Oncolytic Herpes Simplex Virus-1 Lacking ICP34.5 Induces p53-independent Death and Is Efficacious against Chemotherapy-resistant Ovarian Cancer

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ABSTRACT

Replication-restricted herpes simplex virus-1 (HSV-1) strains lacking ICP34.5 are emerging as powerful anticancer agents against several solid tumors including epithelial ovarian cancer (EOC). Although chemotherapy-resistant tumors would be likely candidates for treatment with HSV-1 mutants lacking ICP34.5, the efficacy of these mutants on such tumors is unknown. In the present study, we investigated whether chemotherapy resistance affects the response of ovarian cancer cells to HSV-R3616, an ICP34.5-deficient, replication-restricted HSV-1. Primary EOC cultures obtained from patients who varied in their responses to platinum/paclitaxel induction chemotherapy displayed similar sensitivity to HSV-R3616. Similarly, chemotherapysensitive ovarian cancer cells A2780 and PA-1, possessing wild-type p53, and their respective chemotherapy-resistant clones A2780/200CP, lacking p53 function, and PA-1/E6, permanently expressing the HPV E6 gene, were equally sensitive to HSV oncolysis. Because wild-type HSV can kill cells by apoptosis and nonapoptotic mechanisms, we investigated the involvement of apoptosis and the role of the p53 tumor suppressor gene in oncolysis induced by HSV-R3616. Infection of ovarian cancer cell lines by HSV-R3616 was followed by cell death via apoptosis or nonapoptotic mechanisms as noted by morphology, cell cycle analysis, and in situ TUNEL assay. p53 protein levels remained unchanged, and Bax protein levels decreased in cells possessing intact p53 and that mainly underwent HSV-induced apoptosis. Loss of p53 function did not affect the frequency or rate of apoptosis or the sensitivity of EOC cells to the oncolytic effect of HSV-R3616. These results suggest that recombinant HSV-1 lacking ICP34.5 is capable of killing ovarian cancer cells that lack p53 function, resist apoptosis, and/or are chemotherapy resistant. These data support the hypothesis that HSV-based oncolytic therapy may be efficacious in chemotherapy-resistant tumors, including tumors that are deficient in p53.

INTRODUCTION

Recent advances in molecular virology have allowed for the engineering of attenuated viral particles with promising oncolytic activity. Replication-restricted HSV1-1 mutants have been generated in several laboratories by alteration of genes controlling viral replication such as thymidine kinase (UL23; Refs. 1 and 2) or the ICP6 gene (UL39) encoding the large subunit of HSV ribonucleotide reductase (3). UL23 and UL39 mutants have shown tumor selectivity for neuronal (2, 4, 5) and nonneuronal malignancies (6). HSV oncolytic agents have also been generated by alterations of both copies of the RLI gene (7–9). Its product, the ICP34.5 protein, is important for viral replication (10), viral exit from infected cells (11), prevention of the premature shut-off of protein synthesis in the infected host (12), and neurovirulence (13, 14). ICP34.5- mutants have shown efficacy against several types of CNS malignancies (4, 7, 8, 15–17), experimental s.c. malignant melanoma (18), lung cancer (19), and i.p. human malignant mesothelioma (20) in rodent models. In addition, multi-mutated recombinant HSV-1 lacking ICP34.5 and uracil DNA glycosylase or ribonucleotide reductase have shown efficacy against CNS tumors (9, 21, 22), breast cancer (23), metastatic colon cancer (24), and head and neck squamous cancer (25). Finally, another engineered HSV mutant, R7020, displayed efficacy in human epidermoid carcinoma and prostate adenocarcinoma (26). In the animal studies, no spread of the virus could be documented outside the tumors by immunohistochemistry or PCR after i.p. administration of the virus (20, 27, 28), and no toxicity was seen in treated rodents and nonhuman primates (20, 27, 28). These results suggest that HSV-based oncolytic therapy may represent a potentially pow-
erful and safe tool for the treatment of solid tumors of neuronal and various nonneuronal tissues (29). We reported recently that an ICP34.5− HSV strain displays efficacy against epithelial ovarian cancer in vitro and in vivo in severe combined immunodeficient mouse models (28). A single i.p. administration of 5 × 10⁶ particle-forming units led to dramatic reduction of established i.p. disease and significant prolongation of animal survival. By immunohistochemistry, we demonstrated that the virus had spread within tumor nodules and was still active up to 6 weeks after i.p. administration. Finally, HSV-G207, a strain lacking ICP34.5 and ICP6, displayed efficacy against epithelial ovarian cancer and was shown to be tumor selective in vitro and in vivo, exerting lytic activity against epithelial ovarian cancer cells with significantly lower activity against normal peritoneal mesothelial cells (30).

Although the mechanisms of cell killing by oncolytic HSV-1 mutants have not been characterized, it is known that wild-type HSV-1 can kill cells by apoptosis or cell lysis (31, 32). Several HSV proteins, such as viral tegument-associated proteins, US3 and US5 protein products, and the ICP34.5 protein have been implicated in the induction or prevention of apoptosis (32–35). For instance, ICP34.5 prevents the premature shut-off of protein synthesis and often apoptosis (12) by decreasing the accumulation of phosphorylated eIF2α subunit in the infected host. This counteracts the effect of wild-type HSV infection, which activates PKR (36). PKR is known to phosphorylate eIF2α (36) and p53 (37). Moreover, PKR induction can precede p53 accumulation in some cells destined for p53-dependent apoptosis (38). It is therefore feasible that the p53 functional status of cancer cells may alter the mechanism of cell death induced by ICP34.5-deficient HSV mutants.

