Cancer Chemother Pharmacol (2009) 63:321–330
DOI 10.1007/s00280-008-0742-6

ORIGINAL ARTICLE

Considerations for intravascular administration of oncolytic herpes virus for the treatment of multiple liver metastases

Naohiro Nomura · Hideki Kasuya · Izuru Watanabe · Toshio Shikano · Takashi Shirota · Makoto Misawa · Hiroyuki Sugimoto · Naohito Kanazumi · Shuji Nomoto · Shin Takeda · Akimasa Nakao

Received: 25 November 2007 / Accepted: 17 March 2008 / Published online: 25 June 2008

Abstract
Purpose Oncolytic viral therapy is a newly developed modality for treating tumors. Many clinical trials using oncolytic virus have been performed worldwide, but most of them have used local injection in the tumor. Determination of the effect and safety of intravascular virus injection instead of local injection is necessary for clinical use against multiple liver metastases and systemic metastases.
Methods To evaluate the efficacy and safety of intravascular virus therapy, mice bearing multiple liver metastases were treated by intraportal or intravenous administration of the herpes simplex virus type 1 (HSV-1) mutant, hrR3. Mice treated with hrR3 were killed and organs were harvested for lacZ staining and PCR analysis. Inactivation of oncolytic virus in bloodstream was assessed by neutralization assay in vitro. Infectious activity of hrR3 with vascular endothelial cells was evaluated by replication and cytotoxicity assay.
Results The survival rate of animals treated by hrR3 was significantly improved compared with the untreated group. lacZ staining and PCR analysis demonstrated detectable virus in the tumor but not in normal tissue or other organs except for the adrenal glands. We also showed that vascular endothelial cells allowed virus replication, while normal hepatocytes did not, and human anti-HSV antibody revealed attenuation of the infectious activity of hrR3.
Conclusions Intravascular delivery of hrR3 is effective in treating multiple liver metastases, however, several points must be kept in mind at the time of human clinical trials using intravascular virus administration in order to avoid critical side effects.

Keywords Herpes oncolytic virus · Liver metastases · Intravascular therapy · Adrenal gland · Vascular endothelial cell · Anti-HSV antibody

N. Nomura (✉) · H. Kasuya · I. Watanabe · T. Shikano · T. Shirota · M. Misawa · H. Sugimoto · N. Kanazumi · S. Nomoto · S. Takeda · A. Nakao
Department of Surgery II, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
e-mail: sjypy948@yahoo.co.jp

Introduction

Colorectal cancer is one of the leading causes of cancer death [20], and the liver is the most frequent site of distant metastases. Most patients have multiple metastases and are not candidates for surgical resection [1]. Although various types of chemotherapy have been used to treat patients with unresectable liver metastases, they have had limited success [4, 41]. Oncolytic viral therapy has been viewed as a new strategy for the treatment of advanced cancers.

Researchers have examined the oncolytic activity of several viruses, including adenovirus [36], herpes simplex virus [27], vaccinia virus [28], measles virus [40], reovirus [10], and Newcastle disease virus [25]. Many published reports describe the effectiveness of genetically engineered herpes simplex virus type 1 (HSV-1) [5, 15, 21]. HSV-1 has many advantages over other viruses for cancer gene therapy: (1) it has a broad host range and high efficiency of infection, (2) it has a large genome capacity for delivery of therapeutic transgenes and tumor-specific promoter genes [22, 32], and (3) it can be controlled by anti-herpetic drugs to treat unwanted or uncontrolled viral infection.

Replication-competent HSV-1 infection of cancer cells results in viral replication with cell destruction and liberation of progeny virion that infects adjacent tumor cells. The genetically engineered HSV-1 mutant, hrR3, is defective in

viral ribonucleotide reductase expression as a result of insertion of the *Escherichia coli* lacZ gene into the ribonucleotide reductase gene locus (ICP6) (Fig. 1). Actively dividing cells such as cancer cells express high levels of endogenous ribonucleotide reductase for synthesis of DNA precusors. If hrR3 infects actively dividing cells, viral replication can proceed because the endogenous ribonucletide reductase complements the absence of viral ribonucleotide reductase [14]. The safety and efficacy of this oncolytic viral therapy are dependent on selective viral replication in cancer cells rather than in normal cells.

Most clinical trials using oncolytic viral therapy have been performed using direct intratumor injection. We have performed a Phase 1 clinical trial of the treatment of recurrent breast cancer using intratumor injection of the HSV-1 mutant, HF10 [23, 35, 43]. The results revealed a significant tumor-reducing effect without any side effects, and complete tumor cell disappearance was seen in one of six patients participating in this trial. However, this mode of administration would not be feasible for patients with multiple liver metastases and systemic metastases.

Recently, several studies have reported the efficacy of intravascular therapy using an oncolytic HSV-1 mutant. Pawlik et al. [39] demonstrated that intraportal administration of the HSV-1 mutant, rRp450, effectively treated diffuse hepatocellular carcinoma in Buffalo rats. Several reports have demonstrated the efficacy of intraportal administration of HSV-1 mutants (hrR3, G207, Myb34.5) against established liver metastases from colon carcinoma [8, 24, 34, 50]. Intravenous delivery of G207 has been reported to cause regression of distant subcutaneous prostate and bladder cancers in animal models [38, 46]. Intravenous therapy with IL-12-expressing oncolytic HSV (NV1042) has been reported to significantly improve survival in animals with disseminated pulmonary squamous cell carcinoma [48]. However, we can find only one report to evaluate the efficacy of intravenous therapy using oncolytic HSV against diffuse liver metastases [12].

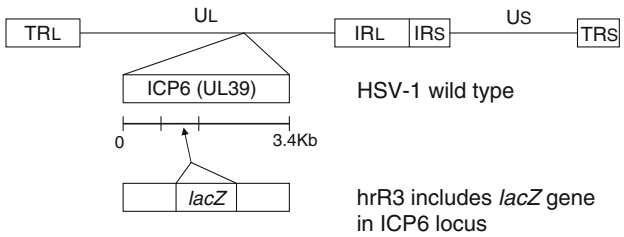


Fig. 1 Schematic gene arrangement of hrR3. hrR3 is a genetically engineered herpes simplex virus type 1 (*HSV-1*) that has the lacZ gene inserted into the viral ribonucleotide reductase gene locus (*ICP6*). Actively dividing cells such as cancer cells express high levels of endogenous ribonucleotide reductase for synthesis of DNA precusors. If hrR3 infects an actively dividing cell, viral replication can be triggered because the endogenous ribonucleotide reductase complements the absence of viral ribonucleotide reductase

HSV-1 is a natural human pathogen and up to 90% of adults have circulating antibodies against HSV [11]. Pre-existing immunity has the potential to attenuate the efficacy of HSV-1 based oncolytic agents. There also remains the possibility that hrR3 can infect and replicate in normally dividing nontumor cells such as vascular endothelial cells, and it has been reported repeatedly that HSV preferentially infects the adrenal gland after intravenous [18], intraperitoneal [19, 42] and intranasal inoculation [33]. In this study, we demonstrate that intravascular delivery of hrR3 is effective in treating multiple liver metastases, although there are several points that should be taken into account in clinical trials using intravascular virus administration.

