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Cancer Gene Therapy Using a Replication-Competent Herpes Simplex Virus Type 1 Vector

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Objective

The authors investigate the efficacy of hrR3, a viral vector derived from herpes simplex virus type 1 (HSV 1), in destroying colon carcinoma cells *in vitro* and *in vivo.* The effect of adding the prodrug ganciclovir in combination with hrR3 infection also is assessed.

Summary Background Data

Most cancer gene therapy strategies use viral vectors that are incapable of replication. The HSV 1 vector hrR3 is capable of replication, and its replication is cytotoxic to cells. hrR3 also possesses the HSV-thymidine kinase gene, which converts ganciclovir into a toxic metabolite. Thus, the addition of ganciclovir to hrR3-infected cells may enhance the ability of hrR3 to destroy tumor cells. To increase specificity for tumor cells, hrR3 has a mutated ribonucleotide reductase gene and replicates selectively in cells with high levels of endogenous ribonucleotide reductase. Actively dividing cells such as tumor cells have high levels of endogenous ribonucleotide reductase for synthesis of DNA precursors. The authors are interested in the use of HSV 1 vectors to treat liver metastases from colorectal cancer.

Methods

Ribonucleotide reductase expression in several colon carcinoma cell lines and in primary cultures of human hepatocytes was determined by Western blot analysis. hrR3-mediated cytotoxicity in the colon carcinoma cell lines was determined using an *in vitro* assay. The human colon carcinoma cell line HT29 was injected into the flanks of nude mice followed by intratumoral injection of hrR3. Tumor growth rate was assessed with and without the addition of intraperitoneal ganciclovir.

Results

Ribonucleotide reductase levels in colon carcinoma cell lines are much higher than in primary cultures of human hepatocytes. hrR3 efficiently destroys colon carcinoma cell lines *in vitro.* A single intratumoral injection of hrR3 into HT29 flank tumors significantly reduces tumor growth rate, and the administration of ganciclovir has no additive effect.

Conclusions

The inherent cytotoxicity of hrR3 replication effectively destroys colon carcinoma cells *in vitro* and *in vivo.* This cytotoxicity is not enhanced *in vivo* by the addition of ganciclovir. In the future, more efficacious and selective HSV 1 vectors may be useful in the treatment of cancer.

Remarkable advances in molecular biology during the past 2 decades have led to the development of novel cancer treatment strategies, and cancer gene therapy represents one such strategy. Numerous approaches to cancer gene therapy have been developed. Researchers have delivered into tumors genes for enzymes that convert inert prodrugs into cytotoxic agents, genes for cytokines or tumor antigens to stimulate an immune response against tumors, tumor-suppressor genes, and genes encoding RNA or DNA sequences that inhibit oncogene expression. Another approach has been to deliver genes not into tumor cells but into immune system cells to promote an antitumor immune response or into bone marrow cells to promote resistance to chemotherapeutic agents.[1–3]

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Most vectors used to introduce foreign genes into cells are modified forms of viruses, with the most common viral vectors derived from retroviruses and adenoviruses.[4] In contrast, we and others have focused our efforts on vectors derived from herpes simplex virus type 1 (HSV 1). Herpes simplex virus type 1 vectors have many advantages for use in cancer gene therapy. Like adenoviral vectors, HSV 1 vectors can be made in high titers and infect a wide range of dividing and nondividing cells. Similar to retroviral vectors, HSV 1 vectors also can be mutated to infect only dividing cells. Herpes simplex virus type 1 also contains the largest foreign gene capacity of all currently used viral vectors, allowing several therapeutic genes to be inserted.[5]

Most retroviral and adenoviral vectors contain a deletion in an essential gene (i.e., a gene required for viral replication) and are therefore unable to replicate in cells. Rather than destroying tumor cells by viral replication, these vectors rely on the delivery of therapeutic genes to destroy tumor cells.[3] In contrast, we and others have explored the use of HSV 1 vectors that are capable of replication (i.e., replication-competent vectors) for cancer gene therapy. One significant advantage of replication-competent HSV 1 vectors is that their replication in tumor cells results in cell lysis.[5] The HSV 1 genome also possesses one of the most commonly used therapeutic genes, HSV-thymidine kinase. Herpes simplex virus-thymidine kinase phosphorylates the prodrug ganciclovir, which is then converted into a toxic metabolite that destroys virus-infected cells.[6] Therefore, there are two mechanisms by which HSV 1 vectors can destroy tumor cells, viral replication and HSV-thymidine kinase conversion of ganciclovir. Although the addition of ganciclovir enhances viral vector cytotoxicity in some model systems, ganciclovir does not enhance the cytotoxicity of replication-competent HSV 1 vectors in certain colon carcinoma cell lines.[7]

