Use of Carrier Cells to Deliver a Replication-selective Herpes Simplex Virus-1 Mutant for the Intraperitoneal Therapy of Epithelial Ovarian Cancer


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ABSTRACT

Epithelial ovarian cancer (EOC) remains localized within the peritoneal cavity in a large number of patients, lending itself to i.p. approaches of therapy. In the present study, we investigated the effect of replication-selective herpes simplex virus-1 (HSV-1) used as an oncolytic agent against EOC and the use of human teratocarcinoma PA-1 as carrier cells for i.p. therapy. HSV-1716, a replication-competent attenuated strain lacking ICP34.5, caused a direct dose-dependent oncolytic effect on EOC cells in vitro. A single i.p. administration of $5 \times 10^6$ plaque-forming units resulted in a significant reduction of tumor volume and tumor spread and an increase in survival in a mouse xenograft model. PA-1 cells supported HSV replication in vitro and bound preferentially to human ovarian carcinoma surfaces compared with mesothelial surfaces in vitro and in vivo. In comparison with the administration of HSV-1716 alone, irradiated PA-1 cells, infected at two multiplicities of infection with HSV-1716 and injected i.p. at $5 \times 10^6$ cells/animal, led to a significant tumor reduction in the two models tested and the significant prolongation of mean survival in one model. Histological evaluation revealed extensive necrosis in tumor areas infected by HSV-1716. Immunohistochemistry against HSV-1 revealed areas of viral infection within tumor nodules, which persisted for several weeks after treatment. Administration of HSV-infected PA-1 carrier cells resulted in larger areas of tumor infected by the virus. Our results indicate that replication-competent attenuated HSV-1 exerts a potent oncolytic effect on EOC, which may be further enhanced by the utilization of a delivery system with carrier cells, based on amplification of the viral load and possibly on preferential binding of carrier cells to tumor surfaces.

INTRODUCTION

Despite the aggressive surgical approaches and combination chemotherapeutic regimens implemented over the past 2 decades, EOC still remains a disease with a grim prognosis. Recent statistics indicate that 25,000 new patients with EOC are diagnosed yearly in the United States; 15,000 deaths occur from this disease yearly (1). Unfortunately, because of the lack of symptoms, the majority of patients are diagnosed at a late stage (2). In addition, although 70% of the patients initially respond to cisplatin-based chemotherapy, the majority of them relapse and develop chemotherapy-resistant disease (3). As a result, the overall 5-year survival is approximately 20% for advanced-stage disease. New therapeutic approaches are, therefore, warranted.

EOC remains localized within the peritoneal cavity in a large proportion of patients, ultimately causing local morbidity and lethal complications (4). Because of its localized nature, EOC lends itself to i.p. approaches of therapy. One such approach is gene therapy. Gene therapy using the HSV\(^{\text{tk}}\) suicide gene followed by ganciclovir (5, 6) or targeting tumor suppressor genes and oncogenes (7, 8) has been tested on experimental ovarian cancer in vitro and in vivo. Sufficient encouraging preclinical results have been obtained to justify initiation of clinical Phase I trials (9, 10). However, results from a recent clinical trial using a HSV\(^{\text{tk}}\)-carrying adenoviral vector applied to mesothelioma localized in the pleural space indicate that gene delivery is restricted to a few superficial cell layers, and treatment of larger three-dimensional tumors may still be inadequate (11).

Replication-competent viral agents may provide a feasible alternative for cancer therapy. Recombinant attenuated forms of HSV-1 represent one family of such agents, which are replication-restricted (12–17). HSV-1 mutants have been generated against HSV-1 revealed areas of viral infection within tumor nodules, which persisted for several weeks after treatment. Administration of HSV-infected PA-1 carrier cells resulted in larger areas of tumor infected by the virus. Our results indicate that replication-competent attenuated HSV-1 exerts a potent oncolytic effect on EOC, which may be further enhanced by the utilization of a delivery system with carrier cells, based on amplification of the viral load and possibly on preferential binding of carrier cells to tumor surfaces.

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3 The abbreviations used are: EOC, epithelial ovarian cancer; pfu, plaque-forming unit(s); MOI, multiplicities of infection; HSV, herpes simplex virus; HSV\(^{\text{tk}}\), HSV-1 thymidine kinase; CNS, central nervous system; SCID, severe combined immunodeficient; CPE, cytopathic effect; FCS, fetal calf-serum; PCR, polymerase chain reaction; OR, odds ratio.
that harbor alterations in genes such as thymidine kinase (tk) or ribonucleotide reductase (RR), exhibiting decreased viral replication in nondoning neuronal cells (13, 17–20). The specificity of the RR− mutants has been shown to be up to 1000-fold higher for malignant rodent cells than endogenous neural cells (21). Another series of HSV-1 mutants has been produced by alterations in both copies of the RL1 gene, a driftpoint fragment of the HSV-1 genome (12, 16, 19, 22, 23). Its product, the ICP34.5 protein, has been implicated in neurovirulence (24–26) and is responsible for preventing apoptosis related to premature shut-off of protein synthesis in the infected host cells (27). ICP34.5-null HSV-1 mutants have been shown to replicate preferentially in tumor cells, causing a direct oncolytic effect but appear to spare normal differentiated tissues (28, 29). These strains have been successfully used to reduce or cure tumors of the CNS in experimental models (12, 13, 15–23, 30, 31).

Previous studies in our laboratories (32) demonstrated the efficacy of HSV-1716, an ICP34.5-null mutant, in reducing tumor burden and conferring survival advantage in an i.p. model of malignant mesothelioma in the SCID mouse. Moreover, these studies suggested that extra-CNS administration of replication-restricted HSV-1 might be safe. HSV-1716 administered i.p. to SCID mice was found to be completely avirulent (32). In fact, there was no viral spread outside the tumors, as was documented by immunohistochemistry and PCR analysis of multiple mucinous tissues, including i.p. and retroperitoneal organs as well as distant organs and the brain. This was not true for wild-type HSV-1, to which SCID mice were found to be extremely sensitive. In fact, i.p. administration of wild-type HSV-1 to SCID mice led to rapid systemic spread of the virus and death of the animals within 1 week (32). Further supporting the safety of HSV-1 mutants, the administration of HSV-1716 to normal human skin in a murine xenograft model was accompanied by no toxicity, whereas administration of a wild-type HSV-1 led to rapid destruction of the xenograft (28). Application of replication-restricted HSV-1 for extra-CNS malignancies was recently extended to other tumors; a RNase reductase-deleted mutant was used in an experimental animal model of metastatic colorectal carcinoma in the liver (33), and a replication-restricted ICP34.5 mutant was used to treat experimental metastatic and s.c. melanoma (23, 34). Moreover, a multiattenuated mutant, HSV-G207, was efficacious in the treatment of breast cancer (35).

In the present study, we sought to define the applicability of HSV-1716 in the treatment of human EOC in vivo. Moreover, we investigated the suitability of PA-1 human teratocarcinoma cells as a carrier system for the delivery of HSV-1716 in vivo. We report that attenuated HSV-1716 exerts a potent oncolytic effect on EOC, which may be enhanced by the utilization of a carrier cell line for the in vivo administration of replication-restricted HSV-1.

MATERIALS AND METHODS

Virus. HSV-1716 was originally isolated in the laboratory of S. M. Brown (Glasgow University, United Kingdom; Ref. 24). The genome of this virus contains a 759-bp deletion located within each copy of the BamHI fragment of the long repeat region of the genome. These deletions remove most of the gene encoding ICP34.5, and the mutant fails to make the protein. The virus used in this study was passed in our laboratory, as originally described (24, 32).

