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A Fusogenic Oncolytic Herpes Simplex Virus for Therapy of Advanced Ovarian Cancer

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14. ABSTRACT
The underlying hypothesis for this project is that incorporation of cell membrane fusion function into an oncolytic herpes simplex virus (HSV) can significantly enhance the anti-tumor effect of the virus. Three specific aims have been proposed and they are: 1) to demonstrate that fusogenic oncolytic HSVs are a potent anti-tumor agent for advanced ovarian cancer; 2) to prove that fusogenic oncolytic HSVs have the same safety profile as their non-fusogenic counterparts; 3) to explore novel delivery strategies that can evade host’s anti-viral immunity for repeated delivery. During the funding period, these aims have been mostly achieved. Our data show that incorporation of a fusogenic glycoprotein (GALV.fus) into an oncolytic herpes simplex virus through a novel controlling mechanism can significantly enhance the antitumor potency of the virus without significantly increasing its toxicity. In the efforts to identify a strategy that can deliver an oncolytic HSV to tumor tissues systemically in the presence of host’s antiviral immunity, we demonstrate that delivery of an oncolytic HSV as a DNA form rather the traditional viral particles represents a possibility. We also show that blood cells such as monocytes/macrophages from STAT1-deficient background could function as cell carriers for systemic delivery of these oncolytic viruses. Several publications have been produced during the funding period. One of the oncolytic viruses that were studied during the funding period is currently being evaluated for translation into clinical testing for treating solid tumors including ovarian cancer.

15. SUBJECT TERMS
Oncolytic virus, advanced ovarian cancer, experimental therapy, syncytial formation

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INTRODUCTION

Because of its inconspicuous early symptoms and the lack of effective screening techniques, ovarian cancer frequently presents at an advanced stage (III/IV), in which the disease has usually spread to the peritoneal cavity. Current therapy for advanced-stage ovarian cancer consists of debulking surgery followed by chemotherapy. Although the clinical response rate is 60–70%, most patients ultimately relapse and succumb to recurrent chemoresistant disease, leading to 14,000 deaths in the United States each year. Hence, there is an urgent need for novel therapeutics that can provide significant clinical benefits or cure for patients with advanced-stage ovarian cancer. Replication-competent herpes simplex virus (oncolytic HSV) holds considerable promise for treating malignant solid tumors such as ovarian cancer, although the potency of the virus needs improvement if its full clinical potential is to be realized. We recently demonstrated that addition of a cell membrane fusion capability to an oncolytic HSV can significantly increase the antitumor potency of the virus. The modified virus kills tumor cells efficiently and directly through both replication and cell membrane fusion. In this funded project, we propose to evaluate the antitumor potency of a newer version of the fusogenic oncolytic HSV, in which two membrane fusion mechanisms were incorporated into a single virus (Synco-2D), against ovarian cancer. Specifically, Three specific aims have been proposed and they are: 1) to demonstrate that fusogenic oncolytic HSVs are a potent anti-tumor agent for advanced ovarian cancer; 2) to prove that fusogenic oncolytic HSVs have the same safety profile as their non-fusogenic counterparts; 3) to explore novel delivery strategies that can evade host’s anti-viral immunity for repeated delivery. As detailed in the following sections, we believe that these aims have been mostly achieved during the funding period.

BODY

1. In vitro antitumor activity of doubly fusogenic oncolytic HSV against human ovarian cancer cells

Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell. When
cells showed complete cytopathic effect, heparin (Sigma, St. Louis, MO) was added to the culture medium at a final concentration of 50 μg/ml) and cells were cultured for another 3 h to release the virus into the medium. The medium was then collected and was subjected to a low speed centrifugation at 1,000 g for 10 min. The cleared supernatant was transferred to another tube and the virus was pelleted through high speed centrifugation (29,000 g for 4 h). The viral pellet was resuspended in PBS containing 10% glycerol and stored at -80°C.

We phenotypically characterized the viruses in greater detail on two human ovarian cancer cell lines (Hey-8 and SKOv3), infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. As shown in Fig. 1, the syncytial plaques after Synco-2D infection differed strikingly from the usual round-cell plaques derived from infection with Baco-1. By 40 h, the Synco-2D-infected tumor cells had fused together, forming a dense material (middle of the infectious focus) that was separated from the boundary of the plaque by a large gap area. This distinctive pattern of syncytial development was especially prominent in Hey-8 cells.

**Fig. 1.** *In vitro* phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.01 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, x200). Arrows indicate a single syncytium.
To determine if the marked syncytial formation resulting from Synco-2D infection would enhance tumor cell killing, we infected ovarian cancer cells with Baco-1 or Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), permitting us to assess both the inherent cytotoxicity of the input virus as well as the ability of the virus to replicate and spread in these cells. The cytotoxic effect of the viruses was quantified by calculating the percentage of viable cells remaining in the wells after infection. The results (Fig. 2) showed a significantly greater tumor cell killing effect by Synco-2D compared to Baco-1 ($p < 0.01$, all comparisons). At 0.01 pfu/cell, Synco-2D reduced the viable cells to less than 40% within 24 h (Fig. 2A). At 0.1 pfu/cell, it completely destroyed the Hey-8 tumor cells within 48 h, a time where there was still more than 30% viable tumor cells in the well infected with Baco-1 (Fig. 2B).

Fig. 2. Comparison of the cytotoxicity of Baco-1 and Synco-2D in cultured ovarian cancer cells. Hey-8 or SKOV3 ovarian cancer cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.01 (A) or 0.1 (B) pfu/cell. Cells were collected at 24 h or 48 h after infection, stained with trypan blue and counted. The percentage of cell viability was determined by dividing the number of viable cells from the infected well by the number of cells from an uninfected well. The data are reported as means ± standard deviations. All comparisons showed a significant advantage in cytotoxicity for Synco-2D ($p < 0.01$).
3. Therapeutic effects of Synco-2D on peritoneal metastases of ovarian cancer

Peritoneal invasion of ovarian cancer is a common and serious clinical problem. It has been reported that about 70% of late-stage ovarian cancer patients have metastatic disease in the peritoneal cavity. We therefore chose a peritoneal metastasis model (xenografted Hey-8 cells) as means to testing the efficacy of Synco-2D against human ovarian cancer. Freshly harvested Hey-8 cells were inoculated into the peritoneal cavities of nude mice at a dose of $3 \times 10^5$ per mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice were injected intraperitoneally with $2 \times 10^7$ pfu/200 µl of either Baco-1 or Synco-2D, or PBS (control), at a site distant from that of tumor cell implantation. A second intraperitoneal injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5±0.7 days (none of the mice survived), there was clear intraperitoneal dissemination of tumor, with an average of 2.5±0.9 tumor nodules found in regions distal from the site of the tumor implantation (Table 1). In the Baco-1 treatment group, 3 mice died before the end of the experiment (first death, 34 days after tumor cell implantation); 5 survivors bore a single large tumor when examined at necropsy. By contrast, none of the Synco-2D-treated mice died or were euthanized during the experiment. Strikingly, 6 of the 8 mice were entirely tumor-free at necropsy by the end of the experiment (Table 1). The other two animals had tumors that are significantly smaller than those in Baco-1-treated mice (p<0.01, Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$N$</th>
<th>No. mice with tumors</th>
<th>No. of nodules</th>
<th>Tumor weight (g)</th>
<th>Death rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>8</td>
<td>8/8</td>
<td>2.5±0.9</td>
<td>1.0±0.6</td>
<td>8/8</td>
</tr>
<tr>
<td>Baco-1</td>
<td>8</td>
<td>8/8</td>
<td>1.0±0.0$^c$</td>
<td>1.5±0.7</td>
<td>3/8$^c$</td>
</tr>
<tr>
<td>Synco-2D</td>
<td>8</td>
<td>2/8$^a$</td>
<td>0.25±0.4$^a$</td>
<td>0.1±0.3$^a$</td>
<td>0/8$^a$</td>
</tr>
</tbody>
</table>

Table 1. Results of Synco-2D therapy for xenografted human ovarian cancer in the peritoneum
\(^a\) \(p<0.01\) as compared with either PBS or Baco-1 treated group.

\(^b\) Means and standard deviations.

\(^c\) \(p<0.01\) as compared with PBS control.

4. In vivo toxicity evaluation

4.1. Intracranial injection. As HSV is a neurotropic pathogen, it is therefore important to evaluate its potential toxicity in the CNS. Although the potential toxic effect of Synco-2D on the brain was evaluated after systemic delivery (see the next section), the presence of the blood-brain barrier may prevent the virus from efficiently getting into the brain from the bloodstream. It is possible that the full extent of its neurotoxicity may not be comprehensively evaluated after systemic delivery of the virus. We therefore injected the viruses directly into mouse brain at a relatively high dose to examine its neurotoxicity in the following experiments.

