Regulation of herpes simplex virus $\gamma_1$34.5 expression and oncolysis of diffuse liver metastases by Myb34.5

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Myb34.5 is a herpes simplex virus 1 (HSV-1) mutant deleted in the gene for ribonucleotide reductase (ICP6). It also carries a version of $\gamma_1$34.5 (a viral gene product that promotes the dephosphorylation of eIF-2α) that is under control of the E2F-responsive cellular B-*myb* promoter, rather than of its endogenous promoter. Myb34.5 replication in tumor cells results in their destruction (oncolysis). $\gamma_1$34.5 expression by HSV-1 subverts an important cell defense mechanism against viral replication by preventing shutoff of protein synthesis after viral infection. Infection of colon carcinoma cells with Myb34.5 results in greater eIF-2α dephosphorylation and viral replication compared with infection with HSV-1 mutants completely defective in $\gamma_1$34.5 expression. In contrast, infection of normal hepatocytes with Myb34.5 results in low levels of eIF-2α dephosphorylation and viral replication that are similar to those observed with HSV-1 mutants completely defective in $\gamma_1$34.5 and ICP6. When administered intravascularly into mice with diffuse liver metastases, Myb34.5 has greater antineoplastic activity than HSV-1 mutants with completely defective $\gamma_1$34.5 expression and more restricted biodistribution compared with HSV-1 mutants with wild-type $\gamma_1$34.5 expression. Myb34.5 displays reduced virulence and toxicity compared to HSV-1 mutants with wild-type $\gamma_1$34.5 expression. Portal venous administration of Myb34.5 significantly reduces liver tumor burden in and prolongs the life of mice with diffuse liver metastases. Preexisting Ab's to HSV-1 do not reduce the antitumor efficacy of Myb34.5 in vivo.

Introduction

The vast majority of cancer gene therapy strategies for transgene delivery rely on genetically modified viruses (1, 2). These viruses generally have been engineered such that they are capable of replication only in special packaging cell lines and incapable of replication in humans. Antineoplastic activity is dependent on transgene delivery and expression. An alternative strategy relies on and exploits viral replication for tumor destruction, whereby infection of tumor cells by virus leads to cell destruction and simultaneous release of progeny virion that can infect adjacent tumor cells. The antineoplastic activity of viral oncolysis is dependent on the very efficient process of viral replication; however, it is critically important to maintain robust viral replication in neoplastic cells and simultaneously attenuate viral replication in non-neoplastic cells.

Several viruses have been examined for their oncolytic activity, including adenovirus (3, 4), herpes simplex virus (5), vaccinia virus (6), and reovirus (7). These viruses have been directly inoculated into tumors, which is an approach that has clinically significant drawbacks specifically for treatment of liver tumors in comparison with intravascular delivery. The first is that this approach requires direct visualization or radiographic imaging of the liver lesions. Both primary and secondary liver tumors are most commonly multifocal, and a majority of patients with these tumors harbor numerous, undetectable foci of hepatic neoplastic cells (8). The inability to precisely determine the boundary between malignancy and normal liver represents another drawback to a strategy that is dependent on direct intratumoral inoculation. Although it is more difficult to demonstrate efficacy following intravascular administration rather than direct intratumoral inoculation of viral vectors, it is important to succeed in this goal.

A modest number of published reports describe the use of genetically engineered herpes simplex virus 1 (HSV-1) for cancer therapy. These reports have generally involved direct intratumoral inoculation of mutant HSV-1 that are defective in expression of thymidine kinase (5), ribonucleotide reductase (9, 10), uracil-*N*-glycosylase (11), or $\gamma_1$34.5 (12). We have demonstrated previously that intravascular administration of an HSV-1 mutant (hrR3) that is defective in the large subunit of ribonucleotide reductase (ICP6) selectively targets liver metastases (13). In this first-generation HSV-1 vector hrR3, the missing viral gene function (ribonucleotide
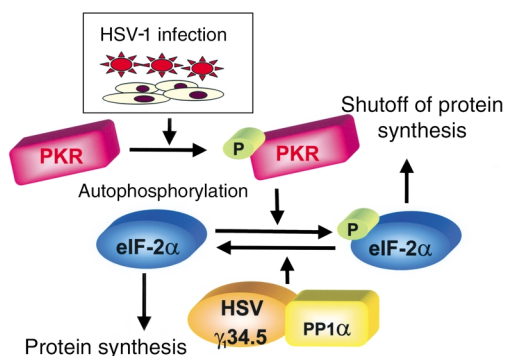
Figure 1

Diagram of the regulation of protein synthesis by $\gamma_1 34.5$ in HSV-1–infected cells. Protein kinase R (PKR) recognizes viral double-strand RNA and is subsequently activated by autophosphorylation. Activated (phosphorylated) PKR phosphorylates eIF-2α, which inhibits initiation of protein translation within the cell and in turn inhibits viral replication. HSV-1 $\gamma_1 34.5$ interacts with cellular protein phosphatase-1α (PP1α) to dephosphorylate eIF-2α, thus allowing continued protein synthesis.

reductase), is effectively complemented by liver metastases but not by normal liver, which results in hrR3 replication preferentially in liver metastases rather than in normal liver. However, hrR3 can replicate at low levels in non-neoplastic cells and demonstrates virulence in vivo at high titer. Accordingly, we have concentrated our efforts on development and characterization of an HSV-1 mutant that is as effective in its oncolytic potential, but less toxic and virulent in vivo.

Myb34.5 is a second-generation replication-conditional HSV-1 mutant in which ICP6 expression is defective and expression of the HSV-1 $\gamma_1 34.5$ gene is regulated by the cellular B-*myb* promoter (14). Following HSV-1 infection, $\gamma_1 34.5$ normally interacts with the cellular protein phosphatase-1α, which leads to eIF-2α dephosphorylation (Figure 1) (15–18). This allows initiation of protein translation to proceed, which is necessary for robust viral replication. HSV-1 mutants with completely defective $\gamma_1 34.5$ expression display significantly attenuated replication in both normal and neoplastic cells. In contrast, regulation of $\gamma_1 34.5$ expression by the cellular B-*myb* promoter following infection by Myb34.5 theoretically permits $\gamma_1 34.5$ expression in cycling cells and in cells with deregulated E2F activation. Accordingly, Myb34.5 replication in quiescent cells should be more attenuated than that of ICP6-defective HSV-1 mutants with normal $\gamma_1 34.5$ expression. And Myb34.5 replication in tumor cells should be greater than that of HSV-1 mutants with completely defective $\gamma_1 34.5$ function.

In the present study we have correlated replication of several HSV-1 mutants that differ in their expression of ICP6 and $\gamma_1 34.5$ with their ability to induce eIF-2α dephosphorylation. We are specifically interested in the applicability of Myb34.5-mediated oncolysis of liver metastases following regional intravascular delivery, and therefore we have examined replication and cyto-

pathic effects in human hepatocytes and colon carcinoma cells. We have demonstrated that (a) the HSV-1 $\gamma_1 34.5$ gene product in Myb34.5 dephosphorylates eIF-2α in infected colon carcinoma cells but not in normal hepatocytes, which represents the fundamental mechanism by which we intended to regulate viral replication in this engineered construct and leads to more restricted biodistribution and less toxicity than hrR3; (b) Myb34.5 replication in colon carcinoma cells is more robust than that of HSV-1 mutants completely defective in $\gamma_1 34.5$ and ICP6, and this is associated with greater antineoplastic activity; (c) portal venous administration of Myb34.5 reduces liver tumor burden more effectively than HSV-1 mutants completely defective in $\gamma_1 34.5$ and ICP6 and produces greater than 50% improvement in median survival of mice despite only a single administration; greater efficacy is observed following multiple Myb34.5 administrations.

