Positron Emission Tomography of Herpes Simplex Virus 1 Oncolyis


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Abstract

Viral oncolysis, the destruction of cancer cells by replicating viruses, is under clinical investigation for cancer therapy. Lytic viral replication in cancer cells both destroys the cells and liberates progeny virion to infect adjacent cancer cells. The safety and efficacy of this approach are dependent on selective and robust viral replication in cancer cells rather than in normal cells. Methods to detect and quantify viral replication in tissues have relied on organ sampling for molecular analyses. Preclinical and clinical studies of viral oncolysis will benefit significantly from development of a noninvasive method to repetitively measure viral replication. We have shown that positron emission tomography (PET) allows for in vivo detection of herpes simplex virus (HSV)-1 replication in tumor cells using 9-(4-[18F]-fluoro-3-[hydroxymethyl]butyl)guanine ([18F]FHBG) as the substrate for HSV thymidine kinase (HSV-TK). As expected, phosphorylated [18F]FHBG is initially trapped within HSV-1–infected tumor cells and is detectable as early as 2 h following virus administration. MicroPET images reveal that [18F]FHBG accumulation in HSV-1–infected tumors peaks at 6 h. However, despite progressive accumulation of HSV-1 titers and HSV-TK protein in the tumor as viral oncolysis proceeds, tumor cell degradation resulting from viral oncolysis increases over time, which limits intracellular retention of [18F]FHBG. These observations have important consequences with regard to strategies to use [18F]FHBG PET for monitoring sites of HSV-TK expression during viral oncolysis. [Cancer Res 2007;67(7):3295–300]

Introduction

Early clinical trials in cancer gene therapy relied on viruses that are genetically engineered to prevent their replication in humans (1). This initial focus on replication-defective viruses resulted from concerns that replication of therapeutic viral vectors in humans would lead to unwanted toxicity. However, infection of tumors with replication-conditional viruses effectively reduces tumor burden as a result of cytopathic effects induced by lytic viral replication (2, 3). Following initial viral infection of tumor cells, progeny virions liberated from lysed tumor cells infect and destroy adjacent tumor cells, thereby resulting in an iterative process referred to as viral oncolysis. The safety and efficacy of this approach are dependent on selective and robust viral replication in cancer cells rather than in normal cells (4). Some replication-conditional viruses display innate tropism for cancer cells (5, 6), whereas others harbor genetically engineered alterations to increase their specificity for replication in tumor cells relative to normal cells (7, 8).

Many viruses have been examined for their potential as oncolytic viruses (9–13), including herpes simplex virus 1 (HSV-1). Several properties of this virus render it well suited to applications of viral oncolysis, including its high transgene capacity, lack of integration into the cellular genome, prevalence in the population, sensitivity to specific antiviral compounds (e.g., acyclovir), and rarity with which it produces severe medical illness. Oncolytic HSV-1 mutants have been engineered that replicate effectively and preferentially in neoplastic cells in vivo (7, 14, 15), reduce tumor burden in animal models (13, 16, 17), and carry therapeutically oriented transgenes that add to antineoplastic efficacy (18, 19). The safety and efficacy of HSV-1 mutants have been examined in clinical trials for patients with brain tumors (20), colon and rectal carcinoma liver metastases (21), and melanoma (22). Information learned from these clinical trials has been limited by the absence of methods to noninvasively determine sites of viral replication.

At present, detection of viral replication requires biopsy of tissue followed by analysis using molecular techniques such as immunohistochemical staining and PCR amplification. This strategy is suited to animal models but not suited to clinical trials. The ability to noninvasively and repetitively measure the magnitude and anatomic sites of viral infection in vivo would dramatically improve the quality of information learned from preclinical and clinical studies of viral oncolysis.