Stereotactic injection of the virus solution (in the volume of 3 μl) into the brains of immune competent mice was done essentially according to the procedure as described \(^3\). Briefly, 8-week-old mice (BALB/c) were anesthetized and placed in a stereotactic frame (Stoelting). A hole was drilled in the skull 1 mm anterior and 2 mm lateral to the bregma with a 0.9-mm burr to expose the dura. The mice were then injected with 3μl of Hanks’ buffered saline containing the designated amount of virus, using a 100μl syringe (Hamilton) fitted with a 26-gauge needle and connected to the manipulating arm of the stereotactic frame. The injection, given over 1 min, was directed to the caudate nucleus at a depth of 3.5 mm from the dura. The needle was left in place for 3 min and then withdrawn slowly over another minute, to prevent reflux of the virus solution. There were 3 groups (n=8) of mice in this experiment: Synco-2D, Baco-1 and PBS. Three mice from each group were sacrificed 2 days after virus injection and the fresh brain tissue was homogenized (in 0.5 ml medium) for virus titration. The remaining five mice from each group were kept for 3 weeks before they were sacrificed. The brain was removed,
fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

The virus titration result of the brain tissues was shown in Table 2. A typical brain section from each group of mice was shown in Fig. 3. The amount of infectious viruses retrieved from the brain tissues of mice receiving either Synco-2D or Baco-1 was significantly lower than the amount of viruses that was initially injected, indicating that the oncolytic HSVs did not replicate significantly in the brain tissue. In addition, there was no significant difference on the amounts of the virus recovered from brain tissues of Synco-2D and Baco-1-treated groups. During the observation period, no animal from either group died. The brain sections show no obvious abnormality at the time (3 weeks) after virus inoculation. Together, these results indicate that Synco-2D was not significantly more toxic than the nonfusogenic Baco-1 when it was inoculated directly into the CNS.

Table 2. Quantification of oncolytic HSVs after intracranial administration

<table>
<thead>
<tr>
<th>Virus titer (pfu/ml)</th>
<th>PBS</th>
<th>Baco-1</th>
<th>Synco-2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10pfu*</td>
<td>1.5±0.2x10^2</td>
<td>2.1±0.3x10^2</td>
</tr>
</tbody>
</table>

* As the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 μl of the homogenized tissue supernatant to 400 μl of DMEM to make a total of 500 μl for infection of Vero cell monolayer seeded in 6-well plates). So if no virus plaque was detected from the well, then the amount of the infectious virus in the organ was <10 pfu/ml.
Fig. 3. Histology of brain tissues after intracranial delivery of oncolytic HSVs. The brain sections were prepared from the indicated mice at 3 weeks after virus delivery. Original magnification: 200X.

4.2. Systemic injection. Even when intratumorally delivered, it is still possible that the oncolytic HSVs may leak from the tumor site into the blood stream. It is therefore important to assess the toxicity of Synco-2D after systemic delivery. Six-week-old immune competent BALB/c mice were injected through the tail vein with either the doubly fusogenic Synco-2D or Baco-1 at two different doses: $1 \times 10^7$ or $1 \times 10^8$ pfu, or merely PBS as a negative control. There were 13 mice in each group, 5 for evaluating virus distribution/histological examination and the other 8 for determining mortality.

Toxicity evaluation criteria:

1) Incidence of mortality. For evaluation of mortality after oncolytic HSV administration, animals were observed for a six-month period. During the observation period, one mouse from the groups receiving the higher dose of Synco-2D and Baco-1 died during the acute phase of virus infection (at day 2 and day 3 after virus infusion, respectively). No serious disease symptom was noticed afterwards during the observation period (Table 3).

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Dose</th>
<th>Route admin.</th>
<th>No. mice</th>
<th>mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baco-1</td>
<td>$1 \times 10^7$</td>
<td>i.v.</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Baco-1</td>
<td>$1 \times 10^8$</td>
<td>i.v.</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Synco-2D</td>
<td>$1 \times 10^7$</td>
<td>i.v.</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Synco-2D</td>
<td>$1 \times 10^8$</td>
<td>i.v.</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>PBS</td>
<td>200 $\mu l$</td>
<td>i.v.</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
2) Histopathological findings after systemic administration of oncolytic HSVs and quantification of retrievable virus from the major organs

For the purpose of determining histological abnormality, mice were euthanized 5 days after virus administration. Major organs were collected and were cut into half. One half was immediately homogenized and the lysate was used for titration on Vero cell monolayers to determine the virus distribution. Another half of the organ tissues was embedded in paraffin and sections were prepared for histological examinations. Only infrequent mild histological abnormality was noticed in liver sections from mice receiving administration of either Baco-1 or Synco-2D (scored as either +). No obvious pathological changes were identified in any other organs (Table 4). The virus titration results from the homogenized organ tissues (Table 5) showed that only a small quantity of infections virus could be detected from liver, which is the major site of virus deposit after systemic delivery. No infectious virus could be detected from any other major organs. These results demonstrate that systemic administration of either the doubly fusogenic Sync-2D or the nonfusogenic Baco-1 only causes a mild and transient liver damage and Synco-2D does not seem to be significantly more toxic than Baco-1 to the liver. This mild liver damage is probably due to the preferred deposit of viruses to the liver after systemic delivery.

Table 4. Results of histological examination after systemic administration of oncolytic HSVs

<table>
<thead>
<tr>
<th>Organs</th>
<th>PBS</th>
<th>Baco-1</th>
<th>Synco-2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100μl</td>
<td>1X10^7</td>
<td>1X10^8</td>
</tr>
<tr>
<td>Liver</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pancreas</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heart</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lung</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Spleen</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Brain</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Table 5. Infectious virus distribution in major organs after systemic administration of oncolytic HSVs

<table>
<thead>
<tr>
<th>Organs</th>
<th>PBS</th>
<th>Baco-1</th>
<th>Synco-2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100μl</td>
<td>1X10⁷</td>
<td>1X10⁸</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;10 pfu/ml †</td>
<td>&lt;10 pfu/ml</td>
<td>22.6±4.5 pfu/ml</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
<tr>
<td>Pancreas</td>
<td>ND*</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
<tr>
<td>Heart</td>
<td>ND</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
<tr>
<td>Brain</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
<td>&lt;10 pfu/ml</td>
</tr>
</tbody>
</table>

†As the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 μl of the homogenized tissue supernatant to 400 μl of DMEM to make a total of 500 μl for infection of Vero cell monolayer seeded in 6-well plates). So if no virus plaque was detected from the well, then the amount of the infectious virus in the organ was <10 pfu/ml;

*Not done, due to bacteria contamination of the samples.

5. Studies of antiviral immune responses and development of strategies to evade them

5.1. Anti-HSV immune serum can’t prevent spread of fusogenic oncolytic HSV in vitro

To compare the effects of anti-HSV immune serum on the spread of fusogenic and non-fusogenic oncolytic HSV in vitro, we initially infected tumor cells (in 12-well plates) with either Synco-2D or Baco-1 at 0.01 pfu/cell for 1 h. Then, medium containing pooled mouse anti-HSV sera at 1:50 dilution, which completely blocked Baco-1 infectivity on Vero cells, was added to the wells. As shown in Fig. 4, inclusion of the immune sera had minimal effect on syncytial formation by Synco-2D, but greatly inhibited the spread of non-fusogenic oncolytic HSV. These findings support the underlying
hypothesis that the antitumor activity of fusogenic oncolytic HSV is less affected by antiviral immunity when compared to a conventional oncolytic HSV once the virus has entered into target cells.

5.2. Liposome formulation of oncolytic HSVs.

Next we tested if formulating oncolytic HSVs with liposomes could evade the host’s humoral antiviral immunity. We initially tested the condition of liposome formulation of oncolytic HSVs. We prepared the virus in 3 different forms: 1) DNA form of viral genome; 2) intact viral particles; 3) de-enveloped viral capsid. We then formulated these different forms of virus preparations with liposomes and examined their infectivity in vitro by counting the plaques after adding them onto Vero cell monolayers. The results showed that viral DNA formulated with liposomes gave the highest number of plaques. Viral capsids formulated with liposome gave the second best result. The intact viral particles formulated with liposome produced the lowest number of viral plaques, possibly because the intact viral particles already contain an outside lipid membrane, which may have prevented liposome formulation.

