Oncolysis of Diffuse Hepatocellular Carcinoma by Intravascular Administration of a Replication-competent, Genetically Engineered Herpesvirus

Timothy M. Pawlik, Hideo Nakamura, Sam S. Yoon, John T. Mullen, Soundararajalu Chandrasekhar, E. Antonio Chiocca, and Kenneth K. Tanabe

Division of Surgical Oncology, Department of Surgery [T. M. P., H. N., S. S. Y., J. T. M., S. C., K. K. T.] and Neurosurgery Service [E. A. C.], Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Abstract

Herpes simplex virus type 1 (HSV-1) replication within tumors can mediate tumor regression (oncolysis). The genetically engineered, HSV-1 mutant rRp450 does not express viral ribonucleotide reductase and is therefore replication conditional. During the course of infection, rRp450 expresses the cytochrome P450 transgene and HSV-1 thymidine kinase gene, thereby enabling it to bioactivate the prodrugs cyclophosphamide and ganciclovir, respectively. rRp450 replication in hepatocellular carcinoma (HCC) cells is cytotoxic and liberates progeny virion that infect adjacent tumor cells. rRp450-mediated oncolysis is enhanced in the presence of cyclophosphamide, whereas it is inhibited in the presence of ganciclovir. As a consequence of defective viral ribonucleotide reductase expression, the yield of rRp450 progeny virions from infection of HCC cells is 3 to 4 log orders greater than that from infection of normal hepatocytes. This is associated with dramatic tumor reduction of diffuse HCC after a single intravascular administration of rRp450. rRp450 holds the promise of the dual therapeutic benefit of selective oncolysis and P450 transgene delivery.

Introduction

HCC is the most common solid malignancy worldwide, with an estimated annual incidence of greater than 1 million new cases per year. The overwhelming majority of patients are not candidates for liver resection, liver transplantation, or percutaneous ethanol injection because of the presence of multifocal disease, inadequate hepatocellular reserve, or tumor involvement of major vascular structures. Despite new insights into the molecular pathogenesis of HCC and improvements in surgery, chemotherapy, and radiation therapy, the median survival of patients with HCC remains dismal.

Gene therapy has been viewed as a new tool that may improve the therapeutic efficacy of currently existing treatments. The overwhelming majority of cancer gene therapy strategies reported to date use replication-defective viruses to serve primarily as vehicles for transgene delivery (4). Numerous cancer gene therapy strategies using these viruses have been explored, including expression of suicide genes, tumor suppressor genes, and genes that influence immune functions (5–8). Several strategies have been developed to render viruses for gene delivery incapable of replication to minimize the risk of viral toxicity or cellular transformation (9). However, viral replication in tumor cells is cytopathic. Accordingly, the paradigm of permitting viral replication to mediate tumor destruction has been explored using adenovirus (10), HSV-1 (11), reovirus (12), and Newcastle disease virus (13). Exploiting the oncolytic effects of HSV-1 replication, Martuza et al. (11) demonstrated that HSV-1 defective in expression of thymidine kinase preferentially replicated in brain tumors. Other oncolytic HSV-1 mutants have been characterized that are defective in expression of viral ribonucleotide reductase (14), γ34.5 (15), or uracyl N-glycosylate (16), and these viruses preferentially replicate in dividing cells rather than quiescent cells.

It is critically important for any gene therapy strategy developed for HCC to be effective against multifocal, diffuse disease. The overwhelming majority of research examining viral vector administration into solid tumors has been performed using direct intratumoral inoculation (4). This mode of administration would not be expected to be effective against either primary or secondary liver tumors, which are commonly multiple, multicentric nodules that are below the limits of radiographic image resolution. Intravascular delivery with specific targeting to divert hepatic malignancies remains an important challenge to meet.

The genetically engineered, replication-conditional HSV-1 mutant rRp450 is constructed such that it is defective in expression of the large subunit of viral ribonucleotide reductase (17). We have tested the hypothesis that the absence of viral ribonucleotide reductase in rRp450 permits selective replication within and destruction of diffuse HCC liver tumors after intravascular administration. During viral replication, rRp450 also expresses the rat cytochrome P450 2B1 (CYP2B1) transgene, which encodes an enzyme responsible for bioactivation of prodrugs, such as cyclophosphamide, into their active cytotoxic metabolites, thus providing a means for intratumoral generation of alkylating metabolites (18). We have examined the ability of rRp450 to selectively destroy diffuse HCC, and we have also examined the effect of rRp450 bioactivation of cyclophosphamide on viral replication.