Methods

Cells and viruses. The Vero African monkey kidney cells, HT29 human colon carcinoma cells, and MC26 mouse colon carcinoma cells were propagated in DMEM with 8% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Primary human and mouse hepatocytes were prepared as described (13). HSV-1 vectors F strain (wild-type HSV-1) and R3616 (defective $\gamma_1 34.5$ expression) (15) were kindly provided by Bernard Roizman (University of Chicago, Chicago, Illinois, USA). The hrR3 (defective ICP6 expression) (19) was kindly provided by S.K. Weller (University of Connecticut, Storrs, Connecticut, USA). Recombinant HSV-1 vectors Myb34.5 (defective ICP6 expression and $\gamma_1 34.5$ expression regulated by the B-*myb* promoter) and MGH1 (defective ICP6 and $\gamma_1 34.5$ expression) were both derived from F strain (14, 20). Heat inactivation of viruses was performed as described (21).

Determination of eIF-2α phosphatase activity. The bacterial expression vectors pQE-eIF-2α and pGEX-PKR were kindly provided by Bryan R.G. Williams (Lerner Research Institute, Cleveland, Ohio, USA) (22). *Escherichia coli* BL21 cells harboring the pQE-eIF-2α and pGEX-PKR expression vectors were grown overnight in 50 ml Luria-Bertani (LB) broth containing 50 µg/ml ampicillin. Following 1:10 dilution in fresh LB broth, cells were grown for 3 hours to an optical density of 0.8, at which time isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a concentration of 1 mM for an additional 4 hours. Bacteria were pelleted and resuspended in modified NTEN (20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1 mM EDTA; 0.2 mM PMSF; 10 µg/ml aprotinin; 10 µg/ml leupeptin). Cells were lysed and sonicated, and lysates were centrifuged at 22,500 g at 4°C for 20 minutes. To purify the His-tagged eIF-2α protein, the supernatants of these lysates were incubated in a 50% Ni-NTA slurry (QIAGEN Inc., Valencia, California, USA) at 4°C for 60 minutes. The lysate–Ni-NTA mixture was loaded into a column and eluted with modified NTEN buffer including 250 mM imida-

zole. To purify the GST-PKR fusion protein the supernatant was incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, USA) for 60 minutes at 4°C, and the lysate–glutathione-Sepharose beads mixture was loaded into a column and eluted with buffer containing 50 mM Tris, pH 8.0; 1 mM EDTA; and 10 μg/ml reduced glutathione. Purified eIF-2α protein (2 μg) was reacted with GST-PKR protein (2 μg) in 20 mM Tris-HCl, pH 7.5; 40 mM KCl; 2 mM $MgCl_2$; and $[\gamma^{32}P]ATP$ (5 Ci) in a final volume of 50 μl for 30 minutes at 32°C to yield phosphorylated eIF-2α. HT29 cells and human hepatocytes were harvested 15 hours after mock infection or infection with 20 plaque-forming units (pfu) of HSV-1 F strain, Myb34.5, or MGH1 per cell, and S10 fractions were prepared from these lysates and diluted to a final volume of 15 μl with 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA. The amount of protein in each sample was quantified by BCA protein assay kit (Pierce Chemical Co., Rockford, Illinois, USA) (17, 23). ATP was added to a final concentration of 0.8 mM. After 30 seconds at 32°C, each sample received 2 μl of eIF-2α-^{32}P and was reincubated at 32°C. The rate of dephosphorylation of eIF-2α-^{32}P was determined by placing 10-μl aliquots into a solution containing SDS at various time points, followed by electrophoresis on 10–20% gradient gels. The ^{32}P remaining in eIF-2α-^{32}P was quantified by an image analyzer (ImageQuant; Molecular Dynamics, Sunnyvale, California, USA) (24).

Viral replication and cytotoxicity assays. Viral replication and viral cytotoxicity assays were performed as described previously (25, 26).

PCR assay. PCR amplification of HSV-1–specific sequences was used to investigate the biodistribution of HSV-1 following administration to mice. Forward oligonucleotide primer 5′-GGAGGCGCCCAAGCGTC-CGGCCG-3′ and reverse oligonucleotide primer 5′-TGGGGTACAGGCTGGCAAAGT-3′ were used to amplify a 229-bp fragment of HSV-1 DNA polymerase gene. BALB/c mouse tissues were incubated in digestion buffer (10 mM Tris–HCl, pH 7.4; 5 mM EDTA; 0.5% SDS; and 200 μg/ml proteinase K, pH 8.0) at 56°C overnight. Following phenol and chloroform (1:1) extraction, DNA was precipitated in 70% ethanol, lyophilized, and resuspended in distilled water. DNA (0.1 μg) was then subjected to PCR amplification. PCR reactions were performed in a 25-μl volume using rTth DNA polymerase per manufacturer's instructions (Perkin-Elmer Applied Biosystems, Foster City, California, USA) with a DNA Thermal Cycler 480 (Perkin-Elmer Applied Biosystems) for 35 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. Appropriate negative controls were used for all PCR reactions, and no contamination of reagents was detected.

Western blot analysis. Measurements of ribonucleotide reductase protein were performed as previously described (10).

Animal studies. BALB/c mice were obtained from Charles River Laboratories Inc. (Wilmington, Massachusetts, USA). Animal studies were performed in accordance with policies of the Massachusetts General Hospital. To assess the therapeutic efficacy of HSV-1 against diffuse liver metastases, a single-cell suspension consisting of 10^5 MC26 cells in 100 μl HBSS without Ca^{2+} or Mg^{2+} was injected into spleens of BALB/c mice, followed 3 days later by 5×10^7 pfu hrR3, MGH1, Myb34.5, or heat-inactivated Myb34.5 in 100 μl media (n = 5 per group). Mice were sacrificed 14 days after tumor implantation, and the livers and spleens were weighed. To assess survival, mice were treated in the same manner and then followed for survival. To examine the therapeutic efficacy of Myb34.5 in HSV-1–vaccinated mice bearing diffuse liver metastases, mice were vaccinated with 10^7 pfu KOS or Myb34.5 in 100 μl HBSS media by subcutaneous flank injection (27). Control mice were vaccinated with mock-infected media. Two mice in each group were sacrificed after 28 days to collect serum for measurement of the presence of Ab's capable of neutralizing Myb34.5-mediated cytotoxicity against HT29 cells. The remaining mice were injected with 10^5 MC26 cells into the spleen 25 days after vaccination and then treated with an intrasplenic injection of 5×10^7 pfu Myb34.5 after 3 more days (n = 6 per group).

To permit multiple intraportal inoculations without repeated laparotomies, we transposed spleens to a subcutaneous position without disruption of the vascular pedicle. We used methylene blue to confirm that injection into the spleen several weeks later still perfused the entire liver. One week after splenic transposition, diffuse liver metastases were established by inoculation of 5×10^3 MC26 cells directly into the mesenteric vein. Mice were then treated with multiple intrasplenic inoculations of hrR3, MGH1, Myb34.5, or heat-inactivated Myb34.5 in the same experiment and followed for survival.

