

## ORIGINAL ARTICLE

## Carrier cell-based delivery of replication-competent HSV-1 mutants enhances antitumor effect for ovarian cancer

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Oncolytic viruses capable of tumor-selective replication and cytolysis have shown early promise as cancer therapeutics. We have developed replication-competent attenuated herpes simplex virus type 1 (HSV-1) mutants, named HF10 and Hh101, which have been evaluated for their oncolytic activities. However, the host immune system remains a significant obstacle to effective intraperitoneal administration of these viruses in the clinical setting. In this study, we investigated the use of these HSV-1 mutants as oncolytic agents against ovarian cancer and the use of human peritoneal mesothelial cells (MCs) as carrier cells for intraperitoneal therapy. MCs were efficiently infected with HSV-1 mutants, and MCs loaded with HSV-1 mutants caused cell killing adequately when cocultured with cancer cells in the presence or absence of HSV antibodies. In a mouse xenograft model of ovarian cancer, the injection of infected carrier cells led to a significant reduction of tumor volume and prolonged survival in comparison with the injection of virus alone. Our results indicate that replication-competent attenuated HSV-1 exerts a potent oncolytic effect on ovarian cancer, which may be further enhanced by the utilization of a carrier cell delivery system, based on amplification of viral load and possibly on avoidance of neutralizing antibodies.

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## Introduction

In Japan, 8000 cases of ovarian cancer are newly diagnosed and more than 4000 women die of this disease every year.<sup>1</sup> Ovarian cancer has a high fatality rate because of the lack of effective screening strategies and the absence of symptoms during the early stage of disease. Thus, most patients with ovarian cancer present with advanced-stage disease in conjunction with intraperitoneal carcinoma. Advanced epithelial ovarian cancer (EOC) is a highly chemosensitive solid tumor with good response rates to first-line chemotherapy. However, the majority of patients eventually relapse, and ultimately die of recurrent chemoresistant disease. Therefore, novel therapeutic approaches are required. EOC remains

localized within the peritoneal cavity in a large proportion of patients, causing local morbidity and lethal complications.<sup>2</sup> Owing to its localized nature, EOC lends itself to intraperitoneal approaches to therapy, including gene therapy.

Oncolytic virotherapy is a promising anticancer therapy because efficient transduction and cancer cell-specific viral replication can boost therapeutic efficacy.<sup>3–7</sup> Therefore, oncolytic viral therapy is viewed as a new strategy for the treatment of advanced cancers. Many published reports describe the effectiveness of genetically engineered herpes simplex virus type 1 (HSV-1). HSV-1 has many advantages over other viruses for cancer gene therapy: (1) it has a broad host range and high efficiency of infection; (2) it has a large genomic capacity and can be engineered to deliver therapeutic transgenes;<sup>8,9</sup> and (3) it can be controlled by anti-herpetic drugs. Unlike retroviruses, the HSV genome does not integrate into the host genome, eliminating concerns of insertional mutagenesis. Clinical trials with several of these agents have been completed, with some efficacy. However, as the majority of those studies have relied on direct administration into target tissue, effective systemic viral delivery is required.

A major theoretical impediment to systemic application of HSV is pre-existing antiviral immunity. Almost all

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individuals >30 years (about 80%) have circulating anti-HSV-1 antibodies in Japan.<sup>10</sup> Virus particles injected into the peritoneal cavity are vulnerable to inactivation by complement proteins, uptake by the reticuloendothelial system and neutralization by circulating antibodies.<sup>11</sup> Of these host defenses, antibodies are likely to be the most restrictive barrier to therapy, as they mediate a long-lasting state of immunity to repeated infections.<sup>12</sup> Several widely differing approaches aimed at protecting viral particles within the circulation and ensuring tumor delivery are currently the focus of intense research. For example, one possibility is modification of the viral coat,<sup>13,14</sup> although such technologies are technically challenging. In addition, cellular carriers could be used as Trojan Horse vehicles to shield oncolytic virus (OV) from neutralization following intraperitoneal administration, and act as *in situ* virus factories once arriving at the tumor site.<sup>15,16</sup>

In this study, we demonstrate that the molecular engineering of cellular carriers can increase their ability to support viral replication, promote direct cell-to-cell viral infection of the tumor, and shield oncolytic virus from neutralizing antibodies during delivery *in vitro*. Furthermore, we show the suitability of human peritoneal mesothelial cells (MCs) as a carrier system for delivery of HF10 and Hh101 to maximize the efficacy of oncolytic virus *in vivo*.

## Materials and methods

### Cell lines and viruses

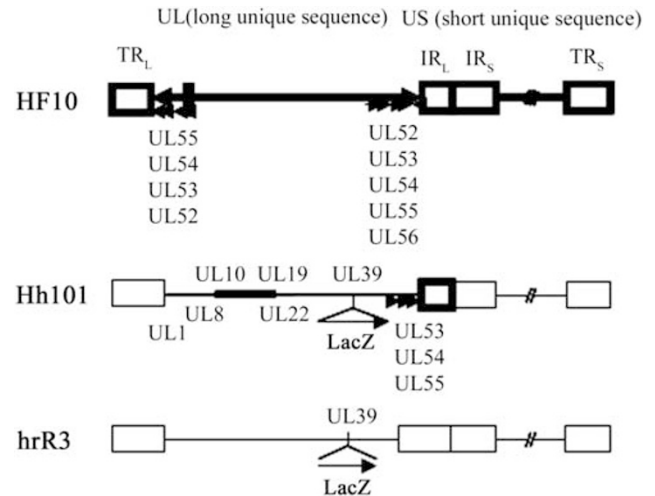
Human ovarian cancer (SKOV3) cells were generously donated by Memorial Sloan-Kettering Cancer Research Laboratory. African green monkey kidney (Vero) cells were obtained from the Riken Cell Bank (Tsukuba, Ibaragi, Japan). SKOV3 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and penicillin–streptomycin. Vero cells were grown in Eagle's minimal essential medium containing 10% calf serum and 1% penicillin–streptomycin. These cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

