

Molecular Imaging of Cardiac Cell Transplantation in Living Animals Using Optical Bioluminescence and Positron Emission Tomography

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Background—The current method of analyzing myocardial cell transplantation relies on postmortem histology. We sought to demonstrate the feasibility of monitoring transplanted cell survival in living animals using molecular imaging techniques.

Methods and Results—For optical bioluminescence charged-coupled device imaging, rats (n=20) underwent intramyocardial injection of embryonic rat H9c2 cardiomyoblasts (3×10^6 to 5×10^5) expressing firefly luciferase (Fluc) reporter gene. Cardiac bioluminescence signals were present for more than 2 weeks with 3×10^6 cells: day 1 ($627\,000 \pm 15\%$), day 2 ($346\,100 \pm 21\%$), day 4 ($112\,800 \pm 20\%$), day 8 ($78\,860 \pm 24\%$), day 12 ($67\,780 \pm 12\%$), and day 16 ($62\,200 \pm 5\%$ $p \cdot s^{-1} \cdot cm^{2-1} \cdot sr^{-1}$). For micro-positron emission tomography imaging, rats (n=20) received cardiomyoblasts (3×10^6) expressing mutant herpes simplex type 1 thymidine kinase (HSV1-sr39tk) reporter gene. Detailed tomography of transplanted cells is shown by 9-(4-[^{18}F]-fluoro-3hydroxymethylbutyl)guanine ([^{18}F]-FHBG) reporter probe and nitrogen-13 ammonia ([^{13}N]-NH₃) perfusion images. Within the transplanted region, there was a 4.48 ± 0.71 -fold increase of in vivo [^{18}F]-FHBG activity and a 4.01 ± 0.51 -fold increase of ex vivo gamma counting compared with control animals. Finally, the in vivo images of cell survival were confirmed by ex vivo autoradiography, histology, immunohistochemistry, and reporter protein assays.

Conclusions—The location(s), magnitude, and survival duration of embryonic cardiomyoblasts were monitored noninvasively. With further development, molecular imaging studies should add critical insights into cardiac cell transplantation biology. (*Circulation*. 2003;108:1302-1305.)

Key Words: transplantation ■ heart failure ■ genes ■ imaging ■ nuclear medicine

Ischemic heart disease accounts for the majority of all cardiovascular deaths. Despite advances in medical therapies, some patients develop refractory symptoms. Over the past decade, growing evidence from animal studies suggests that cell therapy can improve cardiac function.¹ These encouraging data led to recent clinical reports showing beneficial improvement from intramyocardial injections of skeletal myoblasts and transendocardial injections of bone marrow stem cells.² However, the assessment of cell fate and survival in vivo remains elusive. In animal studies, cells labeled with a conventional reporter gene (eg, green fluorescent protein) or a thymidine analogue (eg, bromodeoxyuridine [BrdU]) require postmortem histology at given time points. In clinical studies, the interpretation of therapeutic efficacy often relies on indirect echocardiographic measurements and subjective symptom reports by patients. Thus, there is a strong impetus for developing novel imaging approaches that would allow noninvasive assessment of myocardial response to cell therapy.^{3,4}

Methods

Virus Construction and Amplification

Replication-defective recombinant adenovirus carrying cytomegalovirus promoter driving 2 reporter genes was constructed and amplified as described (Ad-CMV-Fluc and Ad-CMV-HSV1-sr39tk).^{5,6} Henceforth, the Fluc enzyme is referred to as "FL" and the HSV1-sr39tk enzyme as "HSV1-sr39TK."

Cell Culture and In Vitro Assays for FL and HSV1-sr39TK

Embryonic rat H9c2 cardiomyoblasts (American Type Culture Collection) were cultured as described.⁷ Cells were transfected (multiplicity of infection 250) with Ad-CMV-Fluc or Ad-CMV-HSV1-sr39tk overnight. The virus supernatant was removed and the cells washed 3 times with phosphate-buffered saline before adding trypsin for cell collection. Afterward, reporter enzyme activities were confirmed before cell transplantation. The Luciferase Assay Reagent (Promega) was used to determine FL level as described.⁵ Cardiomyoblasts transfected with Ad-CMV-HSV1-sr39tk serve as a negative control. The penciclovir phosphorylation assay was used to deter-