Materials and Methods

Chemicals, Cell Lines, and Viruses. Cell lines derived from human HCC (Hep-3B, Hep-G2, and SK-Hep), mouse HCC (Hep 1-6), rat HCC (McA Rh7777), and the LoVo African Green Monkey kidney (Vero) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM, 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary human hepatocytes were prepared from fresh human liver specimens from the operating room as described previously (19, 20). Primary cultures of mouse and rat hepatocytes were prepared in a similar manner from BALB/c mice and Buffalo rats. Primary hepatocytes were maintained in William’s Medium E containing BSA, insulin, transferrin, selenium, trace elements, dexamethasone, linoleic acid, linolenic acid, glucagon, penicillin, streptomycin, and fungizone on rat tail collagen-coated plates.

The HSV-1 vector rRp450 was constructed as described previously (17). Wild-type HSV-1 strain KOS (kindly provided by Donald Coen, Harvard Medical School, Boston, MA) and rRp450 were propagated and titered on Vero cell cultures as described previously (21). Briefly, confluent Vero cells
were either mock infected or infected with rRp450 using an MOI of 0.005. Media were added to the infected Vero cells 1–2 h after infection. After 72 h, cells and supernatants were harvested, exposed to three freeze/thaw cycles, and centrifuged at 800 g for 10 min. Supernatants were used at various dilutions to infect Vero cell monolayers. Media containing 1% carboxymethylcellulose were added to the infected Vero cells 1–2 h after infection. Plaques were counted 5 days later to determine titers. Titered supernatants were centrifuged at 25,000 g for 1 h, resuspended in DMEM at a concentration of 10^3 pfu/ml, and stored at –80°C.

In Vitro Viral Cytotoxicity and Replication Assays. Viral cytotoxicity was determined as described previously (22). Briefly, 5000 cells/well were plated onto 96-well plates and grown for 36 h. Cells were then infected with either KOS or rRp450 using MOI values ranging from 0.0001 to 100. After 6 days, the number of viable cells was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 6 days later and divided by the number of viable cells treated with mock-infected media. All experiments were performed in quadruplicate.

Viral replication assays were performed by infecting 1 × 10^6 cells with 2 × 10^6 pfu of HSV-1 for 2 h, at which time unadsorbed virus was removed by washing with a glycine-saline solution (pH 3.0). At 48 h after infection, the supernatant and cells were exposed to three freeze/thaw cycles to release virions and titered on Vero cells. For in vitro prodrug bioactivation experiments, 500 μl cyclophosphamide (Sigma Chemical Co., St. Louis, MO) or 50 μg/ml ganciclovir (Syntex Corp., Palo Alto, CA) was added at the start of the incubation period.

Animal Studies. Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Research Animal Care. Buffalo rats weighing 250–300 g (Charles River Laboratories, Wilmington, MA) were anesthetized by i.p. administration of 75 mg/kg ketamine and 1 mg/kg xylazine (Henry Schein, Port Washington, NY). After exposure of the abdominal cavity, a single cell suspension of 8 × 10^6 McA RH7777 cells in 1 ml of PBS was injected into the portal vein over a 1-min period. Animals were treated 5 days later with either 1 × 10^6 pfu of rRp450 or with mock-infected control media by laparotomy and administration into the portal vein. At day 14, animals were killed and livers were harvested. Specimens were soaked in 4% paraformaldehyde in PBS for 24 h, washed with PBS, and then soaked for an additional 48 h in a 30% sucrose solution. Livers were subsequently weighed and photographed.

Results

Oncolytic of HCC by rRp450. We examined the oncolytic effects associated with rRp450 infection of several HCC cell lines. rRp450 was added in increasing MOI values to a human, rat, and mouse HCC cells. rRp450 effectively destroyed the Hep-3B and Hep-G2 human HCC cell lines at an MOI of 0.1, whereas the SK-Hep cell line required a higher MOI of 1–10 for effective tumor destruction (Fig. 1). As expected, the mouse and rat cell lines (Hep-1-6 and McA RH7777, respectively) were less susceptible to the cytopathic effects of rRp450 infection as compared with the Hep-3B and Hep-G2 human cell lines. HSV-1 displays greater tropism for human and primate cells than for mouse and rat cells, and HSV-1 infection, replication, and subsequent cell lysis in the rodent system are known to be less than in the human system. Nonetheless, both the mouse and rat cell lines tested were nearly completely destroyed at an MOI of 10.0. The cytopathic effect of rRp450 was similar to that of its parent (wild-type) KOS virus (data not shown).