hrR3, a ribonucleotide reductase (UL39)-deficient HSV-1 mutant, derived from the parental wild-type strain KOS, was kindly provided by Sandra K Weller (University of Connecticut Health Center, Farmington, CT). HF10 is a non-selected clone derived from HSV-1 strain HF and causes extensive cell membrane fusion in infected cells. Hh101 is a recombinant virus clone isolated from Vero cells co-infected with HF10 and hrR3 (Figure 1). The phenotypes of these viruses have been previously described.<sup>17–19</sup>

To visualize viruses *in vitro* and *in vivo*, the green fluorescent protein (*GFP*) gene was inserted into HF10 under control of the cytomegalovirus (CMV) promoter, in which UL43 was deleted. We named this virus HF-GFP.

### Establishment and characterization of immortalized MCs

Human peritoneal MCs were isolated from surgical specimens of human omentum after obtaining consent from each patient, as described previously.<sup>20</sup> Briefly, small



**Figure 1** Models of the structures of herpes simplex virus type 1 (HSV-1) mutants. A schematic representation of the structure of the Hh101 and HF10 genomes. The locations of deletions and insertions in the genome of HF10 are shown. Expansions indicate the positions of genes within the deletion and insertion regions. Arrows indicate the position and orientation of genes within the expansions.

pieces of omentum were surgically resected under sterile conditions and were trypsinized at 37 °C for 30 min. The suspension was then passed through a 200- $\mu$ m pore nylon mesh to remove undigested fragments, and centrifuged at 2000 r.p.m. for 5 min. The collected cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. In the subsequent experiments, cells were used during the second or third passage after primary culture.

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA). Briefly, hTERT, human cyclin D1 and human mutant Cdk4 (Cdk4R24C: an inhibitor resistant form of Cdk4) were first recombined into entry vectors by BP reaction (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA, by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclin D1 and -hCDK4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously.<sup>21</sup> Following the addition of recombinant viral fluid to MCs in the presence of 4  $\mu$ g ml<sup>-1</sup> polybrene, infected cells were selected in the presence of 250  $\mu$ g ml<sup>-1</sup> of G418, 0.5  $\mu$ g ml<sup>-1</sup> of puromycin, 3  $\mu$ g ml<sup>-1</sup> of blasticidin-S or 50  $\mu$ g ml<sup>-1</sup> of hygromycin-B. These cells are named human omentum mesothelial cells (HOMMCs). The study to establish immortalized MCs was approved by the local ethics committee and institutional review board of our hospital.

### Anti-HSV-1 serum

Anti-HSV-1 serum was obtained from mice or guinea pigs by intravenous injection of HSV-1 grown in Vero cells. The neutralizing capacity of antiserum was determined by mixing about 100 plaque-forming units (PFU) of HSV-1 with serial dilutions of antiserum. The serum titer was

expressed as the dilution causing 50% plaque reduction: the neutralization titers of anti-HSV-1 mouse antiserum and anti-HSV-1 guinea pig antiserum were 1000 and 1600, respectively.

#### *Viral replication assay*

MCs or HOmMCs were plated on 35-mm dishes at a density of  $3.7 \times 10^5$  cells per dish in 2 ml of the growth medium under standard conditions overnight. Cells were then infected with HF10 or Hh101 at multiplicities of infection (MOI) ranging between 0.03 and 3. To measure virus replication, cells were scraped from dishes at the indicated times, lysed by freeze–thaw and centrifuged at 3000 r.p.m. for 5 min. Viral titers were determined from the sample supernatants by plaque assay.

#### *In vitro delivery of HSV using MCs as carriers*

MCs were infected with Hh101 (MOI, 3) for 1 h at 37 °C; free virus was then removed and the cells were washed with phosphate-buffered saline (PBS) three times and resuspended in fresh medium. At 2 h after infection, the infected cells were trypsinized. The suspension was centrifuged at 1300 r.p.m. for 5 min at 4 °C. The collected cells were used as infected carrier cells.

SKOV3 cells were plated on 35-mm dishes at a density of  $5.6 \times 10^5$  cells per dish in 2 ml of the growth medium. After 24 h, Hh101- ( $3 \times 10^5$  PFU) or Hh101-infected carrier cells ( $1 \times 10^5$  cells, MOI, 3) were added to the media, and we observed any resulting cytopathogenic effects (CPEs). At 24 h after infection, viral titers were determined from the sample supernatants by plaque assay.

#### *In vitro effects of anti-HSV-1 antiserum on HF-GFP*

HOmMCs were infected with HF-GFP (MOI, 3) for 1 h at 37 °C; free virus was then removed and the cells were washed with PBS three times and resuspended in fresh medium. At 2 h after infection, the infected cells were trypsinized. The suspension was then centrifuged at 1300 r.p.m. for 5 min at 4 °C. The collected cells were used as infected carrier cells.

SKOV3 cells were plated on 35-mm dishes. After 24 h, HF-GFP ( $10^5$  PFU per dish) or HF-GFP-infected carrier cells ( $10^4$  cells per dish) were added to the media with or without anti-HSV-1 mouse antiserum. At 24 h after infection, SKOV3 cells were photographed using the Leica (Wetglar, Germany) M205FA fluorescence stereomicroscope with a standard GFP filter set. At 30 h, SKOV3 cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet solution. The number of plaques was counted under microscopy. The graphs (Figure 5e) were obtained from two independent experiments.