5.3. Delivery of oncolytic HSVs by carrier cells. We also tested if T-lymphocytes could act as a carrier for oncolytic HSV delivery. As T lymphocytes can freely circulate through the vascular system and can infiltrate to the metastatic tumors, once loaded with the oncolytic HSVs, they may be able to send the virus selectively to the tumor sites. Furthermore, the cell membrane may function as a protective shield to protect the virus from neutralizing antibodies. Human or murine T cells were infected with
Baco-1, which carries the green fluorescent protein (GFP) marker gene in its viral genome and its infectivity can thus be easily identified. The results showed either human or murine T lymphocytes were resistant to infection by oncolytic HSVs. Even at a multiplicity (MOI) of 10, less than 5% of cells showed GFP expression. We then examined NK cells and macrophages, another two cell types that are major components of circulating blood. In addition, both NK cells and macrophages have the ability to infiltrate to tumor tissues. We initially performed a similar in vitro experiment as described above to test the infectivity of the oncolytic Baco-1 on these cells. The results showed that both NK cells and macrophages from human and murine origins were also resistant to infection by Baco-1. At an MOI of 5, less than 10% of cells showed GFP expression.

Recently it has been reported that retrovirus can adhere nonspecifically, or 'hitchhike', to the surface of cytotoxic t lymphocytes (CTLs). CTLs hitchhiked with a retroviral vector can then move to tumor sites and “hand off” the viruses to tumor cells to initiate virus infection. We thus tested this strategy to determine if oncolytic HSV could hitchhike to carrier cells that were found to be non-permissive to infection of HSV vectors in our previous experiments. We incubated either NK cells or macrophages of both human and murine origins ($1 \times 10^5$) with $5 \times 10^5$ plaque-forming-units (pfu) of oncolytic HSV Baco-1 for 1 h. The cells were then gently washed 2 times with PBS and were added to Vero cell monolayers. Viral plaques were examined 48 h later. The results showed that Baco-1 was efficiently loaded to the carrier cells in this way and was then successfully “handed off” to the testing cell monolayers. Since more than $1 \times 10^5$ plaques were obtained from each preparation (with $1 \times 10^5$ carrier cells), it was concluded that approximately 100% of the carrier cells were able to hand off the oncolytic virus. These results indicate that this hitchhike strategy may be a useful way for carrier cell-medicated delivery of oncolytic HSVs for the treatment of metastatic prostate cancer.

**KEY RESEARCH ACCOMPLISHMENTS**

- In vitro work showed that Synco-2D infection induces a distinctive syncytial phenotype and
a significantly enhanced killing activity against human ovarian cancer cells.

- Intraperitoneal injection of Synco-2D had a significantly better therapeutic effect against metastatic human ovarian cancer xenografts than the nonfusogenic HSV, leading to eradication of all tumor masses in 75% of the animals, whereas no animals in the conventional oncolytic HSV treated group was tumor-free.

- Direct injection of the virus to the CNS at a relatively large dose did not cause any mortality. Virus titration from the brain tissue 2 days after virus administration indicates that the viruses had very little replication capability in the brain, which is very important for its potential clinical application, as HSV is considered as neurotropic.

- Systemic administration of oncolytic HSVs caused a mild liver abnormity as identified by histological examination. However, there was no significant difference on the severity of liver toxicity between the doubly fusogenic and the nonfusogenic oncolytic HSVs. This mild liver toxicity is most likely due to the preferred deposit of the systemically delivered viruses to this organ. Virus titration did not detect any residual infectious virus from other major organs.

- Fusogenic oncolytic HSVs have the ability to spread to surrounding tumor cells even in the presence of high concentration of antiviral immune sera, indicating the ability of this unique type of oncolytic HSV to evade the host’s acquired antiviral immunity once the virus has entered into the target cells.

- Oncolytic HSVs could be formulated with liposomes for in vivo delivery. Especially, preparation of oncolytic HSVs in the DNA or de-enveloped capsid form could be more efficiently formulated with liposomes than the intact viral particles. Thus, these two forms of viral preparation will be used in the future in vivo studies.

- Our data demonstrate that macrophages could function as carrier cells for delivery of oncolytic HSVs by the recently reported “Hitchhike” strategy. This will likely represent a
very efficient way of loading oncolytic virus to the carrier cells, which are otherwise non-permissive to infection of oncolytic HSVs.

REPORTABLE OUTCOMES


4. Conference presentation: Dr. Zhang was one of three invited overview speakers at the 29th International Herpesvirus Workshop (held at Reno, Nevada, July 25-30, 2004). Title of talk: HSV vectors for gene therapy of solid tumors and genetic diseases.


7. Dr. Zhang was invited to give a platform presentation at the 4th International Conference on Oncolytic Viruses as Cancer Therapeutics, held at Carefree, Arizona, March 14-17, 2007. He reported the most recent findings from his lab, including studies from this project.

CONCLUSIONS

1. The doubly fusogenic Synco-2D is an effective therapeutic agent against advanced ovarian cancer.

2. Despite its dramatically increased antitumor effect, Synco-2D is as safe as the first generation oncolytic HSV when extensively evaluated in vivo.

3. Novel delivery strategies, such as liposome formulation of viral DNA or capsid instead of intact viral particles, may evade host’s antiviral immunity. Additionally, oncolytic HSVs may be systemically delivered by carrier cells such as lymphocytes and macrophages, in the presence of host’s anti-HSV immunity.

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Effectve Therapy of Metastatic Ovarian Cancer with an Oncolytic Herpes Simplex Virus Incorporating Two Membrane Fusion Mechanisms

Mikihito Nakamori, Xinping Fu, Feng Meng, Aiwu Jin, Lihua Tao, Robert C. Bast, Jr., and Xiaolu Zhang

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ABSTRACT

Purpose and Experimental Design: Replication-competent herpes simplex virus [HSV (oncolytic HSV)] holds considerable promise for treating malignant solid tumors, although the potency of the virus needs improvement if its full clinical potential is to be realized. Incorporation of membrane fusion capability into an oncolytic HSV, either by screening for a syncytial HSV mutant after random mutagenesis or by inserting a hyperfusogenic glycoprotein from gibbon ape leukemia virus into the viral genome, can significantly enhance the antitumor effects of the virus (X. Fu and X. Zhang, Cancer Res., 62: 2306–2312, 2002; X. Fu et al., Mol. Ther., in press, 2003). We reasoned that both fusogenic strategies, incorporated into a single oncolytic HSV, might significantly improve virotherapy for ovarian cancer.

Results: In vitro characterization of a doubly fusogenic oncolytic HSV (Synco-2D) showed that this virus produces a distinctive syncytial phenotype, leading to a significantly increased tumor cell killing ability, compared with that of a nonfusogenic virus. When injected directly into the abdominal cavity of mice bearing human ovarian cancer xenografts, Synco-2D eradicated all tumor masses in 75% of the animals, whereas no animals in the conventional oncolytic HSV-treated group were tumor free.

Conclusions: This newly generated fusogenic oncolytic HSV is a promising candidate for clinical testing against advanced ovarian cancer.

INTRODUCTION

EOC is the leading cause of death from gynecological malignancies. An estimated 23,400 new cases of EOC are diagnosed in the United States each year (1). Because of its inconspicuous early symptoms and the lack of effective screening techniques, this disease frequently presents at an advanced stage (III/IV), in which the disease has usually spread to the peritoneal cavity (2). Current therapy for advanced-stage ovarian cancer consists of debulking surgery followed by chemotherapy (3). Although the clinical response rate is 60–70%, most patients ultimately relapse and succumb to recurrent chemoresistant disease, leading to 14,000 deaths in the United States each year (1). Hence, there is an urgent need for novel therapeutics that can provide significant clinical benefits or cure for patients with advanced-stage EOC.

One experimental therapy that holds great promise for solid tumors such as ovarian cancer is the use of replication-competent (oncolytic) HSV (4, 5). These viruses can infect, replicate in, and kill tumor cells by a direct cytopathic effect, while showing only restricted ability to replicate in normal cells (6–8). In early clinical trials, however, treatment with the current generation of oncolytic viruses did not significantly affect tumor growth or improve prognosis (9, 10). This suboptimal result may reflect viral gene deletions, which can reduce the replicative potential of viruses in tumor cells. For example, deletion of the γ34.5 gene significantly reduced viral growth even in rapidly dividing cells (8, 11–13). Hence, improvements in the potency of these oncolytic viruses are required to obtain clear benefits in cancer patients.