To analyze liver metastases separately from liver tissue for the presence of HSV-1, single liver metastases were established via intrahepatic inoculation of 10^6 MC26 cells during laparotomy. Intrasplenic HSV-1 injections were performed 1 week later, and the animals were sacrificed 3 days later. Tumors were harvested separately from normal liver tissue, and each was processed and examined for HSV-1. For determination of viral titers in liver and tumor, the tissue was weighed, then minced in 1 mg/ml collagenase A in PBS. After centrifugation at 2,000 g, viral plaque-forming units in the supernatants were measured on confluent Vero cell monolayers.

Statistical analysis. Two nonparametric statistical analyses, the log-rank test and Peto-Wilcoxon test, were used to compare survival between groups. Mean liver and spleen were compared using an unpaired two-tailed t test (InStat; Graphpad Software, New York, New York, USA).

Results

eIF-2α phosphatase activity in HSV-1-infected cells. R3616 is a genetically engineered HSV-1 mutant derived from F

Table 1
HSV-1 mutants

Virus	Genotype	ICP6 expression	$\gamma_1 34.5$ Expression	Parental strain	Reference
F strain	Wild-type	Wild-type	Wild-type	F strain	(61)
hrR3	UL39-lacZ$^+$	Absent	Wild-type	KOS	(19)
R3616	$\gamma_1 34.5^-$	Wild-type	Absent	F strain	(15)
MGH1	$\gamma_1 34.5^-$UL39$^-$lacZ$^+$	Absent	Absent	F strain	(20)
Myb34.5	UL39$^-$B-*myb* promoter$^+$-$\gamma_1 34.5^+$	Absent	Regulated by B-*myb* promoter	F strain	(14)

strain and is defective in $\gamma_1 34.5$ expression (Table 1) (15, 16). MGH1 was derived from R3616 and contains the β-galactosidase gene inserted into the ICP6 gene locus (20), thereby disrupting expression of the large subunit of viral ribonucleotide reductase. Myb34.5 was derived from MGH1 and contains the HSV-1 $\gamma_1 34.5$ gene under control of the E2F-responsive, cellular B-*myb* promoter (14). To understand the mechanisms by which HSV-1 regulates protein synthesis in infected hepatocytes and colon carcinoma cells, we examined eIF-2α dephosphorylation following viral infection. Although eIF-2α is normally phosphorylated by PKR in response to HSV-1 infection, HSV-1 $\gamma_1 34.5$ interacts with protein phosphatase-1α to dephosphorylate eIF-2α to block the shutoff of protein synthesis (Figure 1). Proteins in the lysates of human hepatocytes and HT29 colon carcinoma cells infected with F strain, Myb34.5, and MGH1 were examined for their ability to dephosphorylate eIF-2α. Reaction of eIF-2α-^{32}P with the S10 fraction cells or human hepatocytes infected with MGH1 (defective $\gamma_1 34.5$ expression) or mock infected resulted in minimal eIF-2α dephosphorylation (Figure 2). And as expected, the S10 fraction of HT29 cells or human hepatocytes infected with F strain (wild-type $\gamma_1 34.5$ expression) dephosphorylated eIF-2α significantly. Of importance, lysates prepared from HT29 cells infected with Myb34.5 (regulated $\gamma_1 34.5$ expression) significantly dephosphorylated eIF-2α, and lysates prepared from hepatocytes infected with Myb34.5 did not dephosphorylate eIF-2α. In HT29 cells, the eIF-2α phosphatase activity observed following infection with Myb34.5 was more than fourfold higher than that observed following infection with the $\gamma_1 34.5$-deficient MGH1. In hepatocytes, the eIF-2α phosphatase activity observed following infection with Myb34.5 was as low as that observed with MGH1. We concluded that $\gamma_1 34.5$ regulation by the B-*myb* promoter in Myb34.5 is associated with significantly greater eIF-2α dephosphorylation in HT29 cells than in hepatocytes, which represents one of the fundamental mechanisms by which we intended to regulate viral replication in this engineered construct. These data provide strong evidence to support the hypothesis that Myb34.5 will replicate preferentially in liver metastases compared with the normal liver following portal venous administration.

Viral replication. We demonstrated previously that replication of hrR3 (ICP6 defective) is several log orders greater in human colon carcinoma cells than in human hepatocytes (13). This pattern of hrR3 replication is associated with significantly higher levels of cellular ribonucleotide reductase and nucleotide pools in colon carcinoma cells as compared with hepatocytes (26). Myb34.5 is similar to hrR3 in its absence of ICP6 expression and additionally has $\gamma_1 34.5$ regulated by the E2F-responsive, B-*myb* promoter. The pattern of eIF-2α dephosphorylation that we observed in colon carcinoma cells versus hepatocytes following infection with Myb34.5 suggests that $\gamma_1 34.5$ regulation provides an
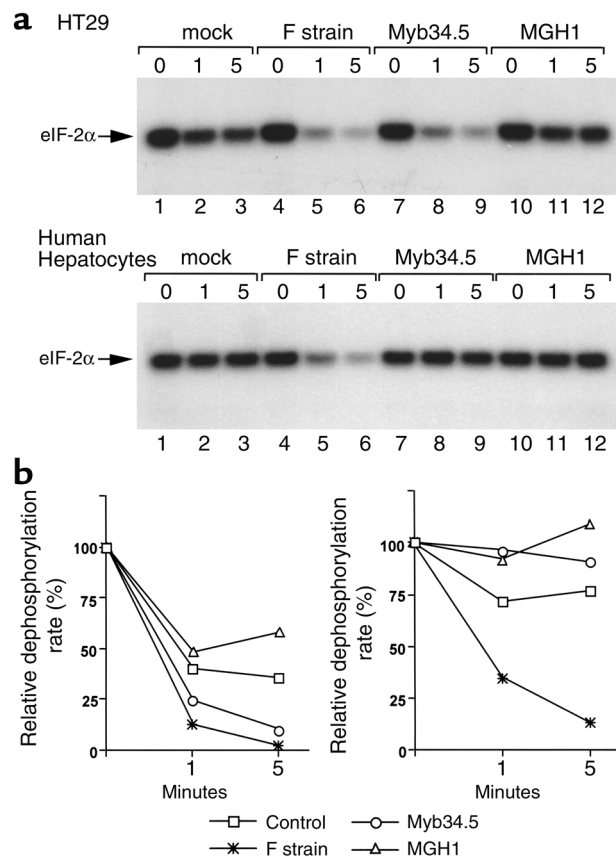

Figure 2
In vitro eIF-2α dephosphorylation assay. (**a**) Autoradiograph of purified phosphorylated eIF-2α reacted with S10 fractions from mock-infected cells (lanes 1–3), F strain–infected cells (lanes 4–6), Myb34.5-infected cells (lanes 7–9), and MGH1-infected cells (lanes 10–12). S10 fractions were collected from HT29 colon carcinoma cells (upper panel) and primary cultures of normal human hepatocytes (lower panel). The reaction time measured in seconds is shown at the top of each panel. (**b**) The eIF-2α dephosphorylation rates of HT29 colon carcinoma cells (left) and human hepatocytes (right) was determined by densitometric quantification of the bands.
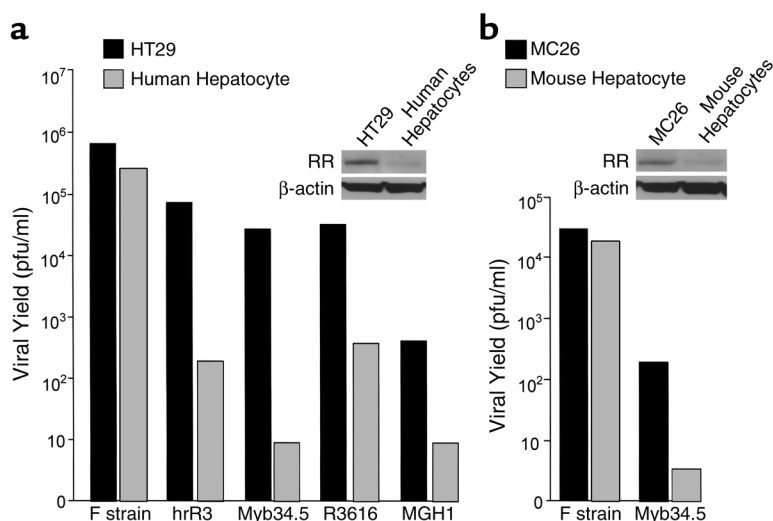
a

b

additional mechanism to attenuate replication in hepatocytes compared with carcinoma cells.