Cells. EOC cell lines SKOV3, NIH:OVCAR3, CaOV3, and human ovarian teratocarcinoma line PA-1 (36) were obtained from the American Type Culture Collection (Manassas, VA). The A2780 EOC line was a kind gift of Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Primary ovarian cultures were obtained from patients with advanced EOC stage III or IV, according to the criteria set by the International Federation of Gynecologists and Obstetricians (FIGO) (4). Malignant effusions, obtained at the time of exploratory laparotomy or diagnostic/therapeutic paracentesis, were centrifuged at 300 × g for 10 min at room temperature, and the cell pellets were collected and seeded in standard tissue culture media (see below). These cells assumed an epithelial phenotype, and their malignant nature was confirmed by a rapid doubling time (average, 18 h) and their immortalized behavior in vitro. EOC cells were passaged 4–5 times before using them in experiments. Normal peritoneal mesothelial cells were obtained from intraoperative peritoneal lavages carried out with normal saline in patients undergoing laparotomy for benign pelvic pathology (pelvic relaxation, uterine myomata). Lavage fluids were centrifuged at 300 × g for 10 min at room temperature, and the cell pellets were collected and seeded in standard media (see below). These cells assumed an epithelial-like phenotype and grew in a cobblestone pattern. Their doubling time was longer than that of primary EOC cultures (average, 36 h) and their nonmalignant nature was confirmed by the fact that they propagated for only a few (4–5) passages even in the presence of growth factors. To eliminate macrophages from primary cultures, culture media were aspirated 30 min after plating, and suspended cells were reseeded in new culture flasks. All of the cell lines and EOC primary isolates were cultured under standard conditions (37°C in a 5% CO₂ atmosphere) and media of RPMI 1640 with 10% heat-inactivated FCS and antibiotics. For normal mesothelial cells, media were supplemented with a mixture of growth factors (SerXtend, Irvine Scientific, Santa Anna, CA) at 0.1% dilution. Institutional Review Board approval had been obtained for the retrieval and utilization of primary cultures.

Assessment of Cytotoxicity in vitro. Cells were plated at a density of 3 × 10⁴ cells/well in 96-well plates and incubated with HSV-1716 at 0.1 and 1 MOI in serum-free media for one h. Serum-enriched media were added subsequently and cultures were followed for 4 days. Cell proliferation assays were performed using a chromogenic kit (CellTiter AQueous96, Promega, Madison, WI) and colorimetric assays were carried out in a microplate ELISA reader (Bio-Tek Instruments, Winooski, VT). CPEs were documented by phase microscopy.

Flow Cytometry Analysis of HSV Antigens. PA-1 cells were cultured in T25 flasks until they reached 60% confluence. They were then infected with 0.5–2.5 MOI of HSV-1716, as above. Cells were harvested at 16 h with a 0.05% trypsin solution, washed once with PBS, and fixed/permeabilized with 70% methanol at -20°C for 20 min. A polyclonal antibody against HSV-1 proteins (American Qualex, La Mirada, CA) was used at a dilution of 1:250, and a secondary antirabbit FITC (FITC)-conjugated antibody (Jackson Immunoresearch Labora-
0.1% trypsin solution, transferred to scintillation vials and subsequently, cells were washed three times with PBS to remove adherent mesothelial or ovarian cancer cells for 30 min.

One-Step Growth Curves. PA-1 teratocarcinoma cells were plated in 6-well plates at a density of $4 \times 10^5$ cells/well under standard conditions overnight and subsequently infected with HSV-1716 at 0.3 MOI. In parallel experiments, PA-1 cells were subjected to a single 2000-cGy dose of ionizing radiation 1 h before exposure to HSV-1716. Cells were harvested in their media by mechanical scraping 1 h after exposure to the virus (0 h) as well as 6, 20, 24, and 48 h later and stored at $-80^\circ$C. One-step growth curves were carried out as described previously (32).

In Vivo Adhesion Assays. PA-1 teratocarcinoma cells were labeled with a rhodamine fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma, St. Louis, MO) as recommended by the manufacturer. Briefly, cells were harvested with a 0.05% trypsin solution, resuspended in PBS and incubated with the dye at 1:250 dilution for 8 min. The reaction was interrupted with the addition of 100% FCS. Cells were then washed, resuspended in RPMI containing 10% FCS, and injected i.p. ($5 \times 10^6$ cells/animal) into SCID mice bearing i.p. SKOV3 tumor (see below). Eight h later, animals were sacrificed, the parietal peritoneum was entirely dissected, and four specimens were prepared containing almost entirely the four different segments of the parietal peritoneum (diaphragmatic, ventral, lateral left, and lateral right). In addition, random biopsies were obtained from the mesentery and the visceral peritoneum. Six-$\mu$m sections were prepared, mounted in Fluoromount medium (Fisher, Pittsburgh, PA) containing 2.5% 1,4-diazabicyclo-(2,2,2)-octane (DABCO, Polyscience, Warrington, PA), to prevent quenching, and examined under a Zeiss microscope.

For quantitative analysis of in vivo adhesion, 20 random fields/slide ($\times40$) were inspected on five slides cut 18 $\mu$m apart from each other from the four different segments of the parietal peritoneum and from two sections of mesenteric areas. Fields were selected in such a way to contain only tumor surface or only tumor-free normal peritoneum. The number of fields containing SKOV3 tumor with or without adhering fluorescent PA-1 cells and those containing normal peritoneum with or without adhering fluorescent PA-1 cells were counted. This experiment was repeated twice.

In Vitro Adhesion Assays. Normal peritoneal mesothelial cells, PA-1 teratocarcinoma cells, and EOC SKOV3, CaOV3, NIH-OVCAR3, and A2780 cells were plated in 48-well plates at a density of $3-4 \times 10^4$ cells/well and allowed to form 100% confluent monolayers. Teratocarcinoma PA-1 cells cultured in T25 flasks were starved with methionine-free media for 2 h and subsequently incubated with $[35S]$methionine media (50 $\mu$Ci/ml) containing 1% dialyzed FCS overnight. Radiolabeled PA-1 cells were harvested by short exposure to 0.05% trypsin solution, washed with serum-free media once, centrifuged at $300 \times g$ for 5 min at room temperature, and resuspended in media containing 1% FCS. PA-1 cells were then seeded at $5 \times 10^3$/well on the different monolayers and allowed to interact with adherent mesothelial or ovarian cancer cells for 30 min. Subsequently, cells were washed three times with PBS to remove nonadherent cells. Adherent cells were harvested with 0.1% trypsin solution, transferred to scintillation vials and counted in a scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). In separate experiments, PA-1 teratocarcinoma cells were radiolabeled with $[35S]$methionine, as described above, and subsequently subjected to ionizing radiation (2000 cGy) and/or infection with HSV-1716 (2 MOI). Cells subjected to different treatments were subsequently plated on the different monolayer substrates, as described above. Adhesion was measured as described above.