Previous experience with chemotherapy drugs demonstrates that many antitumor agents exert their cytotoxic action through induction of apoptosis. Importantly, defective apoptosis has been associated with chemotherapy resistance in EOC and other solid tumors (39, 40). A large proportion of solid tumors displays loss of p53 function attributable to either direct molecular alterations of the gene or inactivation by other pathways (41), accounting for the development of defective apoptosis. EOC provides a good paradigm of a solid tumor with loss of p53 function. Multiple molecular mechanisms may account for the development of chemotherapy resistance in ovarian cancer, including activation of cytoplasmic detoxification pathways, up-regulation of DNA repair enzymes, and possibly acquisition of multidrug resistance (42–45). However, in vitro studies in EOC suggest that alterations in p53 and downstream pathways may also be responsible for the development of resistance to cisplatin and other chemotherapy drugs (42, 43). If p53-dependent pathways play a central role in mediating HSV-induced cell death, the antitumor efficacy of HSV mutants might be significantly impaired in p53-deficient tumors. This may become particularly relevant for clinical trials, where HSV mutants might be tested on patients who have already failed conventional chemotherapy approaches. Studies are therefore warranted to explore the involvement of p53 in cell killing by HSV mutants and its role in affecting cell sensitivity to the oncolytic effect of replication-restricted HSV.

In the present study, we investigated the impact of the acquisition of chemotherapy resistance in ovarian cancer cells on their sensitivity to an attenuated strain of HSV-1 lacking ICP34.5, HSV-R3616, because of the clinical importance of ICP34.5 mutants and further attenuated replication-selective HSV mutants. Given the implication of apoptosis in chemotherapy-induced cytotoxicity and chemoresistance, we also investigated the role of apoptosis in cancer cell killing by HSV-R3616 and we assessed the impact of acquisition of chemotherapy resistance on the mechanisms of cell death induced by HSV-3616. In that context, we analyzed the involvement of p53 tumor suppressor gene in HSV-mediated oncolysis. We report evidence suggesting that the acquisition of chemotherapeutic resistance does not impair EOC cell response to mutant HSV. Moreover, recombinant ICP34.5− HSV-1 kills ovarian cancer cells by mechanisms that are not dependent on the p53 tumor suppressor gene. In some cells, nonapoptotic mechanisms are dominant, whereas apoptotic mechanisms prevail in others. Even in cells dying mainly of apoptosis, p53 is not required. These data support the hypothesis that HSV-based oncolytic therapy may be a biological agent displaying efficacy against solid tumors independently of their p53 status or their sensitivity to chemotherapy.

MATERIALS AND METHODS

Virus Strains. Recombinant HSV R3616, a mutant lacking 1000 bp from the coding domain of each copy of the RL1 gene (14), was generously provided by Bernard Roizman (University of Chicago).

Cell Lines. The EOC cell lines SKOV3, OVCAR-3, and Caov-3 were obtained from the American Type Culture Collection (American Type Culture Collection, Manassas, VA). SKOV3 and Caov-3 cells lack p53 because of a deletion and nonsense mutation, respectively (46), whereas OVCAR-3 cells display aberrant expression of p53 protein because of a missense mutation (47). The epithelial ovarian cancer A2780 cell line and its chemotherapy-resistant clone A2780/200CP were a kind gift of Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA; Ref. 48). Parental A2780 line harbors wild-type p53 gene and is sensitive to cisplatin (49), whereas A2780/200CP, a clone of A2780/70CP, has lost p53 function (50). The human ovarian teratocarcinoma line PA-1 was obtained from the American Type Culture Collection (51). PA-1/E6-10, a PA-1 clone permanently transfected with HPV E6 oncoprotein, and PA-1/neo, a control G418-resistant clone, were generously provided by Dr. Wafik el-Deiry (University of Pennsylvania; Ref. 52). Parental PA-1 and PA-1/neo cells possess wild-type p53 and are sensitive to cisplatin and paclitaxel (49, 52). PA-1/E6-10 cells expressing HPV E6 oncoprotein are chemoresistant and display very low levels of p53 (52), secondary to targeted degradation of p53 attributable to rapid ubiquitin-mediated proteolysis (53). All cells were cultured under standard conditions (37°C in a 5% CO₂ atmosphere) in RPMI 1640 in the presence of 10% heat-inactivated FCS and antibiotics. For PA-1/E6-10 and control PA-1/neo, the above media were supplemented with 200 μg/ml genetecin (Sigma Chemical Co., St. Louis, MO). Cells were infected with HSV-R3616 at various MOIs in serum-free media for 1 h, as described previously (28). Serum-enriched media were added subsequently, and cultures were followed for appropriate intervals. Mock-infected controls were incubated in

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HSV-based Oncolytic Therapy for Ovarian Cancer

3344

serum-free media for 1 h and in serum-enriched media thereafter. Morphological changes were documented by phase microscopy.