We therefore examined the correlation between $\gamma_1 34.5$ function in HSV-1–infected cells and viral replication. We compared the number of infectious virion produced 40 hours after infection by Myb34.5 with that of F strain, hrR3, R3616, and MGH1 in HT29 human colon carcinoma cells and human hepatocytes. Myb34.5 infection of HT29 cells yielded 10,000-fold more infectious progeny virion than did infection of human hepatocytes (Figure 3a). In contrast, the number of infectious virion measured following infection by F strain was nearly identical in HT29 cells and hepatocytes. Furthermore, Myb34.5 replication was similar to that of hrR3 and R3616 in colon carcinoma cells, but more than tenfold less than that of hrR3 or R3616 and identical to that of MGH1 in human hepatocytes. We also compared replication of Myb34.5 and F strain in MC26 mouse colon carcinoma cells and primary hepatocytes. As observed in the human system, F strain replication was nearly identical in colon carcinoma cells and hepatocytes, whereas Myb34.5 replication was four log orders less in the hepatocytes than in the colon carcinoma cells (Figure 3b). HSV-1 displays tropism for human and primate cells relative to murine cells, which results in lower levels of viral replication overall in the

murine cells as compared with human cells. Nonetheless, the same pattern of Myb34.5 replication was observed in the murine tissue system as was observed in the human tissue system. From these data we conclude that (a) Myb34.5 replication is several log orders greater in colon carcinoma cells than hepatocytes in vitro; (b) Myb34.5 replication in colon carcinoma cells is as robust as that of the single mutants hrR3 and R3616; and (c) Myb34.5 replication in hepatocytes is as attenuated as that of the double-mutant MGH1. These data support the notion that Myb34.5 should be equally effective as hrR3 in oncolysis, but less toxic.

Viral cytopathic effects in vitro. To examine the potential for therapeutic oncolysis, we investigated whether Myb34.5 replication is associated with cytopathic
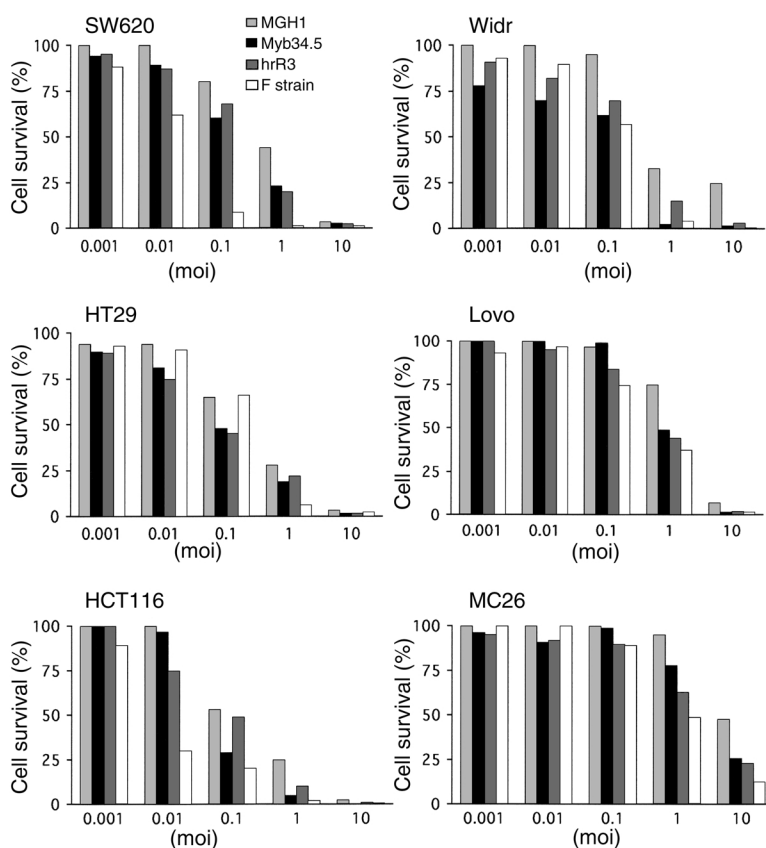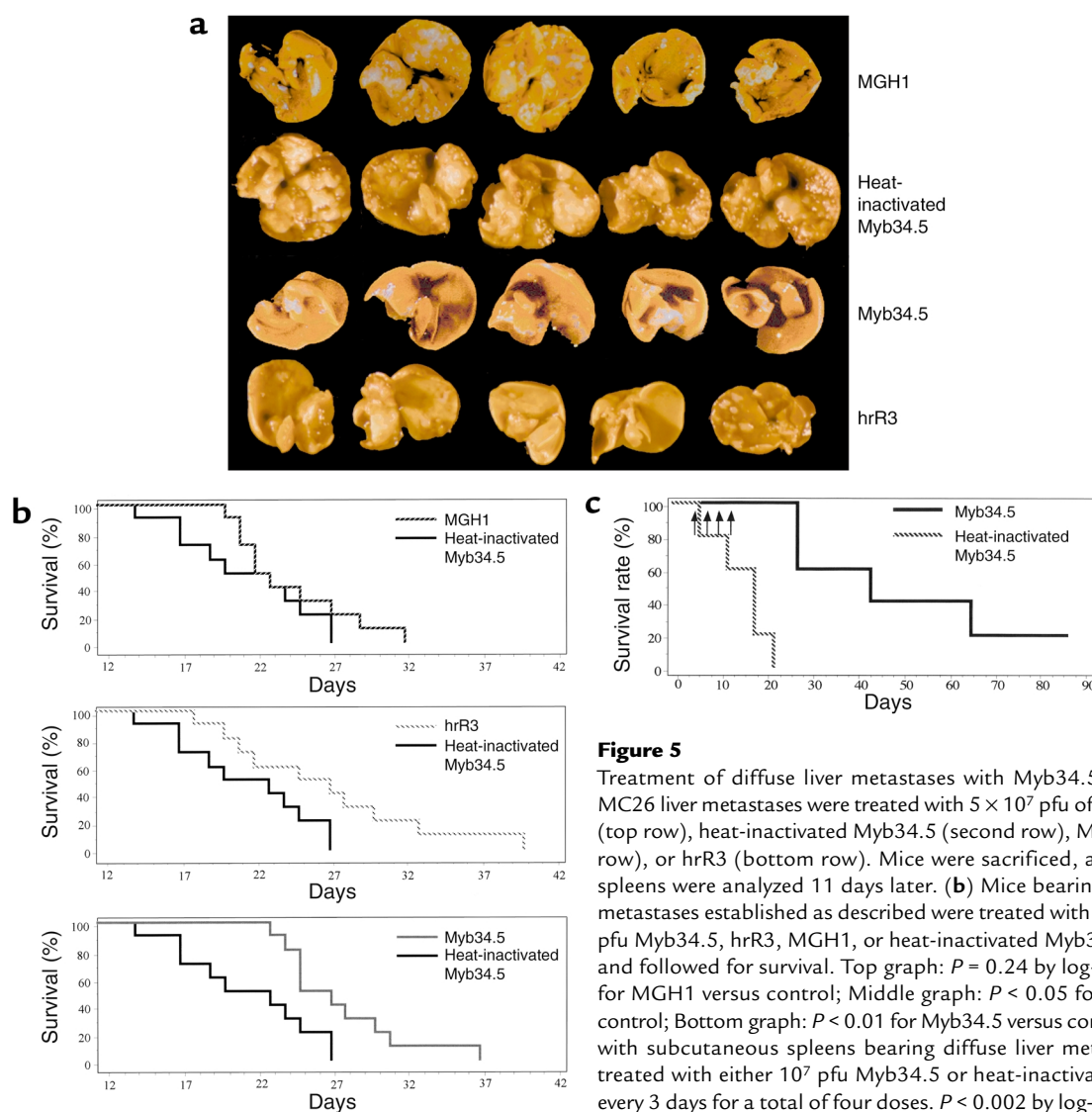
Figure 5
Treatment of diffuse liver metastases with Myb34.5. (**a**) Diffuse MC26 liver metastases were treated with 5×10^7 pfu of either MGH1 (top row), heat-inactivated Myb34.5 (second row), Myb34.5 (third row), or hrR3 (bottom row). Mice were sacrificed, and livers and spleens were analyzed 11 days later. (**b**) Mice bearing diffuse liver metastases established as described were treated with either 5×10^7 pfu Myb34.5, hrR3, MGH1, or heat-inactivated Myb34.5 (control) and followed for survival. Top graph: $P = 0.24$ by log-rank analysis for MGH1 versus control; Middle graph: $P < 0.05$ for hrR3 versus control; Bottom graph: $P < 0.01$ for Myb34.5 versus control. (**c**) Mice with subcutaneous spleens bearing diffuse liver metastases were treated with either 10^7 pfu Myb34.5 or heat-inactivated Myb34.5 every 3 days for a total of four doses. $P < 0.002$ by log-rank analysis.