Materials and methods

Cells and viruses

Vero, African Green monkey kidney cells and HT29, a human colon carcinoma cell line, were obtained from American Type Culture Collection (Manassas, VA). MC26, a mouse colon carcinoma cell line, was obtained from the National Cancer Institute Tumor Repository (Frederick, MD). hNHeps, normal human hepatocytes, and HMVEC-L, normal human lung microvascular endothelial cells, were obtained from Takara Bio Inc. (Otsu, Japan). Vero, MC26 and HT29 were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. hNHeps were maintained in hepatocyte culture medium (HCM) containing fatty acid-free bovine serum albumin (BSA-FAF), insulin, transferrin, hydrocortisone, ascorbic acid, hEGF, gentamicin, and amphotericin B on collagen-coated plates. HMVEC-L were maintained in endothelial cell basal medium-2 containing FBS, hydrocortisone, ascorbic acid, hEGF, hFGF-B, VEGF, R3-IGF, gentamicin, and amphotericin B. The HSV-1 mutant, hrR3 was kindly provided by Sandra K. Weller Ph.D. (University of Connecticut, Storrs, CT) and was propagated and titered on Vero cells and stored in stocks at −80°C before use.

Viral replication assay

MC26, HT29, hNHeps and HMVEC-L cells (10^6 cells) were plated onto 10-cm plates and incubated overnight. The next day, medium was removed and cells were washed with HBSS. The cells were infected with 2×10^6 plaque forming units (pfu) of hrR3 in 200 µl HBSS and the plates were incubated for 1 h and rotated every 20 min. After 1 h of incubation, 5 ml medium was added and the plates were

incubated another 1 h. After 2 h of total incubation time, the medium was removed and unadsorbed virus was removed by washing with a glycine–saline solution (pH 3.0). Five milliliters of medium was then added, and after 40 h of total infection time, the cells and supernatant were harvested, exposed to three freeze–thaw cycles to release virus particles, and titered on Vero cells. The number of plaques was counted after 5 days. The results were divided by the cell number (10^6) and were reported as the mean of three independent experiments.

Viral cytotoxic assay

Cells were plated onto 96-well plates at 5,000 cells per well for 36 h. hrR3 was added at multiplicity of infection (MOI) values ranging from 0.001 to 10 and incubated for 6 days. The number of surviving cells was quantitated by a colorimetric MTT assay, and the percent cell survival was calculated by comparison with the control (mock-infected) cells. Tests were performed in quadruplicate.

Animal studies

BALB/c mice were obtained from Japan CLEA (Hamamatsu, Japan). Animal studies were performed in accordance with the guidelines issued by the Nagoya University Animal Center. Liver metastases models were generated by the injection of a single-cell suspension of 10^6 MC26 cells in 100 μl HBSS into the spleens of BALB/c mice. Mice were randomized into three groups ($n = 18$/group). The first group was left untreated. The second group was treated with intraportal injection (intrasplenic injection) of 10^7 pfu of hrR3 2 days after tumor implantation. The third group was treated with intravenous injection (tail vein injection) of 10^7 pfu of hrR3 2 days after tumor implantation. Six mice in each group were killed 13 days after tumor implantation, and the livers and spleens were weighed. The remaining mice were followed for survival ($n = 12$/group). In another set of experiments, 10^7 pfu of hrR3 was injected into the spleen or tail vein 7 days after tumor implantation. Two days later, mice were killed and organs (liver, spleen, adrenal gland, kidney, lung, and brain) were harvesterd for examination of lacZ expression and analysis of extracted DNA by PCR amplification of HSV-1 sequences.

Histochemical analysis

Harvested tissues were frozen rapidly in OCT compound in liquid nitrogen. Cryostat sectioning of the tissue was performed at a thickness of 10 μm using a microtome. Sections were fixed with 1% glutaraldehyde and evaluated for lacZ expression by staining with X-gal solution.

PCR analysis

PCR amplification of HSV-1-specific sequences was performed to investigate the biodistribution of HSV-1 following intravascular administration in mice. DNA extraction from mouse tissues was performed using a BioRobot EZ1 Workstation (QIAGEN, Tokyo, Japan). The forward oligonucleotide primer, 5′-GGAGGCGCCCAAGCGTCC GGCCG-3′, and the reverse oligonucleotide primer, 5′-TG GGGTACAGGCTGGCAAAGT-3′, were used to amplify a 229-bp fragment of the HSV-1 DNA polymerase gene. We then subjected 0.3 μg of DNA to PCR amplification. PCR reactions were performed in a 25-μl volume using rTth DNA polymerase according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) with a GeneAmp® PCR system 9700 (Applied Biosystems) for 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. HSV-1 genomic DNA was used as a positive control. The amplified fragments were electrophoresed on 3.0% agarose gel and stained with ethidium bromide.

In vitro assay of hrR3 infection in vascular endothelial cells

We determined whether or not hrR3 could infect and replicate in normally dividing nontumor cells, such as vascular endothelial cells, when delivered into the bloodstream.

10^6 HMVEC-L cells were plated onto 10-cm plates and incubated for 24 h. hrR3 in 100 μl of HBSS was infected at an MOI values ranging from 0.01 to 1.0 and the plates were incubated for 1 h and rotated every 20 min. After 24 h of incubation, X-gal staining was performed.

Effect of anti-HSV antibody to hrR3 in the bloodstream

To examine the effect of anti-HSV antibody on hrR3 in the circulation, we performed an in vitro viral neutralization assay using serum of human adults and immunized mice. Serum was harvested from six human adults, three of whom had anti-HSV antibodies (IgG > 4.0) and the other three did not have anti-HSV antibodies (IgG < 2.0). Serum (900 μl) was mixed with 10^4 pfu of hrR3 in 100 μl HBSS. The mixture was incubated for 1 h at 37°C and then hrR3 was titered on Vero cells. The number of plaques was counted after 5 days. BALB/c mice were immunized via intraperitoneal injection with 10^7 pfu of hrR3. Ten days later, mice received a second intraperitoneal injection of the same dose of hrR3. The neutralization assay was performed 10 days after the second immunization. Fifty microliters of serum was harvested from immunized or nonimmunized mice and mixed with 10^3 pfu of hrR3 in 100 μl HBSS. The mixture was incubated for 1 h at 37°C and then hrR3 was titered as described above. All samples were processed in triplicate.

Statistical analysis was performed using an unpaired two-tailed *t* test. The survival data were analyzed using the Kaplan–Meier method and log-rank test.

