Positron-Emission Tomography Reporter Gene Expression Imaging in Rat Myocardium

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Background—This study examines the quantitative accuracy, detection sensitivity, and time course of imaging the expression of a mutant herpes simplex type-1 virus thymidine kinase (HSV1-sr39tk) PET reporter gene in rat myocardium by using the PET reporter probe 9-(4-[18 F]-Fluoro-3-Hydroxymethylbutyl)-Guanine ([18 F]-FHBG) and a small-animal PET (microPET).

Methods and Results—In 40 rats, adenovirus expressing HSV1-sr39tk driven by a cytomegalovirus promoter (Ad-CMV-HSV1-sr39tk, 1×10⁹ to 1×10⁸ pfu) was injected through a thoracotomy directly into the left ventricular myocardium. After 3 days, myocardial perfusion was imaged with [15 N]-ammonia for delineating the left ventricular myocardium, followed by imaging the expression of the reporter gene with intravenous [18 F]-FHBG. The total myocardial [18 F]-FHBG accumulation was quantified in percent of injected dose (%ID). Immunohistochemistry and autoradiography demonstrated HSV1-sr39tk enzyme (HSV1-sr39TK) and accumulation of [18 F]-FHBG in the inoculated myocardium in 3 rats each. In 24 rats with various viral titers, the %ID was correlated with ex vivo well counting (r²=0.981, P<0.0001) and myocardial HSV1-sr39TK activity by tissue enzyme activity assay (r²=0.790, P<0.0001). Myocardial [18 F]-FHBG accumulation was identified at viral titers down to 1×10⁷ pfu. In 6 rats serially imaged up to day 17, myocardial [18 F]-FHBG accumulation on microPET peaked on days 3 to 5 and was no longer identified on days 10 to 17.

Conclusions—HSV1-sr39tk reporter gene expression can be monitored with [18 F]-FHBG and microPET in rat myocardium quantitatively and serially with high detection sensitivity. Cardiac PET reporter gene imaging offers the potential of monitoring the expression of therapeutic genes in cardiac gene therapy. (Circulation. 2003;107:326-332.)

Key Words: nuclear medicine ■ imaging ■ genes ■ myocardium ■ gene therapy
Methods

Study Design
Forty-three male Sprague-Dawley rats (304±56 g, Harlan, Indianapolis, Ind) were studied. In 40 rats, adenovirus carrying HSV1-sr39tk driven by a cytomegalovirus promoter (Ad-CMV-HSV1-sr39tk, 1×10^9 to 1×10^10 pfu) was injected through a thoracotomy directly into the left ventricular (LV) myocardium. Three control rats were similarly injected with Ad-CMV-Firefly luciferase (Ad-CMV-Fluc, 1×10^9 pfu). After 3 days, myocardial perfusion was imaged with [18F]-FDG for delineating the LV myocardium followed by imaging the expression of the reporter gene with [18F]-FHBG, and the total myocardial [18F]-FHBG accumulation was quantified in percent of injected dose (%ID).

Immunohistochemistry and digital autoradiography were performed after microPET imaging in 3 rats each with Ad-CMV-HSV1-sr39tk to demonstrate in transfected myocardium the HSV1-sr39tk enzyme (HSV1-sr39TK) and accumulation of [18F]-FHBG. In 24 rats with various viral titers of Ad-CMV-HSV1-sr39tk, the %ID calculated from microPET images was compared with that from ex vivo well counting and with the myocardial HSV1-sr39TK activity by tissue enzyme activity assay to validate the quantitative accuracy of %ID. The regional myocardial [18F]-FHBG accumulation in the same 24 rats was also analyzed visually to determine the lowest viral titer needed for imaging gene expression and for assessing the detection sensitivity in %ID of myocardial [18F]-FHBG accumulation. The time course of myocardial and hepatic [18F]-FHBG accumulation was examined in 6 rats with Ad-CMV-HSV1-sr39tk by serial microPET imaging up to day 17 after transfection. The study design is summarized in the Table.

This study was approved by the University of California Los Angeles Animal Research Committee and performed in accordance with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and NIH Guidelines for Research Involving Recombinant DNA Molecules.

