Oncolytic recombinant herpes simplex virus for treatment of orthotopic liver tumors in nude mice

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Abstract. Cell-specific, replicating viruses are being developed as a new class of oncolytic agents. A novel approach to viral gene therapy with the use of replication-competent herpes simplex virus has been described; G92A is a replication-competent, multimutant oncolytic herpes simplex virus (HSV) that has been evaluated for anticancer effects and selectivity in the treatment of subcutaneous tumors. G92A replicates efficiently in albumin-producing tumor cell lines but not in non-albumin-producing tumor cell lines, whereas both types are equally susceptible to a non-tissue-specific recombinant HSV, hrR3. In this study, we analyzed the antitumoral efficacy of a single intrasplenic G92A or hrR3 injection in nude mice. In vivo, G92A replicated well in liver xenografts of human albumin-producing hepatoma cells (Hep3B) but not in liver xenografts of a non-albumin-producing malignant colon tumor cell line (HT29), whereas hrR3 replicated well in both tumor types. G92A effectively and selectively replicated throughout liver tumors without apparent hepatotoxicity and inhibited tumor growth, leading to a significantly increased survival time. By monitoring lacZ histochemical staining, we determined the oncolytic potential of recombinant HSV against liver tumors. Our results indicate that G92A warrants further investigation as a clinical therapy against malignant liver tumors.

Introduction

Malignant liver tumor is a leading cause of cancer death, accounting for approximately 600,000 cases worldwide in 2000. In Japan, the incidence has increased. The prognosis of untreated malignant liver tumors is poor. For patients with multi-focal tumors localized to the liver, regional approaches such as orthotopic liver transplantation offer long-term survival. However, the supply of livers available for transplantation is very limited. Given the expected number of patients with liver tumor over the next 20 years, development of more effective treatment modalities is urgently needed.

Several gene therapy strategies have been examined for their potential in the treatment of cancer. Tumor-targeted replication-competent viral agents represent a feasible complement to cancer therapy. In addition to adenovirus, herpes simplex virus (HSV), vaccinia virus and reovirus that have been molecularly engineered to replicate specifically within tumor cells, viruses with inherent tumor specificities are actively being developed as oncolytic agents for cancer treatment (1). Compared to other replication-competent oncolytic vectors, HSV is particularly appealing for its exceptionally rapid replication rate within tumor cells (8-10 h), such that the oncolytic effects may be manifested maximally before the onset of potentially neutralizing antiviral immune responses in the host. HSV infections in humans are asymptomatic in most cases or result in mild febrile illness. G92A is a second-generation replication-conditional HSV-1 mutant that is regulated by the albumin enhancer/promoter (2). G92A efficiently suppressed subcutaneously derived albumin-producing tumor growth in nude mice (3). hrR3 [an HSV-1 strain with a defective ribonucleotide reductase (RR) gene] (4) has also been examined as a replication-competent oncolytic virus because of its preferential replication at low levels in non-neoplastic cells and in vivo virulence at high titer (5,6). The selective replication of viruses bearing mutations of non-essential viral genes can destroy tumor cells. A previous study showed that hrR3 completely eliminates the tumor burden and confers a survival advantage in an intraperitoneal model of pancreatic cancer in athymic mice (7). Toda et al reported that, in vivo, all replication-conditional HSV-1 mutants assayed were effective antitumor agents in experimental models of central nervous system (CNS) tumors and extra-CNS malignancies (8-10). In the present study, we investigated the therapeutic efficacy of G92A and hrR3 in nude mice with malignant liver tumors. We show that intrasplenically inoculated G92A replicated well in liver xenografts of human hepatoma cells (Hep3B) in nude mice but not in human colon carcinoma liver tumors (HT29), whereas hrR3 replicated well in both tumor types. A single dose of the vector was injected intrasplenically into orthotopically implanted malignant liver tumors.
tumor cell lines in nude mice and led to a significantly prolonged survival. The use of replication-competent viral vectors controlled by cell-specific transcriptional regulatory sequences provides a new strategy for tumor therapy with inherent specificity for replication in tumor cells due to their attenuated antiviral responses. Our results show that G92A and hrR3 are effective oncolytic agents against malignant liver tumors in nude mice. In addition, G92A warrants further development as a selective therapy for patients with malignant liver tumors.

