

# Multimodality Therapy with a Replication-conditional Herpes Simplex Virus 1 Mutant that Expresses Yeast Cytosine Deaminase for Intratumoral Conversion of 5-Fluorocytosine to 5-Fluorouracil<sup>1</sup>

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## ABSTRACT

Infection of tumor cells by herpes simplex virus 1 (HSV-1) results in cell destruction and production of progeny virion in a process referred to as viral oncolysis. In this study, an HSV-1 mutant (HSV1yCD) was engineered such that the viral ribonucleotide reductase gene is disrupted by sequences encoding yeast cytosine deaminase, which efficiently metabolizes the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). HSV1yCD-infected cells convert 5-FC to 5-FU, which enhances cytotoxicity without significantly reducing viral replication and oncolysis. Oncolysis by a replicating HSV-1 mutant combined with therapeutic transgene delivery represents a new paradigm; HSV1yCD-infected cells are destroyed by viral replication, and uninfected cells are subjected to bystander killing from both progeny virion and extracellular diffusion of 5-FU. In contrast, HSV1yCD-mediated bioactivation of another prodrug, ganciclovir, impairs viral replication. HSV1yCD administered into the portal venous system replicates preferentially in liver metastases rather than normal liver. The anti-neoplastic activity of HSV1yCD combined with systemic 5-FC administration is greater than that achieved with HSV-1 replication alone. Combination oncolysis and prodrug bioactivation leads to significant prolongation of survival in mice with diffuse liver metastases.

## INTRODUCTION

The overwhelming majority of cancer gene therapy clinical trials in progress today use viruses that have been engineered such that they are incapable of replication in humans (1). Although it has been a long-held belief that replication-defective viruses are safer than replicating viruses for administration into humans, it has become evident that cytopathic effects produced by viral replication efficiently destroy tumors (oncolysis). Viral oncolysis is an efficient mechanism for cancer cell destruction, because as viral replication proceeds and destroys cells, progeny virion are released that infect adjacent cancer cells. Researchers have examined the oncolytic potential of several viruses including adenovirus (2), HSV-1<sup>3</sup> (3), vaccinia virus (4), and reovirus (5).

HSV-1 replication mediates regression of several types of cancer, including hepatocellular carcinoma (6), colon carcinoma (7, 8), brain tumors (3), and prostate carcinoma (9). Because the HSV-1 genome is large (152 kb), the virus is also well suited for transgene delivery. In this study, we report construction of an HSV-1 mutant in which the gene-encoding viral ribonucleotide reductase is inactivated by insertion of transgene sequences encoding yeast CD, which is responsible

for conversion of 5-FC to 5-FU. Experimental results demonstrate that the virus effectively destroys tumor cells and simultaneously induces conversion of the prodrug 5-FC to 5-FU to enhance its antitumor efficacy. The results also demonstrate that 5-FU produced by HSV-1-infected cells induces bystander killing without significantly impairing viral replication and oncolysis. In contrast, HSV1yCD-mediated bioactivation of another prodrug, ganciclovir, impairs viral replication. Intratumoral viral replication combined with 5-FC bioactivation significantly reduces liver tumor burden and prolongs survival in mice.

## MATERIALS AND METHODS

**Cells and Viruses.** Vero African Monkey kidney cells and HT29 human colon carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). MC26 mouse colon carcinoma cells were obtained from the National Cancer Institute Tumor Repository (Frederick, MD). Cells were propagated in DMEM with 8% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Primary human and mouse hepatocytes were prepared as described (10). The HSV-1 vector hrR3 (kindly provided by Dr. E. Antonio Chiocca, Massachusetts General Hospital) is derived from the parental wild-type strain KOS (kindly provided by Dr. Donald Coen, Harvard Medical School).

**Engineering of HSV1yCD Virus Vector.** cDNA-encoding AFP was excised from pQBI25-fC1 plasmid (QUANTUM Biotechnologies, Carlsbad, CA) with *SpeI* and *NotI* and inserted into pCDNA3.1 (Invitrogen, Carlsbad, CA). The resulting expression cassette, including the CMV promoter upstream and polyadenylate tail, was excised as a *PmeI* fragment and cloned into the *SstI* site of pKpX2, which contains the *ICP6* gene (11). A 477-nucleotide fragment of the *CD* gene (12) was PCR amplified using oligonucleotides (forward, 5'-TTCAGTACATGGTGACAGGGGGAATGGCA-3'; reverse, 5'-GCT-GAAGCTTCTACTACCAATATCTTCAAA-3') from the genomic DNA library of *Saccharomyces cerevisiae* S288C (Research Genetics, Huntsville, AL). The amplification product containing the *CD* gene was digested with *NheI-EcoRI* and cloned into pCDNA3.1 downstream from the CMV promoter. The resulting expression cassette including the CMV promoter and polyadenylate splicing signal was excised as a *NruI-PVUII* fragment and subcloned into the *EcoRV* site of pKpX2-AFP to create pKpX2-yCD-AFP (Fig. 1A). This plasmid was linearized with *XbaI* and cotransfected with KOS viral DNA into Vero cells with LipofectAMINE (Life Technologies, Inc.). Cells and media were collected 5 to 7 days after transfection when cytopathic effects were evident. Progeny virion were recovered from cells after three freeze-thaw cycles and then placed onto a monolayer of Vero cells. After overlaying the monolayer with agarose, green fluorescent plaques were observed with fluorescence microscopy and selected as potential recombinants. Isolates were subjected to four rounds of plaque purification before examining their genetic identity by Southern blot analysis.