**Primary EOC Cell Cultures.** Primary ovarian cancers were obtained from two patients with stage IIIc EOC, according to the International Federation of Gynecologists and Obstetricians. Malignant effusions, obtained at the time of debulking, 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). EOC cells were passaged four times prior to using them in experiments. To eliminate macrophages from primary cultures, culture media were aspirated 30 min after plating, and suspended cells were reseeded in new culture flasks. All primary isolates were cultured under standard conditions (37°C in a 5% CO2 atmosphere) in RPMI 1640 in the presence of 10% heat-inactivated FCS and antibiotics. Institutional Review Board approval had been obtained for the retrieval and utilization of primary cultures. Cells were infected with HSV-R3616 as described above.

**Clinical Response to Platinum/Taxol Chemotherapy.** Patients undergoing standard induction chemotherapy with i.v. carboplatin and paclitaxel received treatment every 3 weeks for a total of six courses. Chemotherapy was initiated 2–3 weeks after optimal tumor debulking (<1-cm residual tumor nodules). Tumor response was determined by physical examination, abdominal computed tomography scan and serum CA-125 determination. Serum CA-125 levels were determined routinely in the William Pepper Clinical Laboratory of the University of Pennsylvania Medical Center by RIA using a commercially available kit (Abbott Laboratories, Atlanta, GA).

**MTS Survival Assays.** Cells were plated at a density of 3 × 103 cells/well in 96-well plates and incubated overnight. Cells were infected with HSV-R3616 at 10–2 to 105 MOI in serum-free media (50 μl) for 1 h. Serum-enriched media (50 μl) were added subsequently, and cultures were followed for selected intervals up to 120 h. Cell survival was assessed with colorimetric MTS assays using a chromogenic kit (CellTiter 96; Promega Corp., Madison, WI), and plates were read in a microplate ELISA reader (Bio-Tek Instruments, Winooski, VT).

**Flow Cytometric Cell Cycle Analysis.** Cells were cultured in T-25 flasks and infected with HSV-R3616. At selected intervals, floating cells were collected, and adherent cells were harvested with a 0.05% trypsin-EDTA solution, as above. Collected cells (including floating cells) were fixed in 70% ETOH at −20°C for at least 16 h, treated with RNase A (Sigma; 500 μg/ml for 30 min at room temperature), stained with propidium iodide (20 μg/ml), and analyzed using an EPICS XL flow cytometer (Coulter Corporation). Data were analyzed using a Cellfit program. Cells displaying less than G0/G1 (hypodiploid) DNA content were labeled as being located in sub-G0, and were considered apoptotic (54, 55).

**Fluorescent in situ TUNEL Assay.** Apoptosis was detected by fluorescent *in situ* 3′-end labeling of DNA fragments (*TUNEL* assay) *in vitro*. DNA fragments were labeled and detected by use of the reagents and procedures provided in the *ApopTag in situ* apoptosis detection kit (Oncor, Gaithersburg, MD). Briefly, cells were plated in chamber slides (Nunclon), infected with 1 MOI of HSV R3616, incubated for selected intervals, rinsed twice with PBS, fixed in methanol:acetone (2:1, v/v) at −20°C for 20 min, allowed to air dry, and rinsed with PBS. They were then incubated in a humified chamber at 37°C for 1 h in the presence of terminal deoxynucleotidyl transferase and dUTP and dATP. The cells were washed with buffer and incubated with anti-digoxigenin-fluorescein-conjugated antibody for 30 min at room temperature. The cells were then washed with buffer and observed under epifluorescence and brightfield optics.

**Double Fluorescent TUNEL and Indirect HSV Immunofluorescence.** To assess DNA fragmentation in cells infected with recombinant HSV-1, we performed double fluorescent HSV immunostaining and fluorescent *in situ* 3′-end labeling of DNA fragments. Cells were plated in culture chamber slides (Nunclon) as above and infected with 1 MOI of HSV R3616 as described above. Cells were then rinsed twice with PBS and fixed as above. Indirect immunofluorescence against HSV-1 was performed using a polyclonal antibody against HSV-1 (American Qualex) diluted at 1:250 in 10% goat serum. A rhodamine-conjugated goat antirabbit secondary antibody (Jackson Immunoresearch Laboratories Inc.) was used at 1:250. After immunostaining, a fluorescent TUNEL assay was performed on the same slides, as described above.

**Protein Preparation and Western Blotting.** Cell monolayers were plated in T-25 flasks and infected in serum-free RPMI 1640 with 1 MOI of HSV-R3616 and incubated for the time necessary to reach 50% of cell survival (*T*50). This was at 18 h for PA-1, PA-1/neo, and PA-1/E6-10 and 24 h for A2780 and A2780/200CP. At that time, very few floating cells were noted in the culture plates. Cells were washed with PBS, scraped, and homogenized, and protein was quantified as described previously (56). Equivalent amounts (25 μg) of protein were subjected to a 12% SDS PAGE. Proteins were then transferred to polyvinylidene difluoride transfer membrane. For immunoblotting of p53 and Bax protein, monoclonal antibodies against p53 and Bax protein (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution. An alkaline phosphatase-conjugated antiamouse secondary antibody was used at 1:7500 dilution (Promega Corp., Madison, WI).

**Statistical Analysis.** Multiple comparisons were performed with one-way ANOVA. Post-hoc comparisons of specific paired groups were done with the *t* test. Statistical significance was set at *P* < 0.05. Results are expressed as the mean ± SE.