In Vivo Xenograft Model of EOC. Six-to-eight-week-old female C57 SCID mice (Charles River, Wilmington, MA) were housed in an isolation unit at the Wistar Institute (Philadelphia, PA). Animals were cared for according to protocols approved by the Animal Use Committees of the Wistar Institute and the University of Pennsylvania, in compliance with the Guide for the Care and Use of Laboratory Animals (NIH 85–23, revised 1985). A2780 and SKOV3 cells were cultured under standard conditions until they reached confluence of 70%, were harvested with 0.05% trypsin solution, were washed with serum-enriched media, and centrifuged at 300 $\times g$ for 5 min at 4°C. Cells were injected i.p. (SKOV3, $5 \times 10^6$ cells/animal; A2780, $1 \times 10^6$ cells/animal) in 0.5 ml of RPMI containing 10% FCS and 1% SerXtend® (Irvine Scientific). Five mice were killed at selected times to confirm the presence of i.p. tumors before administering treatment in each experiment. At the end of the experiments, animals were killed, and i.p. tumors were assessed for spread and volume. A semiquantitative scoring system was devised to grade tumor spread. Five areas were screened for the presence of tumor: (a) the injection site; (b) the parietal peritoneum and diaphragm; (c) the small bowel mesentery and omentum; (d) the lesser omentum and hepatic hilum; and (e) the retroperitoneum. Each site received a score of 0–3 as follows:

(a) 0, if no microscopic tumor was seen;
(b) 1, if microscopic tumor was visible with the aid of a dissecting microscope at $\times2.5$;
(c) 2, if tumor nodules less than 5 mm were visible; and
(d) 3, if tumor nodules equal to or more than 5 mm were present.

Moreover, the presence of ascites added one additional point in the scoring system. Thus, the maximum score accumulated in this scale was 16. Subsequently, all of the visible nodules of the tumor were dissected from surrounding normal peritoneum and viscera, and total intra-abdominal tumor was weighed for each animal individually.

Utilization of PA-1 Teratocarcinoma Cells as HSV-1716 Carrier Cells. PA-1 human teratocarcinoma cells were exposed to ionizing radiation at a single dose of 2000 cGy and then infected with HSV-1716 at 2 MOI. Two h after infection, cells were washed with PBS and harvested with 0.05% trypsin solution. Cells were then washed twice in media containing 10% heat-inactivated FCS, centrifuged at $300 \times g$ for 5 min, and resuspended in RPMI containing 1% heat-inactivated FCS. Five $10^6$ cells were injected i.p. into tumor-bearing animals. Control tumor-bearing animals received PA-1 teratocarcinoma cells that had been previously exposed to the same amount of radiation but not infected with HSV-1716. To confirm the lack of tumorigenicity of irradiated PA-1 cells, non-tumor-bearing mice received a flank ($n = 10$) or an i.p. administration ($n = 10$).
of 5 × 10^6 irradiated PA-1 cells and were followed for 16 weeks.

**Application of HSV-1716 in Vivo.** At selected times, animals received a single i.p. dose of HSV-1716 at 5 × 10^6 pfu in 500 µl of serum-free RPMI, a dose found effective in our previous studies with mesothelioma (32). Control animals received a similar volume of media. A separate group of animals received an i.p. injection of 5 × 10^6 irradiated PA-1 teratocarcinoma cells infected with HSV-1716 at 2 MOI as described above, and a second control group received irradiated noninfected PA-1 cells. Animals were killed at selected times, and i.p. tumors were assessed in the different groups. For survival experiments, animals were injected with tumor and treated as above. Animals were followed daily and were removed from the cages if found dead, or were killed if they seemed severely ill. Tumor weight was estimated in killed animals as described above. Survival experiments were repeated twice for each EOC cell line. Kaplan-Meier survival curves were computed using the StatView computer program.

**Histology and Immunohistochemistry.** Tumors obtained from treated and control animals were immediately fixed in formalin (3.7% formaldehyde-PBS) and subsequently embedded in paraffin. Six-μm-thick sections were deparaffinized and stained with H&E. For immunohistochemistry, slides were subjected to antigen retrieval at 105°C for 10 min in 0.1N citric acid and incubated with a monoclonal antibody against HSV-1 (DAKO, Carpenteria, CA) at a dilution of 1:4 for 30 min at 47°C. A horseradish peroxidase-conjugated antimouse antibody (Vectastain, Vector Laboratories, Burlingame, CA) was used at a dilution of 1:400. Slides were then processed according to the manufacturer's instruction using the ABC kit (Vector Laboratories). A mouse preimmune serum was used at a dilution of 1:4 as a negative control (DAKO).

**Statistical Analysis.** Differences in values obtained during in vitro adhesion assays and differences in tumor weights in the animal experiments were determined with one-way ANOVA. Post-hoc comparisons of specific paired groups were done with the t test. The differences in the in vivo adhesion assays were computed with the χ² test for 2 × 2 contingency tables. ORs were computed with the formula OR = a × d/b × c from the 2×2 contingency tables. Survival curves were analyzed with the Mantel-Cox log-rank test. Statistical significance was set at P < 0.05. Results are expressed as the mean ± SE.

**RESULTS**

**HSV-1716 Exerts an Oncolytic Effect on EOC in Vitro.** To assess the oncolytic effect of HSV-1716 on EOC in vitro, primary EOC cultures and established EOC cell lines were exposed to HSV-1716 at 0.1 and 1 MOI. Cell survival was assessed by proliferation colorimetric assays and evaluation of CPEs under phase microscopy. HSV-1716 exerted a direct dose-dependent cytolytic effect on all of the EOC cell lines and on human teratocarcinoma PA-1 cell line (Fig. 1). Primary EOC cultures obtained from patients with advanced disease displayed 10- to 20-fold higher sensitivity compared with established EOC cell lines. A2780 appeared to be the most sensitive EOC cell line, and SKOV3 was the least sensitive. Moreover, teratocarcinoma PA-1 cells displayed a remarkable sensitivity, which was comparable to primary EOC cultures. The above results were also confirmed by phase microscopy: 100% of primary EOC cells, A2780 cells and teratocarcinoma PA-1 cells displayed CPEs within 24–48 h at 1 MOI, whereas 95–99% of NIH:OVCAR3, SKOV3 and CaOV3 cells displayed CPEs by day 4 of culture (data not shown).

**HSV-1716 Exerts an Oncolytic Effect on EOC in Vivo.** To assess the efficacy of HSV-1716 in treating established i.p. EOC, a murine xenograft model was used with two well-characterized EOC cell lines, SKOV3 and A2780, which displayed the lowest and highest sensitivity, respectively, to HSV-1716 in vitro. The oncolytic effect of HSV-1716 was evaluated on different tumor burdens by varying the time of treatment and by using the two aforementioned cell lines, which yield i.p. tumors of different volume.

To assess the effect of HSV-1716 in minimally spread EOC, the virus was administered 1 week after the injection of SKOV3 ovarian cancer cells. Injection i.p. of 5 × 10^6 SKOV3 cells resulted in microscopic tumor at the diaphragm and occasionally the omentum, mesentery, or lesser omentum within 1 week (tumor score, 2/16; tumor weight, 202 ± 7.1 mg; n = 5). At that time, a group of animals received a single dose of HSV-1716 (n = 10), whereas a group of control animals received media only (n = 10). Animals were then sacrificed at 6 weeks (5 weeks later) to evaluate tumors. Animals that received virus displayed significantly smaller tumors compared with control animals (P < 0.001; Table 1). Moreover, HSV-treated animals displayed tumors similar to their pretreatment counterparts (P = 0.78). Tumor spread was advanced in untreated animals (15.5/16), whereas it remained similar to pretreatment in the HSV-treated animals (2.2/16).

