

Effective Therapy of Metastatic Ovarian Cancer with an Oncolytic Herpes Simplex Virus Incorporating Two Membrane Fusion Mechanisms¹

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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

EOC⁴ 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 $\gamma 34.5$ 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 cystadenocarcinoma and has been reported to be moderately resistant to chemotherapy (16, 17). SKOv3 cells were derived from serous cystadenocarcinoma. 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 γ 34.5 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 γ 34.5. 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 from the new virus (Baco-F1) by extracting virion DNA from Vero cells shortly (1 h) after virus infection, as described previously (19). The viral DNA was then transformed into competent *Escherichia coli* cell DH-10B through electroporation, and Baco-F1 DNA was purified from bacterial growth with the use of a Qiagen kit. To insert the hyperfusogenic glycoprotein gene of gibbon ape leukemia virus (GALV.fus) into Baco-F1, we replaced the gene cassette encoding GFP in Baco-F1 with GALV.fus (driven by the conditional UL38 promoter of HSV) through an enforced ligation strategy (15, 20). The ligation mixture was directly transfected into Vero cells using LipofectAMINE (Life Technologies, Inc.) and incubated for 3–5 days to permit the generation of infectious virus. The resultant viruses were subsequently plaque-purified. Viral stocks were prepared by infecting Vero cells with 0.01 pfu/cell. When cells showed complete cytopathic effect, heparin (Sigma, St. Louis, MO) was added to the culture medium at a final concentration

of 50 μ g/ml, and cells were cultured for another 3 h to release the virus into the medium. The medium was then collected and subjected to a low-speed centrifugation at $1,000 \times g$ for 10 min. The cleared supernatant was transferred to another tube, and the virus was pelleted through high-speed centrifugation ($29,000 \times g$ for 4 h). 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 later through trypsinization. The number of viable cells was counted with a hemocytometer after trypan blue staining. 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 well that was left uninfected. 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, 3×10^5 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 μ l. 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 CO_2 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 μ l) 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.

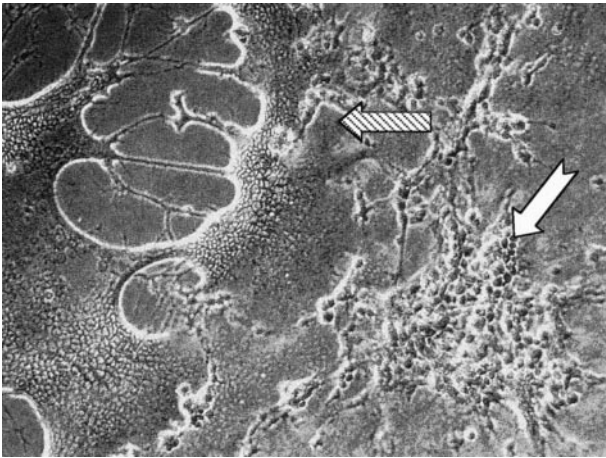


Fig. 1 Phenotypic characterization of Synco-2D in Vero cells. To directly compare the phenotype and plaque size of Baco-1 and Synco-2D, we mixed the two viruses at a 1:1 ratio. The mixture was serially diluted and then used to infect Vero cell monolayers. The photomicrograph, which was taken 40 h after 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, $\times 200$.

Statistical Analysis. Quantitative results are reported as means \pm 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). *P*s $<$ 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, serially diluted, and then used it to infect Vero cell monolayers. Fig. 1 shows a microscopic field containing the plaques from both viruses. The plaque from Synco-2D (*hatched arrow*) was much larger than that of Baco-1 (*open arrow*), so that only a portion of the syncytium could be shown in the photo. The syncytial plaque comprised fused cells with indistinguishable boundaries, whereas the Baco-1 plaque, formed entirely from round cells, was typical of the cytopathic effect produced by conventional HSV infection. The identity of the viruses was