**RESULTS**

**Ovarian Cancer Cells from Patients with Different Sensitivity to Platinum/Taxol Chemotherapy Display Similar Sensitivity to ICP34.5** HSV-1. To test whether the presence of chemotherapy resistance affects the response of ovarian cancer cells to oncolytic HSV-1 mutants, EOC cells were collected from two patients with stage IIIc epithelial ovarian cancer displaying different sensitivities to carboplatin/paclitaxel induction chemotherapy (Fig. 1). One patient (patient A) had a complete response to chemotherapy after optimal debulking, with a rapid decline of serum CA-125 levels and sustained clinical remission. The other patient (patient B) had a poor response to chemotherapy after optimal debulking with incom-
Ovarian Cancer Cell Lines with Different p53 Status Display Similar Sensitivity to ICP34.5+ HSV-1. To test whether chemotherapy resistance affects the sensitivity of established EOC cell lines to HSV-R3616, the effect of HSV-R3616 on chemotherapy-resistant EOC cells (SKOV3, OVCAR-3, and Caov-3) was compared with that of chemotherapy-sensitive cells (A2780). MTS survival assays demonstrated that all cell lines were susceptible to lysis by HSV-R3616 to a similar degree, with survival ranging from $59 \pm 8$ to $70 \pm 12\%$ with 0.01 MOI, from $30 \pm 9$ to $44 \pm 7\%$ with 0.1 MOI, from $12 \pm 4$ to $22 \pm 5\%$ with 1 MOI, and from $0$ to $5 \pm 3\%$ with 10 MOI 72 h after infection.

Kinetics of Cell Death Correlate with Viral Replication. HSV may induce cell death through the action of tegument-associated proteins, in the absence of viral replication, or through the action of viral-encoded proteins during the process of the viral replication cycle (32, 34, 35). To assess whether viral replication is important in the cytotoxic effect of HSV-R3616, we analyzed the magnitude of viral replication and the rapidity of cell killing by the virus in three cell lines. Viral replication at 24 h was analyzed by a one-step growth curve. PA-1 cells demonstrated the highest viral burst size of $105 \pm 13$ virions released/infected cell, whereas A2780 displayed a smaller burst size of $33 \pm 5$ virions/infected cell ($P < 0.05$ versus PA-1 and SKOV3) and SKOV3 displayed $8 \pm 2$ virions/infected cell ($P < 0.01$ versus PA-1). Time-course experiments demonstrated a significant difference in the time required for HSV-R3616 at 1 MOI to achieve $>99\%$ of killing in vitro, which paralleled the burst size in the three lines. The PA-1 line displayed the fastest response to HSV-R3616, with $50 \pm 9\%$ cells killed at 18 h and $99 \pm 0.5\%$ killing achieved within 24 h of exposure to 1 MOI of R3616. A2780 cells also responded rapidly to R3616, with $35 \pm 9\%$ cells killed at 18 h (NS versus PA-1) and $50 \pm 7\%$ cells killed at 24 h ($P < 0.05$ versus PA-1), whereas $99 \pm 0.4\%$ killing was achieved within 48 h. SKOV3 cells displayed the slowest response with $15 \pm 8\%$ cells killed at 18 h ($P < 0.01$ versus PA-1 and A2780), $21 \pm 4\%$ cells killed within 24 h ($P < 0.01$ versus PA-1 and A2780), $39 \pm 7\%$ cells killed at 48 h ($P < 0.01$ versus A2780), $50 \pm 9\%$ cells killed at 60 h, and $99 \pm 0.8\%$ cells killed within 120 h of infection. These results suggest that viral replication might affect the cytotoxic activity of HSV-R3616.

Chemotherapy-resistant Ovarian Cancer Cell Clones Are Sensitive to Recombinant HSV-1. To further test whether the presence of chemotherapy resistance affects the response of ovarian cancer cells to HSV-R3616, we used two pairs of established ovarian cancer cell lines differing in their sensitivity to platinum. The parental A2780 line harbors wild-type p53 (49), which is up-regulated after exposure to ionizing radiation or chemotherapy drugs (57–59). The chemotherapy-resistant clone A2780/200CP is a subclone of A2780/70CP, which has lost p53 function and fails to respond to cisplatin-induced DNA damage with activation of p53 or Bax (60). Ovarian teratocarcinoma clone PA-1/neo harbors wild-type p53 and is sensitive to chemotherapy agents, whereas in the PA-1/E6-10 clone, p53 is rapidly degraded because of sustained expression of the HPV E6 oncogene (52). The latter clone is resistant to paclitaxel (52).