Herpes simplex virus type 1 vectors for cancer gene therapy must be selective for cancer cells. We have centered our investigations around the HSV 1 vector hrR3. hrR3 has the *Escherichia coli* lacZ gene inserted into the viral ribonucleotide reductase gene locus so that the lacZ gene is translated in frame with the amino-terminal portion of ribonucleotide reductase. This insertional mutation essentially deletes hrR3's ribonucleotide reductase expression and adds lacZ expression. Actively dividing cells express high levels of endogenous ribonucleotide reductase for synthesis of DNA precursors. If hrR3 infects an actively dividing cell, viral replication can proceed because the endogenous ribonucleotide reductase complements the absence of viral ribonucleotide reductase. In addition, hrR3-infected cells can be detected by histochemical staining for lacZ expression.[8]

We have been interested in the use of HSV 1 vectors to treat liver metastases from colorectal cancer. Of the approximately 150,000 persons in whom colorectal cancer will develop this year, as many as 25% will have synchronous hepatic metastases and up to 50% will develop metachronous hepatic metastases.[9] Currently, available treatments for patients with unresectable liver metastases offer essentially no hope for a cure.[10] Our preliminary investigations focused on methods to selectively infect diffuse liver metastases with HSV 1 vectors. We previously reported that endogenous ribonucleotide reductase expression in human colorectal carcinoma liver metastases is much higher than in adjacent normal liver and exploited this difference in ribonucleotide reductase expression to target diffuse experimental liver metastases with hrR3. We demonstrated that hrR3 injected into the portal system of nude mice bearing experimental liver metastases selectively replicates in the liver metastases as opposed to the adjacent normal liver.[11]

In the current study, we assessed the efficacy of hrR3 in destroying colon carcinoma cells *in vitro* and *in vivo*. We observed that hrR3 effectively destroys several colon carcinoma cell lines *in vitro* and significantly inhibits growth of the colon carcinoma cell line HT29 in the flanks of nude mice. Systemic administration of ganciclovir did not enhance the antitumor effect of hrR3 *in vivo*.

MATERIALS AND METHODS

Cell Lines and Antibodies

The human colon carcinoma cell lines HT29 and LoVo and the African Green Monkey kidney cell line Vero were obtained from the American Type Culture Collection (Rockville, MD). The human colon carcinoma cell lines SW620 and NCIH508 were provided by Dr. Lee Ellis (M.D. Anderson Cancer Center, Houston, TX). The human colon carcinoma cell line WiDr was provided by Dr. Rei Takahashi (Osaka University, Osaka, Japan). The human colon carcinoma cell line KM12L4 was provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center). The mouse colon carcinoma cell line MC26 was obtained from the National Cancer Institute Tumor Repository (Frederick, MD). All cell lines were maintained in Dulbecco's modified Eagle's medium with 1:1 Hamm's F-12 supplement (DMEM/F-12) and 8% (v/v) fetal calf serum and grown at 37°C in a humidified incubator with 5% carbon dioxide.

The monoclonal antibody MAB3033 (Chemicon International, Temecula, CA) is specific for the M1 subunit of human and mouse ribonucleotide reductase. A-5442 (Sigma Chemical, St. Louis, MO) is a monoclonal antibody specific for β-actin and is conjugated to horseradish peroxidase.