effects. We compared the cytopathic effect of Myb34.5 hrR3, R3616, and F strain infection of five human and one murine colon carcinoma cell lines in vitro. The cytopathic effect of Myb34.5 was consistently greater than that of MGH1 and similar to that of hrR3 and wild-type F strain (Figure 4).

Oncolysis of diffuse colon carcinoma liver metastases. We next examined the antitumor efficacy of Myb34.5 against diffuse experimental liver metastases. Balb/c mice bearing syngeneic MC26 diffuse liver metastases were treated with a single portal venous injection of 5×10^7 pfu of Myb34.5, hrR3, MGH1, or heat-inactivated Myb34.5 (control group). At the time of animal sacrifice 2 weeks later, mice treated with MGH1 or heat-inactivated virus had distended abdomens with bloody ascites. In contrast, mice injected with Myb34.5 or hrR3 appeared healthy and had no ascites. Livers of mice treated with MGH1 or heat-inactivated virus contained numerous (greater than 50) tumor nodules, whereas livers of mice treated with either Myb34.5 or hrR3 contained ten or fewer nodules (Figure 5a). There

was no difference in liver weights between mice treated with MGH1 and mice treated with heat-inactivated (control) virus. However, the liver weights in mice treated with either Myb34.5 or hrR3 were significantly less than those of mice treated with MGH1 or heat-inactivated virus (Table 2, group A). The liver tumor weights in Myb34.5-treated mice were less than those of hrR3-treated mice; however, this difference did not reach statistical significance. There was no statistical difference in spleen weights among these three groups, indicating that the reduction in liver tumor burden did not result from reducing splenic tumor burden.

In a separate experiment we investigated whether these antitumor effects translate to improved survival in the Myb34.5-treated mice. Separate groups of mice were treated with My34.5, hrR3, MGH1, or heat-inactivated Myb34.5 and followed for survival. Mice treated with Myb34.5 or hrR3 demonstrated substantially enhanced survival compared with control mice, with greater than 50% improvement in median survival (Figure 5b). The improvement in survival observed with

Myb34.5 treatment was more statistically significant than the improvement in survival observed with hrR3 treatment. In contrast, no improvement in survival was observed in mice treated with MGH1. The cause of death in all mice was progressive intra-abdominal tumor burden in the liver and spleen (site of tumor inoculation). Mice in these experiments were necessarily treated with only a single administration of virus. Because tumor implantation and subsequent treatment each require a laparotomy, it is not feasible to perform a third laparotomy to administer a second dose of Myb34.5 using this model. Nonetheless, Myb34.5 significantly reduced liver tumor burden and enhanced survival of mice bearing diffuse liver metastases following intravascular delivery of only a single dose.

This commonly used animal model has two significant limitations. The first is that only a single dose can be administered into the portal vein via the spleen. The second limitation is the continued growth of tumor in the spleen at the site of initial tumor inoculation, which limits animal survival. To overcome these problems, we developed a new model in which the spleen is first transposed to a subcutaneous location while keeping its vascular pedicle intact. Subsequently, tumor cells are inoculated into a mesenteric vein to establish diffuse liver metastases. We first used methylene blue to confirm that subsequent injections into the subcutaneous spleen perfused the entire liver. We also determined that mesenteric inoculation of 5×10^3 MC26 cells led to diffuse and rapidly growing liver metastases without growth in the mesentery at the site of tumor injection. We then assessed survival of mice with diffuse liver metastases treated with four injections of Myb34.5 (10^7 pfu) each separated by 3 days. The survival of these mice was markedly prolonged compared with mice treated with similar injections of heat-inactivated Myb34.5, and one mouse remains alive 6 months after treatment (Figure 5c).

Myb34.5 toxicity and biodistribution following administration to mice. Direct comparison of HSV-1 mutants in their ability to replicate in primary hepatocyte cultures suggested that Myb34.5 would be less toxic than F strain or hrR3 following intravascular delivery into the liver (Figure 3). We therefore compared toxicity of F strain, Myb34.5, MGH1, and hrR3 in mice. Humans represent the only species naturally infected by HSV-1, and there are no animal models that accurately recapitulate the natural progression of HSV-1 infection humans. However, mice serve as an excellent model for comparison of the *relative* virulence between different HSV-1 mutants (28–30).

We observed that following subcutaneous inoculation of 10^7 pfu of F strain, mice uniformly developed paralysis followed by death within 25 days. In contrast, no toxicity was observed following subcutaneous inoculation of 10^7 pfu of MGH1 or Myb34.5. To better quantify the extent to which virulence of Myb34.5 is attenuated compared with that of F strain, BALB/c mice were injected with F strain (10^7 pfu, 10^6 pfu, or 10^5 pfu) or

Myb34.5 (10^8 pfu). None of the mice injected with Myb34.5 died in 30 days. In contrast, all of the mice injected with 10^7 pfu F strain died of overwhelming viral infection within 11 days, and 75% of mice inoculated with 10^6 pfu F strain died similarly within 12 days.

