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14. ABSTRACT Currently there is no cure for prostate cancer once the disease has spread beyond the gland. As a large percentage of patients have advanced disease at the time of diagnosis or after the conventional therapy fails, new treatment strategies are urgently needed. We proposed to develop a novel virotherapy for prostate cancer during the funding period. Our working hypothesis was that a fusogenic oncolytic virus would induce a widespread syncytia formation (cell-membrane fusion) in tumor tissues, thus significantly enhancing the antitumor effect. We proposed three tasks to test verify this hypothesis. Task 1 is to initially establish a metastatic prostate cancer model in mice and then to test the potency of antitumor effect of the fusogenic oncolytic HSV. Task 2 is to directly compare the fusogenic oncolytic HSV with a conventional one in vivo for their toxicity. Task 3 is to determine if cell based carriers could function as a delivery vehicle for systemic delivery of the fusogenic oncolytic HSV. Overall these tasks have been largely achieved during the funding period. Specifically, we have demonstrated that the fusogenic oncolytic HSV has a potent antitumor activity against established prostate cancer even after systemic delivery. Additionally, the oncolytic virus also showed an effective therapeutic effect against metastatic prostate cancer, while showing very little toxicity to the experimental animals. The extension of this studies demonstrates that co-administration of fusogenic virotherapy with cyclophosphamide, an approved anticancer chemotherapy drug that also has immunosuppressive activities, can significantly increase the therapeutic effect of virotherapy, possibly by inhibiting the host's innate antiviral activities. These encouraging preclinical results have promoted us to plan for a phase clinical trial of using one of the fusogenic oncolytic HSVs to treat solid tumors including prostate cancer.					
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INTRODUCTION

Prostate cancer remains the most common solid tumor in men, causing an estimated 40,000 deaths per year in the United States. It is the second leading cause of cancer deaths in men, behind cancer of the lung. Current standard therapies are only relatively effective in the short-term. New treatment strategies are clearly needed to improve this situation. Conditionally replicating (oncolytic) viruses offer unique features as anticancer agents. In this funded project, we propose to fully evaluate the antitumor effect and safety of the fusogenic oncolytic HSV against metastatic prostate cancer. We also intend to evaluate if the virotherapy is effective for systemic delivery for treating metastatic diseases, and if necessary, to develop strategies to enhance its systemic delivery.

BODY

Major findings of this project during the funding period are summarized below.

1. Fusogenic oncolytic HSVs can effectively lyse prostate cancer cells when tested in vitro

To determine if fusogenic oncolytic HSVs could effectively lyse prostate cancer cells, we infected PC-3M-Pro4 cells with either the singly fusogenic Synco-2, or the doubly fusogenic Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), allowing us to assess both the inherent cytotoxicity of the input virus and the ability of the virus to replicate and spread in these cells. Baco-1, a nonfusogenic oncolytic HSV, was included as a control. The cytotoxic effect of each virus on the tumor cells was quantified by calculating the percentage of cells that survived after virus infection relative to those surviving in an uninfected well. As compared with Baco-1, both fusogenic oncolytic HSVs had significantly greater cytotoxic activity against this tumor cell line at each test dose and at each of the three harvest times, excluding the earliest time point (24 h) and the lower dose of virus (0.01 pfu/cell) (Fig.1). Direct comparison of Synco-2 and Synco-2D revealed that the doubly fusogenic HSV produced significantly stronger cytotoxicity than the singly fusogenic virus at all time points and at either virus dose ($P < 0.01$). At the lower dose (0.01 pfu/cell), Synco-2D infection reduced the cell

viability to less than 50% within 24 h, in contrast to less than 20% reduction from infection of Synco-2. However, the extent of viable tumor cell reduction was more pronounced at the higher dose. Synco-2D infection completely destroyed the tumor cells by 72 h when the initial virus dose was 0.1 pfu/cell. These results indicate that incorporation of an extra cell-membrane fusion mechanism into a singly fusogenic oncolytic HSV can further enhance the ability of the virus to destroy tumor cells *in vitro*.

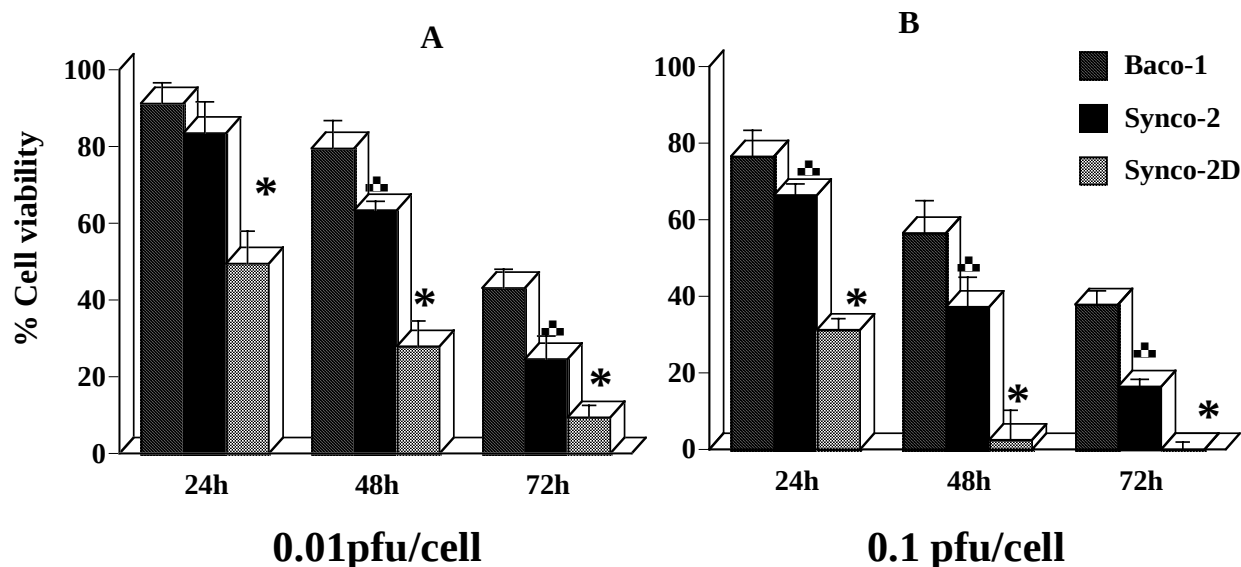


Fig.1. Comparison of the *in vitro* cytotoxicity of Baco-1, Synco-2 and Synco-2D against prostate cancer cells. PC-3M-Pro4 prostate cancer cells were seeded into 12-well plates and infected with Baco-1, Synco-2 or Synco-2D at 0.01 pfu/cell (a) or 0.1 pfu/cell (b), or left uninfected (not shown in figure). Cells were collected 24 h, 48 h or 72 h after infection, and viable cells were counted after trypan blue staining. Percent cell viability was determined by dividing the number of viable cells from the infected wells by the number of cells in the uninfected well. The data are reported as means $\pm$ standard deviations. +, $P < 0.05$ as compared with Baco-1; *, $P < 0.01$ as compared with Synco-2 or Baco-1.

