. We have thus

hypothesized that DCM is associated with secretion of

humoral factors that attempt repair of the damaged myocardium by mobilizing EPC from the bone marrow to the periph-
eral pool. Next, we studied the functional properties of EPC from DCM and healthy rats. To assume a functional pheno-
type, EPC should efficiently attach to matrix, endothelial cells and neighboring cardiomyocytes. We have thus tested in vitro adherence and found that EPC from rats with DCM were signi-

fically compromised in their ability to bind immobilized

Fig. 3. Characterizations of spleen cell EPC. Spleen cell derived MNC were
grown for 5 days on fibronectin coated plates in the presence of endothelial
cell medium and co-stained for anti-BS1 and for Dil-acetylated LDL. Cells
double positive for both markers are regarded as EPC and represent more
that 90% of the total population. B: CFU were stained for CD31, Tie2 and
VEGF-R2 (shown).

Fig. 4. Transfer of EPC improves cardiac performance in rats with DCM. Lewis rats were induced to develop DCM by immunization with myosin and a boost injection as described in methods. EPC from healthy rats or PBS were intravenously infused twice and after 6 weeks, functional assessment of FS was performed by echocardiography (A). Representative echo studies from EPC (B) and PBS (C) infused rats.
fibronectin, cultured endothelial cells and cardiomyocytes as compared with progenitor cells from healthy rats. To prove that the ‘dysfunctional state’ of EPC from the DCM rats is contributory to myocardial damage, we have made the transfusion that essentially reinforced the importance of correcting the quantitative and qualitative defects in EPC.

The presence of the DiI-labeled transfused cells was confirmed in the hearts of the recipient rats, although this finding cannot clarify the mechanism of action. Next, we studied the effects of EPC transfer on vascular density and found no difference between both groups of rats, suggesting that enhancement of the capillary network was not necessarily a major contributor to the beneficial effect.

In conclusion, we show here for the first time, that transfer of EPC is capable of improving myocardial performance in rats with DCM induced by experimental myocarditis. If this finding is further confirmed, clinical studies employing cell therapy may extent to include patients with myocardial dysfunction that does not result from ischemic heart disease.

References


Fig. 5. Histological and serological analyses in PBS and EPC transferred rats. At sacrifice, heart removed from EPC and PBS treated rats were sectioned and stained with H&E for assessment of posterior LV thickness. B. Masson’s trichrome staining was performed to evaluate fibrosis area/total LV area. C. To determine whether vascular area was different between EPC and PBS treated groups, sections were stained with anti-CD31 antibody (methods) and positive cells per field were determined by two blinded expert pathologists. D. To exclude the possibility that EPC transfer influenced myosin specific autoimmune, ELISA was performed in the sera of both treatment groups. * P < 0.01.