The sensitivity of the different cell lines to DNA-damaging

Fig. 1 Ovarian cancer cells from patients with different sensitivity to platinum/Taxol display similar sensitivity to ICP34.5+ HSV-1. Primary ovarian cultures were obtained from ascites of two patients with stage IIIc EOC undergoing optimal debulking (<1-cm residual tumor nodules) who displayed different sensitivity to platinum/paclitaxel. A, serum CA-125 levels during induction chemotherapy with carboplatin/paclitaxel. Patient A had a complete response with a rapid decline of serum CA-125 levels and achieved sustained clinical remission. Patient B had a poor response with incomplete decline of CA-125 and clinical progression of disease at completion of induction chemotherapy, which was accompanied by progressive rise of serum CA-125. Serum CA-125 was determined by RIA and is expressed as the percentage of the baseline prechemotherapy value. B, cells collected from ascites harvested during the debulking laparotomy were exposed to HSV-R3616 after four passages in vitro. Survival was assessed at 96 h with a colorimetric enzymatic method using a commercially available chromogenic kit (CellTiter AQueous96; Promega). No significant differences are noted in the response to HSV-R3616 between the two primary cultures. Bars, SE.
HSV-based Oncolytic Therapy for Ovarian Cancer

agents was assessed by exposing them to increasing doses of CDDP. PA-1/E6-10 displayed significantly lower sensitivity to CDDP compared with control PA-1/neo cells (P < 0.001 both; Fig. 2A). In agreement with others (48), A2780/200CP also displayed significantly lower sensitivity to CDDP compared with its parental line A2780 (Fig. 2B). To assess the efficacy of ICP34.5− HSV in chemotherapy-resistant ovarian cancer, we compared the response to R3616 of the above parental ovarian cancer cells possessing wild-type p53 (p53+) and their clones lacking p53 function (p53−). MTS assays were carried out 4 days after exposure to increasing MOI of the virus. No significant differences in the oncolytic effect of HSV-R3616 were noted between the parental p53+ and their p53− clones (Fig. 2).

Recombinant HSV-1 Induces Cell Death via Apoptosis-dependent and -independent Mechanisms in a Cell-specific Manner. To analyze the type of cell death after infection by HSV-R3616, we tested a p53− cell line (SKOV3) and two p53+ cell lines (A2780 and PA-1). Apoptosis was assessed with phase microscopy (Fig. 3, top), cell cycle analysis (Fig. 3, bottom), and in situ fluorescent TUNEL assay (Fig. 4).

Given the different time of response to HSV-R3616 among different cell lines (not shown), cells were incubated for the time necessary to reach 50% of cell survival compared with normally growing controls (T50), as determined by survival assays. This was at 18 h for PA-1, 24 h for A2780, and 60 h for SKOV3 cells. At that time, very few floating cells were noted in the culture plates. Control PA-1 cells exhibited minimal apoptosis by microscopy (Fig. 3A). PA-1 cells infected at 1 MOI and examined under light microscopy at T50 displayed features of apoptosis (54) including cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation, and apoptotic bodies (Fig. 3B). These features appeared first at 18 h and were widespread by 24 h. By 48 h, >95% of the cells had detached off the culture plates. A2780 cells also exhibited morphological features characteristic of apoptosis within 24 h (not shown), and finally, >95% of the cells detached off the culture plates within 72 h. Control SKOV3 cells exhibited minimal morphological signs of cell death by microscopy (Fig. 3C). In contrast to PA-1 and A2780 cells, HSV-infected SKOV3 cells displayed minimal morphological features of apoptosis. Instead, a large fraction of the cells exhibited giant cell formation within 60–70 h, and >95% of the cells underwent cellular disintegration within 120 h (Fig. 3D). Of note, no syncytia formation was noted with PA-1 or A2780 cells.

Cell cycle analysis was performed in parallel to the above experiments (Fig. 3, bottom). A minimal fraction of uninfected subconfluent PA-1 and SKOV3 cells were located in sub-G1 (Fig. 3, E and G, respectively). Hypodiploid fragmented DNA (sub-G1) compatible with apoptosis was seen in a large fraction of infected PA-1 cells (66 ± 11%). Fig. 3F shows the results of one such experiment. Apoptosis was also seen in a large fraction of infected A2780 cells (96 ± 3%, not shown). On the other hand, a small portion of infected SKOV3 cells was located in sub-G1 (12 ± 7%, P < 0.01 versus PA-1 and A2780 cells). Fig. 3H shows the results of one such experiment. These results suggest that PA-1 and A2780 cells underwent apoptosis,

Fig. 2 Resistance to chemotherapy does not affect the sensitivity of ovarian cancer cells to HSV-R3616. Top, control chemotherapy-sensitive lines with wild-type p53 were compared with chemotherapy-resistant clones lacking p53 for their sensitivity to CDDP. Cells were exposed to increasing concentrations of CDDP for 96 h, and cell survival was assessed by MTS assays. A, p53+ PA-1/E6-10 (PA-1/E6) cells display significant resistance to CDDP compared with parental p53+ A2780. Bottom, the sensitivity of control chemotherapy-sensitive p53+ cell lines and of their chemotherapy-resistant p53− counterparts to HSV-R3616 was assessed by MTS assays. Cells were exposed to increasing MOIs of HSV-R3616 and incubated for 96 h. C, p53− PA-1/E6-10 (PA-1/E6) cells display similar response to HSV-R3616 as the parental p53+ PA-1/neo (PA-1) cells. D, p53− A2780/200CP (A2780/CP) cells display similar response to HSV-R3616 as the parental p53+ A2780 cells.
whereas SKOV3 cells mainly died via nonapoptotic mechanisms (54, 55).

