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In vivo bioluminescence imaging of cord blood derived mesenchymal stem cell transplantation into rat myocardium

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Objective: The conventional method for the analysis of myocardial cell transplantation depends on postmortem histology. Here, we have sought to demonstrate the feasibility of a longitudinal monitoring of transplanted cell survival in living animals, accomplished with optical imaging techniques and pharmacological interventions. ***Methods:*** Human cord blood (50 m*l*) was donated with parental consent. After getting cord blood derived mesenchymal stem cells (CBMSCs), cells were transfected (MOI = 100) overnight with adenovirus encoding firefly luciferase gene (Ad-CMV-Fluc). Our experimental Sprague-Dawley rats (n = 12) were given intramyocardial injections containing 1×10^6 CBMSCs, which had been made to express the firefly luciferase (Fluc) reporter gene. Optical bioluminescence imaging was then conducted using a cooled charged-coupled device (CCD) camera (Xenogen), beginning on the day after the transplantation (day 1). Groups of mice were intraperitoneally injected with cyclosporine (5 mg/kg) or tacrolimus (1 mg/kg), in an attempt to determine the degree to which cell survival had been prolonged, and these values were then compared with the cell survival values of the negative control group. The presence of transplanted CBMSCs on *in vivo* images confirmed by *in situ* hybridization for human specific Alu in the myocardium. ***Results:*** Cardiac bioluminescence signals were determined to be present for 6 days after transplantation: day 1 ($97000 \pm 9100 \times 10^5$ p/s/cm^2/sr), day 3 (9600 ± 1110 p/s/cm^2/sr), and day 5 (3200 ± 550 p/s/cm^2/sr). The six mice that received either cyclosporine or tacrolimus displayed cardiac bioluminescence signals for a period of 8 days after transplantation. We observed significant differences between the treated group and the non-treated group, beginning on day 3 (tacrolimus; 26500 ± 4340 p/s/cm^2/sr, cyclosporine; 27200 ± 3340 p/s/cm^2/sr, non-treated; 9630 ± 1180 p/s/cm^2/sr, $p < 0.01$), and persisting until day 7 (tacrolimus; 12500 ± 2946 p/s/cm^2/sr, cyclosporine; 7310 ± 1258 p/s/cm^2/sr, non-treated; 2460 ± 160 p/s/cm^2/sr, $p < 0.01$). The human-derived CBMSCs were detected in the myocardium 3 days after transplantation by *in situ*

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hybridization. ***Conclusions:*** The locations, magnitude, and survival duration of the CBMSCs were noninvasively monitored with a bioluminescence optical imaging system. We determined that optical molecular imaging expedites the fast throughput screening of pharmaceutical agents, allowing for the noninvasive tracking of cell survival within animals. In rat cardiac CBMSC transplant models, transient immunosuppressive treatment with tacrolimus or cyclosporine was shown to improve donor cell survival.

Key words: cord blood, mesenchymal stem cell, bioluminescence, molecular imaging

INTRODUCTION

ISCHEMIC HEART DISEASE ACCOUNTS for most cardiovascular deaths. Despite recent advances in medical therapies, a significant proportion of such patients remain symptomatic. Therefore, researchers have engaged in extensive investigation, in order to develop alternative treatments for this condition, such as stem cell therapy. Several previous studies have indicated that the implantation of skeletal myoblasts, endothelial progenitor cells, or bone marrow stem cells into an infarcted myocardium can results in improved myocardial function.[1–3] This effects may be related to the secretion of multiple arteriogenic cytokines by stem cells, which would contribute to the formation of a mechanical scaffold or to the recruitment of other beneficial cells to the ischemic region.[4] However, most techniques used for the analysis of stem cell survival in animal models have relied on postmortem histology to determine the fate and migratory behavior of the stem cells. This approach, however, precludes any sort of longitudinal monitoring.[5] An approach which would allow for the monitoring of stem cell activities within the context of the intact whole-body system, rather than with histological slides, would allow us to gain further insights into the underlying biological and physiological properties of stem cells.

