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Imaging a Genetically Engineered Oncolytic Vaccinia Virus (GLV-1h99) using a human norepinephrine transporter (*hNET*) reporter gene

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Abstract

Purpose—Oncolytic viral therapy continues to be investigated for the treatment of cancer and future studies in patients would benefit greatly from a non-invasive modality for assessing virus dissemination, targeting and persistence. The purpose of this study was to determine if a genetically modified vaccinia virus, GLV-1h99, containing a human norepinephrine transporter (*hNET*) reporter gene, could be sequentially monitored by [¹²³I]MIBG γ -camera and [¹²⁴I]MIBG PET imaging.

Experimental Design—GLV-1h99 was tested in human malignant mesothelioma and pancreatic cancer cells lines for cytotoxicity, expression of the hNET protein using immuno blot analysis and [¹²³I]MIBG uptake in cell culture assays. *In vivo* [¹²³I]MIBG γ -camera and serial

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[¹²⁴I]MIBG PET imaging was performed in MSTO-211H orthotopic pleural mesothelioma tumors.

Results—GLV-1h99 successfully infected and provided dose dependent levels of transgene hNET expression in human malignant mesothelioma and pancreatic cancer cells. The time course of [¹²³I]MIBG accumulation showed a peak of radiotracer uptake at 48h after virus infection *in vitro*. *In vivo* hNET expression in MSTO-211H pleural tumors could be imaged by [¹²³I]MIBG scintigraphy and [¹²⁴I]MIBG PET 48 and 72h after GLV-1h99 virus administration. Histological analysis confirmed the presence of GLV-1h99 in tumors.

Conclusion—GLV-1h99 demonstrates high mesothelioma tumor cell infectivity and cytotoxic efficacy. The feasibility of imaging virus-targeted tumor using the hNET reporter system with [¹²³I]MIBG γ -camera and [¹²⁴I]MIBG PET was demonstrated in an orthotopic pleural mesothelioma tumor model. The inclusion of human reporter genes into recombinant oncolytic viruses enhances the potential for translation to clinical monitoring of oncolytic viral therapy.

Keywords

Gene therapy; oncolytic virus; human norepinephrine transporter; [¹²⁴I]MIBG; [¹²³I]MIBG; PET; gamma camera; molecular imaging; reporter gene

Introduction

Malignant pleural mesothelioma and pancreatic cancer are highly aggressive diseases. The annual incidence in the United States was estimated to be ~40,000 cases for pancreatic cancer and ~4,000 cases for malignant mesothelioma in the year 2004 (1). The increasing incidence of mesothelioma worldwide, especially in industrialized nations, is due to the etiology of this disease from asbestos exposure (2). Both of these tumors are highly resistant to current therapy, with 5-year survival rates of only 5% for pancreatic cancer and 9% for mesothelioma (3). Even with combined surgery, chemotherapy and radiation, only a small minority of patients are rendered disease-free for a prolonged period of time (4).

Oncolytic viral therapy has been studied and tested over the past century, and many viral types, including adenovirus, herpes simplex virus, Newcastle disease virus, myxoma virus, vaccinia virus and vesicular stomatitis virus, are being investigated as novel agents for the treatment of human cancer (5). Adenovirus H101 was approved in 2005 for the treatment of head and neck cancer in China (6). Importantly, viruses generally kill cancer cells that are high in ribonucleotide reductase, high in DNA repair enzymes and resistant to apoptosis, characteristics that tend to make tumor cells resistant to chemotherapy and radiation therapy (7).

Vaccinia viruses are particularly attractive agents for oncolytic therapy because versions of this virus have been given to millions of humans in the eradication of smallpox. Vaccinia virus is also an excellent vector because its large genome allows for insertion of multiple foreign genes (8). In addition, it is highly immunogenic and able to induce strong host immune responses against virus-infected cells (9). Although the acceptance of oncolytic viral therapy has been mixed in the medical community, a substantial amount of data has

been reported from clinical trials with vaccinia virus in cancer patients showing good safety and promising responses (9-12).

Future human oncolytic viral therapy studies would benefit greatly from the ability to track and monitor viral distribution, tumor targeting, proliferation and persistence by noninvasive imaging (13). It would provide important safety, efficacy and toxicity correlations. Such real-time tracking would also provide useful viral-dose and administration schedule information for optimization of therapy and would obviate the need for multiple and repeated tissue biopsies. The virus used in the current study is GLV-1h99. This is a recombinant vaccinia virus expressing transgenes for the human norepinephrine transporter (*hNET*) and *β-galactosidase*. *hNET* is a cell-membrane transporter that is highly expressed in many neuroendocrine tumors and can be imaged by radiolabeled metaiodobenzylguanidine (MIBG). The use of *hNET* reporter gene imaging is particularly attractive from a clinical-investigative standpoint because (a) *hNET* is a human protein that should minimize immunogenicity and (b) MIBG can be radiolabeled with ^{123}I or ^{131}I for single-photon emission computed tomography (SPECT) and γ -camera imaging and also with ^{124}I for positron emission tomography (PET) imaging. Currently, [^{123}I]MIBG is a clinically approved radiolabeled probe for imaging *hNET* expression.

