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Intratumoral versus Intravenous Gene Therapy Using a Transcriptionally Targeted Viral Vector in an Orthotopic Hepatocellular Carcinoma Rat Model

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Abstract

Purpose—To evaluate the feasibility of intratumoral delivery of adenoviral vector carrying a bidirectional two-step transcriptional amplification (TSTA) system to amplify transcriptional strength of cancer-specific Survivin promoter in a hepatocellular carcinoma model.

Materials and Methods—MCA-RH7777 cells were implanted in rat liver, and tumor formation was confirmed with [¹⁸F]fluoro-deoxyglucose (18F-FDG) positron emission tomography (PET). The adenoviral vector studied had Survivin promoter driving a therapeutic gene (tumor necrosis factor- α -related apoptosis-inducing ligand [TRAIL]) and a reporter gene (firefly luciferase [FL]; Ad-pSurvivin-TSTA-TRAIL-FL). Tumor-bearing rats were administered Ad-pSurvivin-TSTA-TRAIL-FL intravenously (n = 7) or intratumorally (n = 8). For control groups, adenovirus FL under cytomegalovirus (CMV) promoter (Ad-pCMV-FL) was administered intravenously (n = 3) or intratumorally (n = 3). One day after delivery, bioluminescence imaging was performed to evaluate transduction. At 4 and 7 days after delivery, 18F-FDG-PET was performed to evaluate therapeutic efficacy.

Results—With intravenous delivery, Ad-pSurvivin-TSTA-TRAIL-FL showed no measurable liver tumor FL signal on day 1 after delivery, but showed better therapeutic efficacy than Ad-pCMV-FL on day 7 (PET tumor/liver ratio, 3.5 ± 0.58 vs 6.0 ± 0.71 ; $P = .02$). With intratumoral delivery, Ad-pSurvivin-TSTA-TRAIL-FL showed positive FL signal from all tumors and better therapeutic efficacy than Ad-pCMV-FL on day 7 (2.4 ± 0.50 vs 5.4 ± 0.78 ; $P = .01$). In addition,

intratumoral delivery of Ad-pSurvivin-TSTA-TRAIL-FL demonstrated significant decrease in tumoral viability compared with intravenous delivery (2.4 ± 0.50 vs 3.5 ± 0.58 ; $P = .03$).

Conclusions—Intratumoral delivery of a transcriptionally targeted therapeutic vector for amplifying tumor-specific effect demonstrated better transduction efficiency and therapeutic efficacy for liver cancer than systemic delivery, and may lead to improved therapeutic outcome for future clinical practice.

Gene therapy for cancer can target a variety of pathways to achieve apoptosis, cell lysis, antitumor immunity, or angio-genesis inhibition by transferring genetic materials into targeted cells (1). It requires a series of complex processes for its efficacy, which is affected not only by the selection of the appropriate therapeutic genes, but also by several factors such as vector, delivery method, cell transduction, and transgene expression. Many efforts have been made during the past several years to develop strategies for cancer gene therapy aiming at specific targeting to tumors to avoid unwanted, immunogenic, or toxic transgene expression in normal tissues while achieving high levels of trans-gene expression in cancer cells (1). Transcriptionally targeted cancer gene therapy uses cancer-specific promoters that induce therapeutic gene expression exclusively in cancer cells rather than in normal cells (2). However, the cancer-specific promoters usually demonstrate low levels of transcription and require amplification strategies to reach adequate levels for therapeutic efficacy. We previously developed an adenoviral vector carrying the cancer-specific promoter pSurvivin and a bidirectional dual gene-expression system composed of the therapeutic apoptosis-inducing protein tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL) gene and the reporter firefly luciferase (FL) gene, which has a two-step transcriptional amplification (TSTA) system with GAL4-VP16 fusion protein to amplify the promoter activity (Ad-pSurvivin-TSTA-TRAIL-FL) (3) (Fig 1). The GAL4-VP16 fusion protein amplifies expression of genes under the control of multiple GAL4-binding sites and a thymine-adenine-thymine-adenine minimal promoter (3,4). The ideal gene therapy requires not only efficient targeting but also monitoring the location and extent of therapeutic gene expression, which can be provided by the coexpression of a reporter gene. Our system has been designed so that TRAIL (ie, therapeutic gene) expression can be inferred from the signal generated by FL (ie, reporter gene) expression with bioluminescence imaging (BLI; Fig 1). The expressions of TRAIL and FL in our system were reported to highly correlate in a previous study (3).

