Inhibition of DNA Repair by a Herpes Simplex Virus Vector Enhances the Radiosensitivity of Human Glioblastoma Cells

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Abstract

Expression of the herpes simplex virus (HSV) protein, ICP0, from the viral genome, rendered two radioresistant human glioblastoma multiforme cell lines more sensitive to the effects of ionizing radiation. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and clonogenic survival assays, U87-MG and T98 cell survival was greatly decreased as a function of ionizing radiation dose when ICP0 was expressed in cells compared with when ICP0 was not expressed. Consistent with previous results, we found that the catalytic subunit of DNA-dependent protein kinase was degraded as a function of ICP0 in both cell types. This most likely resulted in the inhibition of DNA repair as inferred by degraded as a function of ICP0 in both cell types. This most likely resulted in the inhibition of DNA repair as inferred by the persistence of γH2AX foci or DNA double-strand breaks. Enhanced apoptosis was also found to occur following irradiation of U87-MG cells preinfected with the ICP0-producing HSV-1 mutant, d106. Our results suggest that expression of ICP0 in human glioblastoma multiforme cells inhibits the repair of DNA double-strand breaks after ionizing radiation treatment, decreasing the survival of these cells in part by induction of apoptosis.

Introduction

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. Despite the use of conventional therapeutic modalities, such as surgery, chemotherapy, and ionizing radiation treatment, the prognosis in patients is poor. Radiation therapy remains the sole agent that increases the survival of patients with GBM but provides modest benefit. Both adult primary (de novo) and secondary GBMs are remarkably resistant to ionizing radiation treatment. The limited efficacy of radiation treatment is believed to arise from the poor apoptotic response to ionizing radiation by the tumor cells. New therapeutic strategies need to be developed for improved long-term management of these tumors. Enhancement of the effects of ionizing radiation, the primary adjuvant treatment for GBM, may increase patient survival and quality of life.

DNA double-strand breaks are known to occur with ionizing radiation exposure of cells. One repaired double-strand break can be sufficient to kill a cell if it inactivates an essential gene or triggers apoptosis. The repair of double-strand breaks is done by two pathways in mammalian cells, nonhomologous end joining, and homologous recombination. The serine/threonine kinase, DNA-dependent protein kinase (DNA-PK), is an important component of nonhomologous end joining, consisting of a large catalytic subunit (DNA-PKcs) and a Ku heterodimer (Ku70 and Ku80 subunits). In response to ionizing radiation, Ku-dependent phosphorylation of DNA-PKcs is required for the repair of double-strand breaks by nonhomologous end joining. DNA-PKcs contributes to the repair of double-strand breaks by assembling the broken ends of DNA molecules and serves as a molecular scaffold for recruiting DNA repair factors to DNA double-strand breaks.

Herpes simplex virus (HSV) is a large, neurotropic DNA virus that affects many aspects of host cell metabolism during infection. The HSV-1 immediate-early protein, ICP0, has been found to induce the degradation of DNA-PKcs by the ubiquitin-dependent proteasome degradation pathway. The extent of DNA-PKcs degradation is cell type specific and it has been shown that HSV replication is increased in cells lacking DNA-PKcs. Therefore, HSV infection may affect nonhomologous end joining.

ICP0 is a promiscuous transactivator shown to enhance the expression of genes introduced into cells by infection or transfection. ICP0 is critical for viral replication in cultured cells infected at low multiplicity, but is not essential in cells infected at high multiplicity. Recently, ICP0 has been shown to be an E3 ubiquitin ligase and has been proposed to promote lytic infections by destabilizing cellular proteins that inhibit the lytic viral life cycle. ICP0 is required for both lytic HSV viral infection and efficient reactivation from latency in vitro and in vivo. In addition to DNA-PKcs degradation, ICP0 has been shown to induce the degradation of proteins associated with nuclear domain 10 bodies in a RING finger–dependent manner. Nuclear domain 10 bodies are discrete nuclear foci where HSV-1 genomes may localize early during infection. Recent studies have suggested that these foci are sites of DNA double-strand break repair and inhibition of HSV genome circularization by ICP0.