We and others have evaluated positron emission tomography (PET) for its usefulness in tracking sites of viral replication. The prototype enzyme-based PET reporter system relies on HSV thymidine kinase (HSV-TK). This viral enzyme is more promiscuous than its mammalian homologue in its spectrum of substrates and can phosphorylate a variety of substrates that are not phosphorylated by mammalian thymidine kinase. These include acycloguanosines [acyclovir; ganciclovir; 9-(4-fluoro-3-[hydroxymethyl]butyl)guanine (FHBG); and 9-[(3-fluoro-1-hydroxy-2-propoxyl)methyl]guanine] and thymidine analogues [5-ido-2′-fluoro-1′β-arabinofuranosyl-uracil (FIAU); ref. 23]. These compounds remain sequestered within cells once they are phosphorylated. Thus, when these compounds are radiolabeled with positron emitting isotopes, PET imaging identifies sites of HSV-TK activity.

We have characterized microPET measurements of HSV-1 oncolysis of murine flank tumors using [18F]FHBG. Importantly, unlike any studies done to date, this study includes a sequential analysis of the evolution of viral oncolysis over time. MicroPET measurements combined with direct measurements of reporter proteins and viral titers reveal an important discordance between levels of viral replication, HSV-TK expression, and microPET measurements. We also conducted microPET imaging studies of metabolic activity of the tumors and tumor cell replication during progression of the viral oncolysis using [18F]fluorodeoxyglucose and [18F]fluorothymidine ([18F]FLT), respectively.
Materials and Methods

**Cells and viruses.** MC26 murine colon carcinoma cells and African green monkey kidney cells (Vero) were maintained in DMEM supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 100 units/mL penicillin (Life Technologies, Inc., Carlsbad, CA), and 100 μg/mL streptomycin (Life Technologies).

HSV-TK-transformed MC26 clones were created by subcloning sr39tk cDNA (kindly provided by Dr. S. Gambhir, Stanford University, Palo Alto, CA) into an expression vector driven by an SV40 promoter. MC26 cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA), and stable G418-resistant clones were isolated and screened for thymidine kinase expression using hypoxanthine-aminopterin-thymidine medium and by selecting their sensitivity to ganciclovir. An sr39tk-expressing MC26 stable transformant was selected that shows 15-fold greater accumulation of 18F]FHBG compared with nontransformed MC26 cells. Methods used to prepare and store virus and methods used to determine viral titers in vitro and in vivo have previously been described (24).

**Animal studies.** Animal studies were done in accordance with policies of the Massachusetts General Hospital Subcommittee on Research Animal Care and the IACUC of the City of Hope (Duarte, CA). All studies involving animals were done in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care and the IACUC of the City of Hope (Duarte, CA). All studies involving animals were done in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care and the IACUC of the City of Hope (Duarte, CA). All studies involving animals were done in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care and the IACUC of the City of Hope (Duarte, CA). All studies involving animals were done in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care and the IACUC of the City of Hope (Duarte, CA).

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**MicroPET imaging of HSV-1 replication in murine tumors.** Once flank tumors reached 5 mm in size, 1 × 10^6 pfu of hrR3 were injected directly into the center of the tumor. Mice were anesthetized via inhalation of isofluorane (1–1.5%) with an oxygen flow rate of 2 L/min. Depth of anesthesia was monitored by respiratory rate and eye and footpad reflex. Catheterization of tail vein was done for administration of radioligand (needle size, 30 gauge; volume of injected activity, 20–60 μL). Heparin sodium (6 units/mL) was inoculated into the tail and upper flanks. Seven days later, 1 × 10^6 plaque-forming units (pfu) of hrR3 in 100-μL PBS were injected into the center of the tumor.

A total of 16 microPET imaging studies were conducted in 10 tumor mice using a viral dose of 1 × 10^6 pfu to investigate HSV-1 replication. MC26 murine colon carcinoma cells stably transformed to express HSV-TK (MC26sr39tk) implanted into the left flanks of BALB/c mice were used to confirm 18F]FHBG expression in vivo.

