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Use of Positron Emission Tomography to Target Prostate Cancer Gene Therapy by Oncolytic Herpes Simplex Virus

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Abstract

Herpes simplex virus (HSV) oncolytic gene therapy is a promising treatment modality against cancer. We have demonstrated that androgen-induced cellular changes enhance oncolytic viral replication and improve efficacy in the treatment of androgen-dependent prostate cancer cell line. Imaging of changes in 18-F deoxyglucose (FDG) uptake by positron emission tomography (PET) is a sensitive method of detecting altered cellular metabolism involved in cancer therapy. We therefore hypothesized that FDG-PET can predict tumor response to oncolytic HSV therapy. In this study, androgen increased cell kill (74%) *in vitro* and enhanced viral yield (2.4-fold) *in vivo* following HSV therapy. This enhanced efficacy was predicted by high FDG accumulation in intact animals compared to low FDG uptake following orchiectomy ($p=0.002$). This proof-of-concept study provides the mechanistic basis for selecting patients for targeted oncolytic viral therapy by means of a noninvasive molecular imaging method in the treatment of prostate cancer.

Keywords

Herpes virus; hormonal therapy; prediction; fluorodeoxyglucose

Background

Over the past decade there has been a rapid expansion in the number and types of agents undergoing clinical development for use in cancer therapy. Success in early-stage trials examining novel anti-cancer therapies however, is not uniformly associated with success in phase III trials, often due to poor selection of patients. As a result, there is a need for techniques which will enable the selection of a subset of patients that are most likely to benefit. This is particularly important for novel gene therapies, where large trials will be associated with prohibitive costs

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Oncolytic viral therapy using multi-mutated replication-competent herpes simplex viruses (HSV) proved to be effective against a number of malignancies in experimental cancer (1–5). Oncolytic HSV are genetically engineered to replicate selectively within tumor cells (6–8). Our laboratory has shown that oncolytic HSV are highly effective against experimental prostate cancer both *in vitro* and *in vivo* (9). Prostate cancer is the most common malignancy among men and the second leading cause of cancer mortality in the USA (10). Current treatment modalities are associated with a high rate of late complications (11–16). Oncolytic HSV therapy is currently under clinical trials for other malignancies, and can be delivered regionally to the prostate, using transrectal ultrasound (TRUS) guidance. A screening method allowing us to predict which prostate cancer patients are likely to respond to oncolytic viral therapy would be beneficial in targeting HSV therapy to selected patients.

Among cancer screening modalities, the role of PET in the diagnosis and management of cancer is rapidly becoming established, due to its sensitivity and diversity of applications. PET is also under investigation for the evaluation of response to treatment (17), and to monitor the delivery of transduced genes during gene therapy (18;19). FDG-PET demonstrates a positive uptake in the primary tumor within a subset of prostate cancer patients (20). Our aim was to discover whether the tumor characteristics that result in a higher FDG uptake are linked to the response to oncolytic HSV treatment.

It is known that cellular proliferation of prostate cancer cell lines as well as other cell lines correlates directly with glucose uptake which can potentially be non-invasively measured by FDG- PET (21). Furthermore, tumor cells with higher S-phase fractions and shorter doubling times have been shown to be more sensitive to oncolytic HSV-1 therapy (22). We therefore hypothesized that FDG-PET can be used as a surrogate marker for predicting tumor response to oncolytic HSV therapy in a prostate cancer xenograft model.

Materials and Methods

Cells

The androgen-sensitive prostate cancer cell line CWR22 (a kind gift of Dr. Zohar Dotan, Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY) was grown in phenol red-free RPMI supplemented with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% charcoal dextran treated fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cells were maintained in a 5% CO₂ humidified incubator at 37°C.

Virus

NV1066 is a replication-competent attenuated herpes simplex-1 mutant virus whose structure has been described in detail previously (23). Briefly, this virus differs from the wild-type in that single copies of the ICP-0, ICP-4, and $\gamma_134.5$ genes have been deleted to increase tumor specificity and to decrease neurovirulence. In addition, NV1066 contains the enhanced green fluorescent protein (eGFP) sequence under the control of a cytomegalovirus (CMV) promoter which serves as a marker of infection. Viral stocks were propagated on Vero cells (American Type Culture Collection, Rockville, MD) and titered by standard plaque assay.

In vitro Cytotoxicity Assay

CWR22 cells were plated (5×10^4 cells per well and 2×10^4 cells per well, respectively) in 12-well flat-bottom plates with either 3 μ M DHT (dihydrotestosterone) or vehicle alone. After incubation for 12 hours, each well was infected with NV1066 at a multiplicity of infection (MOI, or the ratio of viral plaque forming units [PFU] per tumor cell) of 0.005 and 0.01. Every other day after infection, cells were lysed to release intracellular lactate dehydrogenase (LDH).