In recent years, several investigators have attempted to address this issue, using optical reporter gene labeling. This approach was initially intended to allow for the serial tracking and quantification of transplanted stem cells, in a noninvasive and highly sensitive manner.[6–8] Thus, the objective of this study was to determine whether intramyocardially injected bioluminescent cord blood derived mesenchymal stem cells (CBMSCs) in a rat model could be detected and tracked noninvasively with a cooled charged-coupled device (CCD) camera. We also evaluated the efficacy of two potent immunosuppressive agents designed to prolong the survival of stem cells in the rat myocardium.

MATERIALS AND METHODS

Virus Construction and Amplification

We constructed and amplified a replication-defective recombinant adenovirus which harbored the cytomegalovirus (CMV) promoter driving the firefly luciferase reporter gene, as was described previously (Ad-CMV-Fluc).[9] Henceforth, the Fluc enzyme is referred to as 'FL.'

Cord Blood Derived Mesenchymal Stem Cell (CBMSC) Culture

With parental consent, cord blood (50 m*l*) was donated with a heparinized (250 U/m*l*) syringe from the umbilical cord. Cord blood was centrifuged at 800 *g* for 10 min at room temperature, and the serum layer was discarded. The cells were diluted 1:1 with phosphate-buffered saline (PBS) and layered over an equal volume of Ficoll-Paque (1.077 g/m*l*; Amersham Biosciences) and centrifuged at 800 *g* for 30 min. Mononuclear cells were isolated by density gradient centrifugation (300 *g* for 5 min at room temperature), and were seeded at $10^6/cm^2$ in Low-Glucose-Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/m*l* penicillin, 100 μg/m*l* streptomycin, and 2 mM L-glutamine. The cells were cultured at 37°C in 5% CO_2 incubator, and nonadherent cells are removed after 5 days and culture medium are replaced every 3–4 days. When isolated colonies were apparent, the cells were detatched with 0.05% trypsin-EDTA and replated at $2 \times 10^3/cm^2$. After getting the CBMSCs, they were transfected (MOI = 100) with Ad-CMV-Fluc overnight.

Intramyocardial Transplantation of Cord Blood Derived Mesenchymal Stem Cells (CBMSCs)

Twelve Sprague-Dawley rats (each weighing 250 to 350 g) were studied according to protocols which had been previously approved by the Chonnam National University Hospital Animal Research Committee. The rats received a 4:1 mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally for anesthesia, banamine (2.5 mg/kg) for pain relief, atropine (40 μg/kg) to prevent bradycardia, and normal saline (3 to 4 m*l*) for volume replacement. The anesthetized rat were then mechanically ventilated before the performance of a left thoracotomy on each of the rats, under aseptic conditions. Harvested CBMSCs which expressed the FL reporter proteins were maintained on ice for <15 minutes, in order to ensure optimal viability prior to injection. The anterolateral wall of the left ventricular myocardium in each rat was then injected with 1×10^6 pfu of the FL-expressing CBMSCs (n = 9). Sham-operated rats were negative controls (n = 3).