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**Western blot analysis of HSV-TK expression.** Tumor cell lysates containing equal amounts of protein were resolved by PAGE, and proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were incubated overnight at 4°C with rabbit

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After washing thrice with PBS for 1 min each, antidigoxigenin-peroxidase conjugate was added to the slides and incubated for 30 min. The slides were washed twice with PBS and then counterstained with propidium iodide for 15 min. The sections were dehydrated in xylene for 2 min and then mounted with glass coverslip. ApopTag-positive cells were determined under a light microscope with appropriate filters for fluorescence.

**Statistical analysis.** Data, expressed as the mean ± SD, were compared using the unpaired two-tailed t test.

**Results**

Detection of anatomic sites of [18F]FHBG sequestration with microPET. MC26 murine colon carcinoma cells transformed to

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**Figure 1.** Determining the expression of HSV1-TK as a PET reporter probe for imaging hrR3 expression in tumors. A, microPET image of HSV-TK–transformed mouse colon cancer tumor (left) and nontransformed mouse colon cancer MC26 tumor (right) on the flanks radiolabeled with [18F]FHBG. B, time-activity curves of [18F]FHBG accumulation in left tumor (blue curve), right tumor (green curve), heart (red curve), and liver (yellow curve). C, microPET imaging following hrR3 injection correlated with tumor pathology. Row 1, transverse microPET scans of the upper body to include the left and right tumor regions of the mouse at different time points after virus injection. Mice were imaged at 2, 6, 24, 48, and 72 h after virus (hrR3) injection. Row 2, H&E-stained sections of the hrR3-injected (right) tumors. Row 3, X-gal staining for β-galactosidase expression by hrR3. Row 4, immunohistochemical staining for HSV1-TK expression in right tumors. Row 5, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for apoptosis in the hrR3-treated (right) tumors. Row 6, immunohistochemical staining for PCNA in right tumors.
express HSV-TK (MC26sr39tk) were implanted into the left flanks of BALB/c mice and untransformed control tumors (MC26) were implanted into the right flanks. MicroPET imaging with \[^{18}F\]FHBG was done once tumors reached 5 mm. As expected, gradual accumulation of \[^{18}F\]FHBG was observed in the HSV-TK–transformed (left flank) tumor over time (Fig. 1A) as a result of intracellular entrapment of HSV-TK–phosphorylated \[^{18}F\]FHBG. Time-dependent washout of \[^{18}F\]FHBG was observed in the right-flank tumor that was comparable to that of heart and liver (Fig. 1B), which relates to blood flow.

Dynamic \[^{18}F\]FHBG PET study following intratumoral injection of an oncolytic HSV-1 mutant. The progression of HSV-1 oncolysis and HSV-TK activity was studied over time with microPET. Bilateral MC26 tumors were established in BALB/c mice flanks, and once the tumors reached 5 mm in size, \(1 \times 10^9\) pfu of hrR3 were injected into the right-flank tumor. PET scans were obtained with \[^{18}F\]FHBG freshly prepared for each time point: 2, 6, 24, 48, and 72 h after hrR3 administration. As a result of disruption of the gene encoding the large subunit of the viral ribonucleotide reductase, hrR3 replicated preferentially in mitotically active cancer cells compared with quiescent normal cells (14).

Evidence of HSV-TK expression was observed as early as 2 h after viral injection (Fig. 1C). MicroPET scans revealed an area of intense radiolabel uptake in the treated tumor compared with the contralateral untreated tumor. The radiolabel uptake in the right tumor peaked by 6 h after virus injection to gradually decrease its intensity by 72 h. The accumulation of \[^{18}F\]FHBG in the virus-injected right tumor compared with the untreated left tumor was considerable (Fig. 24).