To further define the pattern of cell death induced by HSV-1 mutants in ovarian cancer cells, we assessed DNA fragmentation with the in situ fluorescent TUNEL assay (54). To detect the presence of HSV-R3616 in cells undergoing apoptosis, TUNEL assay was preceded by indirect immunofluorescence for HSV performed on the same cells (Fig. 4). Cells were again incubated for the time necessary to reach 50% of cell survival ($T_{50}$), as above. At that time, very few floating cells were noted in the culture plates. After exposure to 1 MOI, 49% of PA-1 cells exhibited apoptosis by 16 h, as assessed by fluorescent in situ TUNEL assay (Fig. 4A). At that time, >99% of the cells were infected by HSV, as assessed by double HSV immunofluorescence (Fig. 4B). A2780 exhibited a higher rate of apoptosis after infection by HSV-R3616, with 95% of the cells exhibiting DNA fragmentation (Fig. 4C; $P < 0.01$ versus A2780 cells). A2780 cells (>99%) were also infected by HSV-R3616 by 48 h of exposure to the virus at 1 MOI, as assessed by immunofluorescence (Fig. 4D). A significantly lower amount of apoptosis was detected in SKOV3 cells, with only 2% of the cells demonstrating DNA fragmentation at 96 h (Fig. 4E; $P < 0.01$ versus PA-1 and A2780 cells). Using combined HSV immunofluorescence/TUNEL assay, we observed that almost all (>99%) SKOV3 cells were infected by HSV-R3616 (Fig. 4F). These results indicated that HSV-R3616 efficiently infected all ovarian cancer cell lines tested but induced a variable degree of apoptotic cell death, depending on the cell line.

**HSV-induced Apoptosis Is Not Associated with Increase in p53 or Bax Levels in Ovarian Cancer Cells.**

The above results indicate that p53$^+$ A2780 and PA-1 cells promptly undergo apoptosis, whereas p53$^-$ SKOV3 cells fail to undergo apoptosis. These data may suggest an important role of p53 in HSV-induced apoptosis. To assess whether p53 is implicated in apoptosis induced by HSV-R3616, we performed Western blot analysis of p53 protein in the cell lines that undergo apoptosis after infection by HSV-R3616, i.e., A2780 and PA-1/neo, and their chemotherapy-resistant counterparts A2780/200CP and PA-1/E6-10, respectively (Fig. 5, top). Control A2780 cells exposed to lethal ionizing radiation (1500 cGy) displayed an increase in p53 protein levels (Lane R). The baseline level of p53 protein detected in parental A2780 (Fig. 5, Lane 1, −HSV) was similar to that in A2780/200CP (Fig. 5, Lane 2, −HSV), consistent with a missense mutation, as described by others (60). The baseline levels of p53 protein in PA-1/E6-10 (Fig. 5, Lane 4, −HSV) were much lower compared with control PA-1/neo cells (Fig. 5, Lane 3, −HSV), consistent with accelerated degradation of the protein (53). No p53 protein was detected in
SKOV3 cells (not shown). There was no substantial increase in p53 protein levels observed in parental p53
1 A2780 (Fig. 5, Lane 1, +HSV) or PA-1/neo (Fig. 5, Lane 3, +HSV) after infection by HSV-R3616 in multiple experiments. These results suggested that p53 is not up-regulated after infection by HSV-R3616. Interestingly, a decrease in p53 levels was noted in PA-1/E6-10 cells after infection by HSV-R3616 (Fig. 5, Lane 4, +HSV), suggesting accelerated degradation of p53. In one experiment, a slight increase in the levels of p53 protein was noted in A2780/200CP cells after infection with HSV-R3616 (not shown).

As an additional way to confirm the lack of activation of p53-dependent downstream pathways, protein levels of bax, a transcriptional target of p53 gene mediating p53-induced apoptosis (61), were also analyzed in the same cells (Fig. 5, bottom). Control A2780 cells exposed to lethal ionizing radiation (1500 cGy) displayed an increase in bax protein levels (Lane R). A2780/200CP cells displayed significantly lower levels of Bax compared with their chemotherapy-sensitive counterparts, confirming the loss of p53 function in A2780/200CP cells. Further confirming the lack of activation of p53-dependent pathways by HSV-R3616, we did not observe any increase in bax protein in A2780 or PA-1/neo cells after exposure to HSV-R3616. Instead, a slight decrease of bax protein levels was noted in all cell lines after infection by HSV-R3616.

**Loss of p53 Does Not Preclude HSV-induced Apoptosis.** To further assess the involvement of p53-mediated pathways in HSV-induced apoptosis, we analyzed the amount of apoptosis in the two pairs of malignant ovarian cells with different p53 status (Fig. 6). Apoptosis was assessed by fluorescent TUNEL assay,