Results

hrR3 replication in vitro

hrR3 can preferentially replicate in actively dividing cells compared with normal cells because actively dividing cells, such as cancer cells, express high levels of endogenous ribonucleotide reductase. We hypothesized that hrR3 also could replicate in normally dividing nontumor cells such as vascular endothelial cells. Therefore, we compared the number of infectious virus particles produced 40 h after infection by hrR3 in MC26, HT29, hNHeps, and HMVEC-L. In Fig. 2, greater than log 0 means that hrR3 replicated, while less than log 0 means that hrR3 did not replicate. The viral replication assay revealed that hrR3 could not replicate in normal human hepatocytes, however, as we expected, it could replicate in HMVEC-L as in the carcinoma cell lines.

Viral cytotoxity assay in vitro

We examined the cytotoxic effect of hrR3 in the colon carcinoma cell lines (MC26, HT29) and HMVEC-L. The cytotoxity assay revealed that hrR3 had cytotoxic effect in both carcinoma cell lines and vascular endothelial cells (Fig. 3).

Intravascular treatment of diffuse liver metastases by hrR3

We examined the antitumor efficacy of intravascular administration of hrR3 against a diffuse liver metastases model. BALB/c mice bearing MC26 diffuse liver metastases

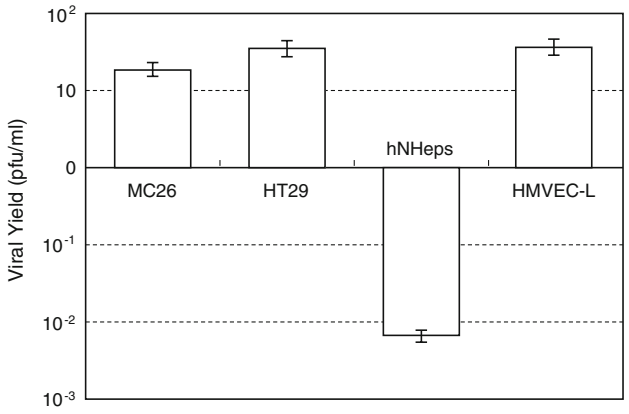


Fig. 2 Replication of hrR3 in colon carcinoma cell lines (*MC26*, *HT29*), normal human hepatocytes, and microvascular endothelial cells (*HMVEC-L*). hrR3 did not replicate in normal hepatocytes, while it replicated in HMVEC-L as much as in colon carcinoma cell lines

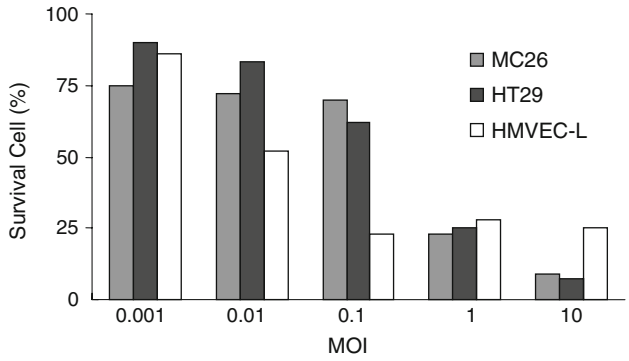


Fig. 3 Cytotoxity assay in vitro. Two colon carcinoma cell lines and (*MC26*, *HT29*) and microvascular endothelial cells (*HMVEC-L*) were infected with hrR3 at several MOI values, and surviving cells were quantitated after 6 days. hrR3 had cytotoxic effects both in carcinoma cell lines and HMVEC-L

were treated by intraportal or intravenous injection of hrR3 and killed 13 days after tumor implantation. Livers of untreated mice had numerous tumor nodules, whereas livers of mice treated by intraportal or intravenous injection of hrR3 were almost normal and the liver weights were significantly less than those of untreated mice (Fig. 4). Spleen weights of mice treated by intravascular injection of hrR3 also were significantly less than those of untreated mice. However, there was no statistical difference in liver and spleen weights between the two treatment groups (Table 1).

Survival rate

Significantly higher survival rates were observed with intraportal and intravenous hrR3-treated animals compared with untreated animals ($P < 0.0001$). There was no statistical difference in survival among the two treatment groups (Fig. 5). None of the mice treated with hrR3 displayed any neurologic symptoms or other toxic side effects after injection. The efficacy of intravenous oncolytic HSV therapy and its potential toxicities have not been well demonstrated. Previous studies have suggested that intraportal delivery of oncolytic HSV is more effective than intravenous systemic delivery [21, 22]. However, our results indicate that intravenous oncolytic virus administration has the potential to become a new solid strategy in the treatment of multiple liver metastases.

X-gal histochemistry

hrR3 possesses the *E. coli* lacZ gene, enabling detection of hrR3-infected cells by histochemical staining for lacZ expression. The mice were killed 2 days after intravascular viral treatment, and organ sections were examined histochemically for lacZ gene expression. When mice were treated with intraportal injection of hrR3, lacZ expression

