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SPECT imaging of cardiac reporter gene expression in living rabbits

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Abstract This work is to demonstrate feasibility of imaging the expression of herpes simplex virus 1-thymidine kinase (HSV1-tk) reporter gene in rabbits myocardium by using the reporter probe ^{131}I-2'-fluoro-2'-deoxy-1-β-$_D$- arabinofuranosyl-5-iodouracil (^{131}I-FIAU) and SPECT. Rabbits of the study group received intramyocardial injection of Ad5-tk and control group received aseptic saline injection. Two sets of experiments were performed on the study group. Rabbits of the 1st set were injected with ^{131}I-FIAU 600 μCi at Day 2 after intramyocardial transfection of Ad5-tk in 1×10^9, 5×10^8, 1×10^8, 5×10^7 and 1×10^7 pfu, and heart SPECT imaging was done at different hours. Rabbits of the 2nd were transferred various titers of Ad5-tk (1×10^9, 5×10^8, 1×10^8, 5×10^7, 1×10^7 pfu) to determine the threshold and optimal viral titer needed for detection of gene expression. Two days later, ^{131}I-FIAU was injected and heart SPECT imaging was performed at 6, 24 and 48 h, before killing them for gamma counting of the hearts. Reverse transcription-polymerase chain reaction (RT-PCR) was used to verify the transferred HSV1-tk gene expression. Semi-quantitative analysis derived of region of interest (ROI) of SPECT images and RT-PCR images was performed and the relationship of SPECT images with *ex vivo* gamma counting and mRNA level were evaluated. SPECT images conformed ^{131}I-FIAU accumulation in rabbits injected with Ad5-tk in the anterolateral wall. The optimal images quality was obtained at 24~48 h for different viral titers. The highest radioactivity in the focal myocardium was seen at 6 h, and then declined with time. The threshold was 5×10^7 pfu of virus titer. The result could be set better in $1\sim5\times10^8$ pfu by SPECT analysis and gamma counting. ROI-derived semi-quantitative study on SPECT images correlated well with *ex vivo* gamma counting and mRNA levels from RT-PCR analysis. The HSV1-tk/^{131}I-FIAU reporter gene/reporter probe system is feasible for cardiac SPECT reporter gene imaging. The optimal Ad5-tk titer is $1\sim5\times10^8$ pfu and optimal imaging time is 24~48 h after transferred Ad5-tk in rabbit. The imaging of transgene expression in heart might be used for noninvasive imaging of gene therapy in cardiac diseases in human.

Key words Reporter gene, Herpes simplex virus 1-thymidine kinase, 2'-fluoro-2'-deoxy-1-β-$_D$-arabinofuranosyl-5-iodouracil, Single photon emission computed tomography, Cardiac

1 Introduction

Cardiac disease remains one of the greatest health problems. Gene therapy is promising a novel approach for treatment of cardiac diseases, such as heart failure and ischemia. It has been reported that phase II clinical trials were conducted with several gene therapeutic approaches[1, 2], most of which, however, ceased owing to lack of decisive proof of therapeutic effects. It is important to develop an accurate, noninvasive and quantitative method for evaluating transfer and expression of therapeutic genes in the myocardium, such as the emerging reporter gene system offered the possibility for noninvasive monitoring the gene therapy.

With reporter genes linked to therapeutic genes that encode proteins for intracellular trapping of sys-

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temically administered reporter probes, one can indirectly infer the location, magnitude, and duration of therapeutic gene expression by imaging the reporter protein and monitoring the therapeutic gene expression[3]. The herpes simplex virus Type 1 thymidine kinase gene (HSV1-tk) used as a reporter gene has been studied extensively. Among various kinds of substrates, radiolabeled 2'-fluoro-2'-deoxy-1-β-D- arabinofuranosyl-5-iodouracil (FIAU) showed high sensitivity and selectivity for detecting HSV1-tk expression[4].

The present study is aimed at demonstrating the feasibility of imaging HSV1-tk/FIAU reporter gene/probe system on heart using SPECT in animal models. The optimal viral titer and imaging time were discussed as basis for human heart reporter gene imaging.