Radiolabeled Compounds
[13N]-ammonia and [18F]-FHBG were produced as described previously.8,9

Recombinant Adenoviruses
HSV1-sr39tk is a PET reporter gene, which leads to phosphorylation and consequent accumulation of [18F]-FHBG in tissues expressing HSV1-sr39tk. Fluc is a reporter gene for optical imaging.10 E1-deleted, replication-defective recombinant Ad-CMV-HSV1-sr39tk and Ad-CMV-Fluc were constructed and purified as described previously.5,10

Animal Preparation
Rats were anesthetized with sodium pentobarbital (50 mg/kg IP). After intravenous injection of [13N]-ammonia (83.1±17.0 MBq), a scout image of myocardial perfusion was first obtained for 4 minutes and immediately reconstructed with filtered back-projection (FBP) algorithms to confirm adequate positioning of the heart in the scanner’s FOV. The heart was repositioned into the FOV if necessary, and myocardial perfusion images were obtained from 10 to 20 minutes after [13N]-ammonia injection. With the animal kept in exactly the same position, [18F]-FHBG (56.5±22.9 MBq) was injected intravenously, and images of [18F]-FHBG accumulation were acquired from 30 to 60 minutes later.

Image Reconstruction
MicroPET image data were reconstructed by using statistical maximum a posteriori probability (MAP) algorithms with a smoothing parameter of 1.0 and a total of 20 iterations into 24 transaxial images on a SPARC Ultra 1 workstation (Sun Microsystems). The reconstructed images were consistent with 128×128 image matrices of 0.40×0.40-mm pixel size and 0.75-mm slice thickness; the spatial resolution is 1.4 mm full-width at half-maximum (FWHM). Photon attenuation was corrected as described previously.11

Image Display
To define the site of [18F]-FHBG accumulation, color-coded transaxial [18F]-FHBG images were superimposed on inverted gray-scaled [13N]-ammonia myocardial perfusion images and reoriented into short-axis images. The reorientation parameters were the same as used for the [13N]-ammonia images. Polar maps were reconstructed from the maximum activity profiles of the 15 contiguous short-axis cuts, including the apex, using standard clinical PET software.7

Summary of Study Design

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Injected Virus</th>
<th>Viral Titer, pfu</th>
<th>No. of Rats</th>
<th>Studies Performed</th>
</tr>
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<tr>
<td>Control study</td>
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<td>3</td>
<td>MicroPET (3)</td>
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<tr>
<td>Immunohistochemistry</td>
<td>Ad-CMV-HSV1-sr39tk</td>
<td>1×10^9</td>
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<td>MicroPET (3), Immunohistochemistry</td>
</tr>
<tr>
<td>Digital autoradiography</td>
<td>Ad-CMV-HSV1-sr39tk</td>
<td>1×10^9</td>
<td>3</td>
<td>MicroPET (3), Digital autoradiography</td>
</tr>
<tr>
<td>Quantitative accuracy and detection sensitivity</td>
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<td>1×10^9</td>
<td>6</td>
<td>MicroPET (3), Well counting, TK assay</td>
</tr>
<tr>
<td>Time course</td>
<td>Ad-CMV-HSV1-sr39tk</td>
<td>1×10^9</td>
<td>6</td>
<td>MicroPET (3, 5, 7, 10, 12, 14, and 17)</td>
</tr>
</tbody>
</table>

Number(s) in parentheses after “MicroPET” show the scanned day(s) after transfection. TK assay indicates tissue enzyme activity assay for HSV1-sr39TK.
Quantitative Analysis

Cross-calibration for conversion of counts per minute to MBq was performed with a cylindrical phantom with a diameter of 35 mm filled with a $^{18}$F solution of known activity concentration. Guided by the $^{[13}\text{N}]$-ammonia images, circular, 1-cm diameter regions of interest (ROI) were placed over the entire LV (ROI_LV) on 12 $^{[18}\text{F}]$-FHBG transaxial images of the mid-LV portion. Background activity was similarly estimated from 12 ROIs of the same size (ROI_BG) assigned to the right lung on the same 12 slices. The total myocardial accumulation of $^{[18}\text{F}]$-FHBG was corrected for background activity in each ROI, summed for the 12 slices, and expressed as %ID:

$$ \frac{\text{mean activity in ROI}_{\text{LV}} (\text{MBq/g})}{\text{injected dose} (\text{MBq})} \times 100. $$