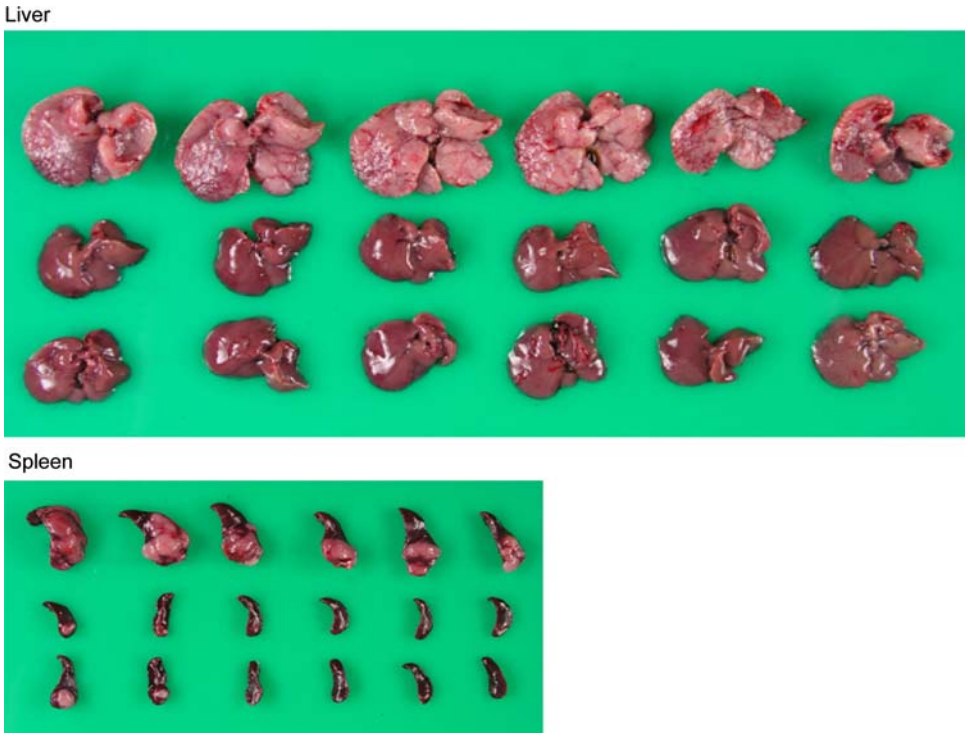


Fig. 4 Treatment of diffuse liver metastases with intravascular administration of hrR3. Mice bearing diffuse liver metastases were treated with intraportal or intravenous administration of 10^7 pfu of hrR3 2 days after tumor implantation. Mice were killed, and livers and spleens were analyzed 13 days after tumor implantation. The control group was left untreated. Livers of untreated mice had numerous tumor nodules, whereas livers of mice treated by intraportal (*middle row*) or intravenous (*lower row*) injection of hrR3 were almost normal

Table 1 Liver and spleen weights following treatment of diffuse liver metastases

Treatment	Liver weight (g) ± SD	Spleen weight (g) ± SD
No treatment	4.55 ± 0.74	1.40 ± 0.49
Intraportal injection	1.60 ± 0.18*	0.22 ± 0.04**
Intravenous injection	1.68 ± 0.15*	0.28 ± 0.12***

* $P < 0.0001$, ** $P = 0.0002$, and *** $P = 0.0003$ when compared with no treatment group

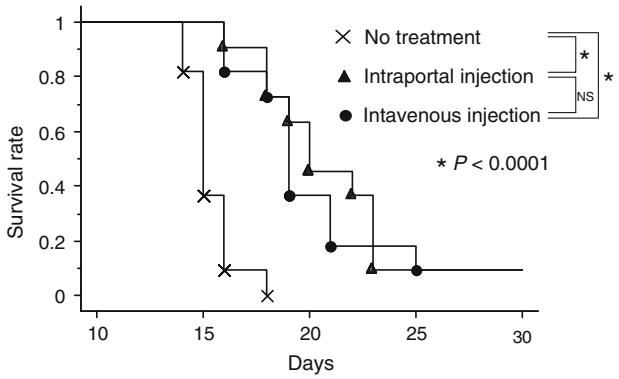


Fig. 5 Survival rates for mice bearing diffuse liver metastases, followed by intraportal or intravenous administration of 10^7 pfu of hrR3 2 days after tumor implantation. The survival rate of treated mice was significantly improved compared with untreated mice ($P < 0.0001$). However, there was no statistical difference in survival between the two treatment groups

was detected in liver tumor, spleen tumor, normal spleen (hrR3 injection site), and adrenal gland. When mice were treated with intravenous injection of hrR3, lacZ expression was detected in liver tumor, spleen tumor, and adrenal gland. The sections of normal liver, brain, lung, and kidney were not stained for lacZ expression. In both treatment groups, sections from the adrenal gland had the strongest staining (Fig. 6).

PCR analysis

We extracted DNA from hrR3-treated mouse tissues and examined biodistribution of HSV by PCR amplification of the HSV-1 DNA polymerase gene. Organs were harvested 2 days after intravascular injection of hrR3 into mice bearing diffuse liver metastases. HSV-1 DNA was detected in the same tissue and organs that lacZ expression was detected. HSV-1 DNA was more strongly detected in adrenal gland rather than in tumor tissue (Fig. 6). The degree of HSV-1 DNA expression in liver and spleen tumor tissue was compared using an image processing and analysis program. The analysis demonstrated that the amount of HSV-1 DNA expression in the tumors from intravenously treated mice was less than that of intraportally treated mice. These results suggest that oncolytic HSV may be consumed and reduced before reaching target cancer cells after intravascular administration, and reduced amounts of virus can reach and infect target cancer cells when the site of virus injection is far from the target cancer.

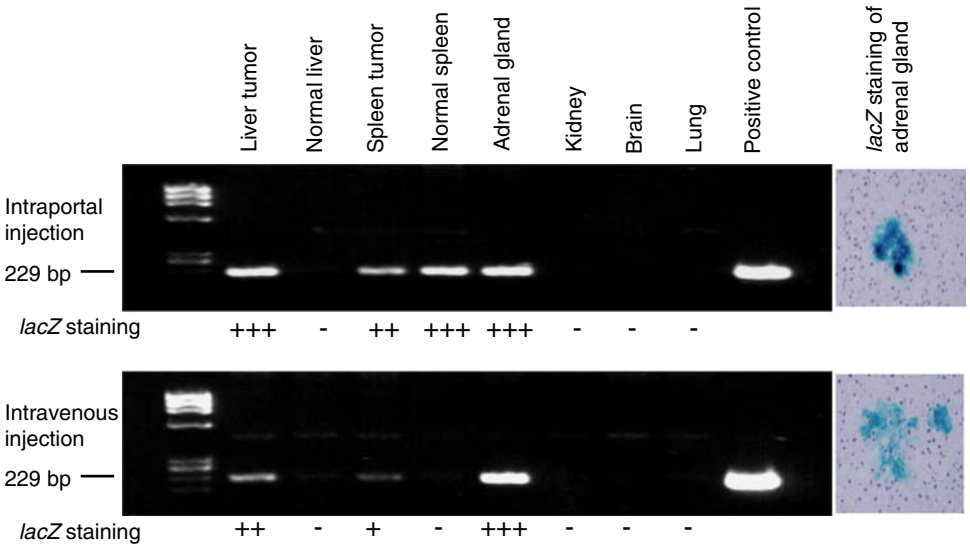


Fig. 6 HSV-1 detection in mice by PCR assay and histochemical lacZ analysis. Mice bearing diffuse liver metastases were treated with intravascular inoculation of 10^7 pfu of hrR3 7 days after tumor implantation. Mice were killed 2 days later to harvest tissue for PCR amplification of a 229-bp portion of the HSV-1 DNA polymerase gene and histochemical examination of lacZ expression. When mice were treated by intraportal injection, HSV-1 DNA and lacZ expression was detected in liver tumor, spleen tumor, normal spleen (hrR3 injection site), and adrenal gland. When mice were treated with intravenous injection, HSV-1 DNA and lacZ expression was detected in tumor but not in normal tissue or organs except for the adrenal gland. In both treatment groups, the sections of adrenal gland had the strongest staining. lacZ staining: *dash* no lacZ staining, *plus* few lacZ staining, *double plus* moderate lacZ staining, *triple plus* strong lacZ staining

In vitro assay of infection of hrR3 in vascular endothelial cells

In vitro infection efficiency was measured by staining for β-galactosidase with X-gal solution 24 h after infection with hrR3 at MOI values ranging from 0.01 to 1.0. Many HMVEC-L cells had positive staining by X-gal histochemistry in a dose-dependent fashion. Stained cells were shrunken or swollen in shape compared with the nonstained cells (Fig. 7). This result indicates that hrR3 can infect vascular endothelial cells and replicate in the cells. We herein hypothesize that if vascular endothelial cells are in an actively dividing state, such as that seen with trauma and inflammation, hrR3 has a possibility to infect vascular endothelial cells when delivered in the bloodstream.