Materials and methods

Cells and viruses. The albumin-expressing Hep3B human hepatoma cell line, the human HT29 colon carcinoma non-albumin-expressing tumor cell line and Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection (Manassas, VA, USA), and E5 cells (Vero cells stably transfected with ICP4) were provided by Neal DeLuca (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Hep3B cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT, USA). HT29 cells were grown in RPMI-1640 medium supplemented with 10% FCS. Vero and E5 cells were grown in DMEM supplemented with 10% newborn calf serum (Hyclone).

The structures of G92A and hrR3 have been described in detail (2,4). hrR3, a non-tissue-specific recombinant HSV defective in RR expression, contains the Escherichia coli lacZ gene inserted into the RR gene locus. The lacZ gene is driven by the ICP6 immediate-early gene promoter. hrR3, kindly provided by Sandy Weller (University of Connecticut Health Center, Farmington, CT, USA), was passaged on parental Vero cells and stored at -80°C until use. G92A (parental strain KOS) was generated from E5 cells at low multiplicity of infection (MOI) of E5 cells. The virus was prepared from infected cells by freeze-thaw/sonication, low-speed centrifugation, ultracentrifugation of supernatant and resuspension of virus pellet in virus buffer (150 mM NaCl/20 mM Tris-HCl, pH 7.5).

Cell destruction assay. Hep3B and HT29 cells were plated onto 6-well dishes (Falcon; Becton-Dickinson, Franklin Lakes, New Jersey). Mice bearing diffuse HT29 and Hep3B liver tumors established as described were treated with 5x10^6 pfu of G92A or hrR3. For mock injection, the same volume of virus buffer was used. Mice were sacrificed 35 days after tumor injection, and livers were analyzed.

Table I. Treatment of diffuse liver tumors with HT29 and Hep3B.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment by orthotopic implantation</th>
<th>Necrotic area (survival rate)</th>
<th>Autopsy day (survival rate)</th>
<th>Liver weight (g ± SD)</th>
<th>No. of mice without liver tumors</th>
</tr>
</thead>
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<tr>
<td>HT29</td>
<td>Mock</td>
<td>35 (100%)</td>
<td>2.17 ± 0.12</td>
<td>0/14</td>
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</tr>
<tr>
<td></td>
<td>hrR3</td>
<td>35 (100%)</td>
<td>1.63 ± 0.07</td>
<td>0/14</td>
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<tr>
<td></td>
<td>G92A</td>
<td>35 (100%)</td>
<td>1.91 ± 0.10</td>
<td>0/14</td>
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<tr>
<td>Hep3B</td>
<td>Mock</td>
<td>35 (50%)</td>
<td>6.07 ± 0.79</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hrR3</td>
<td>35 (67%)</td>
<td>2.99 ± 0.45</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G92A</td>
<td>35 (89%)</td>
<td>2.58 ± 0.32</td>
<td>0/9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. In vitro cell destruction assay. Target cells (1x10^5) of Hep3B (A and C) and HT29 (B and D) were plated onto 6-well dishes 24 h before virus infection. Cells were infected with G92A (C and D) or hrR3 (A and B) at an MOI of 0.07. Infected cells were fixed 72 h after infection and stained with X-gal (black precipitate). Infection with hrR3 resulted in marked cell destruction irrespective of cell type, whereas infection with G92A resulted in replication and destruction of albumin-expressing tumor cells only. Bars, 0.05 mm.
In vitro oncolytic activities of G92A and hrR3. The G92A and hrR3 viruses were replicated in E5 and Vero cells as described in Materials and methods. The cytotoxic ability of both recombinant viruses was then examined. Hep3B and HT29 cells (1x10^5) were plated onto 6-well dishes 24 h before virus infection. To assess whether these viruses have the ability to preferentially replicate and kill malignant tumor cells, cells were examined for cytopathic effects, and viral replication was analyzed by lacZ expression with X-gal staining. Infection with G92A or hrR3 was as follows: Hep3B (Fig. 1A and C) and HT29 (Fig. 1B and D) cells were infected at an MOI of 0.07 with G92A (Fig. 1C and D) or hrR3 (Fig. 1A and B). Plaque formation was observed 72 h after infection; infected cells were fixed and stained with X-gal. The morphology of non-albumin-producing HT29 cells infected with hrR3 showed typical HSV cytopathic effects (Fig. 1B), whereas those infected with G92A were unaltered (Fig. 1D). Albumin-producing Hep3B cells (Fig. 1A and C) became non-viable and completely floated off the tissue culture plates in response to infection with hrHSV. hrHSV infection showed marked cell destruction irrespective of cell type, whereas G92A infection showed replication and destruction only in albumin-expressing cells (Hep3B).