Accumulation of $^{[18}\text{F}]$-FHBG in the liver was also determined from microPET images by placing a ROI to the liver (ROI_LIVER), partially visualized on transaxial $^{[18}\text{F}]$-FHBG images for the heart, and expressed as %ID per gram of liver (specific gravity of 1):

$$ \frac{\text{mean activity in ROI}_{\text{LIVER}} (\text{MBq/g})}{\text{injected dose} (\text{MBq})} \times 100. $$

Immunohistochemistry

Rats were killed immediately after the microPET imaging, and the hearts were rapidly dissected and fixed in 10% formalin. Heart sections were prepared at 10-μm thickness perpendicular to the LV axis with a cryomicrotome (Leica) and placed on glass slides coated with Vectabond (Vector Laboratory). Each tissue was hydrated with PBS and preincubated at room temperature with 5% horse serum for 60 minutes to prevent nonspecific accumulation. After removal of horse serum, sections were incubated with rabbit anti-thymidine kinase antibody (courtesy of M.E. Black, University of Washington, Seattle, Wash) at 1/1000 dilution at room temperature for 60 minutes, washed with 3 rinses of PBS for 5 minutes each, and air-dried. Sections were then incubated with fluorescein anti-rabbit IgG antibody (Vector Laboratory) at 1/200 dilution at room temperature for 60 minutes and rinsed in 3 successive PBS washes before glycerol mount and cover slipping. Immunofluorescent images were visualized with UV-excitation by a Nikon Optiphot microscope (Nikon).

Digital Autoradiography

Rats were killed immediately after microPET imaging and rapidly frozen with liquid nitrogen. The excised chest was embedded in 5% carboxymethylcellulose (Sigma Aldrich). The block was equilibrated with liquid nitrogen and sectioned in transaxial cuts with a slice thickness of 45 μm, using a PMV cryomicrotome. The cryosections were exposed directly onto digital plates for up to 12 hours. All digital plates were scanned on a Fuji Bas 5000 digital autoradiographic system (Fuji photo film) at a resolution of 25 μm.

Gamma Well Counting

Rats were killed immediately after microPET imaging, and the hearts were rapidly dissected, rinsed from blood, weighed, and counted for $^{18}$F radioactivity in a gamma well counter (Cobra II Auto-Gamma, Packard). The total myocardial accumulation of $^{[18}\text{F}]$-FHBG was expressed as %ID.

Tissue Enzyme Activity Assay for HSV1-sr39TK

Myocardial HSV1-sr39TK activity was determined as described previously. Results are expressed as percent conversion of 8-3H-PCV in (dpm/μg protein per minute of tissue extract)/(dpm of control sample)×100.

Statistical Analysis

Mean values are given with standard deviations. One-way ANOVA was used for statistical analyses among 4 groups with different viral titers, and Fisher’s PLSD for following post hoc tests. Receiver operating characteristics (ROC) curve analysis was used for visual detection of myocardial $^{[18}\text{F}]$-FHBG accumulation. Linear and non-linear correlation and nonparametric Spearman’s correlation coefficient were applied to the relation between %ID calculated from $^{[18}\text{F}]$-FHBG microPET images and that from well counting or myocardial HSV1-sr39TK activity. Probability values <0.05 were considered statistically significant.

Results

Quality of MicroPET Images

The left and right ventricular myocardium was clearly visualized on $^{[13}\text{N}]$-ammonia microPET images in all animals.
Localized tracer accumulation was detected on \[^{18}\text{F}\]-FHBG images in rats injected with Ad-CMV-HSV1-sr39tk; its location in the LV anterolateral wall was verified through superimposition on the \[^{13}\text{N}\]-ammonia images (Figure 1). Moreover, short-axis images and polar map displays allowed improved localization of the myocardial \[^{18}\text{F}\]-FHBG accumulation and of its extent and distribution (Figure 2).