Another strategy for gene targeting and amplification is a localized gene delivery. When therapeutic genes for cancer are delivered by intravenous injection, undesirable interactions between the administered genes and blood components or nontarget cells would be expected (5), and induce more loss of the DNA complex into the systemic circulation. In addition, although the intravenously administered genes targeting liver cancer enter the liver efficiently, it has been shown that most of them are completely trapped by Kupffer cells, thereby making it impossible to direct genes to tumor cells (6). Therefore, it would be desirable to administer the genes in a way that avoids the barriers that can be expected with systemic delivery. Local delivery can increase the direct uptake of the DNA complex into the target tissue compared with systemic delivery, because the local delivery of genes helps in bypassing multiple anatomic and physiologic barriers to a target, leading to higher target

concentration with lower systemic exposure (7). Various methods available to accomplish this strategy include direct intratumoral injection or transarterial injection through the vessels that directly feed the tumor. We hypothesize that combining the transcriptional targeting strategy with local delivery techniques may lead to even more specific and amplified transgene expression in cancer gene therapy than using either strategy alone. In this study, we used molecular imaging to compare the transgene expression and therapeutic efficacy of intratumoral versus intravenous delivery of Ad-pSurvivin-TSTA-TRAIL-FL.

MATERIALS AND METHODS

Tumor Model

Cell preparation—McA-RH7777, a syngeneic rat hepatocellular carcinoma (HCC) cell line, was obtained from American Type Culture Collection (Manassas, Virginia) and cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator with 5% CO₂ in atmosphere in air at 37°C. A total of 10⁶ cells suspended in 100 µL of phosphate-buffered saline (PBS) solution were prepared for injection into each animal.

Animal preparation—Animal experiments (Fig 2) were approved by our institutional animal care and use committee. Twenty-three Buffalo rats weighing 300–350 g (Charles River Laboratory, Wilmington, Massachusetts) were used for the orthotopic tumor model (8), and kept separately during the experiment with ad libitum access to standard laboratory diet and water. Animals were anesthetized with isoflurane inhalation: for induction, 5% isoflurane was mixed in 1 L O₂/min, and, for maintenance, approximately 2%–4% isoflurane was mixed in 1 L O₂/min. Analgesia was provided with a subcutaneous injection of buprenorphine (0.01–0.05 mg/kg) and an intramuscular injection of flunixin meglumine (2.5 mg/kg). After laparotomy, the liver was mobilized to expose the left lobe, onto which a total of 10⁶ cells suspended in 100 µL of PBS solution were injected slowly (typically over a period of 30–60 s) (9). To prevent extrahepatic cell spillage, a cotton applicator was applied over the needle insertion site for 2–3 minutes, followed by application of 70% ethanol to the peritoneal cavity. The incision was then closed in layers with suture. Fourteen days after tumor implantation, animals were anesthetized for [¹⁸F]fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) imaging, which was performed with a Concorde R4 microPET system (Siemens, Malvern, Pennsylvania) after tail-vein injection of approximately 500 µCi of 18F-FDG. A 15-minute static scan with an approximate resolution of 2 mm in each axial direction was obtained 1 hour after 18F-FDG injection. Images were reconstructed with the ordered-subsets expectation maximization algorithm. Hypermetabolic foci within the liver verified the presence of the orthotopic HCC on 18F-FDG-PET imaging (Fig 3).

Gene Delivery

Construction of adenoviral vector—All adenoviruses are first-generation E1-, E3-deleted. The bidirectional TSTA ad-enovirus, Ad-pSurvivin-TSTA-TRAIL-FL, was cloned by Vector Biolabs (Philadelphia, Pennsylvania) as previously described (3). The control adenovirus encoding a cytomegalovirus (CMV) promoter and FL (Ad-pCMV-FL) was

constructed by using the AdEasy kit (Agilent Technologies, Santa Clara, California) according to the manufacturer guidelines. In short, all gene sequences were inserted into the pShuttle intermediate plasmid and then recombined into the adenoviral backbone in bacteria. Viruses were later packaged and amplified in HEK-293 cells, and titers were determined by using an adenovirus titer immunoassay kit (QuickTiter; Cell Biolabs, San Diego, California).