#### *Animal studies*

Animal studies were performed in accordance with guidelines issued by the Animal Center at Nagoya University School of Medicine. Female Balb/c slc nu/nu mice (5 to 6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). For surgical procedures, mice were anesthetized with an intraperitoneal injection of 7.2% chloral hydrate in sterile PBS ( $0.005 \text{ ml g}^{-1}$  body weight).

#### *Subcutaneous tumor model*

To determine the therapeutic efficacy of HF10, we used a subcutaneous (s.c.) tumor model. SKOV3 cells were cultured and passaged twice *in vitro*, and  $5 \times 10^6$  cells were injected s.c. into the flanks of 5-week-old nude mice. At 8 days after tumor challenge, when s.c. tumors were ~10–15 mm in diameter, mice were treated with intratumoral (i.t.) injection of HF10 ( $1 \times 10^7$  PFU). Animals in the first group were injected on days 8, 10 and 12. Animals in the second group were injected on days 8, 10, 12, 18, 20 and 22. Control mice were treated with i.t. injection of 1 ml PBS. Tumor volume was monitored for the indicated number of days after treatment.

#### *Intraperitoneal tumor model*

We confirmed that intraperitoneal (i.p.) injection of SKOV3 cells into 6-week-old female Balb/c nude mice resulted in peritoneally disseminated tumors, ascites, cachexia and death. To assess the efficacy of Hh101, this murine xenograft model was used. Nude mice ( $n=27$ ) were engrafted i.p. with  $2 \times 10^6$  SKOV3 cells. HOmMCs were infected for 2 h with MOI=3 of Hh101 and were used as carriers. After freezing and thawing at 2 h after infection,  $\sim 5 \times 10^4$  PFU of Hh101 were detected as infectious viruses. To estimate the effect of Hh101 in minimally spread ovarian cancer, HOmMCs ( $3 \times 10^6$  cells) infected with Hh101 were injected i.p. on days 3, 6 and 9. The control groups received 1 ml PBS or Hh101 ( $5 \times 10^4$  PFU  $\text{ml}^{-1}$ ) by i.p. injection on the same days. Mice of each group were followed up to record survival times.

To evaluate the role of Hh101 in more advanced disease, SKOV3 tumors were allowed to grow for 6 days before they were treated. Female Balb/c nude mice (6 weeks old) ( $n=10$ ) were engrafted i.p. with  $2 \times 10^6$  SKOV3 cells, and this animal group was treated with repeated injection on days 6, 9, 12, 15 and 18. Animals were followed up daily to record survival times.

#### *Localization of virus-associated GFP expression in mice with disseminated ovarian cancer*

Female Balb/c nu/nu mice (6 weeks old) were engrafted i.p. with  $2 \times 10^6$  SKOV3 cells. On day 30, mice were randomized into two cohorts (control, HF-GFP treatment group). The control group received i.p. administration of 1 ml PBS. The HF-GFP treatment group was given  $10^7$  PFU of HF-GFP i.p. After 24 h, mice were killed and tumors were examined using the Leica M205FA automated fluorescence stereomicroscope with a standard GFP filter set.

To assess the effect of anti-HSV-1 antiserum by using infected carrier cell, we injected control serum or anti-HSV-1 guinea pig antiserum of (500  $\mu\text{l}$  per each mouse,  $\times 1/100$  dilution) i.p. into each mouse at 1 h before treatment. HOmMCs were preinfected with HF-GFP (MOI, 3) for 2 h and were washed in PBS. For this experiment, a disseminated ovarian cancer model ( $1 \times 10^7$  SKOV3 cells, i.p.) was established in 6-week-old female Balb/c nu/nu mice. On day 14, mice were randomized into control, HF-GFP and HF-GFP-infected HOmMCs groups. The HOmMCs group was treated with  $10^7$  cells of



HOMMC-infected HF-GFP. Mice were killed after 24 h and the intra-abdominal images were obtained by the fluorescence stereomicroscope. For each experiment, images were captured under identical exposure settings. Overlays were generated using Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

#### Statistical methods

Data were analyzed using the StatView statistical software package (SAS Institution, Cary, NC). The survival data were analyzed using the Kaplan–Meier method and the log-rank test. Differences in tumor volumes between the treated and control groups were analyzed by the Student's *t*-test. *P*-values <0.05 were considered statistically significant.