**Southern Blot Analysis.** Viral DNA was isolated after lysis of infected Vero cells with 0.5% SDS and proteinase K (500 µg/ml) by repeated phenol-chloroform extraction and ethanol precipitation. DNA was digested with *NruI*, separated by agarose gel electrophoresis, and transferred to a nylon membrane (Amersham Corp., Arlington Heights, IL). A *BamHI* fragment from pKpX2 containing *ICP6* sequences was labeled, hybridized to the membrane, and detected with an enhanced chemiluminescence system (Amersham Corp.).

**CD Functional Analysis.** CD activity was quantified by measuring conversion of [6-<sup>3</sup>H]5-FC to [3<sup>3</sup>H]5-FU. HT29 cells infected with either HSV1yCD

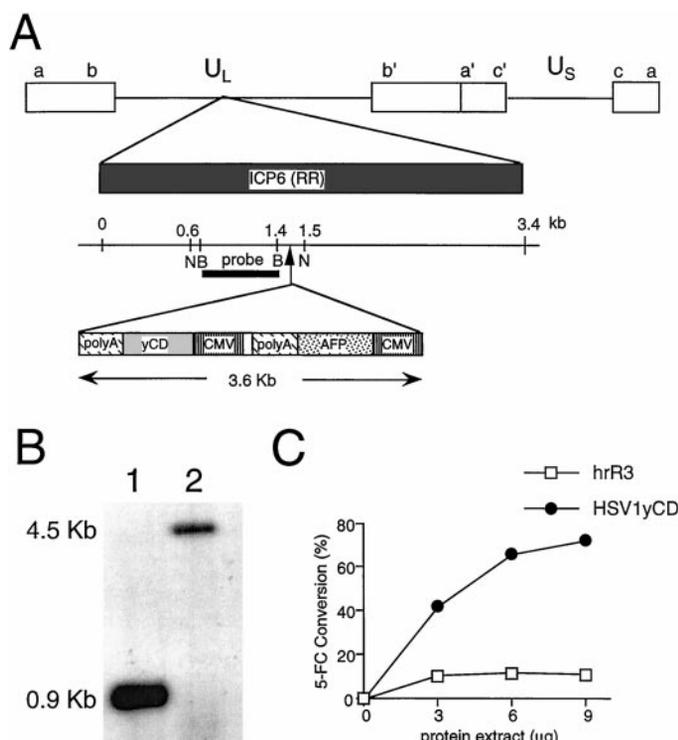
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<sup>3</sup> The abbreviations used are: HSV-1, herpes simplex virus 1; CD, cytosine deaminase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; pfu, plaque-forming units; moi, multiplicity of infection; CMV, cytomegalovirus; AFP, autofluorescence protein.



**Fig. 1.** Construction of HSV1yCD. **A**, expression cassettes for the genes encoding AFP and yeast CD (*yCD*) were cloned into plasmid pKpX2, which contains the *ICP6* gene. HSV1yCD resulted from homologous recombination between KOS and pKpX2 sequences, with resulting inactivation of *ICP6* and insertion of the yeast *CD* and *AFP* genes regulated by CMV promoters. *N*, *NruI*; *B*, *Bam*HI, and *CMV*, CMV promoter. **B**, Southern blot analysis performed on DNA prepared from KOS (Lane 1) or HSV1yCD (Lane 2) digested with *NruI* using a 800-bp *Bam*HI fragment of *ICP6* revealed hybridization between the probe and a 900-bp fragment from KOS that is expected in the absence of homologous recombination. The 4.5-kb fragment observed from HSV1yCD results from integration of the 3.6-kb sequence containing yeast CD and AFP into the *ICP6* locus. **C**, increasing amounts of cell extracts from HT29 cells infected with either HSV1yCD or hrR3 were incubated with [ $^3$ H]5-FC, and [ $^3$ H]5-FU was isolated by elution from a SCX Bond Elute column and counted.

or hrR3 were harvested and subjected to three freeze-thaw cycles in 100 mM Tris (pH 7.8) and 1 mM EDTA. Cell extracts were incubated with 1  $\mu$ Ci/mmol [ $^3$ H]5-FC (Moravek Biochemicals, Brea, CA) in a 30- $\mu$ l reaction volume for 2 h at 37°C. The produced [ $^3$ H]5-FU was isolated by elution from a SCX Bond Elute column (Varian, Harbor City, CA) and counted. Also, total [ $^3$ H]5-FC was counted to calculate the percentage conversion.

