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PRINCIPAL INVESTIGATOR: Xiaoliu Zhang, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

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6. AUTHOR(S)
Xiaoliu Zhang, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

E-Mail: xzhang@bcm.tmc.edu

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The tasks that were originally planned for the first year of this 3 year project are to demonstrate that the fusogenic oncolytic herpes simplex viruses are potent anti-tumor agents for advanced ovarian cancer. Specifically we planned to: 1) produce a large quantity of high grade and high titer viral stocks for the in vivo animal experiments: 2) establish advanced ovarian cancer in the animal model: 3) to therapeutically administer virus, and to evaluate the therapeutic results. We have now successfully finished these tasks. We initially prepared a large stock of the doubly fusogenic oncolytic HSV Synco-2D. We then characterized Synco-2D in vitro and the result showed that its infection produced a distinctive syncytial phenotype, leading to a significantly increased tumor cell killing ability, compared with that of a nonfusogenic virus (Baco-1). Next we directly injected both Synco-2D and Baco-1 (as control) 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 Baco-1-treated group was tumor-free. These results demonstrate that the doubly fusogenic Synco-2D is indeed an effective therapeutic agent against advanced ovarian cancer. We are now planning to execute the tasks planned for year 2.

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INTRODUCTION

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancies. 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. Current therapy for advanced-stage ovarian cancer consists of debulking surgery followed by chemotherapy. Although the clinical response rate is 60–70%, most patients ultimately relapse and succumb to recurrent chemoresistant disease, leading to 14,000 deaths in the United States each year. Hence, there is an urgent need for novel therapeutics that can provide significant clinical benefits or cure for patients with advanced-stage EOC. Replication-competent herpes simplex virus (oncolytic HSV) holds considerable promise for treating malignant solid tumors such as ovarian cancer, although the potency of the virus needs improvement if its full clinical potential is to be realized. We recently demonstrated that addition of a cell membrane fusion capability to an oncolytic HSV can significantly increase the antitumor potency of the virus [1,2]. The modified virus kills tumor cells efficiently and directly through both replication and cell membrane fusion. In this funded project, we propose to evaluate the antitumor potency of a newer version of the fusogenic oncolytic HSV, in which two membrane fusion mechanisms were incorporated into a single virus (Synco-2D), against ovarian cancer. Specifically, we planned to conduct the following studies in year 1 of this three year project: 1) to produce a large quantity of high grade and high titer viral stocks for the in vivo animal experiments: 2) to establish advanced ovarian cancer in the animal model: 3) to therapeutically administer virus, and to evaluate the therapeutic results. As detailed in the following sections, we believe we have successfully finished these tasks.

BODY

1. Stock preparation and in vitro characterization of doubly fusogenic oncolytic HSV

Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell. When cells showed complete cytopathic effect, heparin (Sigma, St. Louis, MO) was added to the culture medium at a final concentration of 50 μg/ml) and cells were cultured for another 3 h to release the virus into the medium. The medium was then collected and was subjected to a low speed centrifugation at 1,000 *g* for 10 min. The cleared supernatant was transferred to another tube and the virus was pelleted through high speed centrifugation (29,000 *g* for 4 h). The viral pellet was resuspended in PBS containing 10% glycerol and stored at -80^{0}C.

We phenotypically characterized the viruses in greater detail on two human ovarian cancer cell

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

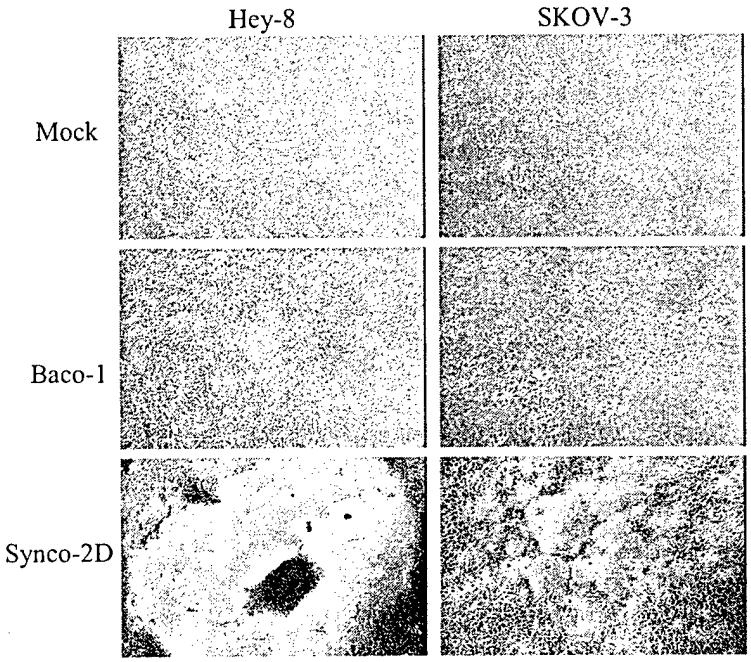

Fig. 1. *In vitro* phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.01 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, x200). Arrows indicate a single syncytium.