2. Fusogenic oncolytic HSVs have a potent antitumor effect against prostate cancer in vivo

To evaluate the potency of these fusogenic oncolytic HSVs against human prostate cancer, we established both primary and metastatic xenografts in SCID mice by orthotopic and systemic injection

of PC-3M-Pro4 cells. The resultant animal model bears more relevance to patients with advanced prostate cancer than do models in which the tumor cells are implanted subcutaneously. Eight days after tumor cell implantation, 5 mice were examined surgically and all were found to have primary tumors with diameters of approximately 2 mm. The mice were then given their first intravenous injection (through the tail vein) of oncolytic virus (Baco-1, Synco-2 or Synco-2D) at a dose of 2×10^7 , followed in 1 week by a second injection of the same virus dose. PBS-treated mice served as controls. Forty days after the first inoculation of PC-3M-Pro4 cells, orthotopic tumors were explanted and weighed. Metastases in the regional lymph node or lung were identified and counted with a dissecting microscope. Three mice from the PBS-treated group died before the end of the experiment (on days 33, 35 and 36), but were examined in the same manner as other mice and included in the overall analysis.

Systemic delivery of oncolytic HSVs had a significant therapeutic effect on the growth of established prostate cancer at primary sites. By the time the animals were sacrificed (or died due to tumor overgrowth), the orthotopic tumors in PBS-treated mice had attained relatively large volumes (mean weight, 2.17 ± 0.59 mg; Fig.2). Systemic administration of a conventional oncolytic HSV, Baco-1, produced noticeable suppression of tumor growth. The mean weight of orthotopic tumors in this treatment group was 1.57 ± 0.36 mg, representing approximate 30% reduction in tumor volume by comparison with the PBS control group. The therapeutic benefit derived from fusogenic oncolytic HSVs was even more profound: the mean tumor weights of mice treated with either Synco-2 or Synco-2D were less than half those of Baco-1-treated mice. Comparison of mean tumor volumes in the fusogenic virus-treated groups suggested a stronger oncolytic effect from Synco-2D (0.45 ± 0.21 mg Vs. 0.64 ± 0.22 mg for Synco-2), although the difference did not achieve statistical significance ($P=0.287$). These findings show that both Synco-2 and Synco-2D are significantly more effective than Baco-1 in reducing the orthotopic tumor burden after systemic administration, while Synco-2D

appears only marginally better than Synco-2 against established orthotopic tumor.

Systemic administration of oncolytic HSVs also produced striking effects on the lung metastases of prostate cancer (Table 1). Compared with PBS-treated mice, which had a mean 25.4 ± 12.2 tumor nodules per lung, mice receiving an oncolytic HSV showed significant reductions in metastatic lesions: Baco-1, 12.5 ± 3.1 ($P < 0.01$); Synco-2, 6.8 ± 2.2 ($P < 0.01$); Synco-2D, 1.1 ± 1.6 ($P < 0.01$). Moreover, the mean number of lung nodules in the Synco-2D-treated animals was significantly lower than in the Synco-2 group ($P < 0.05$), while both Synco-2 and Synco-2D produced better therapeutic effects than Baco-1 ($P < 0.01$). Tumor metastasis to the local draining lymph nodes was another endpoint of treatment. Administration of Baco-1 or either of the two fusogenic oncolytic HSVs effectively prevented lymph node invasion of the orthotopic tumor; lymph node metastases were detected only in the PBS control group (Table 1). Taken together, these results provide compelling evidence that the doubly fusogenic oncolytic HSV is more potent than the singly fusogenic oncolytic HSV against lung metastases of prostate cancer after systemic administration of virus, in agreement with finding in Fig.1.

Table 1. Therapeutic effect of oncolytic HSVs on lung metastases of PC-3M-Pro4 tumor cells

Treatment	PBS (control)	Baco-1	Synco-2	Synco-2D
Lung metastases	25.4 ± 12.2	12.5 ± 3.1^a	$6.8 \pm 2.2^{a,b}$	$1.1 \pm 1.6^{a,b,c}$
Lymph node metastases	1.6 ± 0.9	ND	ND	ND

Lung metastases were established by tail vein injection of PC-3M-Pro4 tumor cells ($1 \times 10^5/100\mu\text{l}$) 1 day after orthotopic tumor inoculation. The treatment plan was the same as described in the legend to Fig.2. Forty days after orthotopic tumor inoculation, mice were euthanized by CO₂ inhalation and their lungs were resected, washed in saline, and placed in Bouin's fixative. Lung metastases were counted with the aid of a dissecting microscope 24 h later. The findings are reported as means and standard deviations. ND = not detected.

^a $P < 0.01$ as compared with control.

^b $P < 0.01$ as compared with Baco-1.

^c $P < 0.05$ as compared with Synco-2.