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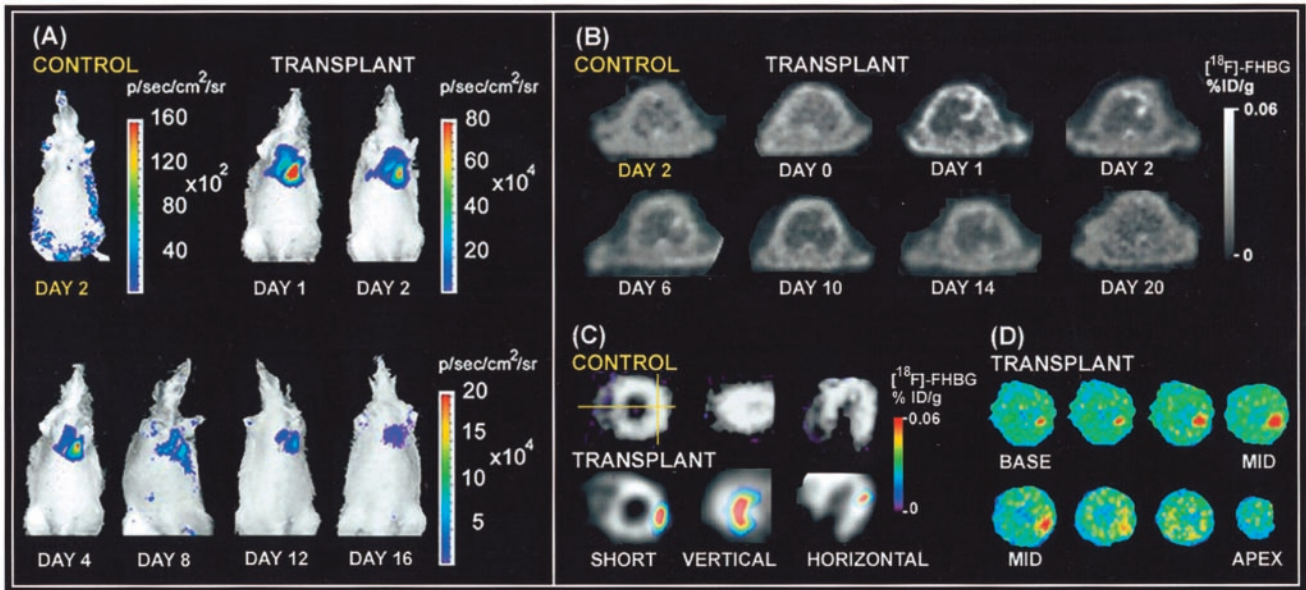


Figure 1. Molecular imaging of cardiac cell transplantation in living animals. A, Optical imaging shows a representative rat transplanted with embryonic cardiomyoblasts expressing Fluc reporter gene that emits significant cardiac bioluminescence activity at days 1, 2, 4, 8, 12, and 16 ($P < 0.05$ vs control). Control rat has background signal only. B, MicroPET imaging shows a representative rat transplanted with cardiomyoblasts expressing HSV1-sr39tk reporter gene, whereby the location(s), magnitude, and survival duration were monitored by longitudinal imaging of [^{18}F]-FHBG reporter activity (gray scale). C, Detailed tomographic views of cardiac microPET images are shown in short, vertical, and horizontal axis. At day 2, a representative transplant rat has significant activity at the lateral wall, as shown by the [^{18}F]-FHBG reporter activity image (color scale) overlaid on the [^{13}N]- NH_3 perfusion image (gray scale). In contrast, control rat has homogeneous [^{13}N]- NH_3 perfusion but background [^{18}F]-FHBG reporter activity. D, Autoradiography of the same study rat confirms trapping of [^{18}F] radioactivity by transplanted cells at the lateral wall at finer spatial resolution ($\approx 50 \mu\text{m}$).

mine HSV1-sr39TK level as described.⁶ Cardiomyoblasts transfected with Ad-CMV-Fluc served as a negative control. All experiments were performed in triplicates.

Intramyocardial Transplantation of Embryonic H9c2 Cardiomyoblasts

Female nude athymic rats (weighing 200 to 300 g; Charles River Laboratories, Wilmington, Mass) were studied under protocols approved by the UCLA Animal Research Committee. Harvested cardiomyoblasts expressing either FL or HSV1-sr39TK reporter proteins were kept on ice for < 15 minutes for optimal viability before injection ($n = 50$ rats). The aseptic lateral thoracotomy and postoperative care procedure have been described previously.⁶ The surgical mortality rate was $\approx 15\%$.