In this study we show that insertion of the *hNET* reporter gene into a recombinant vaccinia virus does not alter tumor killing; GLV-1h99 retains excellent tumor specificity and cytotoxic efficacy. In addition, we demonstrate the feasibility of using the *hNET* reporter system for *in vivo* noninvasive imaging of oncolytic viral therapy in an orthotopic pleural mesothelioma tumor model by [^{123}I]MIBG γ -camera imaging and [^{124}I]MIBG PET imaging.

Material and Methods

Cell lines

Human pancreatic carcinoma cell lines PANC-1, BxPC-3, HS766T and MiaPaCa-2, the mesothelioma cell line MSTO-211H, and the human neuroblastoma cell line SK-N-SH, which expresses *hNET*, were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). JMN cells were a kind gift from Dr. Frank Sirotnik from Memorial Sloan-Kettering Cancer Center (New York, NY, USA). H-2052 and H-2373 cell lines were a kind donation from Dr. Pass from the Karmanos Cancer Institute (Wayne State University, Detroit, MI, USA).

All cells were grown in appropriate media, maintained in a humidified incubator at 37 °C supplied with 5% CO₂ and subcultured twice weekly.

Virus strains

GLV-1h68 (non-*hNET* containing virus) is a replication competent, recombinant vaccinia virus derived from the LIVP strain (Lister strain from the Institute for Research on Virus Preparations, Moscow) and its construction was previously described (14).

GLV-1h99 (hNET-expressing virus) was derived from GLV-1h68 by replacing the *Renilla* luciferase-green fluorescent protein (Ruc-GFP) expression cassette at the F14.5L locus with a hNET expression cassette through *in vivo* homologous recombination.

Cytotoxicity assay

Cells were plated at 2×10^4 per well in 12-well plates in 1 ml of media per well. After incubation for 6 hours, cells were infected with GLV-1h99 or GLV-1h68 at MOI's (multiplicity of infection) of 1.0, 0.10, and 0.01 and 0 (control wells). Viral cytotoxicity was measured daily for 7 days. Cells were washed with PBS and lysed in 200 μ l per well of 1.5% Triton X (Sigma, St. Louis, MO) to release intracellular lactate dehydrogenase, which was quantified with a Cytotox 96 kit (Promega, Madison, WI) on a spectrophotometer (EL321e, Bio-Tek Instruments) at 490 nm. Results are expressed as the percentage of surviving cells. This percentage was determined by comparing the measured lactate dehydrogenase of each infected sample to that in uninfected, control cells. All samples were analyzed in triplicate.

Immunoblot analysis

To evaluate the level of hNET protein expression in cells (H2052, MSTO211H, PANC1) infected with virus (GLV-1h99 or GLV-1h68) and in the neuroblastoma cell line, SK-N-SH, at 12, 24 48 and 72h after infection, immunoblot analysis was performed. A purified mouse antibody against hNET (NET17-1, Mab Tech Inc. GA, USA) was used at a final dilution of 1:500 and incubated for 12 hours at +4° C. The secondary antibody (peroxidase conjugated anti-mouse IgG (Vector labs Inc., CA, USA)) exposure was for 1 hour at a 1:2,000 dilution. Peroxidase-bound protein bands were visualized using the ECL method (Amersham Pharmacia Biotech, Little Chalfont, UK).

In vitro radiotracer assay

[¹²³I]MIBG radiotracer uptake studies were performed in MSTO-211h and PANC1 cells after infection with virus (GLV-1h99 or GLV-1h68) as well as in the neuroblastoma cell line, SK-N-SH, using previously described methods (15). Briefly, cells were plated at 1×10^6 per well in 6-well plates in 2 ml of media per well. After incubation for 6 hours, cells were infected with GLV-1h68 or GLV1h99 at MOIs of 1.0 and 0 (control wells). Following 12-, 24-, 48- and 72-h incubation periods with virus at 37°C and 5% CO₂, the medium was aspirated and the cells were washed with PBS (pH 7.4). [¹²³I]MIBG uptake was initiated by adding 2 ml of DME containing 0.0185 MBq/ml (0.5 μ Ci/ml) carrier-free [¹²³I]MIBG. Cells were harvested after a 60-minute incubation period, and the cell pellet-to-medium activity ratio (cpm/gm of pellet/cpm/ml of medium) was calculated from the radioactivity measurements assayed in a gamma counter (Packard, United Technologies). All studies were performed in triplicate.

