Selectivity of an Oncolytic Herpes Simplex Virus for Cells Expressing the DF3/MUC1 Antigen

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ABSTRACT

Replication-conditional viruses destroy tumors in a process referred to as viral oncolysis. An important prerequisite for this cancer therapy strategy is use of viruses that replicate preferentially in neoplastic cells. In this study the DF3/MUC1 promoter/enhancer sequence is used to regulate expression of γ134.5 to drive replication of a Herpes simplex virus 1 (HSV-1) mutant (DF3γ34.5) preferentially in DF3/MUC1-positive cells. HSV-1 γ134.5 functions to dephosphorylate elongation initiation factor 2α, which is an important step for robust HSV-1 replication. After DF3γ34.5 infection of cells, elongation initiation factor 2α phosphatase activity and viral replication were observed preferentially in DF3/MUC1-positive cells but not in DF3/MUC1-negative cells. Regulation of γ134.5 function results in preferential replication in cancer cells that express DF3/MUC1, restricted biodistribution in vivo, and less toxicity as assessed by LD50. Preferential replication of DF3γ34.5 was observed in DF3/MUC1-positive liver tumors after intravascular perfusion of human liver specimens. DF3γ34.5 was effective against carcinoma xenografts in nude mice. Regulation of γ134.5 by the DF3/MUC1 promoter is a promising strategy for development of HSV-1 mutants for viral oncolysis.

INTRODUCTION

Viruses have evolved extremely efficient mechanisms to infect cells, subvert cell defenses, deliver their genetic payload, express viral genes, and produce progeny virions. Most viruses used in clinical trials of gene therapy have been rendered replication-defective by genetic engineering such that they serve mainly as gene delivery vehicles and do not replicate outside of specialized packaging cell lines (1). There has been growing interest, however, in relying on the efficiency of viral replication itself as a means to destroy cancer cells in a process referred to as viral oncolysis (2). The safety and efficacy of this approach are dependent on selective viral replication in cancer cells rather than in normal cells.

Several strategies have been explored to restrict viral replication to neoplastic cells, including use of tumor-associated promoters to regulate expression of genes critical for viral replication. A promoter/enhancer sequence for the prostate-specific antigen (PSA) gene has been used to regulate adenoviral E1A expression to restrict its replication to PSA-positive cells (3). A promoter sequence of the α-fetoprotein gene has been used to regulate expression of both E1A and E1B55kDa in an adenoviral mutant that replicates selectively in tumors that express α-fetoprotein (4). The E2F-responsive B-myb promoter has been used to regulate expression of a gene critical for replication of Herpes simplex virus 1 (HSV-1; Ref. 5). DF3/MUC1 is a tumor-associated antigen that is overexpressed on many human carcinomas, including breast, pancreatic, and colon cancer (6–8). DF3/MUC1 transcript overexpression is observed in breast cancer (9), and the 5′ flanking region of the gene has been characterized (10). DF3/MUC1 gene expression is regulated by sequences between positions −598 and −485 bp upstream from the transcription start site. This promoter/enhancer has been used to regulate expression of E1A in an adenoviral mutant, Ad.DF3-E1, which replicates preferentially in DF3/MUC1-positive cancer cells (11).

HSV-1 is an effective oncolytic virus in animal models (12–14), and clinical studies of HSV-1 for oncolysis have been conducted. G207 is a replication-conditional HSV-1 mutant that has been administered to patients with recurrent malignant glioma (15). The HSV-1 mutant 1716 is defective in expression of HSV-1 γ134.5 (16) and has been administered to patients with recurrent malignant glioma in a clinical trial (17).

We have previously demonstrated that HSV-1 mutants defective in viral ribonucleotide reductase replicate preferentially in colon cancer liver metastases rather than normal liver because of higher mitotic activity and higher levels of functionally complementing cellular ribonucleotide reductase in the metastases (18). The destruction of these liver tumors is a result of viral replication rather than host-immune responses (19, 20). In this study we constructed and characterized a mutant HSV-1 in which the γ134.5 gene is regulated by a DF3/MUC1 promoter. Regulation of HSV-1 γ134.5 function results in preferential viral replication and oncolysis in cancer cells that express DF3/MUC1, restricted biodistribution in vivo, and less toxicity as assessed by LD50. This HSV-1 mutant was effective against carcinoma xenografts in nude mice.