Viral Vector Preparation

The HSV 1 vector hrR3 was provided by Dr. Sandra Weller (Connecticut Medical School, New Haven, CT). hrR3 was grown in Vero cells and titered as previously described.[12] Briefly, confluent Vero cells were infected with hrR3 at a multiplicity of infection (MOI) (i.e., number of plaque-forming units per cell) of two. One to 2 hours after infection, DMEM/F-12, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin were added to the

infected Vero cells. After 24 to 48 hours, cells and supernatant were harvested, exposed to three freeze—thaw cycles, and centrifuged at 800 × g for 10 minutes. The supernatant was used at various dilutions to infect confluent Vero cells. One to 2 hours after infection, 50% DMEM/F-12, 4% fetal calf serum, and 0.75% agarose were added to the infected Vero cells. Plaques were counted 1 week later, and titer was determined. The titered supernatant was centrifuged at 25,000 × g for 1 hour, resuspended in DMEM at a concentration of 10^{10} plaque-forming units per ml and stored at −80°C.

Primary Hepatocyte Culture

Human hepatocytes were isolated with perfusates I and II as previously described[13] with minor modifications. Briefly, fresh liver specimens from the operating room were perfused with perfusate I followed by perfusate II at 40°C. The digested specimens were gently agitated in perfusate II to release the dissociated hepatocytes, filtered through four layers of gauze, and centrifuged at 50 × g for 5 minutes. Dissociated hepatocytes were washed three times in perfusate II, counted on a hemacytometer, and resuspended in William's medium E containing 10% bovine serum albumin, insulin, transferrin, selenium, trace elements, dexamethasone, linoleic acid, linolenic acid, glucagon, nicotinamide, penicillin, streptomycin, and fungizone at a density of 3×10^6 cells per 100 mm collagen-coated plate. One hundred millimeter plates were coated overnight with 5 $\mu g/cm^2$ rat tail collagen (Collaborative Biomedical Products, Bedford, MA). Hepatocytes were incubated at 37°C, and the medium was changed 2 hours after plating, the next morning, and every 2 days subsequently.

Western Blot Analysis

Cells were harvested with 0.05% trypsin and 0.53 mM EDTA in phosphate-buffered saline (PBS), washed once with PBS, resuspended in lysis buffer (1% Triton X-100, 50 mM Tris [pH = 8.0], 150 mM sodium chloride 0.2% sodium azide, 0.1% mammalian protease inhibitor cocktail [Sigma Chemical]) at a concentration of 10^8 cells/ml, and incubated on ice for 30 minutes. Total protein concentrations were measured using the bicinchoninic acid Protein Assay following manufacturer's instructions (Pierce Chemical, Rockford, IL). Lysates containing equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions and electrotransferred onto nitrocellulose filters. Filters were blocked with 5% milk in PBS overnight at 4°C, washed briefly with 1% milk containing 0.1% Tween-20 in PBS, incubated with 50 μg/ml MAB3033 for 1 hour, washed three times with 1% milk containing 0.1% Tween-20 in PBS, incubated with A-5442 diluted 1:4000 for 1 hour, and washed three times with 150 mM sodium chloride, 10 mM Tris (pH = 8.0), and 0.05% Tween-20. Specific proteins were detected using an enhanced chemiluminescence system following manufacturer's instructions (Amersham Life Science, Arlington Heights, IL).

In Vitro Cell Infection Assay

Viral cytotoxicity was determined as previously described.[11] Briefly, 5000 cells per well were plated onto 96-well plates and grown for 36 hours. The medium was replaced with 50 μl DMEM/F-12 containing hrR3 at MOI ranging from 0.0001 to 1.0. Cells were incubated at 37°C for 45 minutes with gentle shaking every 15 minutes. Fifty microliters DMEM/F-12 with 16% fetal calf serum, 200 U/ml penicillin, and 200 mg/ml streptomycin were then added, and cells were incubated at 37°C. After 6 days, the medium was replaced with 0.5 mg/ml thiazolyl blue (MTT, Sigma Chemical) in RPMI 1640 without phenol red, and cells were incubated for 2 hours at 37°C to allow formazan crystals to form. The medium was replaced with 50 μl dimethylsulfoxide, and the plates were shaken vigorously. The optical density (OD) of each well was measured using an automatic plate reader (Anthos HTS, Anthos Labtes Instruments, Salzburg, Austria) at a wavelength of 550 nm with a reference wavelength of 650 nm. Percentage cell survival was determined by dividing the $OD_{550/650}$ of hrR3-infected cells with the $OD_{550/650}$ of mock-infected cells. All experiments were performed in quadruplicate.

