Oncolytic Using Herpes Simplex Virus Type 1 Engineered to Express Cytosine Deaminase and a Fusogenic Glycoprotein for Head and Neck Squamous Cell Carcinoma

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Objective: To determine if prodrug conversion of fluorocytosine to fluorouracil by an engineered herpes virus, OncoVEXGALV/CD, enhances oncolytic therapy of head and neck squamous cell carcinoma.

Design: We assessed the ability of OncoVEXGALV/CD and OncoVEXGFP to infect, replicate within, and lyse 4 head and neck squamous cell carcinoma lines in vitro. The effects of adding fluorocytosine with OncoVEXGALV/CD were evaluated.

Results: Head and neck squamous cell carcinoma was permissive to green fluorescent protein expression in 100% of cells by OncoVEXGFP at a multiplicity of infection of 1 after 48 hours and supported logarithmic viral replication. Virus caused more than 60% cell death 6 days after exposure to virus at a multiplicity of infection of 0.1 in 3 of the 4 cell lines. Fluorocytosine did not enhance cytotoxicity induced by OncoVEXGALV/CD at a multiplicity of infection of 0.1. However, for the least-sensitive SCC25 cell line, virus at a multiplicity of infection of 0.01 was cytotoxic to only 4% of cells after 6 days but was cytotoxic to 35% of cells with fluorocytosine.

Conclusions: OncoVEXGALV/CD efficiently infects, replicates within, and lyses head and neck squamous cell carcinoma at relatively low viral doses. Prodrug conversion by cytosine deaminase did not enhance therapy at viral doses that cause efficient cytotoxicity but may have beneficial effects in less-sensitive cell lines at low viral doses.


THE PROGNOSIS OF PATIENTS with head and neck squamous cell carcinoma (HNSCC) has remained essentially unchanged during the past 30 years, with approximately 60% of all patients dying within 5 years.1 Clinical research investigating combined radiotherapy and chemotherapy regimens has led to improved organ preservation but has not changed overall survival.2-5 Concurrent and adjuvant chemoradiotherapy regimens used to achieve these goals are associated with functional morbidity, leading to xerostomia, loss of taste, and alterations in speech and swallowing.6,7 Local and regional recurrence remains the primary source of failure for these patients.7-8 There is a need for novel therapeutics aimed at improving local and regional control while minimizing toxic effects.

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus that can be genetically manipulated to have an attenuated ability to infect healthy cells but retain the ability to infect and kill cancer cells. It has been shown to be effective at infecting and lysing a variety of human cancer cell lines, including HNSCC. Because of a large, nonessential genome, HSV-1 can be extensively genetically altered. These alterations have included the insertion of reporter genes such as GFP (green fluorescent protein) or lacZ (β-galactosidase) for fluorescent or histologic detection or genes to improve the infectivity and cytotoxicity of oncolytic HSV-1, including the insertion of genes encoding interleukins,9-14 granulocyte-macrophage colony-stimulating factor,14,15 cytosine deaminase (CD),17,18 and fusogenic proteins.18,19

Cytosine deaminase is a pyrimidine salvage enzyme derived from Escherichia coli that, through hydrolytic deamination, converts cytosine to uracil. It also converts the antifungal fluorocytosine to the chemotherapeutic agent fluorouracil. The cytotoxic effect of fluorouracil is principally achieved by the conversion of fluorouracil to 5-fluoro-dUMP by uracil phospho-
Fluorouracil is used in head and neck cancer therapy and has activity as a radiosensitizer. Adverse effects, particularly mucositis, can be increased with combined modality therapy. Such adverse effects might theoretically be diminished by localiz

The fusogenic membrane glycoprotein of the gibbon ape leukemia virus (GALV) may enhance cytotoxicity by forming a large multinucleated syncytia without impairing viral replication.18,19,22 Oncolytic HSV-1 with genetic insertions of CD/UPRT or GALV demonstrates cytotoxicity in human malignant cell lines.17-20,22,23 Recently, a mutant HSV-1 was constructed that expresses GALV and CD/UPRT (OncoVEXGALV/CD) and that demonstrates cytotoxicity in colon, lung, and pancreatic cancer cell lines. In this study, we assess the utility of OncoVEXGALV/CD with and without fluorocytosine for treating HNSCC.