To assess the role of HSV-1716 in more advanced disease, SKOV3 tumors were allowed to grow for 3 weeks before they were treated with virus. At 3 weeks, larger i.p. tumors were observed (tumor score, 3.5/16; tumor weight, 837 ± 9.1 mg; n = 5; Table 1). Treated animals received a single dose of i.p. virus, whereas control animals received media only. Animals were then killed at 6 weeks (3 weeks later) to evaluate tumors. Animals that received HSV-1716 (n = 10) displayed signifi-
significantly smaller tumors compared with control animals (n = 10; P < 0.001). Moreover, HSV-treated animals displayed tumors similar to their pretreatment counterparts (P = 0.86). Tumor spread was again advanced in untreated animals (score, 16/16), whereas it remained similar to tumor spread at pretreatment in the HSV-treated animals (3/16).

To assess the role of HSV-1716 in bulky EOC, we used the A2780 model. Administration of 1 × 10⁶ A2780 cells resulted in bulky i.p. tumors with 0.5–2 mm tumor nodules spread throughout the abdominal cavity within 1 week (score, 16/16; tumor weight, 1860 ± 532 mg; n = 5; Table 1). At that time, a group of animals received a single dose of HSV-1716 (n = 10), and a group of control animals received media only (n = 10). Animals were then killed at 6 weeks (5 weeks later) to evaluate tumors. HSV-treated animals displayed significantly smaller tumors compared with control animals that received saline at week one (P < 0.001; n = 10; Table 1). In addition, no difference in tumor weight was detected between the HSV-treated animals and their pretreatment counterparts (P = 0.86; n = 10).

To assess the role of HSV-1716 in more bulky i.p. disease, A2780 tumors were allowed to grow for 3 weeks after cell injection. At that time, wide-spread tumor nodules, 4–14 mm in size (score 16/16; tumor weight, 8150 ± 912 mg; n = 5), were noted (Table 1). Administration of a single dose of HSV-1716 at 3 weeks resulted in the arrest of tumor growth at 6 weeks (3 weeks later) compared with their pretreatment counterparts (Table 1). HSV-treated animals displayed significantly smaller tumors at 6 weeks, compared with control animals that received media (P < 0.05; n = 10). In addition, no significant differences in tumor weight were observed between the animals treated with HSV-1716 on week 3 compared with their pretreatment counterparts (P = 0.66; n = 10).

**HSV-1716 Efficiently Infects and Replicates in Irradiated PA-1 Cells.** One of the hypotheses of the present study was that i.p. administration of PA-1 cells previously infected with HSV might more effectively deliver virus to the tumor in vivo. One of the difficulties of comparing the effect of virus administered alone versus carrier cells administered after being infected in vitro with HSV lies in the different amounts of particles potentially inoculated. To minimize the difference in the amount of viral load initially administered, we determined the lowest MOI at which 100% of PA-1 cells were infected with HSV-1716. PA-1 cells were incubated with increasing doses of the virus, and the rate of infection of the cells was determined by flow cytometry (Fig. 2A). These experiments indicated that approximately 100% of PA-1 cells were infected at an MOI of 2. At this MOI, each PA-1 cell presumably was initially infected by one or, at most, two viral particles.

We hypothesized that carrier cells would lead to significant amplification of the viral load delivered i.p. to the animals in vivo. To assess the magnitude of viral replication in PA-1 cells in vitro, we determined the viral burst size (Fig. 2B). The burst size of HSV-1716 in PA-1 teratocarcinoma cells was 200 in the

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**Table 1** Effect of HSV-1716 on tumor burden

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Pretreatment weight</th>
<th>Wk of treatment</th>
<th>Weight of tumor at 6 wk</th>
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<tr>
<td>SKOV3</td>
<td>202 ± 7</td>
<td>1</td>
<td>1,147 ± 11</td>
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<tr>
<td></td>
<td>837 ± 9</td>
<td>3</td>
<td>1,249 ± 13.2</td>
</tr>
<tr>
<td>A2780</td>
<td>1,860 ± 532</td>
<td>1</td>
<td>18,705 ± 2,120</td>
</tr>
<tr>
<td></td>
<td>8,150 ± 912</td>
<td>3</td>
<td>19,100 ± 1,115</td>
</tr>
</tbody>
</table>

*a* P < 0.001 versus untreated.

*b* Nonsignificant versus pretreatment.

**Fig. 2** HSV-1716 infection of PA-1 cells. A, infection rate of PA-1 teratocarcinoma cells 16 h after exposure to increasing MOI of HSV-1716. Analysis was carried out by flow cytometry using a polyclonal antibody against HSV-1 antigens. B, single step-growth curve of HSV-1716 in control nonirradiated human teratocarcinoma PA-1 cells (solid) and cells exposed to ionizing radiation (20 Gy) before the infection (■). PA-1 cells support viral replication even after exposure to a lethal dose of radiation. Cells were incubated with 0.3 MOI, which was 1000 pfu of virus.
Fig. 3 PA-1 producer cells adhere preferentially to tumor nodules rather than to normal peritoneum. PA-1 cells were labeled with fluorescent dye, and $5 \times 10^6$ cells were injected i.p. in SKOV3 tumor-bearing mice. Animals were killed 8 h later, and multiple biopsies were obtained from tumor-bearing areas and normal peritoneum. Representative areas of the abdomen covered by tumor and diaphragm are shown. A, H&E staining of a diaphragmatic area demonstrates the presence of several layers of SKOV3 tumor cells adhering on the surface (arrowheads) of the diaphragm (d). A layer of cells with darker nuclei on the surface represent PA-1 cells (arrows); $\times 60$. B, H&E staining of an area of the diaphragm (d) that is not covered by tumor. The peritoneum is free of tumor; two cells are noted on the peritoneal surface, probably representing PA-1 cells (arrow). C, a continuous layer of fluorescent PA-1 cells (arrows) is adherent to the tumor cells coating the diaphragm (rhodamine). Arrowheads, the margin of the diaphragm; $\times 40$. D, examination of an adjacent area of the diaphragm (d) that is not covered by tumor. Fluorescent PA-1 cells are absent from the peritoneum. E, in the same section as C, tissue autofluorescence (blue filter) reveals several layers of SKOV3 tumor cells covering the diaphragm under the PA-1 layer. Arrowheads, the margin of the diaphragm; $\times 40$. F, in the same section as D, no SKOV3 tumor cells are detected in this area of the diaphragm by tissue autofluorescence; $\times 40$. 
Table 2  Preferential binding of PA-1 cells to EOC in vivo

To assess the behavior of PA-1 carrier cells in our murine xenograft model, we injected fluorescent PA-1 cells i.p. into SKOV3-tumor-bearing mice. Eight h later, animals were killed, the parietal peritoneum was entirely dissected, and four specimens were prepared containing almost entirely the four different segments of the parietal peritoneum (diaphragmatic, ventral, lateral left, and lateral right). In addition, random biopsies were obtained from the mesentery and the visceral peritoneum. Twenty random fields/slide (×40) were inspected on five slides cut 18 µm apart from each other from the four different segments of the parietal peritoneum and from two sections of mesenteric areas. Fields were selected in such a way to contain only tumor surface or tumor-free normal peritoneum. The number of fields containing SKOV3 tumor with or without adhering fluorescent PA-1 cells and those containing normal peritoneum with or without adhering fluorescent PA-1 cells were counted. $P_i$ were computed with the $χ^2$ test for $2 \times 2$ contingency table.