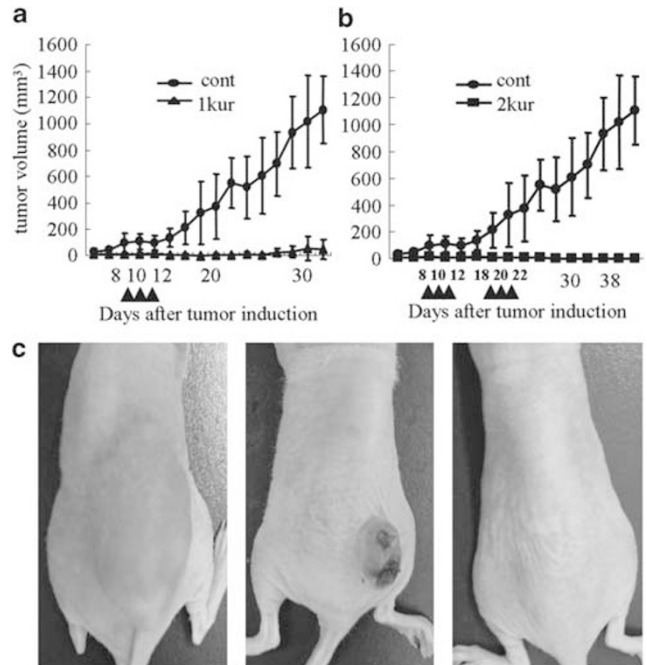
## Results

### *Intratumoral Administration of HSV mutants suppresses s.c. tumor growth of human ovarian cancer cells in nude mice*

We examined the ability of HF10 to control tumor cell growth in an *in vivo* model. We used an s.c. tumor model, because HF10 is fatal to immunodeficient animals when it is administered intravenously or intraperitoneally. The flanks of Balb/c-nu mice were s.c. injected with  $5 \times 10^6$  SKOV3 cells. When tumors were palpable (day 8), i.e. injections of PBS or HF10 ( $1 \times 10^7$  PFU) were made on days 8, 10 and 12 for group one, and on days 8, 10, 12, 20 and 22 for group two. Injections (i.t.) with HF10 significantly reduced tumor growth compared with PBS-injected control animals (Figure 2a). Moreover, in group two, complete disappearance of tumors was observed in some animals (Figure 2b). Representative pictures of control and HF10-injected mice are shown (Figure 2c).

### *In vitro replication of HSV mutants in MCs, immortalized HOMMCs and SKOV3 cells*

To determine which cell line might be adequate for use as a carrier, we next tested the ability of HF10 and Hh101 to replicate in MCs, immortalized human omentum MCs and in SKOV3 cells. Human MCs may pose considerable advantages as vehicles for oncolytic virotherapy for ovarian cancer. First, MCs can be isolated from patients and grown in culture relatively easily. In addition, if isolated from the same patient that will be treated, autologous transplantation overcomes the difficulties related to immune rejection of the transplanted cells. Cells were infected at MOIs of 3 or 0.03. At MOI of 3, virus titers were ~10-fold higher in HOMMCs than in MCs or SKOV3 cells at 24 h after infection (Figures 3e, f). This correlated with the observation of more extensive and rapid CPEs in HOMMCs than SKOV3 cells (Figure 3c, d). Moreover, viral titers of MCs and SKOV3 cells infected at MOI of 3 and HOMMCs infected at MOI of 0.03 increased equally with time. Virus replication was the most efficient in HOMMCs; thus these findings suggested that HOMMCs would be suitable for use as carrier cells.



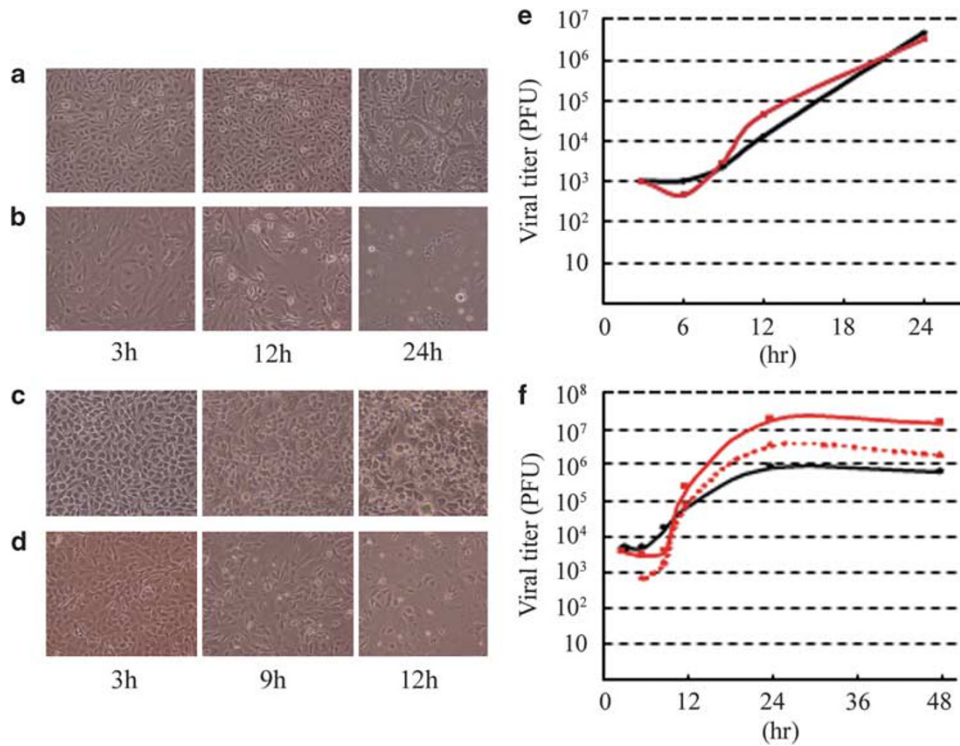
**Figure 2** HF10 reduces tumor growth in a subcutaneous (s.c.) ovarian cancer model. (a) In all,  $5 \times 10^6$  SKOV3 cells were s.c. implanted into the flank of 5-week-old nude mice. When s.c. tumors were approximately 10–15 mm in diameter, phosphate-buffered saline (PBS) (control) or  $1 \times 10^7$  plaque-forming units HF10 were injected intratumorally on days 8, 10 and 12. (b) Group two was injected on days 8, 10, 12, 18, 20 and 22. Tumor volume was monitored for the indicated days after treatment ( $P < 0.01$ ; control vs HF10 treatment group). Bars represent means + s.e.m. of each group. (c) Representative pictures of control (right), group one (middle) and group two (left) at day 30.