In Vivo Studies

Animal studies were performed in accordance with the guidelines issued by the Massachusetts General Hospital Subcommittee on Research Animal Care. Before viral inoculation, mice were kept in a barrier facility. Viral inoculations and care of mice harboring virus were performed in approved viral vector rooms. Pathogen-free adult male athymic BALB/c nude mice weighing 15 g to 20 g were allowed to acclimate for at least 1 week. HT29 cells were detached from plates in 0.05% trypsin and 0.53 mM EDTA in PBS and resuspended in Hank's balanced salt solution (HBSS) free of calcium and magnesium to create a single-cell suspension. Mice were given the inhalational anesthetic metofane (Mallinckrodt Veterinary, Mundelein, IL), and 5×10^6 cells in 100 μl HBSS were injected subcutaneously into the right flank. Mice were randomized 10 days later to receive either 1×10^8 plaque-forming units hrR3 in 50 μl HBSS or 50 μl HBSS alone injected into the center of the tumors. Seven days later, mice were again randomized to receive daily intraperitoneal injections of either 50 mg/kg ganciclovir in 100 μl PBS or 100 μl PBS alone for 7 consecutive days. Tumors were measured beginning 10 days after tumor cell injection and subsequently every 3 or 4 days.

Tumor mass was calculated by the following formula:

$$\text{tumor mass} = \frac{(\text{length} \times \text{width})^2}{2}$$

Percentage tumor growth was calculated on posttumor injection day (PTD) X by the following formula:

$$\text{percentage tumor growth} = \frac{\text{mass on PTD X}}{\text{mass on PTD 10}}$$

Twelve mice were used in each of the four groups.

Liver Sections and Histochemical Staining

Additional mice were subjected to the same protocol of tumor inoculation in the flank followed by intratumoral hrR3 injection, except that intratumoral hrR3 injection was performed on PTD 7 and repeated on PTD 10 and PTD 13. On PTD 14 and 54, the mice were killed, and tumor, liver, and brain specimens were obtained. Specifically, metofane inhalational anesthesia was administered, the inferior vena cava was lacerated, and PBS was administered by intracardiac injection followed by 3% paraformaldehyde in PBS. Tumor and liver specimens were dissected, soaked in 3% paraformaldehyde for 1 to 2 hours, washed with PBS, soaked in 30% sucrose for 24 to 48 hours, and snap-frozen in OCT Compound (Miles, Elkhart, IN) in liquid nitrogen. Brain specimens were treated similarly except that they were fixed in 3% paraformaldehyde for 24 hours. Five μM sections were cut on a cryostat and placed onto slides. Slides were washed with PBS, incubated for 24 to 48 hours in a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, Sigma Chemical Co.), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS, washed again with PBS, and counter-stained with cresyl violet.

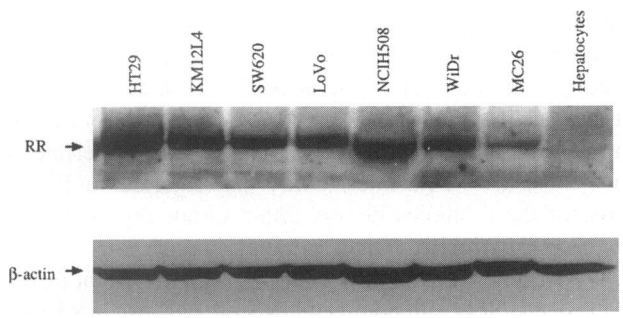

Figure 1. Ribonucleotide reductase expression in colon carcinoma cell lines and hepatocytes. Western blot analysis was performed for expression of ribonucleotide reductase (RR) and β-actin in colon carcinoma cell lines and primary cultures of human hepatocytes.

