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Imaging Survival and Function of Transplanted Cardiac Resident Stem Cells

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Objectives The goal of this study is to characterize resident cardiac stem cells (CSCs) and investigate their therapeutic efficacy in myocardial infarction by molecular imaging methods.

Background CSCs have been isolated and characterized in vitro. These cells offer a provocative method to regenerate the damaged myocardium. However, the survival kinetics and function of transplanted CSCs have not been fully elucidated.

Methods CSCs were isolated from L2G85 transgenic mice (FVB strain background) that constitutively express both firefly luciferase and enhanced green fluorescence protein reporter gene. CSCs were characterized in vitro and transplanted in vivo into murine infarction models. Multimodality noninvasive imaging techniques were used to assess CSC survival and therapeutic efficacy for restoration of cardiac function.

Results CSCs can be isolated from L2G85 mice, and fluorescence-activated cell sorting analysis showed expression of resident CSC markers (Sca-1, c-Kit) and mesenchymal stem cell markers (CD90, CD106). Afterwards, 5×10^5 CSCs ($n = 30$) or phosphate-buffered saline control ($n = 15$) was injected into the hearts of syngeneic FVB mice undergoing left anterior descending artery ligation. Bioluminescence imaging showed poor donor cell survival by week 8. Echocardiogram, invasive hemodynamic pressure-volume analysis, positron emission tomography imaging with fluorine-18-fluorodeoxyglucose, and cardiac magnetic resonance imaging demonstrated no significant difference in cardiac contractility and viability between the CSC and control group. Finally, postmortem analysis confirmed transplanted CSCs integrated with host cardiomyocytes by immunohistology.

Conclusions In a mouse myocardial infarction model, Sca-1–positive CSCs provide no long-term engraftment and benefit to cardiac function as determined by multimodality imaging. (J Am Coll Cardiol 2009;53:1229–40) © 2009 by the American College of Cardiology Foundation

Recent years have brought stem cell therapy for heart disease to the forefront. Several clinical trials have shown beneficial effects of stem cell transplantation to improve cardiac function after myocardial infarction (MI) (1–3). However, the mechanism(s) behind this benefit is not clearly understood. Although numerous studies have suggested the cardioprotective benefits from various stem cell types, they also carry limitations including the potential of tumorigenicity with embryonic stem cells (4), arrhythmogenicity with skeletal myoblasts (5), as well as existing controversies surrounding transdifferentiation of bone marrow-derived stem cells (6,7).

The identification of undifferentiated cells with stem cell-like properties derived from the myocardium has renewed the excitement in this field. A number of recent studies have confirmed the presence of cells bearing stem cell markers in the myocardium, which were successfully terminally differentiated into cell lineages that can benefit myocardial function (8–12). A recent study even used resident cardiac stem cells (CSCs) derived from human myocardial biopsy specimens and injected them into mice, showing engraftment and improved myocardial function (12).

We have previously described the L2G85 transgenic mice, which constitutively express both the firefly luciferase (Fluc) and enhanced green fluorescence protein (eGFP) genes (13,14). Cells derived from these mice provide the

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Abbreviations and Acronyms

BLI = bioluminescence imaging
BM-MSC = bone marrow-derived mesenchymal stem cell
CSC = cardiac stem cell(s)
DAPI = 4′6-diamidino-2-phenylindole
DiI-ac-LDL = DiI acetylated low-density lipoprotein
EF = ejection fraction
eGFP = enhanced green fluorescence protein
FBS = fetal bovine serum
Fluc = firefly luciferase
FS = fractional shortening
%ID/g = injected dose per gram of heart
LAD = left anterior descending artery
LVEDd = left ventricular end-diastolic diameter
LVESd = left ventricular end-systolic diameter
MI = myocardial infarction
MRI = magnetic resonance imaging
PBS = phosphate-buffered saline
PET = positron emission tomography
PV = pressure-volume
ROI = region of interest
RT-PCR = reverse transcription-polymerase chain reaction
[^{18}F]-FDG = fluorine-18-fluorodeoxyglucose

advantage of being "trackable" over time. Using the previously described methods for CSC isolation and proliferation (10), we now provide in detail the survival kinetics of transplanted CSCs in infarcted murine hearts. In contrast to previous studies (9,10,12,15,16), our results suggest that these CSCs do not constitute long-term improvement in myocardial function. We also show definitive evidence via bioluminescence imaging (BLI) that the majority of these cells are not viable by 8 weeks post-transplantation.