LDH was then quantified with a Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI). Results are expressed as the surviving fraction of cells as determined by the measured absorbance of each sample relative to control, untreated cell lysates. Additionally we quantified cellular proliferation and response to treatment in the presence or absence of androgen by determining the absolute numbers of cells after seven days under each condition. Viable cells from individual wells were counted after trypsinization and staining with trypan blue. All samples were analyzed in triplicate.

Animal Studies

All animal procedures were performed with the approval of the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Eight-week old male athymic rats (National Cancer Institute, Bethesda, MD) were used for all *in vivo* experiments and were housed in a temperature- and light-controlled animal facility. Food and water were permitted *ad libitum*. In all PET imaging studies, as well as surgical procedures, animals were anesthetized with inhalational methoxyflurane and were sacrificed by CO₂ inhalation at the termination of the experiment.

Flank Tumors

Flank tumors were established in all experiments by injecting 1×10^7 CWR22 cells suspended in 100 μ L solution (50% PBS, 50% Matrigel; BD Biosciences, Bedford, MA) into the subcutaneous flanks of athymic rats. In all experiments, tumors were measured three times weekly after implantation, and tumor volumes were recorded.

¹⁸FDG-PET Imaging

Radiopharmaceutical Preparation

The fluorine-18 labeled 2-[¹⁸F]-fluoro-2-deoxy-D-glucose (¹⁸FDG) was prepared using the General Electric PETtrace Microlab. All radiopharmaceuticals were subjected to stringent quality control assays including sterility and apyrogenicity testing of batches ¹⁸FDG was formulated in phosphate buffered saline (PBS).

PET Imaging

¹⁸FDG accumulation was measured *in vivo* using a whole-body General Electric Advance PET scanner (Milwaukee, WI). Flank tumors were established in the subcutaneous flanks of 7 athymic rats (three tumors per animal) as described above. Baseline PET imaging was performed two weeks after tumor cell implantation with an average tumor volume of 596 mm³. Animals were fasted for 6 hours before PET imaging, with the exception of water intake *ad libitum*, and were anesthetized with inhalational methoxyflurane for the study. Rats were positioned prone and a 2 minute scout transmission was performed to verify correct positioning. 2.2 mCi ¹⁸FDG in 100 μ L PBS was then injected into the penile vein over 30 seconds and whole-body activity was recorded. The PET dynamic scan consisted of 10 one minute frames, 10 two minute frames, and 3 five minute frames. All scans were reconstructed using vendor provided iterative reconstruction software.

After obtaining baseline PET images, animals were randomized to undergo either orchiectomy (n=4 rats, 10 tumors) or sham-operation (n=3 rats, 9 tumors). PET imaging was then repeated 108 hours after operation using the same protocol.

Data Analysis

Regions of interest (ROI) were drawn around the tumors on the final summed 20 minute frames of the ¹⁸FDG scans. These same ROIs were then copied onto all dynamic frames, allowing the generation of physical decay corrected time activity curves. Vendor provided software on the

GE Advance scanner was used to calculate maximum standardized uptake values (SUV_{max}). All values were normalized to the SUV_{max} of both the liver and chest wall. A Wilcoxon test was used to determine the difference between the means of the two determinations.

Real-Time Quantitative PCR Analysis for Determination of Viral Titer

CWR22 flank tumors were established in the bilateral flanks of 14 athymic rats. Seven days after tumor implantation, animals were randomized to undergo either orchiectomy or sham-operation. After an additional two weeks, based on predetermined size criteria, tumors with an average diameter between 7 and 17 mm were selected (n=15 tumors; 8 in orchiectomy group, 7 in sham-operation group) and were treated with a single intratumoral injection of 1×10^7 PFU of NV1066 in 50 μ L PBS. Two additional tumors from each group were treated with PBS alone to serve as a negative control. Seventy-two hours after treatment, animals were sacrificed and tumors were harvested, weighed, and snap frozen in liquid nitrogen for subsequent analysis.

Genomic DNA was isolated per standard protocol (DNeasy Tissue Kit, Qiagen Inc., Valencia, CA). SYBR green-based real-time quantitative polymerase chain reaction (PCR) was performed with a Bio-Rad iCycler thermal cycler and iQ detection system (Bio-Rad Laboratories, Hercules, CA). Forward (5'-ATGTTTCCCGTCTGGTCCAC-3') and reverse (5'-CCCTGTGCGCCTTACGTGAA-3') primers were designed for a 111-base pair (bp) fragment of the HSV-1 ICP-0 immediately-early gene. Forward (5'-GTAACCCGTTGAACCCCAT-3') and reverse (5'-CCATCCAATCGGTAGTAGCG-3') primers were also designed for an 87-bp coding sequence of the 18S housekeeping gene to normalize the amount of DNA present.