Viral injection—On day 15, 1 day after tumor growth was confirmed by PET, 10^9 pfu of Ad-pSurvivin-TSTA-TRAIL-FL in 300 μ L of PBS solution was administered into the rats by intravenous tail vein injection ($n = 7$) or intratumoral injection ($n = 8$). Laparotomy was done for direct intratumoral injection under visual guidance. To establish control groups matched to either delivery method, 10^9 pfu of Ad-pCMV-FL, which has neither tumor-specific promoter nor therapeutic gene, were administered by intravenous ($n = 3$) or intratumoral injection ($n = 3$).

Evaluation of Transgene Expressions

At day 1 after gene delivery, BLI was performed by using an IVIS Spectrum imaging system (Xenogen/Perkin-Elmer, Alameda, California). Animals were anesthetized as described earlier, and 45 mg of D-luciferin (Xenogen/Perkin-Elmer) was administered by intraperitoneal injection. Five minutes later, animals were placed in a light-tight chamber, and photons emitted were collected with a cooled charge-coupled device camera. All images were acquired with an integration time of 60 seconds, and regions of interest (ROIs) of equal size were drawn within the tumor to measure average radiance (expressed as photons/s/cm²/sr).

Evaluation of Therapeutic Responses

To quantify the therapeutic response, 18F-FDG-PET images obtained 14 days after tumor cell implantation and 1 day before gene delivery served as a baseline study. Follow-up 18F-FDG-PET images were serially obtained at days 4 and 7 after gene delivery. Regional tumor radioactivity concentrations (in percent injected dose per gram) were determined from ROIs placed around areas of 18F-FDG-related radioactivity within the tumor. To subtract background activity, ROIs were also drawn within the normal liver parenchyma on the same image sets. The net activity at the tumor was therefore calculated according to the following formula:

$$\text{Tumor:liver ratio} = (\text{activity at ROI}_{\text{tumor}} - \text{activity at ROI}_{\text{liver}}) / \text{activity at ROI}_{\text{liver}}$$

Statistical Analysis

Statistical analysis to compare FL and PET activity between experimental groups was performed by the Mann-Whitney test (SPSS version 11.0 [SPSS, Chicago, Illinois] for Windows [Microsoft, Redmond, Washington]). A nominal *P* value of less than .05 was considered to indicate a statistically significant difference.

RESULTS

Confirmation of Tumor Formation

Tumors were implanted in 23 animals, but two animals among them showed no tumor growth on PET at 14 days after tumor implantation. Therefore, 21 animals were suitable for inclusion in this study.

Transgene Expression

Intravenous administration—In the Ad-pSurvivin-TSTA-TRAIL-FL group, BLI performed 1 day after viral delivery showed no measurable FL signal within the liver or tumor. Conversely, in the Ad-pCMV-FL group, there was strong FL signal throughout the liver parenchyma, but discrete tumor signal or lack of tumor signal could not be appreciated (Fig 4). Therefore, FL signal from the tumors could not be measured in intravenous delivery for those two vectors.

Intratumoral administration—Intratumoral delivery resulted in positive FL signal from all tumors in Ad-pSurvivin-TSTA-TRAIL-FL (1.74×10^5 p/s/cm²/sr $\pm$ 1.07×10^4) and Ad-pCMV-FL groups (1.61×10^5 p/s/cm²/sr $\pm$ 1.12×10^4 ; Fig 5), but there was no statistical difference in FL activity between Ad-pSurvivin-TSTA-TRAIL-FL and Ad-pCMV-FL.

Therapeutic Response

Intravenous administration—Seven days after gene delivery, there was significantly less 18F-FDG-PET activity, a surrogate for tumor growth, in the Ad-pSurvivin-TSTA-TRAIL-FL group compared with the Ad-pCMV-FL group (PET liver/tumor ratios of 3.5 ± 0.58 vs 6.0 ± 0.71 ; $P = .02$; Fig 6).