Malignant pleural mesothelioma xenograft model

Athymic *nu/nu* female mice were purchased from the National Cancer Institute (NCI, MD) and were housed five per cage and allowed food and water *ad libitum* in the MSKCC Vivarium for 1 week before tumor cell implantation. All animal studies were performed in compliance with all applicable policies, procedures and regulatory requirements of the

Institutional Animal Care and Use Committee (IACUC), the Research Animal Resource Center (RARC) of MSKCC and the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals". All animal procedures were performed under anesthesia induced by inhalation of 2% isoflurane. After the studies all animals were sacrificed by CO₂ asphyxiation.

An incision 3 to 5 mm in length was made over the fourth to fifth intercostal space of the right chest. The underlying inflating and deflating lung was thereby easily visualized through the thin fascia. Slowly, 100 µl of MSTO-211H malignant mesothelioma cellular suspension (5×10^6 cells) were injected. After the injection the skin was closed with surgical staples and mice were returned to their cages.

Intrapleural treatment with virus was performed in a similar fashion as described above 10 days after tumor cell instillation into the pleural cavity. GLV-1h99 or GLV-1h68 (1×10^7 pfu) was administered in 100 µL PBS and animals were gently rotated from side to side to help distribute the virus throughout the pleural cavity. Control animals (no virus) received only 100 µL PBS.

MIBG Synthesis

Clinical grade [¹²³I]MIBG was obtained from MDS Nordion (Canada). The average radiochemical purity was in excess of 97% (determined by MDS Nordion using the Sep Pak cartridge method), and the specific activity ~320 MBq/µmol (8.7 mCi/µmol) according to the vendor.

[¹²⁴I]MIBG was prepared using minor modifications to the reported nucleophilic isotopic exchange method (16), following a procedure previously reported by Moroz and coworkers (17). The radiochemical purity of the final product was >95% with an overall yield of >80% and the specific activity 18.5 ± 5.2 MBq/µmol (0.5 ± 0.14 mCi/µmol). The maximum specific activities (no carrier-added synthesis) for the [¹²³I]- and [¹²⁴I]-labeled compounds were 8.9 and 1.2 TBq/µmol (241 and 33 Ci/µmol), respectively, due to the 7.4-fold difference in the decay rate of the two isotopes.

Clinical grade [¹⁸F]FDG was obtained from IBA Molecular (Somerset, NJ) with a specific activity >41 MBq/µmol (>11 mCi/µmol) and a radiochemical purity of >98%.

[¹²³I]MIBG γ -camera *in vivo* imaging

Each animal was injected intravenously with ~18.5 MBq (500 µCi) of [¹²³I]MIBG 48h after intrapleural GLV-1h99 injection and imaged on a X-SPECT™ dedicated small-animal gamma camera SPECT-CT scanner (Gamma Medica, Northridge, CA). A photopeak energy window of 143-175 keV and a low-energy high-resolution (LEHR) parallel-hole collimator was used to acquire ten-minute ¹²³I images at 2hours post- [¹²³I]MIBG administration.

The X-SPECT™ gamma camera system was calibrated by imaging a mouse-size (30-ml) cylinder filled with an independently measured concentration (MBq/ml) of technetium-99m using a photopeak energy window of 126-154 keV and LEHR collimation. The resulting ^{99m}Tc images were exported to Intefile and then imported into the ASIPRO™

(Siemens Pre-clinical Solutions, Knoxville, TN) image-processing software environment. By region of interest (ROI) analysis, a system calibration factor (in cpm/pixel per MBq/ml) was derived. Animal images were likewise exported to Interfile and then imported into ASIPro™ and parameterized in terms of the decay-corrected percent injected dose per gram (%ID/gm) based on the foregoing calibration factor, the administered activity, the time post-administration of imaging, and the image duration. Implicit in the foregoing analysis is the reasonable assumption that the sensitivities of the X-SPECT™ gamma camera system for ¹²³I and ^{99m}Tc are comparable.

[¹²⁴I]MIBG microPET *in vivo* imaging

In a group of 5 animals (10 days after MSTO-211H tumor cell instillation into the pleural cavity), each animal was injected via the tail vein with 9.25 MBq (250 μCi) of [¹⁸F]FDG. [¹⁸F]FDG PET scanning was performed 1h after tracer administration using a 10-minute list-mode acquisition. Animals were fasted 12h before tracer administration and kept under anesthesia between FDG injection and imaging.

In a group of 16 animals, 4 sub-groups of 3-5 animals each were studied (5 animals in sub-group 1 and 2; 3 animals in sub-group 3 and 4). Each animal was injected via the tail vein with 9.25 MBq (250 μCi) of [¹²⁴I]MIBG. Animals in sub-groups 1 and 2 were injected with GLV-1h99 48 and 72h prior to [¹²⁴I]MIBG administration. Sub-group 3 animals received GLV-1h68 48h prior to radiotracer administration; sub-group 4 animals was not injected with virus, receiving only 100μl PBS). Potassium iodide was used to block the uptake of radioactive iodine by the thyroid. [¹²⁴I]MIBG PET was performed for 10 minute 1, 2, and 4h after tracer administration, for 15 minute at 12h, for 30 minute at 24h, and for 60 minute at 48h. After tracer administration and between imaging time points, the animals were allowed to wake up and maintain normal husbandry.