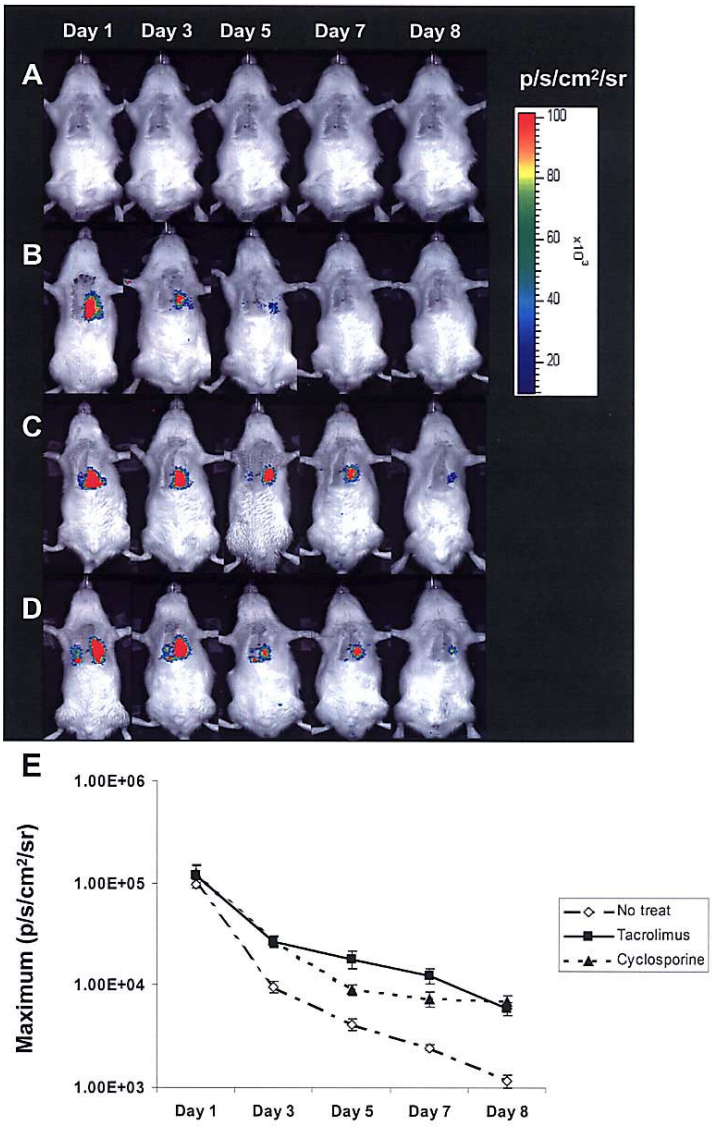

Fig. 1 Bioluminescence optical imaging of cord blood derived mesenchymal stem cell (CBMSC) transplantation in living rats. (A) Negative control rats that received saline injections into the myocardium exhibited no imaging signals coming from the anterior chest. (B) Rats were injected with CBMSCs, which expressed the firefly luciferase gene, into the anterolateral wall of the myocardium. Imaging signals were apparent from day 1. Signal intensity disappeared completely on day 7 after transplantation (C, D). Rats were treated with tacrolimus (C, 1 mg/kg/day) or cyclosporine (D, 5 mg/kg/day) from –3 day to 8 days of transplantation. Imaging signals were apparent from day 1 to day 7. Signal intensity disappeared almost completely on the 8th day after transplantation. (E) We determined there to be a significant difference in imaging signal intensity between the non-treated group and the treated group (tacrolimus or cyclosporine) from day 3 ($p < 0.01$) to day 7 ($p < 0.05$).

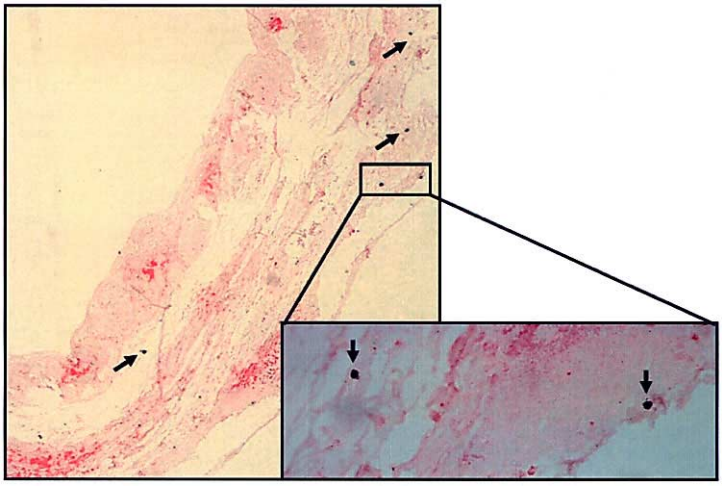

Fig. 2 *In situ* hybridization for human specific Alu in the myocardium. The presence of transplanted human-derived CBMSCs on *in vivo* images was confirmed by *in situ* hybridization for human specific Alu in the myocardium. The CBMSCs were detected in the myocardial region 3 days after transplantation. Black dots representing human specific Alu gene are indicated by black arrows.

Pharmaceutical Intervention for the Prolongation of Cell Survival

From day −3 to 8, the rats were treated daily with intraperitoneal injections of either cyclosporine (5 mg/kg/day; n = 3) or tacrolimus (1 mg/kg/day; n = 3), both of which inhibit calcineurin and IL-2, which are prerequisites for the activation of lymphocytes. Total volume of intraperitoneally injected tacrolimus or cyclosporine was 80 μl. The non-treated animals received saline injections (1 m*l*/kg/day; n = 3). Cell survival was assessed by using optical bioluminescence imaging, beginning on the day after the transplantation (day 1) and ending on day 8.

