Noninvasive Imaging of Transgene Expression by Use of Positron Emission Tomography in a Pig Model of Myocardial Gene Transfer

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Background—Radionuclide imaging of reporter gene expression may be useful for noninvasive monitoring of clinical cardiac gene therapy. Experience until now, however, has been limited to small animals.

Methods and Results—To evaluate feasibility in a clinically applicable setting, pigs were studied by conventional positron emission tomography (PET) 2 days after regional intramyocardial injection of control adenovirus or adenovirus carrying herpesviral thymidine kinase reporter gene (HSV1-tk). Myocardial blood flow was quantified by use of [13N]ammonia. Subsequently, kinetics of the reporter substrate [124I]-2′-fluoro-2′-deoxy-5-iodo-1-β-D-arabino-furanosyluracil (FIAU) were assessed over a period of 2 hours. Areas infected with adenovirus expressing HSV1-tk showed significantly elevated FIAU retention during the first 30 minutes after injection. At later times, washout was observed, and retention was not different from that in areas infected with control virus or remote myocardium. Early in vivo FIAU uptake correlated with ex vivo images, autoradiography, and immunohistochemistry for reporter gene product after euthanasia. After intramyocardial injection of both adenoviruses, myocardial blood flow was mildly elevated compared with that in remote areas, consistent with histological signs of regional inflammation.

Conclusions—In vivo quantification of regional myocardial transgene expression is feasible with clinical PET methodology, the radioiodinated reporter probe FIAU, and the HSV1-tk reporter gene. Radioactivity efflux after specific initial uptake was not observed previously in tumor studies, suggesting that tissue-specific differences in nucleoside metabolism influence reporter probe kinetics. By coregistering reporter gene expression with additional biological parameters such as myocardial blood flow, PET allows for noninvasive characterization of the success of cardiac gene transfer along with its functional correlates. (Circulation. 2003;108:2127-2133.)

Key Words: imaging ■ gene therapy ■ radioisotopes ■ genes

Cardiac gene therapy is promising for treatment of various diseases, including heart failure and ischemia. Although some gene therapeutic approaches have reached clinical phase II trials, analysis of the success of gene delivery is still limited to assessment of improvement of symptoms or routine test results. Thus, there is increasing emphasis on development of imaging strategies that allow for noninvasive evaluation of transgene expression in the heart.

Assessment of cardiac reporter gene expression has recently been achieved by use of optical bioluminescence imaging. This technique, however, remains limited to small animals and does not allow for regional myocardial evaluation. Positron emission tomography (PET) is well suited for imaging of biological processes, not only because of high sensitivity for tracer detection but also because quantitative tomographic information is provided. The principle of nuclear imaging of transgene expression is based on reporter genes expressing a gene product that is normally absent in host tissue and that results in specific accumulation of a radioactive reporter probe. Imaging of cardiac reporter gene expression was recently performed in living rats with dedicated micro-PET technology. Compared with clinical PET scanners, micro-PET has superior sensitivity and spatial resolution, but it is limited to small animals. Whether in vivo imaging of cardiac reporter gene expression can be transferred to conventional PET, which would be required for future human application, has not yet been demonstrated.
Thus, we evaluated the feasibility of cardiac reporter gene imaging in a large-animal model with conventional PET methodology. The herpesvirus type 1 thymidine kinase reporter gene (HSV1-tk) was adenovirally transduced and traced by the radiiodinated reporter substrate 2'-fluoro-2'-deoxy-5-iodo-1-β-D-arabinofuranosyluracil (FIAU). In addition, myocardial reporter probe kinetics were investigated, and functional effects of gene transfer were determined noninvasively by quantifying myocardial blood flow during the same PET session.

Methods

Experimental Protocol
The experimental protocol was approved by the regional governmental commission for animal protection (Regierung von Oberbayern).

Five young domestic pigs (Versuchstation Thalhausen, Germany) underwent left thoracotomy under anesthesia (isoflurane 0.4% to 1.5% vol/vol, propofol 7 to 10 mL/h IV, fentanyl IV as required). The pericardium was opened, and gene transfer was performed with 1 × 10^10 pfu of replication-defective type 5 adenovirus prepared in 8 to 10 syringes containing equal amounts of vector (~200 μL volume). Those were injected consecutively into an area of ~3 × 3 cm of anterior/anterolateral left ventricular myocardium. The first-generation, E1 region-deleted adenoviral vector either carried HSV1-tk under transcriptional control of human cytomegalovirus promoter (AdΔR71E1) or carried no exogenous gene (AdΔd70E1). Injection was slightly modified between animals. Whereas the first 2 pigs received AdΔR71E1 into the basal anterolateral wall, only AdΔd70E1 as control was injected in pig 3. In the remaining 2 animals, both AdΔR71E1 and AdΔd70E1 were injected into different areas in basal or apical myocardium. Injection sites were switched between pigs 4 (AdΔd70E1 into basal, AdΔR71E1 into apical wall) and pig 5 (AdΔR71E1 into basal, AdΔd70E1 into apical wall) to control for effects of localization on imaging.