MATERIALS AND METHODS

Cells and Viruses. Vero African Green Monkey kidney cells were obtained from American Type Culture Collection (Manassas, VA). MC26 mouse colon carcinoma cells were obtained from the National Cancer Institute Tumor Repository (Frederick, MD). A375 human melanoma cells were provided by Isaiah Fidler (M. D. Anderson Cancer Center, Houston, TX), and MCF-7 cells were provided by Donald Kufe (Dana-Farber Cancer Institute, Boston, MA). SW1990 and CAPAN2 human pancreatic carcinoma cells were provided by Andrew Warshaw (Massachusetts General Hospital). Primary human hepatocytes were prepared as described previously (21). Human umbilical vascular endothelial cells were obtained from Cell Applications, Inc. (San Diego, CA). HSV-1 viruses F strain (wild-type HSV-1) and R3616 (defective γ134.5 expression) were provided by Bernard Roizman (University of Chicago, Chicago, IL; Ref. 22). MGH1 is a HSV-1 mutant defective in thymidine kinase (TK) expression and viral ribonucleotide reductase (ICP6) expression and was provided by E. Antonio Chiocca (Massachusetts General Hospital). Viruses were propagated and titered on Vero cells, and heat-inactivation of virus was performed as described (23).

Replication-Conditional HSV-1 Mutant with DF3/MUC1 Promoter. The coding sequence of the γ134.5 gene was isolated from pBGL34.5 (5) as a NcoI-Sacl fragment and cloned into plLmutf28 (New England BioLabs, Beverly, MA) by use of the same restriction sites. The DF3/MUC1 promoter, provided by Donald Kufe (Dana-Farber Cancer Institute, Boston, MA), was isolated from pDF3 (11) as a SpeI-XhoI fragment and subcloned into pCRY (Invitrogen, San Diego, CA) in the same sites. A Nhel-SpeI fragment containing autofluorescence protein (AFP) regulated by a cytomegalovirus (CMV) promoter was isolated and subcloned into the SpeI locus of this plasmid. A
The presence of 2,5-diphenyltetrazolium bromide assay. Experiments were performed three times in quadruplicate, and results of representative experiments are shown. Two, five thaw cycles to release virions, and titered on Vero cells. Viral cytotoxicity was assessed by a single tail vein inoculation of 10^8 pfu of virus for 2 h, at which time unadsorbed virus was removed by washing with a glycine–plaque forming units (pfu) of virus for 6 days. The number of values ranging from 0.0001 to 10 and incubated for 6 days. The number of surviving cells was quantitated by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Experimental values were obtained from statistical analysis. RESULTS

HSV-1 mutant with γ34.5 regulated by a DF3/MUC1 promoter. To develop a HSV-1 mutant with attenuated replication in MUC1-negative cells, we used a DF3/MUC1 promoter sequence to regulate γ34.5 expression. We first constructed a plasmid containing an expression cassette in which γ34.5 expression is regulated by a strong immediate-early CMV promoter (Fig. 1). Using homologous recombination techniques, we introduced this dual gene cassette into the TK locus of R3616, which is a HSV-1 mutant that harbors deletions in both γ34.5 loci (22). After four rounds of plaque purification, one isolate was selected and designated DF3γ34.5. The genotype of this mutant was confirmed by Southern blot analysis.