2 Materials and methods

2.1 Materials

2.1.1 Reagents

FAU(2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyluacil), Moravek Biochemicals Co., USA; Iodogen (1,3,4,6-tetrachloro-3alpha, 6alpha-diphenylglucoluril), Sigma Co., USA; Sep-Pak C18 reverse-phase column, Waters Milford M.A.USA; [^{131}I]NaI, Beijing Atomic High Tech Co., China; Taq DNA polymerase chain reaction kit, Promega Corp., USA; primer of PCR, Shanghai Yingjun Genetech Co., China; sumianxin II, Military Veterinarian Institute, China.

2.1.2 Instruments

Discovery VG SPECT, GE, USA; GC-1200 γ radioimmunoanalyzer, Keda Co.; Biophotometer, Eppendorf Co., Germany.

2.1.3 Animals

Chinese rabbits weighing 2.0~2.6kg were supplied by Animal Laboratory Center of Tongji Medical College.

2.2 Methods

2.2.1 Construction of recombinant adenoviruses (Ad-CMV-HSV1-tk)

The plasmid vector, pDC316- tk, and the virus vector, E1/E3-deleted replication-defective recombinant adenovirus Type 5 (Ad5-tk), carrying the HSV1-tk gene under the transcriptional control of the cytomegalovirus (CMV) promoter, were constructed and purified at Vector Gene Technology Company Ltd., China. The HSV1-tk gene in the recombinant vectors was identified by PCR. The viral titer of Ad5-tk was 1.6×10^{10} IU/mL as determined by TCID50 method.

2.2.2 Prepared radioiodination of FAU

FAU was labeled referring to a modified method in Ref.[5]. Briefly, the dissolved FAU (1 μmol) in 50 μL PBS (pH 7.4) was added to an Eppendorf tube coated with 250 μg Iodogen and combined with 200 μL no-carrier-added [^{131}I] NaI (185 MBq). After 8 min at 70°C, the solution was cooled on ice for 30 s. It was placed at room temperature for 5 min, and removed from the solid oxidizing reagent. The product, FIAU, was fixed on a reverse-phase Sep-Pak C-18 column, washed with 4 mL water and eluted with 4 mL methanol. The fractions were collected for measuring the radioactivity and labeling efficiency. The peak fractions were identified for the radiochemical purity assessment. The ^{131}I-FIAU was dissolved with sterile, passing through a sterile nonpyrogenic 0.22 μm Millipore filter. The products were diluted to 37 MBq/mL for animal studies.

2.2.3 Animal preparation and myocardial injection of Adenoviruses

Rabbits were anesthetized with sumianxin Ⅱ (0.2 mL/kg body weight) into the hind leg. They were fixed on operating-table for supine position and sterilized in front of the chest with Iodophors. The left third and fourth rib cartilage were disconnected along left sternal border, opening and exposing the mediastinum and heart, cutting-off the pericardium, and maintaining the pleura integrated and breathe steady, as possible as we could.

Various viral titers of Ad5-tk and asepsis saline were injected into anterolateral wall of left ventricular using 1 mL syringe containing 100 μL of viral volume. The needle was left for 10 s to prevent reflux of the inoculum and to ensure its resorption. The chest wound was sutured, with negative pressure applied to the chest cavity by a thin sterile tube connected to a syringe to prevent pneumothorax. The surgical procedures were performed under aseptic conditions. All rabbits were intramuscular injection of benzylpenicillin sodium for prevention of infection. Two days before ^{131}I-FIAU injection, all animals received oral administration of aqueous iodine solution to block thyroid uptake of radioactive iodide.

2.2.4 Experimental groups

Rabbits of the study group received Ad5-tk injection and the control group received asepsis saline injection. Two sets of experiments were performed on the study group.

The first set of experiments was performed to assess the feasibility and sensitivity expression of HSV1-tk in transferred myocardium by ^{131}I-FIAU imaging. Suitable time course of ^{131}I-FIAU imaging was evaluated. The rabbits were injected with ^{131}I-FIAU 600 μCi through ear vein at Day 2 after intramyocardial transfection of different titers of Ad5-tk (1×10^9, 5×10^8, 1×10^8, 5×10^7 and 1×10^7 pfu). Heart SPECT imaging was performed at 6, 24, 48 and 72 h. The rabbit with intramyocardial transfection of 1×10^9 pfu Ad5-tk continued the imaging at 96 and 120 h.