Quantification was performed by spiking a 25 mg sample of mouse kidney with a known amount of NV1066 (9×10^7 PFU) to serve as a positive control. Total DNA was isolated and used to construct a standard curve via serial dilutions. A value of PFU per tumor was then obtained via comparison with standard curve. Quantitative PCR was repeated twice for tumor samples with similar results.

Results

In vitro

The effect of androgen presence or absence on cell proliferation was determined in CWR22, a hormone-sensitive prostate cancer cell line. After 7 days in culture, there was a 2.64 fold increase of cells grown in 3 μ M DHT as compared to cells grown without testosterone ($p < 0.01$, t-test) (Figure 1A). Subsequently the effect of androgen on cell kill was assessed. The presence of androgen increased cytotoxicity markedly. Compared to untreated cells, CWR22 cells treated with MOI of 0.005, demonstrated 74% cell kill in the presence of androgen ($\pm 1.7\%$) and no cell kill in the absence of androgen ($110\% \pm 1.7\%$, $P < 0.01$) seven days after infection. Significant differences between the treatment arms were also present at the higher MOI of 0.01 (Figure 1B).

The effect of androgen on cell proliferation and cell kill was also noted in absolute numbers (Figures 1C). CWR22 cells in the presence of DHT grew faster compared to those grown in an androgen-free environment, making the overall number of cells higher ($p < 0.01$). However by day 7, the absolute number of cells was significantly lower for cells treated with NV1066 in the presence of androgen compared to those without ($p < 0.01$).

In vivo

These experiments demonstrate the effect of the endocrine environment on oncolytic HSV treatment efficacy in an androgen-sensitive prostate cancer cell line. Furthermore, androgen

withdrawal gives us a tool to manipulate the cell line in terms of susceptibility to HSV treatment. Previous studies demonstrated the efficacy of HSV treatment for prostate cell line tumors without taking into account the endocrine environment (2;9;24).

We next sought to observe the effect of androgen manipulation on FDG-PET imaging *in vivo* to examine whether it correlated with the response to treatment *in vitro*.

FDG-PET—Flank tumors were established in the subcutaneous flanks of seven athymic rats, three tumors per animal. Baseline PET imaging was performed two weeks after tumor cell implantation with an average tumor volume of 596 mm³. After obtaining baseline PET images, animals were randomized to undergo either orchiectomy (n=4 rats, 10 tumors) or sham-operation (n=3 rats, 9 tumors). PET imaging was then repeated 108 hours after operation using the same protocol. All values were normalized to the SUV_{max} of the liver. A Wilcoxon test was used to determine the difference between the means of the two determinations.

Figure 2 demonstrates the decrease in FDG uptake between baseline and day 5 PET scan. We observed a statistically significant decrease in FDG uptake in the orchiectomized animals at the time of second PET scan. Orchiectomized rats had a 32% decreased SUV_{max} compared to 12% increased uptake in intact animals (Figure 2a, P=0.002). We have therefore demonstrated that as early as 5 days after orchiectomy, FDG-PET exhibits a different metabolic profile for these otherwise identical tumors. Next, we attempted to demonstrate that a decrease of both *in vitro* HSV cytotoxicity and FDG uptake following androgen deprivation translates to lower *in vivo* efficacy of HSV treatment.

NV1066 treatment—Achieving a reliable androgen withdrawal while having a small enough tumor to be treated with a single dose of 1×10^7 PFU of NV1066, did not allow us to use the animals who had undergone the PET study (tumor size was too large to achieve homogenous spread necessary for representative result at real time quantitative PCR). We therefore implanted bilateral CWR22 flank tumors in a separate cohort of 14 athymic rats. Seven days after tumor implantation, animals were randomized by total tumor volume to undergo either orchiectomy or sham-operation. After an additional two weeks, based on predetermined size criteria, tumors with an average diameter between 7 and 17 mm were selected (n=15 tumors; eight in orchiectomy group; mean tumor volume 734 mm³, seven in intact group; mean tumor volume 590 mm³). These were treated with a single intratumoral injection of 1×10^7 PFU of NV1066. Two additional tumors from each group were treated with PBS alone to serve as a negative control. Seventy-two hours after treatment, animals were sacrificed and tumors were harvested. Real-time quantitative PCR for the viral gene ICP-0 was performed. The mean value of PFU per mg tumor obtained for intact rats was 400 ± 94 , median 390 (range 89–690) compared to a mean of 110 ± 60 , median 47 (range 1 to 507) in orchiectomized rats. This represents a 4-fold increase in viral production/mg tumor (p=0.02 by Mann-Whitney U).

Orchiectomy therefore reduces FDG uptake and viral yield post inoculation, suggesting FDG uptake may be a useful predictor of patients in whom viral therapy may be most efficacious.