**Control Study**

In 3 control rats injected with Ad-CMV-Fluc, no retention of \[^{18}\text{F}\]-FHBG was noted on the microPET images. This effectively excluded nonspecific \[^{18}\text{F}\]-FHBG accumulation in myocardium inoculated with a control transgene. Further, estimates of the %ID of myocardial \[^{18}\text{F}\]-FHBG accumulation in the control rats averaged 0.000\pm0.003% and thus approached zero, supporting the validity of the approach used for correction of background activity.

**Immunohistochemistry**

Immunohistochemistry in 3 rats revealed HSV1-sr39TK activity in the inoculated anterolateral but little if any in the inferior and posterior walls and the septum. No inflammatory response such as increased leukocytic infiltration was observed microscopically (Figure 3).

**Digital Autoradiography**

Digital autoradiography revealed \[^{18}\text{F}\]-FHBG accumulation in the inoculated anterolateral wall of all 3 rats. The myocardial distribution of \[^{18}\text{F}\]-FHBG accumulation as demonstrated on the autoradiographs was similar to that seen on the microPET images obtained in the same animals (Figure 4).

**Quantitative Accuracy for \[^{18}\text{F}\]-FHBG MicroPET Imaging**

The %ID for myocardial \[^{18}\text{F}\]-FHBG accumulation determined in vivo from microPET images was highly correlated with that determined postmortem by well counting. The %ID also significantly correlated with the myocardial HSV1-sr39TK activity, although not as closely as with well-counted activity (Figure 5).

**Detection Sensitivity for HSV1-sr39tk Gene Expression**

Myocardial \[^{18}\text{F}\]-FHBG accumulation was visually identified in all rats at viral titers of \(1\times10^8\) and \(1\times10^9\) pfu and in 4 of 6 rats at titers of \(1\times10^7\) pfu but in none at titers of \(1\times10^6\) pfu. ROC curve analysis suggests a cutoff %ID value of 0.006% for visually identifiable myocardial \[^{18}\text{F}\]-FHBG accumulation (Figure 6).

**Time Course of HSV1-sr39tk Gene Expression**

Serial imaging demonstrated myocardial \[^{18}\text{F}\]-FHBG accumulation on days 3 and 5, less on day 7, and no longer on days 10 to 17. Significant \[^{18}\text{F}\]-FHBG uptake was observed in the liver on day 3 but had declined on day 5 (Figure 7).

**Discussion**

In rats after intramyocardial injection of Ad-CMV-HSV1-sr39tk, \[^{18}\text{F}\]-FHBG microPET images demonstrated the PET reporter gene expression in the inoculated myocardium as delineated by \[^{13}\text{N}\]-ammonia perfusion imaging and polar map displays. Absence of detectable \[^{18}\text{F}\]-FHBG accumulation in the myocardium of control animals inoculated with Ad-CMV-Fluc indicates that \[^{18}\text{F}\]-FHBG accumulation was specific to the expressed reporter gene. Immunohistochemistry and digital autoradiography confirmed the expression of the enzyme induced by the PET reporter gene and accumulation of the PET reporter probe in the inoculated myocardial wall.
The close correlation of %ID for total myocardial $[^{18}F]$-FHBG accumulation derived in vivo by microPET with that by ex vivo well counting and tissue HSV1-sr39TK activity indicates that tracer activity concentrations can be accurately and noninvasively measured with microPET. The sensitivity study using various viral titers showed high detectability of the gene expression; serial imaging revealed the persistence of gene expression for about 1 to 2 weeks after transfection.