### *In vitro delivery of HSV using MCs as carrier cells*

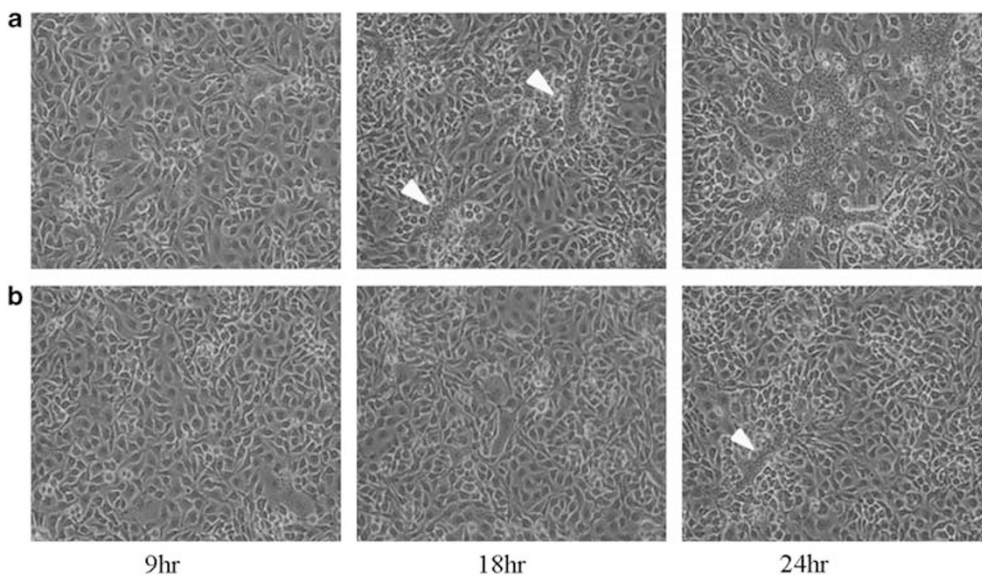
We estimated the efficacy of tumor killing caused by virus-loaded carrier cells *in vitro*. To this end, the oncolytic effects of tumor cells cocultured with virus-loaded MCs was compared with direct infection by virus. We administered Hh101- ( $3 \times 10^5$  PFU) or Hh101-infected carrier cells ( $1 \times 10^5$  cells; MOI of 3) to the media of SKOV3 cells. At 24 h after infection, virus titers in the media were  $6.3 \times 10^4$  PFU ml<sup>-1</sup> when Hh101-infected carrier cells were administered, and only  $2.1 \times 10^2$  PFU ml<sup>-1</sup> when Hh101 virus was administered. Moreover, CPE was observed ~12 h after infection when Hh101-infected carrier cells were administered (Figure 4a), whereas it was observed 24 h after infection with Hh101 virus alone (Figure 4b). CPE was spread more rapidly and extensively in the case of Hh101-infected carrier cells (Figure 4a). Carrier cells therefore supported sufficient viral replication and could contact target cancer cells efficiently.

### *In vitro immune evasion by cell-based delivery of HSV*

As the majority of human adults has been exposed to HSV and has anti-HSV antibodies, it is theoretically possible that oncolytic virus would be attenuated by circulating antibodies. To examine the effect of antibodies



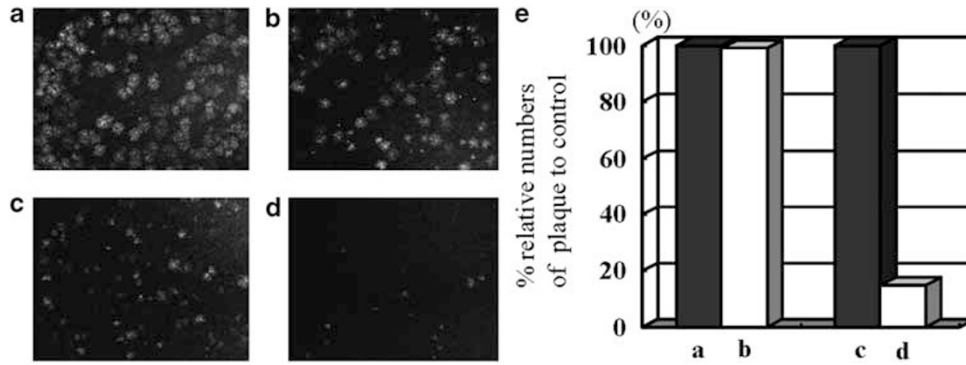
**Figure 3** HF10 and Hh101 replication in mesothelial cells (MCs), human omentum mesothelial cells (HOMMCs) and SKOV3 cells. SKOV3 cells (a, black line) and MCs (b, red line) were infected with HF10 at multiplicities of infection (MOI) 3. SKOV3 cells (c, black line) and HOMMCs (d, red line) were infected with Hh101 at MOI 3. Representative cytopathogenic effects (CPE) are shown as time series. Cells were harvested and virus titer was determined by plaque assay. The red dotted line shows virus titers in MCs infected with Hh101 at MOI 0.03 (e, f). The values represent the mean of samples tested in triplicate. PFU, plaque-forming unit.