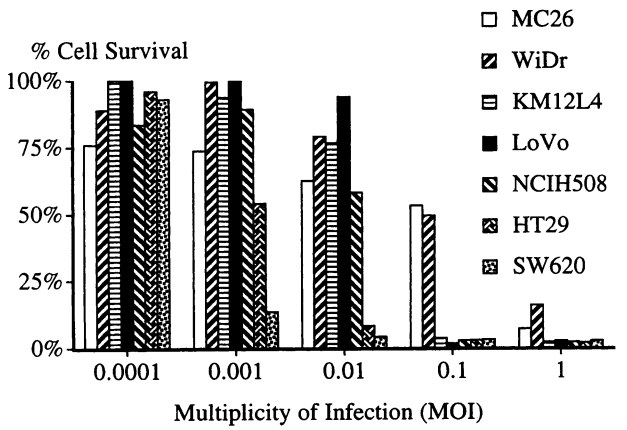

Figure 2. hrR3-mediated cytotoxicity *in vitro*. Colon carcinoma cell lines were plated in 96 well plates and infected with hrR3 at various multiplicities of infection. Six days after infection, the number of surviving cells was quantitated by MTT assay (see Methods section). Percentage cell survival was determined compared with mock-infected cells.

RESULTS

Ribonucleotide Reductase Expression in Human and Mouse Colon Carcinoma Cell Lines and Primary Human Hepatocytes

We examined ribonucleotide reductase expression in colon carcinoma cell lines and normal human hepatocytes by Western blot analysis. All six human colon carcinoma cell lines and one mouse colon carcinoma cell line expressed significantly higher levels of cellular ribonucleotide reductase than did primary cultures of human hepatocytes (Fig. 1). Ribonucleotide reductase was undetectable in primary cultures of human hepatocytes by Western blot analysis. Samples also were probed for β-actin to ensure equal protein loading between samples. These data support the feasibility of selectively targeting diffuse liver metastases from colorectal carcinoma with hrR3.

Herpes Simplex Virus 1 Vector hrR3-mediated Lysis of Colon Carcinoma Cell Lines and Primary Human Hepatocytes *In Vitro*

We have previously shown that hrR3 effectively destroys the colon carcinoma cell lines HT29, KM12L4, and KM12C6 *in vitro*.[7] To examine hrR3 efficacy in additional cell lines, hrR3 cytotoxicity was tested in human and mouse colon carcinoma cell lines using MOI ranging from 0.0001 to 1.0. As shown in Figure 2, hrR3 effectively destroyed most of the colon carcinoma cell lines at a MOI of less than 1.0. SW620 cells were nearly completely destroyed using a MOI as low as 0.001. The mouse colon carcinoma cell line MC26 and the human colon carcinoma cell line WiDr were

the most resistant cell lines. Nonetheless, both of these cell lines were nearly completely destroyed at a MOI of 1.0. These data suggest that hrR3 is effective against a wide variety of colon carcinoma cell lines, but that there is some heterogeneity between cell lines in their susceptibility to hrR3.

Herpes Simplex Virus 1 Vector hrR3-mediated Inhibition of HT29 Human Colon Carcinoma Cell Tumors *In Vivo*

Next, we determined the efficacy of hrR3 in destroying tumor cells *in vivo* with and without the systemic administration of ganciclovir. Given the difficulty in accurately measuring tumor growth at several time points in the liver, we chose to demonstrate *in vivo* destruction of colon carcinoma cells in nude mice bearing HT29 flank tumors. Five $\times$ 10^6 HT29 cells were injected subcutaneously into the flanks of nude mice. Ten days later, 1 X 10^8 plaque-forming units hrR3 or HBSS was injected in the center of the developing tumors. Seven days later, mice were given daily intraperitoneal injections of either ganciclovir or PBS for 7 days. Tumor size was measured every 3 to 4 days. Significant tumor growth inhibition was observed with a single intratumoral injection of hrR3 (Fig. 3A). By 28 days after HT29 injection, the percentage tumor growth of treated tumors was 857%, whereas the percentage tumor growth of untreated controls was 2033% ($p = 0.0033$). Neither of these two groups of mice received ganciclovir. The addition of intraperitoneal ganciclovir to mice treated with an intratumoral injection of hrR3 or HBSS had no effect on tumor growth (Fig. 3B). There was no observed toxicity in hrR3-treated mice during this period.