The second set of experiments was to evaluate viral dose-dependent to determine the detection threshold and the suitable viral titer for imaging gene expression. The Ad5-tk virus was administered at doses of 1×10^9, 5×10^8, 1×10^8, 5×10^7, and 1×10^7 pfu. Two days later, ^{131}I-FIAU was injected through ear vein and heart SPECT imaging was performed at 6, 24 and 48h. The rabbits were killed for gamma counting and RT-PCR analysis. Each group contained three animals.

2.2.5 Cardiac SPECT reporter gene imaging

After animals were anesthetized by muscles injection of sumianxin Ⅱ (0.2 mL/kg body weight), the rabbits were fixed on the examination couch of GE Discovery SPECT, which is featured by its dual-head, high energy, parallel hole collimators and the energy window centered at 364 keV with 20% width. Planar images were obtained in 5 min and reconstructed to a 128 × 128 matrix. The myocardium tomography images were obtained, in a procedure identical to conventional myocardium tomography. The tomography images were reconstructed to short axis, vertical long axis, horizontal long axis and polar map. Region of interest (ROI) technique was used to analyze the planar images. The ratio of the heart site to upper limb site (T/NT) was calculated.

2.2.6 Gamma counting of rabbit myocardium tissue

Rabbits were killed after SPECT imaging. Hearts were rapidly dissected, washed with physiologic saline to rinse blood. The hearts were weighed, and the ^{131}I radioactivity was counted with a radio-immune gamma counter. The total myocardial accumulation of ^{131}I-FIAU was expressed as percent of injection dose per gram of myocardium tissues (%ID/g).

2.2.7 RT-PCR assay

Reverse transcriptase-polymerase chain reaction assay (RT-PCR) was carried out to determine the HSV1-tk gene expression in myocardium tissues. (HSV1-tk refers to the gene, and HSV1-TK to the corresponding enzyme.) The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control. Primer sequences were as follows:

HSV1-tk,

5’-CTCACCCTCATCTTCGACCG-3’ (forward),

5’-CCTGCAGATACCGCACCGTA-3’ (reverse),

the predicted product size was 282 bp;

GAPDH,

5’-CCATGGAGAAGGCTGGGG-3’ (forward),

5’-CAAAGTTGTCATGGATGACC-3’ (reverse),

the predicted product size was 195 bp.

PCR was performed in a DNA thermal cycler with a Taq DNA polymerase. PCR conditions were as follows: a hot start after 5 minutes at 94°C; 34 cycles consisting of denaturing for 30 s at 94°C, annealing for 30 s at 57°C, and elongation for 60 s at 72°C; and a final extension period of 10 min at 72°C. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The integrated density value (IDV) of each PCR band was measured and analyzed with GelDoc XP gel imaging system and Quantity One software (Bio-Rad, USA). The amount of mRNA products for HSV1-tk was expressed as the ratio to GAPDH mRNA product.

2.2.8 Statistical analysis

Numeric data were expressed as mean ± SD. Linear regression analysis was performed to assess linearity between two variables,quantifying the strength of correlation in terms of r^2, the square of Pearson product moment correlation coefficient. Data were analyzed with the SPSS11.0 code. $P<0.05$ was considered statistically significant.

3 Results

3.1 Labeling efficiency and radiochemical purity

The radiolabeling efficiency of [^{131}I] FIAU was (53.82±2.05)% (n=5). After purification on Sep-pak

C-18 column, the radiochemical purity of the final product [^{131}I] FIAU was (94.85±1.76)% (n=5).

3.2 SPECT images of cardiac reporter gene expression

After administration of Ad5-tk to the focal myocardium, localized tracer accumulation was observed on ^{131}I-FIAU SPECT images. Ad5-tk-injected rabbits showed significant ^{131}I-FIAU activity in the anterolateral wall compared with the control rabbits, suggesting local expression of HSV1-tk after adenoviral gene transfer. the tomography images and polar maps allowed improved localization of the myocardial ^{131}I-FIAU accumulation (Fig. 1 and Fig. 2).

In the first experiment set, serial imaging demonstrated myocardial ^{131}I-FIAU accumulation in 72 h, less at 96 h, and naught at 120 h (Fig. 1). The highest radioactivity in the focal myocardium could be seen at 6 h, and then declined with time. The optimal images quality was at 24~48 h for different viral titers.