**Flow Cytometry.** Cells were trypsinized and fixed with 0.5% formaldehyde and permeabilized with 0.1% Triton X-100. They were stained with 10 mg/ml propidium iodide in the presence of 100 mg/ml RNase at 4°C overnight. Cell cycle analysis was performed by FACScan using ModFit LT software (Becton Dickinson, Franklin Lakes, NJ). To examine cells not infected by HSV1yCD, HT29 cells were infected at moi = 0.005, and 48 h later only FL1-H-negative cells were analyzed for cell cycle distribution.

**In Vitro Cell Culture Studies.** Cells ( $1 \times 10^6$ ) were infected with  $2 \times 10^6$  pfu of virus for 2 h, at which time unabsorbed virus was removed by washing with a glycogen-saline solution (pH 3.0). The supernatant and cells were harvested after culture for 40 h in the presence or absence of prodrugs, exposed to three freeze-thaw cycles to release progeny virions, and titered on Vero cell monolayers. The results represent the mean of three independent experiments. To confirm secretion of 5-FU by HSV1yCD-infected cells, HT29 cells were infected with either HSV1yCD or hrR3, and 5-FC was added 24 h later. Conditioned media were collected 72 h later and incubated at either 37°C or 60°C for 10 min to inactivate HSV-1. These conditioned media were added to freshly prepared HT29 cells, which were subsequently counted and analyzed for cell cycle distribution.

**Animal Studies.** Studies on BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA) were performed in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care. To assess the specificity of HSV1yCD infection in animals bearing diffuse liver

metastases, a single cell suspension of  $1 \times 10^5$  MC26 cells in 100  $\mu$ l of HBSS without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  were injected into spleens of BALB/c mice, followed 7 days later by intrasplenic injection of  $1 \times 10^8$  pfu HSV1yCD in 100  $\mu$ l of media. Livers were harvested 3 days later for frozen section analysis. To assess the therapeutic efficacy of HSV1yCD injected into flank tumors, a single cell suspension of  $1 \times 10^6$  MC26 cells in 100  $\mu$ l of HBSS without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  was injected into right flank of BALB/c mice, followed by intratumoral injections of  $1 \times 10^8$  pfu HSV1yCD, hrR3, or heat-inactivated HSV1yCD in 100  $\mu$ l of media ( $n = 5$ /group) 3, 5, and 7 days later. Mice received i.p. injections of 750 mg/kg 5-FC or saline on days 4, 6, 8, 9, 10, 11, 12, and 13. Tumor volumes were recorded every 3 days. To assess survival after treatment of diffuse liver metastases with HSV1yCD and 5-FC, diffuse MC26 liver metastases were established as described above, followed 3 days later by intrasplenic injection of  $1 \times 10^8$  pfu HSV1yCD, hrR3, or heat-inactivated HSV1yCD in 100  $\mu$ l of media ( $n = 5$ /group). Mice were randomized to receive daily i.p. injections of either 5-FC (750 mg/kg) or saline for 10 days starting on the 5th day after viral administration. The distribution of the intervals until death was determined by the method of Kaplan and Meier.

**Statistical Analysis.** A nonparametric statistical analysis, generalized Wilcoxon test was used to compare survival between groups (InStat; Graphpad Software, New York, NY).