**METHODS**

**CELL LINES**

Four human HNSCC cell lines, SCC15, SCC25, QLL1, and QLL2, were studied. Cells were grown in minimal essential medium (MEM) with 10% fetal calf serum and a combination of penicillin and streptomycin. Cells were maintained at 37°C in 5% carbon dioxide.

**VIRUSES**

OncoVEXGFP and OncoVEXGALV/CD have previously been described. Briefly, OncoVEXGALV/CD and OncoVEXGFP were constructed from the same wild-type HSV-1 strain, JS-1, with the genes encoding ICP34.5 and ICP47 completely deleted. The inserted genes replace both copies of ICP34.5 and are expressed from cytomegalovirus or cytomegalovirus and respiratory syncytial virus promoters in OncoVEXGFP and OncoVEXGALV/CD, respectively.

**VIRAL ENTRY**

The SCC15, QLL1, and QLL2 cells were plated at 3 × 10^4 cells per well in 2 mL of medium in 6-well plates overnight. OncoVEXGFP at a multiplicity of infection (MOI) of 1.0 was added in 100 µL of modified media, and cells were digitally imaged along a time course using an inverted microscope (Nikon Eclipse TS100; Nikon Inc, Melville, New York) with a green fluorescent filter and a digital camera (SPOT RT Slider; Diagnostic Instruments Inc, Sterling Heights, Michigan) until 100% of the cells expressed GFP.

**VIRAL CYTOTOXICITY**

Cell lines QLL1, QLL2, SCC15, and SCC25 were plated on 12-well plates at 2 × 10^4 cells per well in 1 mL of media overnight. Cells were infected with OncoVEXGFP or OncoVEXGALV/CD at MOIs of 0, 0.1, 0.01, and 0.001 in 100 µL of medium. Daily, supernatants were removed for viral titers, and cells were washed with a phosphate-buffered saline (PBS) solution and were lysed with Triton-X 1.35%. Lactate dehydrogenase (LDH) was quantified using a commercially available kit (Cytotox 96 kit; Promega, Madison, Wisconsin) and spectrophotometry (EL321e; Bio-Tek Instruments, Winooski, Vermont) at 490 nm. On the fourth day after infection, 1 mL of media was added. Results are expressed as the percentage of cells surviving compared with the untreated (MOI of 0) control cells.

**VIRAL REPLICATION**

Supernatants from the cytotoxicity experiments were removed and were frozen at −80°C for later plaque assays. Serial dilutions of supernatants were later added to confluent vero cells on 6-well plates for 4 hours. Supernatants were removed, and the cells were washed with 1 mL of media. Two milliliters of 1% agarose was added to each well. After 48 hours, 2 mL of 2% neutral red was added. After 24 hours, plaques were counted. Experiments were performed in triplicate for each condition.

**VIRAL CYTOTOXICITY PLUS PRODRUG ACTIVATION**

The QLL1 and QLL2 cells were plated at 2 × 10^4 cells per well in 2 mL of MEM on 12-well plates overnight. Cells were infected with OncoVEXGALV/CD at an MOI of 0 or 0.1. Fluorocytosine was added to a final well concentration of 600 µmol/L, or PBS was added to control wells. The LDH assays were performed on days 3 to 6 as described previously herein.

To determine whether the timing of fluorocytosine administration affects cytotoxicity, the QLL1 cells were infected with OncoVEXGALV/CD at an MOI of 0.01. Fluorocytosine was added to a final concentration of 2400 µmol/L 0, 12, 24, and 48 hours after viral exposure, or PBS was added to control wells. The LDH assays were performed on days 3 to 6.