We recently reported that addition of a cell membrane fusion capability to an oncolytic HSV can significantly increase the antitumor potency of the virus (14, 15). The modified virus kills tumor cells efficiently and directly through both replication and cell membrane fusion. These two cytolytic mechanisms may also produce a synergistic effect through syncytial formation that facilitates the spread of the oncolytic virus in tumor tissue. The fusogenic activity of the virus was generated by random mutagenesis of a well-characterized oncolytic HSV (14) or by inserting a hyperfusogenic membrane glycoprotein from gibbon ape leukemia virus (GALV.fus) into the viral genome (15). In either case, the fusogenic oncolytic HSV showed strikingly enhanced antitumor activity, compared with that of the nonfusogenic virus, when tested against locally established liver cancer (15) or lung metastases of breast cancer (14).

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4 The abbreviations used are: EOC, epithelial ovarian cancer; HSV, herpes simplex virus; pfu, plaque-forming unit(s); FBS, fetal bovine serum; BAC, bacterial artificial chromosome; GFP, green fluorescent protein.
We report here the properties of a newer version of the fusogenic oncolytic HSV, in which two membrane fusion mechanisms were incorporated into a single virus (Synco-2D). In vitro characterization showed that Synco-2D has a distinctive pattern of syncytial formation, leading to a significant increase in tumor cell killing. When injected directly into the abdominal cavities of mice bearing human ovarian cancer xenografts, Synco-2D induced complete remissions in 75% of the treated animals, in contrast to partial remissions in the group treated with a conventional oncolytic HSV. These results suggest that this newly generated fusogenic oncolytic HSV is a promising candidate for in vivo virotherapy against advanced ovarian cancer.

MATERIALS AND METHODS

Cell Lines and Mice. African green monkey kidney cells (Vero) were purchased from American Type Culture Collection (Manassas, VA). The Hey-8 cell line was established from a peritoneal deposit of a moderately differentiated papillary ovarian cystoadenocarcinoma and has been reported to be moderately resistant to chemotherapy (16, 17). SKOv3 cells were derived from serous cystoadenocarcinoma. All cells were cultured in DMEM containing 10% FBS. Female BALB/c mice or Hsd athymic (nu/nu) mice [obtained from Harlan (Indianapolis, IN)] were housed under specific pathogen-free conditions and used in experiments when they attained the age of 7–8 weeks.

Construction of Fusogenic HSV Vectors. All oncolytic HSVs were derived from fHSV-Δ-pac, a BAC-based construct that contains a mutated HSV genome, in which the diploid gene encoding γ34.5 and both copies of HSV packaging signal have been deleted (18). Infectious HSV cannot be generated from this construct unless an intact HSV packaging signal is provided in cis; otherwise, the virus will be replication conditional due to the deletion of both copies of γ34.5. Baco-1 was constructed by inserting a DNA sequence containing a HSV packaging signal and an enhanced GFP gene cassette into the unique PacI restriction site located in the BAC sequence in fHSV-Δ-pac, as described previously (15). To generate Synco-2D, we initially subjected Baco-1 to random mutagenesis (14). The syncytial phenotype was identified by screening the mutagenized virus on Vero cells. The circular form of viral DNA was then obtained with high-speed centrifugation (29,000 g) for 10 min. The viral pellet was resuspended in PBS containing 10% glycerol and stored at −80°C.

Characterization of Newly Generated HSV Vector and in Vitro Cell Killing Assay. For phenotypic characterization of the new virus, Hey-8 or SKOv3 ovarian cancer cells were seeded into 6-well plates and then infected the following day with Baco-1 or Synco-2D at a dose of 0.01 pfu/cell. Cells were cultured in a maintenance medium (containing 1% FBS) and left for up to 2 days to allow the fusion pattern and plaques to develop.

For viral growth characterization, Vero cells seeded in triplicate in 6-well plates were infected with the viruses at 0.01 or 0.1 pfu/cell for 1 h. Cells were washed once with PBS to remove unadsorbed and uninternalized viruses before 1 ml of fresh medium was added. Cells were harvested at 0, 12, 24, 36, and 48 h after infection. Viruses were released by repeated freezing and thawing and sonication. Virus titers were determined on Vero cells by a plaque assay.

To measure in vitro killing effect of viruses, Hey-8 or SKOv3 tumor cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.1 and 0.01 pfu/cell or left uninfected. Cells were harvested 24 or 48 h after infection. Viruses were released by repeated freezing and thawing and sonication. The percentage of viable cells was calculated by dividing the number of cells that excluded trypan blue from the infected well by the number of cells that excluded trypan blue from the infected well as the viable cells. The experiments were done in triplicate, and the averaged numbers were used for the final calculation.

Animal Studies. Hey-8 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% EDTA. Trypsinization was stopped with medium containing 10% FBS, and cells were washed once in serum-free medium and resuspended in PBS. Only single cell suspensions with >95% viability were used for in vivo injection. Briefly, on day 0, 3 × 10^7 viable Hey-8 cells were inoculated into the peritoneal cavities of 8-week-old female nude mice. The mice were then randomly divided into three groups of eight mice each. On days 14 and 28 after tumor inoculation, the mice in groups 1 and 2 received i.p. injection with either Baco-1 or Synco-2D at a dose of 2 × 10^7 pfu in a total volume of 200 μl. Mice in group 3 received the same volume of PBS as the control. The therapeutic injection was given at a site distant from the area of tumor cell inoculation. On day 42 after tumor cell inoculation, all surviving mice were euthanized by CO₂ exposure and evaluated macroscopically for the number and size of tumor nodules in the abdominal cavity. For toxicity evaluation, 7–8-week-old BALB/c mice received injection with two different doses of viruses (5 × 10^7 or 3 × 10^8) or the same volume (200 μl) of PBS as a control. There were 5 mice/group. The viruses were prepared with serum-free medium, and the injection was given through the tail vein at a speed of 2 min/injection.
Statistical Analysis. Quantitative results are reported as means ± SDs. The statistical analysis was performed by Student’s t test or one-way ANOVA, with the exception of the survival data, which were analyzed by the Kaplan-Meier method and the log-rank (Mantel-Cox) test using Statview 5.0 software (SAS Institute, Inc., Cary, NC). Ps < 0.05 were considered significant.

RESULTS

Generation and Characterization of Doubly Fusogenic Oncolytic HSV. Membrane fusion capability was first introduced into a conventional oncolytic HSV, Baco-1 (15), through random mutagenesis of incorporation of the thymidine analogue bromodeoxyuridine during viral replication in Vero cells (14). The virus was phenotypically identified and purified to homogeneity. The gene encoding the hyperfusogenic GALV.fus (21), driven by the promoter of the strict-late gene-UL38, was then cloned into the BAC-based viral genome through an enforced ligation strategy (15) to replace the GFP gene of Baco-1. One of the plaque-purified viruses, designated Synco-2D, was chosen for further characterization.

To directly compare the phenotypes and plaque size of Baco-1 and Synco-2D, we mixed the two viruses at a 1:1 ratio. The mixture was serially diluted and then used to infect Vero cell monolayers. The photomicrograph, which was taken 40 h after virus infection, shows an area containing plaques from both viruses. The plaque from Synco-2D, in which only a portion is shown, is indicated by the hatched arrow; the open arrow indicates the plaque from Baco-1. Original magnification, ×200.

We phenotypically characterized the viruses in greater detail on two human ovarian cancer cell lines (Hey-8 and SKOv3) infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. As shown in Fig. 2, bottom panels, the syncytial plaques after Synco-2D infection differed strikingly from the usual round cell plaques derived from infection with Baco-1. By 40 h, the Synco-2D-infected tumor cells had fused together, forming a dense material (middle of the infectious focus) that was separated from the boundary of the plaque by a large gap area. This distinctive pattern of syncytial development was especially prominent in Hey-8 cells.

Comparison of Tumor Cell Killing in Vitro. To determine whether the marked syncytial formation resulting from Synco-2D infection would enhance tumor cell killing, we infected ovarian cancer cells with Baco-1 or Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), permitting us to assess both the inherent cytotoxicity of the input virus and the ability of the virus to replicate and spread in these

Fig. 1 Phenotypic characterization of Synco-2D in Vero cells. To directly compare the phenotype and plaque size of Baco-1 and Synco-2D, we mixed the two viruses at a 1:1 ratio. The mixture was serially diluted and then used to infect Vero cell monolayers. The photomicrograph, which was taken 40 h after virus infection, shows an area containing plaques from both viruses. The plaque from Synco-2D, in which only a portion is shown, is indicated by the hatched arrow; the open arrow indicates the plaque from Baco-1. Original magnification, ×200.