Imaging was performed using a Focus 120 microPET™ dedicated small-animal PET scanner (Concorde Microsystems Inc, Knoxville, TN). Three-dimensional (3D) list-mode data were acquired using an energy window of 350-700 keV for ¹⁸F and 410-580 keV for ¹²⁴I, respectively, and a coincidence timing window of 6 ns. These data were then sorted into two-dimensional (2D) histograms by Fourier re-binning. The image data were corrected for (a) non-uniformity of scanner response using a uniform cylinder source-based normalization, (b) dead time count losses using a singles count rate-based global correction, (c) physical decay to the time of injection, and (d) the ¹²⁴I branching ratio. The count rates in the reconstructed images were converted to activity concentration (% of injected dose per gram of tissue, %ID/g) using a system calibration factor (MBq/ml per cps/voxel) derived from imaging of a mouse-size phantom filled with a uniform aqueous solution of ¹⁸F.

Image analysis was performed using ASIPro™ (Siemens Pre-clinical Solutions, Knoxville, TN). At all acquired scans (including [¹⁸F]FDG PET, serial [¹²⁴I]MIBG PET and [¹²³I]MIBG γ-camera) ROI's were manually drawn over tumor, lung, liver and skeletal muscle. For each tissue and time point post-injection, the measured radioactivity was expressed as %ID/g. The maximum %ID/g value was recorded for each tissue and from these tumor-to-organ ratios for lung, liver and skeletal muscle were then calculated.

Immunohistochemistry

After the final image, the animals were sacrificed and the tumors harvested and frozen in Tissue-Tek Optimal Cutting Temperature (O.C.T.) Compound (Sakura Finetek USA, Inc., Torrance, CA). Tissues were cut into 5- μ m thick sections and mounted on glass slides. Cryosections were fixed and stained with hematoxylin and eosin (H & E) and 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal; 1 mg/ml) in an iron solution of 5 mmol/l $K_4Fe(CN)_6$, 5 mmol/l $K_3Fe(CN)_6$, and 2 mmol/l $MgCl_2$, as previously described (18), to identify virally mediated *lacZ* expression.

Statistics

A two-tailed unpaired t-test was applied to determine the significance of differences between values using the MS Office 2003 Excel 11.0 statistical package (Microsoft, Redmond, Washington, USA).

Results

Cytotoxicity assays *in vitro* demonstrated dose dependent lytic activity

Four mesothelioma and four pancreatic cancer cell lines demonstrated lytic cytotoxicity following exposure to GLV-1h99 (hNET-expressing virus) and to GLV-1h68 (non-hNET containing virus). Similar cytotoxicity was observed with GLV-1h99 and GLV-1h68 at a MOI of 1.0 (Figure 1A) and a dose-dependent lytic effect was also demonstrated (Figure 1B). At a MOI of 0.1, all MSTO-211H and H2052 mesothelioma cells as well as 80% of the PANC1 pancreatic cancer cells were dead at day 7. Oncolysis appeared to be more gradual over time in PANC1 cells, compared to the more sigmoidal lytic-time profile in MSTO-211H cells (Figure 1). The mesothelioma cell line JMN and the pancreatic cancer cell line HS766T were more resistant, and showed only 80% cell death by day 7 at a MOI of 1.0 (Figure 1C). MiaPaCa2 and BxPC3 (pancreatic cancer cell lines) and H2373 (mesothelioma cell line) were sensitive to the virus only at a higher MOI of 10 (data not shown).

Immunoblot analysis confirmed dose dependent levels of transgene hNET expression

The two most sensitive mesothelioma cell lines (MSTO-211H and H2052) and the most sensitive pancreatic cancer cell line (PANC1), based on the cytotoxicity assays, were chosen for immunoblot analysis and compared to the endogenous hNET-expressing neuroblastoma cell line, SK-N-SH. The levels of hNET expression 24 hours after GLV-1h99 (hNET-expressing virus) viral infection at a MOI of 1.0 were investigated (Figure 2A). In addition to the ~80-kD hNET band, two low-molecular weight immunoreactive bands (~50-55 kD and ~37-40 kD, respectively) are seen in the blots of the GLV-1h99-infected cells; these two low-molecular weight bands are barely visible in the blots of the SK-N-SH neuroblastoma cells. Similar to the cytotoxicity assay, there was a viral dose-dependent expression of hNET at different MOI's (0.1, 1.0, 5 and 10) (shown for MSTO-211H cells in Figure 2B). Strong hNET expression was found in the MSTO-211H and PANC1 cell lines by 12 hours after GLV-1h99 viral infection, peaking at 24 hours followed by a gradual decline over 72 hours (shown for MSTO-211H cells in Figure 2C and 2D). A similar pattern of hNET expression

was observed in the other cell lines, although the hNET immunoblot bands were less intense (data not shown). The neuroblastoma cell line (SK-N-SH), expressing endogenous hNET, served as a positive control for the immunoblot analysis and radiotracer uptake studies; the GLV-1h68 virus-infected (non-hNET containing virus) and the uninfected mesothelioma and pancreatic cancer cell lines served as negative controls (Figure 2B).