Intratumoral administration—Seven days after gene delivery, there was significantly less 18F-FDG-PET activity in the Ad-pSurvivin-TSTA-TRAIL-FL group compared with the Ad-pCMV-FL group (PET liver/tumor ratios of 2.4 ± 0.50 vs 5.4 ± 0.78 ; $P = .01$; Fig 7).

Intratumoral delivery of Ad-pSurvivin-TSTA-TRAIL-FL demonstrated significantly lower 18F-FDG-PET activity compared with intravenous delivery (PET tumor/liver ratios of 2.4 ± 0.50 vs 3.5 ± 0.58 ; $P = .03$; Fig 8) 7 d after gene administration.

DISCUSSION

The treatment of liver cancer, especially advanced HCC, remains difficult because of advanced tumor stage and associated liver cirrhosis at initial presentation. However, a host of emerging therapeutic strategies for HCC are now being explored, and gene therapy is included in the innovative therapies (10). Adenoviral vectors are well suited for gene therapies, as they can carry large DNA inserts (as much as approximately 7.5 kb) and transduce dividing and nondividing cells (11). They have also been shown to be efficient gene transfer vectors for liver by local delivery (12). However, they are naturally hepatotropic, as they bind to the hepatocyte Coxsackie and adenovirus receptor, which mediates cellular uptake (13,14). This can lead to excessive transgene expression in the normal liver parenchyma and result in potential hepatotoxicity (15,16). In the present study,

the hepatotropism of adenovirus was also observed with strong diffuse FL signal over the entire liver after intravenous administration of Ad-pCMV-FL (Fig 4). In the context of HCC, the ability to avoid expression of cytotoxic genes in normal hepatocytes is particularly important because of the impaired liver function typically seen in this patient population (9). To that end, we focused on developing a gene therapy agent that would have molecular specificity and then combining it with local delivery.

There are two key findings from this work. First, intravenous administration of Ad-pSurvivin-TSTA-TRAIL-FL impaired tumor growth compared with the control vector. We previously demonstrated, by ex vivo imaging and tissue lysates, that intravenous administration of Ad-pSurvivin-TSTA-TRAIL-FL had greater reporter gene expression in the tumor than in the normal liver. We also previously demonstrated that the reporter gene expression within the tumor was equivalent between intravenous administration of Ad-pCMV-FL and Ad-pSurvivin-TRAIL-FL (9). Additionally, the Survivin promoter-driven TSTA system has demonstrated lower FL expression in normal liver after intravenous delivery in healthy mice compared with a CMV-promoter driven system (3). Nevertheless, it is the work of the present study that first demonstrated superior tumor growth inhibition of intravenous administration of Ad-pSurvivin-TSTA-TRAIL-FL versus Ad-pCMV-FL (Fig 6).

Transcriptionally targeted gene therapy is a promising approach that uses tumor-specific promoters to induce therapeutic gene expression exclusively in cancer cells (2). However, one challenge with this strategy is the low level of transcription by the promoters. To overcome this limitation, we have previously developed and validated a TSTA system (4,17). Survivin, a member of the apoptosis inhibitor family, is overexpressed in many cancers, including HCC, but not in normal tissues (18,19). Therefore, pSurvivin is well suited for a transcriptional targeting system for the cancer tissue, and has been used for this purpose in various cancer models (20–22). In a previous study, by taking advantage of overexpressed α -fetoprotein in HCC, specific reporter and therapeutic genes were placed under the control of α -fetoprotein regulatory sequences, leading to expression of these genes only in cells producing α -fetoprotein (23).

Second, intratumoral administration caused partial tumor regression compared with intravenous administration of Ad-pSurvivin-TSTA-TRAIL-FL in killing tumor cells. There are several experimental studies that demonstrated the benefits of local gene delivery versus systemic delivery for liver tumors, but most studies used nonspecific promoters, which can target normal cells as well as tumor cells (24,25). Although some researchers used tumor-specific promoter for HCC gene therapy, cell targeting with a cancer-specific promoter also induced a decrease in the antitumoral efficacy as a result of the low transcriptional activity of the promoter (26,27). They suggested that repeated interventions would be a possible solution to overcome the weak activity of the promoter. pSurvivin activity was enhanced by the TSTA system, and a single intratumoral injection of Ad-pSurvivin-TSTA-TRAIL-FL induced a better therapeutic effect than intravenous injection.