Fig. 2 In vitro phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, ×200). Arrows indicate a single syncytium.
cells. The cytotoxic effect of the viruses was quantified by calculating the percentage of viable cells remaining in the wells after infection. The results (Fig. 4) showed a significantly greater tumor cell killing effect by Synco-2D compared with Baco-1 ($P < 0.01$, all comparisons). At 0.01 pfu/cell, Synco-2D reduced the viable cells to $<40\%$ within 24 h (Fig. 4A). At 0.1 pfu/cell, it completely destroyed the Hey-8 tumor cells within 48 h, a time at which there was still $>30\%$ viable tumor cells in the well infected with Baco-1 (Fig. 4B). The degree of tumor cell killing appeared to correlate with the extent of syncytial formation (as seen in Fig. 2); that is, both the cytolytic and syncytium-inducing effects of Synco-2D were greater in Hey-8 cells than in SKOv3 cells.

Therapeutic Effects of Synco-2D on Peritoneal Metastases of Ovarian Cancer. Peritoneal invasion of ovarian cancer is a common and serious clinical problem. It has been reported that about 70\% of late-stage ovarian cancer patients have metastatic disease in the peritoneal cavity (2). We therefore chose a peritoneal metastatic model (xenografted Hey-8 cells) as a means to test the efficacy of Synco-2D against human ovarian cancer. Freshly harvested Hey-8 cells were inoculated into the peritoneal cavities of nude mice at a dose of $3 \times 10^5$ cells/mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice received i.p. injection with $2 \times 10^7$ pfu/200 µl of either Baco-1, Synco-2D, or PBS (control) at a site distant from that of tumor cell implantation. A second i.p. injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5 $\pm$ 0.7 days (none of the mice survived); there was clear i.p. dissemination of nude mice at a dose of $3 \times 10^5$ cells/mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice received i.p. injection with $2 \times 10^7$ pfu/200 µl of either Baco-1, Synco-2D, or PBS (control) at a site distant from that of tumor cell implantation. A second i.p. injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5 $\pm$ 0.7 days (none of the mice survived); there was clear i.p. dissemination of tumor (Fig. 5A), with an average of 2.5 $\pm$ 0.9 tumor nodules found in regions distal from the site of the tumor implantation (Table 1). In the Baco-1 treatment group, three mice died before the end of the experiment [first death, 34 days after tumor cell implantation (Fig. 6)]; five survivors bore a single large tumor (Fig. 5B) when examined at necropsy. By contrast, none of the Synco-2D-treated mice died or were euthanized during the experiment. Strikingly, six of the eight mice were entirely tumor free at necropsy by the end of the experiment (Table 1 and Fig. 5C). The other two animals had tumors that are significantly smaller than those in Baco-1-treated mice ($P < 0.01$; Table 1).

Toxicity Evaluation of Fusogenic Oncolytic HSV. As a first step toward evaluating the toxicity of this doubly fusogenic
oncolytic HSV, we injected either Baco-1 or Synco-2D at relatively high doses (5 × 10^7 and 3 × 10^8 pfu) into immuno-
competent BALB/c mice through the tail vein (n = 5 mice/ dose). The dose of 3 × 10^8 pfu in 200 μl of solution is the
maximum that could be injected systemically into mice, given
the modest scale of virus preparation (maximum titer, <2 × 10^9
pfu/ml). The mice were then observed for 6 weeks. Shortly after
virus injection, two mice receiving the higher dose of Baco-1
became inactive, but they recovered in less than 3 h. Otherwise,
there were no animal deaths or evidence of disease during the
observation period. These results indicate that, despite its high
antitumor potency, Synco-2D is probably not much more toxic
than its parental nonfusogenic virus.

**DISCUSSION**

In our previous study, we had shown that a syncytial
mutant (Fu-10) selected from the well-characterized oncolytic
G207 virus through random mutagenesis had potent antitumor
activity against lung metastases of breast cancer (14). We also
showed that insertion of the hyperfusogenic glycoprotein from
gibbon ape leukemia virus into a conventional oncolytic HSV
can significantly increase the antitumor potency of that virus
(15). Here, we created Synco-2D, a modified version of the
fusogenic oncolytic HSV, by incorporating both fusion mecha-
nisms into a single oncolytic HSV. In vitro characterization
studies showed that infection with Synco-2D promotes ex-
tremely strong syncytial formation in both Vero and human
ovarian tumor cell lines. The i.p. administration of this virus to
animals bearing established human ovarian cancer in the ab-
dominal cavity produced striking therapeutic effects: six of eight
mice became totally tumor free, in contrast to the presence of
bulky tumors in all mice treated with the conventional oncolytic
virus. This result suggests that strategies similar to ours would
significantly increase the likelihood of success in clinical trails
of oncolytic HSVs in patients with advanced ovarian cancer and
perhaps other malignant solid tumors as well.

Although we did not directly compare the doubly fusogenic
virus with either of the two previously described HSVs with a
single membrane fusion capability (14, 15), syncytial formation
was clearly greater with Synco-2D, suggesting enhancement
of antitumor activity. Additionally, virotherapy with Synco-2D

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Table 1  Results of Synco-2D therapy for xenografted human ovarian cancer in the peritoneum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>No. of mice with tumors</th>
<th>No. of nodules</th>
<th>Tumor weight (g)</th>
<th>Death rate</th>
</tr>
</thead>
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<tr>
<td>PBS</td>
<td>8</td>
<td>8/8</td>
<td>2.5 ± 0.9</td>
<td>1.0 ± 0.6</td>
<td>8/8</td>
</tr>
<tr>
<td>Baco-1</td>
<td>8</td>
<td>8/8</td>
<td>1.0 ± 0.0b</td>
<td>1.5 ± 0.7</td>
<td>3/8b</td>
</tr>
<tr>
<td>Synco-2D</td>
<td>8</td>
<td>2/8c</td>
<td>0.25 ± 0.4c</td>
<td>0.1 ± 0.3c</td>
<td>0/8c</td>
</tr>
</tbody>
</table>

* Means and SDs.

* P < 0.01 as compared with PBS control.

* P < 0.01 as compared with either the PBS- or Baco-1-treated group.

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**Fig. 5**  Effects of Synco-2D in xenograft model of i.p. ovarian cancer. Human ovarian cancer xenografts were established through i.p. injection of 3 × 10^5 Hey-8 cells into nude mice. The mice then received i.p. injection, on days 14 and 28, with either PBS (control) or 2 × 10^7 pfu of Baco-1 or Synco-2D. Forty-two days after tumor implantation, any mice that were still alive were euthanized. All mice were examined for the presence of tumor nodules in the abdominal cavity. A (PBS), B (Baco-1), and C (Synco-2D) show the gross pathological findings in a representative mouse from each group.

**Fig. 6**  Prolonged survival of mice treated with Synco-2D. The figure shows the survival curve on Kaplan-Meier plots of data from the experiment described in Fig. 5. The asterisk denotes a significant difference between the Synco-2D group and either the PBS or Baco-1 group (P < 0.01).
should reduce the emergence of treatment resistance. This is because syncytial formation mediated by fusogenic glycoproteins relies on the initial binding of fusogenic glycoproteins with their specific receptors on target cells, which then induces ordered structural changes of the membrane lipid bilayers, leading in turn to lipid mixing and eventually to membrane fusion either between viral and cellular membranes or among cellular membranes (22). The envelope glycoprotein of GALV is the only viral protein required for both viral and cellular membrane fusion. The cellular receptor for GALV has been identified as Pit1, a type III sodium-dependent phosphate transporter (23, 24). The membrane fusion induced by HSV is more complex, requiring the participation of multiple viral glycoproteins and at least two specific receptors on the cell surface, heparan sulfate and the newly discovered HVEM (herpesvirus entry mediator (25, 26)). Therefore, it is possible that tumor cells that become resistant to one fusion protein (e.g., due to the lack of a requisite cellular receptor) will still be destroyed by syncytial formation resulting from expression of the other fusion protein, which uses a totally different cellular receptor to initiate the membrane fusion process. Our recent finding that infection with Synco-2D but not with Fu-10 or Synco-2 can cause syncytia formation in several murine tumor cell lines and one human tumor line supports this possibility.5

The peritoneal xenograft model of human ovarian cancer used to evaluate the efficacy of Synco-2D has definite clinical relevance. Although patients with stage I EOC (localized to ovaries) have a 5-year survival rate of 90% after surgical resection (27), those with tumor spreading beyond the ovaries, often to the peritoneal cavity, usually have very poor prognosis. Because the disease frequently remains confined to the peritoneal surfaces in these patients (28), successful management of peritoneal metastases can directly improve prognosis. There are also several additional advantages to administering oncolytic viruses i.p. The peritoneal cavity serves as a restricted compartment, allowing maximal contact and interaction between cancer cells and therapeutic agents. It also circumvents encounters with neutralizing antibodies and reduces toxicity by comparison with systemic administration. To closely mimic clinical situations, we administered the oncolytic viruses at a relatively late stage after tumor cell implantation, when all of the mice had tumors that were palpable through the abdominal wall. The viral dose of 2 × 10^7 pfu, which eradicated tumor in 75% of the mice, is equivalent to 6 × 10^10 pfu in humans, a dose similar to the highest dose of G207 administered in a Phase I study for malignant gliomas (10). Bearing in mind that the peritoneal cavity represents a substantially larger area than the brain and is probably less vulnerable to any potential toxic effects of the virus, we believe that such a virus dose should be safe and well tolerated in patients. Thus, direct administration of Synco-2D, alone or in association with a surgical debulking procedure to remove gross recurrent disease, is a reasonable strategy for clinical testing.