In the second experiment set, the ^{131}I-FIAU accumulation in the transfected myocardium was dose-dependent. This can be clearly seen in Fig. 2. Myocardial ^{131}I-FIAU accumulation was visually identified in all rabbits at viral titers of 1×10^8 pfu to 1×10^9 pfu and in 2 of 3 rabbits at titers of 5×10^7 pfu, but not at titers of 1×10^7 pfu. The semi-quantitative T/NT (Table 1) was the highest (6.17±0.35) for administration of 1×10^9 pfu Ad5-tk. It was lower for the administration of 5×10^8 pfu to 5×10^7 pfu, and the lowest with 1×10^7 pfu. A strong linear relationship was observed between the T/NT ratio and the Ad5-tk dose in myocardium (r^2=0.978, P<0.01).

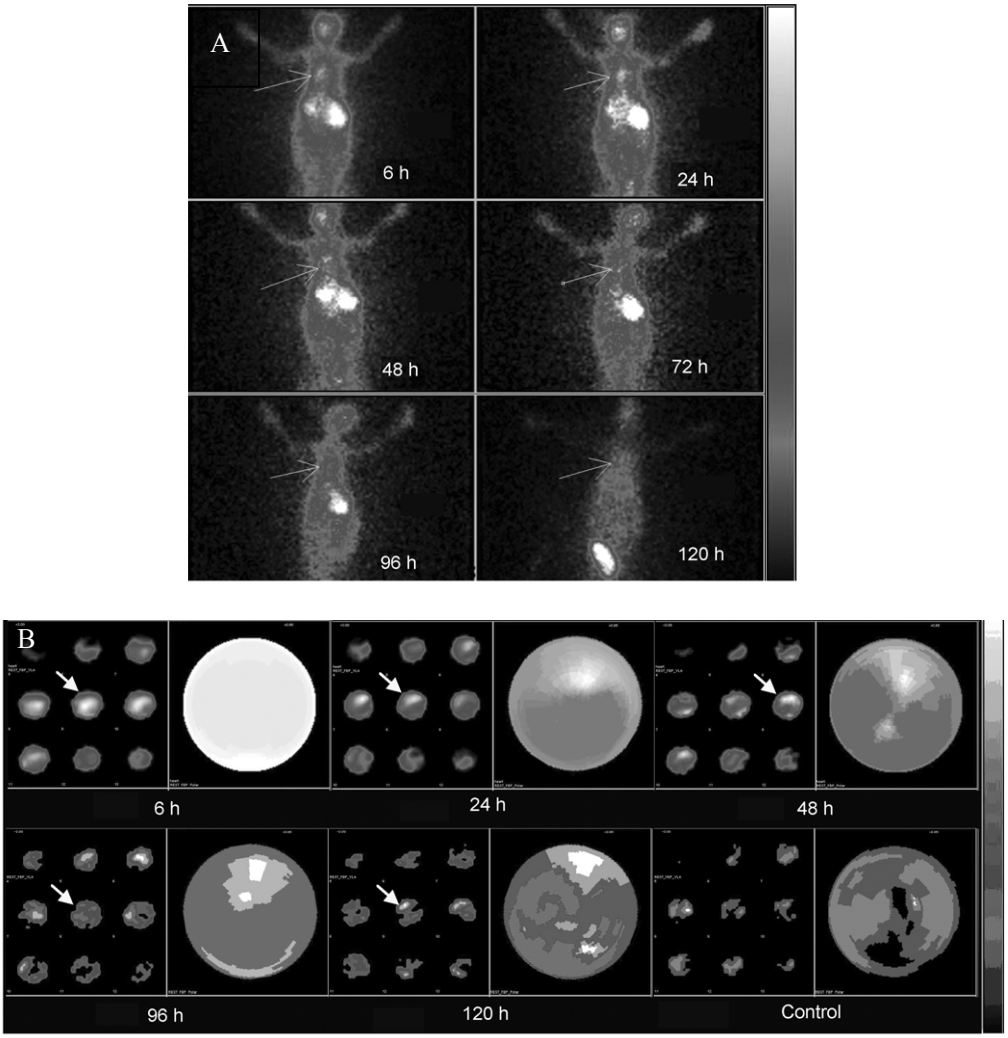

Fig.1 SPECT images of cardiac gene expression of a representative rabbit at different hours after transferring 1×10^9 pfu Ad5-tk and saline into myocardium. A. SPECT planar images; B. SPECT tomography images and polar map. The arrows indicate the sites where HSV1-tk transferred.