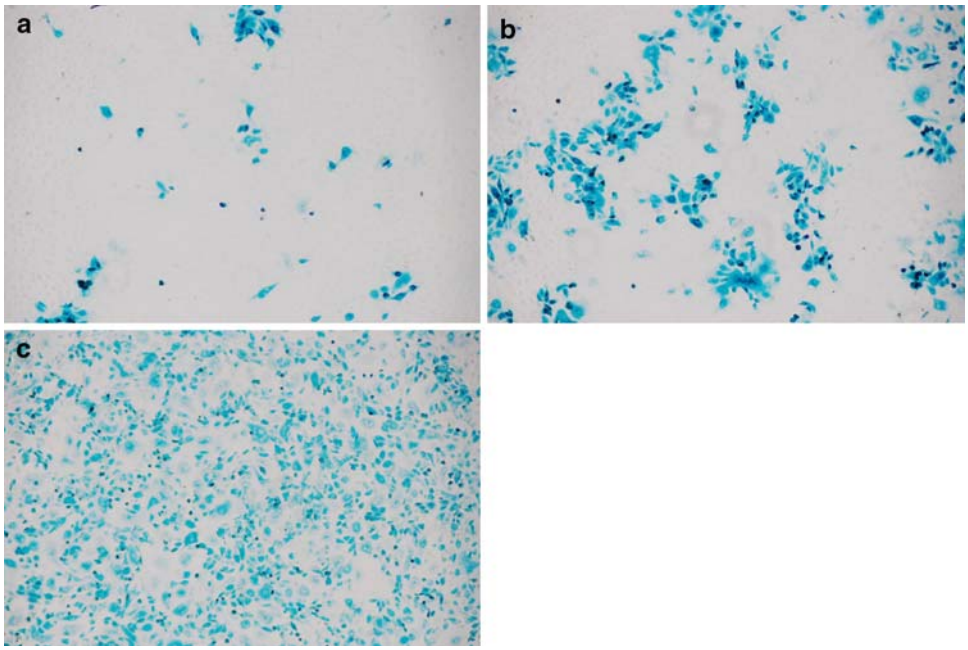


Fig. 7 In vitro infection efficiency was measured by staining for β-galactosidase with X-gal solution 24 h after infection with hrR3 at MOI values ranging from 0.01 to 1.0. Many HMVEC-L cells stained blue by X-gal histochemistry in a dose-dependent fashion (**a** MOI = 0.01, **b** MOI = 0.1, **c** MOI = 1.0). Stained cells were shrunken or swollen in shape compared with the nonstained cells (×200)

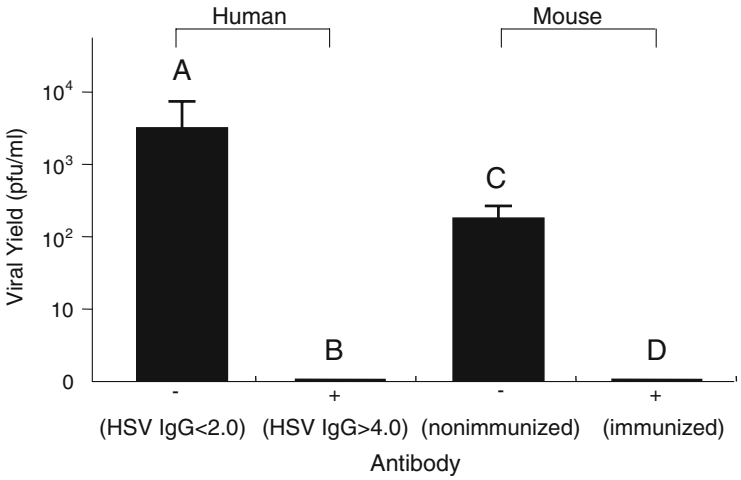
Effect of anti-HSV antibody on hrR3 in circulation

Most human adults possesses anti-HSV antibodies, so it is theoretically possible that oncolytic virus is attenuated by circulating antibodies when delivered by intravascular injection in humans. To examine the effect of antibodies against hrR3 in the bloodstream, we performed an in vitro viral neutralization assay. The values of anti-HSV IgG tested by enzyme immunoassay (EIA) were >92, >92, and 9.4, respectively, in the anti-HSV antibody-positive individuals, and <2.0 in all antibody-negative individuals. When hrR3 was mixed with anti-HSV antibody-negative serum, the titer of hrR3 was $4.1 \pm 5.1 \times 10^3$ pfu. In contrast, when hrR3 was mixed with anti-HSV antibody-positive serum, the titer of hrR3 was 0 pfu for all three serum samples (Fig. 8). Next we performed viral neutralization assay using immunized mice. When hrR3 was mixed with serum from nonimmunized mice, the titer was $2.0 \pm 1.0 \times 10^2$ pfu. In contrast, when hrR3 was mixed with serum from immunized mice, the titer was 0 pfu in all samples. These results demonstrated that the infectious activity of hrR3 could be significantly attenuated by anti-HSV antibodies in serum of human and immunized animals.

Discussion

Genetically engineered, conditionally replicating HSV-1 is a promising therapeutic agent for cancer therapy. Because the HSV-1 mutant, hrR3, lacks a functional ribonucleotide reductase gene, it preferentially replicates in cells with high levels of endogenous ribonucleotide reductase activity. The main antitumor mechanism of oncolytic viruses results from viral replication within infected tumor cells, resulting in cell destruction, and liberation of progeny virus particles that can directly infect adjacent tumor cells. Recently, the antiangiogenic activity of oncolytic HSV was reported as an additional antitumor mechanism [3, 31, 47]. Angiogenesis, the process through which new blood vessels develop from preexisting vascular endothelial cells, plays a crucial role in solid tumor growth [13]. Because one endothelial cell can support 5–50 tumor cells [30], disruption of vasculature is an efficient strategy to control tumor growth and metastases. Benencia et al. [3] demonstrated that HSV-1716 lacking ICP34.5 could infect and kill tumor endothelial cells and exert antiangiogenic effects by intravascular inoculation in an ovarian cancer animal model.