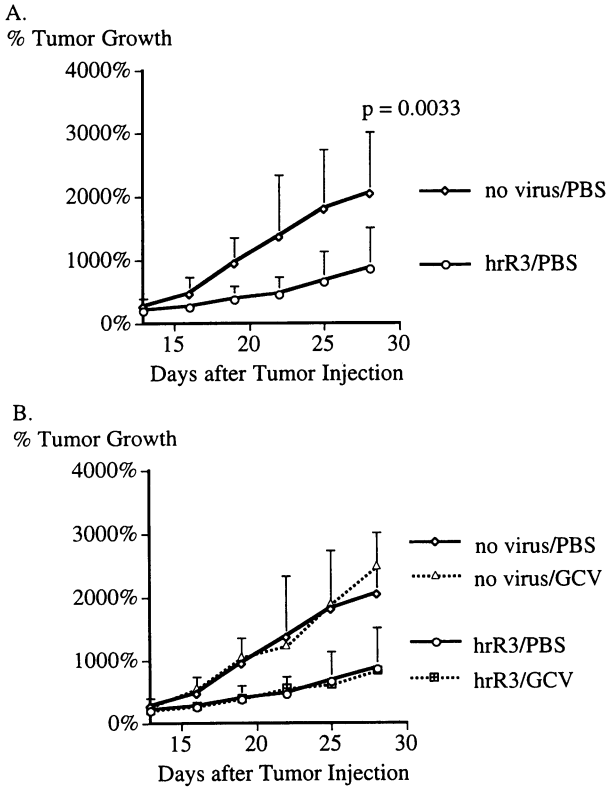

Figure 3. hrR3 inhibition of flank tumor growth. HT29 cells were injected in the flanks of nude mice. Ten days later, mice received an intratumoral injection of either hrR3 or saline (no virus). Seven days later, mice received daily intraperitoneal injections of either phosphate buffered saline (PBS) or ganciclovir (GCV) for 7 consecutive days. Percentage tumor growth was determined as compared with tumor size 10 days after initial tumor injection. Figure 3A demonstrates the tumor growth curves for mice receiving saline followed by intraperitoneal PBS (no virus/PBS) and mice receiving hrR3 followed by intraperitoneal PBS (hrR3/PBS). In Figure 3B, two tumor growth curves have been added to the curves from Figure 3A, the tumor growth curve for mice receiving saline followed by intraperitoneal GCV (no virus/GCV) and mice receiving hrR3 followed by intraperitoneal GCV (hrR3/GCV). Standard deviation bars are shown.

Histochemical Staining of Tumors

hrR3 possesses the *E. coli* lacZ gene, enabling detection of hrR3-infected cells by histochemical staining for lacZ expression. As seen in Figure 4A, tumors stained for lacZ expression 1 day after the last of three intratumoral injections of hrR3 show intense blue staining throughout much of the tumor. Grossly, more than 75% of the tumor sections stained positively for lacZ expression. At 41 days after the last intratumoral injection of hrR3, only small islands of blue-staining cells were observed, with one such island shown in Figure 4B. Grossly, less than 10% of the tumor sections stained positively for lacZ expression. Staining of brain and liver specimens from these mice revealed no lacZ expression (data not shown).

DISCUSSION

Several investigators have examined gene therapy as a strategy to treat liver metastases. Chen et al. described the treatment of liver metastases from colon carcinoma in a mouse model using adenoviral vectors to deliver either the HSV-thymidine kinase gene or interleukin-2 gene.[14] Caruso et al. repeated the study using a similar model with an adenoviral vector containing mouse interleukin-12.[15] Both of these studies involved direct intratumoral injection of the vector, a strategy that is not feasible for diffuse liver metastases. Hurford et al. delivered retroviral producer cells by intrasplenic injection in mice to transduce experimental liver metastases with interleukin-4 and interleukin-2.[16] All three of these studies demonstrated tumor regression and increased survival in treated animals.