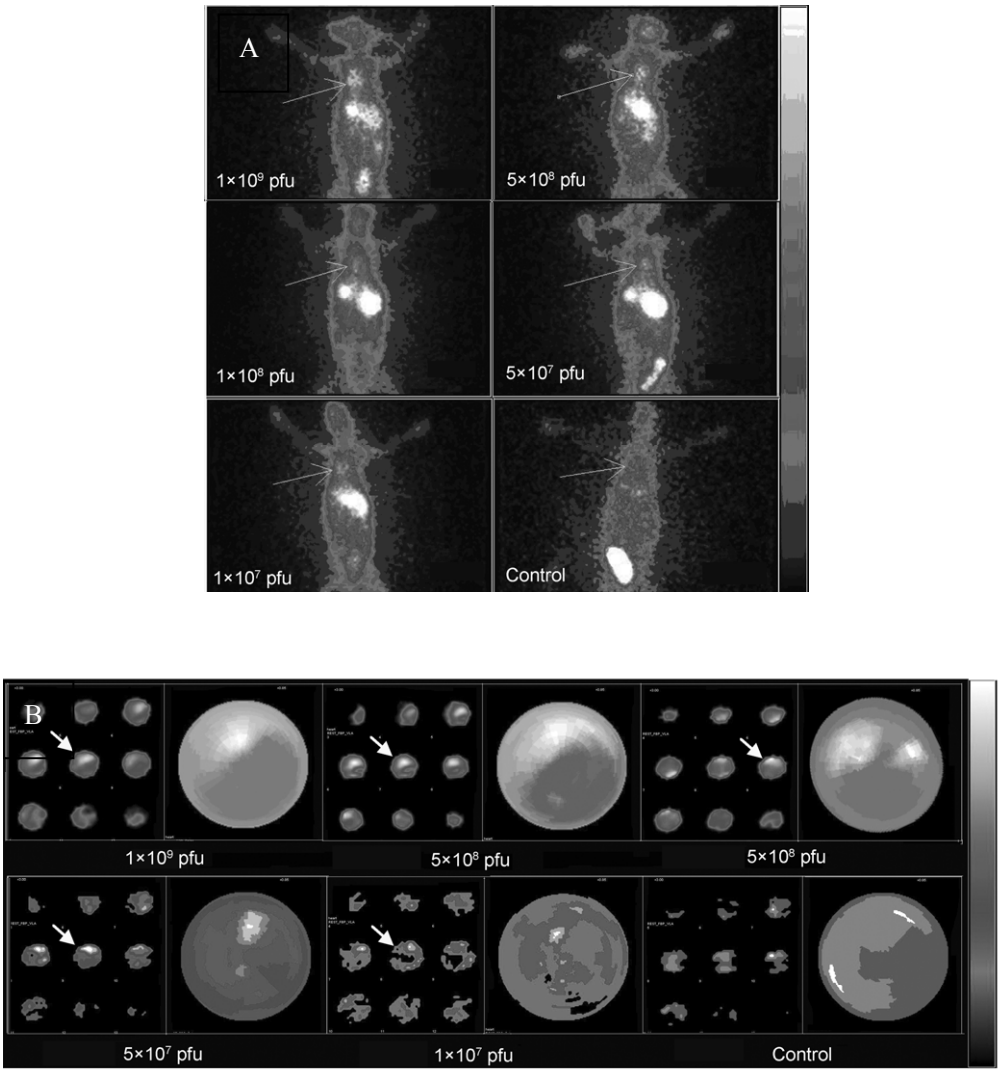

Fig.2 The 24-h SPECT images of cardiac gene expression of representative rabbits at different viral titers after transferring Ad5-tk and saline into myocardium. A. SPECT planar images; B. SPECT tomography images and polar map. The arrows indicate the sites where HSV1-tk transferred.