Two days later, pigs were anesthetized and underwent PET. After PET, animals were killed (KCl 15 mg IV, sodium pentobarbital [Narcoren] 10 mL IV). Hearts were excised, rinsed, and sliced into transmural myocardial specimens were embedded in paraffin. From each frozen tissue sample, 40-μm transmural slices were prepared (HM500OM microtome, Microm). Digital autoradiography was performed (Sykam), acetonitrile was removed in vacuum. Aliquots (400 μL) were injected onto a Nucleosil 100 5-C18 column (CS-Chromatographie Service, Langerwehe/Germany). The gradient was 0% to 40% acetonitrile in water containing 0.1% trifluoroacetic acid over 20 minutes. One-minute fractions were collected and gamma-counted.

Ex Vivo Imaging
After euthanasia, cardiac short-axis slices were placed in the scanner. A 10-minute transmission scan was obtained, followed by a 30-minute emission scan. In attenuation-corrected images, regions of interest were defined manually for 9 myocardial areas similar to in vivo analysis, and regional tracer concentration was calculated as a percentage of the average of all regions.

Autoradiography
From each frozen tissue sample, 40-μm transmural slices were prepared (HM500OM microtome, Microm). Digital autoradiography (Phosphorimager 445SI, Molecular Dynamics) was performed for all slices from an animal on the same imaging plate, and ratios of maximal counts in virus injection areas relative to remote area were calculated.

Histology and Immunohistochemistry
Formalin-fixed myocardial specimens were embedded in paraffin. For histological examination, paraffin sections (2 μm) were stained with hematoxylin-eosin. For immunohistochemistry, sections were deparaffinized, dehydrated, and pressure-cooked in citrate buffer, followed by blocking of endogenous peroxidase (3% H2O2/methanol). The streptavidin–horseradish peroxidase technique was used for immunostaining, with incubation of slides first with polyclonal rabbit–anti–HSV1-tk antibody (1:200) and consecutively with ready-to-use anti-rabbit antibody (Dako ChemMate Detection Kit K5001).

Statistical Analysis
Data were analyzed with StatView5.0 software (SAS Institute). Values are expressed as mean ± SD. Differences were assessed by paired/unpaired t test as appropriate or (for multiple comparisons) by 1-way ANOVA with post hoc Scheffe’s F test. Simple linear regression was used to describe relations between pairs of continuous variables. A 2-sided probability value of P < 0.05 was defined as significant.

Results

Static PET Imaging
Myocardial perfusion was mildly heterogeneous in all animals, but no perfusion defects were observed. At visual...
analysis of early FIAU images, significant accumulation was detectable in all myocardial areas infected with AdHCMVTk. No accumulation was observed in areas infected with Adnull or remote myocardium. Two hours after FIAU injection and at 24-hour and 48-hour PET imaging (pig 1), HSV1-tk–expressing cardiac areas could no longer be separated from the blood pool (Figure 1).

Reporter probe uptake in other organs may indicate extramyocardial vector leakage. Standardized uptake values (=activity concentration/×body weight/injected activity), derived from regions of interest in early transaxial FIAU images, were low overall for the lungs (0.31±0.06) as major targeting organ for systemic adenoviral leakage compared with nonmanipulated myocardium (0.87±0.21) and liver (1.14±0.44). Values in pig 3, which received control vector only, were in the range of the other animals. Taken together, these data suggest little influence of extramyocardial vector leakage in the present study.

Analysis of Cardiac $^{124}$I FIAU Kinetics In Vivo

Retention
Regional myocardial radioactivity retention at various times after injection is depicted in Figure 2. In areas infected with AdHCMVTk, retention was significantly higher at early times, up to 30 minutes. Values in areas infected with Adnull were comparable to those in remote myocardium. Overall, significant tracer wash-out was observed.

Plasma Metabolites
At high-performance liquid chromatography of venous blood, a peak suggestive appearance of free iodine was observed, which increased over time but remained lower than the peak of labeled FIAU. The percentage of free iodine relative to labeled FIAU was 22±18% at 7 minutes, 39±33% at 30 minutes, and 56±13% at 120 minutes. No other peaks were detected, suggesting the absence of additional metabolites.