We are interested in using HSV 1 vectors to treat diffuse liver metastases from colorectal cancer. Because hrR3 lacks a functional ribonucleotide reductase gene, it preferentially replicates in cells with high levels of endogenous ribonucleotide reductase. In this study, we demonstrated that the

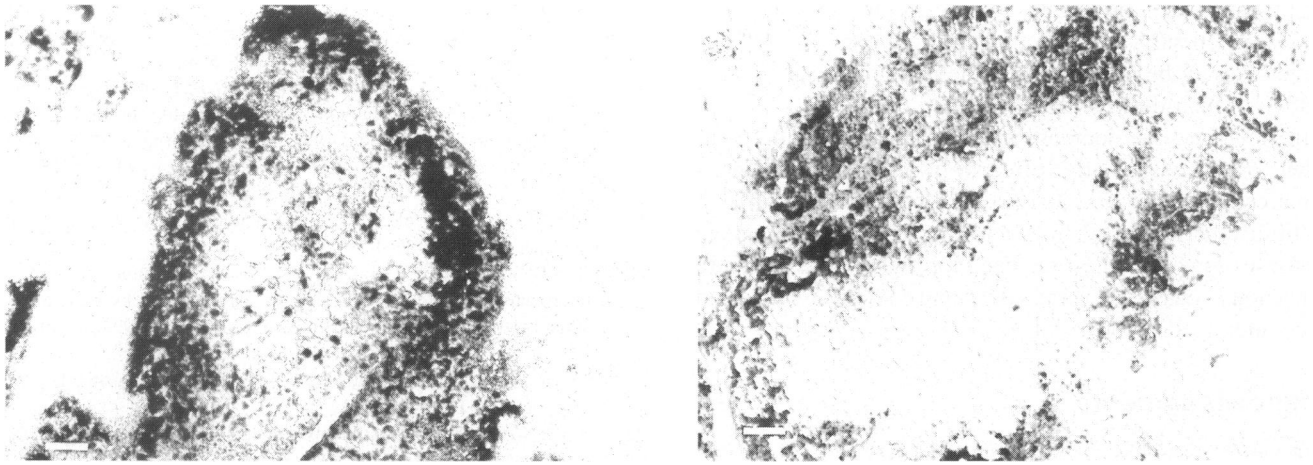

Figure 4. LacZ expression in HT29 flank tumors after hrR3 inoculation. HT29 cells were injected in the flanks of nude mice. On posttumor injection day (PTD) 7, 10, and 13, mice received intratumoral injections of hrR3. Mice were sacrificed on PTD 14 and 54, and tumor sections were stained for lacZ expression. Tumor sections from mice sacrificed on PTD 14 (Fig. 4A) and PTD 54 (Fig. 4B) are shown. Blue-staining cells are positive for lacZ expression. Bar = 100 μM.

expression of ribonucleotide reductase is much higher in colon carcinoma cell lines than in primary cultures of human hepatocytes. This is consistent with our previous finding that human colon carcinoma liver metastases in surgical specimens have higher ribonucleotide reductase expression than adjacent normal liver.[11]

Using an *in vitro* cytotoxicity assay, we showed that hrR3 efficiently destroys a wide variety of colon carcinoma cell lines, with some heterogeneity in susceptibility to hrR3-mediated cytotoxicity between cell lines. This variability could be due to differences between cell lines in viral adsorption and entry, viral gene expression, or viral protein translation. Susceptibility to HSV 1 replication does not appear to be related to the host cell's expression of either wild-type or mutant p53 (manuscript in preparation). It is not surprising that hrR3 replicates in the mouse colon carcinoma cell line MC26 less efficiently than in most human colon carcinoma cell lines given that hrR3 is derived from HSV 1, whose normal host is human cells.

We also demonstrated that hrR3 injection into HT29 flank tumors in nude mice significantly reduces tumor growth rate. Staining of flank tumors for lacZ expression revealed that hrR3 initially infects tumor cells diffusely and over the course of several weeks persistently infected a minority of surviving tumor cells. Hematoxylin and eosin staining of tumors after hrR3 injection did not show a significant number of inflammatory cells so the reduction of tumor growth rate is unlikely due to a nonspecific inflammatory response (data not shown).