## RESULTS

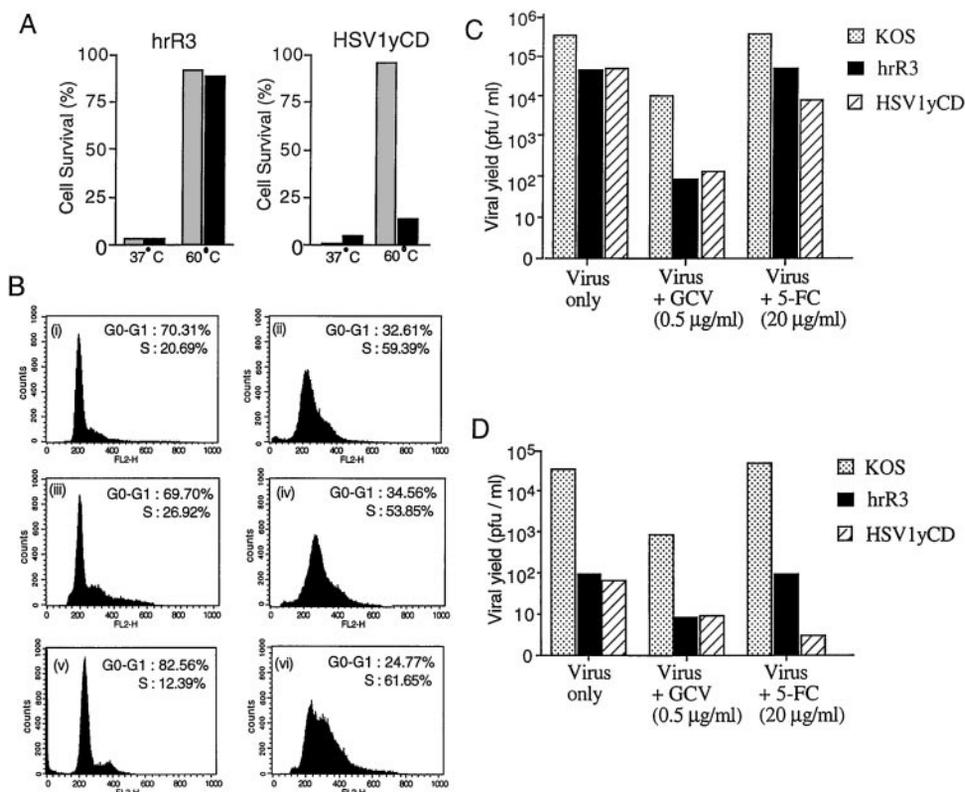
**Construction of a Replication-conditional HSV-1 Mutant That Expresses Yeast CD and AFP.** A plasmid containing the AFP marker gene and yeast CD gene regulated by a CMV promoter was constructed for homologous recombination in the *ICP6* locus of wild-type HSV-1 strain KOS (see "Materials and Methods"; Fig. 1A). After four rounds of plaque purification, one HSV-1 mutant, designated HSV1yCD, was selected for analysis by Southern blot. HSV1yCD DNA digested with *NruI* and probed with a 800-bp radiolabeled fragment representing a *Bam*HI fragment of *ICP6* demonstrated findings consistent with the desired homologous recombination (Fig. 1B). In addition, PCR amplification of yeast CD sequences from HSV1yCD indicated the presence of this gene within the viral genome (data not shown).

To confirm function of the yeast CD gene, cell extracts from HT29 cells infected with either HSV1yCD or a control virus (hrR3) that is defective in *ICP6* were incubated with 5-FC and assayed for conversion to 5-FU. Mammalian cells convert 5-FC to 5-FU extremely inefficiently; however, cells transduced with the yeast CD gene will rapidly convert 5-FC to 5-FU (12, 13). We observed that only cell extracts prepared from HSV1yCD-infected cells converted 5-FC to 5-FU, indicating that the yeast CD gene in HSV1yCD is functional during viral infection (Fig. 1C).

**Combined Oncolysis and Prodrug Bioactivation.** 5-FU is a freely diffusible metabolite that should exert cytotoxic effects and be recoverable in the media from HSV1yCD-infected cells exposed to 5-FC. 5-FU that diffuses extracellularly may induce bystander killing of uninfected cells. To examine this hypothesis, HT29 cells were infected with HSV1yCD or control virus hrR3 (moi = 1) in the presence or absence of 5-FC. The conditioned media were then recovered after 72 h and placed on fresh HT29 cells. Because the conditioned media also contain infectious HSV1yCD or hrR3, the media were incubated at 60°C for 10 min to inactivate infectious virus without inactivating 5-FU (or 37°C for control experiments). Medium conditioned by HSV1yCD-infected cells exposed to 5-FC was highly cytotoxic to HT29 cells despite successful heat inactivation of HSV-1, indicating conversion of 5-FC to 5-FU and diffusion into the medium (Fig. 2A).

To examine bystander killing resulting from HSV1yCD-mediated intracellular conversion of 5-FC to 5-FU, it was necessary to develop an assay to separately measure the cytotoxic effects of viral replication and the cytotoxic effects of 5-FU exposure. Cells that are sensi-

Fig. 2. Combined HSV1yCD-mediated oncolysis and prodrug bioactivation. **A**, HT29 cells were infected with hrR3 or HSV1yCD in the presence (black bars) or absence (gray bars) of 5-FC. Conditioned media were then placed at either 37°C or 60°C for 10 min before placing on fresh HT29 cells. Cells were counted 5 days later. **B**, FACS was used to analyze cell cycle distribution of HT29 cells grown in media (i) without 5-FU, (ii) with 5-FU, (iii) conditioned by HSV1yCD-infected cells cultured without 5-FC, or (iv) conditioned by HSV1yCD-infected cells cultured with 5-FC. To examine bystander effects, HT29 cells were infected with HSV1yCD (moi = 0.005) in the absence of 5-FC (v) and presence of 5-FC (vi), and cell cycle analysis was performed on the nonfluorescent cells. **C**, the titer of infectious virion recovered was determined 40 h after infection of HT29 cells with KOS, hrR3, or HSV1yCD in the presence or absence of ganciclovir (GCV) or 5-FC. **D**, the titer of infectious virion recovered was determined 40 h after infection of human hepatocytes with KOS, hrR3, or HSV1yCD in the presence or absence of ganciclovir (GCV) or 5-FC.