Table 1 Semi-quantitative data of different viral titer of Ad5-tk (mean ± SD)

Titer / pfu		T/NT ratio from SPECT images	Gamma counting /%ID·g^{-1}	HSV1-tk/GAPDH mRNA ratio
Ad5-tk	1×10^9	6.17±0.35	1.71±0.02	0.77
	5×10^8	4.44±0.11	1.43±0.03	0.70
	1×10^8	3.77±0.07	0.99±0.09	0.61
	5×10^7	3.11±0.01	0.60±0.00	0.42
	1×10^7	1.82±0.11	0.39±0.00	0.19
Saline		1.09±0.03	0.26±0.01	—

3.3 Confirmation of SPECT images with ex vivo gamma counting

Ex vivo gamma counting data were shown in Table 1. Over the range of viral titers tested ($1\times10^9 \sim 1\times10^7$pfu), the gamma counting declined with the viral titers. The highest was found in transfecting 1×10^9pfu Ad5-tk myocardium, i.e. (1.71±0.02)% ID/g, and the lowest in transfecting 1×10^7pfu Ad5-tk myocardium, (0.39±0.00) % ID/g. A strong linear relationship was observed between the gamma counting and Ad-tk dose in myocardium (r^2=0.972, P<0.01). Importantly, ROI-derived T/NT ratio from the SPECT images provided the information about ^{131}I-FIAU uptake in focal myocardium, correlated well with *ex vivo* gamma counting (r^2=0.933, P<0.01).

3.4 Confirmation of SPECT with RT-PCR

RT-PCR showed an obvious mRNA expression of

HSV1-tk in the cardiac myocardium transferred with Ad5-tk, while the saline control group revealed no HSV1-tk mRNA expression (Fig. 3). The positive results of RT-PCR amplification generated fragments near 300 bp. Data of the intensity ratio of the HSV1-tk band to internal control GAPDH band were shown in Table 1. As expected, the ratio increased with viral titer. Likewise, over the range of viral titer tested, a high correlation existed between the T/NT ratio from SPECT images and RT-PCR (r^2=0.877, P<0.01).

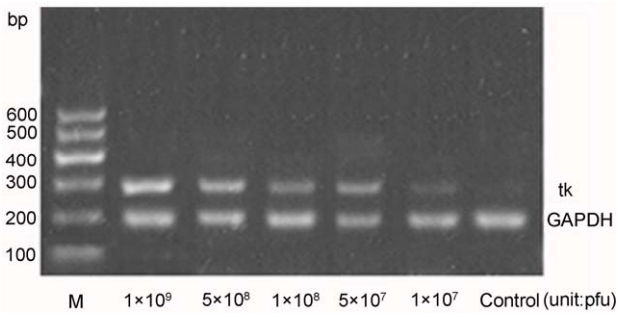

Fig. 3 RT-PCR images of cardiac gene expression at different viral titers after transferring Ad5-tk and saline into myocardium.

4 Discussion

HSV1-tk/FIAU is the reporter gene/reporter probe system commonly used in molecular imaging studies, among several potentially suitable combinations of marker genes and substrates for imaging. HSV1-tk is normally not present in host tissue, and it encodes for an enzyme catalyzing phosphorylation and, thus, intracellular accumulation of marker substrates. Previously, most applications were targeted toward imaging transgene expression in tumor models[5-7]. There were only a few studies of this system on heart and principally focused on using PET and dedicated small animal PET (microPET)[8-11]. Bengel *et al.*[12] applied the system to the heart firstly and demonstrated that HSV1-tk can be expressed in a manner similar to tumor cells using an adenoviral vector, and specific accumulation of the radioiodinated FIAU in the area of in vivo myocardial gene transfer was demonstrated at ARG. Recently, it has been shown that results obtained in rats using dedicated small field-of view microPET can be transferred to a clinical setting using conventional PET scanner[9]. Areas injected with adenovirus expressing HSV1-tk were specifically visualized in the pig heart using ^{124}I-FIAU and were validated vs. *ex vivo* count rates and immunohistochemistry. But PET and micro PET are expensive, much requirement to the probes and hardly widespread. If reporter gene imaging is to become a routine clinical strategy for monitor cardiac therapeutic gene expression, development of a clinically applicable approach for the noninvasive imaging of cardiac transgene expression is needed. Single-photon emission computed tomography (SPECT) is sensitive, objective, semi-quantitative, and widely applicable in clinic cardiac nuclear. However, the feasibility, sensitivity and the time course of SPECT imaging reporter expression are uncertain.