Liver sections and histochemical staining. To analyze liver tumors and liver tissue for the presence of rHSV, direct injection of 1x10^7 Hep3B or HT29 cells was performed during laparotomy. Intrapleural rHSV injections were performed 2 weeks after tumor implantation, and animals were sacrificed 3 days after virus challenge. Livers were resected after a PBS flush and fixed with 2% paraformaldehyde, 5 mM Ethylene Glycol Bis (β-aminoethylether)-N, N', N'-tetraacetic acid and 2 mM MgCl₂ in 0.1 M Pipes buffer (pH 7.3) overnight. Whole livers were cut into 20-μm sections by cryostat and stained histochemically in X-gal solution with 0.02% NP-40 and 0.01% sodium deoxycholate. Paraffin-embedded sections were subjected to hematoxylin-and-eosin staining for histo-analytical analysis.

Results

In vivo target cell specificity. To investigate the potential of G92A and hrR3 for use in therapy of malignant tumors, we tested the viruses in orthotopic liver tumors in nude mice. To produce diffuse liver tumors, 1x10^7 Hep3B cells and HT29 cells was injected directly into the right hepatic lobe of nude mice. Animals then received intrasplenic treatment on days 7 with either G92A (5x10^6 pfu for Hep3B, n=9, group B; HT29, n=9, group C) or hrR3 (5x10^6 pfu for Hep3B, n=9, group C; HT29, n=9, group F). Control animals received virus buffer alone (Hep3B, n=5, group A; HT29, n=5, group D). Curves represent the sum of two experiments carried out under identical conditions. Survival was evaluated using the Kaplan-Meier method and statistical differences in survival were determined by log-rank analysis. Statistical significance was set at p<0.05. (group A vs group B, p<0.05; group A vs group C, p<0.05; group D vs group E, not significant; group D vs group F, p<0.05).

Animal studies

Hepatic tumor model and treatment with HSV vector. All procedures involving animals were approved by the guidelines of the Institutional Animal Care and Use Committee of Osaka Medical College. Pathogen-free, 4- to 5-week-old female BALB/c nude mice were allowed to acclimate for 1 week. Mice were anesthetized with a 0.35-ml i.p. injection of 84% bacteriostatic saline, 10% pentobarbital (1 mg/kg; Abbott Laboratories, Chicago, IL, USA) and 6% ethyl alcohol for all surgical procedures. For liver tumor formation, a 1-cm subcostal incision was made to divide the peritoneum and expose the liver. Mice received an orthotopic implant of 1x10^7 heterogeneous Hep3B or HT29 cells in a 50-μl single-cell suspension injected directly into the right hepatic lobe with a Hamilton syringe (Reno, NV, USA) and 30 G needle. One week later, 5x10^7 plaque-forming units (pfu) of G92A or hrR3 in 50-μl virus buffer was injected into the spleen, or an equivalent volume of virus buffer alone was injected as a control. To assess tumor response and virus efficacy, animals were sacrificed 35 days after tumor injection, and livers were removed for examination (Table I). Data were analyzed using the SPSS statistical software package. In addition, groups of animals were followed up for survival. Survival was evaluated using the Kaplan-Meier method and log-rank test. Statistical significance was set at p<0.05.
Figure 3. Comparison of G92A and hrR3 viral therapy in Hep3B liver tumors. A, mice (a-c) treated with a single intrasplenic injection of buffer (a and d), hrR3 (5x10⁶ pfu) (b and e) or G92A (c and f) and livers (d-f). G92A- and hrR3-injected mice showed significant differences compared to buffer-injected mice. Bars, 5 mm. B, tumor burdens were assessed by weight. Mice were sacrificed 35 days after tumor injection, and livers were analyzed. p<0.01 for hrR3 versus control and for G92A versus control; p=0.32 for hrR3 versus G92A. NS, not significant.