tive to 5-FU ultimately undergo apoptosis after several days of drug exposure *in vitro*. However, if HSV1yCD-infected cells are observed for several days, the cells are destroyed solely as a consequence of viral replication, thereby precluding an analysis of the effects of 5-FU. Therefore, we instead examined the effects of 5-FU exposure on cell cycle distribution. Cells exposed to 5-FU accumulate in S-phase before undergoing apoptosis (14, 15). We confirmed this in a control experiment, in which we observed that exposure of HT29 cells to media containing 5-FU resulted in characteristic pre-apoptotic cell cycle changes, with accumulation of cells in S-phase (Fig. 2B; Panels i and ii). HT29 cells exposed to media conditioned by HSV1yCD-infected cells in the presence of 5-FC showed identical cell cycle changes (despite heat-inactivation of virus) because of 5-FU in the media (Fig. 2B; Panels iii and iv). As a negative control experiment, we demonstrated that conditioned media of hrR3-infected cells cultured in the presence of 5-FC do not exhibit this S-phase accumulation pattern (data not shown). To examine bystander killing, we infected HT29 cells with HSV1yCD (moi = 0.005) in the presence or absence of 5-FC and then examined the uninfected cell population 72 h later by gating out cells expressing green fluorescence. When cultured in the presence of 5-FC, this uninfected cell population (representing 99.5% of cells) demonstrated cell cycle changes identical to cells exposed to 5-FU, indicative of bystander effects on these uninfected cells (Fig. 2B; Panels v and vi). When cultured in the absence of 5-FC, the uninfected cell population showed no cell cycle changes (data not shown).

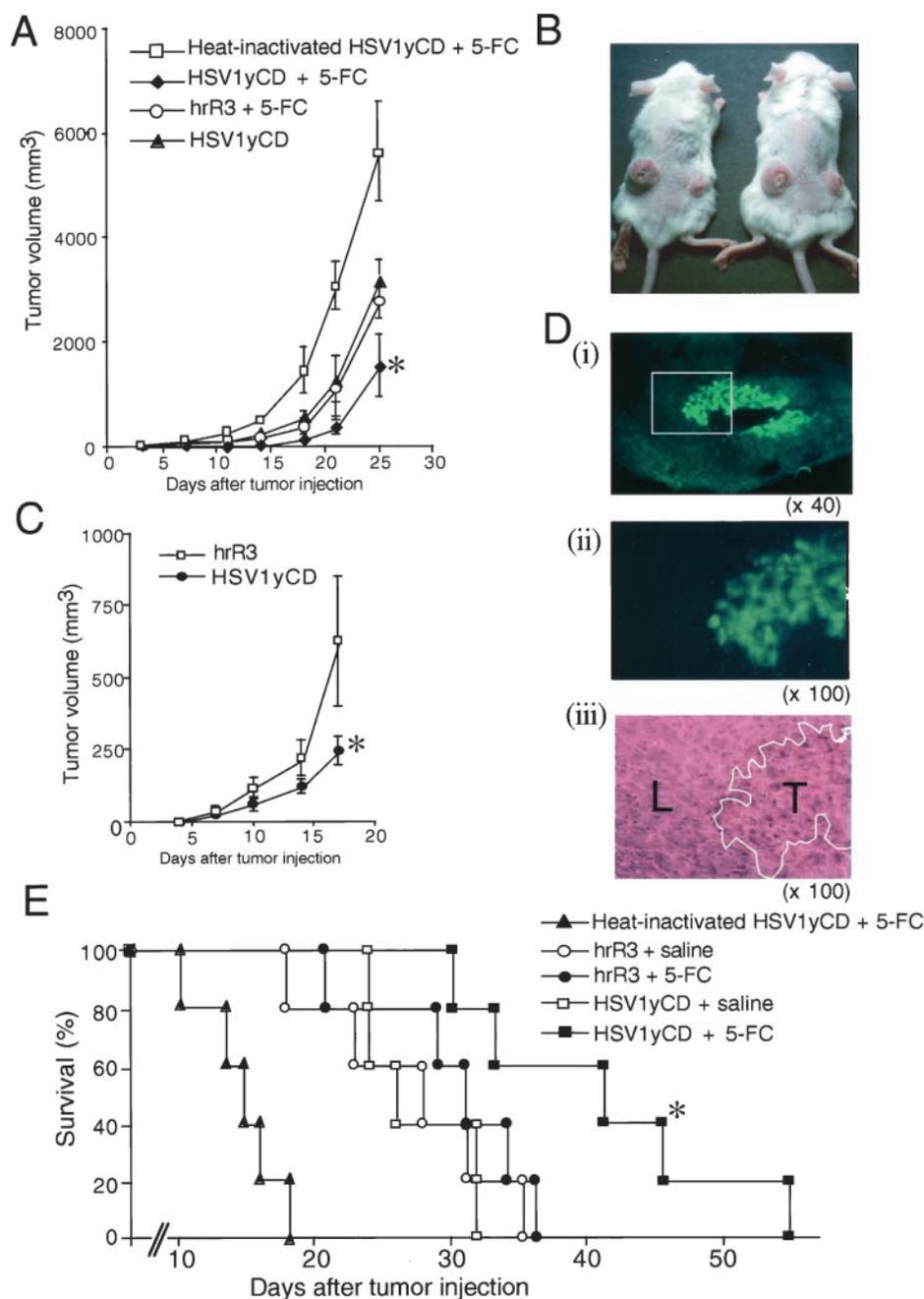
By modulating viral replication, intratumoral prodrug bioactivation during the course of HSV-1-mediated lytic replication may either augment or reduce overall anti-neoplastic efficacy (6, 16). Accordingly, we measured HSV1yCD replication in the presence or absence of either ganciclovir or 5-FU. In the absence of either prodrug, replication of HSV1yCD in HT29 cells was equivalent to that of another ICP6-defective mutant, hrR3, and was only one log order attenuated compared with wild-type strain KOS (Fig. 2C). As ex-