***In vitro* [¹²³I]MIBG uptake showed peak of radiotracer uptake 48h after virus infection**

The time course of [¹²³I]MIBG accumulation was studied in PANC1 and MSTO-211H cells following infection with the GLV-1h99 (hNET-expressing virus) virus at a MOI of 1.0. [¹²³I]MIBG accumulation in non-infected MSTO-211H and PANC1 cells was low (Figure 3A and 3B, respectively). There was no significant increase in radiotracer uptake 24h after infection of the cells with GLV-1h68 (non-hNET containing virus, negative control). In contrast, there was a significant ($p < 0.01$) increase in [¹²³I]MIBG accumulation in both cancer cell lines at all time points (12h, 24h, 48h and 72h) after infection with GLV-1h99 (Figure 3A and B). Peak radiotracer uptake was observed at 48h after virus infection in both cell lines. The natural hNET-expressing neuroblastoma cell line (SK-N-SH) served as a positive control. Total cell protein in the [¹²³I]MIBG uptake assays were unchanged over the first 24 hours following GLV-1h99 infection, compared to uninfected cells. At 48 and 72h after viral infection, there was a decrease in measured cell protein (Figure 3C and 3D).

hNET expression imaging by [¹²³I]MIBG scintigraphy and [¹²⁴I]MIBG PET

Following direct injection of hNET-expressing GLV-1h99 virus into MSTO-211H orthotopic pleural tumors, viral localization was visualized by [¹²⁴I]MIBG PET imaging of hNET expression in pleural tumors (Figure 4A). [¹²⁴I]MIBG was intravenously administered 48 or 72 hours after intrapleural virus injection, and sequential PET imaging was performed 1 to 48 hours after radiotracer administration. Tumor radioactivity values (%ID/g) were measured and tumor-to-organ ratios were calculated. The highest levels of radioactivity in the pleural tumors were found 48h after injection of GLV-1h99 (hNET-expressing virus), followed by tumors that were injected with GLV-1h99 72h prior to [¹²⁴I]MIBG administration. Low levels of radioactivity were observed in tumors that were injected with GLV-1h68 (non-hNET containing virus) and in tumors that were not injected with virus (Figure 4B). Maximum activity in both the pleural tumors and remote organs (background) were observed at the time of the initial measurement, 1 hour after radiotracer administration. Tumor and remote organ activity decreased over time (1 to 72 hours) in all four groups of animals. The decrease in tumor activity was more rapid over the first 12 hours after [¹²⁴I]MIBG administration in the two control groups; tumors injected with GLV-1h68 (non-hNET containing virus) or no virus.

Tumor-to-organ (lung, liver, muscle) ratios were calculated from the PET image data (as described in the Material and Methods section) and the highest values were obtained for the group of animals that were infected with GLV-1h99 (hNET-expressing virus) 48h before radiotracer administration (Figure 5). Comparing the animals that were treated with GLV-1h99 48h before [¹²⁴I]MIBG administration to the animals that received no virus, the ratio differences were highly significant ($p < 0.01$) at the 2h imaging time point and significant ($p < 0.05$) at the 1h imaging time point. Nearly the same low tumor-to-organ

ratios were found for the two control groups of animals and the tumor-to-organ ratios decreased over time.

For localization of the tumors and for comparison to a clinically used imaging technique, [^{18}F]FDG PET imaging was also performed. [^{124}I]MIBG PET and [^{18}F]FDG PET imaging were compared (Figure 4 and 5). The pleural tumors were visualized by [^{18}F]FDG PET imaging, but image contrast at 48 and 72 hours after GLV-1h99 virus (hNET-expressing virus) injection was greater with [^{124}I]MIBG PET compared to [^{18}F]FDG PET. The [^{124}I]MIBG and [^{18}F]FDG tumor-to-lung, tumor-to-liver and tumor-to-muscle ratios in control animals were similar.

In vivo hNET expression in the pleural tumors after GLV-1h99 (hNET-expressing) virus administration could also be imaged by [^{123}I]MIBG planar scintigraphy. All GLV-1h99 injected animals showed localized accumulation of [^{123}I]MIBG radioactivity in the virus-injected pleural tumors compared to the control animals that received no virus (Figure 6). The tumor-to-background ratios for the GLV-1h99 infected animals was with 2.4 ± 0.2 , significantly ($p < 0.01$) higher compared to the group that received no virus, 1.5 ± 0.1 .

