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ABSTRACT

The safety of the first generation oncolytic HSVs (e.g., G207) has been well documented and are currently in clinical trials. However, the available evidence so far indicates that, although safe, they may have only limited antitumor activity on their own. We have demonstrated that a doubly fusogenic oncolytic HSV, Synco-2D, is extremely effective at destroying metastatic ovarian cancer. However, maintaining the safety profile of the first generation oncolytic HSVs is mandatory for the potential application of Synco-2D in the clinics. We therefore devoted the second year of this project to evaluate the toxicity of Synco-2D. Our results show that: 1) Direct injection of the virus to the CNS at a relatively large dose did not cause any mortality. 2) Systemic administration of oncolytic HSVs caused a mild liver abnormality 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. Together, these results confirm our original hypothesis that due to the unique design of the doubly fusogenic Synco-2D, it is not significantly more toxic than the nonfusogenic counterpart despite its dramatically enhanced antitumor activity.

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INTRODUCTION

We demonstrated in our earlier studies that incorporation of cell-membrane activity into an oncolytic herpes simplex virus (HSV) could increase the antitumor activity of the virus (1). To further increase the antitumor potency of the virus, we subsequently constructed a newer version of fusogenic HSV by incorporating two membrane fusion mechanisms into a single oncolytic HSV, generating Synco-2D. This was achieved through a two-step procedure. Initially, membrane fusion capability was first introduced into Baco-1, the nonfusogenic oncolytic HSV, through random mutagenesis (2). Then the hyperfusogenic *GALV.fus* gene, driven by the strict late promoter of the *UL38* gene, was cloned into the BAC-based viral genome through an enforced ligation strategy to replace the enhanced green fluorescent protein gene of Baco-1 (3). The antitumor potency of Synco-2D has been assessed against metastatic ovarian cancer in the abdominal cavity. As the data presented in our year-1 report showed, intraperitoneal administration of Synco-2D at a moderate dose at a site distant from that of tumor cell implantation has a dramatic therapeutic effect on established ovarian cancer, rendering 75% of animals tumor-free. By contrast, none of the animals treated with Baco-1 were tumor-free; most of the mice in the PBS-control group died or became ill and were euthanized during the experiment.

As the expression of the hyperfusogenic gene *GALV.fus* contained in Synco-2D can also cause syncytia formation in normal cells, uncontrolled expression of the gene even in the context of a tumor-restricted oncolytic virus can still pose a safety concern. This is particularly true when systemic administration is required, in cases of metastatic diseases, for example. Therefore, during the construction of Synco-2D, we tailored the expression of *GALV.fus* with the ability of oncolytic HSV to selectively replicate in tumor cells by driving its expression with a strict late viral promoter (*UL38p*). This is because HSV genome transcription is a tightly regulated molecular cascade in which early and late phases of gene expression are separated by viral DNA replication (4). In particular, some of the

late transcripts (e.g., UL38 gene) can be characterized as strict-late, whose expression depends rigorously on the initiation of viral DNA replication. As such, the promoters of strict-late viral genes (e.g., UL38p that was used in Synco-2D) will be extremely active in tumor tissue, where the oncolytic virus can fully replicate, but silent in normal cells if these are nondividing or post-mitotic, since viral replication would be limited (2). We therefore expect that, although extremely potent at destroying tumor cells, Synco-2D would probably be not significantly more toxic than the nonfusogenic HSVs to the normal tissues. The proposed task for year 2 of this project was to test this hypothesis.

BODY

We evaluated the toxicity of Synco-2D by injecting the virus into immune competent animals in two different routes: intracranially and systemically. We directly compared the results between Synco-2D and the non-fusogenic Baco-1.

Experimental design and procedures

1) Preparation of highly purified virus stocks for the toxicity studies. As the viruses would be injected intracranially and systemically into the animals, the quality of virus stocks prepared from routine procedure of harvesting virus from cell lysates wouldn't be good enough. Therefore we used a modified procedure of virus stock preparation. Initially the viruses were released into the culture medium by incubating the infected cells with heparin (50 µg/ml of culture medium) for 3h. The culture supernatant was harvested (without collecting cells, therefore significantly reduced the contamination of cellular components in the virus stocks). The virion particles were then spun down through 2h centrifugation at 12,000g. The virus pellet was resuspended in either PBS or Hanks medium and stored in -80°C. The virus stocks prepared from this procedure was found to have much higher titer and less cellular contaminants than the prepared from traditional way of collecting cell lysates.

2) Virus injection schemes

a. 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 blood stream. 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 μ l) into the brains of immune competent mice was done essentially according to the procedure as described (5). 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 3 μ l of Hanks' buffered saline containing the designated amount of virus, using a 100 μ l 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 1. A typical brain section from each

group of mice was shown in Fig. 1. 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 1. Quantification of oncolytic HSVs after intracranial administration

	PBS	Baco-1	Synco-2D
Virus titer (pfu/ml)	<10pfu*	$1.5 \pm 0.2 \times 10^2$	$2.1 \pm 0.3 \times 10^2$

* As the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 μ l of the homogenized tissue supernatant to 400 μ l of DMEM to make a total of 500 μ l 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.