Optical Bioluminescence and Micro-Positron Emission Tomography Imaging of Cardiomyoblast Transplantation

Optical bioluminescence imaging was performed using the charged-coupled device camera (Xenogen).⁵ After intraperitoneal injection of reporter substrate D-Luciferin (375 mg/kg body weight), rats were imaged for 30 minutes using 30×1 -minute acquisition scans. The same rats were scanned every 1 to 4 days for more than 2 weeks. Bioluminescence was quantified in units of photons \cdot second⁻¹ \cdot centimeter⁻² \cdot steradian⁻¹.⁴ Cardiac positron emission tomography (PET) imaging was acquired using a prototype microPET scanner.⁶ Perfusion tracer [^{13}N]- NH_3 (2.35 ± 0.35 mCi) was injected via the tail vein and rats imaged for 20 minutes. The rats were then injected with [^{18}F]-FHBG (2.55 ± 0.51 mCi), and images from 60 to 80 minutes after injection were reconstructed by filtered back-projection algorithm.⁶

Gamma Well Counting and Digital Autoradiography

After imaging, explanted hearts were counted for [^{18}F] radioactivity in a gamma well counter (Cobra II Auto-Gamma).⁶ Afterward, hearts

were frozen in liquid nitrogen and embedded in 5% carboxymethylcellulose. The cryosections were exposed directly onto digital plates and scanned on a Fuji Bas 5000 digital phosphorimager as described.⁴

Histology and Immunohistochemistry

Explanted hearts underwent formalin fixation, paraffin sectioning, hematoxylin and eosin staining, and immunohistochemistry. Sections were incubated with primary antibody for troponin T, BrdU (10 mmol/L; overnight incubation in cell culture), and α -smooth muscle actin, followed by secondary antibody (rat adsorbed anti-mouse, 1:1000 dilution). Visualization was accomplished using an avidin-biotin-peroxidase complex as described by manufacturer (Vector Labs).

Data Analysis

Data are given as mean \pm SD. For statistical analysis, the 2-tailed Student's *t* test was used. Differences were considered significant at $P < 0.05$.

Results

Optical Bioluminescence Imaging of Cell Transplantation

To understand the physiological pattern of cell survival, transplanted cardiomyoblasts (3×10^6) expressing Fluc reporter gene were imaged repetitively over a 2-week period in the same animals ($n = 5$). Control rats transplanted with cardiomyoblasts expressing HSV1-sr39tk show background signal only ($n = 5$) (Figure 1A). To assess the detection threshold, different numbers of cardiomyoblasts (2×10^6 , 1×10^6 , and 5×10^5 ; $n = 5$ each) were also injected. Cardiac signals were clearly present even in the 5×10^5 group: day 1 ($112\,500 \pm 17\%$), day 2 ($59\,670 \pm 15\%$), day 4 ($21\,420 \pm 10\%$), day 8 ($18\,660 \pm 18\%$), day 12 ($9123 \pm 4\%$),

and day 16 ($7070 \pm 6\%$) versus day 2 control rat ($1306 \pm 13\% \text{ p} \cdot \text{s}^{-1} \cdot \text{cm}^2 \cdot \text{sr}^{-1}$) ($P < 0.01$). Drastic reduction of signal intensity was seen within the first 1 to 4 days for all groups of animals, especially those that received larger doses of cells.

MicroPET Imaging of Cell Transplantation

To assess the feasibility of monitoring cell transplant using the radionuclide approach, transplanted cardiomyoblasts (3×10^6) expressing HSV1-sr39tk reporter gene were imaged longitudinally in the same animals ($n=20$). Control rats transplanted with cardiomyoblasts expressing Fluc show background signal only ($n=5$) (Figure 1B). MicroPET imaging shows detailed tomographic location of transplanted cells when the [^{18}F]-FHBG reporter activity image is overlaid on the [^{13}N]- NH_3 perfusion image (Figure 1C). At day 2, regions of interest drawn over [^{18}F]-FHBG activity show significant differences at the transplanted region (0.056 ± 0.014) compared with its contralateral septum (0.019 ± 0.002) and control rats (0.013 ± 0.001 % injected dose/g) ($P < 0.0001$).

Validation of In Vivo Imaging Results With Traditional Ex Vivo Assays

The presence of transplanted cardiomyoblasts on in vivo images was confirmed by several independent methods. First, the ex vivo gamma well counting showed a 4.01 ± 0.51 -fold increase of trapped [^{18}F] radioactivity in study rats (0.038 ± 0.017) compared with control rats (0.009 ± 0.005 % ID/g) ($P < 0.05$), consistent with the in vivo region of interest analysis. Second, the high-resolution ex vivo autoradiography confirmed the anatomic location of transplanted cells on microPET image (Figure 1D). Third, the in vitro HSV1-sr39TK reporter protein assay in the transplanted region (0.420 ± 0.111) was significantly higher compared with its contralateral septum (0.057 ± 0.011) and control rats (0.020 ± 0.010 % conversion/ μg per min) ($P < 0.05$). Finally, the microPET location of transplanted cardiomyoblasts correlated with hematoxylin and eosin stain (Figure 2A). Numerous cells undergoing apoptosis can be seen at higher power of magnification (Figure 2B). Immunohistochemical studies of these embryonic cardiomyoblasts stained negative for troponin T (marker for adult heart muscle), positive for α -smooth muscle actin (marker for embryonic heart muscle), and positive for BrdU (marker for DNA synthesis) (Figures 2C through 2F).