**Figure 4** *In vitro* delivery of herpes simplex virus (HSV) using mesothelial cells as carriers. Hh101-infected carrier cells (a) or cell-free Hh101 (b) were added to the media of SKOV3 cells. Representative cytopathic effects (CPEs) are shown as time series. The white arrow heads show small CPEs.

against virus delivery by carrier cells, we performed *in vitro* experiments. We plated SKOV3 cells on 35-mm dishes, and after 24h, HF-GFP ( $10^5$  PFU per dish) or HF-GFP-infected carrier cells ( $10^4$  cells per dish) were

added to the culture media containing with anti-HSV-1 antiserum or control serum. As evidenced by virus-associated fluorescence, extensive replication was seen in SKOV3 cells 24h following administration of HF-GFP-



**Figure 5** *In vitro* effects of anti-herpes simplex virus type 1 (HSV-1) antibody on HF-green fluorescent protein (GFP). SKOV3 cells were plated for 24 h, and HF-GFP-infected carrier cells (a, b) or HF-GFP (c, d) were added to the media containing control serum (a, c) or anti-HSV-1 antiserum (b, d). Anti-HSV-1 antiserum was added to the media to give a final dilution of 1:50. Representative cytopathogenic effect (CPE) images are taken at 24 h using the Leica M205FA fluorescence stereomicroscope with a standard GFP filter set. SKOV3 cells were then fixed, stained with 0.2% crystal violet solution and observed at 30 h. The number of plaques was counted and expressed as a percentage of number obtained in control cultures (e).

infected carrier cells in spite of anti-HSV-1 antiserum. In the absence of anti-HSV-1 antiserum (Figure 5a), however, virus-associated fluorescence was larger and brighter than in the presence of anti-HSV-1 antiserum (Figure 5b). In contrast, little virus-associated GFP was observed in SKOV3 cells 24 h following administration of HF-GFP in the presence of anti-HSV-1 antiserum (Figure 5d), although a number of GFP-expressing cells were detectable in the absence of anti-HSV-1 antiserum (Figure 5c). These results indicated that cellular carriers can efficiently shield oncolytic virus from neutralizing antibodies.

#### Localization of virally infected cell delivery in the presence of anti-HSV-1 antiserum

In order to visualize the distribution of the cellular vehicles in mice, we used HF-GFP, which allowed us to follow the biodistribution of virus-associated fluorescence using an *in vivo* imaging system. To assess the localization of intraperitoneally injected virus, we established a mouse model using ovarian cancer cells, in which  $2 \times 10^6$  SKOV3 cells were inoculated into the peritoneal cavity of nude mice, leading to the formation of peritoneal disseminations. HF-GFP was injected into the peritoneal cavity 30 days after the initial inoculation of cancer cells. At 24 h after HF-GFP injection, nearly all visible tumor nodules in the peritoneal cavity were GFP positive. We detected GFP expression even in small tumors and the brightness of GFP varied in each tumor (Figure 6b). In contrast, no GFP expression was seen in the tumors in animals of the control group (Figure 6a). The GFP expression persisted for 7 days after HF-GFP injection; however, the brightness of GFP weakened as time passed. GFP expression persisted longer in mice injected with HF-GFP-infected carrier cells than in mice injected with HF-GFP alone. These findings suggested that viruses injected into the peritoneal cavity exhibited preferential and specific distribution in disseminated cancer foci in HSV-1 naïve animals.

Next, to examine the impact of a pre-existing immune response, we used the passive immunization method in

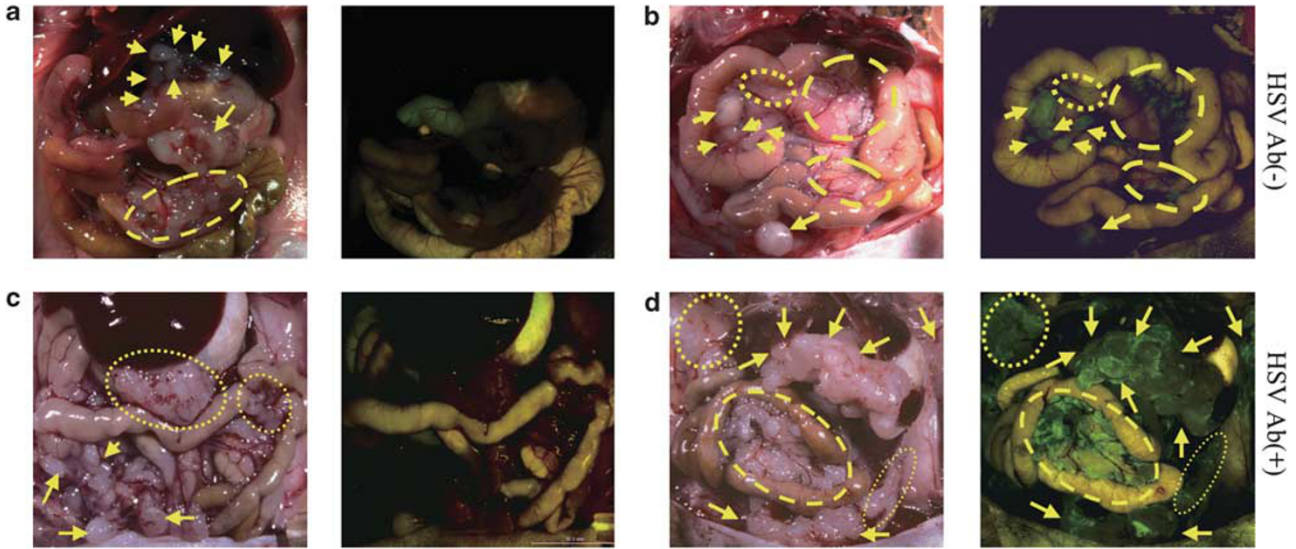
which anti-HSV-1 antiserum was injected *i.p.* into mice. Intraperitoneal tumor-bearing mice were given cell-free HF-GFP or HF-GFP-infected HOMMCs *i.p.* at 1 h after treatment with anti-HSV-1 antiserum or control serum. No clear GFP signal was observed in disseminated tumors when HF-GFP was given to mice pretreated with anti-HSV-1 antiserum (Figure 6c). In contrast, a significant GFP signal was detected in peritoneal tumors in spite of pretreatment with anti-HSV-1 antiserum, suggesting that infected carrier cells could bypass circulating antibodies and transfer virus to intraperitoneally disseminated ovarian tumors (Figure 6d).