Cyclophosphamide Potentiates the Cytotoxic Effects of rRp450 in Vitro. rRp450 contains the transgene encoding rat cytochrome P450 2B1 (CYP2B1), which bioactivates the prodrug cyclophosphamide (17). As a vector derived from HSV-1, rRp450 also expresses viral thymidine kinase, which serves as a suicide gene by virtue of its ability to phosphorylate ganciclovir. rRp450 was engineered to destroy tumors primarily by viral replication. Although this mechanism of tumor destruction can theoretically be enhanced by the addition of either cyclophosphamide or ganciclovir, addition of either of these produgs could also theoretically reduce the extent of cell killing by reducing rRp450 replication.

We examined the effects of ganciclovir and cyclophosphamide on rRp450-infected HCC cells by comparing rRp450-mediated cytotoxicity in the presence and absence of each of the produgs. To observe the effects of prodrug treatment, we infected each HCC cell line with rRp450 using an MOI that produced approximately 50% cell destruction for that particular cell line. Infected cells were then incubated for 6 days in media containing cyclophosphamide (500 μM), media containing ganciclovir (50 μM), or media alone. We have previously demonstrated that cells infected with rRp450 efficiently bioactivate cyclophosphamide (17, 23). Cyclophosphamide alone had a negligible effect on cell survival, which is consistent with the known need for cyclophosphamide to be converted to active metabolites for it to exert its cytotoxic effects (Fig. 2A). In contrast, when cyclophosphamide was added to rRp450-infected cells, the increase in cell destruction beyond that observed with rRp450 infection alone ranged from 23–31%. Destruction of rRp450-infected HCC cells in the presence of cyclophosphamide was nearly complete. Ganciclovir alone had a negligible effect on HCC cells, which is consistent with the known need for ganciclovir to be phosphorylated for it to exert its cytotoxic effects. However, in contrast to cyclophosphamide, when ganciclovir was added to cells infected with rRp450, cytotoxicity was reduced compared to that seen in cells infected with rRp450 alone.

These results indicate that cyclophosphamide and ganciclovir differ substantially in their ability to potentiate rRp450-mediated oncolysis. We presumed that this difference was caused by a reduction in rRp450 replication in the presence of ganciclovir. HSV-1 thymidine kinase phosphorylates ganciclovir, thereby initiating its bioactivation into a metabolite that inhibits both viral and cellular DNA synthesis (24). To examine this hypothesis, we compared rRp450 replication in Hep-G2...
HCC cells in the presence of either cyclophosphamide or ganciclovir. The presence of cyclophosphamide did not inhibit rRp450 replication, whereas the presence of ganciclovir reduced rRp450 replication by 3 log orders (Fig. 2B). This notable difference between the effects of cyclophosphamide and ganciclovir on rRp450 replication may explain in part their different effects on rRp450-induced oncolysis of HCC.

Comparison of Selective rRp450 Replication in HCC and Hepatocytes. Because rRp450 is engineered to be defective in its expression of ICP6, its replication is significantly attenuated. ICP6 serves as the large subunit of viral ribonucleotide reductase. We presumed that this virus would replicate more robustly in cells with high levels of cellular ribonucleotide reductase and correspondingly high levels of intracellular deoxyribonucleotides. HCC expresses significantly higher levels of ribonucleotide reductase compared to surrounding normal liver (25), and we therefore reasoned that rRp450 replication would proceed more robustly in HCC cells than in hepatocytes. To test this hypothesis, we compared replication of rRp450 with that of KOS (wild-type HSV-1) in primary cultures of human hepatocytes and in Hep-G2 HCC. rRp450 replication in human HCC was nearly as robust as that of the KOS wild-type strain (Fig. 3A). Of note, rRp450 replication was 3–4 log orders lower in human hepatocytes than in HCC cells, whereas KOS replication was not nearly as attenuated in the hepatocytes. We performed a similar analysis in both a mouse model and a rat model, comparing the replication of both viruses in primary cultures of mouse hepatocytes versus Hep 1-6 as well as primary cultures of rat hepatocytes versus McA RH7777 (Fig. 3, B and C). As was observed in the human tissue system, rRp450 replication was similar to that of KOS in the HCC cell lines but was 3 log orders lower than that of KOS in both primary mouse and rat hepatocytes. As expected, the overall efficiency of rRp450 replication was less in the two rodent systems than in the human system. Nonetheless, the magnitude of the difference between rRp450 replication in hepatocytes versus HCC was similar in the human, mouse, and rat models. Most importantly, these results support the feasibility of treating diffuse liver tumors by injection of rRp450 into the portal blood stream because of the preferential replication of rRp450 in HCC rather than normal hepatocytes.