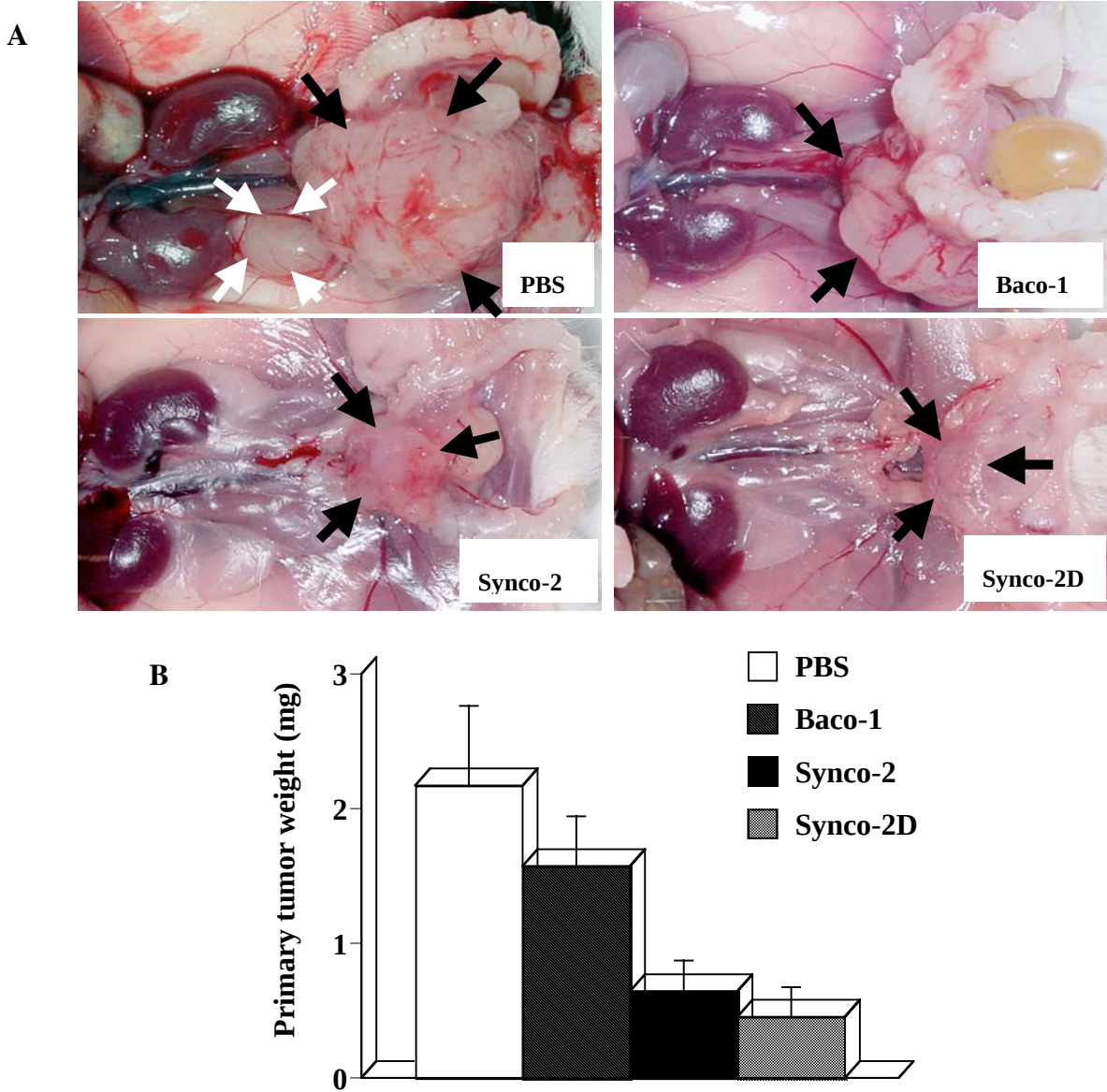


Fig.2. Therapeutic effect of the fusogenic oncolytic HSVs against orthotopic tumors. Human prostate cancer xenografts were established in the primary site through orthotopic inoculation of PC-3M-Pro4 cells. Eight and 15 days after tumor cell inoculation, mice received intravenously administered of oncolytic HSVs at a dose of 2×10^7 pfu at a volume of 100 μ l through the tail vein. Forty days after orthotopic tumor inoculation, mice that

were still alive were euthanized and examined for the presence of tumor masses at the original injection site, as well as lymph node metastases. (A) Typical photos of pathologic specimens from 1 mouse per treatment group showing localized tumor and lymph node metastasis (seen only in PBS-treated group). Orthotopic tumors are indicated by hatched arrows and the single lymph node metastasis by closed arrows; (B) Mean weights ($\pm$ 1 SD) of orthotopic tumors excised from the mice described in panel A.

3. In vivo toxicity evaluation

We conducted experiments to directly compare the toxicity of the fusogenic and non-fusogenic oncolytic HSVs by injecting the viruses systemically into immune-competent mice at escalating doses.

3.1 Experimental design and procedure. Six-week-old immune competent BALB/c mice were injected through the tail vein with either the fusogenic Synco-2 or Baco-1 at a low and high doses: 5×10^7 or 3×10^8 pfu (the dose of 3×10^8 in 200 μ l of solution is the maximum that could be injected systemically into mice, given the modest scale of virus preparation). Mice in negative control group were given the same volume of PBS.

3.2 Incidence of mortality. For evaluation of mortality after oncolytic HSV administration, animals were observed for a six-month period. 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 in either groups.

3.3. Histopathological findings after systemic administration of oncolytic HSVs. For determining histopathological abnormalities, mice were killed 1 week after virus administration. Following euthanasia, a complete necropsy was performed. Tissue samples were obtained from major organs including brain, heart, intestines, kidney, liver, lung, pancreas, spleen, and stomach. About one-third of the freshly obtained organ tissues were immediately frozen for determining virus distribution. Tissue sections were prepared from the remaining organ mass by the College's pathology core facilities and then examined for signs of histopathological changes. Only mild histological

abnormality was occasionally seen in liver sections from mice receiving administration of either Baco-1 or Synco-2. There was no major difference on the severity of this abnormality among the fusogenic and nonfusogenic oncolytic HSVs. However, there was a slight difference on the severity of the liver pathological abnormality in animals receiving low and high doses of Synco-2. No obvious pathological changes were identified in any other organs from mice in all the three treatment groups. During the microscopic examinations, particular attention was made to look for syncytial formation from tissue sections prepared from mice received Synco-2 administration. No syncytial formation was visible, even in liver sections where mild tissue abnormality was noticed.

4. Strategies to facilitate systemic delivery of fusogenic oncolytic HSVs

4.1. Liposome formulation of oncolytic HSVs. To test 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.

4.2. Delivery of oncolytic HSVs by carrier cells. We initially tested the infectivity of oncolytic HSVs on T lymphocytes. The 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 an 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 (1). We thus tested this strategy to determine if oncolytic HSV could hitchhike to carrier cells that were found to be nonpermissive to infection of HSV vectors in our previous experiments. We incubated either NK cells or macrophages of both human and murine origins (1×10^5) with 5×10^5 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 1×10^5 plaques were obtained from each preparation (with 1×10^5 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-mediated delivery of oncolytic HSVs for the treatment of metastatic prostate cancer.

5. Co-administration of cyclophosphamide can dramatically enhance the antitumor effect of a fusogenic oncolytic HSV.

One of the major obstacles facing the successful application of virotherapy such as the fusogenic oncolytic HSVs is the host's immune defense mechanisms, which can restrict the ability of the virus to replicate and spread within tumors. In one of the extended studies of this project, we exploited the potential of cyclophosphamide, a cancer chemotherapeutic drug that also inhibits innate immune

responses, to enhance the activity of oncolytic viruses. Our data showed that co-administration of cyclophosphamide with a fusogenic oncolytic HSV could significantly prolong the virus replication in tumor tissues, thus leading a better antitumor effect. Moreover, it also potentiated the ability of oncolytic HSV to induce tumor-specific immune responses. Together, our results suggest that coadministration of oncolytic HSV with cyclophosphamide would be a feasible way to enhance the antitumor effects, a strategy that may be implemented into the planned clinical trial.