To directly address whether Myb34.5 is less toxic than the HSV-1 single-locus–mutant hrR3 following portal venous inoculation, we examined survival of mice following intrasplenic inoculation of either Myb34.5 (10^8 pfu and 10^9 pfu) or hrR3 (10^8 pfu and 10^9 pfu). None of the mice that received Myb34.5 died, whereas three of four mice that received 10^9 pfu hrR3 died, and one of four mice receiving 10^8 pfu hrR3 died within 5 days. These results are consistent with those observed in vitro and suggest that because Myb34.5 replication is more attenuated than that of hrR3 in normal hepatocytes, it is less toxic than hrR3 following intraportal administration.

In a separate experiment we compared biodistribution of F strain, Myb34.5, MGH1, and hrR3 by examining mouse tissues for the presence of HSV-1 DNA by PCR amplification of the HSV-1 DNA polymerase gene. When mice were sacrificed 8 days after subcutaneous inoculation, HSV-1 DNA was detected in all tissues examined (brain, lung, spleen, liver, colon) from mice injected with F strain; however, it was not detected in any tissues of the mice injected with Myb34.5, hrR3, or MGH1 (Figure 6a). When mice were sacrificed 10 days after portal venous inoculation, HSV-1 DNA was detected in all tissues examined from mice injected with 10^5 pfu F strain, but only in the spleen and livers from mice infected with Myb34.5, hrR3, or MGH1, even though they were injected with 500-fold more virus (5×10^7 pfu) (Figure 6b). To determine whether replication in liver metastases contributes to persistence of Myb34.5 in other sites, the experiment was repeated using mice bearing diffuse MC26 liver metastases. Notably, no evidence of Myb34.5, hrR3, or MGH1 was identified in tissues outside the liver and spleen (Figure 6c), and this biodistribution was similar to that observed in mice without liver metastases (Figure 6b). These data suggest that Myb34.5 replication in tumor nodules in the liver does not contribute to seed-

Table 2

Liver and spleen weight following treatment of MC26 liver metastases

Group	Immunization	Treatment	Number	Liver weight (g) ± SD
A	None	Mock	5	2.73 ± 0.27
	None	MGH1	5	2.99 ± 1.26
	None	hrR3	5	1.50 ± 0.20[A]
	None	Myb34.5	5	1.24 ± 0.17[A, B]
B	Myb34.5	Mock	6	2.50 ± 0.80
	Myb34.5	Myb34.5	6	1.63 ± 0.28[C, D]
	Mock	Myb34.5	6	1.70 ± 0.19[C]

Group A: No immunization prior to tumor implantation. Livers and spleens were weighed 14 days after tumor implantation. Group B: Immunization was performed 25 days before tumor implantation. [A]$P < 0.01$ when compared with nonimmunized mice treated with either MGH1 or mock virus. [B]$P > 0.05$ when compared with nonimmunized hrR3-treated animals. [C]$P < 0.05$ when compared with Myb34.5-immunized mock-treated animals. [D]P value not significant when compared with mock-immunized Myb34.5-treated animals.
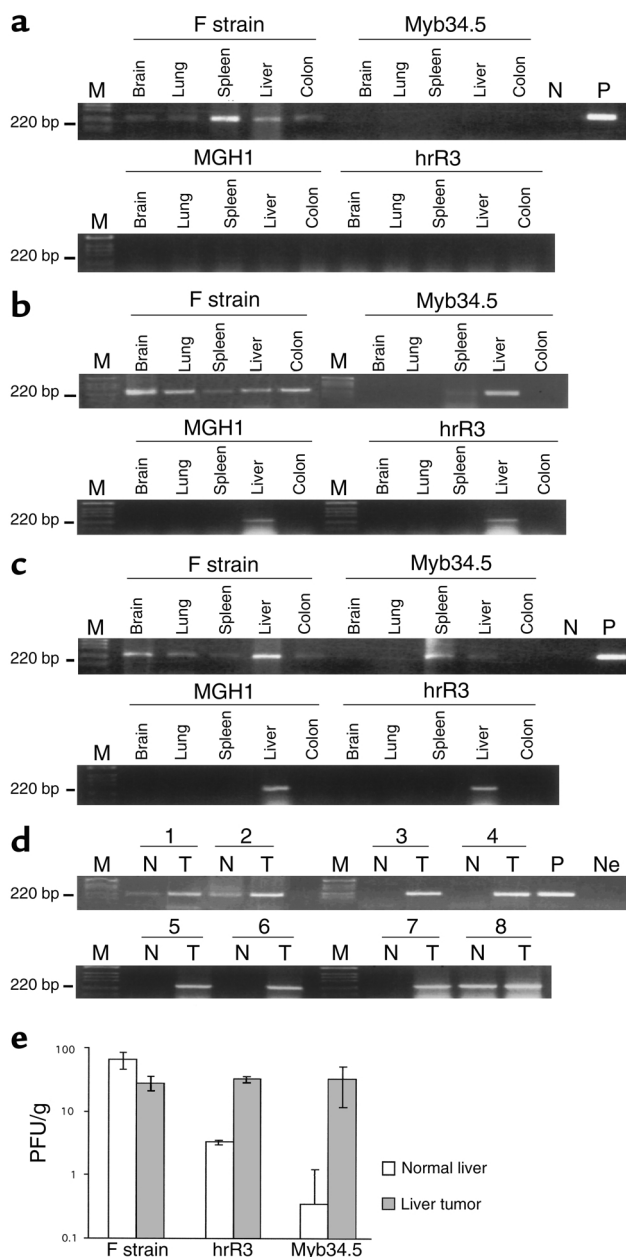
a F strain / Myb34.5 / MGH1 / hrR3

b F strain / Myb34.5 / MGH1 / hrR3

c F strain / Myb34.5 / MGH1 / hrR3

d 1 2 3 4 5 6 7 8

e

Figure 6

HSV-1 detection in mice by PCR assay. (**a**) Mice inoculated subcutaneously with F strain, Myb34.5, MGH1, or hrR3 were sacrificed 8 days later to harvest tissues for PCR amplification of a 220-bp portion of the HSV-1 DNA polymerase gene. A 1-kb marker is shown in lane M, with the location of the 220-bp bands indicated. A negative control reaction without template is shown in lane N, and a positive control reaction using HSV-1 genomic DNA as template is shown in lane P. (**b**) Mice inoculated intrasplenically with either 10^5 pfu F strain or 5×10^7 pfu Myb34.5, MGH1, or hrR3 were sacrificed 10 days later to harvest tissues for PCR analysis as above. (**c**) Mice bearing diffuse liver metastases were treated with an intrasplenic inoculation of either 10^5 pfu F strain or 5×10^7 pfu Myb34.5, MGH1, or hrR3, as above, and sacrificed 10 days later to harvest tissues for PCR analysis. In these animals, analysis of liver tissue included normal liver homogenized together with liver metastases. (**d**) Mice bearing a single liver metastases were treated with an intrasplenic inoculation of either 10^5 pfu F strain (1 and 2) or 5×10^7 pfu Myb34.5 (3 and 4), MGH1 (5 and 6), or hrR3 (7 and 8). Mice were sacrificed 12 days (rather than 8 days) later to harvest tissue for PCR analysis of either normal liver (N) or liver tumor (T). Positive (P) and negative (Ne) controls are shown. (**e**) Mice bearing a single liver metastases that had been treated with an intrasplenic injection of either 10^5 pfu F strain (*n* = 3), 5×10^7 pfu hrR3 (*n* = 3), or 5×10^7 pfu Myb34.5 (*n* = 6). Normal liver and metastases were harvested separately and titered for the presence of HSV-1. The differences in viral titers in liver tumors are not statistically significant. The difference in viral titer in normal liver are statistically significant by *t* test as follows: F strain versus hrR3, *P* = 0.03; F strain versus Myb34.5, *P* = 0.03; hrR3 versus Myb34.5, *P* = 0.04.

and hrR3 in tumor nodules were identical to that of F strain (Figure 6e). The titer of Myb34.5 in normal liver was significantly less than those of hrR3 and F strain, despite having inoculated 500-fold more Myb34.5 into the spleen than F strain. In concert, these results suggest that the toxicity and virulence of Myb34.5 and MGH1 are attenuated compared with F strain and hrR3. Myb34.5 and MGH1 biodistribution are significantly more restricted and appear to be limited to liver tumors after portal venous administration, and these viruses persist for a longer period of time in liver metastases compared with normal livers.