eIF-2α Phosphatase Activity in HSV-1-Infected Cells. To assess γ34.5 function in HSV-1-infected cells, we analyzed eIF-2α dephosphorylation in infected cells. eIF-2α is normally phosphorylated by PKR in response to HSV-1 infection, and HSV-1 γ34.5 interacts with cellular protein phosphatase 1α to dephosphorylate eIF-2α to block the shutoff of protein synthesis (Fig. 2). We compared γ34.5 function in cells infected by F strain (wild-type HSV-1), R3616 (γ34.5-deficient), and DF3γ34.5 (γ34.5 expression regulated by the DF3/MUC1 promoter). A375 melanoma cells and Vero cells are DF3/MUC1-negative, whereas CAPAN2 cells are DF3/MUC1-positive (Fig. 3A).
also followed a pattern that correlated with DF3/MUC1 expression. Cells were infected with each of the HSV-1 mutants at increasing multiplicity of infection values, and cell survival was assessed 6 days later. In the DF3/MUC1-positive cells, DF3γ34.5 was as oncolytic as RH105 (γ34.5+) and more oncolytic than R3616 (γ34.5−). In the DF3/MUC1-negative cells, DF3γ34.5 was more attenuated than both RH105 and R3616 and as attenuated as the double mutant MGH1 at low multiplicity of infection values (Fig. 3D). These data are consistent with DF3γ34.5 replication and oncolysis being dependent on DF3/MUC1 expression.

It was not possible to examine the specificity of the human DF3 promoter in mouse models; accordingly, we devised an assay to examine in human tissues how this promoter regulates DF3γ34.5 replication. Human liver biopsy specimens, each containing a small colon carcinoma metastasis and surrounding normal liver, were harvested fresh from the operating room. As expected, immunohistochemical staining revealed DF3/MUC1 expression in the metastases and not in the normal liver (data not shown). Small blood vessels were cannulated to inject either F strain or DF3γ34.5. The specimen was then cut into pieces measuring 5 mm in thickness and incubated in medium for 72 h; at which time examination of the slices infected with DF3γ34.5 revealed green fluorescence (indicative of viral replication and marker transgene expression) preferentially in the metastases rather than the normal liver (Fig. 4A). Moreover, although the titers of F strain recovered from normal liver tissue were similar to titers recovered from liver metastases, titers of DF3γ34.5 were two log orders lower in the normal liver than in the metastases (Fig. 4B). These data demonstrate preferential replication of DF3γ34.5 in liver metastases compared with normal liver after intravascular perfusion of human liver specimens.

HSV-1 Oncolyis of Flank Tumor Xenografts. We examined antineoplastic efficacy in vivo against DF3/MUC1-positive CAPAN2 flank tumors and DF3/MUC1-negative A375 flank tumors. Tumors were directly inoculated with virus every third day for a total of four inoculations, and tumor sizes were measured every 5 days. In both tumor models, DF3γ34.5 demonstrated greater antineoplastic effects than that of mock-infected medium (control mice; Fig. 5). In addition, the antineoplastic effect of DF3γ34.5 was greater than that of the control RH105 virus in DF3/MUC1-positive CAPAN2 tumors and less than that of RH105 in DF3/MUC1-negative A375 tumors. As expected in the A375 tumors, DF3γ34.5 was as attenuated as the double mutant MGH1. These data are consistent with our in vitro...
observations and provide further evidence that DF3y34.5 replication and consequent antineoplastic activity are regulated by the DF3 promoter.

**LD** and **Biodistribution in Mice.** On the basis of our observation that DF3y34.5 replication is more attenuated than that of R3616 and RH105 in MUC1-negative cells, we examined whether this correlated with a lower LD after i.v. inoculation. Cohorts of mice received injections of F strain, DF3y34.5, R3616, or RH105. Mice receiving the higher doses commonly developed paralysis followed by death within 5 days (Table 2). As has been demonstrated previously, wild-type F strain was the most virulent, with all mice rapidly dying.
at the lowest dose examined, whereas the single-site mutants R3616 and RH105 were more attenuated in virulence. Notably, the LD_{50} of DF3/H9253^34.5 was one-half to one full log order greater than that of mutants R3616 and RH105. This reduction in pathological virulence of DF3/H9253^34.5 observed after i.v. inoculation was associated with a more restricted biodistribution as assessed by PCR analysis of harvested organs (Table 3).

