Molecular Imaging of the Kinetics of Vascular Endothelial Growth Factor Gene Expression in Ischemic Myocardium

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Background—Angiogenic gene therapy is a promising treatment paradigm for patients with ischemic heart disease. In this study, we used micro–positron emission tomography (microPET) to monitor the transgene expression, function, and effects in a whole-body system.

Methods and Results—Adenovirus with cytomegalovirus promoter driving an angiogenic gene (vascular endothelial growth factor [VEGF]) linked to a PET reporter gene (herpes simplex virus type 1 mutant thymidine kinase; Ad-CMV-VEGF121-CMV-HSV1-sr39tk) was used to transfect rat embryonic cardiomyoblasts in vitro. Expression of both genes correlated strongly ($r=0.98; P<0.001$). Afterward, rats underwent ligation of the left anterior descending artery followed by injection of $1 \times 10^{10}$ pfu of Ad-CMV-VEGF121-CMV-HSV1-sr39tk (study; $n=35$) or Ad-null (control; $n=15$) at the peri-infarct region. Noninvasive microPET imaging was used to assess the uptake of $9-\left(4-{\begin{smallfbox}}\text{F}\end{smallfbox}}\right)$-fluorohydroxymethylbutyl)guanine (${\begin{smallfbox}}\text{F}\end{smallfbox}}$-FHBG) PET reporter probe by cells expressing the HSV1-sr39tk PET reporter gene. Cardiac transgene expression peaked at day 1 and declined over the next 2 weeks. Repeat adenoviral injections at day 60 yielded no detectable signal. The in vivo reporter gene expression (% injected dose/g of $\text{F}$-FHBG) correlated well with ex vivo gamma counting ($r=0.92$), myocardial tissue HSV1-sr39TK enzyme activity ($r=0.95$), and myocardial tissue VEGF level ($r=0.94; P<0.001$ for all). The VEGF121 isoform induced significant increases in capillaries and small blood vessels. However, the level of neovascularization did not translate into significant improvements in functional parameters such as myocardial contractility by echocardiography, perfusion by nitrogen-13 ammonia imaging, and metabolism by $\text{F}$-fluorodeoxyglucose imaging.

Conclusions—Taken together, these findings establish the feasibility of molecular imaging for monitoring angiogenic gene expression with a PET reporter gene and probe noninvasively, quantitatively, and repetitively. The principles demonstrated here can be used to evaluate other therapeutic genes of interest in animal models before future clinical trials are initiated. (Circulation. 2004;110:685-691.)

Key Words: gene therapy • angiogenesis • imaging • myocardium • nuclear medicine

Ischemic heart disease is a leading cause of morbidity and mortality in the United States. Despite recent advances in medical therapies, a significant proportion of patients remain symptomatic. Thus, an alternative treatment approach collectively termed “therapeutic angiogenesis” has undergone intense investigation. One of the most widely studied angiogenic factors is the vascular endothelial growth factor (VEGF), a homodimeric 34- to 46-kDa heparin-binding glycoprotein. As a result of posttranscriptional mRNA splicing, mature monomeric VEGF transcripts contain 121-, 165-, 189-, or 206-amino acid residues. VEGF121 and VEGF165 are soluble proteins that can diffuse extracellularly, whereas VEGF189 and VEGF206 are bound to heparin-containing proteoglycans on the cell surface or in the basement membrane. In animal models and phase 1 clinical trials, VEGF therapy (delivered as protein, plasmid, or adenovirus) significantly improved myocardial perfusion and function. In contrast, recent double-blind, randomized clinical trials have shown less consistent if not disappointing results. Potential reasons for these discrepancies include (1) increased resistance to angiogenic stimulation in the selected patient population, (2) short half-life of angiogenic growth factors, resulting in regression of newly formed capillaries, and (3) lack of available imaging methods to carefully track cardiac transgene expression, function, and effects.
Over the past decade, advances in imaging technologies have allowed investigators to visualize targeted cellular processes at a molecular or genetic level in whole-body living subjects. Most applications were targeted toward imaging transgene expression in tumor models. Similar development of noninvasive surrogate markers for monitoring cardiac transgene expression would be a major advance for cardiology. Recently, we demonstrated the feasibility of imaging the location, magnitude, and duration of a single PET (positron emission tomography) reporter gene (HSV1-sr39tk) expression in the myocardium of living rats using a dedicated small-animal microPET with high spatial resolution (2 mm$^3$) compared with 6 mm$^3$ for clinical PET).