Methods

Isolation and culture of CSCs. Animal protocols were approved by the Stanford University Animal Care and Use Committee. The L2G85 transgenic mice of FVB background with beta-actin promoter driving Fluc-eGFP have been described previously (14,17). Normal adult female FVB mice were used as recipient controls. CSCs were isolated from 6- to 12-week-old L2G85 mice as described with some modifications (10,11). In brief, myocardial tissue was cut into a 1- to 2-mm piece, washed with Hanks' balanced salt solution (Invitrogen, Carlsbad, California), and incubated with 0.1% collagenase II for 30 min at 37°C with frequent shaking. Cells were then filtered through 100-μm mesh. The cells obtained were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 0.1 mmol/l nonessential amino acids, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mmol/l glutamine, and 0.1 mmol/l beta-mercaptoethanol. After 2 to 3 weeks, a population of phase-bright cells appeared over the adhered fibroblast-like cells. These phase-bright cells were collected by 2 washes with phosphate-buffered saline (PBS), and 1 wash with cell dissolution buffer (Gibco, Grand Island, New York) at room temperature under microscopic monitoring, and subcultured in poly-lysine-coated plates (BD Pharmingen, San Jose, California) with the same medium (Online Videos 1 and 2).

Immunofluorescence staining of cultured CSCs. The cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with antibodies to Sca-1 (BD Pharmingen) in situ. Briefly, cells were incubated with rat antimouse Sca-1 for 1 h at room temperature and incubated with goat antirat Alexa Fluor 594 (Invitrogen). 4′6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining.

Flow cytometry analysis. Fluorescence-activated cell sorting (FACS) analysis of the bright cells and the cells subcultured on poly-lysine-coated plates were carried out, and the third passage of bone marrow-derived mesenchymal stem cells (BM-MSCs) from L2G85 mice was used as control cells. The isolation and culturing techniques of BM-MSCs were similar to a previous report (18). Antibodies used in this study were phycoerythrin-conjugated anti-CD34, CD29, CD90, CD44, CD54, Flk1, Sca-1, allophycocyanin-conjugated anti-CD31 and c-Kit, allophycocyanin-conjugated antirat IgG2a, and rat antimouse CD62E, CD62P, Tie-2, CD144 (all from BD Pharmingen). The stained cells were analyzed using BD LSR (BD Pharmingen). Dead cells stained by propidium-iodide were excluded from the analysis. Isotype-identical antibodies served as controls (BD Pharmingen). FlowJo software (Tree Star Inc., Ashland, Oregon) was used for followed data analysis.

In vitro differentiation of CSC. For cardiac and smooth muscle differentiation, CSCs were cultured in poly-lysine-coated plates in differentiation medium containing 35% Iscove's modified Dulbecco's medium with 10% FBS/65% Dulbecco's modified Eagle medium-Ham F-12 mix containing 2% B27, 0.1 mmol/l 2-mercaptoethanol, 10 ng/ml epidermal growth factor (R&D Systems Inc., Minneapolis, Minnesota), 20 ng/ml basic fibroblast growth factor (R&D Systems Inc.), 40 nmol/l cardiotrophin-1 (R&D Systems Inc.), 40 nmol/l thrombin (Sigma-Aldrich, St. Louis, Missouri), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mmol/l glutamine (10). After 1 week, the cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with antibodies specific to cardiac troponin T, myocyte enhancer factor 2C, connexin 43, and α-smooth muscle actin (all from Santa Cruz Biotech, Santa Cruz, California) in situ. Briefly, cells were incubated with first antibody for 1 h at room temperature and incubated with goat antirabbit or donkey antimouse Alexa Fluor 594. DAPI was used for nuclear counterstaining. For endothelial differentiation, the phase-bright cells were cultured on fibronectin-coated plates with EGM-2 (Cambrex, Walkersville, Maryland) with an extra 20 ng/ml of vascular endothelial growth factor. DiI acetylated low-density lipoprotein (DiI-ac-LDL) uptake assay and Matrigel assay were used to confirm endothelial cell phenotype differentiation. For DiI-ac-LDL uptake assay, cells were incubated with 10 μg/ml of DiI-ac-LDL (Molecular Probes, Eugene, Oregon) at 37°C for 6 h. After washing with PBS twice, the slides were fixed and detected with fluorescence microscopy as described (19,20). The formation of endothelial tubes was assessed by seeding cells in 24-well plates coated with