The observation that \[^{18}F\]FHBG peaked as early as 6 h following injection of virus into the tumor was unexpected considering the kinetics of viral replication and the process of iterative infection of tumor cells by progeny virion liberated from the first wave of virus-infected cells. We therefore measured hrR3 titers in infected tumors and observed that the viral titer peaked 24 h after hrR3 administration. Peak hrR3 at this time point was 4-fold greater than the titer recovered immediately after intratumoral injection (Fig. 2B). Thus, the discordance between the time to peak \[^{18}F\]FHBG accumulation and time to peak hrR3 titers in treated tumors may be a result of tumor cell lysis taking place following viral oncolysis.

Pathologic evaluation of hrR3-treated tumors. To further investigate the kinetics of intratumoral \[^{18}F\]FHBG accumulation and viral titers, HSV-TK expression following hrR3 injection was assessed by immunohistochemical staining of sections from harvested tumors. These studies showed gradually increasing expression of HSV-TK over 72 h (Fig. 1C). Similar results were observed in an analysis of HSV-TK in tumor by Western blot (Fig. 2C). Similarly, expression of the lacZ transgene seemed to increase over 72 h following hrR3 administration. These data suggest that the decline in \[^{18}F\]FHBG accumulation in tumors starting after 24 h post intratumoral injection of hrR3 is not caused by a decline in HSV-TK expression.

Histologic examination of hrR3-treated flank tumors revealed small regions of tumor destruction at the earliest time point of viral infection (2 h), which continued to increase in size over time.
(Fig. 1C). Tumor destruction following viral oncolysis was characterized by cellular degradation, nuclear polymorphism, and loss of cellular outline together with nuclear fragmentation leading to necrosis. Apoptosis continued to increase over the 72-h duration of the study. Of note, these destroyed cells were unable to retain phosphorylated [18F]FHBG intracellularly.

Measurement of tumor cell proliferation during viral oncolysis with hrR3 by microPET imaging with [18F]FLT. The effect of viral oncolysis on tumor cell proliferation was studied by microPET imaging of proliferating cells using [18F]FLT. FLT data obtained in untreated tumors showed considerable radiolabel uptake by both left and right tumors indicative of robust tumor cell proliferation at a rate of 0.012 ± 0.004% per hour, corresponding to a tumor doubling time of 58 ± 16 h. After virus injection into the right tumor, FLT uptake in the right tumor decreased whereas the FLT uptake continued to increase in the untreated left tumor (Figs. 2D and 3). These measurements were in accordance with results of immunohistochemical staining for PCNA in virus treated tumors, which revealed a decrease in the percentage of proliferating cells after viral oncolysis. PCNA is a nuclear polypeptide whose synthesis correlates with G1 and S phases of the cell cycle in proliferating cells. The percentage of PCNA-positive cells decreased from 60% at 2 h after virus infection to 37% at 72 h.

Discussion

Based largely on promising results of viral oncolysis observed in preclinical models, several replication-conditional viruses are currently under examination in clinical trials (20, 22, 28–30). Notably, the elements of success observed in preclinical models are both efficacy in reduction of tumor burden and preferential infection of tumor cells rather than normal cells. This latter observation in animal models has generally been studied by molecular analyses of potential sites of viral infection; harvested organs from sacrificed animals are analyzed by PCR, immunohistochemistry, or fluorescent microscopy for evidence of transgene expression or viral gene expression using immunohistochemical staining, PCR, and fluorescent microscopy (16, 19, 31–34). An assessment of sites and magnitude of viral replication in vivo is absolutely necessary to correlate vector design as well as dose schedule with specificity of infection, toxicity, and antineoplastic efficacy.

For these same reasons, determining the sites and magnitude of viral replication during viral oncolysis of tumors is important in clinical trials. Clearly, organ sampling for molecular analyses is much less feasible in clinical studies compared with animal models, as these procedures are invasive, cannot be done repetitively in multiple organs, and are subject to sampling errors. It is therefore critically important to develop techniques for noninvasively imaging viral replication.