Uncontrolled induction of syncytial formation by fusogenic oncolytic HSVs is a potential safety concern. We believe that both of the fusion mechanisms in Synco-2D are tumor specific. First, the syncytial formation from mutagenized HSVs is mainly due to aberrant expression of several viral glycoproteins, such as gB and gK (29–32). Because these glycoproteins are encoded by late genes whose expression depends upon viral DNA replication, such oncolytic viruses will retain the safety of the original virus because syncytial formation will only occur in tumor cells (where virus can undergo a full infection cycle) and not in normal nondividing cells (where virus replication is restricted, and very low levels of glycoproteins are expressed). To control the fusogenic activity from GALV.fus, we used a strict late viral promoter to direct gene expression, the activity of which has been shown to remain confined to the tumor tissue after systemic administration with an oncolytic HSV (20). Thus, GALV.fus-mediated syncytial formation was linked to the virus to replicate conditionally in tumor cells. The demonstrations in our previous studies that blocking viral DNA replication completely abolishes syncytia-forming ability of Fu-10 (the selected syncytial mutant from G207; Ref. 14) and that Synco-2 (containing GALV.fus driven by UL38p) cannot induce syncytial formation in nondividing cells (15) strongly suggest that Synco-2D retains the safety profile of a conventional oncolytic HSV. This notion is supported by the observation that i.v. injection of Synco-2D in relatively large doses (up to 3 × 10^8 pfu) is well tolerated in mice.

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REFERENCES


5 Unpublished observation.
An oncolytic virus derived from type 2 herpes simplex virus has potent therapeutic effect against metastatic ovarian cancer

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Oncolytic viruses derived from herpes simplex virus (HSV) have shown considerable promise as antitumor agents against solid tumors including ovarian cancer. The current group of oncolytic HSVs was constructed exclusively from type 1 HSV. To exploit further the therapeutic potential of replication-selective viruses, we constructed an oncolytic virus from type 2 HSV by deleting the protein kinase domain of the viral \textit{ICP10} gene, which targets the activated Ras signaling pathway in tumor cells. In the study reported here, we administered this HSV-2-derived virus intraperitoneally (i.p.) to nude mice bearing metastatic human ovarian tumor xenografts, evaluated its oncolytic activity, and compared with to that of a virus constructed from HSV-1. Two injections of the HSV-2-derived virus (3×10\textsuperscript{6} pfu per dose) led to complete eradication of disseminated tumors in the peritoneal cavity in more than 87% of the mice, whereas the HSV-1-based oncolytic virus, administered at the same dose and on the same schedule, eradicated tumor nodules in only 12% of mice (\(P<0.01\)). We conclude that i.p. administration of this HSV-2-based oncolytic virus may provide effective treatment for metastatic human ovarian cancer.


Keywords: oncolytic virus; HSV-2; ovarian cancer; metastatic; Ras signaling pathway

Introduction

Ovarian cancer remains the leading cause of death in patients with gynecological malignancies in the United States. Because of ineffective screening strategies and inconspicuous early symptoms, these tumors are usually detected late in the clinical course, when metastases are present. The primary site of metastatic spread is within the peritoneal cavity. Despite improved surgical techniques and chemotherapy, the mortality rate for ovarian cancer has remained largely unchanged over the last decade,\textsuperscript{1,2} mandating the development of new treatment strategies.

Replication-selective viruses afford a promising treatment for malignant solid tumors. Oncolytic viruses derived from herpes simplex virus (HSV) were initially designed and constructed for the treatment of brain tumors.\textsuperscript{3,4} Subsequently, they have proved effective against a variety of other human solid tumors, including ovarian cancer.\textsuperscript{5,6} The current group of oncolytic HSVs were constructed exclusively from type 1 virus (HSV-1), most often by deleting the \textit{g34.5} gene, which encodes a neurovirulent factor, and/or insertional mutation of the \textit{ICP6} gene, which encodes the large subunit of ribonucleotide reductase.\textsuperscript{3,4,7–9} Inactivation of either or both of these genes enables the virus to replicate selectively in dividing cells whereas sparing normal *nondividing cells.\textsuperscript{10–12}

We have recently constructed a new oncolytic virus from type 2 HSV (HSV-2) to exploit a unique feature of the viral \textit{ICP10} gene, which contains in its N terminus a well-defined region that encodes a serine/threonine protein kinase (PK) activity.\textsuperscript{13} As this PK domain can bind and phosphorylate the GTPase-activating protein Ras-GAP, which leads to activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK)/MAPK mitogenic pathway and c-Fos induction and stabilization, it is required for efficient HSV-2 replication.\textsuperscript{14,15} A mutant HSV-2 virus (FusOn-H2), deleted for this PK domain, replicated selectively in and thus lysed human tumor cells. Infection with this virus also induced syncytia formation in tumor cells, providing an additional oncolytic mechanism that enhanced its antitumor effect overall.\textsuperscript{16}

Here, we report the therapeutic effects of FusOn-H2 against metastatic human ovarian cancer xenografts growing in the peritoneal cavity of nude mice. Our results indicate that this mutant virus may be an effective oncolytic agent in the treatment of metastatic
human ovarian cancer. Indeed, two intraperitoneal (i.p.) injections of the virus at a moderate dose completely eradicated disseminated tumors in the peritoneal cavity of most animals, in contrast to the weak activity of a conventional oncolytic virus derived from type 1 HSV.

**Materials and methods**

**Cell lines and viruses**

African green monkey kidney (Vero) cells were obtained from American Type Culture Collection (Rockville, MD). SKOV3 cells derived from a serous cystoadenocarcinoma were kindly provided by Dr Robert Bast (M D Anderson Cancer Center). All cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS). Female Hsd athymic (nu/nu) mice (obtained from Harlan, Indianapolis, Indiana) were kept under specific pathogen-free conditions and used in experiments when they attained the age of 7–8 weeks.

Construction of the HSV-1-derived oncolytic virus Baco-1 is described elsewhere,6,17 as is construction of FusOn-H2.16 Briefly, Baco-1 was constructed from a bacterial artificial chromosome (BAC)-based construct that contains a mutated HSV genome, in which the diploid gene encoding γ34.5 and both copies of the HSV packaging signal (pac) have been deleted.18 The green fluorescent protein (GFP) gene driven by the cytomegalovirus (CMV) promoter, together with a copy of the HSV-1 pac, were inserted into the unique PacI site of fHSV-delta-pac by direct ligation, to generate the infectious HSV (Baco-1). Thus, Baco-1 has both copies of the γ34.5 gene deleted and contains the GFP marker gene.17

For construction of FusOn-H2, the ICP10 gene, in which the protein kinase domain (equivalent to nucleotide span 86999–88228) were amplified by PCR. These PCR products were cloned together into pNeb193, generating pNeb-ICP10-deltaPK. Hence, the new plasmid contains a mutated ICP10 gene, in which the protein kinase domain (equivalent to nucleotide span 86999–88228) is deleted. Then, the DNA sequence containing the CMV promoter-EGFP gene was PCR amplified from pSZ-EGFP, and the PCR-amplified DNA was cloned into the deleted PK locus of pNeb-ICP10-deltaPK, generating pNeb-PKF-2. During the design of PCR-amplification strategies, the EGFP gene was fused in-frame with the remaining RR domain of the ICP10 gene, so that the new protein product of this gene contained the intact EGFP. The modified ICP10 gene was inserted into the genome of wt186 by homologous recombination. The recombinant virus (FusOn-H2) was identified by screening for GFP-positive plaques. Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell, harvesting the virus after 2 days, and storing it at −80°C.