Immunohistochemistry confirmed viral presence in tumors

All animals were sacrificed and examined to confirm the presence of pleural tumors. All pleural lesions were shown to be malignant pleural mesothelioma on H & E staining (Figure 7A and B). In addition, all tumors infected with vaccinia virus stained positive for lacZ, confirming the presence of the virus in tumors and indicating that all tumors visualized by [^{124}I]MIBG PET or [^{123}I]MIBG scintigraphy reflect GLV-1h99 expression of a functional hNET transporter protein (Figure 7C and D).

Discussion

Oncolytic viral therapy dates back more than a century (19), and has had a mixed and fluctuating level of acceptance in the medical community. Nevertheless, it still holds promise as a biological treatment for some standard therapy resistant cancers (11). A number of viruses (e.g. adenovirus, herpes simplex virus, Newcastle disease virus, myxoma virus, vaccinia virus and vesicular stomatitis virus) have been shown to infect and replicate in cancer cells and to selectively kill them (oncolysis). Oncolytic viral therapy has been used in cancer treatment and has evolved from the use of wild-type viruses to genetically engineered viruses that express therapeutic transgenes. Clinical and preclinical trials involving different viral strains and constructs have demonstrated to be safe and to have potent antitumor effects (10). Oncolytic viruses have also demonstrated enhanced efficacy involving combination regimens with approved chemotherapeutics and radio-therapy (20). Two oncolytic viruses (G207 and H101) have entered randomized phase III clinical testing (10), and marketing approval was obtained for H101 in 2005 (6). Future studies are likely to focus on optimization of viral doses and administration routes, interaction with the immune system and *in vivo* monitoring through imaging. The ability to noninvasively and repetitively identify anatomic sites of viral targeting and to measure the magnitude of viral infection could provide important safety, efficacy, and toxicity information during clinical studies of viral oncolysis.

Vaccinia virus is perhaps the most widely administered medical product in history; it is certainly the most successful biological product. Vaccinia also displays many of the qualities thought necessary for an effective antitumor agent and it is particularly well characterized in humans due to its role in the eradication of smallpox. Vaccinia has a short life cycle and spreads rapidly; it has inherent systemic tumor targeting, a high propensity to induce cell lysis, well-defined biology, and a large cloning capacity. (12). The large insertional cloning capacity allows for the inclusion of several functional and therapeutic transgenes. With the insertion of reporter genes not expressed in uninfected cells, viruses can be localized and the course of viral therapy can be monitored. A noninvasive, clinically applicable method for imaging viruses in target tissue or specific organs of the body would be of considerable value during oncolytic viral therapy in patients.

In this study we describe the use of a genetically modified vaccinia virus, GLV-1h99, that has been engineered for specific targeted treatment of cancer and for non-invasive imaging. GLV-1h99 is able to efficiently infect, replicate in, and lyse a variety of human pancreatic and mesothelioma cancer cell lines. The oncolytic potency of GLV-1h99 was shown to be similar to the non-hNET-containing parent virus, GLV-1h68, in eight pancreatic and mesothelioma cancer cell lines. GLV-1h68 has also been shown to successfully treat an orthotopic animal model of mesothelioma with pleural disease (21).

The reporter gene chosen for insertion into GLV-1h99 was based on the very favorable PET and SPECT imaging characteristics of the hNET-MIBG reporter imaging system (17) and because [^{123}I]MIBG is an approved radiopharmaceutical for clinical imaging of neuroendocrine tumors (22, 23). In contrast to a study published by McCart et al. (24) using an oncolytic vaccinia virus expressing the human somatostatin receptor SSTR2, hNET is a transporter based reporter gene systems. Receptors usually have a 1:1 binding relationship with a radiolabeled ligand; transporters provide signal amplification through transport-mediated concentrative intracellular accumulation of the radiolabeled substrate. hNET is a transmembrane protein that mediates the transport of norepinephrine, dopamine, and epinephrine across the cell membrane (25). It is one of several human reporter genes that are currently being used in preclinical studies (17, 26) and has a high potential for rapid translation into clinical reporter gene imaging studies (27, 28).

The hNET immunoblots showed protein expression in all infected cell lines and the expression was time and dose dependent. The antibody used recognizes a degraded, or less glycosylated form of the protein (~50-55 kD and ~37-40 kD) as well as a more highly glycosylated hNET protein (~80 kD). Interestingly, the low-molecular weight bands were more intense early (12-24h) after GLV-1h99 infection, compared to later time points (48-72h) (Figure 2). These bands also appear in SK-N-SH neuroblastoma cells and in hNET-transduced cell lines (17), but at much lower intensity. We suspect that the prominence of the low-molecular weight bands is the effect of viral infection, replication and lysis, and that the low-molecular weight-immunoreactive protein may be non-functional with respect to MIBG transport and accumulation.