rRp450 Effectively Treats Diffuse HCC in Vivo. To examine the oncolytic efficacy of rRp450 in vivo, we used a well-described model of experimental HCC involving tumor cell inoculation into the portal vein (26). Diffuse HCC was established in syngeneic Buffalo rats as described, and the rats were treated with a single intraportal inoculation of mock-infected media or $1 \times 10^8$ pfu rRp450 five days later. Rats were sacrificed 14 days after initial tumor cell implantation. At that time, all of the control rats treated with mock-infected media had distended abdomen with bloody ascites, and some were moribund. In contrast, the rRp450-treated animals all appeared healthy. Tumor nodules in the mock-infected group were innumerable, whereas the number of nodules in the rRp450 group was less than 10 nodules/animal (Fig. 4). The marked difference in liver tumor burden resulting from a single administration of virus also yielded a marked difference in liver tumor weights, with control livers weighing three times that of rRp450-treated animal livers ($33.56 \pm 6.67$ g versus $11.62 \pm 1.31$ g; $P < 0.05$).

Discussion

Replication-conditional viruses hold promise as potentially effective therapeutic agents. The principal antitumor activity of replication-conditional HSV-1 results from viral replication within tumor cells, resulting in cell destruction, as well as production of progeny virion that can directly infect adjacent tumor cells. Various strategies may be used to restrict viral replication to specific types of cells. One approach involves genetic engineering of the virus to knock out proteins...
that are critical for replication, thereby restricting viral replication to cells that can complement the missing viral proteins. The complementing functions that the cells provide would ideally be present only in neoplastic cells and not in normal cells. A significant difference between normal liver and HCC that can be exploited by rRp450 is the level of mitotic activity. The absence of viral ribonucleotide reductase in rRp450 greatly attenuates its ability to establish a lytic infection unless it infects a mitotically active cell. However, there are several cell populations outside the liver that are actively dividing and would therefore also be susceptible to lytic infection by rRp450. Previous studies performed with rRp450’s parent virus, hrR3, which contains the lacZ marker gene, revealed no evidence of β-galactosidase staining in the brain or lungs of animals exposed to intraportal inoculations of this HSV-1 mutant (27). The 3–4 log order difference in rRp450 replication observed in human hepatocytes as compared with human HCC suggests the presence of a relatively large therapeutic window. The nearly complete first-pass clearance of HSV-1 by the liver after portal venous administration combined with this therapeutic window may be sufficient to avoid toxicity associated with unwanted rRp450 replication in nonneoplastic cells. Others have corroborated this inability of an ICP6-defective HSV-1 mutant to infect and replicate in quiescent organs (14, 28).

Another approach that may be used to restrict viral replication to neoplastic cells involves exploitation of genetic alterations found specifically in cancer cells. For example, Bischoff et al. (10) described a replication-conditional adenovirus mutant that is defective in E1B expression. These investigators have proposed that the absence of E1B restricts its replication to cells with absent or mutant p53, although the basis for the tumor specificity of this virus has recently been challenged (29). Reovirus has also been examined as an oncolytic virus because it replicates preferentially in cells with activated ras (12).

rRp450 is a second-generation HSV-1 mutant that was derived from hrR3. ICP6 expression is defective in hrR3 by virtue of an insertional mutation in which the lacZ gene was inserted into the ICP6 locus by homologous recombination (17). None of the ICP6 gene has been deleted from hrR3, and, accordingly, this virus is not safe for clinical trials; theoretically, wild-type virus could be reconstituted simply by spontaneous expulsion of the lacZ gene. rRp450 is similar to hrR3 in its deficiency of ICP6 expression; however, most of the ICP6 coding region has been deleted from rRp450, thereby dramatically reducing the risk of spontaneous reversion to a wild-type virus. Furthermore, the CYP2B1 transgene that was recombined into this locus confers increased cyclophosphamide sensitivity to rRp450-infected tumor cells (17, 23).