KEY RESEARCH ACCOMPLISHMENTS

- Both in vitro and in vivo studies show that the fusogenic oncolytic HSVs are potent antitumor agents against either primary or metastatic prostate cancer.
- Direct in vivo comparison show that, despite their increased antitumor activity, fusogenic oncolytic HSVs are as safe as the nonfusogenic first generation oncolytic HSV.
- Both liposome formulation and cell-based carriers may be suitable for systemic delivery of fusogenic oncolytic HSVs in the presence of host's anti-HSV immunity.
- Co-administration of fusogenic oncolytic HSV-based virotherapy with cyclophosphamide may further enhance its therapeutic outcome in the planned clinical trials.

REPORTABLE OUTCOMES

1. Conference presentation: The 6th Annual Meeting of the American Society of Gene Therapy (6/5-9/2003, Washington, DC)

Title of abstract: Systemic Delivery of Fusogenic Oncolytic Herpes Simplex Viruses for Advanced Prostate Cancer.

2. Conference presentation: The 9th Annual meeting of Japanese Society of Gene Therapy (7/19-20/2003, Tokyo, Japan).

Title of abstract: Enhancement of the Therapeutic Efficacy of an Oncolytic Herpes Simplex Virus

(HSV) by Two Membrane-Fusion Mechanisms: Comparison with a Conventional HSV Therapy.

3. Publication: Mikihiro Nakamori, Xinping Fu, Curtis A. Pettaway and Xiaoliu Zhang (2004). Potent antitumor activity after systemic delivery of a doubly fusogenic oncolytic herpes simplex virus against metastatic prostate cancer. *Prostate*. 60(1): 53-60.

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.

5. 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.

6. 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

Fusogenic oncolytic HSVs are potent yet safe antitumor agents that warrant clinical evaluations in patients suffering from late stage prostate cancer.

REFERENCES

1. Cole C, Qiao J, Kottke T, Diaz RM, Ahmed A, Sanchez-Perez L, Brunn G, Thompson J, Chester J, Vile RG. Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. *Nat Med* 2005;11(10):1073-1081.

APPENDICES:

A re-print from *Prostate*

Potent Antitumor Activity After Systemic Delivery of a Doubly Fusogenic Oncolytic Herpes Simplex Virus Against Metastatic Prostate Cancer

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BACKGROUND. Although conventional radiation therapy and surgery are potentially curative treatments for organ-confined prostate cancer, there are few effective treatments for metastatic disease. Oncolytic viruses have shown considerable promise for the treatment of solid tumors including prostate cancer. We recently demonstrated that incorporation of a cell membrane fusion capability into an oncolytic herpes simplex virus (HSV) can significantly increase the antitumor potency of the virus.

METHODS. We used a mouse model of primary and metastatic human prostate cancer established from PC-3M-Pro4 to evaluate three different types of oncolytic HSVs: non-fusogenic Baco-1, singly fusogenic Synco-2, and doubly fusogenic Synco-2D.

RESULTS. Our results show that Synco-2D has greater oncolytic activity than either Baco-1 or Synco-2 virus. Against lung metastases of human prostate cancer xenografts, intravenous administration of Synco-2D had produced a significant reduction of tumor nodules by day 40 post-inoculation as compared with Synco-2 ($P < 0.05$), Baco-1 ($P < 0.01$), and PBS control ($P < 0.01$).

CONCLUSIONS. We conclude that the doubly fusogenic Synco-2D is an effective therapeutic agent for human metastatic prostate cancer. *Prostate* 60: 53–60, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: oncolytic; HSV; fusogenic; GALV; prostate cancer; systemic

INTRODUCTION

Prostate cancer remains the most common solid tumor in men, causing an estimated 40,000 deaths per year in the United States. It is the second leading cause of cancer deaths in men, behind cancer of the lung [1]. Current standard therapies for organ-confined prostate cancer include radiation or surgery and, in some circumstances, neoadjuvant or adjuvant hormonal therapy. While these treatments are relatively effective in the short-term, a significant proportion of patients who initially present with localized disease ultimately relapse. Moreover, each of these therapies may produce unwanted side effects. The major risk faced by patients with prostate cancer is the development of metastatic disease. The prognosis for patients with locally relapsed or metastatic prostate cancer is very poor, even when they respond initially to androgen deprivation. Metastatic prostate cancer is essentially

resistant to systemic cytotoxic chemotherapy, while external beam and radioisotope radiation therapies offer only palliative relief of symptoms. New treatment strategies are clearly needed to improve this situation [2].

Conditionally replicating (oncolytic) viruses offer unique features as anticancer agents. First, unlike conventional cytotoxic drugs, they target cancer cells specifically, because of their restricted ability to replicate in

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normal cells [3–5]. Second, unlike replication-defective vectors, oncolytic viruses can spread from initially infected tumor cells to surrounding tumor cells. Consequently, even when only small populations of tumor cells are initially infected/transduced, it may be possible to achieve a large volume of distribution and enhanced anticancer effects due to viral replication and dissemination.

Several human viruses, including herpes simplex virus (HSV), adenovirus, and reoviruses, have been modified for oncolytic purposes, and some have already been moved into clinic trials. Early clinical experience indicates that these oncolytic viruses are safe, but may have only limited antitumor activity when used as monotherapy [6–9]. A variety of strategies are being pursued to enhance the potency of oncolytic viruses. In one such approach, oncolytic virotherapy is combined with standard radiotherapy or chemotherapy, resulting in synergistic effect [10,11]. Another strategy is to clone therapeutic genes into the oncolytic virus to arm the virus with additional cytotoxic mechanisms that augment the direct lytic functions of the virus [12]. Particularly attractive in this context are cytotoxic mechanisms with potent bystander effects capable of eliminating tumor cells that the virus cannot reach.

We recently demonstrated that incorporation of cell membrane fusion capability into an oncolytic HSV can significantly increase the antitumor potency of the virus [13–15]. These fusogenic oncolytic HSVs were constructed by three different methods: (1) screening for the syncytial phenotype after random mutation of a well-established oncolytic HSV (to obtain Fu-10) [13]; (2) insertion of the gene encoding the hyperfusogenic membrane glycoprotein of gibbon ape leukemia virus (GALV.fus) into the genome of an oncolytic HSV (to generate Synco-2) [14]; and (3) incorporation of both of these two membrane fusion mechanisms into a single oncolytic HSV (to generate Synco-2D) [15]. Regardless of the method used, the fusogenic oncolytic HSV showed a strikingly enhanced antitumor activity when compared with the nonfusogenic virus.