<table>
<thead>
<tr>
<th>Tumor present</th>
<th>Tumor absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diaphragmatic peritoneum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-1 present</td>
<td>82 (91.1)</td>
<td>102</td>
</tr>
<tr>
<td>PA-1 absent</td>
<td>8 (8.9)</td>
<td>98</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>90 (100)</td>
<td>200</td>
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<tr>
<td><strong>Nondiaphragmatic peritoneum</strong></td>
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<td></td>
</tr>
<tr>
<td>PA-1 present</td>
<td>67 (75.3)</td>
<td>101</td>
</tr>
<tr>
<td>PA-1 absent</td>
<td>22 (24.7)</td>
<td>229</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>89 (100)</td>
<td>330</td>
</tr>
</tbody>
</table>

* Percentages represent number of observations over total for each column.

absence of ionizing radiation. The administration of the HSV-1 infected carrier cells into humans will most likely require a safeguard such as radiation to eliminate risks from the administration of potentially uninfected carrier cells. Therefore, PA-1 cells were subjected to a single (lethal) dose of 20 Gy 1 h before injection, and viral replication was assessed. Burst size experiments indicated that irradiated PA-1 cells supported viral replication with a burst size of 70.

**PA-1 Carrier Cells Preferentially Bind to EOC Surface Compared with Normal Peritoneum in Vitro.** To assess the behavior of the carrier cells in our murine xenograft model, we injected fluorescent PA-1 cells i.p into SKOV3-tumor-bearing mice. Overall, we observed that a large number of fluorescent PA-1 cells adhered to areas of the diaphragm or other peritoneal surfaces that were covered by tumor. In some areas, PA-1 cells almost formed a monolayer covering the SKOV3 preexisting tumor surfaces that were covered by tumor. In some areas, PA-1 cells adhered to areas of the diaphragm or other peritoneal surfaces that were covered by tumor. Twenty random fields/slide (×40) were inspected on five slides cut 18 µm apart from each other from the four different segments of the parietal peritoneum and from two sections of mesenteric areas. Fields were selected in such a way to contain only tumor surface or tumor-free normal peritoneum. The number of fields containing SKOV3 tumor with or without adhering fluorescent PA-1 cells and those containing normal peritoneum with or without adhering fluorescent PA-1 cells were counted. $P_i$ were computed with the $χ^2$ test for $2 \times 2$ contingency table.

**In vitro** adhesion of PA-1 cells on human normal mesothelium or ovarian cancer. PA-1 cells were radiolabeled and plated on monolayers of normal mesothelium obtained from three women with benign pelvic pathology ($M1$, $M2$, and $M3$) or on monolayers of established ovarian cancer cell lines (PA-1, A2780, SKOV3, OVCAR3, and CaOV3) or primary EOC cultures obtained from two patients with advanced EOC (OV1 and OV2). Results are expressed as percentage of cells that were adherent 30 min after the plating. With the exception of A2780 cells, a 5- to 8-fold higher affinity of PA-1 cells is noted for ovarian cancer monolayers (striped columns) compared with normal human mesothelial cells (black columns).

**PA-1 Carrier Cells Effectively Deliver HSV-1716 in Vivo.** To assess the suitability of carrier cells for the delivery of HSV-1716 in vivo, we compared the effects of HSV-1716 administered directly with the effects of HSV-1716 delivered via PA-1 cells. One week after the administration of A2780 tumor cells, SCID mice received a single i.p. administration of
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Fig. 5 In vivo effect of HSV-1716 on A2780 tumor growth. SCID mice received injections of $1 \times 10^6$ A2780 cells i.p., and control animals were killed 1 week later to assess i.p. tumors (Pretreatment, black bar; n = 5). At that time, animals received treatment with i.p. HSV-1716 at $5 \times 10^5$ pfu/animal (HSV1716; n = 10) or with radiated PA-1 cells infected with HSV-1716 [5 × 10^6 cells/animal, infected at 2 MOI (PA-1 HSV1716); n = 10]. Control animals received i.p. media (Medium; n = 10) or radiated PA-1 cells (PA-1; n = 10). There was a significant reduction in tumor growth 4 weeks after a single i.p. administration of the virus compared with controls treated with media (P < 0.01) and an even larger reduction after the administration of PA-1 cells infected with HSV-1716 compared with their respective controls (P < 0.001). The progression of disease was completely stabilized 4 weeks after a single administration of HSV-infected PA-1 cells. (*, P < 0.01; **, P < 0.001).

virus alone or HSV-infected PA-1 cells (Fig. 5). Control animals received media or uninfected/irradiated PA-1 cells, respectively. Four weeks later, a significant increase in tumor weight was noted in control animals receiving media (20 ± 2.5 g; n = 20) or irradiated PA-1 cells (22.3 ± 3 g; n = 20) compared with pretreatment animals (19 ± 0.1 g; n = 5; P < 0.001, both). The administration of HSV-1716 alone (n = 20) allowed for a small increase in tumor weight (3.4 ± 0.2 g) compared with pretreatment animals (P < 0.05), whereas the injection of PA-1 infected with HSV-1716 (n = 20) resulted in the stabilization of disease (2 ± 0.2 g) compared with pretreatment animals (P = 0.198). Tumors in HSV-treated animals or in those treated with HSV-infected carrier cells were significantly smaller compared with those of their control counterparts receiving media (P < 0.01) or receiving uninfected PA-1 cells (P < 0.001), respectively. Moreover, tumors in animals treated with HSV-infected carrier cells were significantly smaller compared with those receiving HSV-1716 alone (P < 0.05).

Similar results were obtained in experiments carried out with SKOV3 cells infected i.p. (5 × 10^6/animal) into SCID mice (Fig. 6). One week after the injection of tumor cells, control animals received media (group 1, n = 20) or irradiated PA-1 cells (group 2, n = 20), whereas treated animals received a single i.p. injection of virus (group 3, n = 20) or HSV-infected PA-1 carrier cells (group 4, n = 20), respectively (Table 3). Animals from each group were killed at 4 and 7 weeks after treatment. Control animals from group 1 receiving media alone (n = 10) displayed a 6-fold increase in tumor weight (P < 0.01) and an extensive spread of disease at 4 weeks (score, 15.5/16) compared with pretreatment animals killed at 1 week (n = 5; tumor score, 2.5/16). A 10-fold increase in tumor weight (P < 0.001 versus pretreatment) and further tumor spread (score, 16/16) was noted at 7 weeks (n = 10). Control animals from group 2, receiving irradiated PA-1 cells (n = 10), displayed a 6.5-fold increase in tumor weight (P < 0.001 versus pretreatment) and extensive tumor spread (score, 15.9/16) at 4 weeks (Table 3). A 12-fold increase in tumor weight (P < 0.001 versus pretreatment) and diffuse spread (score, 16/16) were noted at 7 weeks (n = 10). There was no significant difference in tumor growth or tumor spread between the control groups receiving media or irradiated PA-1 cells. Animals treated with HSV-1716 (group 3, n = 10) displayed significantly less tumor growth (P < 0.001) and spread (score, 2.5/16) at 4 or 7 weeks compared with control group 1 (Table 3). Group 3 displayed no difference in tumor weight at 7 weeks from pretreatment animals (P = 0.61), which suggests a stabilization of disease. Animals receiving HSV-infected carrier cells (group 4) also had significantly smaller tumors (P < 0.001) and less tumor spread (score, 2/16) at 4 and 7 weeks than their control group 2 (Table 3). Group 4 showed no tumor progression at 4 weeks (P = 0.43) and significant tumor regression by week 7 (P < 0.05 versus pretreatment). In addition, group 4 had a significantly smaller tumor burden at 7 weeks than HSV-1716-treated animals (group 3, P < 0.05; Table 3). Remarkably, in the group receiving HSV-infected PA-1 producer cells, 20% of the animals displayed no detectable disease either grossly or under the dissecting microscope at 4 weeks (n = 4 of 20) or at 7 weeks (n = 4 of 20).