Figure 4. Comparison of G92A and hrR3 viral therapy in HT29 liver tumors. A, HT29 liver tumors treated with 5x10⁶ pfu of G92A or hrR3. Mice were sacrificed 35 days after tumor injection, and livers were analyzed. Representative livers from mice treated with a single intrasplenic injection of buffer (control), hrR3 or G92A are shown. hrR3-injected mice showed significant differences compared to buffer-injected mice. G92A-injected mice showed no remarkable differences compared to buffer-injected mice. Bars, 5 mm. B, tumor burden was assessed by weight. Mice were sacrificed 35 days after tumor injection, and livers were analyzed: p<0.01 for hrR3 versus control; there was no significant difference for G92A versus control or for hrR3 versus G92A. NS, not significant.

Figure 5. Microscopic visualization of virus spread. Tumors were inoculated with 5x10⁶ pfu of G92A (C and D) or hrR3 (A and B). Three days after virus inoculation, livers were removed after PBS flush and stained histochemically with X-gal. G92A spread well in Hep3B tumor nodules (C) but not at all in HT29 tumor nodules (D). hrR3 showed effective viral spread in Hep3B and in HT29 tumor nodules (A and B). Bars, 0.5 mm.
significantly inhibited by G92A or hrR3 injection compared to that in control animals injected with injection buffer alone, whereas non-albumin-producing tumor growth was inhibited only by hrR3 injection (Figs. 3 and 4). Differences in survival were statistically significant in Hep3B-injected mice between those untreated and those treated with hrR3 or G92A at 35 days after Hep3B injection (p<0.05 by log-rank test). Whereas, there was significant difference in survival between untreated HT29-injected mice and those treated only with hrR3 compared with those treated with G92A at 49 days after HT29 injection (p<0.05 by log-rank test) (Fig. 2).

Hep3B injection models were monitored for 35 days. None of the mice receiving viral therapy developed neurological symptoms or other adverse effects. Results of G92A and hrR3 viral therapies in Hep3B liver tumors are shown in Fig. 3A. Representative mice and livers after treatment with a single intrasplenic injection of virus buffer alone (a and d), hrR3 (b and e) or G92A (c and f) are shown. Mice were sacrificed 35 days after tumor injection, and livers were analyzed (Fig. 3B). A total of 5x10^6 pfu of G92A or hrR3 showed significant differences compared to buffer injection. Tumor burden was assessed by weight. The control group (n=9) had a mean weight of 6.07 g, the hrR3-treated group (n=9) had a mean weight of 2.99 g (p<0.01; unpaired t-test) and the G92A-treated group (n=9) had a mean weight of 2.58 g (p<0.01; unpaired t-test). HT29 liver tumors treated with 5x10^6 pfu of G92A or hrR3 are shown in Fig. 4A. Mice were sacrificed 35 days after tumor injection, and livers were analyzed. Livers from mice treated with a single intrasplenic injection of buffer (a), hrR3 (b) or G92A (c) are shown. hrR3-injected mice showed significant differences compared to buffer-injected mice. G92A-injected mice showed no remarkable differences compared to buffer-injected mice. G92A and hrR3 viral therapy for HT29 liver tumors was also assessed by weight (Fig. 4B). The control group (n=14) had a mean weight of 2.17 g, the hrR3-treated group (n=14) had a mean weight of 1.63 g (p<0.01; unpaired t-test) and the G92A-treated group (n=14) had a mean weight of 1.91 g (no significant difference; unpaired t-test).

We next sought to determine whether G92A or hrR3 replicates selectively in diffuse malignant liver tumors. To produce diffuse liver tumors, 1x10^5 Hep3B or HT29 cells was injected into the right hepatic lobe of nude mice. Thirty days after tumor injection, 5x10^6 pfu of G92A or hrR3 was injected intrasplenically. Animals were sacrificed 3 days after virus inoculation, livers were removed after a PBS flush and sectioned, and sections were stained with X-gal for examination of lacZ gene expression. G92A infection spread well in Hep3B tumor nodules (Fig. 5C) and not at all in HT29 tumor nodules and normal liver (Fig. 5D). hrR3 showed effective viral spread in Hep3B and HT29 tumor nodules with partial spread in normal liver (Fig. 5A and B). Spleen, intestine and kidney specimens from the same mice showed no lacZ staining (data not shown). Mice injected with virus buffer showed no lacZ staining (data not shown).