We subsequently showed that the expression of 2 PET reporter genes (HSV1-sr39tk and mutant dopamine 2 receptor) linked together by an internal ribosomal entry site had good correlations in both cell assays ($r=0.98$, $P<0.001$) and rat myocardium ($r=0.86$, $P<0.001$). Therefore, the present study is a logical progression of our previous works on cardiac gene imaging. In a rat model of myocardial infarction (MI), we hypothesize that by substituting one of the PET reporter genes with a therapeutic gene, we can monitor the therapeutic gene of interest (VEGF$_{121}$) by imaging the linked reporter gene (HSV1-sr39tk).

### Methods

#### Virus Construction and Amplification

A replication-defective recombinant adenovirus carrying dual-expression cassettes separated by a polyA tail with cytomegalovirus (CMV) promoters driving the expression of human VEGF$_{121}$ gene and HSV1-sr39tk reporter gene (Ad-CMV-VEGF$_{121}$-CMV-HSV1-sr39tk) was constructed, purified, and amplified as described previously. The Ad-null and single-cassette Ad-CMV-HSV1-sr39tk served as negative and positive control viruses, respectively. Henceforth, “HSV1-sr39tk” refers to reporter gene and “HSV1-sr39TK” refers to reporter enzyme.

#### Cell-Transfection Studies

Rat embryonic H9c2 cardiomyoblasts (American Type Culture Collection) were cultured as described previously. Cells were transfected with Ad-CMV-VEGF$_{121}$-CMV-HSV1-sr39tk at various plaque forming units (pfu): 0, $1 \times 10^2$, $5 \times 10^3$, $1 \times 10^4$, $5 \times 10^4$, and $1 \times 10^5$ pfu) for 90 minutes. H9c2 cells transduced with equal titers of Ad-null served as negative controls. After 48 hours, the total amount of VEGF$_{121}$ in the cell medium was measured quantitatively by dividing the number of pixels in the ROI by the total number of pixels in the polar map. Image analysis was performed by an investigator blinded to the study conditions (I.Y.C. and M.S.).

#### Western Blot Analysis

Cell lysates were prepared from H9c2 cells transfected with Ad-null, Ad-CMV-HSV1-sr39tk, or Ad-CMV-VEGF$_{121}$-CMV-HSV1-sr39tk as described previously. Mouse monoclonal IgG anti-human VEGF antibody (Oncogene Research) and rabbit polyclonal antibodies against simplex virus (HSV) type 1 thymidine kinase (HSV1-TK) antibody (kind gift from Dr Margaret Black, Washington State University) were used to qualitatively determine VEGF$_{121}$ and HSV1-sr39TK levels, respectively.

### Induction of MI

Adult female Sprague-Dawley rats (weight 200 to 300 g; Charles River Laboratories, Waltham, Mass.) received isoflurane (2%) for general anesthesia during mechanical ventilation. MI was induced by ligation of the left anterior descending coronary artery 2 to 3 mm from the tip of the left auricle with a 6-0 polypropylene suture. This resulted in myocardial blanching and ST-segment elevation on an ECG monitor (Sillogic EC-60 model). Afterward, 3 separate sites around the peri-infarct region were injected with a total of 50 μL of viral volume. The study was performed in accordance with protocols approved by the University Animal Research Committee.