#### Intraperitoneal administration of HSV-1 mutant-infected carrier cells improved survival of mice with ovarian cancer

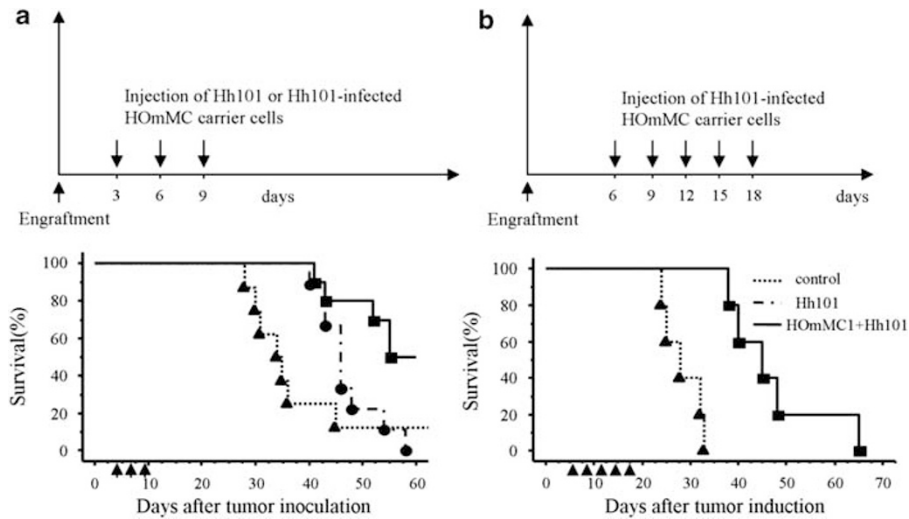
To assess the suitability of carrier cells for the delivery of HSV-1 mutants *in vivo*, we conducted survival experiments to compare the effects of Hh101 administered directly with the effects of Hh101 delivered via HOMMCs. Because HF10 is lethal to immunodeficient animals, we utilized Hh101, which is a recombinant virus clone isolated from HF10 and hrR3 and is less virulent than HF10. PBS, Hh101 or Hh101-infected carrier cells were injected three times 3 days after the *i.p.* injection of  $2 \times 10^6$  SKOV3 cells, a time at which tumors would be invisible to the naked eye. Three repeated therapeutic injections of Hh101-infected HOMMCs significantly improved the mean survival time of ovarian cancer-engrafted nude mice (55 days,  $n = 10$ ) compared with the administration of Hh101 alone (46 days,  $n = 9$ ;  $P < 0.05$ ) (Figure 7a). Two-tenth of the animals in the carrier cell-treated group were completely protected from relapse of peritoneal tumor and ascites.

Next, PBS or Hh101-infected carrier cells were injected five times 6 days after the injection of tumor cells, a time at which numerous macroscopic white, 2 mm diameter tumors were seen at the diaphragm, at the mesentery and occasionally at the omentum. As shown in Figure 7b, all mice, irrespective of treatment, developed macroscopic





**Figure 6** *In vivo* visualization of virally infected cell delivery in the presence of anti-herpes simplex virus type 1 (HSV-1) antiserum. Mice were bearing disseminated SKOV3 ovarian tumors. Mice were randomized into non-treatment group (a, b) and treatment group with anti-HSV-1 antiserum (c, d). Intraperitoneal tumor-bearing mice were given phosphate-buffered saline (PBS) (a), cell-free HF-green fluorescent protein (GFP) (b, c) or HF-GFP-infected human omentum mesothelial cells (HomMCs) (d) intraperitoneally 1 h after treatment. Representative photographs showing between tumor location and GFP signal. Each picture taken 24 h after viral injections is shown. The yellow arrows and dotted-line circles indicate disseminated ovarian tumor.



**Figure 7** Therapeutic effects of Hh101-infected carrier cells in an ovarian cancer model. Nude mice were engrafted intraperitoneally with SKOV3 cells and (a) treatment was started 3 days later using three repeated i.p. injections. Three repeated therapeutic injections of Hh101-infected human omentum mesothelial cells (HOMMCs) improved the survival of ovarian cancer-engrafted nude mice. Injections of Hh101-infected carrier cells were more effective than Hh101 injections alone. Some of the mice treated with Hh101-infected carrier cells survived without symptoms or site injection tumors for >80 days. (b) Therapy was started on day 6 with five repeated i.p. injections. Median survival was significantly ( $P=0.0018$ ) prolonged for the group of carrier-cell-treated animals compared with the control group (median survival, 45 days vs 28 days).

tumors in the peritoneal cavity and subsequently died. However, the survival time was extended by treatment with Hh101-infected carrier cells. This resulted in an approximate doubling of the median survival time (45 days;  $n=5$ ) compared with that for control animals receiving PBS alone (28 days;  $n=5$ ;  $P<0.01$ ). Thus, in both experiments the prognosis was significantly improved by treatment with Hh101-infected carrier cells.