Here, we report on the antitumor efficacy of these different versions of oncolytic HSVs in a murine model bearing both primary prostate cancer xenografts and lung metastases, established through simultaneous orthotopic and systemic injection of a human prostate cancer cell line, PC-3M-Pro4. The study had a twofold objective. First, we sought to determine whether the fusogenic oncolytic HSVs could gain access to and kill distant tumors after systemic administration and if they are potent enough to produce a significant antitumor effect on metastatic prostate cancer, which is currently incurable with conventional therapies. Second, we directly compared the antitumor activities of

the different fusogenic oncolytic HSVs to determine if the doubly fusogenic virus (Synco-2D) is more potent than the singly fusogenic virus (Synco-2). Our results demonstrate that Synco-2D has the most potent therapeutic activity in this tumor model, based on significantly greater reductions in lung nodules after intravenous administration of the virus. We conclude that systemic administration of a Synco-2D-like HSV may provide effective treatment for metastatic human prostate cancer.

MATERIALS AND METHODS

Cell Lines and Viruses

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO₂ atmosphere. The highly metastatic prostate cancer cell line PC-3M-Pro4 [16] was grown in RPMI 1640 supplemented with 10% FBS. For in vivo inoculation, the cells were harvested from culture flasks after a brief period of trypsinization. Only single-cell suspensions of >95% viability (trypan blue exclusion) were used for tumor inoculation.

The oncolytic HSVs were derived from fHSV-delta-pac, a bacterial artificial chromosome (BAC)-based HSV construct; the details of their construction are described elsewhere [14,15]. Briefly, to generate Baco-1 and Synco-2, we ligated the enhanced green fluorescent protein gene (EGFP, for Baco-1) or GALV.fus (for Synco-2), together with the HSV packaging signal sequence, into the unique PacI site in fHSV-delta-pac. The ligation mixture was subsequently transfected into Vero cells for virus production. Synco-2D was generated by subjecting Baco-1 to random mutagenesis, followed by screening for the syncytial phenotype [13]. Then the circular form of viral DNA was obtained by extracting virion DNA from Vero cells shortly (1 hr) after virus infection, using a previously described method [17]. The viral DNA was then transformed into competent *E. coli* cell DH-10B through electroporation. The gene cassette encoding EGFP in the viral genome was then cut out with PacI and replaced with GALV.fus (driven by the conditional UL38 promoter of HSV) by use of an enforced ligation strategy, as described [15]. The ligation mixture was directly transfected into Vero cells using LipofectAMINE (GIBCO-BRL), and the cells were incubated for 3–5 days to allow infectious virus to be generated. The virus was subsequently plaque purified. Viral stocks were prepared by infecting Vero cells with the viruses at 0.01 plaque-forming units (pfu) per cell, harvested after 2 days and stored at –80°C. Viral titers were quantified by plaque assay and are reported as plaque-forming units (pfu).

In Vitro Phenotypic Characterization and Cell Killing Assay

To characterize the phenotypes of the fusogenic oncolytic HSVs in vitro, we seeded PC-3M-Pro4 cancer cells into 6-well plates. The cells were infected the following day with serially diluted viruses (Baco-1, Synco-2, or Synco-2D) and cultured in DMEM containing 1% FBS for up to 3 days to allow fusion to occur and plaques to develop. To evaluate the cytotoxicity of each virus in vitro, we plated PC-3M-Pro4 cells in 12-well plates at 5×10^4 cells/well. Cells were infected with Baco-1, Synco-2, or Synco-2D at 0.01 or 0.1 pfu/cell and harvested at 24-hr intervals. Cell viability was determined using trypan blue staining. The percentage of cell viability was calculated by dividing the number of viable cells from the infected well by the number of cells from the uninfected well. All experiments were performed in triplicate with mean numbers used in the final calculation.

Animal Studies

BALB/cByJlcrSmnHsd-scid mice (5–6 weeks old) were obtained from Harlan (Indianapolis, IN) and were kept in groups of four or fewer under specific pathogen-free conditions. All animal studies were approved by Baylor College of Medicine Animal Care and Use Subcommittee and performed in accordance with its policies. For surgical procedures, all the mice were anesthetized with an intraperitoneal injection of mixture solution containing 2.5% 2,2,2-tribromoethanol and *tert*-amylalcohol (1:1). Orthotopic inoculation was performed according to a previously described procedure [18]. Briefly, a transverse incision was made in the lower abdomen. After the abdominal wall muscles were split, the bladder and seminal vesicles were exposed and retracted anteriorly to reveal the dorsal prostate. Then, 2×10^5 PC-3M-Pro4 cells suspended in 10 μ l of PBS were carefully injected under the prostate capsule using a 30-gauge needle and a glass Hamilton syringe (Hamilton Syringe Co., Reno, NV). The formation of a bulla indicated a satisfactory injection. The incision was closed with a single layer of surgical clips (Autoclip; Clay Adama, Parsippany, NJ). Lung metastases of prostate cancer were established through tail vein injection of PC-3M-Pro4 cells (1×10^5 /100 μ l) on the day after orthotopic tumor inoculation [19]. Mice were then randomly divided into 4 groups ($n = 8$) and were injected in tail vein twice with either PBS (control) or 2×10^7 pfu of viruses (Baco-1, Synco-2, or Synco-2D, at a volume of 100 μ l), on days 7 and 14 after the tumor inoculation. Forty days after orthotopic tumor inoculation, the mice were euthanized by CO₂ inhalation. Primary tumors were excised and weighted. At the same time, animal lungs were resected, washed in

saline, and placed in Bouin's fixative. Lung metastases were counted with the aid of a dissecting microscope 24 hr later, as described previously [20].