Optical Bioluminescence Imaging of Cord Blood Derived Mesenchymal Stem Cell (CBMSC) Transplantation

Optical bioluminescence imaging was conducted with a CCD camera (Xenogen, Alameda, CA). After the intraperitoneal injection of the D-Luciferin reporter substrate (375 mg/kg body weight), each of the rats was imaged for 30 minutes, using 30 × 1-minute acquisition scans. The same rats were then scanned every day until the imaging signals had completely disappeared. Bioluminescence was then quantified in units of photons·second^{-1}·centimeter$^{2-1}$·steridian^{-1}.

Tissue Processing and In Situ *Hybridization*

Explanted hearts underwent formalin fixation, paraffin sectioning and *in situ* hybridization. The oligodeoxynucleotide probes corresponding to the most conserved areas of human Alu sequences were DIG-labeled with the PCR DIG probe synthesis kit (Roche). Sections were deparaffinized in xylene and rehydrated in PBS, and then incubated with TE buffer containing 2 mg/m*l* proteinase K for 30 min at 37°C. After pre-hybridization with hybridization buffer [50% formamide (Sigma) in 5 × SSC, 0.1% sodium-lauroylsarcosine (Sigma), 0.02% SDS (Sigma), 2% blocking reagent (Roche)] for 3 h at 85°C, slides were incubated with fresh hybridization buffer containing the denatured DIG-labeled DNA probe (10–200 ng/m*l*) for further 10 min at 94°C. Then slides were transfered to ice for 10 min and incubated overnight at 42°C. Pre-hybridization and hybridization steps were performed in a moist chamber containing 50% formamide. After hybridization, slides were briefly rinsed in 2 × SSC at room temperature and three times in 0.1 × SSC for 15 min at 42°C. Visualization of DIG-labeled DNA probe was performed according to the protocol of the DIG nucleic acid detection kit (Roche).

Data Analysis

All data in this study are expressed as means ± SD. We used ANOVA (Analysis of Variance) tests for statistical analysis. A p value of <0.05 was considered to be statistically significant.

RESULTS

Optical Bioluminescence Imaging of Cell Transplantation

In order to gain understanding into the physiological patterns of cell survival, the transplanted Fluc reporter-expressing CBMSCs (1×10^6) were imaged repetitively over a 1-week period in the same set of animals (n = 3). The negative control rats that had received transplantated CBMSCs which did not express FL, exhibited a background signal only (n = 3) (Fig. 1A). In the transplanted rats, cardiac signals were apparent: day 1 (97000 ± 9100 p/s/cm^2/sr), day 3 (9600 ± 1110 p/s/cm^2/sr), day 5 (3200 ± 550 p/s/cm^2/sr), and day 7 (2500 ± 160 p/s/cm^2/sr). Signal intensity completely disappeared by day 7 in all animals.

Pharmaceutical Intervention for the Prolongation of Cell Survival

In order to determine the role of immunosuppressive agents with regard to the prolongation of cell survival, we intraperitoneally applied cyclosporine and tacrolimus to rats, both before and after the transplantation of CBMSC. The six mice who had received cyclosporine (5 mg/kg/day) or tacrolimus (1 mg/kg/day) evidenced strong cardiac bioluminescence signals for 8 days after transplantation. We detected significant differences between the treated and non-treated groups from day 3 (tacrolimus; 26500 ± 4340 p/s/cm^2/sr, cyclosporine; 27200 ± 3340 p/s/cm^2/sr, non-treated; 9630 ± 1180 p/s/cm^2/sr, $p < 0.01$) to day 7 (tacrolimus; 12500 ± 2946 p/s/cm^2/sr, cyclosporine; 7310 ± 1258 p/s/cm^2/sr, non-treated; 2460 ± 160 p/s/cm^2/sr, $p < 0.05$). Bioluminescence signals were observed with the cooled CCD camera until the 8th day after the transplantation (Fig. 1B–E).

Validation of In Vivo *Imaging Results with* In Situ *Hybridization*

The presence of transplanted CBMSCs on *in vivo* images was confirmed by *in situ* hybridization for human specific Alu in the myocardium. The human-derived CBMSCs were detected in the myocardium 3 days after implantation. Black dots representing human specific Alu gene are shown in Figure 2 (*black arrow*).