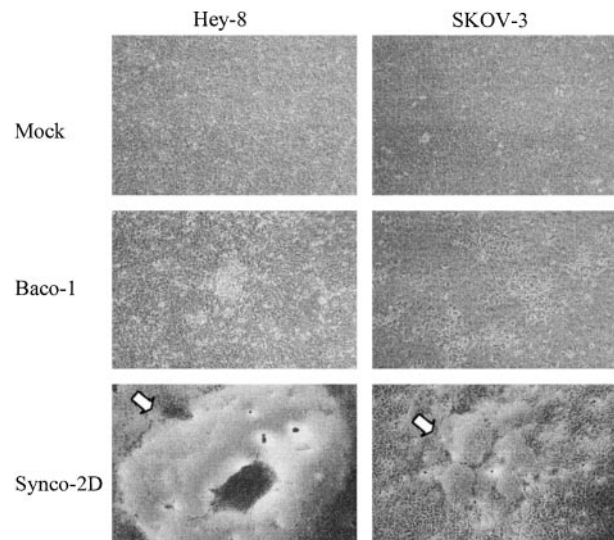


Fig. 2 *In vitro* phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOV3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, $\times 200$). *Arrows* indicate a single syncytium.

confirmed by the presence (Baco-1) or absence (Synco-2D) of GFP expression under the fluorescent microscopy (data not shown).

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.

We also compared replication properties of Synco-2D with those of Baco-1 and a wild-type HSV (strain 17) on Vero cells. The cells were infected with the viruses at either 0.1 or 0.01 pfu/cell. The viruses were harvested at 12-h intervals after infection and quantified through plaque assay. The results showed that at either dose, the viral titers from Synco-2D had almost 10-fold enhanced ability to replicate relative to Baco-1 in Vero cells (Fig. 3). This result is consistent with our earlier observation on the growth properties of Fu-10, a fusogenic oncolytic HSV selected from G207 through a similar strategy (14). However, the titer of Synco-2D was substantially lower than that of the wild-type HSV strain 17.

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

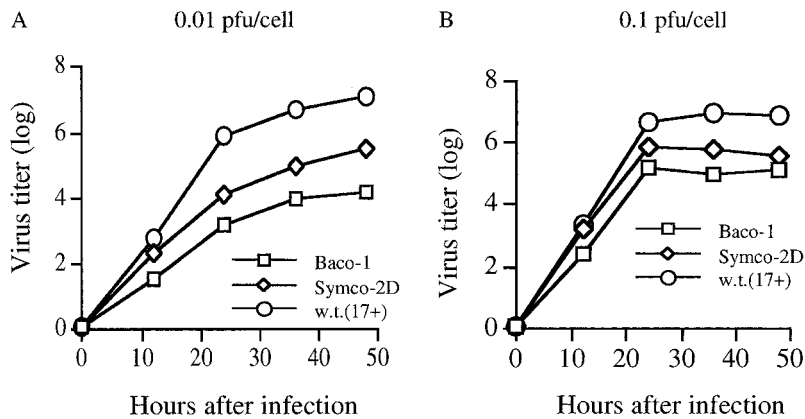


Fig. 3 Replicative properties of Synco-2D. Vero cells were seeded into 6-well plates and infected with the viruses at 0.01 pfu/cell (A) or 0.1 pfu/cell (B). The viruses were harvested at 0, 12, 24, 36, and 48 h after infection and titrated by plaque assay, and the results were plotted against the time after infection.

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 metastasis 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 3×10^5 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 2×10^7 pfu/200 μ l 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 ± 0.7 days (none of the mice survived); there was clear i.p. dissemination of tumor (Fig. 5A), 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, 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