Statistical Analysis

All quantitative results are reported as means $\pm$ standard deviations. The statistical analysis was performed by one-way ANOVA using Statview 5.0 software (Abacus Concepts, Berkeley, CA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Syncytial Formation by Fusogenic Oncolytic HSVs in PC-3M-Pro4 Prostate Cancer Cells

We chose the human prostate cancer cell line PC-3M-Pro4 for both in vitro and in vivo testing of our fusogenic oncolytic HSVs. PC-3M-Pro4 was selected from PC-3M cell line through repeated cycles of orthotopic inoculation/harvest in athymic mice, and has been shown to efficiently produce lung metastases after intravenous injection into immune-deficient mice [16]. To characterize and compare the phenotypic properties of Baco-1, Synco-2, and Synco-2D, we infected PC-3M-Pro4 cells with serially diluted virus and at different times after infection, photographed the plaques typically formed by virus infection. As shown in Figure 1, Baco-1 produced plaques consisting of round cells, while plaques resulting from Synco-2 or Synco-2D infection were composed entirely of fused cells. By 48 hr after infection, a single Synco-2D plaque was too big to be photographed within a single microscopic field, in contrast to that resulting from Synco-2 infection, indicating that Synco-2D may have greater tumor cell-killing ability than the singly fusogenic oncolytic HSV Synco-2.

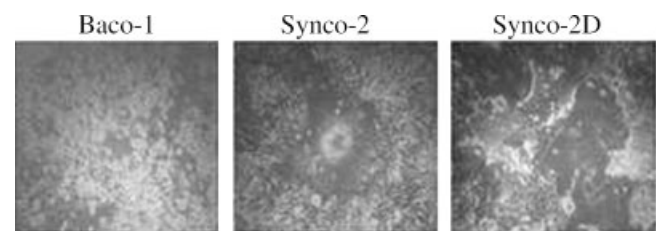


Fig. 1. Phenotypic characterization of fusogenic oncolytic HSV in a prostate cancer cell line, PC-3M-Pro4. PC-3M-Pro4 cells were infected with either nonfusogenic (Baco-1) or fusogenic (Synco-2 or Synco-2D) oncolytic HSVs. Each panel shows an area containing a single plaque, photographed at 48 hr post-infection (original magnification, 200 $\times$).

Direct Comparison of the Prostate Cancer Cell-Killing Activity of Singly and Doubly Fusogenic Oncolytic HSVs

To determine if the enhanced ability of Synco-2D to induce syncytial formation correlates with an increased ability to destroy tumor cells, we infected PC-3M-Pro4 cells with Baco-1, Synco-2, or Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), allowing us to assess both the inherent cytotoxicity of the input virus and the ability of the virus to replicate and spread in these cells. The cytotoxic effect of each virus on the tumor cells was quantified by calculating the percentage of cells that survived after virus infection relative to those surviving in an uninfected well. As compared with Baco-1, both fusogenic oncolytic HSVs had significantly greater cytotoxic activity against this tumor cell line at each test dose and at each of the three harvest times, excluding the earliest time-point (24 hr) and the lower dose of virus (0.01 pfu/cell) (Fig. 2). Direct comparison of Synco-2 and Synco-2D revealed that the doubly fusogenic HSV produced significantly stronger cytotoxicity than the singly fusogenic virus at all time points and at either virus dose ($P < 0.01$). At the lower dose (0.01 pfu/cell), Synco-2D infection reduced the cell viability to less than 50% within 24 hr, in contrast to less than 20% reduction from infection of Synco-2. However, the extent of viable tumor cell reduction was more pronounced at the higher dose. Synco-2D infection completely destroyed the tumor cells by 72 hr when the initial virus dose was 0.1 pfu/cell. These results indicate that incorporation of an extra cell-membrane fusion mechanism into a singly fusogenic oncolytic HSV can further enhance the ability of the virus to destroy tumor cells in vitro.

Therapeutic Efficacy Against Primary Tumor After Systemic Delivery

To evaluate the potency of these fusogenic oncolytic HSVs against human prostate cancer, we established both primary and metastatic xenografts in SCID mice by orthotopic and systemic injection of PC-3M-Pro4 cells. The resultant animal model bears more relevance to patients with advanced prostate cancer than do models in which the tumor cells are implanted subcutaneously. Eight days after tumor cell implantation, five mice were examined surgically and all were found to have primary tumors with diameters of approximately 2 mm (data not shown). The mice were then given their first intravenous injection (through the tail vein) of oncolytic virus (Baco-1, Synco-2, or Synco-2D) at a dose of 2×10^7 , followed in 1 week by a second injection of the same virus dose. PBS-treated mice served as controls. Forty days after the first inoculation of PC-3M-Pro4 cells, orthotopic tumors were explanted and weighed. Metastases in the regional lymph node or lung were identified and counted with a dissecting microscope. Three mice from the PBS-treated group died before the end of the experiment (on days 33, 35, and 36), but were examined in the same manner as other mice and included in the overall analysis.

Systemic delivery of oncolytic HSVs had a significant therapeutic effect on the growth of established prostate cancer at primary sites. By the time, the animals were sacrificed (or died due to tumor overgrowth), the orthotopic tumors in PBS-treated mice had attained relatively large volumes (mean weight, 2.17 ± 0.59 mg; Fig. 3). Systemic administration of a conventional oncolytic HSV, Baco-1, produced noticeable suppression of tumor growth. The mean weight of

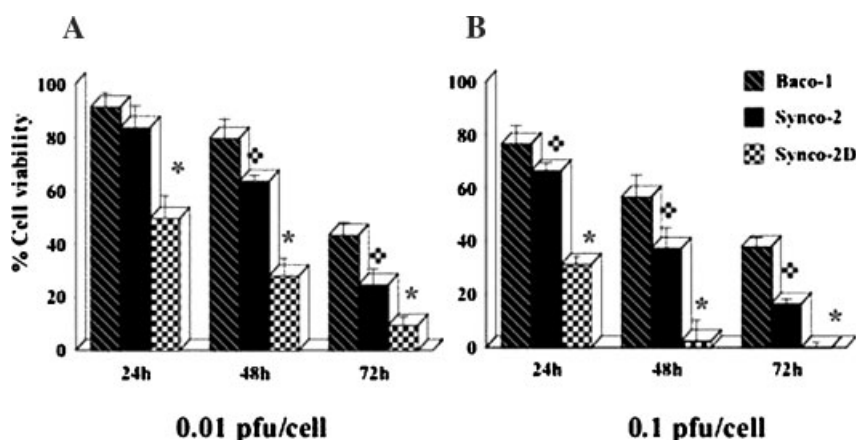


Fig. 2. Comparison of the in vitro cytotoxicity of Baco-1, Synco-2, and Synco-2D against prostate cancer cells. PC-3M-Pro4 prostate cancer cells were seeded into 12-well plates and infected with Baco-1, Synco-2, or Synco-2D at 0.01 pfu/cell (A) or 0.1 pfu/cell (B), or left uninfected (not shown in figure). Cells were collected 24, 48, or 72 hr after infection, and viable cells were counted after trypan blue staining. Percent cell viability was determined by dividing the number of viable cells from the infected wells by the number of cells in the uninfected well. The data are reported as means $\pm$ standard deviations. ✕, $P < 0.05$ as compared with Baco-1; *, $P < 0.01$ as compared with Synco-2 or Baco-1.