In vitro [^{123}I]MIBG uptake studies also showed time-dependent radiotracer uptake, peaking at 24-48h after viral infection (~5-fold above control) for MSTO-211H cells. The uptake

levels were lower in the GLV-1h99 infected cells compared to the hNET-expressing neuroblastoma cells. In the viral-treated cultures, it is likely that not all of the cells are infected with virus and therefore not all express the reporter gene during the early phase of viral infection. In addition, the hNET protein may not have been translocated and inserted into the cell membrane to form a functional transporter during the initial 12-24h period after infection. During the late, pre-lytic phase of viral infection (72h and beyond), the hNET transporter could be impaired, and following cell lysis the accumulated MIBG radiotracer would be lost. Thus, there appears to be a relatively narrow window, ~ 24-48 hours after viral infection of MSTD-211 H cells, during which the hNET reporter is maximally functional. These results reflect the dynamic state between viral infection, replication, and lysis of tumor cells.

This dynamic state of viral infectivity and functional hNET expression was also observed in the *in vivo* imaging studies. It should be noted that the whole tumor is only partially infected with virus and tumor cells are at different stages of virus infection at any given time (shown by immunohistochemistry). Timing of [124 I]MIBG PET imaging after GLV-1h99 virus injection was very important in the *in vivo* studies. Better imaging results were obtained at 48h compared to 72h after viral injection. MIBG uptake in GLV-1h99 infected cells, both *in vitro* and *in vivo*, is not exactly comparable with MIBG uptake in SK-N-SH neuroblastoma cells or in cells transduced with constitutive hNET expression cassettes (17) where expression levels are constant. Nevertheless, the quantitative [124 I]MIBG PET and [123 I]MIBG SPECT studies demonstrated that imaging of GLV-1h99 viral infection of MSTO-211H pleural tumors is feasible after direct tumor injection.

Similarly, the timing of PET imaging after [124 I]MIBG i.v. injection was also shown to be important. Radioactivity levels (% dose/cc) as well as tumor-to-organ ratios in GLV-1h99-infected tumors were highest during the first four-hour period after tracer administration. This differs from the findings of Moroz et al. (17) in a xenograft model, where constitutive expression of the hNET reporter occurred in all tumor cells and optimal imaging results were obtained at late time points (48 and 72h after tracer administration). This time-dependent difference probably reflects the effect of increasing cell death resulting from viral oncolysis subsequent to [124 I]MIBG injection. Oncolysis will result in a loss of [124 I]MIBG from the infected tumor cells and tumor, consistent with the rapid decrease in the PET signal after 4 hours (Figure 4B). A similar pattern was observed in the *in vitro* immunoblot analysis and [123 I]MIBG uptake studies, showing decreasing hNET expression and radiotracer uptake at later time points after GLV-1h99 infection (Figure 3B).

Pancreatic cancer and malignant pleural mesothelioma remain largely unresponsive to standard treatments and are rapidly fatal diseases in most cases. Thus alternate treatment options must be considered. Preclinical studies have shown the efficacy of oncolytic herpes simplex viruses in treatment of pancreatic cancer (29) and malignant mesothelioma (4). Oncolytic viral therapy has also been shown to be synergistic with radiation and chemotherapy (30, 31). Kelly et al. (21) has demonstrated effective killing in malignant mesothelioma cell lines and xenografts using a genetically modified oncolytic vaccinia virus, GLV-1h68, the parent virus of GLV-1h99.

Conclusions

We have demonstrated cytotoxic efficacy *in vitro* and tumor-specific imaging following GLV-1h99 infection of an orthoptic mesothelioma tumor model. GLV-1h99 expresses the hNET human reporter gene which was imaged with a clinically approved radiopharmaceutical, [¹²³I]MIBG, and with a positron emitting analog, [¹²⁴I]MIBG. This imaging paradigm could be directly translated to human studies, and clinical trials of oncolytic viral therapy would benefit from this noninvasive imaging paradigm.

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Statement of Translational Relevance

Oncolytic viral therapy dates back more than a century, but has had a mixed and fluctuating level of acceptance in the medical community. Nevertheless, it continues to be investigated and still holds promise as a biological treatment for resistant cancers, such as malignant pleural mesothelioma and pancreatic cancer. Future oncolytic viral trials in patients would benefit greatly from a non-invasive imaging modality for assessing virus dissemination, targeting and persistence. The purpose of this study was to determine if a genetically modified vaccinia virus, GLV-1h99, containing a human norepinephrine transporter (*hNET*) reporter gene, could be sequentially monitored by [^{123}I]MIBG γ -camera and [^{124}I]MIBG PET imaging in an orthotopic pleural mesothelioma animal model. This imaging paradigm could be directly translated to human studies.