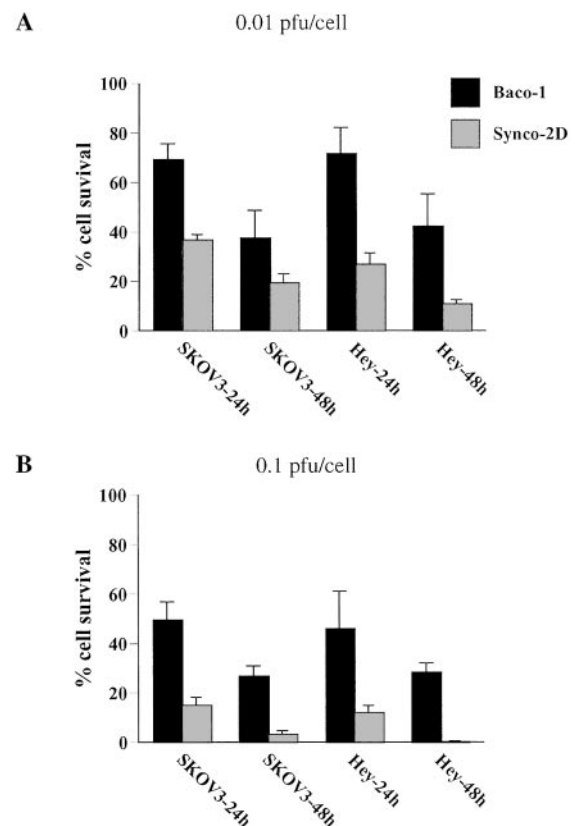


Fig. 4 Comparison of the cytotoxicity of Baco-1 and Synco-2D in cultured ovarian cancer cells. Hey-8 or SKOV3 ovarian cancer cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.01 (A) or 0.1 (B) pfu/cell. Cells were collected at 24 or 48 h after infection, stained with trypan 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 \pm SD. All comparisons showed a significant advantage in cytotoxicity for Synco-2D ($P < 0.01$).

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

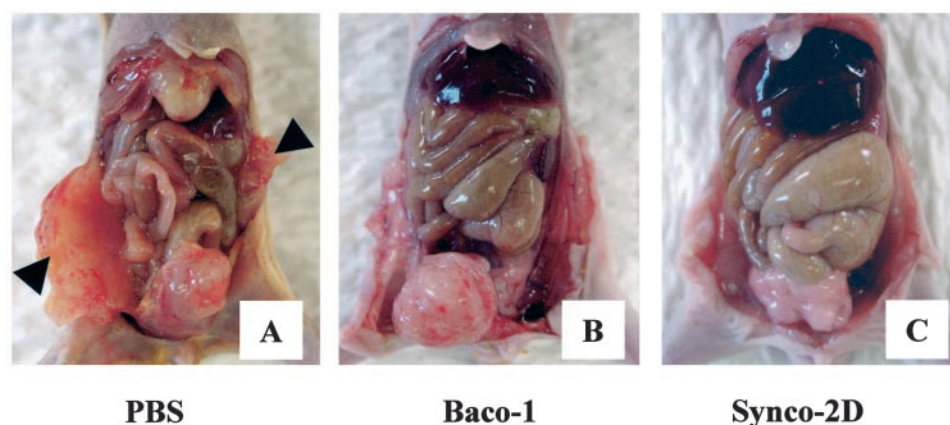


Fig. 5 Effects of Synco-2D in xenograft model of i.p. ovarian cancer. Human ovarian cancer xenografts were established through i.p. injection of 3×10^5 Hey-8 cells into nude mice. The mice then received i.p. injection, on days 14 and 28, with either PBS (control) or 2×10^7 pfu of Baco-1 or Synco-2D. 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.

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

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

^a Means and SDs.

^b $P < 0.01$ as compared with PBS control.

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

oncolytic HSV, we injected either Baco-1 or Synco-2D at relatively high doses (5×10^7 and 3×10^8 pfu) into immunocompetent BALB/c mice through the tail vein ($n = 5$ mice/dose). The dose of 3×10^8 pfu in 200 μ l of solution is the maximum that could be injected systemically into mice, given the modest scale of virus preparation (maximum titer, $<2 \times 10^9$ 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-

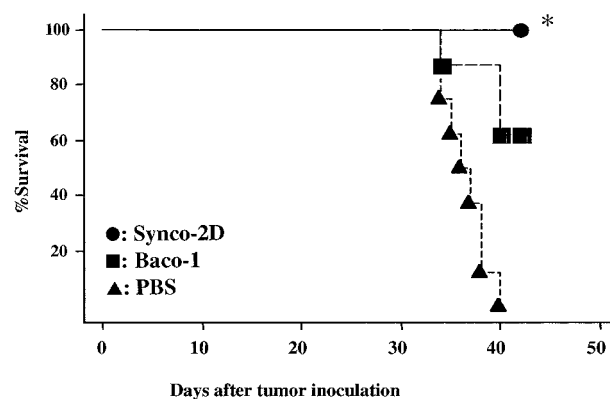


Fig. 6 Prolonged survival of mice treated with Synco-2D. The figure shows the survival curve on Kaplan-Meier plots of data from the experiment described in Fig. 5. The asterisk denotes a significant difference between the Synco-2D group and either the PBS or Baco-1 group ($P < 0.01$).