**Phenotypic characterization and oncolytic activity of FusOn-H2 against a human ovarian cancer cell line**

To phenotypically characterize the new virus, we seeded SKOV3 ovarian cancer cells into six-well plates, and then infected them on the following day with Baco-1, wt186 or FusOn-H2 at a dose of 0.01 pfu/cell. Cells were cultured in a maintenance medium (containing 1% FBS) and were incubated for up to 2 days to allow the fusion pattern and plaques to develop. To measure the *in vitro* killing effect of the viruses, we seeded SKOV3 tumor cell lines into 24-well plates and infected them with Baco-1 or FusOn-H2 at 0.01 and 0.001 pfu/cell, or left them uninfected. Cells were harvested 24, 48 or 72 h later by trypsinization, and the number of viable cells determined with a hemocytometer after Trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells excluding Trypan blue in the infected well by the number excluding the stain in the well that was left uninfected. The experiments were performed in triplicate, with mean cell numbers used for the final calculation.

**Animal studies**

SKOV3 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in phosphate-buffer saline (PBS). Only single cell suspensions with >95% viability were used for *in vivo* injection. Briefly, on day 0, 2 × 10⁶ SKOV3 cells were inoculated into the peritoneal cavities of 8-week-old female nude mice. The mice were then randomly divided into three groups of eight mice each. On days 8 and 15 after tumor inoculation, the mice in groups 1 and 2 were injected i.p. with either Baco-1 or FusOn-H2 at a dose of 3 × 10⁶ pfu in a total volume of 200 μl. Mice in group 3 received the same volume of PBS as the control. The therapeutic injection was given at a site distant from the area of tumor cell inoculation. On day 36 after tumor cell inoculation, all surviving mice were euthanized by CO₂ exposure and evaluated macroscopically for the number and size of tumor nodules in the abdominal cavity.

**Systemic and intracerebral virus injections**

For systemic administration, the virus was diluted in 100 μl of serum-free medium and injected into the animal through the tail-vein as described.19 Intracranial injection was performed essentially as described.20 Briefly, mice were anesthetized and placed in a stereotactic frame (Stoelting). A burr hole was drilled in the skull 1 mm anterior and 2 mm lateral to the bregma with a 0.9 mm burr to expose the dura. The mice were injected with 3 μl of serum-free medium containing the virus, by use of a 100 μl syringe (Hamilton) fitted with a 26-gauge needle and connected to the manipulating arm of the stereotactic frame. The injection was directed to the caudate nucleus at a depth of 3.5 mm from the dura and given over 2.5 min. The needle was left in place for 3 min and then withdrawn slowly over another minute, to prevent reflux of the virus solution.
Immunohistochemical staining for HSV antigens in liver sections

Livers were removed 3 or 4 days after systemic administration of HSVs, fixed with 4% paraformaldehyde overnight at 4°C, and treated with 25% sucrose overnight at 4°C. Ten micrometer sections were then prepared, air-dried at room temperature overnight and treated with 4% paraformaldehyde for 2 min, before being treated with 1% NP40/PBS for 5 min. This procedure was followed by several washes with PBS. The liver sections were then incubated with a rat anti-HSV serum (prepared by our own lab at a 1:100 dilution), at 37°C for 60 min. After washing, fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG antibody (Sigma-Aldrich, St Louis, MO), diluted at 1:200, was added to the tissue section and incubated at 37°C for 60 min. After two washes with PBS, the slides were dehydrated in an ascending series of ethanol for 2 min, cleared in xylene for 2 min, mounted in DPX mounting solution, and observed under a fluorescence microscope.

Statistical analysis

Quantitative results are reported as means±s.d. The statistical analysis was performed by Student’s t-test or one-way ANOVA. P-values of less than 0.05 were considered significant.

Results

Growth property and phenotype of FusOn-H2 in human ovarian cancer cells

We initially compared the growth of FusOn-H2 with that of its parental wt186 virus and Baco-1. Cells seeded in six-well plates were infected with the viruses at a relatively low dose (0.01 pfu/cell) and harvested at 48 h post-infection, enabling us to assess both the inherent infectivity of the input virus and its ability to replicate and spread in these cells. The results showed that FusOn-H2 replicated well in SKOV3 cells, reaching a titer of almost $1 \times 10^5$ pfu/ml (Figure 1). Overall, the titer was about 1 log lower than that of the wt186 or Baco-1 virus, most likely because of the induction of apoptosis in FusOn-H2-infected cells (Fu et al., unpublished data).

FusOn-H2 has a fusogenic phenotype in the human breast cancer cell line MDA-MB-435. To determine if it retains this phenotype in human ovarian cancer cells, we infected SKOV3 cells in a six-well plate with either the parental wt186 or FusOn-H2 virus, and monitored the cultures for cytopathic effects and syncytia formation. At 24 h postinfection (Figure 2), syncytia formation was clearly present in cultures infected with FusOn-H2, whereas neither the parental wt186 nor Baco-1 virus produced fusogenic phenotype. Instead, they induced a typical cytopathic effect, represented by cell rounding and swelling. At 48 h after infection, most of the SKOV3 cells infected with FusOn-H2 had detached from the plate, leaving fusion bodies and other cell debris floating in the medium. There was some minimal syncytia formation in the well infected with wt186, but the overall effect differed markedly from that of FusOn-H2 observed at either 24 or 48 h postinfection. At the later stage of infection, the...
plaques in the well infected with Baco-1 virus were becoming larger, but there were still no sign of syncytia formation (Figure 2).

Efficiency of FusOn-H2-induced killing of human ovarian cancer cells in vitro
To assess the cytolytic activity of FusOn-H2, we infected SKOV3 cells seeded in 24-well plates with this virus, wt186 and Baco-1 at a dose of either 0.01 or 0.001 pfu/cell. The cells were harvested at different times after infection, and cell viability was determined by Trypan blue staining. At 0.01 pfu/cell (Figure 3a), infection with FusOn-H2 killed significantly more tumor cells by 24 h postinfection than did either wt186 or Baco-1 ($P < 0.05$). The extend of FusOn-H2-induced killing was similar to that of wt186 at 48 and 72 h postinfection, but remained significantly greater than that of Baco-1 at these two later time points ($P < 0.05$). At 0.001 pfu/cell (Figure 3b), the oncolytic effect of FusOn-H2 was equivalent to that of wt186 at all the time points, whereas both viruses were significantly more oncolytic than Baco-1 ($P < 0.05$). We also evaluated the in vitro oncolytic activity of FusOn-H2 against another human ovarian cancer line, Hey8. Overall, the antitumor effect was essentially the same as obtained with SKOV3 cells (data not shown).

Evaluation of FusOn-H2 oncolytic activity against metastatic ovarian cancer
We chose a peritoneal metastasis model to evaluate the antitumor effects of FusOn-H2 against ovarian cancer. This model is clinically relevant, as peritoneal invasion by ovarian cancer is common and usually represents a serious complication. Freshly harvested SKOV3 cells were inoculated into the peritoneal cavities of nude mice at $2 \times 10^6$ cells/mouse. Eight days later, the mice were i.p. injected with $3 \times 10^6$ pfu of either Baco-1 or FusOn-H2 or PBS (control) at a site distant from that of tumor cell implantation. At the time of injection, animals lacked any sign of ascites, but they had either visible or microscopic tumors in the peritoneal cavity.

This injection was repeated at the same dose 1 week later. Results from the PBS control group (Figure 4, PBS) showed that implantation of these tumor cells in the specified quantify leads to the formation of disseminated metastatic tumor nodules. On average, there were more than 15 tumor nodules in each mouse, and the tumors from each mouse weighed more than 1 g. Direct injection of FusOn-H2 into the peritoneal cavity produced a highly significant therapeutic effect, completely eradicating all tumor nodules in seven of eight mice (Figure 4, FusOn-H2). The one mouse that was not tumor-free had only a single nodule. Although Baco-1 showed significant oncolytic activity by comparison with the PBS control (Figure 4, also Table 1, $P < 0.01$), it was clearly less effective than FusOn-H2, in terms of both the percentage of tumor-free animals (12.5 vs 87.5%, $P < 0.01$) and the weight of the remaining tumor nodules (0.7 ± 0.4 g vs 0.02 ± 0.05 g, $P < 0.01$). These results clearly demonstrate the ability of FusOn-H2 to eradicate or control metastatic...
ovarian cancer established in the peritoneal cavity of mice, even when it is administered with limited frequency and in a very moderate dose.