Discussion

This experiment was conducted on a prostate cancer model. Prostate cancer is a challenging malignancy that affects a large population, being the second leading cause of cancer mortality in the USA and the most common among men (10). Gene therapy can be applied directly to the prostate, using transrectal ultrasound (TRUS) guidance. Oncolytic HSV is an attractive agent for treatment of this cancer because of the selectivity of these viruses for the killing of cancer. Theoretically these replication-competent viruses can infect the cancerous prostate, replicate, and with release of progeny virus further infect neighboring tumor cells to extend

antitumor effects. These viruses have also been engineered to spare normal neural tissues and therefore would theoretically spare important pelvic nerves that are often injured by alternative surgical or radiation therapy. There have been a number of investigators who have attempted to use HSV for treatment of this malignancy (2;9;24). The current study confirms the high sensitivity of this tumor type to viral therapy. Furthermore, we have demonstrated the androgen influence on this sensitivity and the ability of PET scanning in predicting biologic activity.

Others have shown changes in FDG metabolism to be an early indicator of response to therapy (17). The approach to use FDG-PET as a predictor to therapy response, as described in the current study, is to our knowledge a new one. The rationale for this was that FDG uptake and sensitivity to viral therapy of a tumor were both described to correlate tightly with cell proliferation in a direct pattern(21;22). In order to prove our hypothesis and to combine these observations from different studies into one study we decided to use a well established mono systemic model with manipulatable cell proliferation rates.

The current data suggest that the FDG uptake of the tumor may reflect metabolic changes favorable to viral replication. These data would encourage clinical investigation of this non-invasive modality as a method for selecting patients for trials with HSV. More broadly, FDG-PET might predict the response of other highly prevalent and FDG-PET-detectible malignancies (breast cancer, non-small cell lung cancer, and colorectal cancer) to oncolytic HSV therapy.

As more cancer treatment modalities are available to the physician, cancer is more commonly treated in a tailor made approach. Currently, breast cancer tumors are examined both for the hormone receptor and for the human epidermal-growth factor receptor 2 (HER2) status in order to predict sensitivities to currently available treatment modalities (25). The epidermal growth factor receptor (EGFR) mutation, discovered recently in non-small cell lung cancer patients, predicts a dramatic response to gefitinib treatment in the 10% of patients whose tumor carry the mutation (26). The ultimate goal is a non-invasive study that predicts response to specific therapies. Such a test would allow rational patient selection for therapy, as well as decreased cost and increased yield of clinical trials.

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C

Figure 1. Cell proliferation can be influenced by androgen levels and correlates directly with cytotoxicity of oncolytic herpes simplex viral therapy in vitro

A. Androgen-sensitive CWR22 cells were plated at 2×10^4 per well and allowed to proliferate either with 3 μ M dihydrotestosterone (DHT) (-■-) or vehicle alone (-□-). Cell number was assessed by LDH assay, with results compared to readings of a known number of cells.

B. Androgen-sensitive CWR22 cells were plated at 2×10^4 per well either with 3 μ M DHT (-■-) or vehicle alone (-□-) and subsequently infected with NV1066 at a MOI of 0.01. Results of LDH cytotoxicity assays are expressed as the surviving fraction of cells compared to untreated cells grown with 3 μ M DHT (-■-) or vehicle alone (-□-).

C. Absolute numbers of cells were counted after seven days grown with 3 μ M DHT (shaded bars) or vehicle alone (clear bars), either untreated or treated with MOIs of 0.005 and 0.01. MOI (multiplicity of infection) represents the ratio of the number of viral particles to tumor cells.

Figure 2. Hormonally influenced tumor metabolism can be measured by dynamic FDG-PET and correlates with sensitivity to oncolytic viral infection in vivo

A. FDG-PET of CWR22 Flank tumors

Tumors were established in the subcutaneous flanks of seven athymic rats (three tumors per animal). Baseline PET imaging was performed at an average tumor volume of 596 mm³. Animals were randomized to undergo either orchietomy (n=4 rats, 10 tumors) or sham-operation (n=3 rats, 9 tumors). PET imaging was then repeated 108 hours after surgery using

the same protocol. All values were normalized to the SUV_{max} of both the liver and chest wall (data not shown). A Wilcoxon test was used to determine the difference between the means of the two determinations. Tumors in orchiectomized animals demonstrated a decrease in FDG uptake between baseline and day 5 PET scan. Normalized to the liver, the FDG uptake in orchiectomized rats had a 32% decreased SUV_{max} compared to 12% increased uptake in intact animals ($P=0.002$). When normalized to the chest wall, we obtained similar results.

B. FDG-PET of CWR22 Flank tumors before and after orchiectomy

FDG-PET scan demonstrating two flank tumors before and 5 days after orchiectomy: the arrows point to the flank tumors. A decrease in uptake is demonstrated for both tumors.