**DISCUSSION**

Replication-competent viruses have many advantages over replication-defective viruses for cancer therapy applications. Because progeny virions can infect adjacent cells, it is not necessary to infect all tumor cells initially, and vector distribution increases over time (24). In contrast, the distribution of transgene expression is much more restricted after direct intratumoral inoculation of replication-defective vectors. Another benefit of replication-competent viruses is that the maximum “dose” is greater than the input dose as a result of in vivo amplification. Incorporation of a therapeutic transgene within the genome of a replication-competent virus permits a two-pronged cancer therapy strategy: oncolysis by viral replication together with the effects of therapeutic transgene expression (25, 26). However, careful selection of therapeutic transgenes is necessary to avoid the problem of antagonism between transgene expression and viral replication (25, 27). The combination of viral oncolysis with therapeutic transgene expression may reduce the risk of emergence of tumor cell resistance to therapy.

One of the greatest challenges faced in the field of viral oncolysis is the development of successful strategies to maximize viral replication in tumor cells and minimize replication in normal cells. Several approaches to restrict viral replication to cancer cells have been examined. The simplest strategy is to inoculate the virus directly into the tumor. This approach has several drawbacks, including the inability to treat radiographically or visually occult lesions and the inability to distribute the virus homogeneously throughout the tumor. Another strategy involves modulation of the interaction between virus and cell surface receptors to permit viral entry into tumor cells but not normal cells. The most well-known example of this strategy is modification of the adenovirus fiber to overcome tumor cell down-regulation of the viral entry receptor CAR (28). A third strategy involves exploitation of the natural properties of some viruses to infect and replicate...
specifically within cancer cells. The natural selectivity of Newcastle disease virus and vesiculostomatitis virus for cancer cells appears to be a result of defects in the IFN signaling pathways that are commonly present in cancer cells but intact in normal cells (29, 30). Another strategy involves removal of genes from a virus that are critical for replication in normal cells but whose absence is functionally complemented in neoplastic cells. The E1b 55-kDa protein is not expressed in cells infected by the adenoviral mutant Onyx-015 (31). In the absence of E1B55kD protein, viral replication is attenuated except in cells in which the p53 pathway is already disrupted.

In this study we restricted viral replication by regulation of the HSV-1 γ34.5 gene by a promoter sequence for a tumor-associated antigen. γ34.5 plays a critical role in aiding HSV-1 to subvert an important cellular defense after infection, i.e., PKR activation (32, 33). The importance of this defense mechanism against viral infection has been affirmed by the observation that most viruses have incorporated strategies to overcome the shutoff of protein translation that accompanies PKR activation. Adenovirus expresses VAI RNA to inhibit PKR activation (34). Similarly, human immunodeficiency virus produces TAR RNA, which performs a function similar to that of VAI RNA (35). Influenza virus stabilizes a cellular inhibitor of PKR that forms after infection, thereby functionally inhibiting PKR (36, 37), and as another example, the E3L and NS5A proteins that are expressed by HCV are known inhibitors of PKR (38). HSV-1 circumvents the consequences of PKR activation by expression of γ34.5 (Fig. 2), which has sequences homologous with the GADD34 protein (39).

DF3/MUC1 overexpression is observed in many human carcinomas, and mRNA overexpression has been observed in breast carcinomas (40). Abe and Kufe (10) identified elements in the DF3/MUC1 promoter is not expected to function in mice as it does in humans. The hypothesis that attenuated toxicity of DF3/y34.5 in humans mirrors that observed in mice necessarily requires examination in a clinical trial. Human gene therapy phase trials are exceedingly costly; we therefore developed an assay to examine viral replication in a clinical trial. Human gene therapy phase trials are exceedingly costly; we therefore developed an assay to examine viral replication in human tissues. Our observation that DF3/y34.5 replication is attenuated in normal human liver relative to colon cancer liver metastases after perfusion of a portion of the organ lends credence to the notion that DF3/y34.5 would behave similarly after intravascular administration into patients’ livers. We used this organ perfusion experimental model to examine HSV-1 replication; however, it is clearly applicable to examination of other viruses and other therapeutic agents. Conceivably, similar models using portions of other human organs can be developed.