To assess prodrug activation at conditions adverse to viral oncolysis, the more-resistant SCC25 was exposed to OncoVEXGALV/CD at an MOI of 0 or 0.05. Either PBS as control or fluorocytosine was added to a final concentration of 2400 µmol/L. The LDH assays were performed on days 3 to 6. Each condition was assayed in triplicate.

**PRODRUG ACTIVATION ASSAY**

The conversion of fluorocytosine to fluorouracil was indirectly assayed by assessing the cytotoxic activity of conditioned media after viral inactivation. The SCC25 cells were plated at 2.5 × 10^4 cells per well in 6-well plates in 2 mL of medium and were incubated overnight. OncoVEXGALV/CD was added at an MOI of 0.01 in 100 µL of media for 24 hours. Fluorocytosine was added to a concentration of 1200 µmol/L, or PBS was added to control wells. Supernatants were removed after 24 hours and were centrifuged at 800 rpm for 5 minutes. To inactivate virus, supernatant samples were heated to 90°C for 1 hour and were treated with UV light at 10 pulses of 300 mJ/cm². The conditioned media was assayed as described previously herein.

Untreated SCC25 cells were plated at 2 × 10^4 cells per well in 12-well plates in 0.5 mL of medium and were incubated overnight. An additional 0.5 mL of each supernatant sample was then added. The LDH assays were performed on the indicated days using untreated cells in MEM plus 10% fetal calf serum as 100% viable controls. Samples were assayed in triplicate.
STATISTICAL ANALYSIS

All of the data are expressed as mean (SEM). Comparisons between 2 groups were made using the 2-tailed t test.

RESULTS

ONCOVEXGFP EFFICIENTLY INFECTS AND EXPRESSES GFP IN HNSCC

Equal numbers of SCC15, QLL1, and QLL2 cells exposed to OncoVEXGFP at an MOI of 1.0 were imaged using bright and darkfield photography. The GFP was first visualized in SCC15 cells by 2 hours (data not shown). Progressively increasing percentages of GFP expression were observed under fluorescent microscopy (×100) across time. By 48 hours, 100% of the remaining viable cells were fluorescent for all 3 cell lines, demonstrating efficient viral infection and gene expression by OncoVEXGFP (Figure 1). Viral cytotoxic effects also caused significant declines in viable cell numbers by 48 hours, most pronounced for SCC15, followed by QLL1.

ONCOVEXGFP AND ONCOVEXGALVCD REPLICATE EFFICIENTLY IN HNSCC

The SCC15, SCC25, QLL1, and QLL2 cell lines were treated with OncoVEXGFP or OncoVEXGALVCD at an MOI of 0.01, and supernatants were collected for plaque assays. All 4 HNSCC lines supported logarithmic replication by both viruses (Figure 2). SCC15 supported the most robust viral replication, with OncoVEXGFP titers rising dramatically from 12.5 plaque-forming units (pfu) to 2.88 × 10^6 pfu during a 3-day interval. Viral replication progressed slightly slower for QLL1, peaking on day 4, and was the latest for the least-sensitive SCC25 cell line on day 5. Early viral replication was most pronounced for QLL2, reaching 3.43 × 10^4 pfu in 48 hours, but it quickly leveled out in the subsequent days.

Growth curves demonstrate an early advantage to the OncoVEXGALVCD virus over OncoVEXGFP for SCC15 and SCC25 in the first viral replication cycle (days 1-3), although differences were no longer observed once peak titers were reached. Both viruses replicated at similar logarithmic rates in QLL1 and QLL2.