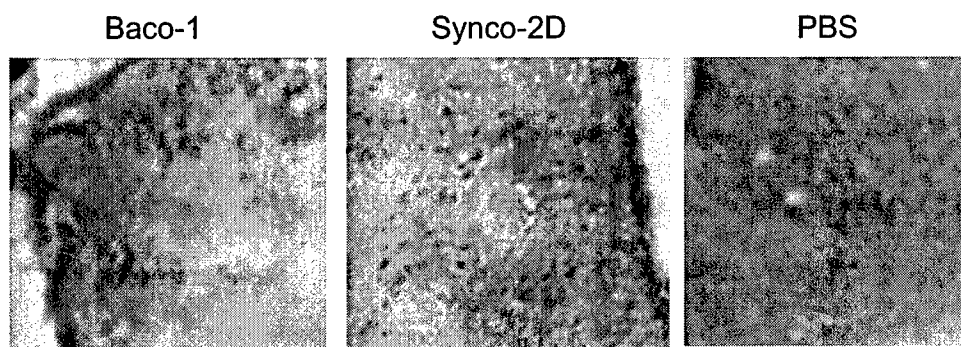


Fig. 1. Histology of brain tissues after intracranial delivery of oncolytic HSVs. The brain sections were prepared from the indicated mice at 3 weeks after virus delivery. Original magnification: 200X.

b. 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: 1×10^7 or 1×10^8 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 2).

Table 2. Mortality after systemic administration of oncolytic HSVs

Viruses	Dose	Route admin.	No. mice	mortality
Baco-1	1×10^7	i.v.	6	0
Baco-1	1×10^8	i.v.	6	1
Synco-2D	1×10^7	i.v.	6	0
Synco-2D	1×10^8	i.v.	6	1
PBS	200 μ l	i.v.	6	0

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 3). The virus titration results from the homogenized organ tissues (Table 4) showed that only a small quantity of infectious 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 Synco-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 3. Results of histological examination after systemic administration of oncolytic HSVs

Organs	PBS	Baco-1		Synco-2D	
	100 μ l	1X10 ⁷	1X10 ⁸	1X10 ⁷	1X10 ⁸
Liver	—	+	+	—	+
Kidney	—	—	—	—	—
Pancreas	—	—	—	—	—
Heart	—	—	—	—	—
Lung	—	—	—	—	—
Spleen	—	—	—	—	—
Brain	—	—	—	—	—

Table 4. Infectious virus distribution in major organs after systemic administration of oncolytic HSVs

Organs	PBS	Baco-1		Synco-2D	
	100 μ l	1X10 ⁷	1X10 ⁸	1X10 ⁷	1X10 ⁸
Liver	<10pfu/ml ^ψ	<10pfu/ml	22.6±4.5pfu/ml	<10pfu/ml	18.3±5.7pfu/ml
Kidney	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Pancreas	ND*	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Heart	ND	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Lung	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	ND
Brain	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Spleen	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml

^ψAs the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 μ l of the homogenized tissue supernatant to 400 μ l of DMEM to make a total of 500 μ l 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.

KEY RESEARCH ACCOMPLISHMENTS

In these studies, the potential toxicity of the doubly fusogenic oncolytic HSV Synco-2D was evaluated through two routes of administration: intracranial and systemic. The results obtained from these in vivo toxicity studies demonstrate the following

- 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 abnormality 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.
- Together, these results confirm our original hypothesis that due to the unique design of the doubly fusogenic Synco-2D, it is not significantly more toxic than the nonfusogenic counterpart despite its dramatically enhanced antitumor activity.

REPORTABLE OUTCOMES

1. Dr. Zhang and his associates were invited to contribute a book chapter on their work on developing fusogenic oncolytic HSVs for treatment of solid tumors including prostate and ovarian cancers. X. Fu, M. Nakamori and X. Zhang. (2005). Fusogenic Oncolytic Herpes Simplex Virus. In: Virus Therapy of Human Cancers. Sinkovics & Horvath eds. (New York: marcel dekker Inc.). pp713-738.

2. 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.

3. Invited corporate seminar presentation: Dr. Zhang was invited by Immusol, Inc (San Diego, USA) to deliver a seminar, on September 23, 2004. Title of talk: Oncolytic virotherapy for solid tumors including prostate and ovarian cancer.

CONCLUSIONS

The safety of the first generation oncolytic HSVs (e.g., G207) has been well documented and are currently in clinical trials. However, the available evidence so far indicates that, although safe, they may have only limited antitumor activity on their own. We have demonstrated that incorporation of cell-membrane fusion function into the virus can significantly enhance its antitumor potency. Among the series of fusogenic oncolytic HSVs constructed by our lab, the doubly fusogenic Synco-2D is the most potent and has been demonstrated to be extremely effective at destroying metastatic ovarian cancer. However, maintaining the safety profile of the first generation oncolytic HSVs is mandatory for the potential application of Synco-2D in the clinics. We therefore devoted the second year of this project to evaluate the toxicity of Synco-2D. Our results demonstrate that, due to its unique design and construction, Synco-2D is not significantly more toxic than the first generation oncolytic HSVs, therefore warranting its potential in human usage.

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APPENDICES:

None