#### Animal Study Design

Figure 1 depicts the vector construct and research design. Animals were divided into the study group receiving $1 \times 10^{10}$ pfu of Ad-CMV-VEGF$_{121}$-CMV-HSV1-sr39tk (n = 35) or the control group receiving Ad-null (n = 15) injection. The overall surgical mortality was ~22% (8 in the VEGF study group and 3 in the control group). To ensure equal randomization, all animals underwent echocardiography, $[^{13}N]$-NH$_3$, and $[^{18}F]$-fluorodeoxyglucose ($[^{18}F]$-FDG) imaging at baseline (day 2). Animals without clear evidence of MI were categorized into the sham surgery group (~20%; 7 in the VEGF group and 3 in the control group). Subsequently, 20 animals in the VEGF study group and 9 in the Ad-null control group with similar degrees of MI were included for analysis.

### Imaging the Kinetics of Cardiac Transgene Expression With MicroPET

The pharmacokinetics of VEGF$_{121}$ therapeutic gene expression were monitored by imaging the linked HSV1-sr39tk reporter gene expression every 1 to 3 days in 6 study animals with the $9\times4\cdot[$$[^{18}F]$]-fluorodeoxyglucose ($[^{18}F]$-FDG) radiotracer. Three of these animals underwent a second intramyocardial injection ($1 \times 10^{10}$ pfu of Ad-CMV-VEGF$_{121}$-CMV-HSV1-sr39tk) at day 60 and were scanned on days 62 and 64. Images from 50 to 60 minutes after $[^{18}F]$-FHBG injection were reconstructed by filtered back-projection (FBP) and the maximum a posteriori probability (MAP) algorithm. Tracer activity (mCi/g) was divided by the injected dose (mCi) to obtain a tissue uptake index, expressed as percentage injected dose per gram of tissue (% ID/g) as described previously. Image acquisition was performed by 2 investigators blinded to the study conditions (I.Y.C. and M.S.).

### Assessment of Myocardial Perfusion and Metabolism With MicroPET

Animals were injected with $[^{13}N]$-NH$_3$ radiotracers (1.42±0.28 mCi; T$_{1/2}$ = 9.5 minutes) via tail vein and imaged for 15 minutes to assess perfusion deficits after MI. Afterward, $[^{18}F]$-FDG radiotracers (1.39±0.22 mCi; T$_{1/2}$ = 110 minutes) were injected to identify viable and nonviable tissues as described previously. Axial microPET image data were resliced into 3 cardiac axes with CAPP software (CTI). Regions of interest (ROIs) were drawn around the perfusion and metabolism defects by visual inspection with Image J software (National Institutes of Health). The percentage defect was calculated by dividing the number of pixels in the ROI by the total number of pixels in the polar map. Image analysis was performed by an investigator blinded to the study conditions (J.R.T.).

### Assessment of Left Ventricular Contractility With Echocardiography

Rats were sedated with tribromoethanol (0.25 mg/g IP). Echocardiographic images were obtained with an Acuson Sequoia C256 equipped with a 15-MHz probe. Left ventricular ejection fraction and percent fractional shortening were calculated as described previously. All measurements were averaged on 3 consecutive cardiac cycles and analyzed by 2 investigators blinded to the study conditions (A.C. and J.-J.M.).
Ex Vivo Validation With Gamma Well Counting and Enzyme Assays

After imaging, explanted hearts \((n=29)\) were counted for \([^{18}\text{F}]\) radioactivity in a gamma well counter (Cobra II Auto-Gamma). The total myocardial accumulation of \([^{18}\text{F}]\)-FHBG was expressed as % ID/g tissue. Afterward, hearts \((n=12)\) were homogenized to determine VEGF~121~ and HSV1-sr39TK enzyme activities by ELISA and penciclovir phosphorylation assays, respectively, as described previously.10