ONCOVEXGFP AND ONCOVEXGALVCD ARE CYTOTOXIC TO HNSCC IN VITRO

The HNSCC lines QLL1, QLL2, SCC15, and SCC25 were treated with OncoVEXGFP or OncoVEXGALVCD at varying MOIs, and cell viability was assessed by means of LDH assay. Viral dose-dependent cytotoxicity was noted for all the cell lines. SCC15 was most susceptible to viral cytotoxicity, followed by QLL1, QLL2, and, finally, SCC25, which was the least susceptible. Except for SCC25, cell lines showed greater than 60% cytotoxicity by 6 days after exposure to either virus at an MOI of 0.1 (Figure 3). At a low viral dose of an MOI of 0.01, greater than 70% of the highly sensitive SCC15 cells were dead by 6 days. OncoVEXGFP and OncoVEXGALVCD exhibited similar cytotoxicity profiles.

Crystal violet staining of the remaining viable cells on day 6 after exposure to virus at an MOI of 0.1 qualita-

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Figure 1. Equal numbers of cells from 3 head and neck squamous cell carcinoma lines were infected with OncoVEXGFP at a multiplicity of infection of 1. Progressively increasing percentages of green fluorescent protein expression were observed under fluorescent microscopy (×100) across time. By 48 hours, 100% of the remaining viable cells were fluorescent for all 3 cell lines, demonstrating efficient viral infection and gene expression by OncoVEXGFP. Viral cytotoxic effects also caused significant declines in viable cell numbers by 48 hours, most pronounced for SCC15, followed by QLL1.
OncoVEXGALV/CD at an MOI of 0.1 on day 6 yielded a syncytial phenotype in QLL2 cells seen with crystal violet staining, in sharp contrast to the OncoVEXGFP virus. However, the identical cytotoxicity curves between the 2 viruses for these 4 HNSCC cell lines demonstrate that GALV expression did not induce any additional overall cytotoxic benefit.

**FLUOROCYTOSINE ENHANCES ONCOVEXGALV/CD CYTOTOXICITY OF SCC25 AT LOW MOI**

The addition of the prodrug fluorocytosine at 600 µmol/L to QLL1 cells infected with OncoVEXGALV/CD at an MOI of 0.1 did not enhance cytotoxicity compared with cells that did not receive fluorocytosine (Figure 4A). QLL2 cells treated identically demonstrated a subtle trend toward increased cytotoxicity with the fluorocytosine on days 4 and 5, but this difference was not statistically significant. We considered the possibility that early fluorouracil production interfered with viral replication and offset any additional benefit from drug production. We, therefore, next delayed the timing of fluorocytosine addition to allow for viral replication, and then we increased its concentration to 2400 µmol/L. The addition of fluorocytosine to QLL1 cells infected at an MOI of 0.01 showed no differences in cytotoxicity when the fluorocytosine is added at 12, 24, or 48 hours compared with controls not receiving fluorocytosine.

We reasoned that the high sensitivity of SCC15, QLL1, and QLL2 to direct viral oncolysis might limit the ability of infected cells to adequately express CD/UPRT before cell death. To account for this possibility, we treated the least-sensitive SCC25 cell line with OncoVEXGALV/CD at an MOI of 0.05, either with or without fluorocytosine at a concentration of 2400 µmol/L. Under these conditions, direct viral oncolytic effects were minimal. We observed a statistically significant enhancement of cytotoxicity by day 6, with an increase from 4% to 35% cytotoxicity appreciated with the addition of the fluorocytosine (Figure 4A).

**ONCOVEXGALV/CD-INFECTED CELLS CONVERT FLUOROCYTOSINE TO FLUOROURACIL**

To indirectly assess the production of active fluorouracil by OncoVEXGALV/CD, we quantified the cytotoxic activity of conditioned media on SCC25 cells after...
viral inactivation. The LDH assays demonstrated 80% less cell viability by day 4 for the cells treated with supernatant collected from SCC25 cells treated with OncoVEX\textsuperscript{GALV/CD} and fluorocytosine (Figure 4B). In contrast, supernatant from SCC25 cells treated with virus alone or fluorocytosine alone demonstrated approximately 20% to 25% less viability, with statistically significant differences at all time points compared with the fluorocytosine samples ($P < .05$). The reduced viability induced by the virus-alone or fluorocytosine-alone samples most likely relates to SCC25 growth rate reduction from addition of the nutrient-depleted media compared with control SCC25 wells, which received fresh media and were considered 100% viable for these comparative assays. Collectively, these studies suggest that there is active fluorouracil produced by OncoVEX\textsuperscript{GALV/CD} plus fluorocytosine in SCC25 cells under these conditions.