Several imaging modalities have been tested for imaging cancer gene therapy, including PET, optical imaging, single-photon emission computed tomography, and magnetic resonance imaging (26, 35). PET has many advantages, including high spatial resolution and methods for imaging molecular processes that are quantitative, repeatable, and three-dimensional. As a single imaging modality, PET allows for accurate tumor localization as well as determination of response to treatment. Thus, successful development of PET for imaging of viral replication would theoretically enable PET to simultaneously acquire information on tumor location, sites, and magnitude of viral replication, and response to therapy. Importantly, concepts developed in preclinical studies with microPET are easily translated to clinical PET studies.

Previously published studies have reported on nuclear imaging of HSV-1 viral oncolysis. For example, HSV-1 has been labeled passively with [111In-oxine, and viral biodistribution in animals was then assessed with gamma camera imaging (36). One drawback to this approach is that the process of passive labeling with [111In-oxine labels both infectious and noninfected viral particles. Moreover, this technique measures only the initial biodistribution of viral particles and is not capable of imaging successive waves of viral infection and replication. In another study, [124I]FIAU was used for PET imaging of HSV-TK expression following HSV-1 infection of tumors (37). The investigators showed that different HSV-1 mutant viruses produced different levels of [124I]FIAU accumulation in vivo following infection of cells. [124I]FIAU label accumulation in vivo correlated with the amount of virus inoculated directly into a tumor, but, importantly, these investigators did not study HSV-1 replication within the same tumor over time (e.g., repetitive imaging for quantification in the same animal). In a separate study, HSV-TK reporter was imaged with PET using [124I]FIAU following direct inoculation of hrR3 into rat 9L gliosarcoma tumors (38). The rate of FIAU accumulation in hrR3-infected rat gliosarcoma cells was correlated with the levels of HSV-TK gene expression. These studies involved selection of a single time point to carry out PET imaging and did not attempt to study viral replication in a single tumor over time.

Our imaging studies show the ability to detect HSV-TK phosphorylation of [18F]FHBG as early as 2 h after administration of 1 × 10⁶ pfu into a tumor. Unlike previous studies, we have imaged the treated tumors sequentially over time. Radiolabel accumulation is seen to peak by 6 h after virus injection. As viral replication gathers momentum and viral oncolysis proceeds, HSV-TK increases in the tumor. However, with increasing cell lysis resulting from viral oncolysis, [18F]FHBG does not remain sequestered within the cells and PET signal within the tumor declines. While it is possible that the nature of the tumor microenvironment at later time points of viral oncolysis impedes...
[18F]FHBG access to the remaining viable tumor cells, PET studies in HSV-1–treated tumors with [18F]fluorodeoxyglucose argue against this hypothesis (data not shown). On the other hand, [18F]FLT data suggest a decrease in viable tumor cells following virus treatment over time, which is indicative of viral effects.

We initiated this study with the prospect of imaging sites and magnitude of viral replication during viral oncolysis of tumors over time. Our data suggest that viral replication in tumors can be imaged by microPET during the early stages of viral oncolysis. However, as viral oncolysis creates tumor destruction, the imaging system fails to correlate with the magnitude of viral replication. An intact cell is required for intracellular sequestration of phosphorylated [18F]FHBG, but cell lysis, which is the hallmark of viral oncolysis, makes imaging the latter stages of viral oncolysis in tumors unachievable. The important interaction between HSV-1 oncolysis and PET imaging of phosphorylated [18F]FHBG that we have described herein does not eliminate PET as a useful modality for imaging viral oncolysis. PET retains its usefulness for identification of sites of initial HSV-1 infection, and a reduction in [18F]FHBG accumulation following an early peak correlates with effective oncolysis. Additional experiments are necessary to determine whether PET imaging of HSV-1 infection is applicable following intravascular administration (rather than intratumoral administration). Clinical trials of viral oncolysis would benefit significantly from noninvasive measurements of these variables.

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