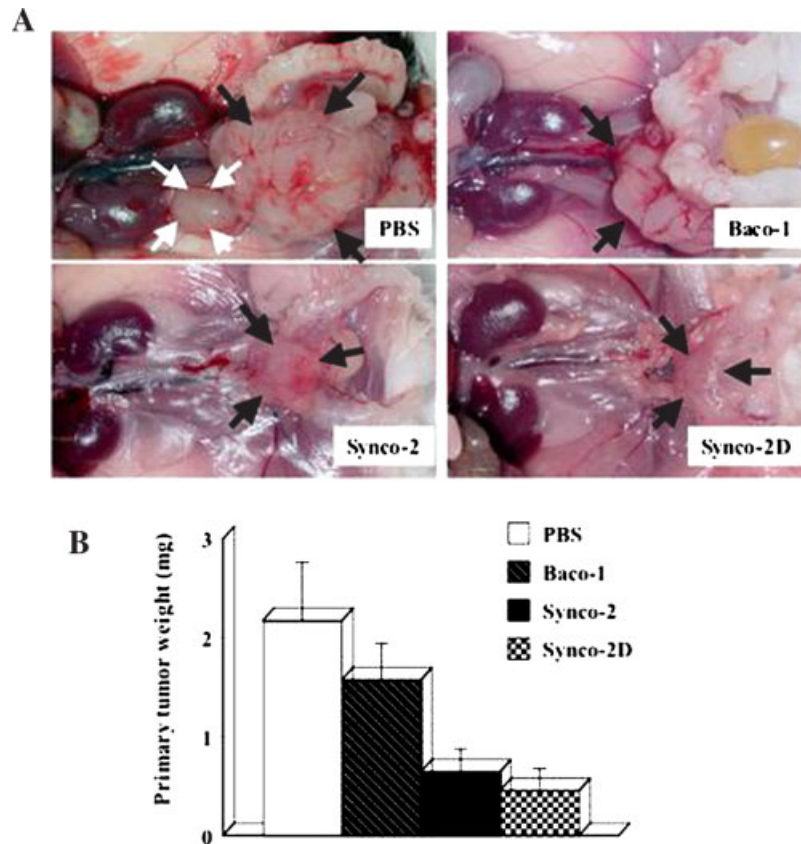


Fig. 3. Therapeutic effect of the fusogenic oncolytic HSVs against orthotopic tumors. Human prostate cancer xenografts were established in the primary site through orthotopic inoculation of PC-3M-Pro4 cells. Eight and 15 days after tumor cell inoculation, mice received intravenously administered of oncolytic HSVs at a dose of 2×10^7 pfu at a volume of 100 μ l through the tail vein. Forty days after orthotopic tumor inoculation, mice that were still alive were euthanized and examined for the presence of tumor masses at the original injection site, as well as lymph node metastases. **A:** Typical photos of pathologic specimens from 1 mouse per treatment group showing localized tumor and lymph node metastasis (seen only in PBS-treated group). Orthotopic tumors are indicated by hatched arrows and the single lymph node metastasis by closed arrows; **(B)** Mean weights ($\pm$ SD) of orthotopic tumors excised from the mice described in Panel A.

orthotopic tumors in this treatment group was 1.57 ± 0.36 mg, representing approximate 30% reduction in tumor volume by comparison with the PBS control group. The therapeutic benefit derived from fusogenic oncolytic HSVs was even more profound: the mean tumor weights of mice treated with either Synco-2 or Synco-2D were less than half those of Baco-1-treated mice. Comparison of mean tumor volumes in the fusogenic virus-treated groups suggested a stronger oncolytic effect from Synco-2D (0.45 ± 0.21 mg vs. 0.64 ± 0.22 mg for Synco-2), although the difference did not achieve statistical significance ($P = 0.287$). These findings show that both Synco-2 and Synco-2D are significantly more effective than Baco-1 in reducing the orthotopic tumor burden after systemic administration, while Synco-2D appears only marginally better than Synco-2 against established orthotopic tumor.

Systemic administration of oncolytic HSVs also produced striking effects on the lung metastases of pro-

state cancer (Table I). Compared with PBS-treated mice, which had a mean 25.4 ± 12.2 tumor nodules per lung, mice receiving an oncolytic HSV showed significant reductions in metastatic lesions: Baco-1, 12.5 ± 3.1 ($P < 0.01$); Synco-2, 6.8 ± 2.2 ($P < 0.01$); Synco-2D, 1.1 ± 1.6 ($P < 0.01$). Moreover, the mean number of lung nodules in the Synco-2D-treated animals was significantly lower than in the Synco-2 group ($P < 0.05$), while both Synco-2 and Synco-2D produced better therapeutic effects than Baco-1 ($P < 0.01$). Tumor metastasis to the local draining lymph nodes was another endpoint of treatment. Administration of Baco-1 or either of the two fusogenic oncolytic HSVs effectively prevented lymph node invasion of the orthotopic tumor; lymph node metastases were detected only in the PBS control group (Table I). Taken together, these results provide compelling evidence that the doubly fusogenic oncolytic HSV is more potent than the singly fusogenic oncolytic HSV against lung metastases of

TABLE I. Therapeutic Effect of Oncolytic HSVs on Lung Metastases of PC-3M-Pro4 Tumor Cells

Treatment	PBS (control)	Baco-1	Synco-2	Synco-2D
Lung metastases	25.4 ± 12.2	12.5 ± 3.1*	6.8 ± 2.2***	1.1 ± 1.6***,***
Lymph node metastases	1.6 ± 0.9	ND	ND	ND

Lung metastases were established by tail vein injection of PC-3M-Pro4 tumor cells (1×10^5 /100 μ l) 1 day after orthotopic tumor inoculation. The treatment plan was the same as described in the legend to Figure 3. Forty days after orthotopic tumor inoculation, mice were euthanized by CO₂ inhalation and their lungs were resected, washed in saline, and placed in Bouin's fixative. Lung metastases were counted with the aid of a dissecting microscope 24 hr later. The findings are reported as means and standard deviations. ND, not detected.

* $P < 0.01$ as compared with control.

** $P < 0.01$ as compared with Baco-1.

*** $P < 0.05$ as compared with Synco-2.

prostate cancer after systemic administration of virus, in agreement with the findings in Figures 1 and 2.