Recent advances in molecular therapies have created new opportunities for interventional radiologists, who are expected to play an important role in the targeted delivery of the novel

therapeutic agents that will need to be translated from the laboratory to the bedside. Therefore, many of these new treatments may ultimately rely on interventional radiologists for their precise delivery.

The use of TRAIL, a member of the tumor necrosis factor family, adds another level of molecular tumor specificity to this treatment. TRAIL rapidly induces apoptosis in a wide variety of cancer cells upon interaction with specific receptors, but has minimal effect on normal cells. Normal cells have decoy receptors that competitively bind TRAIL but do not initiate the apoptosis cascade (28). TRAIL is a promising “suicide gene” therapy for cancer (29,30), as it has a “bystander effect” (31–33), and TRAIL gene therapy has proven to be more efficacious for HCC treatment than the TRAIL protein administered in its soluble form (34). We previously demonstrated that our construct, Ad-pSurvivin-TRAIL-FL, expressed highly correlative levels of TRAIL and FL in vitro and in vivo (3). Similarly, on intratumoral administration, we showed robust FL expression by BLI (Fig 5) and tumor growth inhibition by PET (Fig 7).

In systemic gene or drug delivery for liver cancer, there are multiple anatomic and physiologic barriers to lower the efficiency of targeting (35): (i) interaction with blood components, (ii) filtration by pulmonary vascular bed, (iii) uptake by reticuloendothelial system or nontarget cells, (iv) limited single-path proportion, and (v) no specificity in cellular uptake and intracellular targeting. Because of these limitations and toxicities of systemic exposure, the target concentration is usually low and the efficiency of delivery is limited. The main advantage of interventional local delivery of therapeutic agents is that it can bypass multiple anatomic and physiologic barriers to a target. In conventional transcatheter arterial chemoembolization therapy for HCC, chemotherapeutic agents can be localized within a tumor in high concentrations that are as many as 100 times greater than those achievable with systemic chemotherapy (36). Direct injection of the vector into the tumor is another preferred way to achieve an efficient transduction in patients with liver cancer (37). A gene can be delivered to a targeted area of an organ precisely under image guidance by radiologic devices such as fluoroscopy, ultrasound, computed tomography, or magnetic resonance imaging, which can help to preserve hepatic function, reduce systemic toxicity, and increase local effects, and thereby improve the therapeutic outcome. In the present study, direct visual observation of the tumor after laparotomy was preferred over image-guided intratumoral delivery because of the small size of the tumors and the necessity to avoid extra-hepatic spillage of virus and unwanted viral transduction in the peritoneal cavity.

The present study has several limitations. First, we were able to detect initial gene expression on day 1 by using BLI. However, we did not perform serial BLI imaging because our construct was a suicide gene. Instead, our endpoint was ¹⁸F-FDG-PET activity to determine the therapeutic effect of our gene construct. This was of particular interest in the intravenous TRAIL group, in which we did not detect BLI signal on day 1, but did see a therapeutic benefit, which suggests that we were unable to detect very low levels of transgene expression. Second, although we have demonstrated the efficacy of intratumoral delivery in a single-tumor orthotopic model, intratumoral delivery would be impractical for multiple tumors or metastatic disease. Moreover, large tumors would not be treated

effectively because it would be impossible to achieve homogenous distribution of the genes throughout the heterogeneous tissue matrix of the targeted tumor. To overcome this problem, multiple interventions with the use of vectors replicating selectively in the tumor cells have been proposed, as well as systems enabling the transgenic product to diffuse and penetrate neighboring cells (38). One example of the latter strategy is the fusion of p53 to VP22, a protein from herpes simplex virus type 1 with the remarkable property of being transported through cell boundaries (39).

In conclusion, intravenous delivery of Ad-Survivin-TRAIL-FL showed a therapeutic effect, but intratumoral injection demonstrated superior transduction efficiency and therapeutic efficacy for animal HCC. Targeting efficiency of gene or drug delivery to the liver cancer can be remarkably improved by intratumoral administration. Therefore, local delivery might be a promising approach for amplifying the tumor-specific effect of transcriptionally targeted therapeutic vectors, and the combination of these strategies may lead to improved therapeutic outcomes for future clinical practice.