nisms into a single oncolytic HSV. *In vitro* characterization studies showed that infection with Synco-2D promotes extremely 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 abdominal 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 trials 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

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 virus *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 HSV 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

- Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. *CA Cancer J. Clin.* 51: 15–36, 2001.
- Buy, J. N., Moss, A. A., Ghossain, M. A., Sciote, C., Malbec, L., Vadrot, D., Paniel, B. J., and Decroix, Y. Peritoneal implants from ovarian tumors: CT findings. *Radiology*, 169: 691–694, 1988.
- Ozols, R. F. Management of advanced ovarian cancer consensus summary. Advanced Ovarian Cancer Consensus Faculty. *Semin. Oncol.* 27: 47–49, 2000.
- Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science (Wash. DC)*, 252: 854–856, 1991.
- Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D., and Martuza, R. L. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat. Med.*, 1: 938–943, 1995.
- Bolovan, C. A., Sawtell, N. M., and Thompson, R. L. ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J. Virol.*, 68: 48–55, 1994.
- Chou, J., and Roizman, B. The γ 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA*, 89: 3266–3270, 1992.
- McKie, E. A., MacLean, A. R., Lewis, A. D., Cruickshank, G., Rampling, R., Barnett, S. C., Kennedy, P. G., and Brown, S. M. Selective *in vitro* replication of herpes simplex virus type 1 (HSV-1) ICP34.5 null mutants in primary human CNS tumours: evaluation of a potentially effective clinical therapy. *Br. J. Cancer*, 74: 745–752, 1996.
- Rampling, R., Cruickshank, G., Papanastassiou, V., Nicoll, J., Hadley, D., Brennan, D., Petty, R., MacLean, A., Harland, J., McKie, E., Mabbs, R., and Brown, M. Toxicity evaluation of replication-competent

⁵ Unpublished observation.

- herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.*, 7: 859–866, 2000.
10. Markert, J. M., Medlock, M. D., Rabkin, S. D., Gillespie, G. Y., Todo, T., Hunter, W. D., Palmer, C. A., Feigenbaum, F., Tornatore, C., Tufaro, F., and Martuza, R. L. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a Phase I trial. *Gene Ther.*, 7: 867–874, 2000.
 11. Kramm, C. M., Chase, M., Herrlinger, U., Jacobs, A., Pechan, P. A., Rainov, N. G., Sena-Esteves, M., Aghi, M., Barnett, F. H., Chiocca, E. A., and Breakefield, X. O. Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. *Hum. Gene Ther.*, 8: 2057–2068, 1997.
 12. Todo, T., Martuza, R. L., Rabkin, S. D., and Johnson, P. A. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. *Proc. Natl. Acad. Sci. USA*, 98: 6396–6401, 2001.
 13. Andreansky, S., Soroceanu, L., Flotte, E. R., Chou, J., Markert, J. M., Gillespie, G. Y., Roizman, B., and Whitley, R. J. Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors. *Cancer Res.*, 57: 1502–1509, 1997.
 14. Fu, X., and Zhang, X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. *Cancer Res.*, 62: 2306–2312, 2002.
 15. Fu, X., Tao, L., Jin, A., Vile, R., Brenner, M., and Zhang, X. Expression of a fusogenic membrane glycoprotein by an oncolytic herpes simplex virus provides potent synergistic anti-tumor effect. *Mol. Ther.*, in press, 2003.
 16. Selby, P. J., Thomas, J. M., Monaghan, P., Sloane, J., and Peckham, M. J. Human tumour xenografts established and serially transplanted in mice immunologically deprived by thymectomy, cytosine arabinoside and whole-body irradiation. *Br. J. Cancer*, 41: 52–61, 1980.
 17. Buick, R. N., Pullano, R., and Trent, J. M. Comparative properties of five human ovarian adenocarcinoma cell lines. *Cancer Res.*, 45: 3668–3676, 1985.
 18. Saeki, Y., Ichikawa, T., Saeki, A., Chiocca, E. A., Tobler, K., Ackermann, M., Breakefield, X. O., and Fraefel, C. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in *Escherichia coli*: rescue of replication-competent virus progeny and packaging of amplicon vectors. *Hum. Gene Ther.*, 9: 2787–2794, 1998.
 19. Zhang, X., Efstathiou, S., and Simmons, A. Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology*, 202: 530–539, 1994.
 20. Fu, X., Meng, F., Tao, L., Jin, A., and Zhang, X. A strict late viral promoter is a strong tumor-specific promoter in the context of an oncolytic herpes simplex virus. *Gene Ther.*, in press, 2003.
 21. Fielding, A. K., Chapel-Fernandes, S., Chadwick, M. P., Bullough, F. J., Cosset, F. L., and Russell, S. J. A hyperfusogenic gibbon ape leukemia envelope glycoprotein: targeting of a cytotoxic gene by ligand display. *Hum. Gene Ther.*, 11: 817–826, 2000.
 22. Lentz, B. R., Malinin, V., Haque, M. E., and Evans, K. Protein machines and lipid assemblies: current views of cell membrane fusion. *Curr. Opin. Struct. Biol.*, 10: 607–615, 2000.
 23. Johann, S. V., van Zeijl, M., Cekleniak, J., and O'Hara, B. Definition of a domain of GLVRI which is necessary for infection by gibbon ape leukemia virus and which is highly polymorphic between species. *J. Virol.*, 67: 6733–6736, 1993.
 24. Kavanaugh, M. P., Miller, D. G., Zhang, W., Law, W., Kozak, S. L., Kabat, D., and Miller, A. D. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proc. Natl. Acad. Sci. USA*, 91: 7071–7075, 1994.
 25. Terry-Allison, T., Montgomery, R. I., Warner, M. S., Geraghty, R. J., and Spear, P. G. Contributions of gD receptors and glycosaminoglycan sulfation to cell fusion mediated by herpes simplex virus 1. *Virus Res.*, 74: 39–45, 2001.
 26. Terry-Allison, T., Montgomery, R. I., Whitbeck, J. C., Xu, R., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. HveA (herpesvirus entry mediator A), a coreceptor for herpes simplex virus entry, also participates in virus-induced cell fusion. *J. Virol.*, 72: 5802–5810, 1998.
 27. Rubin, S. C., Randall, T. C., Armstrong, K. A., Chi, D. S., and Hoskins, W. J. Ten-year follow-up of ovarian cancer patients after second-look laparotomy with negative findings. *Obstet. Gynecol.*, 93: 21–24, 1999.
 28. Pickel, H., Lahousen, M., Girardi, F., Tamussino, K., and Stettner, H. Intraperitoneal and retroperitoneal spread of ovarian cancer. *In: F. Sharp, W. P. Mason, and R. E. Leake (eds.), Ovarian Cancer: Biological and Therapeutic Challenges*, pp. 171–178. London: Chapman & Hall Medical, 1989.
 29. Read, G. S., Person, S., and Keller, P. M. Genetic studies of cell fusion induced by herpes simplex virus type 1. *J. Virol.*, 35: 105–113, 1980.
 30. Bond, V. C., Person, S., and Warner, S. C. The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. *J. Gen. Virol.*, 61: 245–254, 1982.
 31. Pogue-Geile, K. L., Lee, G. T., Shapira, S. K., and Spear, P. G. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. *Virology*, 136: 100–109, 1984.
 32. Person, S., Kousoulas, K. G., Knowles, R. W., Read, G. S., Holland, T. C., Keller, P. M., and Warner, S. C. Glycoprotein processing in mutants of HSV-1 that induce cell fusion. *Virology*, 117: 293–306, 1982.