C

	Cell lysis at day 7 with a MOI 0.1 % $\pm$ SD	Cell lysis at day 7 with a MOI 1.0 % $\pm$ SD
Mesothelioma cell lines		
H2372	8.8 $\pm$ 3.2	15.9 $\pm$ 4.8
JMN	31.3 $\pm$ 2.3	78.2 $\pm$ 7.5
H2052	93.9 $\pm$ 1.1	97.7 $\pm$ 0.6
MSTO-211H	97.6 $\pm$ 1.8	98.3 $\pm$ 1.1
Pancreatic cancer cell lines		
BxPC3	2.7 $\pm$ 3.3	7.4 $\pm$ 1.5
MiaPaCa2	3.2 $\pm$ 2.3	18.3 $\pm$ 5.4
HS766T	16.8 $\pm$ 11.9	83.9 $\pm$ 3.9
PANC1	66.3 $\pm$ 2.3	90.6 $\pm$ 2.0

Figure 1. GLV-1h99 (hNET-expressing virus) and GLV-1h68 (non-hNET-containing virus) cytotoxicity in PANC1 and MSTO-211H cells

Comparative GLV-1h99 and GLV-1h68 cytotoxicity at a MOI of 1.0 (A). The % cell survival of PANC1 and MSTO-211H cells at MOI's of 0.01, 0.10 and 1.0 (B). Cell lysis in % $\pm$ SD of the four mesothelioma and four pancreatic cancer cell lines at day 7 with a MOI of 0.1 and 1.0 (C).

Figure 2. Immunoblot analysis in PANC1 and MSTO-211H cells

hNET expression in SK-N-SH cells, as well as in H2052, MSTO-211H and PANC1 cells 24h after GLV-1h99 (hNET-expressing virus) infection at a MOI of 1.0 (A). hNET expression in MSTO-211H cells at various time points after GLV-1h99 infection at a MOI of 1.0 (B); hNET expression 24h after GLV-1h99 infection at MOI's of 0.1, 1.0, 5 and 10 in MSTO-211H cells (C) and PANC1 (D) (Immunoblots on Figure A were not run on the same gel, but were normalized to β -actin expression; the immunoblots on B, C and D were run on the same gel).

Figure 3. *In vitro* [¹²³I]MIBG uptake in PANC1 and MSTO-211H cell

[¹²³I]MIBG uptake in MSTO-211H (A) and PANC1 (B) cells at various times after infection with GLV-1h99, 48h after infection with GLV-1h68 (non-hNET containing virus) and in SK-N-SH neuroblastoma cells. Corresponding total cell protein from [¹²³I]MIBG uptake in MSTO-211H (C) and PANC1 (D).

Figure 4. [^{124}I]MIBG and [^{18}F]FDG-PET imaging of MSTO-211H pleural tumors (A) Axial, coronal and sagittal views of [^{124}I]MIBG-PET 48h after GLV-1h99 (hNET-expressing virus) injection (images at 2h and 4h after radiotracer administration) and a pre-treatment [^{18}F]FDG-PET (images at 1h after tracer administration). (B) Tumor radioactivity (%ID/g) of tumors injected with GLV-1h99 48h and 72h before radiotracer administration (red and orange squares) as well as injected with GLV-1h68 (non-hNET containing virus) or no virus (light and dark blue symbols respectively). (C) Photograph of a MSTO-211H pleural tumor; the white plaques on the lung surface and chest wall are malignant pleural mesothelioma (the heart is removed in this photograph).

Figure 5. Tumor-to-organ radioactivity ratios

Tumor-to-organ (lung, liver, muscle) ratios calculated from PET image data (as described in the Material and Methods section) were obtained 1h, 2h, 4h and 12h after [^{124}I]MIBG injection. GLV-1h99 (hNET-expressing virus) injected 48h and 72h before radiotracer administration as well as GLV-1h68 (non-hNET containing virus) injected 48h before radiotracer administration and no virus studies at 1h, 2h, 4h and 12h after [^{124}I]MIBG injection are presented and are compared to 1h [^{18}F]FDG studies.

Figure 6. [^{123}I]MIBG scintigraphy of MSTO211H pleural tumors
 (A) Photograph of a MSTO-211H pleural tumor bearing mouse. [^{123}I]MIBG-scintigrams (2h after tracer administration) (B). The GLV-1h99 virus injected tumor is visualized (oval outline); the non-injected control tumor is not visualized. (C) The tumor-to-background radioactivity ratios were measured in the scintigrams; GLV-1h99 injected tumors (orange column), non-injected control tumors (blue column).

Figure 7. Immunohistochemistry

H&E staining (upper row) confirms the histological diagnosis of malignant pleural mesothelioma. LacZ staining (lower row) demonstrates vaccinia viral infection of the tumor cells.