In this study, we demonstrated the efficacy of intravascular oncolytic virus therapy for the treatment of multiple colorectal liver metastases. We established a diffuse liver



	Viral Yield (pfu/ml)		
A	1.7×10^3	0.7×10^3	10×10^3
B	0	0	0
C	2×10^2	3×10^2	1×10^2
D	0	0	0

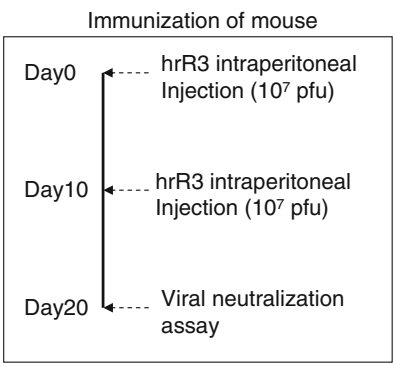


Fig. 8 Anti-HSV antibody inactivates oncolytic HSV. Serum was harvested from six human adults, three of whom had anti-HSV antibodies (IgG > 4.0) and three who did not (IgG < 2.0). When hrR3 was mixed with anti-HSV antibody-negative serum, the titer of hrR3 was $4.1 \pm 5.1 \times 10^3$ pfu. In contrast, when hrR3 was mixed with anti-HSV antidody positive serum, the titer of hrR3 was 0 pfu in all three samples. Mice were immunized via intraperitoneal injection with 10^7 pfu of hrR3, and serum was harvested 10 days after the second immunization. When hrR3 was mixed with serum from nonimmunized mice, the titer was $2.0 \pm 1.0 \times 10^2$ pfu. In contrast, when hrR3 was mixed with serum from immunized mice, the titer of hrR3 was 0 pfu in all three samples

metastases model by splenic injection of MC26 colon carcinoma cells. We performed intraportal or intravenous therapy using hrR3, and compared the therapeutic efficacy between the two intravascular delivery systems. Both intraportal and intravenous treatments with hrR3 demonstrated significant tumor growth inhibition and prolongation of survival, and hrR3-treated mice developed no clinical side effects attributable to hrR3 administration. However, there was no statitistical difference in liver weights or survival rates among the two treatment groups. The effect of the oncolytic virus administered in blood vessels may depend not only on the direct cytotoxic effect of the virus but also on the antiangiogenic effect exerted as a result of direct infection of tumor endothelial cells.

In a separate experiment we compared the biodistribution of hrR3 by examining mouse tissue for the presence of the HSV-1 DNA polymerase gene. HSV-1 DNA was detected in liver tumor but not in normal liver after either intraportal or intravenous injection, but the amount of HSV-1 DNA expressed in liver tumor was significantly less after intravenous injection compared with intraportal injection. This result indicates that the greater the distance from the injection site to the target tumor, the less the amount of virus can reach and infect the target cancer cells.

We assumed that hrR3 was consumed and reduced in the bloodstream before reaching target cancer cells. Because hrR3 preferentially replicates in dividing cells, there still remains the possibility that hrR3 can replicate in normally dividing nontumor cells, such as vascular endothelial cells, intestinal epithelial cells and bone marrow cells [49]. Yoon et al. [50] reported that hrR3 could replicate in regenerating mouse liver cells after partial hepatectomy. Miyatake et al. [29] reported that hrR3 could replicate in the proliferative phase of vascular smooth muscle cells after balloon angioplasty and prevent restenosis.

We showed that hrR3 can infect and replicate in human lung microvascular endothelial cells (HMVEC-L) in vitro. In normal vessels, vascular endothelial cells are stable and not actively dividing, so that hrR3 cannot infect stable endothelial cells. However, if the vessels are injured by trauma, inflamation or oxidative stress, vascular endothelial cells of the injured vessels may be in an actively dividing state to heal the injury and be easily infected by hrR3. Several reports indicate that HSV infection of endothelial cells attracts leukocytes with subsequent inflammatory damage, activates procoagulant changes in endothelium with increased thrombin generation and platelet adhesion, changes the interaction of endothelium with extracellular matrix proteins, and leads to atherosclerosis [26, 44, 45]. If oncolytic virus infects some locus of vascular endothelial cells before reaching target tumor and replicating, the virus would be detected for 2–3 days and then wiped out by human immunity. We think that virus cannot maintain a continuous infection on the vascular endothelial cells, and releasing the progeny to a targeted tumor. That means the amount of virus that reaches target organs would be reduced if the virus were to infect vascular endothelial cells en route.

HSV-1 is a natural human pathogen, and the majority of adults have circulating anti-HSV antibodies. Preexisting immunity is a theoretical obstacle for virus-based therapies. Herrlinger et al. [17] demonstrated that gene transfer to brain tumors using hrR3 was greatly reduced, but not completely abolished in the setting of strong anti-HSV-1 immunity and that there was no difference in the efficacy of tumor treatment whether or not animals were seropositive. Chahlavi et al. [9] showed that the HSV-1 mutant vector, G207, inhibited tumor growth to a similar extent whether the mice had anti-HSV immunity or not, and multiple treatments with G207 were far superior to a single treatment. However, these results were induced by intratumor inoculations of oncolytic HSV mutants. It is conceivable that oncolytic HSV mutants administered by intravascular injection may be more easily blocked by anti-HSV immunity compared with intratumor injection. Results from our in vitro viral neutralization assay showed that anti-HSV immunity greatly attenuated infectious activity of hrR3 delivered into blood vessels. Delman et al. [12] tested the anticancer effects of NV1020 or G207, two multimutated HSV-1 oncolytic viruses, in immunocompetent mice previously immunized with wild-type HSV-1. They treated multiple hepatic metastatic cancers in immunized mice by intraportal or intravenous injection of oncolytic viruses. Intraportal viral therapy in immunized mice significantly reduced hepatic tumor nodule counts, and low-dose portal therapy with 10^6 pfu yielded results similar to those of high-dose therapy with 10^7 pfu. When virus is administered intravenously, high-dose therapy significantly reduced tumor nodule counts, whereas low-dose therapy did not show a significant reduction. Their results indicate that when the distance from the site of viral administration to target cells is long, virus is likely to be affected by circulating antibodies, but that when given at appropriate doses, circulating antibodies have minimal measurable effects on viral oncolytic therapy. On the other hand, pre-existing anti-HSV-1 immunity is considered to serve a positive role in preventing the nonspecific toxicity of the vector [17]. Thus, in clinical trials using HF10 for breast cancer, we chose patients who had serum anti-HSV antibodies [35].