The therapeutic strategy that we have explored involves two mechanisms of tumor cell destruction: (a) viral oncolysis; and (b) prodrug activation. There are several advantages of a strategy that combines the lytic replication of HSV-1 in tumor cells with cyclophosphamide activation by CYP2B1. First, the combined effects of two different mechanisms of tumor cell destruction are more effective than either mechanism alone. Second, combined modality treatment using therapies with different mechanisms of resistance dramatically reduces the risk of emergent resistant clones of tumor cells. Treatment strategies that rely solely on in vivo activation of a single prodrug for treatment of solid tumors would very likely fail due to the emergence of drug-resistant clones. Even the incorporation of a second prodrug activation gene to allow in vivo activation of two prodrugs runs the significant risk of failure due to the presence of drug-resistant tumor clones. Notably, patients with solid tumor metastases are presently treated with multiple-agent chemotherapy regimens but are never cured due to the eventual emergence of drug-resistant tumor cells (30). The long history of failures using numerous polychemotherapy regimens in an attempt to cure patients with solid tumor metastases argues strongly that strategies that rely solely on activation of a single prodrug or even multiple prodrugs are doomed to failure in such patients because of the emergence of drug-resistant clones (30). In contrast, a strategy that combines two completely different mechanisms of antitumor activity, such as prodrug activation and lytic viral replication, may reduce the risk of tumor cell resistance. We have previously shown regression of 9 L glioma tumors after multimodal treatment by rRp450 viral oncolysis combined with cyclophosphamide/CYP2B1 and ganciclovir/HSV-TK gene therapies. In contrast, treatment with single agents alone produced only tumor growth retardation (23). Another combination therapy that has been investigated to minimize the risk of tumor cell resistance is that of radiation therapy and HSV-1-induced viral oncolysis (31, 32).

Many investigators have cloned prodrug-activating genes (e.g., suicide genes) into replication-defective viral vectors for cancer therapy (4). However, expression of prodrug activation enzymes in replication-conditional vectors for combined viral oncolysis and prodrug activation represents a significantly more difficult therapeutic approach to model. If viral replication is impeded by metabolites gen-

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**Fig. 3. Replication of rRp450 and KOS in HCC cell lines and primary hepatocytes.**

A. Human HCC (Hep-G2) or human hepatocytes (HH) were infected with rRp450 using an MOI of 2. Virus recovered from cells and media 40 h later were titrated on confluent layers of Vero cells. B. Mouse HCC cells (Hep 1-6) versus mouse hepatocytes (MH) as well as (C) rat HCC cells (McA RH7777) versus rat hepatocytes (RH) were similarly examined for titers of rRp450. Experiments were performed three times, and representative results are shown.

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erated as a result of prodrug activation, the overall effect of combined prodrug activation and viral oncolysis may be less than that seen with either one alone. Accordingly, each combination must be examined empirically. We observed that the presence of ganciclovir reduced rRp450 replication in human HCC cells, thereby reducing viral oncolysis. In contrast, cyclophosphamide had minimal impact on rRp450 replication and substantially increased overall cytotoxicity. The explanation for this finding may be related to differences in the mechanisms of action between the active metabolites. HSV-1 thymidine kinase phosphorylates ganciclovir, which is then converted to metabolites that act as false nucleotides that cause premature termination of replicating DNA strands. This affects both viral and genomic DNA synthesis. In contrast, the active metabolite of cyclophosphamide, phosphoramide mustard, can damage DNA only during mitosis, when multiple DNA strand breaks occur at the cross-link sites (33). Nonmitotic cross-linked viral DNA may be spared from extensive damage and may thus be repaired more readily than genomic DNA.

Whereas the therapeutic implications of these findings are straightforward, the importance of retaining an intact thymidine kinase gene in HSV-1 vectors such as rRp450 should not be overlooked. rRp450 clearly retains its susceptibility to ganciclovir, which is an important safety feature to enable treatment with ganciclovir (or acyclovir) to terminate unwanted viral replication.

Although we observed striking antitumor activity of rRp450 in the Buffalo rat model of HCC, we did not observe any complete responses. In addition, the tumor burden at the time of treatment was less than that frequently observed in patients. However, it is noteworthy that the antitumor activity observed was produced after a single treatment with rRp450. Based on our experience with single injection versus multiple injections of HSV-1 vectors into flank tumors, we believe that multiple intraportal inoculations will produce more significant antitumor effects than a single injection. However, multiple dosing is technically difficult to examine in the current model. It should also be emphasized that HSV-1 replication is several orders of magnitude more robust in human cancer cells than in rodent cancer cells. Accordingly, HSV-1 oncolytic therapy may be more effective in patients than in rodents, despite the higher liver tumor burdens observed in patients.

We did not observe any toxicity secondary to rRp450 infusion into the liver. None of the animals developed signs or symptoms of encephalitis. The significantly attenuated replication of rRp450 in hepatocytes presumably provides a relatively large therapeutic window. We did not formally examine rRp450-induced toxicity in this Buffalo rat HCC model because rodent models in general are not suitable for determining the relationship between HSV-1 dose schedule and toxicity. HSV-1 infection of owl monkeys is accepted as the most suitable model to examine the safety and toxicity of HSV-1 vectors (34, 35). We are presently examining rRp450 toxicity after intravascular administration to owl monkeys.

References

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