DF3/y34.5 itself is not suitable for examination in clinical trials because it is not susceptible to ganciclovir or acyclovir as a result of inactivation of its TK gene. Sensitivity to these therapeutic agents is an important safety feature to limit unwanted viral replication. We selected the TK locus for homologous recombination because of the ease with which recombinants can be selected with ganciclovir and because we are interested mainly in testing principles. Despite the availability of other antivirals to which these TK-defective viruses should be sensitive, DF3/y34.5 is not suitable for clinical trials without repair of the TK gene.

The strategic decision of which promoter to use is important to the success of this strategy. For this study, we selected a DF3/MUC1 promoter sequence that has previously been demonstrated to effectively regulate adenoviral replication (11). The choice of location in the HSV-1 genome in which to place the heterologous promoter is equally important. Use of a carboxyembryonic antigen (CEA) promoter in the U/39 locus to regulate ICP4 expression does not result in preferential HSV-1 replication in CEA-positive cells (42). cis interactions in the region of the promoter may affect the specificity of transcriptional regulation. Others have successfully regulated gene

### Table 2: Survival after tail vein inoculation of Herpes simplex virus-1

<table>
<thead>
<tr>
<th>Survival (%)</th>
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<tbody>
<tr>
<td>1 × 10⁵ pfu*</td>
</tr>
<tr>
<td>F strain</td>
</tr>
<tr>
<td>R3616</td>
</tr>
<tr>
<td>RH105</td>
</tr>
<tr>
<td>DF3y34.5</td>
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</tbody>
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* pfu, plaque-forming units; ND, not done.

### Table 3: Sites of Herpes simplex virus-1 infection as assessed by PCR

<table>
<thead>
<tr>
<th>Positive/tested (n)</th>
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<tbody>
<tr>
<td>Brain Colon Liver Lung Spleen Tumor</td>
</tr>
<tr>
<td>F strain 3/3 2/3 2/3 0/3 3/3 3/3</td>
</tr>
<tr>
<td>R3616 1/3 0/3 0/3 0/3 3/3 3/3</td>
</tr>
<tr>
<td>DF3y34.5 0/3 0/3 0/3 0/3 0/3 0/3</td>
</tr>
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Fig. 5. Herpes simplex virus-1 (HSV-1) oncolysis of flank tumor xenografts. Tumor volumes were measured in BALB/c (nu/nu) mice bearing either A375 or CAPAN2 flank tumors that were inoculated once with DF3y34.5 (○), RH105 (□), MGH1 (△), or mock-infected medium (PBS, ▲). For A375 (top), P = 0.002 for DF3y34.5 versus RH105; and P = 0.32 for DF3y34.5 versus MGH1. For CAPAN2 (bottom), P = 0.013 for DF3y34.5 versus RH105; and P = 0.002 for DF3y34.5 versus MGH1. Bars, SD.
expression within the $U_{123}$ (TK) locus (43), and this observation strongly influenced our decision to use this locus. Finally, the strategic decision of which HSV-1 gene to regulate with the heterologous promoter is important; the gene product ideally should be one whose absence is not effectively complemented in normal cells.

Replication-competent viruses offer several advantages over replication-defective viruses for cancer gene therapy applications. The success with which replication-competent viruses can treat cancer will very likely be dependent on the ability to achieve replication preferentially in neoplastic cells rather than normal cells. Our results demonstrate that the $DF3/MUC1$ promoter regulates $\gamma_{34.5}$ expression within the context of HSV-1 replication in a manner that effectively attenuates viral replication in $DF3/MUC1$-negative cells but permits effective destruction of tumors. Because $DF3/MUC1$ is overexpressed in a broad spectrum of carcinomas, this approach to viral oncolysis may be broadly applicable.

REFERENCES

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