We demonstrated the ability of 2 attenuated, replication-competent, oncolytic herpes viruses (OncoVEX\textsuperscript{GFP} and OncoVEX\textsuperscript{GALV/CD}) to infect and lyse HNSCC lines in vitro. At an MOI of 1, OncoVEX\textsuperscript{GFP} completely infected all the cells within 48 hours and induced a significant amount of cell death for the SCC15 and QLL1 cell lines in this short period. Furthermore, both viruses exhibited efficient, logarithmic replication after infecting cells at an MOI of 0.01. SCC15 supported the most rapid viral proliferation, followed by QLL1 and SCC25, although all supported similar peak titers. Even the least-sensitive QLL2 permitted a greater than 170-fold increase in viral titers over a 2-day period, demonstrating a highly favorable host cancer cell environment for viral production. From a clinical standpoint, this finding implies that high levels of prog-
Figure 4. Cytotoxicity of combination fluorocytosine with oncolytic herpes simplex virus type 1. A, The addition of the prodrug fluorocytosine (FC), 600 µmol/L, to QLL1 cells infected with OncoVEX\textsuperscript{GALV/CD} at a multiplicity of infection (MOI) of 0.1 did not enhance cytotoxicity compared with cells that did not receive FC. B, QLL2 cells treated identically demonstrated a subtle trend toward increased cytotoxicity with FC on days 4 and 5, but this difference was not statistically significant. C, To account for the possibility that fluorouracil production interfered with viral replication, the timing of the FC addition to QLL1 at an MOI of 0.01 was varied, and the FC concentration was increased to 2400 µmol/L. There were still no differences in cytotoxicity when the FC was added at 12, 24, or 48 hours compared with controls not receiving FC. D, Because direct viral oncolysis might limit the ability of infected cells to adequately express cytosine deaminase/uracil phosphoribosyltransferase before cell death, we treated the least-sensitive SCC25 cell line with OncoVEX\textsuperscript{GALV/CD} at an MOI of 0.05, with or without FC at 2400 µmol/L. Under these conditions, direct viral oncolytic effects were minimal, but we observed a significant enhancement of cytotoxicity by day 6, with an increase from 4% to 35% cytotoxicity appreciated with the addition of FC (\(P < .01\)). 

E, To assess the production of active fluorouracil by OncoVEX\textsuperscript{GALV/CD}, we quantified the cytotoxic activity of conditioned media from SCC25 cells exposed to OncoVEX\textsuperscript{GALV/CD} and FC after viral inactivation. Lactate dehydrogenase assays on SCC25 cells demonstrated 80% cytotoxicity by day 4 for cells treated with conditioned media collected from cells treated with OncoVEX\textsuperscript{GALV/CD} and FC. There were significant differences at all time points (\(P < .01\)) compared with the FC-alone or virus-alone control samples. The slightly reduced viability detected from the FC-alone or virus-alone samples likely results from growth rate reduction effects from nutrient-depleted media. Error bars represent SEM.
eny virus could be produced and released to infect and lyse adjacent cancer cells.