We did not observe any complete responses after a single injection of hrR3. Methods to enhance the antitumor effect of hrR3 include multiple injections of hrR3, rather than a single injection, and the addition of therapeutic genes to the HSV 1 vector. The HSV 1 genome already encodes for one commonly used therapeutic gene, HSV-thymidine kinase, and the administration of ganciclovir can enhance virus-mediated killing of noninfected adjacent tumor cells by the "bystander effect."[17] However, we previously have shown that this bystander effect does not occur *in vitro* in some colon carcinoma cell lines including HT29, even at high ganciclovir concentrations.[7] Consistent with this previous report, we observed in the present study that the addition of ganciclovir does not enhance HT29 tumor destruction *in vivo*. The maximum nontoxic dose of ganciclovir (50 mg/kg) was administered.[18] Possible explanations for the lack of bystander effect in our animal model include low levels of gap junctional communication between HT29 cells, lack of specific apoptosis pathways in HT29 cells, and deficient immunologic response to tumors in nude mice.[19]

Other therapeutic genes that could be inserted in HSV 1 vectors to increase their antitumor activity include genes for other prodrug-converting enzymes such as cytosine deaminase,[20] genes to stimulate the immune system's antitumor response,[21] or genes to prevent tumor angiogenesis.[22]

We chose to examine the efficacy of hrR3 against a human colon carcinoma cell line growing on the flanks of nude mice so that tumor size could easily be assessed. The efficacy of hrR3 in experimental liver metastases currently is being evaluated.

Because hrR3 preferentially replicates in dividing cells, there still remains the possibility that hrR3 can replicate in normally dividing nontumor cells such as intestinal epithelial cells and bone marrow cells. It also is conceivable that hrR3 may replicate, albeit inefficiently, in nondividing cells. The degree to which hrR3 replicates in nontumor cells currently is under investigation, because this relates directly to anticipated toxicity of treatment with hrR3. We did not detect lacZ expression in brain and liver specimens in mice inoculated with hrR3, although histochemical staining is not the most sensitive method of detection.[23] In addition, we did

not examine sections of intestinal epithelium or bone marrow. Theoretically, any infection of nontumor cells by HSV 1 vectors may be attenuated by the administration of ganciclovir, which destroys HSV-infected cells.[6]

In summary, we demonstrated that the replication-competent HSV 1 vector hrR3 effectively destroys colon carcinoma cells *in vitro* and *in vivo*. The heterogeneity in susceptibility between cell lines and the absence of complete responses *in vivo* suggest the need for higher doses or more efficacious vectors. Several safety and toxicity issues need to be addressed before clinical trials.

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Discussion

DR. TIMOTHY J. EBERLEIN (Boston, Massachusetts): Metastatic disease from colorectal cancer, especially to the liver, is a major source of mortality. Chemotherapy is not effective, and therefore a new systemic type of therapy is required. This study attempts to address the problem by identifying a new treatment utilizing gene therapy. This study is a specific follow-up to Dr. Tanabe's earlier observations that colorectal carcinoma liver metastases have a much higher level of expression of ribonucleotide reductase compared to relatively nondividing human hepatocytes.

In these experiments the authors utilize a herpes simplex virus 1 vector deficient of ribonucleotide reductase. Since the vector lacks a functional ribonucleotide reductase gene, it preferentially will replicate in cells with high levels of endogenous ribonucleotide reductase. In this set of experiments Dr. Tanabe has shown reduction in tumor growth rate utilizing direct tumor injection. There appeared to be relatively little toxicity. However, this line of experiments raises several questions.

First, would a different route of injection, for example intravenous, reduce anti-tumor efficacy yet enhance toxicity, especially to gut epithelial cells and bone marrow cells, since they would very likely be affected by this vector?

Along the same line, most humans will possess antibodies to HSV 1 and therefore unless the vector is substantially changed will host immune response attenuate the efficacy of this vector in humans?

There appear to be a variability and susceptibility with this vector between different colon cancer cell lines. Do you have an explanation? Is this related to the particular cell line or the degree of differentiation of tumor, or perhaps the level of ribonucleotide reductase in the tumor?

In previous experiments as well as the experiments just provided, this vector therapy caused significantly reduced tumor growth and destruction of the tumor cells *in vitro*. Is this mechanism of destruction known? Specifically, how does this relate to the observation and the set of experiments that treatment with ganciclovir does not enhance reduction of tumor growth rate even in the control group? Is this a function of the tumor line chosen, the