## Discussion

Genetically engineered, conditionally replicating HSV-1 is a promising therapeutic agent for cancer therapy. The main antitumor mechanism of oncolytic viruses results from viral replication within infected tumor cells, resulting in cell destruction, and liberation of progeny virus particles that can directly infect adjacent tumor cells.<sup>7</sup>

Most clinical trials using oncolytic viral therapy have been performed using direct i.t. injection. However, almost all HSV-1 mutants were not so effective as expected when used clinically as antitumor cytolytic agents.<sup>22–24</sup> In an effort to develop more effective, well-tolerated, novel viral therapeutic agents, we have focused a highly attenuated oncolytic HSV-1 mutant, which lacks four accessory genes (*UL56*, *UL43*, *UL49.5* and *UL55*) and LAT (latency-associated transcript).<sup>17–19</sup> Our previous observation have shown that the HF10 is a potent novel agent for oncolytic therapy that is safe and effective for colon cancer, sarcoma and melanoma treatment in mouse models.<sup>25–27</sup> We have also performed a clinical trial of the treatment of recurrent breast cancer and head and neck cancer using i.t. injection of HF10.<sup>28–30</sup> These results revealed a potent oncolytic effect of HF10 without any side effects in human. Currently HF10 is being tested in the United States for the patients with advanced head and neck cancer in a Phase I clinical trial. Here, we have examined the ability of HF10 to control ovarian cancer in order to apply the HF10 therapy for peritoneally disseminated ovarian cancer. Firstly, we performed i.t. therapy of s.c. xenograft tumors using HF10, and tumor growth was significantly reduced. Moreover, in animals treated with six injections, a complete disappearance of the tumor was observed in some animals.

As a strategy to potentially enhance the delivery of HSV to disseminated tumors and to protect the virus from inhibitory factors (complement, anti-HSV antibodies) in the peritoneal cavity, we and others are exploring the use of carrier cells as Trojan Horses to deliver virus to tumors. The optimal carrier cell should be highly susceptible to HSV infection, not be rapidly killed by the virus, traffic to tumors and transfer the viral infection to the tumor cells via cell-to-cell heterofusion and/or by production of virus progeny.<sup>11,31,32</sup> An assortment of cells have been explored in this regard, including tumor cells,<sup>24,33–35</sup> outgrowth endothelial cells<sup>36</sup> and T cells.<sup>31,37</sup> In this work, we observed an ~10-fold higher amplification of the virus in HOMMCs than SKOV3 cells *in vitro*. Virus replication was the most efficient in HOMMCs, so we decided to use these cells as HSV carrier cells. Next, we estimated the efficacy of tumor killing caused by virus-loaded carrier cells *in vitro*. Our *in vitro* studies clearly demonstrated the efficacy of spread of infection between tumor cells and carrier cells. The transfer and spread of infectivity by MCs derived from the omentum was much higher than infectivity transfer by cell-free viruses. Taken together, these findings suggested that HOMMCs would be suitable for use as carrier cells to treat peritoneally disseminated ovarian cancer. However, the ultimate fear of carrier cells after intraperitoneal inoculation may pose some safety concerns. In this study, we immortalized normal human peritoneal MCs with non-viral human genes (mutant Cdk4, cyclin D1 and hTERT) and utilized as carrier cells. Such as the case, a carcinogenic potential of HOMMCs would not emerge thus far (data not shown). In the previous study,<sup>21</sup> we have developed immortalized ovarian surface epithelium with the same gene sets, and we did not observe any tumorigenesis up to

doublings 60. The possibility of clinical application of carrier cells warrants that safety would be ensured.

Ascites frequently accumulate in patients who have tumor spread in the peritoneal cavity, and this fluid is expected to be rich in anti-HSV antibodies because the immunoglobulin G content of ascites fluid is known to reflect that of blood.<sup>38</sup> Also, it has been shown that pre-existing neutralizing antibodies in ascites may prevent initial adenovirus vector delivery in ovarian cancer patients.<sup>39</sup> Thus, carrier cells are expected to be useful not only for systemic virus delivery but also for intraperitoneal administration in patients with peritoneal metastases and pre-existing humoral immune response. We also examined the effect of antibodies against virus delivery by carrier cells. Our *in vitro* data demonstrated that direct cell-to-cell transfer of infectivity by HOMMCs was five to six times more resistant to neutralizing antibodies than infectivity transfer by naked virus. Thus, once infection is successfully transferred to the tumor, it is expected that antibodies will not stop i.t. virus spread. We also showed that HOMMCs infected with HF-GFP could target pre-established ovarian tumor nodules in mice (Figure 6), and this result is consistent with that of measles virus-infected cell carriers.<sup>40</sup> These data support the potential use of HSV oncolytic therapy using carrier cells in humans with pre-existing immunity to HSV.

This study supports the concept that the utilization of carrier cells may have a role in HSV-based oncolytic therapies. Inoculation of HOMMCs infected at MOI 3 or the equal titer of HSV particles should represent comparable viral loads initially administered to the animals. In the SKOV3 model, the carrier cell strategy led to a significant prolongation of animal survival compared with virus alone. Earlier treatment (3 days after engraftment) with infected carrier cells was even more effective. Hh101-infected carrier cells rescued few of the animals, because Hh101 is more attenuated than HF10, which is lethal for immunodeficient mice. If we use HF10 for carrier cell-based therapy in an immunocompetent model, we anticipate that the therapeutic effect would be better and be enhanced. Moreover, the immunogenicity of carrier cells may enhance therapy, as the activation of antitumor immunity during virotherapy appears to contribute to some degree to eliminating tumors and may help to protect from disease. To estimate the role of the immune response in oncolytic viral therapy, we would need to investigate this theory in a syngeneic immunocompetent mouse model of disseminated peritoneal ovarian carcinoma.

In conclusion, we establish that human peritoneal MCs are useful for carrier cells of oncolytic HSV in treating peritoneally disseminated ovarian cancer. Infected MCs and HOMMCs have the unique ability to produce a burst of virus upon delivery to the tumor site. In addition, this strategy allowed oncolytic HSV to escape neutralization by antibodies and complement, and subsequently to transfer the virus to tumor cells by *in situ* cell fusion. These findings may have significant implications for oncolytic virotherapy for ovarian cancer.



## Conflict of interest

The authors declare no conflict of interest.

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