To assess the carcinogenic potential of PA-1 carrier cells, 6–8-week-old SCID mice (n = 10) were given i.p. injections of $5 \times 10^5$ irradiated PA-1 cells infected with HSV-1716 at 2 MOI. Animals were followed for 16 weeks and then killed to evaluate the presence of i.p. tumors. No microscopic or gross tumor was detected. In addition, 10 animals were given injections in the flank with $5 \times 10^6$ PA-1 cells, which were prepared in a similar manner. Similarly, no tumor was detected over 16 weeks.

The Use of HSV-infected Carrier Cells Prolongs the Survival of SKOV3 Tumor-bearing Animals. Survival experiments carried out in animals bearing SKOV3 tumors confirmed the oncolytic activity of HSV-1716 on EOC (Fig. 7). A single dose of HSV-1716 was administered 4 weeks after the injection of tumor cells, a time in which there would be a sizable i.p. tumor burden (Table 3). This resulted in the approximate doubling of the mean survival time (17 weeks; n = 20) compared with that for control animals receiving media alone (9 weeks; n = 20; P < 0.001). Moreover, the administration of HSV-infected PA-1 cells (n = 20) allowed for a slightly longer mean survival time compared with the administration of HSV-1716 (20 weeks; P < 0.01 versus HSV-1716 alone).

Similar experiments were performed in animals bearing A2780 tumors: the injection of a single dose of HSV-1716 4 weeks after the injection of tumor cells, a time in which there would be extensive i.p. tumor, also led to the doubling of animal survival (13 weeks; n = 20) compared with controls (6 weeks, n = 20; not shown). PA-1 cells infected with HSV-1716 did not result in any further increase in mean animal survival time (13.5 weeks, n = 20) compared with the administration of HSV-1716 alone (P = 0.987). However, when some of the moribund animals in the treated groups were killed, the amount of i.p. tumor encountered was minimal in HSV-treated animals (1.9 ± 0.4 g; n = 4) and even smaller in PA-1/HSV-treated animals.
mean weighed. Weights are expressed in mg and values are expressed as the mean ± SE. Pretreatment weight = 0.214 ± 0.032 mg.

### Table 3 Enhancement of HSV-1716 treatment by carrier cells in vivo

To assess the efficacy of HSV-1716 to treat EOC and the suitability of carrier cells for the delivery of HSV-1716 in vivo, we compared the effects of HSV-1716 administered directly i.p. with the effects of HSV-1716 delivered via PA-1 carrier cells injected i.p. after radiation and in vitro infection. All of the animals (SCID mice) received a single i.p. injection of 5 × 10⁶ SKOV3 cells. Control animals received media (group 1, n = 20) or irradiated PA-1 cells (group 2, n = 20) 1 week later. Treated animals received a single i.p. injection of virus (group 3, n = 20) or HSV-infected PA-1 carrier cells (group 4, n = 20) 1 week later. Animals from each group were killed at 4 and 7 weeks after treatment. Tumors were dissected and weighed. Weights are expressed in mg and values are expressed as the mean ± SE. Pretreatment weight = 0.214 ± 0.032 mg.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Weight at posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media</td>
<td>1.225 ± 0.09&lt;sup&gt;a&lt;/sup&gt; 2.256 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Irradiated PA-1</td>
<td>1.412 ± 0.01&lt;sup&gt;b&lt;/sup&gt; 2.494 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>HSV-1716</td>
<td>0.225 ± 0.03&lt;sup&gt;c&lt;/sup&gt; 0.380 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>PA-1/HSV-1716</td>
<td>0.185 ± 0.04&lt;sup&gt;c&lt;/sup&gt; 0.131 ± 0.09&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01 versus pretreatment.
<sup>b</sup> P < 0.001 versus pretreatment.
<sup>c</sup> P < 0.001 versus control group 1 at 4 weeks.
<sup>d</sup> P < 0.001 versus control group 1 at 7 weeks.
<sup>e</sup> P < 0.01 versus control group 2 at 4 weeks.
<sup>f</sup> P < 0.001 versus control group 2 at 7 weeks; P < 0.05 versus pretreatment; P < 0.05 versus group 3 (HSV-1716 alone) at 7 weeks.

*Fig. 6 In vivo effect of HSV-1716 on SKOV3 tumor growth. SCID mice were given i.p. injections of 5 × 10⁶ SKOV3 cells and 1 week later received treatment with i.p. HSV-1716 (5 × 10⁶ pfu) or PA-1 cells (5 × 10⁶ cells) previously radiated and infected with HSV-1716 (2 MOI). Control animals received i.p. media or radiated PA-1 cells. Animals were killed 4 weeks later. A, large tumor nodules extend throughout the entire surface of the diaphragm in control animals (yellow arrows) after ascites was removed. Arrowhead, nodule at the hepatic hilum. B, microscopic evaluation reveals solid tumor sheets. C, a significant reduction in tumor growth is seen after a single i.p. administration of HSV-1716; only a few tumor nodules are present on the diaphragm (yellow arrows) and in other peritoneal areas. D, microscopic evaluation reveals tumor nodules with necrosis and cell vacuolization surrounded by connective tissue (yellow arrows), which suggests a desmoplastic reaction to HSV-infected tumor. Solid tumor sheets have grown around this nodule. E, a more pronounced tumor reduction is documented after the administration of HSV-infected PA-1 cells. No large tumor nodules are noted. Occasional miliary nodules are seen on the diaphragm (open arrow); a nodule at the hepatic hilum is also noted (arrowhead). Similar reduction was observed in other areas of the peritoneal cavity. F, microscopic evaluation reveals few layers of tumor cells on the diaphragm in areas with miliary nodules.

*Fig. 7 In vivo effect of HSV-1716 on the survival of SCID mice bearing SKOV3 tumors. SKOV3 cells (5 × 10⁶) were injected i.p., and 4 weeks later, animals received treatment (arrow) with HSV-1716 (5 × 10⁶ pfu) or HSV-infected PA-1 cells infected with HSV-1716 at 2 MOI (5 × 10⁶) i.p. control; n = 20, □). Control animals (n = 20, ■) received media alone. Curves represent the sum of two experiments carried out under identical conditions. A significant prolongation of survival is noted after i.p. injection of HSV-1716 (P < 0.001 versus controls). An even more dramatic prolongation is noted after the administration of HSV-infected PA-1 cells (P < 0.001 versus control; P < 0.01 versus HSV-1716 alone). (0.9 ± 0.4, n = 3). Of note, these animals displayed large tumors at the abdominal injection site, which were ulcerated and necrotic. These findings suggested that death may have ensued because of infectious or tumor-related complications stemming from the injection.
Fig. 8  Microscopic features of tumor infected by HSV-1716.  

a, H&E staining of control untreated A2780 tumor reveals solid sheets of tumor cells with well-established vascularization (v); ×10.  
b, higher magnification (×100) reveals numerous mitoses (white arrows) and capillaries (v).  
c, H&E staining of SKOV3 tumor treated with HSV-1716—delivered i.p. directly or via PA-1 producer cells—results in extensive tumor necrosis; ×10.  
d, higher magnification (×100) reveals marked cell vacuolization, suggestive of HSV-induced cytopathic effects. Occasional polymorphonucleates are seen in the tumor (black arrow).  
e, HSV-treated tumor with extensive vacuolization incubated with preimmune rabbit serum (negative control); ×100.  
f, HSV-treated tumor displaying extensive vacuolization incubated with a polyclonal antibody against HSV-1 antigens (×100). Intense HSV staining is noted in most cells displaying vacuolization. Some cells show features of necrosis but do not stain for HSV (arrows), which suggests possible bystander killing mechanisms.  
g, low-power view of SKOV3 tumor treated with a single i.p. dose of PA-1 cells (5 × 10⁶ pfu/SCID mouse) infected with HSV-1716 at 2 MOI; ×6. Extensive areas of the tumor show strong staining for HSV, indicating deep penetration of the virus within the nodule.  
h, low-power view of SKOV3 tumor treated with a single i.p. dose of HSV-1716 (5 × 10⁶ pfu/SCID mouse); ×6. Immunohistochemistry for HSV-1 antigens reveals areas of viral infection penetrating deep in the nodule. Application of HSV-1716 alone is followed by less extensive tumor infection...
from the injection-site tumors. This was in sharp contrast to the untreated animals, which carried very bulky i.p. tumors (22.7 ± 3.1 g, n = 4), most of which were associated with hemorrhagic ascites.