DISCUSSION

In this study, we have demonstrated the feasibility of the monitoring of cells which have been transplanted into the myocardia of living animals, using reporter gene imaging technology. The presence of transplanted CBMSCs on *in vivo* images was confirmed by *in situ* hybridization for human specific Alu in the myocardium. The location, magnitude, and survival duration of these cells were monitored for 1 week, under real-time physiological conditions. Drastic reductions were noted in signal

intensity, however, within the first 1 to 4 days after transplantation, and this is probably due to human derived CBMSC death as the result of immune reactions. Under the conditions as set up in this experiment, optical imaging was found to be extremely sensitive with regard to the detection of signals from the relevant cells.

Several imaging strategies are currently under active investigation, including radionuclide labeling, ferromagnetic labeling, and reporter gene labeling.[10] In a study of radionuclide labeling, Aicher et al.[6] injection indium-111 oxine-labeled endothelial progenitor cells into the infarcted myocardia of nude rats, and imaged them at 24 to 96 hours, using a gamma camera. The main limitation associated with this approach is that radionuclides have physical half-lives, making it possible to monitor cell distribution only for a limited number of days.

In a study of ferromagnetic labeling, Kraitchman et al.[7] injected mesenchymal swine stem cells, which contained ferrumoxide particles, into the hearts of pigs. After 24 hours, these injected sites became ovoid hypoenhancing lesions, with sharp borders. One to 3 weeks after the cells had been injected, the borders became less clearly delineated, due to the degradation of the ferrumoxide particles. As the ferrumoxide particles continue to register magnetic resonance signals, even when the injected cells have undergone apoptosis or cell death, it becomes more difficult to correlate the magnetic resonance signal with the actual number of viable cells.

During the process of reporter gene labeling, the cells are transfected with reporter genes before being implanted into the myocardium.[8] In cases in which the cells remain alive, the reporter gene will be expressed. In cases in which the cells are dead, the reporter gene will not be expressed. Employing this approach, Wu et al.[8] recently used embryonic cardiomyoblasts which express herpes simplex virus type I thymidine kinase (HSV1-tk) or firefly luciferase (Fluc) reporter genes, which they then noninvasively tracked using either micro-PET or bioluminescence optical imaging. Drastic reductions were noted in signal intensity within the first 1 to 4 days, and this was tentatively attributed to acute donor cell death as the result of inflammation, adenoviral toxicity, ischemia, or apoptosis.

Molecular imaging techniques for the noninvasive tracking of cell survival within an animal could clearly prove quite useful for the screening of pharmaceutical agents. In cardiac transplant animals to whom tacrolimus or cyclosporine was administered, signal intensity was determined to be significantly higher than in the group which had not been treated. This indicates the improvement of cell survival subsequent to immunosuppressive treatment. Tacrolimus, like cyclosporine, inhibits the generation of cytokines, including IL-2. Both of these compounds also inhibit the expression of IL-2 receptors, and block cell division. Although tacrolimus and cyclosporine exhibit similar properties, tacrolimus is 10 to 100 times more potent, on a per gram basis, than is cyclosporine. According to the results of our current study, bioluminescence signals were substantially higher in the tacrolimus group than in the cyclosporine group, from day 5 to day 7 after transplantation. Recently, Wu et al.[11] reported that tacrolimus treatment resulted in significant but modest improvements in cell survival at Days 2 and 10 in mice in which embryonic rat cardiomyoblasts had been transplanted into the thigh muscles.

In our current study, we initially suggested a potential role for a molecular imaging technique for the investigation of unresolved issues in stem cell transplantation using cooled CCD imaging, to wit; what is the optimal cell type, cell dosage, and delivery route? How long do cells survive after transplantation? What pharmaceutical agents are capable of improving cell survival durations? What percentage of injected cells was successfully delivered to the area of interest? In summary, *in vivo* cardiac cell transplantation imaging provides obvious advantages over traditional techniques. Such noninvasive approaches will allow for the rapid evaluation of many of the important parameters denoted above. In cardiac transplant models, the transient use of immunosuppressants, such as tacrolimus and cyclosporine, was determined to effect improvements in the survival of the donor cells.

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