**FusOn-H2 toxicity in vivo**

Our previous data showed that FusOn-H2 could not replicate in normal nondividing cells such as primary hepatocytes, indicating that it is probably a safe oncolytic agent for in vivo use. To assess its toxicity in vivo more extensively, we injected the virus systemically (via the tail vein) into immune-competent Balb/c mice at two different doses: \(1 \times 10^6\) and \(1 \times 10^7\) pfu. We included the parental wt HSV-2 as a control, but in doses 2 logs lower than that used with FusOn-H2. Toxicity was assessed by two criteria: mortality and liver function measured by monitoring levels of alanine transaminase (ALT) and aspartate transaminase (AST) activity in blood samples collected at different times after virus administration. Liver function was chosen as an endpoint because oncolytic HSV particles are distributed to the liver after systemic delivery, and infection of both human and murine liver by HSV can cause hepatitis. All animals receiving the wt HSV-2 died before day 10 after virus injection, in contrast to the survival of the entire group receiving FusOn-H2, even at significantly high doses (Table 2). When autopsied, the animals killed by wt HSV-2 had extensive gross necrotic foci in their livers, whereas in mice euthanized at day 4 after FusOn-H2 administration, the livers appeared normal (Figure 5a). Histologic examination of liver tissue sections confirmed these gross observations. Hepatic structures from FusOn-H2-treated mice were intact with no obvious cytopathic effects, whereas liver tissue from wt HSV-2-treated mice showed extensive necrosis and complete destruction of structural elements (Figure 5b). Immunohistochemical staining revealed widespread evidence of

![Figure 5](image)

**Table 1** Mean number and weight of tumor nodules after virotherapy

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment groups</th>
<th>PBS</th>
<th>Baco-1</th>
<th>FusOn-H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. tumor nodules</td>
<td></td>
<td>6.0 ± 5.4^a</td>
<td>0.1 ± 0.3^b</td>
<td>15.5 ± 8.4</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td></td>
<td>1.2 ± 0.6</td>
<td>0.7 ± 0.4^a</td>
<td>0.02 ± 0.05^b</td>
</tr>
</tbody>
</table>

Abbreviation: PBS, phosphate-buffer saline. Tumor-free mice/total mice 0/8 1/8 7/8. ^aP<0.01 as compared with PBS control. ^bP<0.01 as compared with Baco-1.

**Table 2** Mortality of Balb/c mice after virus injection

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (pfu)</th>
<th>Injection site</th>
<th>No. surviving mice/no injected</th>
<th>Time to death (days p.i.)</th>
<th>Observation time (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt186</td>
<td>(1 \times 10^4)</td>
<td>i.v.</td>
<td>0/5</td>
<td>6, 7, 7, 7, 8</td>
<td>1</td>
</tr>
<tr>
<td>wt186</td>
<td>(1 \times 10^5)</td>
<td>i.v.</td>
<td>0/5</td>
<td>6, 6, 6, 7, 9</td>
<td>1</td>
</tr>
<tr>
<td>FusOn-H2</td>
<td>(1 \times 10^6)</td>
<td>i.v.</td>
<td>5/5</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>FusOn-H2</td>
<td>(1 \times 10^7)</td>
<td>i.v.</td>
<td>5/5</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>wt186</td>
<td>(1 \times 10^4)</td>
<td>i.c.</td>
<td>0/5</td>
<td>3, 3, 4, 4, 5</td>
<td>2</td>
</tr>
<tr>
<td>wt186</td>
<td>(1 \times 10^5)</td>
<td>i.c.</td>
<td>0/5</td>
<td>2, 2, 2, 3, 7</td>
<td>2</td>
</tr>
<tr>
<td>FusOn-H2</td>
<td>(1 \times 10^6)</td>
<td>i.c.</td>
<td>5/5</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>FusOn-H2</td>
<td>(1 \times 10^7)</td>
<td>i.c.</td>
<td>4/5</td>
<td></td>
<td>58</td>
</tr>
</tbody>
</table>

Abbreviations: p.i., postinjection; i.v., intravenous; i.c., intracranial.
Table 3  Liver function assay after virus administration

<table>
<thead>
<tr>
<th>Days</th>
<th>ALT (U)</th>
<th>ALT (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FusOn-H2</td>
<td>wt186</td>
<td>FusOn-H2</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
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</tr>
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<td>10</td>
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<td>74</td>
</tr>
<tr>
<td>30</td>
<td>34</td>
<td>78</td>
</tr>
</tbody>
</table>

Abbreviation: ALT, alanine transaminase.

aThe figures represent the average of ALT and AST levels obtained from five randomly bled mice before the animals were grouped.
bAs all mice receiving wild-type virus died before the second time point, only blood samples from day 3 were available for this group.
cSignificantly increased over pretreatment value ($p<0.01$).

disease. Because of the relatively confined space it occupies and its ready accessibility for therapeutic intervention, the peritoneal cavity also represents an especially attractive site for investigative virotherapy. In our previous studies,6 we used the human ovarian cancer cell line Hey8 to establish ovarian tumor xenografts in the peritoneal cavity for evaluation of potentially useful oncolytic viruses. Implantation of Hey8 cells led to the formation of fewer (1 or 2) but larger tumor nodules in the peritoneal cavity than did implantation of SKOV3 cells, which led to widespread dissemination of relatively small tumor nodules across the pelvic and abdominal surfaces, more closely resembling the clinical scenario. In the present study, two injections of FusOn-H2 at a moderate dose into the peritoneal cavity produced a clear antitumor effect, leading to complete eradication of metastases in over 87% of the animals. In sharp contrast, treatment with Baco-1, an oncolytic virus derived from HSV-1, yielded only a modest antitumor effect (one tumor-free mouse among eight tested). These results indicate that FusOn-H2 is an effective oncolytic agent for the treatment of metastatic human ovarian cancer in the peritoneal cavity.

What could account for the superior oncolytic effect of this HSV-2-derived virus? FusOn-H2, but not its parental wt virus, showed a clear fusogenic phenotype in the human breast cancer cell line MDA-MB-435.16 Pheno-typic characterization of this virus, wt186 and Baco-1, an HSV-1-based virus, on SKOV3 cells at 24 h after infection demonstrated syncytia formation by FusOn-H2 only, although by 48 h wt186 also induced weak cell-membrane fusion. As neither Baco-1 nor its wt HSV-1 parent was fusogenic in these cells6 it appears that HSV-2 is probably inherently more fusogenic than HSV-1 in this human ovarian cancer cell line and that the mutagenesis procedure for the deletion of the PK domain enhanced the fusogenic property of the virus. Work by us and others has demonstrated that incorporation of cell membrane fusion activity into an oncolytic virus can significantly enhance its antitumor effect.19,25 Hence we attribute the potent antitumor activity of FusOn-H2 in this metastatic ovarian cancer model, at least in part, to the presence of a fusogenic property related to deletion of the PK domain from the parental HSV-2 virus.

Although the genomes of HSV-1 and HSV-2 are largely collinear and their sequences share a high homology, there are certain differences among the functions of the gene products. For example, several proteins encoded by HSV-2, including the secreted form of glycoprotein G and the virion host shutoff protein, but not their counterparts in HSV-1, are involved in evading the host’s innate immunity against viral infection.26,27 Both NK cells and interferons are key components of the host’s innate immunity, which plays a crucial role in controlling HSV infection.28–30 Thus, the HSV-2-derived FusOn-H2 mutant might have gained a therapeutic advantage over its HSV-1 counterpart (Baco-1) by virtue of mechanisms that protect it from the innate immune response of the host. This would help to explain the observation that FusOn-H2 had significantly better antitumor efficacy.
than Baco-1, even though its replication capacity in tumors was about 1 log lower.

The replication capacity of FusOn-H2 depends not only on the proliferative status of the host cell, as do HSV-1-based oncolytic viruses, but also on the activation status of the Ras signaling pathway. Although Ras gene mutations in ovarian cancer cells are not common, the Ras signaling pathway in such cells is frequently activated through other mechanisms, such as phosphoinositide-3 kinase or protein kinase B upregulation, making ovarian cancer an attractive target for FusOn-H2 treatment.

Indeed, by exploiting both Ras signaling and cell-cycle status for its oncolytic activity, FusOn-H2 would be expected to show enhanced tumor cell-specificity and thus increased safety in patients with ovarian cancer, a prediction that is supported by the in vivo toxicity data collected after systemic and intracranial inoculations of FusOn-H2. Administration of the virus by either route at a relatively large dose (up to \(1 \times 10^7\) pfu) was considerably less toxic than the parental wt virus given at a much lower dose, and was well-tolerated by the animals. These results indicate that FusOn-H2 is severely attenuated as compared with its parental wt186 virus and likely retains the safety profile of a conventional oncolytic HSV.

Acknowledgements

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References