Results of X-gal histochemistry and PCR analysis indicated that hrR3 could selectively replicate within cancer cells but not within normal cells except for the adrenal gland. It is well known that the adrenal gland is an important target for HSV infection [11, 18, 19, 33]. High concentrations of corticosteroid hormone in the adrenal cortex are supposed to suppress local immunity and increase the sensitivity to HSV infection [37]. Moreover, macrophages serve as a frontal

barrier to acute HSV infection, but the number of macrophages in the adrenal gland is lower than the number found in the liver or spleen [2, 16]. Previous studies involving adrenalectomy in mice have demonstrated that the adrenal gland is the main source of virus entering the central nervous system [18]. After replication in the adrenal gland, virus enters the preganglionic nerve fibers, which supply the medulla, and spreads through tracts of autonomic fibers in the ventral white matter of the thoracic spinal cord [6, 7]. Such involvement of the spinal cord is associated with demyelination and bilateral hind limb paralysis. In our experiments, no mice showed any complications, such as paralysis or convulsions, after intravascular injection of hrR3. Furthemore, neither HSV-1 DNA nor lacZ expression were detected in the brains of hrR3-treated mice. We hypothesize that because adrenal cells are normal and nondividing, hrR3-infected adrenal cells fall into apoptosis preventing viral replication. In our clinical experiments with intratumor injection of HF10, there seemed to be no evidence of histochemical damage in the adrenal glands and irregular corticosteroid hormone from the adrenal glands. However, before starting clinical trials of intravascular injections, attention should be paid to avoid harmful side effects.

In conclusion, the present study supports the feasibility of intravascular administration of oncolytic HSV-1 mutants for the treatment of multiple liver metastases with several caveats: (1) HSV oncolytic virus can reach the target tumor and reduce tumor growth after intravascular administration, but anti-HSV antibodies may attack and attenuate the virus; (2) vascular endothelial cells might be damaged by oncolytic virus if they are in the dividing state; and (3) adrenal glands might be infected by virus. These points should be taken into account in clinical trials using intravascular virus administration. Although the most common route of delivery of oncolytic HSV-1 has been a direct intratumor inoculation, an intravascular, especially intravenous, delivery would further broaden the clinical application of oncolytic HSV-1 vectors and may be used as a form of combination therapy together with chemotherapy or radiation in the near future. Preclinical studies of the possible toxicity after intravascular administration of oncolytic HSV will be nessesary in order to consider this therapeutic strategy for human use.

Acknowledgment This work was supported by the Takeda Science Foundation[I] 2006.

References

1. Adam R, Pascal G, Castaing D, Azoulay D, Delvart V, Paule B, Levi F, Bismuth H (2004) Tumor progression while on chemotherapy: a contraindication to liver resection for multiple colorectal metastases? Ann Surg 240:1052–1064
2. Aita K, Irie H, Koyama AH, Fukuda A, Yoshida T, Shiga J (2001) Acute adrenal infection by HSV-1: role of apoptosis in viral replication. Arch Virol 146:2009–2020
3. Benencia F, Courreges MC, Conejo-Garcia JR, Bucknovich RJ, Zhang L, Carroll RH, Morgan MA, Coukos G (2005) Oncolytic HSV exerts antiangiogenic activity in ovarian carcinoma. Hum Gene Ther 16:765–778
4. Biasco G, Derenzini E, Grazi GL, Ercolani G, Ravaioli M, Pantaleo MA, Brandi G (2006) Treatment of hepatic metastases from colorectal cancer: many doubts, some certainties. Cancer Treat Rev 32:214–228
5. Breakefield XO, Kramm CM, Chiocca EA, Pechan PA (1996) Herpes simplex virus vectors for tumor therapy. In: Sobel RE, Scanlon KJ (eds) The internet book of gene therapy. Cancer therapeutics. Appleton & Lange, Hartford, pp 41–56
6. Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ, Kalsbeek A (1999) Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur J Neurosci 5:1535–1544
7. Burgos JS, Ramirez C, Sastre I, Bullido MJ, Valdivieso F (2002) Involvement of apolipoprotein E in the hematogenous route of herpes simplex virus type 1 to the central nervous system. J Virol 76:12394–12398
8. Carroll NM, Chiocca EA, Takahashi K, Tanabe KK (1996) Enhancement of gene therapy specificity for diffuse colon carcinoma liver metastases with recombinant herpes simplex virus. Ann Surg 224:323–330
9. Chahlavi A, Rabkin SD, Todo T, Sundaresan P, Martuza RL (1999) Effect of prior exposure to herpes simplex virus 1 on viral vector-mediated tumor therapy in immunocompetent mice. Gene Ther 6:1751–1758
10. Coffey MC, Strong JE, Forsyth PA, Lee PEK (1998) Reovirus therapy of tumors with activated ras pathway. Science 282:1332–1334
11. Corey L, Spear PG (1986) Infection with herpes simplex viruses. N Engl J Med 314:686–691
12. Delman KA, Bennett JJ, Zager JS, Burt BM, McAuliffe PF, Petrowsky H, Kooby DA, Hawkins WG, Horsburgh BC, Johnson P, Fong F (2000) Effects of preexisting immunity on the response to herpes simplex-based oncolytic viral therapy. Hum Gene Ther 11:2465–2472
13. Folkman J (2003) Fundamental concepts of the angiogenic process. Curr Mol Med 3:643–651
14. Goldstein DJ, Weller SK (1998) Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. Virology 166:41–51
15. Hamel W, Magnelli L, Chiarugi VP, Israel MA (1996) Herpes simplex virus thimidine kinase/ganciclovir-mediated apoptotic death of bystander cells. Cancer Res 56:2697–2702
16. Harada Y, Kurokawa E, Endo H, Kimura T, Saito M, Sugawara Y, Irie H, Ito K, Fujii M, Shimizu Y, Kumagai K (1989) Protection of OK-432, streptococcus pyogenes preparation, against lethal infection of mice with herpes simplex virus. Microbiol Immunol 33:467–477
17. Herrlinger U, Kramm CM, Aboody-Guterman KS, Silver JS, Ikeda K, Johnston KM, Pechan PA, Barth RF, Finkelstein D, Chiocca EA, Louis DN, Breakefield XO (1998) Pre-existing herpes simplex virus 1 (HSV-1) immunity decreases, but does not abolish, gene transfer to experimental brain tumor by a HSV-1 vector. Gene Ther 5:809–819
18. Hill TJ, Yirrell DL, Blyth WA (1986) Infection of the adrenal gland as a route to the central nervous system after viraemia with herpes simplex virus in the mouse. J Gen Virol 67:309–320
19. Irie H, Harada Y, Kurokawa E, Saito M, Sugawara Y, Ohami H, Mori W (1987) Early adrenal infection by herpes simplex virus type-1 (Miyama + GC strain): special reference to inoculation dose and spread from the adrenal to the central nervous system. Virchows Arch B Cell Pathol Incl Mol Pathol 53:325–331

20. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007) Cancer Statistics, 2007. CA Cancer J Clin 57:43–66
21. Kasuya H, Nishiyama Y, Nomoto S, Hosono J, Takeda S, Nakao A (1999) Intraperitoneal delivery of hrR3 and ganciclovir prolongs survival in mice with disseminated pancreatic cancer. J Surg Oncol 72:136–141
22. Kasuya H, Pawlik TM, Mullen JT, Donahue JM, Nakamura H, Chandrasekhar S, Kawasaki H, Choi E, Tanabe KK (2004) Selectivity of an oncolytic herpes simplex virus for cells expressing the DF3/MUC1 antigen. Cancer Res 64:2561–2567
23. Kimata H, Imai T, Kikumori T, Teshigahara O, Nagasaka T, Goshima F, Nishiyama Y, Nakao A (2006) Pilot study of oncolytic viral therapy using mutant herpes simplex virus (HF10) against recurrent metastatic breast cancer. Ann Surg Oncol 13:1078–1084
24. Kooby DA, Carew JF, Halterman MW, Mack JE, Bertino JR, Blumgart LH, Federoff HJ, Fong Y (1999) Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1 (G207). FASEB J 13:1325–1334
25. Lorence RM, Roberts MS, O'Neil JD, Groene WS, Miller JA, Mueller SN, Bamat MK (2007) Phase 1 clinical experience using intravenous administration of PV701, an oncolytic Newcastle disease virus. Curr Cancer Drug Targets 7:169–174
26. MacGregor RR, Friedman HM, Macarak EJ, Kefalides NA (1980) Virus infection of endothelial cells increases granulocyte adherence. J Clin Invest 65:1469–1477
27. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM (1991) Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science 252:854–856
28. McCart JA, Ward JM, Lee J, Hu Y, Alexander HR, Libutti SK, Moss B, Bartlett DL (2001) Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. Cancer Res 61:8751–8757
29. Miyatake S, Yukawa H, Toda H, Matsuoka N, Takahashi R, Hashimoto N (1999) Inhibition of rat vascular smooth muscle cell proliferation in vitro and in vivo by recombinant replication-competent herpes simplex virus. Stroke 30:2431–2439
30. Modzelewski RA, Davies P, Watkins SC, Auerbach R, Chang MJ, Johnson CS (1994) Isolation and identification of fresh tumor-derived endothelial cells from a murine RIF-1 fibrosarcoma. Cancer Res 54:336–339
31. Mullen JT, Donahue JM, Chandrasekhar S, Yoon SS, Liu W, Ellis LM, Nakamura H, Kasuya H, Pawlik TM, Tanabe KK (2004) Oncolysis by viral replication and inhibition of angiogenesis by a replication-conditional herpes simplex virus that expresses mouse endostatin. Cancer 15:869–877
32. Mullen JT, Kasuya H, Yoon SS, Carroll NM, Pawlik TM, Chandrasekhar S, Nakamura H, Donahue JM, Tanabe KK (2002) Regulation of herpes simplex virus 1 replication using tumor-associated promoters. Ann Surg 236:502–513
33. Nachtigal M, Caulfield JB (1984) Early and late pathogenic changes in the adrenals of mice after infection with herpes simplex virus type 1. Am J Pathol 75:175–185
34. Nakamura H, Kasuya H, Mullen JT, Yoon SS, Pawlik TM, Chandrasekhar S, Donahue JM, Chiocca EA, Chung RY, Tanabe KK (2002) Regulation of herpes simplex virus $\gamma_1$34.5 expression and oncolysis of diffuse liver metastases by Myb34.5. J Clin Invest 109:871–882
35. Nakao A, Kimata H, Imai T, Kikumori T, Teshigahara O, Nagasaka T, Goshima F, Nishiyama Y (2004) Intratumoral injection of herpes simplex virus HF10 in recurrent breast cancer. Ann Oncol 15:987–991
36. Nemunaitis J, Khuri F, Ganly I, Arseneau J, Posner M, Vokes E, Kuhn J, McCarty T, Landers S, Blackburn A, Romel L, Randlev B, Kaye S, Kirn D (2001) Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. J Clin Oncol 19:289–298
37. Nishiyama Y, Rapp F (1979) Regulation of persistent infection with herpes simplex virus in vitro by hydrocortisone. J Virol 31:841–844
38. Oyama M, Ohigashi T, Hoshono M, Nakashima J, Tachibana M, Murai M, Uemura K, Yazaki T (2000) Intravesical and intravenous therapy of human bladder cancer by herpes vector G207. Hum Gene Ther 11:1683–1693
39. Pawlik TM, Nakamura H, Yoon SS, Mullen JT, Chandrasekhar S, Chiocca EA, Tanabe KK (2000) Oncolysis of diffuse hepatocellular carcinoma by intravascular administration of a replication-competent, genetically engineered herpes virus. Cancer Res 60:2790–2795
40. Peng KW, TenEyck CJ, Galanis E, Kalli KR, Hartmann LC, Russell SJ (2002) Intraperitoneal therapy of ovarian cancer using an engineered measles virus. Cancer Res 62:4656–4662
41. Penna C, Nordlinger B (2002) Colorectal metastasis (liver and lung). Surg Clin North Am 82:1075–1090
42. Potratz D, Brake B, Dienes HP, Schulz TF, Hosp M, Dierich MP, Falke D (1986) Herpes simplex virus type 1 and 2 in the adrenal glands: replication and histopathology. Arch Virol 90:207–222
43. Teshigahara O, Goshima F, Takao K, Kohno S, Kimata H, Nakao A, Nishiyama Y (2004) Oncolytic viral therapy for breast cancer with herpes simplex virus type 1 mutant HF10. J Surg Oncol 85:42–47
44. Visser MR, Vercellotti GM (1993) Herpes simplex virus and atherosclerosis. Eur Heart J 14:39–42
45. Visser MR, Vercellotti GM, McCarthy JB, Goodman JL, Herbst TJ, Furcht LT, Jacob HS (1989) Herpes simplex virus inhibits endothelial cell attachment and migration to extracellular matrix proteins. Am J Pathol 134:223–230
46. Walker JR, McGeagh KG, Sundaresan P, Jorgensen T, Rabkin SD, Martuza RL (1999) Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Hum Gene Ther 10:2237–2243
47. Wong RJ, Chan M, Yu Z, Ghossein R, Ngai I, Adusumilli PS, Stiles BM, Shah JP, Singh B, Fong Y (2004) Angiogenesis inhibition by an oncolytic herpes virus expressing interleukin 12. Clin Cancer Res 10:4509–4516
48. Wong RJ, Chan M, Yu Z, Kim TH, Bhargava A, Stiles BM, Horsburgh BC, Shah JP, Ghossein RA, Singh B, Fong Y (2004) Effective intravenous therapy of murine pulmonary metastases with an oncolytic herpes virus expressing interleukin 12. Clin Cancer Res 10:251–259
49. Yoon SS, Carroll NM, Chiocca EA, Tanabe KK (1998) Cancer gene therapy using a replication-competent herpes simplex virus type 1 vector. Ann Surg 228:366–374
50. Yoon SS, Nakamura H, Carroll NM, Bode BP, Chiocca EA, Tanabe KK (2000) An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma. FASEB J 14:301–303