Host immune effects on Myb34.5-mediated oncolysis. HSV-1–mediated oncolysis may theoretically be influenced by host immune responses to the virus (31). A large percentage of patients have Ab's to HSV-1 (32), and the presence of these Ab's might decrease the efficiency of viral infection and oncolysis. On the other hand, it has been reported that the host immune response can enhance the antitumor effects of HSV-1–mediated oncolysis (33, 34). Accordingly, we investigated how the presence of neutralizing Ab's affects Myb34.5-mediated oncolysis of diffuse liver metastases. Mice were subcutaneously vaccinated with mock-infected media, 10^7 pfu of KOS, or 10^7 pfu of Myb34.5. Two mice in each group were sacrificed after 28 days to collect serum for measurement of the presence of Ab's capable of neutralizing Myb34.5 cytotoxicity against HT29 cells. Sera from the mice vaccinated with mock-infected media had little neutralizing effect, whereas

ing of the virus to distant sites. Finally, we established single liver tumors by direct subcapsular tumor cell inoculation, and then treated the mice 7 days later with an intrasplenic injection of either 10^5 pfu F strain or 5×10^7 pfu Myb34.5, MGH1, or hrR3. HSV-1 DNA polymerase was amplified from both the liver tumors and normal liver tissue 12 days after F strain or hrR3 administration (Figure 6d). In contrast, Myb34.5 and MGH1 were detectable only in the metastases and not in normal liver. We examined liver tumors and normal liver at an earlier time point (3 days after viral inoculation) and quantitated infectious virus on Vero cell monolayers. The process of viral recovery from tissues is inefficient, and even when tissue is harvested immediately after viral inoculation, we measure 100- to 1000-fold less virus than inoculated. The titers of Myb34.5

the sera from mice vaccinated with either Myb34.5 or KOS significantly reduced Myb34.5-mediated cytotoxicity (data not shown). MC26 liver metastases were established in mice that had been vaccinated with either Myb34.5 or mock-infected media. The mice were then treated with a single portal venous injection with either Myb34.5 or mock-infected media. We sacrificed the mice 14 days after tumor implantation and examined the livers. Livers from mice treated with a single portal venous injection of Myb34.5 had fewer tumor nodules and significantly less tumor burden (Figure 7). The liver tumor burden in Myb34.5-treated mice was the same whether or not the mice had been vaccinated to produce neutralizing anti–HSV-1 Ab's (Table 2, group B). These results indicate that pre-existing Ab's to HSV-1 neither enhance nor attenuate Myb34.5-mediated oncolysis.

Discussion

Viruses have developed efficient mechanisms to infect cells, subvert cellular defenses, express viral genes, and produce progeny virion. Scientists have exploited these natural processes for purposes of therapeutic gene delivery, and genetically engineered viruses are presently the most commonly used gene delivery vehicles in clinical trials today (1, 2). Viruses that serve solely as gene delivery vehicles have been genetically modified such that they are incapable of replication to minimize the risk of uncontrolled replication in the host. However, because viral replication results in tissue destruction, several investigators have examined strategies of injecting replicating viruses into tumors to destroy the tumors as a result of viral replication (3–7, 35). The concept of viral inoculation to destroy tumors was described nearly a century ago (36).

Oncolysis achieved by viral replication has several potential advantages compared with traditional cancer therapies (for review see ref. 35). Viral replication within a tumor amplifies the administered dose, because tumor cells produce progeny virion that infect adjacent tumor cells. Cell-to-cell propagation of virus enhances infection throughout the tumor. This is in contrast to vascular spread, as occurs with traditional therapeutic agents, in which it has been observed that high interstitial pressures and dysregulation of tumor neovasculature combine to limit delivery of these agents to tumor cells (37). Another advantage of replicating viruses is their ability to serve dual roles: induction of oncolysis by viral replication and delivery of transgenes for added therapeutic efficacy (38).

The safety of replication-conditional viruses used in humans for cancer therapy remains of paramount importance, and these viruses should meet specific safety criteria. First, viral replication should be limited to neoplastic cells, with negligible if any replication in normal cells. Second, the potential for spontaneous reversion to a virulent strain should be minimized. Third, an effective antidote (antiviral agent) should be safe to administer and readily available if needed. Finally, the therapeutic window should be large.

We reported previously our observations on the preferential localization of hrR3 within diffuse liver metastases following portal venous administration (10). Based on these initial observations, we subsequently examined strategies to engineer an HSV-1 mutant that displays an enhanced safety profile, with attenuation in pathologic virulence but equally effective oncolytic activity. Myb34.5 is unique because of its regulation of $\gamma_1 34.5$ by a heterologous promoter. Only two other HSV-1 mutants have been described in which replication is regulated by a heterologous promoter (39, 40). In both of these mutants the heterologous promoter regulates ICP4 expression; however, we have observed that some cells effectively complement the absence of ICP4 (data not shown). In addition, the HSV-1 thymidine kinase gene is disrupted in both of these ICP4-locus mutants such that they are not suitable for clinical trials. The absence of a functional HSV-1 thymidine kinase gene destroys sensitivity to acyclovir, which is an effective therapeutic agent for HSV-1 infection in clinical use today (41). Myb34.5 retains an active HSV-1 thymidine kinase gene and is sensitive to acyclovir. Another safety issue addressed in Myb34.5 is its con-

Figure 7

Treatment of diffuse liver metastases with Myb34.5 in mice with neutralizing Ab's to HSV-1. Mice were vaccinated with either Myb34.5 (top and middle row) or mock-infected media (lower row) 25 days before intrasplenic inoculation of MC26 cells. After confirmation of the presence of neutralizing Ab's to HSV, mice were treated with 5×10^7 pfu Myb34.5 3 days after tumor implantation and sacrificed 14 days after tumor implantation (middle and lower row). To serve as a control group, mice vaccinated with Myb34.5 were treated with heat-inactivated Myb34.5 (upper row).
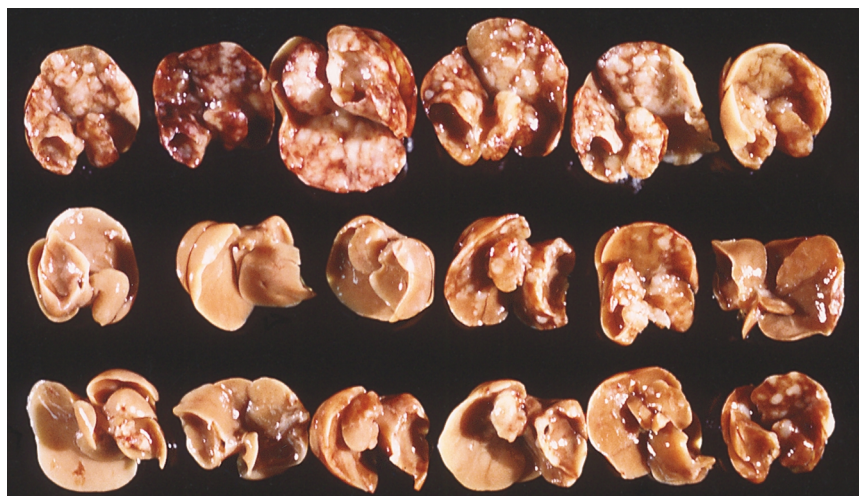
struction with multiple genetic alterations, thereby dramatically reducing the risk of spontaneous reversion of Myb34.5 to a wild-type virus.