pected, because both hrR3 and HSV1yCD are defective in ICP6 expression, their replication was three log orders more attenuated in human hepatocytes compared with HT29 colon carcinoma cells (Fig. 2D). Preferential HSV1yCD replication in HT29 cells compared with hepatocytes is a result of ICP6 inactivation, because wild-type KOS virus does not display this pattern of replication (7). Of note, replication of all of the viruses in the presence of ganciclovir was significantly attenuated. In contrast, replication of HSV1yCD cells was only minimally attenuated in the presence of 5-FC. These data demonstrate the important point that the combination of oncolysis induced by HSV1yCD replication and prodrug activation can be significantly antagonistic, as in the case of ganciclovir bioactivation. However, 5-FC bioactivation is associated with only a minimal decrease in HSV1yCD replication (less than one log order attenuation). These data indicate that in the context of HSV-1 replication-mediated oncolysis, it is more logical to pursue 5-FC bioactivation by yeast CD than ganciclovir bioactivation by HSV-1 thymidine kinase for combination therapy.

**Effect of HSV1yCD Replication and 5-FC Bioactivation on Tumor Growth.** We examined the effect of HSV1yCD replication combined with 5-FC bioactivation by directly inoculating virus into MC26 tumors growing on flanks of BALB/c mice and administering 5-FC i.p. The reduction in tumor growth observed after administration of HSV1yCD and 5-FC was significantly greater than that observed after administration of HSV1yCD alone or heat-inactivated HSV1yCD (Fig. 3A). As expected, the antitumor effect of HSV1yCD administration alone was identical to the effect of administration of hrR3 combined with 5-FC, because hrR3 is incapable of 5-FC bioactivation.

We next examined whether intratumoral 5-FU production results in any systemic effects. We established MC26 tumors on both the left and right flanks of BALB/c mice and inoculated the right flank tumors with HSV1yCD and the left flank tumors with the same titer of hrR3. i.p. 5-FC was administered to all of the mice. HSV-1-mediated on-

Fig. 3. Effect of HSV1yCD replication and 5-FC bioactivation on tumor growth. *A*, MC26 tumors growing on the flanks of BALB/c mice received injections with hrR3, HSV1yCD, or heat-inactivated HSV1yCD and then treated with i.p. injections of 5-FC or saline. \*,  $P < 0.001$  for HSV1yCD + 5-FC compared with heat-inactivated HSV1yCD and  $P < 0.005$  for HSV1yCD + 5-FC compared with HSV1yCD alone. *B*, mice with bilateral MC26 flank tumors were treated with HSV1yCD injection into the right flank tumor and hrR3 injection into the left flank tumor, followed by i.p. administration of 5-FC. Two representative mice are shown. *C*, tumor volume of the right and left flank tumors are shown. \*,  $P < 0.01$ . *D*, mice with diffuse liver metastases received  $1 \times 10^8$  pfu HSV1yCD into the spleen and were sacrificed 3 days later. The location of HSV1yCD is indicated by green fluorescence in a section of liver viewed under lower power (*i*) and high power (*ii*), with the location of tumor (*T*) and normal liver (*L*) outlined (*iii*). *E*, BALB/c mice bearing diffuse liver metastases were treated with a single intrasplenic inoculation of  $1 \times 10^8$  pfu hrR3, HSV1yCD, or heat-inactivated HSV1yCD. Mice received daily i.p. injections of 5-FC or saline for 10 days. \*,  $P < 0.01$  for HSV1yCD + 5-FC compared with heat-inactivated HSV1yCD + 5-FC and  $P < 0.05$  for HSV1yCD + 5-FC compared with HSV1yCD + saline.



colysis should be identical in each flank because both vectors are ICP6-defective HSV-1 mutants. However, the right flank tumors were exposed to intratumoral conversion of 5-FC to 5-FU, whereas the left flank tumors were exposed to 5-FU that diffused from the right flank tumors into the systemic circulation. We observed that the tumors infected with HSV1yCD were significantly smaller than those infected with hrR3 (Fig. 3, *B* and *C*), indicating that the anti-neoplastic effects of intratumoral conversion of 5-FC to 5-FU are greater than those associated with the 5-FU that diffuses out of the tumor to distant sites.