Immunohistochemical Stainings

Explanted hearts \((n=17)\) were processed for routine light microscopy and immunohistochemistry as described previously. The following primary antibodies and dilutions were used: mouse monoclonal IgG anti-human VEGF antibody at a dilution of 1:50; rabbit polyclonal HSV1-TK antibody at 1:500; mouse monoclonal anti-smooth muscle actin (α-SMA; Sigma Chemical) at 1:400; and goat polyclonal anti-CD31 antibody (Santa Cruz Biotechnology) at 1:200. To estimate capillary density, 5 randomly selected fields near the peri-infarct region were evaluated in 10 sections per heart. Computerized images were captured at a magnification of \(\times200\). Capillary density (positive CD31 stain) was defined as the mean number of capillaries per square millimeter. The number of small blood vessels (positive α-SMA stain) was counted in a similar fashion.

Statistical Analysis

Data are expressed as mean±SEM. A probability value of \(<0.05\) was considered statistically significant. Linear regression analysis was performed to assess the linear relationship between 2 variables. The significance of correlation was obtained by performing the 2-tailed Student’s \(t\) test against the null hypothesis that the correlation coefficient was zero.

Results

Cell Assays Showed Strong Correlations Between Reporter and Therapeutic Genes

For H9c2 cardiomyoblasts transfected with Ad-CMV-VEGF~121~-CMV-HSV1-sr39tk, both the VEGF~121~ and HSV1-sr39TK proteins were recognized at \(\approx18\) and \(\approx46\) kDa by their antibodies on Western blots (Figure 2A). The wider band for HSV1-sr39TK was due to nonspecific binding by the rabbit polyclonal HSV1-TK antibody. Precise quantification of the therapeutic and reporter gene activities was analyzed by ELISA and penciclovir phosphorylation enzyme assays, respectively. Over the range of viral titers tested, a high correlation \((r=0.98; P<0.001)\) existed between the expressions of the 2 linked genes (Figure 2B).
Noninvasive Monitoring of Cardiac Transgene Expression

As determined by myocardial uptake of $^{[18F]}$-FHBG reporter probe, cardiac transgene expression peaked at day 1 (0.311 ± 0.021% ID/g), progressively declined afterward, and was no longer detected by day 14 (Figure 3A). To address the clinical question of whether repeated injections are effective for increasing therapeutic efficacy, we evaluated transgene expression in a subset of the animals (n = 3) that underwent a second intramyocardial injection of Ad-CMV-VEGF121-CMV-HSV-sr39tk at day 60. Scans performed on days 62 and 64 showed no detectable $^{[18F]}$-FHBG signal, thus indicating the absence of HSV1-sr39tk gene expression (Figure 3B). As expected, control rats injected with Ad-null showed background cardiac $^{[18F]}$-FHBG activity (0.033 ± 0.005% ID/g; data not shown).

In Vivo Gene, Perfusion, and Metabolism Imaging With MicroPET

Figure 4 shows the $^{[14}N$-NH$_3$ perfusion, $^{[18F]}$-FDG metabolism, and overlaid $^{[18F]}$-FHBG reporter probe images. As expected, control and sham animals injected with Ad-null showed only background $^{[18F]}$-FHBG activity that faintly outlined the shape of the chest cavity in blue (0.025 to 0.035% ID/g). In contrast, study animals injected with Ad-CMV-VEGF121-CMV-HSV1-sr39tk showed intense $^{[18F]}$-FHBG signal around the region of perfusion and metabolism defects in red (0.300 to 0.350% ID/g). Importantly, in vivo ROI-derived $^{[18F]}$-FHBG activity correlated well with ex vivo $^{[18F]}$ gamma counting of the explanted heart ($r=0.92$, P<0.001), ex vivo tissue HSV1-sr39TK enzyme activity ($r=0.95$, P<0.001), and ex vivo tissue levels of VEGF$_{121}$ ($r=0.94$, P<0.001). Likewise, ex vivo tissue levels of VEGF$_{121}$ and HSV1-sr39TK showed a strong correlation with each other ($r=0.90$, P<0.001).