Discussion

Increased understanding of the factors responsible for HSV virulence and replication has revealed a potential role of HSV mutants in treating malignancies (11). Neurological tumors were first targeted due to natural HSV neurotropism. Various recombinant, replicating HSV mutants have since been tested as therapies for extra-CNS malignancies (5,10,12). Varghese et al reported the safety and effectiveness of rHSV 1716, harboring a homozgous deletion of ICP34.5, against human malignant mesothelioma cell lines in vitro and in a mouse model of epithelial ovarian cancer (13-15). The rHSV mutant G207 has a deletion in ICP34.5 and a lacZ gene insertion inactivating the ICP6 gene (8). Both rHSV 1716 and G207 were effective in animal models of malignant peritoneal disease induced by injection of human ovarian cancer cells (14,16). Intraperitoneal administration of G207 was also reported to have therapeutic potential against peritoneal tumors (17). rHSV R3616, an ICP34.5 deficient, replication-restricted HSV-1 was equally effective against chemotherapy-sensitive and -resistant ovarian cancer cells in vitro and in vivo (16). Thus, rHSV mutants may be effective in the treatment of malignant tumors. Despite promising findings, few phase 1 or 2 studies have been reported (18,19).

Our study examined the effects of two types of HSV mutant on malignant liver tumors in nude mice. G92A and hrR3 prolonged animal survival and appeared to significantly effect tumor regression in albumin-producing tumors (Hep3B), whereas G92A had no effect on non-albumin-producing tumors (HT29), indicating that the oncolytic effect of G92A is more selective than that of hrR3.

The precise mechanism of viral cytotoxicity remains to be elucidated. The antitumor effect of rHSV is reported to be much greater than its evident distribution throughout tumors (20-25). HSV infection induces apoptosis in adjacent, uninfected cells in vitro and in vivo (26). The route of administration will be a critical determining factor in the efficacy of viral therapy. At first, we examined the efficacy of intraportal delivery of hrR3 via splenic injection. The efficacy and specificity of lacZ gene expression in tumor cells were striking. The level of lacZ expression in tumor nodules was extremely high compared to that in normal hepatic parenchyma. Very low incidence but distinct blue-stained cells were observed in normal liver by hrR3, whereas no blue staining of cells was observed in normal livers by the same delivery of G92A (data not shown). Thus, hrR3 also has the possibility of spreading in normal liver. As expected, G92A is more safe and has more feasibility of therapeutic strategy.

Further studies are warranted to assess the level of lacZ expression at various time intervals after infection and to examine the immune responses to G92A and hrR3 when they are injected into the spleen. In the present study, we observed efficient rHSV vector infection and replication in human hepatoma (Hep3B) and colon cancer (HT29) cell lines. In contrast, normal mouse hepatocytes are resistant not only to virus infection but also to replication. A single intrasplenic injection of rHSV into malignant liver tumors showed potent antitumor activity. The ability of rHSV to replicate intratumorally in vivo was shown by X-gal staining of lacZ (5,27-29). Our data indicate that intratumoral HSV replication led to slower tumor growth, tumor destruction and prolonged survival. These results are consistent with those reported previously for subcutaneous injections of rHSV against malignant tumors in nude mice (3). There was presumably no
uncontrolled viral replication in treated animals because it has been reported that viral replication is limited to the tumor bed and decreased to negligible levels after 7 days (8,30,31). We did not observe any adverse effects of virus in animals treated with the indicated vector doses. In conclusion, HSV replication is tumor-selective in vitro as well as in vivo and produces significant destruction of large tumors before the onset of potentially neutralizing antiviral immune responses in the host. A single intrasplenic injection of virus can significantly prolong the survival of tumor-bearing animals. This oncolytic virus system warrants further development as a therapeutic option for clinical treatment of malignant liver tumors.

**References**