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**Fig. 4** Cell death after infection by HSV-R3616 may occur via apoptosis or apoptosis-independent mechanisms. Double fluorescent DNA *in situ* labeling (FITC; A, C, and E) and HSV immunofluorescence (rhodamine; B, D, and F) was carried out in PA-1, A2780, and SKOV3 cell lines after infection of the cells with HSV-R3616 at 1 MOI. Cells were incubated for the time necessary to reach *T*_{50}. A different response to HSV-R3616 is noted in the different cell lines. PA-1 cells display DNA fragmentation 49 ± 5% (A, ×100), whereas >99% of the cells are infected by HSV (B, ×100). A2780 cells display DNA fragmentation in 95 ± 2% (C, ×60), and >99% of the cells are infected by HSV (D, ×60). By contrast, SKOV3 cells display DNA fragmentation in 2 ± 1.5% (E, ×60), whereas >99% of the cells are infected by HSV (F, ×60). To further demonstrate the paucity of DNA fragmentation in HSV-infected SKOV3 cells, cells were photographed with both filters for rhodamine and fluorescein open (G, ×60), clearly showing the presence of only one single cell (arrow) undergoing DNA fragmentation, despite that all cells are infected. This cell can be traced in panels E–H. Giant cell formation and cell fragmentation can be appreciated by phase microscopy (H, ×60) in HSV-infected SKOV3 cells. PA-1 cells were infected with HSV-R3616 (I, ×60) and incubated with preimmune rabbit serum (J, ×60) as negative controls. A negative control for TUNEL assay, prepared in the absence of dUTP and dATP, can be seen in K (×60). The difference in the amount of apoptosis detected in the different cell lines is illustrated by the graph (bottom left). Data represent results from analysis of 50 high power fields from three different experiments. Mock-infected controls; □, HSV-infected cells. Bars, SE.
as described above, and quantified by counting the number of cells displaying DNA fragmentation over the total number of cells/field in 50 intermediate power fields (×40). Exposure of A2780 and A2780/200CP cells to 1 MOI of HSV-R3616 for 48 h resulted in similar amount of apoptosis (95 ± 4% and 89 ± 4% of cells, respectively; P = 0.981). Exposure of PA-1/E6-10 and control PA-1/neo to 1 MOI of HSV-R3616 for 36 h also resulted in a similar amount of apoptosis (52 ± 3% and 49 ± 5% of cells, respectively; P = 0.862). These results indicate that p53 is not required for apoptosis induced by the ICP34.5-deleted HSV-1 and that chemotherapy resistance did not alter the mechanism of HSV-induced cell death. It is likely that the differences observed among the different cell lines are related to cell line-specific factors, as described previously for wild-type HSV-1 (32).

**DISCUSSION**

HSV-1 strains engineered through deletions or insertional mutations of the ICP34.5 region have been shown previously to exert a strong oncolytic effect on experimental tumors of the CNS, both of neuronal origin (4, 7, 8, 15–17, 21) as well as metastatic melanoma (16). Promising preclinical data resulted in the initiation of two Phase I clinical trials of stereotactic intra-tumoral injection of recombinant HSV-1 for the treatment of intracerebral malignant gliomas (62). The potential clinical applications of ICP34.5-deficient HSV-1 strains, however, may extend to extra-CNS malignancies (29), as a continuously expanding list of tumors are proving sensitive to HSV oncolysis, including mesothelioma (20), malignant melanoma (18), metastatic colon carcinoma (6, 24), head and neck squamous cancer (25), breast cancer (23), lung cancer (19), and prostate cancer (26). We reported recently that ovarian cancer is highly sensitive to ICP34.5- HSV-1 mutant 1716 (28), and that HSV-G207 displays tumor selectivity against EOC cells with significantly lower toxicity against peritoneal mesothelium (30). In the present study, we provide evidence that HSV-based oncolytic therapy is effective against chemotherapy-resistant EOC. This was demonstrated both on primary cultures from patients who displayed different responses to platinum/paclitaxel therapy and on established cell lines displaying cross-resistance to various chemotherapy agents, including platinum. Because chemotherapy resistance represents a major cause of treatment failure in ovarian cancer and other solid tumors, the present results provide potentially critical information for the clinical application of recombinant HSV-1.

Although multiple molecular mechanisms may account for the development of chemotherapy resistance in ovarian cancer (42–45), defective apoptosis is emerging as an additional important mechanism. Extensive studies have indicated that cisplatin, Adriamycin, etoposide, and other chemotherapy agents trigger p53-mediated apoptosis (63). In ovarian cancer cells, exposure to cisplatin (49, 58–60) or paclitaxel (59, 64) results in up-regulation of p53 and some of its transcriptional targets. Importantly, ovarian cancer cells developing resistance to cisplatin (50, 57, 65) or other commonly used chemotherapeutic agents (65, 66) in vitro display loss of p53 or its transcriptional targets such as p21WAF1/CIP1 or bax. Furthermore, introduction of a wild-type p53 transgene restores chemosensitivity (50). Although more controversial (64, 67), loss of p53 has also been reported to decrease the sensitivity of cancer cells to Taxol (52). Similar associations between loss of p53 and loss of chemo-
sitivity to most of the commonly used chemotherapy drugs have been made in many other solid tumors (68–71). Major investigational efforts are presently being made to identify antitumor agents for the treatment of chemotherapy-resistant, p53-deficient malignancies (72). Examples include p53-based gene therapy (73) and the use of an E1B-deleted/E1A-expressing adenovirus, Addl520, which has been renamed as ONYX-015 (74, 75). Our present data clearly indicate that HSV-1 lacking ICP34.5 is equally efficacious in chemothera-apy-resistant ovarian cancer cells as in chemotherapy-sensitive cells in vitro and kills ovarian cancer cells independently of their p53 status, similar to the results observed in colorectal cancer (76). These results are in agreement with our previous observation that HSV-1716, another ICP34.5 mutant, was efficacious in vivo both against chemotherapy-sensitive A2780 and chemotherapy-resistant SKOV3 EOC i.p. xenografts in the severe immunodeficient mouse model (28). Taken together, these studies are in agreement with the recent observation by Advani et al. (26) that HSV mutant R7020 was efficacious in a radiation- and chemotherapy-resistant human epidermoid carcinoma and a hormone-refractory prostate adenocarcinoma line. Recombinant HV mutants are therefore emerging as possibly powerful antitumor agents that may hold promise in chemotherapy-resistant solid tumors. In addition, HSV may also be used in an adjuvant setting, because it enhances the sensitivity to chemotherapy in a synergistic manner in several non-small cell lung cancer lines (19) and head and neck cancers (77).