DISCUSSION

We recently demonstrated that a syncytial mutant (Fu-10) selected from the well-characterized oncolytic HSV G207 through random mutageneses has a dramatically enhanced antitumor effect on lung metastasis of breast cancer compared with that of a conventional non-fusogenic virus [13]. Fusogenic oncolytic HSVs constructed by inserting a hyperfusogenic glycoprotein into a conventional oncolytic HSV can also significantly increase the antitumor effect of that virus [14]. Most recently, we constructed a newer fusogenic oncolytic HSV, in which both fusion mechanisms were incorporated into a single virus (Synco-2D). Intraperitoneal administration of this virus to mice with disseminated ovarian cancer led to eradication of tumors in 75% of mice [15].

In the studies reported here, we directly compared the singly and doubly fusogenic HSVs for their ability to kill human prostate cancer cells in vitro and in vivo. Synco-2D was significantly more oncolytic than Synco-2 against cultured PC-3M-Pro4 cells, indicating that the additional cell-membrane fusion mechanism contained in the doubly fusogenic virus had indeed increased the antitumor potency of the virus. This impression was supported by data from our animal model of prostate cancer, in which Synco-2D showed a significantly better therapeutic effect than Synco-2 against lung metastases. However, Synco-2D and Synco-2 were approximately equivalent in their lytic activities against orthotopic tumor. This differential effect on primary and metastatic tumors can probably be attributed to the route of virus administration and the discrepancy between tumor volumes. By the time the viruses were

administered, the orthotopic tumor volume was relatively bulky (diameters of approximately 2 mm) and was likely substantially larger than the lung nodules. Although we did not verify the size of lung metastatic lesions, we assume that they were still quite small when the oncolytic viruses were administered. Indeed, only relatively small nodules were seen in PBS-treated animals on completion of the experiment. Since systemic administration of oncolytic HSVs can distribute only a limited amount of virus to large tumor masses, the enhanced oncolytic activity of a doubly fusogenic virus would not be expected to yield a noticeably better therapeutic effect than a singly fusogenic virus. Thus, in future application of fusogenic HSVs, it may be preferable to resect bulky tumors before attempting virotherapy. Additionally, due to the rich blood supply and filtering capability of lung tissue, the nodules may have received a larger distribution of systemically administered virus than did the orthotopic tumors. Whatever the explanation, this outcome of virotherapy agrees with our earlier study of Fu-10, which produced a striking therapeutic effect on lung metastases of breast cancer [13]. It will be important to confirm the preferential distribution of oncolytic HSVs to the lungs as a first step in developing guidelines for treating either primary or metastatic tumors in this organ with oncolytic virotherapy.

Syncytial formation mediated by fusogenic glycoproteins relies on the initial binding of fusogenic glycoproteins to their specific receptors on target cells, which induces ordered structural changes of the membrane lipid bilayers, leading in turn to lipid mixing and eventual fusion of either viral and cellular membranes or cellular membranes alone [21]. The cellular receptor for GALV.fus has been identified as PiT1, a type III sodium-dependent phosphate transporter [22,23]. However, the membrane fusion induced by HSVs is

more complex, requiring the participation of multiple viral glycoproteins and at least two specific cellular receptors on the cell surface [24–27]. Thus, besides their quantitative advantages over singly fusogenic oncolytic HSVs, doubly fusogenic viruses such as Synco-2D may reduce the emergence of therapy-resistant tumor cells. That is, tumor cells resistant to syncytial formation mediated by one membrane-fusion mechanism could still be destroyed by syncytial formation resulting from another mechanism. Our recent finding that Synco-2D infection, but not infection due to Fu-10 or Synco-2, can cause syncytial formation in several murine and one human tumor cell line supports this possibility (Nakamori et al., unpublished data).

Several previous publications demonstrated that oncolytic HSVs can inhibit prostate tumor growth both *in vitro* and *in vivo* [28–31]. However, most of these studies were conducted with subcutaneously established prostate cancer xenografts treated by intratumor injection of the viruses. If virotherapy is to succeed as an effective means of treating prostate cancer, it will be critical to demonstrate its efficacy against multifocal disease. Our results showing significant therapeutic effect of moderate-dose, intravenous administered Synco-2D against lung metastases in mice indicate that this doubly fusogenic virus may become an important tool in efforts to destroy established human prostate cancer metastases.

Although a modified version of our current protocol may be applicable to the treatment of clinical prostate cancer patients, some precautions need to be observed. Even though oncolytic HSVs replicate only in tumor cells, the syncytia produced by these fusogenic viruses are potentially toxic to normal cells and, if uncontrolled, could pose a safety concern. This is particularly true when the viruses are systemically administered, as in the present study. During the construction of Synco-2D, we employed several strategies to restrict syncytial formation to tumor tissues. First, we used UL38p, a strict-late viral promoter, to direct GALV.fus gene expression; because its activity remains confined to the tumor tissue after systemic administration with an oncolytic HSV [32], providing a link between conditional replication of the virus in tumor cells and GALV.fus-mediated syncytial formation. Second, the syncytial formation from mutagenized HSV is mainly due to aberrant expression of several viral glycoproteins such as gB and gK [33–36]. As these glycoproteins are encoded by late genes whose expression depends upon viral DNA replication, the cell membrane fusion mediated by this mechanism would only occur in tumor cells (where the virus can undergo a full infectious cycle) but not in normal nondividing cells (where virus replication is restricted and very low levels of glycoproteins are expressed). Our previous

demonstration that blocking viral DNA replication completely abolishes syncytia-forming ability of Fu-10 (a selected syncytial mutant of G207) [13], and that Synco-2 (containing GALV.fus driven by UL38p) cannot induce syncytial formation in nondividing cells [14] strongly suggest that Synco-2D retains the safety profile of a conventional oncolytic HSV. More comprehensive toxicity studies on these fusogenic oncolytic HSVs will strengthen their potential as clinically relevant antitumor agents.

In conclusion, we directly compared the antitumor potency of three different types of oncolytic HSVs in a clinically relevant tumor xenograft model bearing both primary and metastatic prostate cancer. The data showed that systemic delivery of both the singly and doubly fusogenic oncolytic HSVs had a significant therapeutic effect on growth of prostate cancer confined to the primary site. Against the lung metastases, the doubly fusogenic Synco-2D is significantly more effective than either the singly fusogenic Synco-2 or the non-fusogenic Baco-1. We conclude that a doubly fusogenic oncolytic HSV similar to Synco-2D may provide substantial clinical benefits to patients of late stage prostate cancer, which is incurable with the current treatment modalities.

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