Figure 1. PET slices and polar maps of myocardial perfusion and $^{124}$I FIAU distribution 2 days after injection of AdHCMVTk (pig 2, left) or control virus (Adnull, pig 3, right; arrows mark injection site) into basal anterolateral myocardium. Perfusion in areas of vector injection is mildly increased for both animals. Specific uptake of $^{124}$I FIAU is observed in early images in AdHCMVTk animal. At late imaging, this area is no longer separated from blood pool. LA indicates left atrium; RA, right atrium; LV, left ventricle; and RV, right ventricle.
Simplified Compartmental Modeling
Analysis was restricted to the first 20 minutes to minimize the effects of free iodine. In areas of HSV1-tk expression, wash-in of tracer into tissue ($K_1$) was significantly higher than wash-out from tissue ($k_2$), yielding significantly higher VD ($K_1/H_1=0.29/0.14$ vs $0.22/0.10$; $P<0.05$) compared with areas infected with control adenovirus or remote areas ($K_1/H_1=0.33/0.10$ vs $0.25/0.10$; $P<0.05$).

Effects of Gene Transfer on Myocardial Blood Flow
Reporter substrate accumulation and myocardial blood flow were coregistered and regionally matched. At intraindividual comparison, resting flow was mildly but significantly higher in areas of virus injection than in remote areas ($0.99\pm0.23$ vs $0.85\pm0.26$ mL·min$^{-1}$·g$^{-1}$; $P=0.045$). Comparison between areas of $Ad_{hCMVTK}$ and $Ad_{null}$ injection, however, revealed no significant difference ($0.97\pm0.28$ versus $1.02\pm0.20$ mL·min$^{-1}$·g$^{-1}$; $P=0.81$).

Ex Vivo Imaging/Autoradiography
PET imaging of macroscopic heart slices revealed increased radioactivity in areas of $Ad_{hCMVTK}$ injection only (Figure 3A). Segmental ex vivo tracer concentration correlated significantly with early myocardial in vivo tracer uptake ($r=0.80$; $P<0.001$). Autoradiography confirmed higher radioactivity in $Ad_{hCMVTK}$ areas. Count ratio relative to remote myocardium was $2.50\pm0.94$ compared with $1.01\pm0.16$ for samples from $Ad_{null}$ areas ($P=0.046$). Consistent with the vector administration technique, epicardium showed higher accumulation than endocardium in $Ad_{hCMVTK}$ areas (Figure 3B).

Histology/Immunohistochemistry
Histology revealed various degrees of global inflammatory reaction in pericardial layers of all tissue samples as a consequence of the surgical procedure. Signs of intramural lymphocytic infiltration were observed in samples from areas of both $Ad_{hCMVTK}$ and $Ad_{null}$ injection but not in remote areas (Figure 4).

At immunohistochemistry, HSV1-TK enzyme was identified in nuclei and in cytoplasm of cells in all areas of $Ad_{hCMVTK}$ injection but not in other areas (Figure 5).

Discussion
In this study, regional myocardial transgene expression was quantified in vivo for the first time by use of clinical PET, the HSV1-tk reporter gene, and the radiolabeled nucleoside FIAU. There is considerable experience for nuclear imaging of reporter gene expression in oncology, and initial
human experience in brain tumors has already been reported. Reporter gene imaging has been transferred from tumors to the heart only recently by use of ex vivo autoradiography followed by in vivo static imaging with micro-PET technology. The present data not only confirm that noninvasive imaging of cardiac gene expression is transferable from experimental high-sensitivity and high-resolution micro-PET to conventional, clinically applicable PET but also give insights into reporter probe dynamics, which have not been obtained previously in small animals. For the first time, the presence of tissue-specific myocardial properties influencing kinetics of radiolabeled nucleosides is suggested.

**Issues Related to Myocardial FIAU Kinetics**

In previous studies in HSV1-tk–transduced tumors, kinetic analysis showed that FIAU uptake increased rapidly within the first hour and then remained stable until 24 hours after injection. In tumors, FIAU is thought to be taken up by cells, phosphorylated intracellularly by reporter gene product, and then incorporated into host DNA. Wash-out of radioactivity after initial specific uptake in HSV1-tk–transduced tissue seems to be specific for myocardium. Contributing factors may be related to (1) limited influx, (2) intracellular accumulation, and (3) enhanced FIAU-derived radioactivity efflux.

FIAU influx to myocardium is influenced by blood flow and systemic metabolic degradation. Quantification of myocardial blood flow suggested mild elevation of resting flow in adenovirally infected areas, which may be explained by well-known and histologically confirmed inflammatory reaction. Regionally enhanced FIAU uptake in HSV1-tk–expressing areas cannot be attributed to elevated flow, because FIAU uptake was absent in areas infected with control adenovirus despite the presence of inflammation and similar degrees of flow elevation. Because blood flow to myocardium is higher than that to tumors, more rapid delivery may explain the detectability of specific FIAU uptake at earlier times than previously reported for tumors, but issues related to flow are unlikely to explain subsequent radioactivity wash-out.