The mechanisms mediating cell killing by HSV are a matter of intense investigation. Galvan and Roizman (32) first showed that wild-type HSV-1 may induce cell death by apoptosis or nonapoptotic death, depending on the cell type. The finding that apoptosis can be triggered by conditionally replicative HSV-1 mutants suggest that structural proteins of the viral capsid may be involved in initiating apoptosis in some instances (32, 78), but HSV replication is required in the induction of apoptosis in some cell lines (35). Although the pathway of HSV-induced apoptosis in normal activated peripheral T lymphocytes was independent of Fas (79), activation of caspase-3 may play a role in HSV-induced apoptosis in some cells. Galvan et al. (80, 81) reported that the HSV dl20 mutant, which lacks ICP4, induced apoptosis in human SK-N-SH cells and Hep-2 cells using caspase-3-independent and -dependent pathways, respectively. The Bcl-2 family plays a major in modulating apoptosis in all systems studied to date, and Bcl-2 proto-oncogene may also modulate HSV-induced apoptosis because its induction can prevent apoptosis induced by HSV-2 (82) and the HSV-1 mutant dl20 (80).

In the present study, we focused selectively on the mechanisms of cell killing pertinent to replication-restricted ICP34.5-deficient HSV-R3616, given its antitumor activity and its potential therapeutic applications. In particular, we were interested to assess whether oncolytic HSV-induced cancer cell apoptosis relies on the host cell apoptotic machinery. This is a critical question, given that chemothera-apy-resistant cancer cells often display deficient apoptotic pathways, including p53 and downstream genes. Recently, the cytotoxic activity of HSV-1 vectors against colorectal cancer cell lines was found to be unaffected by the p53 gene, because stable transfectants that expressed no p53, wild-type p53, mutant p53, or both wild-type p53 and mutant p53 were equally susceptible to HSV-1 vector cytotoxicity (76). ICP34.5 can inhibit apoptosis in some infected cells, and ICP34.5 mutants may induce apoptosis in some cells (14). Interestingly, cell lines were observed to undergo a variable degree of apoptosis, similar to the cell specific response of wild-type HSV (32). Some cells, like SKOV3, did not display any appreciable apoptosis, whereas others, such as PA-1 and A2780, displayed features of apoptosis by morphological analysis, cell cycle analysis, and in situ TUNEL assays (54, 55). At this time, it is unclear why some cells preferentially undergo apoptosis while others die of nonapoptotic death after infection by mutant HSV-1. This phenomenon suggests that endogenous factors promoting programmed cell death or cell survival likely modulate cell response to HSV. Despite the facts that PKR can affect p53 (37, 38) as well as the ICP34.5 target eIF2a, our results clearly indicate that apoptosis induced by recombinant HSV-R3616 does not require endogenous p53 of the host. Furthermore, chemotherapy resistance does not impact on the mechanism of cell death. The possibility that other proteins of the apoptotic pathway downstream of p53 such as caspase-3 and Bcl-2 family members may be involved in HSV-3616-induced programmed cell death is not excluded. Interestingly, SKOV3 cells were reported to overexpress Bcl-xL, another member of the Bcl-2 family of survival proteins (83). It is unlikely that the structural proteins of the viral capsid (32, 78) were the major trigger of cell death in our study because the kinetics of cell death correlated with the degree of viral replication, suggesting that ICP34.5 HSV-induced cell death was dependent on factors produced during viral replication. In addition, infection of nondividing normal fibroblasts (84) or peritoneal mesothelial cells with ICP34.5 mutants yielded poor viral replication and significantly reduced cell death (30) in comparison to cancer cells. Further studies will be necessary to elucidate these molecular mechanisms of wild-type and mutant HSV-induced cell death. However, from the therapeutic point of view, our data suggest that even cells that resisted apoptosis induced by HSV-1 were killed by nonapoptotic mechanisms. Furthermore, both chemosensitive and chemotherapy-resistant cell lines were equally sensitive to HSV oncolysis and used similar modes of cell death.

In conclusion, the present study demonstrates that HSV-R3616, a recombinant HSV-1 lacking ICP34.5, kills ovarian cancer cells regardless of their p53 status. The oncolysis occurred via nonapoptotic mechanisms or via p53-independent apoptosis, in a cell-specific manner. Ovarian cancer clones lacking p53 and displaying resistance to platinum displayed the same rate of apoptosis and the same sensitivity to HSV-R3616 as their chemosensitive parental lines possessing wild-type p53. These results suggest that recombinant oncolytic HSV-1 lacking ICP34.5 may be an emerging powerful oncolytic agent, which may be used in the adjuvant treatment of chemotherapy-resistant solid tumors, including those displaying alterations of p53-dependent pathways.

REFERENCES


HSV-based Oncolytic Therapy for Ovarian Cancer


Oncolytic Herpes Simplex Virus-1 Lacking ICP34.5 Induces p53-independent Death and Is Efficacious against Chemotherapy-resistant Ovarian Cancer

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