The relative sensitivity by these different cell lines to viral replication was similarly reflected in the viral cytotoxicity studies. Both viruses demonstrated significant cytotoxicity in 3 of the 4 cell lines at a relatively low concentration of an MOI of 0.1 and showed cytotoxic effects in a dose-response manner. Furthermore, the highly sensitive SCC15 cell line was susceptible to nearly 80% cytotoxicity by day 6 at a very low MOI of 0.01. The SCC25 cell line showed the least sensitivity to viral oncolysis. However, it is likely that higher viral MOIs than those tested (≥0.1) would yield more rapid and potent cytotoxicity given the dose-dependent effects observed and the high degree of infection achieved at an MOI of 1.

We did not note any difference between the oncolytic effects of OncoVEX<sup>GFP</sup> and OncoVEX<sup>GALV</sup>. The GALV fusogenic membrane glycoprotein promotes syncytia formation, and we identified multinucleated cells in samples infected by OncoVEX<sup>GALV</sup> during the cytotoxicity studies with crystal violet staining. Although GALV glycoprotein expression has been shown to enhance viral cytotoxicity in some cell lines, we did not observe any difference with GALV expression in these HNSCC lines.

The application of OncoVEX<sup>GALV</sup> in the presence of fluorocytosine did not seem to offer a significant cytotoxic benefit over OncoVEX<sup>GFP</sup> at MOIs that can induce significant cell death. There are several potential explanations for this finding. The most likely possibility is that the sensitive cells are infected and lysed before fluorouracil, which requires cell division, has a chance to exert its cytotoxic effect. The second possibility is that fluorouracil may be exerting an inhibitory effect on viral DNA replication. The CD/UPRT gene is under the control of a constitutively expressed early cytomegalovirus promoter, so infected cells will express these proteins before the first replication cycle of the viral genome. Because fluorouracil inhibits DNA synthesis and RNA production, viral protein production and genome replication could, therefore, be inhibited. Finally, fluorouracil may not have particular activity for these 4 HNSCC lines. Fluorouracil is not typically used in isolation for HNSCC, and it is most commonly used as a radiosensitizer combined with a platinum-based chemotherapeutic agent. Fluorouracil is effective as single-agent therapy in only a small percentage of patients with HNSCC.

To account for the first possibility, we tested OncoVEX<sup>GALV</sup> with fluorocytosine in the least-sensitive HNSCC line, SCC25, at a low MOI. At these conditions, where the virus by itself could induce only minimal cytotoxicity, we observed a modest enhancement of cytotoxicity with the addition of fluorocytosine. Prodrug assays of conditioned media suggested that active fluorouracil had been produced. These findings imply that the addition of fluorocytosine to OncoVEX<sup>GALV</sup> might be most beneficial in conditions where viral dosing must be limited. Such low-dose viral application may be attractive from the standpoint of minimizing the potential for any viral-induced toxicity; the virus alone would be unable to induce cytotoxic effects but combined with fluorocytosine could elicit a therapeutic benefit. This strategy might also apply to clinical scenarios where enhancement of therapy is necessary for less virally sensitive tumor targets.

One obvious advantage of treating tumor sites directly with OncoVEX<sup>GALV</sup> combined with fluorouracil is the localization of active fluorouracil to these tumor sites and the potential avoidance of toxic effects associated with its systemic distribution. Similarly, the potential local administration of radiotherapy, which has enhanced efficacy combined with fluorouracil, seems to be an attractive strategy to apply combined with OncoVEX<sup>GALV</sup> with fluorocytosine. Such applications of these viruses, using in vivo models, are potential future avenues of investigation.

This study demonstrates potent oncolytic efficacy by OncoVEX<sup>GFP</sup> and OncoVEX<sup>GALV</sup> in HNSCC in vitro. We show the potential benefits and limitations of using a prodrug conversion strategy with OncoVEX<sup>GALV</sup> in these cell lines. Although a benefit was observed only at conditions of minimal direct viral oncolysis, these findings may have potential application in clinical scenarios where minimal viral dosing is a goal or in treating tumor targets that are less sensitive to viral oncolysis. These findings support the further investigation of these novel viruses for HNSCC and their potential application in vivo and combined with radiotherapy.

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