With ^{131}I labeled FAU, we had labeling rate of (53.82±2.05)% and radiochemical purity of (94.85±1.76)%. This provided the prerequisite to explore the expression of HSV1-tk/FIAU in myocardium using SPECT.

In this study, in rabbit after intramyocardial was transfected HSV1-tk gene, ^{131}I-FIAU SPECT images demonstrated the reporter gene expression in the inoculated myocardium as delineated by tomography images and polar map displays. Absence of detectable ^{131}I-FIAU accumulation in the myocardium of control animals inoculated with saline indicates that ^{131}I-FIAU accumulation was specific to the expressed reporter gene. Myocardial ^{131}I-FIAU uptake in experimental rabbits is confirmed by *ex vivo* gamma counts, and the expression of myocardial HSV1-TK protein specific for rabbits injected with Ad5-tk was confirmed by RT-PCR analysis. Our results suggested that the HSV1-tk gene might be used as a reporter gene and that radiolabeled FIAU might be used as a reporter probe for the image of successful gene transfer and expression in cardiac tissue.

Our serial imaging revealed the persistence of gene expression for about 4 days after gene transfection. The optimal images quality was obtained at 24~48 h for different viral titers. The time was shorter than 7 days by Inubushi *et al.*[8] having reportered, who used the PET reporter probe 9-(4-[18F]-Fluoro-3-Hydroxymethylbutyl)-Guanine ([18F]-FHBG) and a microPET to detect the expression of a mutant herpes simplex type-1 virus thymidine kinase (HSV1-sr39tk) PET reporter gene. The possible causes are that the tracer early wash-out from myocardium and microPET offers better spatial resolution over SPECT for imag-

ing reporter gene expression. However, the special radiopharmaceutical preparation and expensive imaging instruments limit microPET application. SPECT imaging is also high sensitive and tomographic. More importantly, it is extensive application in clinical studies and it is much cheaper than microPET.

The sensitivity study using various viral titers showed high detectability of the gene expression. The average level of ^{131}I-FIAU accumulation in transfected myocardium correlated strongly with the dose (pfu) of injected Ad vector. However, the viral titer of 1×10^7 pfu obviously could not get clear image. We considered that the threshold of Ad viral titer should be at least 5×10^7 pfu, and better to be $1\sim5\times10^8$ pfu, but not 1×10^9 pfu. Although more clear images and higher semi-quantitative data were obtained from semi-quantitative analysis, more mortality rabbit was seen in this subgroup (data not shown). The possible reasons may be animal host immune response against adenovirus and is also expected to occur in human studies[13].

SPECT imaging myocardial reporter gene expression can offer additional information. Firstly, SPECT is semi-quantitative. The close correlation of the ratio of T/NT gained from SPECT images for myocardial ^{131}I-FIAU accumulation derived *in vivo* with that by *ex vivo* gamma counting and mRNA degree from RT-PCR indicates that tracer activity concentrations can be accurately and noninvasively measured with SPECT. Secondly, SPECT can offer the information of reporter probe distribution to all over of body. In our study, ^{131}I-FIAU was ingested by not only the myocardium but also the stomach and liver. Accumulation of radioactivity in stomach is related to ^{131}I-FIAU deiodination and non-specificity uptake. Significant ^{131}I-FIAU uptake within the liver was likely due to the quality of cardiac injection with egress of adenovirus from the myocardium into the systemic circulation and eventual binding to coxsackie-adenovirus receptors on hepatocytes. Substituting a cardiac tissue-specific promoter (eg, myosin light chain kinase)[14] for the constitutive CMV promoter may diminish extra-cardiac activity. Although not a major focus in the present study, potential systemic toxicity can be assessed by imaging extramyocardial leakage through reporter probe accumulation in other organs.

5 Conclusion

The present study confirmed the feasibility of HSV1-tk as reporter gene and ^{131}I-FIAU as reporter probe for cardiac reporter gene imaging. The optimal Ad5-tk titer for imaging is $1\sim5\times10^8$ pfu and the optimal imaging time is 24~48 h after gene transferred. Thus, the imaging of transgene expression in the heart is feasible and may be used for the noninvasive imaging of gene therapy in human.

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