**HSV-1716 Penetrates Deeply in Tumor Nodules and Causes Extensive Necrosis in a Dose-dependent Manner.** Microscopic examination of control untreated tumors revealed solid sheets of tumor with well-established vascularization (Fig. 8a), and examination under higher magnification revealed numerous mitoses (which indicated rapid tumor growth), many capillaries, and no inflammatory infiltrate (Fig. 8b). On the contrary, tumors collected from animals treated with HSV-1716 or with HSV-infected producer cells revealed extensive necrosis (Fig. 8c). Examination at high power revealed extensive cell vacuolization, suggestive of HSV-induced CPE (Fig. 8d). Occasionally, polymorphonuclear leukocytes were also visible within the necrotic areas of the tumor. Immunohistochemistry against HSV-1 revealed the presence of virus within the necrotic areas, in which many vacuolized cells stained strongly for HSV-1 (Fig. 8f). Interestingly, some nearby cells displaying features of cell death did not stain for HSV-1, which suggests the existence of bystander killing mechanisms. In treated animals, the virus invaded deeply in solid tumor nodules, within which distinct areas of viral spread were noted several weeks after a single i.p. injection (Fig. 8, g and h). Immunohistochemistry evaluation of tumors obtained from animals treated with HSV-infected PA-1 cells revealed even larger areas of viral infection penetrating deeply within the tumor (Fig. 8g) compared with animals treated with HSV-1716 alone (Fig. 8h). Areas of viral infection were primarily found in the interface between uninfected tumor and areas of necrosis, which suggests that, by virtue of its replication, the virus advanced into the tumor leaving behind an area of necrosis (Fig. 8i). In keeping with data reported previously with HSV-1716 (32), no HSV-1 staining was detected in normal murine tissues (Fig. 8, k, l, and m) such as liver, pancreas, kidney, adrenal, spleen, small bowel myenteric plexus, or brain of treated animals.

**DISCUSSION**

In this study, we sought to determine the efficacy of attenuated HSV-1716 in treating human EOC. ICP34.5 mutants such as HSV-1716 have proven effective in treating experimental CNS malignancies (12, 15, 16, 19, 20, 22, 30, 31), s.c. melanoma, and metastatic melanoma to the brain (23, 34). Moreover, it was previously shown to exert an oncolytic effect on malignant mesothelioma, a localized peritoneal tumor (32). In the present study, we show that i.p. administration of HSV-1716 to tumor-bearing animals resulted in significant reduction or arrest of tumor growth. The effect of a single i.p. dose to stabilize i.p. disease was noted even with bulky tumors and with different cell lines. Further confirming the effect of the virus, a single administration of HSV-1716 resulted in significant survival advantage in both of the models studied.

The mechanism by which HSV stabilized tumor growth in the SCID model is attributable to its direct oncolytic effect. In *vitro*, this virus was shown to exert a direct lytic effect on EOC cell lines and primary cultures. i.p. tumors in treated animals displayed large areas of necrosis, which were related to infection by the replication-competent HSV-1 mutant. Our immunohistochemistry studies confirmed that the virus penetrated deeply into the tumor tissue and was present within, or adjacent to, areas of necrosis. In those areas, tumor cells displaying fragmentation, necrosis, or CPE, stained positive for HSV. Of note, within the areas of necrosis, many necrotic-appearing cells did not display immunopositivity for HSV-1 antigens. Although HSV antigens may have degraded, another interpretation is that cell death may have been caused by different mechanisms than direct HSV-1 infection. Recently, Brandt *et al.* (37) reported that the antitumor effect of a recombinant-attenuated HSV-1/HSV-2 strain was disproportionately extensive compared with viral spread in the tumor. Taken together, these data suggest that bystander killing mechanisms may also mediate the oncolytic effects of HSV-1 mutants. Similar bystander mechanisms inducing apoptosis *in vitro* and *in vivo* were recently reported for wild-type HSV-1 (38, 39).

HSV-1716 actively infected tumor nodules, but there was no evidence of viral spread within normal murine tissues by immunohistochemistry. Although there is no current mechanistic explanation for the tumor selectivity of ICP34.5-deficient HSV mutants, this selectivity has been documented in multiple models *in vitro* and *in vivo* (15, 18–20, 23, 26, 28, 30–32, 34). Brown *et al.* (40), reported that HSV-1716 replication is severely restricted in terminally differentiated cells compared with tumor cells, whereas wild-type HSV-1 can usually replicate equally well in both cell types. Moreover, human keratinocytes were shown to support HSV-1716 replication much less than wild-type HSV *in vitro* and in normal human skin xenografts (28). Our group previously reported that i.p. inoculation of HSV-1716 into SCID mice did not result in viral spread to normal murine tissues by immunohistochemistry and PCR analysis (32). It is possible that different susceptibility to HSV infection between murine and human cells might have partly affected these results. Nevertheless, the SCID mouse is very sensitive to wild-type HSV-1 (32), suggesting that the lack of spread of HSV-1716 is due to its attenuation. Additional experiments may be needed to confirm the tumor selectivity of these mutants in the human.

*Our in vitro* results indicated that there was a clear dose-related effect of the virus. Because large-scale production of mutant HSV may offer significant technical difficulties, meth-
odologies to amplify the viral load delivered to the animals will be very useful. We hypothesized that the use of carrier cells could represent one such methodology. With this approach, appropriate cells supporting HSV replication (carrier cells) would be infected in vitro with HSV virions and subsequently injected in proximity of the tumor, where they would eventually burst, releasing a large amount of active viral particles. Cell-based therapies using homologous or heterologous cells have been designed previously for tumor vaccines (41–44). In the present work, we elected to use the human teratocarcinoma PA-1, a well-characterized cell line (36), as an HSV carrier cell line. This line has been approved by the Food and Drug Administration for i.p. use in humans and is already being used in a Phase 1 clinical trial for vaccine therapy of ovarian cancer (45). In vitro, we observed a 70-fold amplification of the virus in irradiated PA-1 cells within 24 h. The efficacy of PA-1 cells to deliver HSV was then tested in vivo. To minimize the artifact potentially produced by the administration of different viral loads between animals receiving virus alone and animals receiving infected carrier cells, the minimal amount of viral particles capable of infecting 100% of PA-1 cells was estimated by flow cytometry. Our results indicated that 100% of PA-1 cells were infected at two MOI. Theoretically, carrier cells were initially infected on average by one or at most two viral particles at two MOI. Therefore, inoculation of an equal number of HSV particles or PA-1 cells infected at two MOI should represent comparable amounts of viral loads initially administered to the animals. HSV-1716 administered via carrier PA-1 cells led to a reduction in tumor volume and tumor spread that was significant compared with virus alone in both of the models used. In the SKOV3 model, the carrier cell strategy also led to a significant prolongation of animal survival compared with virus alone. In the A2780 model, mean animal survival was not significantly prolonged by the utilization of the carrier cells. However, survival may not have been the best end point to assess the effect of the i.p. treatment in this model. In our experiments, moribund A2780-bearing animals that had been treated with virus harbored minimal i.p. tumors, and animals treated with carrier cells harbored even smaller tumors, whereas untreated animals harbored large i.p. tumors. On the other hand, all of the A2780-bearing animals carried large s.c. tumors that were not affected by the i.p. treatment and may have impacted survival (32). It is possible that survival would have been different in the absence of these s.c. tumors. Nevertheless, it is impossible to exclude other reasons for the failure of HSV-infected carrier cells to improve survival in comparison with virus treatment alone in A2780 tumors.