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ABBREVIATIONS

Ad-pCMV-FL	adenovirus encoding a cytomegalovirus promoter and firefly luciferase
Ad-pSurvivin-TSTA-TRAIL-FL	adenoviral vector carrying two-step transcriptional amplification of Survivin promoter driving tumor necrosis factor- α -related apoptosis-inducing ligand and firefly luciferase
BLI	bioluminescence imaging
CMV	cytomegalovirus
^{18}F-FDG	[^{18}F]fluorodeoxyglucose
FL	firefly luciferase
HCC	hepatocellular carcinoma
PBS	phosphate-buffered saline
PET	positron emission tomography
ROI	region of interest
TRAIL	tumor necrosis factor- α -related apoptosis-inducing ligand
TSTA	two-step transcriptional amplification

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Figure 1.

Schematic illustration of the Survivin-targeted bidirectional TSTA system. In the first step, pSurvivin is used to drive the expression of the transactivator fusion protein Gal4-VP16, composed of a Gal4-DNA-binding domain (*DBD*) fused in frame to two copies of the VP16-transactivation domain. The use of the GAL4-VP16 fusion helps in amplifying the expression of tumor-specific promoter to induce high level of the target protein ultimately. In the second step, the transactivator fusion protein Gal4-VP16 binds to Gal4-binding sites situated in between two thymine-adenine-thymine-adenine promoters, each of which simultaneously drives a downstream gene (TRAIL or FLuc). Therefore, this system is helpful in indirectly imaging the expression of TRAIL (ie, therapeutic gene) by monitoring the level of expression of the transcriptionally coupled FLuc (ie, reporter gene). (Available in color online at www.jvir.org.)

Figure 2.

Overview of experiment schedule. (Available in color online at www.jvir.org.)

Figure 3. ^{18}F fluorodeoxyglucose PET imaging obtained at 14 days after tumor implantation. Transverse (left) and coronal (right) images show hypermetabolic foci (arrows), verifying successful tumor formation in the liver. Arrowhead represents ^{18}F -FDG uptake in the normal myocardium.

Figure 4.

BLI obtained at 1 day after intravenous delivery of Ad-pSurvivin-TSTA-TRAIL-FL (left) and Ad-pCMV-FL (right). Ad-pSurvivin-TSTA-TRAIL-FL shows no FL activity, whereas Ad-pCMV-FL shows diffuse FL activity from the entire liver as a result of the strong hepatotropic feature of adenovirus.

Figure 5.
BLI obtained at 1 day after intratumoral delivery of Ad-pSurvivin-TRAIL-FL (left) and Ad-pCMV-FL (right). Both groups show strong FL activities from the orthotopic HCC, which verifies viral transduction and gene expression.

Figure 6.

Analysis of ^{18}F -FDG-PET imaging in intravenous delivery. Ad-pSurvivin-TRAIL-FL shows less increase of tumor activity than Ad-pCMV-FL during the follow-up period and, at day 7 after gene delivery, the tumor activity in the Ad-pSurvivin-TRAIL-FL group is significantly lower than that in the Ad-pCMV-FL group (3.5 ± 0.58 vs 6.0 ± 0.71 ; $*P = .02$). (Available in color online at www.jvir.org.)

Figure 7.

Analysis of ^{18}F -FDG-PET imaging in intratumoral delivery. Ad-pSurvivin-TRAIL-FL shows less increase of tumor activity at day 4 after gene delivery than Ad-pCMV-FL and a decrease of tumor activity at day 7, at which time the difference of tumor activities is significant between Ad-pSurvivin-TRAIL-FL and Ad-pCMV-FL (2.4 ± 0.50 vs 5.4 ± 0.78 ; $*P = .01$). (Available in color online at www.jvir.org)

Figure 8.

Comparison of ^{18}F -FDG-PET imaging between intravenous and intratumoral delivery of Ad-pSurvivin-TRAIL-FL. Although the tumor activities in both delivery methods are almost the same at day 4 after gene delivery, intratumoral delivery shows a statistically significant decrease of tumor activity at day 7 compared with intravenous delivery (2.4 ± 0.50 vs 3.5 ± 0.58 ; $*P = .03$). (Available in color online at www.jvir.org.)