Myb34.5 displays a larger therapeutic window than hrR3 or MGH1 as assessed by measurements of viral replication in colon carcinoma cells versus hepatocytes and attenuated virulence and toxicity in vivo. Moreover, it retains equivalent oncolytic activity and significantly enhances survival in mice bearing diffuse liver metastases following administration of only a single dose. Long-term survival was observed following administration of multiple doses. The genetic modifications in Myb34.5 are designed to attenuate its replication in hepatocytes without attenuating replication in neoplastic cells. These modifications alone would not be expected to enhance its destruction of neoplastic cells compared with either wild-type HSV-1 or hrR3. A separate strategy, such as expression of transgenes (e.g., suicide genes, cytokine genes), is required to enhance the endogenous oncolytic potential of HSV-1 mutants (42). Not surprisingly, Myb34.5 replication in vitro is not as robust as that of F strain in some cell lines. However, studies quantifying replication in liver metastases demonstrate equivalent titers of either virus 3 days after intraportal inoculation. The factors governing replication in vivo are numerous and complex.

Humans represent the only species that is naturally infected by HSV-1. Both rodent and nonhuman primate models for comparing the relative virulence of HSV-1 and HSV2 strains have been described (27–29, 43–47). None of these models, including *Aotus* monkey models, fully recapitulate HSV-1 infection observed in humans. *Aotus* monkeys are both expensive and difficult to acquire for research purposes. The mouse model has several advantages compared with the *Aotus* monkey model, including the absence of any ambiguity over the strain or age of the animals (44, 45), ability to examine toxicity in several animals for each dose level and each strain of HSV-1, significantly greater research experience with this model, and the absence of significant logistical or financial barriers. We demonstrated that the LD_{50} of Myb34.5 is at least one log order greater than that of hrR3 and at least two log orders greater than that of wild-type F strain in a mouse model. The observed biodistribution of Myb34.5 compared with F strain as assessed by PCR amplification provides additional evidence that Myb34.5 replication is highly specific for liver metastases, whereas PCR evidence of F strain replication was detected in all of the tissues examined.

PKR activation in response to viral infection remains an important cellular defense (48, 49). The importance of this mechanism is underscored by the observation that most viruses have incorporated strategies to overcome the shutoff of protein translation that accompanies PKR activation. For example, adenovirus produces VAI RNA to associate with PKR to inhibit its activation (50). Similarly, HIV produces TAR RNA, which performs a function similar to VAI RNA (51). P58^{IPK} is an inhibitor of PKR that is constitutively expressed in mammalian cells and is normally inactivated by specific inhibitory molecules. Influenza virus disrupts this P58^{IPK}-inhibitory molecule complex following infection, thereby functionally activating the PKR inhibitory activity of P58^{IPK} (52, 53). As another example, the E3L and NS5A proteins that are expressed by HCV are known inhibitors of PKR (54, 55).

HSV-1 circumvents the consequences of PKR activation by expression of $\gamma_1 34.5$ (Figure 1). HSV-1 $\gamma_1 34.5$ contains sequences that share homology with the GADD 34 protein (18), which normally interacts with protein phosphatase-1α to dephosphorylate eIF-2α. Following HSV-1 infection, $\gamma_1 34.5$ interacts with cellular protein phosphatase-1α to dephosphorylate eIF-2α, which prevents shutoff of cellular protein synthesis (18). Consequently, HSV-1 mutants that are defective in $\gamma_1 34.5$ expression display attenuated replication and virulence (15). For example, HSV-1 mutants R3616 and MGH1 are both completely defective in their expression of $\gamma_1 34.5$, and the magnitude of their replication is significantly attenuated compared with that of wild-type HSV-1 (15, 20). MGH1 is essentially identical to another HSV-1 mutant, G207 (45, 56, 57), which has been examined in a clinical trial for brain tumor patients (58) and is presently under examination for treatment of liver tumors. These HSV-1 mutants are defective in both $\gamma_1 34.5$ and viral ribonucleotase expression. In a direct comparison of MGH1 and Myb34.5, we observed that their replication in hepatocytes is similar, yet Myb34.5 replication in carcinoma cells is substantially greater than that of MGH1. This difference correlates with the greater efficacy observed in vivo of Myb34.5 compared with MGH1. Our data suggest that this difference in oncolytic potential between MGH1 and Myb34.5 results from the difference in $\gamma_1 34.5$ expression and function between these two mutants following infection of carcinoma cells. In addition, Myb34.5 replication in hepatocytes was more attenuated than that of the mutant hrR3, which is defective only in ICP6 expression. This resulted in a higher LD_{50} in mice for Myb34.5 compared with hrR3, yet Myb34.5 retains similar oncolytic efficacy as hrR3 against diffuse liver metastases.

A large percentage of patients with colorectal carcinoma liver metastases develop metastases only in the liver and without evidence of disease in other sites (59). Because of this tumor biology, several approaches to regional (hepatic) therapy for colorectal carcinoma liver metastases have been examined in clinical trials, including hepatic arterial and portal venous infusion (60). We examined portal venous viral inoculation because it is not possible to cannulate the hepatic artery of mice. The hepatic tumor burden in patients with colon carcinoma liver metastases is generally greater than that which was treated in this study. However, Myb34.5 replication is two to three log orders greater in human colon carcinoma cells than in murine colon carcinoma cells.

Both innate and elicited antiviral responses reduce the efficiency of HSV-1–mediated oncolysis of tumors following intravascular delivery in the brain (31). However, delivery through the vasculature into the liver is likely to be much more efficient than delivery into the brain. Biodistribution studies of HSV-1 after intravenous administration demonstrate that most of the virus localizes to the liver (61). It is thus conceivable that the effect of innate and elicited antiviral responses may not be as limiting for tumors in the liver as for tumors in the brain. We observed equally impressive Myb34.5-mediated oncolysis of liver metastases in naive mice as in mice with neutralizing Ab's to HSV-1. This is an important finding, given that the prevalence of Ab's to HSV-1 in the United States is as high as 80% in some populations (32). We have demonstrated previously that the HSV-1–mediated antineoplastic activity observed in this liver metastases model is dependent on viral replication and not dependent on host immune responses (10). The importance of this lies in the observation that responses to immunotherapy observed in mouse models are often not reproducible in humans.

Myb34.5 effectively destroys diffuse liver metastases following intravascular administration. It is attenuated in vitro and in vivo compared with wild-type strains and HSV-1 vectors harboring a single mutation and displays a much more restricted biodistribution following portal venous administration. These encouraging results warrant pursuit of clinical studies of Myb34.5 in the treatment of patients with liver metastases.

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