VEGF$_{121}$ Induces Neovascularization in Ischemic Myocardium

The left ventricular infarct areas seen on in vivo perfusion and metabolism images were confirmed by ex vivo hematoxylin-and-eosin and trichrome stains (Figure 5, a through d). Expression of the 2 linked genes was also demonstrated immunohistochemically. The HSV1-TK stained positive around the needle track where the virus was injected in the epicardium (Figure 5, e and f). The VEGF$_{121}$ stained positive transmurally because it is a soluble protein that can diffuse extracellularly (Figure 5, g and h). As expected, control rats injected with Ad-null had negative HSV1-TK staining and a basal level of VEGF$_{121}$ staining (data not shown). Around the peri-infarct region, the mean densities of capillaries (747 ± 104 versus 450 ± 101 per mm$^2$; Figure 5, i and j) and small blood vessels (8.1 ± 0.8 versus 5.1 ± 1.2 per mm$^2$; Figure 5, k and l) were significantly higher in the study group than in the control group (P<0.05 for both). We did not notice any evidence of hemangioma-like proliferations by gross or microscopic examination.

New Microvessels Do Not Translate Into Significant Functional Improvement

To address whether this level of neovasculature was physiologically relevant, we performed detailed functional imaging
studies in sham (failed induction of MI; n=10), control (Ad-null; n=6), and study (Ad-CMV-VEGF121-CMV-HSV1-sr39tk; n=10) animals at baseline (day 2) and at week 10. At baseline, there was no significant difference in left ventricular ejection fraction for study and control rats. After 10 weeks of VEGF121 treatment, left ventricular ejection fraction showed mild improvement in the study group (43.4±8.1% to 47.3±12.5%) compared with the control group (47.5±9.3% to 45.2±8.4%), but this did not reach statistical significance. Similarly, fractional shortening showed no significant changes in the study group (18.8±5.4% to 21.4±8.4%) or control (21.5±6.2% to 20.0±5.6%) groups. However, owing to the small size of rat myocardium, we were unable to accurately and consistently determine any significant changes in regional contractility involving the left anterior wall. For comparison of [13N]-NH3 and [18F]-FDG imaging, the VEGF-treated study animals showed an encouraging trend toward lower perfusion defects (15.2±3.1% to 13.8±2.6%) and metabolism deficits (12.7±4.3% to 11.5±4.6%), but the changes were not statistically significant. The control group also did not show any significant changes in perfusion (14.0±4.0% to 15.3±4.1%) or metabolism (13.4±2.3% to 15.1±3.0%) scores (P=NS).

**Discussion**

This is the first cardiac study to validate the novel approach of linking a therapeutic gene (VEGF121) with a PET reporter gene (HSV1-sr39tk). Our findings are as follows: (1) the dual-cassette adenoviral vector can efficiently transfect rat embryonic cardiomyoblasts (in vitro) and rat myocardium (in vivo), with strong correlations between the 2 genes; (2) embryonic cardiomyoblasts (in vitro) and rat myocardium (in vivo), with strong correlations between the 2 genes; (2) and consistently determine any significant changes in regional contractility involving the left anterior wall. For comparison of [13N]-NH3 and [18F]-FDG imaging, the VEGF-treated study animals showed an encouraging trend toward lower perfusion defects (15.2±3.1% to 13.8±2.6%) and metabolism deficits (12.7±4.3% to 11.5±4.6%), but the changes were not statistically significant. The control group also did not show any significant changes in perfusion (14.0±4.0% to 15.3±4.1%) or metabolism (13.4±2.3% to 15.1±3.0%) scores (P=NS).