As suggested by plasma metabolite analysis, systemic degradation of labeled FIAU, eg, via thymidine phosphorylase or dihydropyrimidine dehydrogenase, may result in deiodination, as previously suggested in tumor-bearing rats, by accumulation of radioactivity in stomach and thyroid and...

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**Figure 4.** Hematoxylin-eosin–stained slices from a remote area (A and B) and an area of adenovirus injection (AdHCMVTK, C and D). Presence of lymphocytic infiltration indicates inflammatory reaction after adenoviral infection in C and D, which was present after AdHCMVTK and Adnull injection.
may be a contributor to the disappearance of specific tracer uptake. Available labeled FIAU, however, still exceeded free iodine in plasma at 2 hours, where specific myocardial uptake was no longer detectable, suggesting that systemic degradation is not a major contributor to cardiac tracer efflux.

Accumulation of FIAU in HSV1-tk–transduced tissue is dependent on expression of the reporter gene product, the HSV1-TK enzyme. Although the transgene product was well identified by immunohistochemistry, it needs to be considered that overall expression per volume of target tissue may be lower than in previous studies, in which either stable ex vivo–transduced tumor cells were used or adenovirus was injected into much smaller target volumes in rats. Finally, because of little proliferation in myocardium, DNA incorporation of phosphorylated FIAU in HSV1-tk-transduced cells may be low, facilitating intracellular degradation and subsequent tracer efflux. The most likely explanation for rapid degradation of phosphorylated FIAU in myocardium, and thus for radiotracer efflux, is tissue-specific expression of 5′-nucleotidases. Human cytosolic 5′-nucleotidase I, eg, has recently been cloned and characterized. This enzyme is present at high levels in myocardium, where it is thought to be responsible for production of adenosine, a vasodilatory and cardioprotective agent, from AMP. Other nucleotidases have been identified in mitochondria, in which they are thought to protect against overproduction of deoxyribonucleoside triphosphates, which are toxic at high levels. Nucleotidase-mediated dephosphorylation of FIAU monophosphate may thus explain tracer wash-out from myocardium, in contrast to observations in tumors and liver. Further supportive evidence for this tissue-specific metabolic pathway also comes from tragically ended trials of hepatitis therapy with pharmacological doses of FIAU, in which severe toxicity to liver, muscle, and other organs but not to myocardium was reported.

**Approaches for PET Reporter Gene Imaging**

In this study, we obtained quantitative parameters of reporter gene expression from dynamic PET. A FIAU retention index allowed for reliable identification of regional myocardial HSV1-tk expression. In addition, compartmental modeling suggested an effect of HSV1-tk on FIAU distribution volume, although the model simplifies reporter probe kinetics and is
susceptible to statistical uncertainty. Especially in areas not expressing reporter gene, myocardial tracer kinetics hardly differ from those of blood and surrounding tissue, limiting the feasibility of fitting algorithms.

Quantitative correlation between cellular FIAU uptake and HSV1-tk expression has been demonstrated previously in tumors,¹³ in which FIAU as pyrimidine derivative was the most efficient probe among several reporter substrates for HSV1-tk.¹³,¹⁶ Another approach is to use the acycloguanosine derivative 9-(4-[¹²⁴I]Fluoro-3-hydroxymethyl-butyl)guanine (FHBG) as reporter probe, which however showed markedly lower initial uptake and faster clearance from HSV1-tk-transduced tissue.¹⁰ FHBG is thus used together with a mutant herpesviral thymidine kinase reporter gene, which enhances its accumulation. This yielded high-quality in vivo images of cardiac reporter gene expression in rats,⁴ in which a quantitative correlation between reporter probe accumulation and reporter gene product has been demonstrated.³ The myocardial kinetics of FHBG, which may have different affinity to, eg, nucleotidases, have not been evaluated to date. Other reporter gene/reporter probe combinations, such as dopamine D₂ receptor and [¹²⁴I]Fluoroethylpiperone or sodium iodide symporter and radioiodine, are available but have not been applied in the cardiac setting.²² Future studies will need to define an optimal approach for imaging of cardiac transgene expression.

Monitoring Delivery and Expression of Therapeutic Genes by PET

On the basis of the present and previous data, PET holds great promise for monitoring of cardiac gene therapy: by coexpressing reporter gene and therapeutic gene, not only location but also magnitude and persistence of transgene expression can be determined serially in vivo. 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. If reporter gene imaging is combined with other tracers as in the present study, the functional effects of gene therapy on microcirculation and metabolism can be determined in parallel and correlated with transgene expression. Furthermore, the principle of reporter gene imaging is independent of the applied vector. It may be used with less immunogenic adenovirus-associated or lentivirus or for cell-based gene transfer. Monitoring of tissue-specific transgene expression²³ or of expression of intrinsic genes,²⁴ as demonstrated in tumors, may allow for even more detailed and specific molecular characterization of myocardium in the future.

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References

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