2. Comparison of tumor cell killing in vitro

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

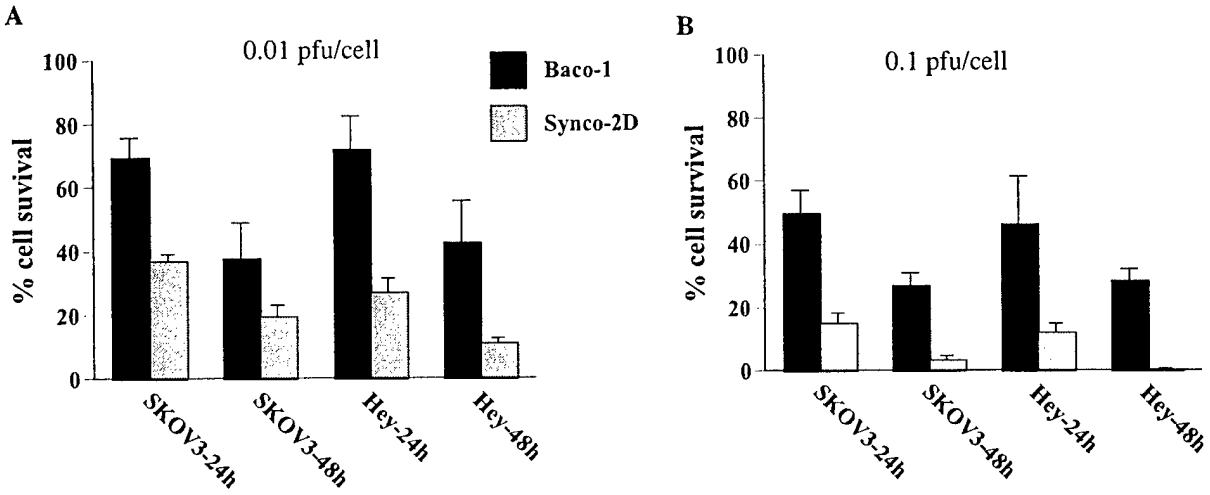

Fig. 2. Comparison of the cytotoxicity of Baco-1 and Synco-2D in cultured ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.01 (A) or 0.1 (B) pfu/cell. Cells were collected at 24 h or 48 h after infection, stained with trypan blue and counted. The percentage of cell viability was determined by dividing the number of viable cells from the infected well by the number of cells from an uninfected well. The data are reported as means ± standard deviations. All comparisons shoed a significant advantage in cytotoxicity for Synco-2D ($p < 0.01$).

3. Therapeutic effects of Synco-2D on peritoneal metastases of ovarian cancer

Peritoneal invasion of ovarian cancer is a common and serious clinical problem. It has been reported that about 70% of late-stage ovarian cancer patients have metastatic disease in the peritoneal cavity. We therefore chose a peritoneal metastasis model (xenografted Hey-8 cells) as means to testing the efficacy of Synco-2D against human ovarian cancer. Freshly harvested Hey-8 cells were inoculated into the peritoneal cavities of nude mice at a dose of 3×10^5 per mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice were injected intraperitoneally with 2×10^7 pfu/200 µl of either Baco-1 or Synco-2D, or PBS (control), at a site distant from that of tumor cell implantation. A second intraperitoneal injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5±0.7 days (none of the mice survived), there was clear intraperitoneal dissemination of tumor, with an average of 2.5±0.9 tumor nodules found in regions distal from the site of the tumor implantation (Table 1). In the Baco-1 treatment group, 3

mice died before the end of the experiment (first death, 34 days after tumor cell implantation); 5 survivors bore a single large tumor when examined at necropsy. By contrast, none of the Synco-2D-treated mice died or were euthanized during the experiment. Strikingly, 6 of the 8 mice were entirely tumor-free at necropsy by the end of the experiment (Table 1). The other two animals had tumors that are significantly smaller than those in Baco-1-treated mice ($p<0.01$, Table 1).

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

Treatment	*N*	No. mice with tumors	No. of nodules	Tumor weight (g)[b]	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[c]	1.5±0.7	3/8[c]
Synco-2D	8	2/8[a]	0.25±0.4[a]	0.1±0.3[a]	0/8[a]

[a] $p<0.01$ as compared with either PBS or Baco-1 treated group.

[b] Means and standard deviations.

[c] $p<0.01$ as compared with PBS control.

KEY RESEARCH ACCOMPLISHMENTS

- Accomplished high titer stock preparations of the doubly fusogenic oncolytic HSV (Synco-2D) with cell culturing facilities in an academic lab.
- In vitro work showed that Synco-2D infectin induces a distinctive syncytial phenotype and a significantly enhanced killing activity against human ovarian cancer cells.
- Demonstrated that intraperitoneal injection of Synco-2D had a significantly better therapeutic effect against metastatic human ovarian cancer xenografts than the nonfusogenic HSV, leading to eradication of all tumor masses in 75% of the animals, whereas no animals in the conventional oncolytic HSV treated group was tumor-free.

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: Effective Therapy of metastatic ovarian cancer with an oncolytic herpes simplex virus incorporating two membrane-fusion mechanisms.

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

CONCLUSIONS

The results we have obtained so far on this funded project clearly demonstrate that the doubly fusogenic Synco-2D is indeed an effective therapeutic agent against advanced ovarian cancer. Now it is important to confirm the safety of Synco-2D in a suitable animal model, a task that has been planned for year 2 of this project.

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