Several features of our research deserve further discussion. First, 10 of 50 surgical animals (≈20%) showed no wall-motion abnormality on echocardiography and no defects on [13N]-NH3 and [18F]-FDG imaging. This unsuccessful occlusion of the left anterior descending coronary artery is consistent with 2 large studies that reported 17% to 36% failure rates in rat and mouse models.18,19 We strongly believe that early identification during baseline scans and subsequent exclusion of these sham animals are important. If categorized into the study group, these sham animals can favorably bias the interpretation of therapeutic efficacy by VEGF121 treatment. Second, the noninvasive nature of gene imaging allowed us to perform repetitive assessment of transgene expression, even after second adenoviral injections within the same animals. The significant reduction of [18F]-FBHB activity observed over time is likely due to animal host immune response against adenovirus and is also expected to occur in human studies.7 This short period of adenovirus-mediated gene expression may limit its efficacy, because Dor et al16 have shown that premature cessation of the VEGF stimulus can cause regression of newly acquired vessels. Third, we delivered a single VEGF121 gene at 1×1010 pfu because it was the comparable gene and dosage used in several clinical trials.1,5 However, Whitlock et al14 recently showed that adenovirus-mediated transfer of a minigene expressing multiple isoforms of VEGF (121, 165, and 189) is more effective at inducing angiogenesis than individual VEGF cDNAs. Finally, we assessed the functional effects of VEGF in a rat MI model. In contrast, VEGF treatment is likely to be more efficacious during chronic myocardial ischemia, as recently demonstrated by Hughes et al20 in porcine myocardium using regional perfusion and segmental wall-motion analysis.

Despite the lack of functional improvement with the VEGF121 gene, our primary goal was to assess the feasibility of monitoring the magnitude, location, and duration of the angiogenic gene expression indirectly by imaging the linked PET reporter gene. We believe similar imaging approaches can and should be applied toward validation of other potential therapeutic genes such as fibroblast growth factor, nitric oxide, and hypoxia-inducible factor-1α before proceeding to clinical studies.1,2 These therapeutic genes can be linked to a
variety of reporter genes (HSV1-sr39tk, transferrin, or firefly luciferase), under the control of specific promoters (cardiac tissue specific or tetracycline inducible), delivered by less immunogenic vectors (lentivirus or gutless adenovirus), and monitored by multimodality imaging approaches (PET, MRI, or optical). In doing so, one can expeditiously evaluate the optimal combinations of angiogenic factors, delivery strategy, and duration of gene expression that are needed to induce therapeutic angiogenesis while minimizing potential toxicities.

In theory, microPET imaging of therapeutic gene expression can be achieved by either a direct or an indirect approach. The direct approach involves imaging radioactive probes that target therapeutic gene products such as mRNA or enzyme. However, this approach requires synthesizing a customized probe for every therapeutic gene of interest. In contrast, the indirect approach, which involves a reporter gene and reporter probe, is more generalizable for gene therapy application. The reporter probe itself does not have to be changed if one wishes to study a new biological process, which saves valuable time needed to synthesize, test, and validate new radiotracer agents. In addition to quantitative gene imaging, PET imaging offers functional information on physiological variables such as perfusion and metabolism. In post-MI rats, Kudo et al showed that microPET imaging of perfusion defects and metabolic deficits as measured by uptake of $^{[13]}$N-$\mathrm{NH}_3$ and $^{[18]}$F-FDG radiotracers, respectively, correlated well with postmortem histology ($r=0.93$ and $r=0.97; P<0.01$).

In summary, gene transfer holds great promise for the treatment of cardiovascular diseases. For cardiac gene therapy to be successful, biological issues related to pharmacokinetics and functional and physiological effects of gene expression will need to be fully evaluated before clinical trials. Molecular imaging is an extremely powerful technique that has the potential of analyzing these factors noninvasively, repetitively, and quantitatively in an intact, living whole-body system. In addition, it can be used to track cell survival, monitor endogenous transcriptional regulation, screen transgenic animal phenotypes, study protein-protein interactions, and expedite novel drug discovery. Because microPET can be scaled up to a clinical PET scanner, the present study should have direct translational relevance. Eventually, the goal is to use molecular imaging to help develop gene therapy protocols that are safe, beneficial, and quantifiable.

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**References**


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