HSV1yCD replication is substantially greater in carcinoma cells than in hepatocytes, presumably because carcinoma cells are better able to complement the absence of viral ribonucleotide reductase than quiescent hepatocytes (Ref. 17; Fig. 2, *C* and *D*). When HSV1yCD is administered into the portal venous system of mice bearing diffuse liver metastases, fluorescence indicative of the presence of HSV1yCD

was identified specifically in the metastases and not in normal liver 48 h after administration of virus (Fig. 3*D*).

We examined the efficacy of treating diffuse liver metastases with a combination of HSV1yCD and systemic 5-FC administration. BALB/c mice bearing diffuse liver metastases were treated with a single portal venous injection of  $5 \times 10^7$  pfu HSV1yCD or media. Livers of mice in the control group contained numerous (greater than 50) tumor nodules, whereas livers of mice treated with HSV1yCD contained fewer than five (data not shown). The liver weights in the HSV1yCD-treated mice were significantly less than those of the control group mice (mean value of  $1.63 \pm 0.16$  grams versus  $3.03 \pm 0.19$  grams;  $P = 0.017$ ). We did not have the capability to compare tissue levels of 5-FU in normal liver and tumor nodules. However, the promoter-regulating AFP expression is identical to the promoter-regulating yeast CD expression. On the basis of the distribution of green fluorescence, it is reasonable to assume that similar to

AFP, yeast CD is preferentially expressed in the liver metastases rather than in normal liver.

The reduction in liver tumor burden after administration of a single dose of HSV1yCD is substantial enough that at the time of animal sacrifice, it would be difficult to measure any additional benefit that might result from intratumoral generation of 5-FU combined with viral oncolysis mediated by HSV1yCD. Therefore, to examine for any incremental benefit of prodrug activation in a model of diffuse liver metastases, we instead evaluated survival of mice treated with an ICP6-defective virus with or without 5-FC bioactivation. Mice bearing diffuse liver metastases were treated with HSV1yCD, hrR3, or heat-inactivated HSV1yCD. Mice were also randomized to receive either 5-FC or saline. The median survival of mice treated with HSV1yCD and 5-FC was nearly three times that of mice that received no virus (Fig. 3E). The cause of death of all of the mice was intra-abdominal tumor progression, and none of the mice developed signs of encephalitis or hepatitis. All of the mice died; however, the median survival of mice treated with HSV1yCD and 5-FC was also significantly greater than that of mice that received only HSV1yCD or hrR3 and 5-FC and was three times that of untreated controls. These results indicate that intratumoral generation of 5-FU enhances the anti-neoplastic effects of HSV-1-mediated oncolysis of diffuse liver metastases.

## DISCUSSION

HSV-1 mutants that are defective in expression of thymidine kinase (3), ribonucleotide reductase (7, 18), uracil-*N*-glycosylase (19), or  $\gamma_{134.5}$  (20) reduce tumor burden after direct intratumoral inoculation. Because each of these mutants lack specific viral genes, its pathological virulence is attenuated, which enhances its safety for clinical application. However, replication and oncolytic efficiency of these mutants are attenuated compared with wild-type HSV-1; therefore, these attenuated mutants will not reduce tumor burden to a greater extent than wild-type HSV-1. Moreover, replication of even wild-type HSV-1 produces only limited anti-neoplastic effects. We examined the antitumor efficacy of wild-type HSV-1 (F strain) administered into the portal venous system of mice bearing diffuse liver metastases and did not observe complete tumor regression in any of the mice (data not shown). We concluded that it would be difficult if not impossible to achieve complete tumor regression by relying solely on oncolysis by attenuated HSV-1 mutants.

Therefore, we have explored strategies to enhance viral oncolysis by expression of transgenes, such as prodrug-activating genes. The delicate balance between the potentially conflicting goals of achieving robust viral replication in cancer cells and simultaneously destroying the cancer cells by intratumoral generation of cytotoxic metabolites complicates this strategy. If the effect of the cytotoxic metabolites reduces the robustness of viral replication, then the combined effects will be antagonistic rather than additive or synergistic. Although many prodrug-activation strategies have been described using replication-defective vectors, the combination of prodrug activation by a replicating HSV-1 mutant is a new paradigm, and we have identified important interactions between the two modalities. A greater understanding of the interactions between cellular response to prodrug activation and HSV-1 replication is required for both rational design of oncolytic viral mutants and rational design of clinical trials.