**Correlation in Quantitative Accuracy Study**

The %ID for myocardial $[^{18}F]$-FHBG accumulation determined by microPET was highly correlated with that by well counting. This close correlation was maintained even when data from only 12 rats with $1 \times 10^5$ and $1 \times 10^6$ pfu of viral titers and thus with a more suitable range for reporter gene expression imaging were analyzed ($y=0.78x-0.0036$, $r^2=0.957$, $P<0.0001$). The less-than-unity slope of the regression line indicates a modest systematic underestimation of the %ID by microPET, possibly because of an underestimation of the calibration factor. Although the %ID by microPET did not correlate with myocardial HSV1-sr39TK activities as closely as with well-counted activity, the correlation remained statistically significant. Possible reasons for this somewhat poorer correlation might include that the net $[^{18}F]$-FHBG accumulation (a) was calculated for the entire heart, whereas the enzyme activity is only calculated per microgram of protein; (b) reflects the mass flux of substrate (ie, product of enzyme activity and substrate) as compared with the phosphorylating enzyme activity only; and (c) depends on intracellular concentrations of thymidine competing with $[^{18}F]$-FHBG for phosphorylation. The correlation analysis also revealed possible “plateauing” of $[^{18}F]$-FHBG accumulation relative to HSV1-sr39TK activity, which is similar to the relation between $[8\text{-}^{14}C]$-ganciclovir uptake and HSV1-TK enzyme activity observed previously in the liver of mice.\(^4\)

**Issues Specific for PET Reporter Gene Expression Imaging of the Heart**

Although imaging of PET reporter gene expression in rodents has been reported for oncological or neurological applications,\(^4,5\) information on its applications in the heart has
remained limited. This may be because of the need for special experimental skills, but also because of complexities related to imaging the small and thin-walled rodent heart. In the present study, we acquired images of the myocardial uptake of \[^{13}\text{N}\]-ammonia and of \[^{18}\text{F}\]-FHBG while the animal remained in the same position. Images of gene expression thus could be superimposed on images of myocardial perfusion and the site and extent of gene expression in the myocardium be displayed on polar maps. Furthermore, the image data were reconstructed with MAP algorithms, which improved the volumetric resolution to 1.43 (±2.7) mm\(^3\) as compared with 2.7 (±8) mm\(^3\) with the conventional FBP algorithms. Furthermore, the “total” myocardial accumulation of the PET reporter probe was measured with a background-subtraction method developed for the present study. This subtraction method offered a means for estimating the magnitude of gene expression and for overcoming problems related to partial volume effects caused by the thin wall of the rat heart and the limited spatial resolution of microPET relative to size of the heart. The approach required no assumptions for partial volume because it accounted for all activity within the confines of the myocardium and in excess of background activity. Finally, \[^{18}\text{F}\]-FHBG uptake occurred also in the liver, seen best on day 3 and declining on day 5, and probably was related to egress of adenovirus from the myocardium into the systemic circulation and eventual binding to coxsackie-adenovirus receptors on hepatocytes. Substituting cardiomyocyte-specific promoters, such as myosin light chain kinase, for constitutive CMV promoter may diminish extracardiac activity.

**Impact of PET Reporter Gene Expression Imaging on Human Cardiac Gene Therapy**

Gene therapy entails multiple complex steps including delivery and expression of the therapeutic gene and action of the therapeutic protein leading to therapeutic benefits. For gene therapy to be efficacious, the transgene expression must be of sufficient magnitude and duration, both of which may vary between individuals, as also seen in the present study. Only when sufficient therapeutic gene expression is confirmed through imaging of PET reporter gene expression can the efficacy of the gene therapy be evaluated appropriately in any given subject. Therefore, identification of gene expression noninvasively may prove critical for optimization of gene therapy. Further, if the magnitude of expression of the therapeutic gene determines its therapeutic effects, then PET reporter gene imaging may be useful for predicting its therapeutic effect. Additionally, unnecessarily long-term lo-
cal expression of, for example, cardiac angiogenic therapeutic genes may lead to undesirable formation of hemangiomas. Therefore, monitoring of delivery and expression of cardiac therapeutic genes noninvasively by PET reporter gene imaging may become useful. Since PET imaging can be performed in subjects ranging from rodents to humans, images of cardiac PET reporter gene expression could aid in the design of strategies for gene therapy through animal studies and for evaluating the effect of human cardiac gene therapy.

Conclusions
After intramyocardial injection of Ad-CMV-HSV1-sr39tk, PET reporter gene expression can be monitored with a PET reporter probe $[^{18}F]${\textsubscript{F}}HBG and microPET in the rat myocardium noninvasively, quantitatively, and repeatedly at high detection sensitivities. Cardiac PET reporter gene imaging offers the potential of monitoring the expression of therapeutic genes in cardiac gene therapy in animals and humans.

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References
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