Although additional studies are needed to fully explore the benefits of using carrier cells, the present study supports the concept that the utilization of carrier cells may have a role in HSV-based oncolytic therapies. Our results indicate that carrier cells successfully deliver the virus i.p. and result in tumor reduction that is at least equivalent to, or better than, virus alone in the immunodeficient host. Our immunohistochemistry studies showed that the utilization of carrier cells resulted in larger areas of i.p. tumors being infected by the virus and in deeper penetration of the virus within tumor nodules compared with virus administered directly. Two possible mechanisms may account for this: (a) based on our in vitro observations, the lysis of infected carrier cells within the peritoneal cavity may have released high amounts of viral particles within the peritoneal fluid; and (b) because HSV is known to propagate also by direct cell-to-cell spread (46, 47), carrier cells may have promoted direct cell-to-cell viral infection of the tumor by directly binding to EOC cells. It is attractive to speculate that by virtue of viral amplification and through direct adhesion of carrier cells to tumor cells, a significantly larger amount of virus actually infected the i.p. tumors. Although immune neutralization of HSV was not an issue with the SCID model, this may occur in the peritoneal cavity of an immunocompetent host by complement or by neutralizing antibodies (48) that may be present in the peritoneal fluid. In fact, antiviral antibodies are highly prevalent in the adult population (49). The utilization of a cell carrier system to deliver the virus may partly circumvent this barrier because cell-to-cell spread is less affected by neutralizing antibodies than infection by free virus (46, 47).

The in vivo distribution of carrier cells after i.p. administration is intriguing. PA-1 cells seemed to adhere predominantly to areas of peritoneum that were covered by established tumor and not to close-by areas of normal peritoneum. There was a close correlation between the presence of tumor and PA-1 cells as calculated by ORs. It is possible that the physical forces governing fluid migration within the peritoneal cavity were partially responsible for this effect or that species-related differences in the affinity of interaction may have influenced these findings. However, our in vitro studies performed with human normal mesothelial cells showed that PA-1 cells bound predominantly to EOC surfaces compared with normal human mesothelium. This finally suggests that cell-cell interactions may also play a role in promoting the adhesion of PA-1 producer cells to tumor compared with normal mesothelium. The molecular mechanisms underlying these interactions were beyond the focus of the present study. Other cell lines may support HSV replication well and also display high affinity of interaction with ovarian cancer cells. These issues are currently being investigated.

The ultimate fate of carrier cells after i.p. inoculation may pose some safety concerns. In a clinical context, there must be certainty that carrier cells will not survive. In the present study, we “sterilized” carrier cells with lethal ionizing radiation. An argument could be made that this extra step was not necessary insomuch as these cells are very sensitive to HSV-induced killing. In fact, radiation decreased viral amplification and may have adversely affected efficacy. However, the possibility of clinical applications of carrier cells warrants that safety be ensured. If few carrier cells escape infection by HSV in vitro, a carcinogenic potential would emerge. Recently, Advani et al. (50) reported that malignant glioma cells displayed higher viral replication of HSV R3616, another ICP34.5− mutant, after exposure to similar amounts of ionizing radiation. Although the molecular mechanisms underlying this enhancement are unknown, the authors speculated that ionizing radiation may induce specific cellular genes, complementing the defects resulting from the deletion of ICP34.5 in the virus and leading to an increased ability of the virus to replicate in the host cells. Although radiation-induced enhancement may not occur in the case of PA-1 cells infected with HSV-1716, there may be other cell lines that respond to radiation in a manner similar to glioma.
cells and may become more suitable for HSV-1716 carrier strategies. It should be noted, also, that R3616 and 1716 are derived from two different wild-type prototypes, namely HSV(F) and Glasgow HSV171 strains, respectively, which differ in several genes including ICP4 (51, 52). These intrinsic differences between the two viruses may account for the observed different response to radiation.

A potential advantage offered by the utilization of a carrier cell line in HSV-based oncolytic therapy relates to the possibility of manipulating these cells as follows:

(a) the identification of molecules mediating the interaction between carrier cells and tumor cells or normal human mesothelial cells may allow for the engineering of carrier cells that exhibit enhanced preferential binding to tumor cells (but not to normal mesothelium) or that promote greater cell-to-cell viral spread;

(b) molecular engineering of carrier cells may increase their ability to support viral replication;

(c) molecular manipulation of carrier cells could aid in circumventing natural or acquired host immune mechanisms. For example, carrier cells could be manufactured to secrete truncated HSV-like glycoproteins that inactivate complement (gC) or antibodies (gE) (53–55). Carrier cells could also be engineered to secrete immunomodulatory cytokines. Interleukin-12 (IL-12) and IL-4 were recently shown to enhance the efficacy of HSV-based oncolysis (56, 57); and

(d) a vaccine antitumor response in selective patients might be generated. It is interesting to note that an antitumor vaccine clinical trial for the treatment of advanced EOC has been initiated using HSVtk-enriched PA-1 cells (45). The lysis of PA-1 cells after the administration of ganciclovir is anticipated to trigger an antitumor immune response. Similarly, HSV-infected carrier cells may lyse on the surface of i.p. tumors, increasing the recruitment of antigen-presenting cells and mononuclear cells to the site of injection (58). This may augment the immediate oncolytic effect of the virus and, by unmasking tumor antigens, possibly lead to a sustained vaccine effect.

In conclusion, the present study demonstrates that EOC is extremely sensitive to attenuated HSV-1. These findings may have significant implications for both oncolytic as well as gene therapy of EOC. In fact, attenuated HSV-1 mutants may be used as gene therapy vectors with significant advantages over replication-incompetent oncolytic or retroviral vectors (21). In addition, oncolytic attenuated HSV-1 strains are being engineered with progressively increased specificity (59, 60), and the insertion of immunomodulatory (56, 57) or corrective genes (59, 60) may further increase the efficacy of these agents. The present study also investigated the use of carrier cells and showed that this methodology may be useful in viral-based i.p. therapies. Theoretical advantages offered by a carrier-cell approach include amplification of the viral load and possibly enhanced local delivery afforded by carrier cells binding to the tumor and mediating direct cell-to-cell viral delivery. Additional potential advantages of a carrier cell system may relate to the possibility of manipulating the cells to maximize viral replication, to enhance binding to tumor surfaces, and to mediate evasion from immune-mediated viral inactivation as well as to possibly generate an antitumor vaccine effect.

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Use of Carrier Cells to Deliver a Replication-selective Herpes Simplex Virus-1 Mutant for the Intraperitoneal Therapy of Epithelial Ovarian Cancer

George Coukos, Antonis Makrigiannakis, Eugene H. Kang, et al.


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