For example, our data indicate that ganciclovir activation by HSV-1 thymidine kinase significantly inhibits HSV-1 replication, and consequently the combination of HSV-1-mediated oncolysis and ganciclovir bioactivation produces results that are no better than oncolysis alone. In contrast, the combination of HSV-1-mediated oncolysis and intratumoral conversion of 5-FC to 5-FU augments anti-neoplastic

efficacy compared with HSV-1-mediated oncolysis alone. The explanation for differences between the effect of ganciclovir and 5-FC is presumably related to differences in the mechanism of action between their respective active metabolites. Phosphorylated ganciclovir serves as a false nucleotide that produces premature termination of replicating DNA strands. This affects both viral and genomic DNA synthesis. The mechanism of 5-FU-mediated cytotoxicity is less clear, because it is converted to several metabolites that each have different biochemical actions (21). However, much interest has been placed in the 5-FU metabolite 5-fluorodeoxyuridylate, which inhibits thymidylate synthase. This presumably affects cellular DNA synthesis more than viral DNA synthesis.

The combination of HSV-1-mediated lytic replication and intratumoral conversion of 5-FC to 5-FU for treatment of colorectal carcinoma liver metastases has many theoretical benefits. First, 5-FU is one of the most active and commonly used chemotherapy agents used to treat colorectal carcinoma liver metastases (22). Second, combined modality treatment using therapies with different mechanisms of antitumor activity such as prodrug bioactivation combined with lytic viral replication reduces the risk that emergence of resistant tumor cells will lead to treatment failures. And third, our data demonstrate that when combined with oncolysis, the antitumor effects associated with intratumoral production of 5-FU are greater than those associated with 5-FU leakage to separate tumors in other sites. Another combination therapy that may minimize the risk of tumor cell resistance is radiation therapy combined with HSV-1-induced viral oncolysis (23, 24).

HSV1yCD-mediated oncolysis and intratumoral conversion of 5-FC to 5-FU each produce bystander killing, because each therapy destroys tumor cells that were not initially infected by HSV1yCD. In the case of HSV1yCD-mediated oncolysis, tumor cells that initially escape viral infection are secondarily infected by progeny virion that are released from infected cells. And in the case of conversion of 5-FC to 5-FU, uninfected tumor cells are exposed to the chemotherapeutically active 5-FU that diffuses out from infected cells. We developed an assay to enable us to experimentally isolate the two mechanisms of tumor cell destruction and specifically measure 5-FU-mediated bystander killing of uninfected tumor cells. The importance of bystander killing lies in the realization that no gene delivery vehicles can transduce 100% of cells within a tumor. Bystander killing is necessary to achieve complete tumor destruction despite transduction of only a fraction of the tumor cells (25).

We observed that HSV1yCD replication is only minimally affected by 5-FC and significantly inhibited by ganciclovir. Although the therapeutic implications of these findings are straightforward, the importance of retaining an intact thymidine kinase gene in HSV-1 vectors such as HSV1yCD should not be overlooked. HSV1yCD clearly retains its susceptibility to ganciclovir, which is an important safety feature that permits effective therapy with ganciclovir (or acyclovir) to terminate unwanted viral replication.

Direct intratumoral inoculation of flank tumors is the model used most commonly by investigators to demonstrate efficacy of cytotoxic gene therapy strategies. However, most patients with primary or secondary liver tumors harbor multiple neoplastic foci such that therapies that rely upon accurate intratumoral inoculation of each and every lesion are doomed to failure. It is necessary to develop agents that can be administered intravascularly to target all of the neoplastic foci. In the case of replication-conditional viruses, it is critically important to develop viruses that replicate preferentially in carcinoma cells rather than normal hepatocytes. HSV-1 mutants that are defective in ICP6 expression are ideally suited to target diffuse liver metastases via portal venous administration (10, 17). Replicating viruses offer many advantages over replication-defective viruses;

however, oncolysis alone may be inadequate to completely eliminate tumor burden. Expression of therapeutic transgenes combined with oncolysis can be more effective than either approach alone. The interaction between viral replication and transgene function may be antagonistic, and each potential combination must be examined empirically. Although the anti-neoplastic effects of HSV-1 lytic viral replication combined with 5-FC bioactivation proved to be additive in these experiments, tumor growth was only slowed, and no animals with liver metastases were cured after a single viral inoculation. Additional studies examining the relationship between HSV1yCD dose-schedule and response as well as 5-FC dose-schedule and response are required to gain a better understanding of the optimal efficacy of the combination of HSV1yCD and 5-FC.

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## Multimodality Therapy with a Replication-conditional Herpes Simplex Virus 1 Mutant that Expresses Yeast Cytosine Deaminase for Intratumoral Conversion of 5-Fluorocytosine to 5-Fluorouracil

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