Discussion

This is the first study to demonstrate the feasibility of monitoring transplanted cells in the myocardium of living animals using reporter gene imaging technology. The location(s), magnitude, and survival duration under real-time physiological influences were monitored over 2 weeks. Drastic reductions of signal intensity within the first 1 to 4 days after transplantation were noted, likely because of acute donor cell death from inflammation, ischemia, and apoptosis as shown on histology.^{1,3} These survival kinetic curves are consistent with other studies using serial terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay, TaqMan polymerase chain reaction, and immunohistological samples obtained from a large

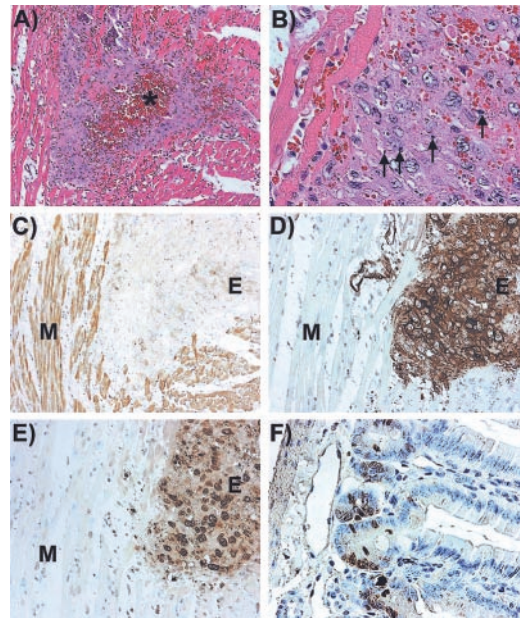


Figure 2. Validation of in vivo images with ex vivo histology and immunohistochemistry. A, Low-power hematoxylin-and-eosin stain shows transplanted cardiomyoblasts surrounding a central area of hemorrhage at the site of injection (*). B, High-power hematoxylin-and-eosin stain shows multiple multinucleated cells consistent with embryonic cell morphology. Numerous cells undergoing apoptosis are also evident by their condensed nuclei (arrow). At day 2, transplanted cells stain negative for troponin T (C), positive for α -smooth muscle actin (D), and positive for BrdU (E). F, The proliferating intestinal villi cells of the same rat serve as positive control for BrdU stain. E indicates embryonic cardiomyoblasts; M, adult host cardiomyocytes.

number of animals euthanized at different time points.^{8,9} Under the given conditions, optical imaging has a very high detection threshold, whereas microPET imaging provides detailed anatomic locations of transplanted cells. Most importantly, in vivo imaging results were confirmed by ex vivo gamma counting and enzyme assays and correlated to autoradiography, histology, and immunohistochemistry.

Several imaging strategies are being actively investigated. Kraitchman et al¹⁰ recently demonstrated the feasibility of monitoring magnetically labeled mesenchymal stem cells using MRI. In the present study, our group has focused on labeling transplanted cardiac cells with different reporter genes for multimodality imaging purposes. Several other reporter genes, such as the dopamine type 2 receptor, somatostatin receptor, and synthetic renilla luciferase, have already shown promising results for labeling tumor cells in animal studies using PET, single photon-emission computed tomography, and optical bioluminescence imaging.⁴ Thus, the wide assortment of reporter genes opens up the possibility for further cardiac studies whereby different stem cells can be labeled with different reporter genes and their engraftment, survival, and differentiation analyzed individually.^{1,3,4}

In our proof-of-principle study, rats underwent intramyocardial injection of embryonic cardiomyoblasts. Issues such as the optimal cell type, cell number, and delivery route will need to be addressed.^{1,3} Likewise, detection sensitivity and signal quantification for each imaging modality will have to

be elucidated. With transient adenoviral transfection, the in vitro reporter protein activity declines after 5 days, which may have further contributed to signal reduction.⁷ Ongoing studies will involve stable transfection of stem cells.⁴ This approach will need to be carefully investigated before application in human disease. Encouragingly, use of genetically modified stem cells secreting vascular endothelial growth factor has been shown to add additional cardioprotective benefits.¹¹ This ex vivo manipulation allows one to link any therapeutic gene of interest to a reporter gene.⁴ Thus, reporter gene imaging may potentially be used to monitor combined cell transplantation and gene therapy.

In summary, in vivo imaging of cardiac cell transplantation provides obvious advantages over traditional techniques. These noninvasive approaches will allow investigators to rapidly evaluate many important parameters, determine the efficacy of repeated transplantations, and screen for novel drugs designed to reduce acute donor cell death or apoptosis. With further refinement, molecular imaging studies will likely contribute to the goal of clinical transplantation protocols that are reproducible, beneficial, and quantifiable.

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