Replication-defective mutant HSV-1 viruses were used to study the effect of ICP0 on DNA repair after ionizing radiation in human GBM cells. The mutant virus, d106, is defective in the expression of all of the immediate-early viral genes except that which encodes ICP0. The mutant virus d106 is an isogenic mutant of d106 and does not express any of the immediate-early viral genes necessary for HSV-1 genome expression. Both of these mutant viruses were used to infect human GBM cells before irradiation to determine the effect of ICP0 on cell survival, proliferation, DNA-PKcs protein levels, DNA double-strand break repair, and apoptosis.

Materials and Methods

Cells and viruses. The human GBM cell lines T98 and U87-MG were obtained from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in DMEM supplemented with sodium pyruvate, nonessential amino acids, antibiotics, and 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). The HSV-1 immediate-early mutant viruses, d106...
and d109, are derived from the wild-type strain KOS and have been previously described (21). Each mutant was grown and assayed for infectivity [plaque-forming unit (pfu)/mL] in the appropriate complementing cell lines (F06 and E11) as previously described (21). Ads.I.E4(ICP0), an adenovirus vector that expresses ICP0, and Ads.S11D, the empty adenoviral vector, were provided by Douglas Brough (GenVec, Gaithersburg, MD) and have been described (22).

*Herpes simplex virus-1 adenovirus infection, ionizing radiation, and cell survival/proliferation.* Tumor cells were seeded in triplicate at 2 × 10^5 cells/well (0.1 mL) in 96-well flat-bottomed plates and incubated overnight at 37°C. Confluent monolayers of T98 and U87-MG cells were infected with the HSV-1 viruses, d106 or d109, at a multiplicity of infection (MOI) of 10 for 24 hours before irradiation. U87-MG cells were infected with 10^6 particles per cell with the adenoviruses Ads.S11E4(ICP0) or Ads.S11D. Ionizing radiation treatment was delivered at room temperature in a 137Cs irradiator (Gammacell40, Atomic Energy of Canada Limited, Mississauga, Ontario, Canada) at a dose rate of 0.87 Gy/minute. Cells were subsequently returned to the incubator.

To assess cell survival and proliferation after viral infection and ionizing radiation treatment, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (ATCC; ref. 23) was done at 0, 2, 4, 6, and 8 days after cell irradiation. MTT reagent was added (0.02 mL) to each well and cells were placed in the incubator for 1 hour. A solubilizing cell detergent (ATCC) (0.1 mL) was added to the cells and the formazan reaction product was measured with a microtiter plate reader (Dynatek, Alexandria, VA). Absorbance values are presented as the mean of three wells per treatment ± SD.

Clonogenic survival assays were done on U87-MG cells after d106 infection and ionizing radiation treatment. Exponentially growing cells were seeded at a density of 10^5 cells/well in six-well flat-bottomed plates overnight. Cells were either mock-infected or infected with d106 at a MOI of 10 pfu/cell for 24 hours. Cells were irradiated at 10 Gy and placed in the incubator overnight. Trypsinized cells were serially diluted and plated in triplicate on six-well plates. Cells were left to grow colonies for 2 weeks with routine changing of serum-containing medium twice a week. Cells were fixed and stained with a crystal violet/formalin solution and colonies were counted. Cell survival was estimated from the number of colonies (defined as a cluster of >50 cells) formed and expressed as a fraction of the number of cells seeded multiplied by the plating efficiency (PE). The PE was determined by the number of colonies formed in the mock infected group of cells that did not receive ionizing radiation and expressed as a fraction of the number of cells seeded (PE = 0.9).

**Western blot analysis of catalytic subunit of DNA-dependent protein kinase and cleaved caspase-3.** For Western blot analysis of DNA-PKcs, U87-MG and T98 cells were infected with either d109 or d106 and cell lysates were harvested at 6 and 24 hours postinfection. T98 and U87-MG cells were mock- or d106-infected for 6 hours and then irradiated (0 or 10 Gy). DNA-PKcs (mouse monoclonal at a dilution of 1:1000 in 5% nonfat dry milk/TBS-Tween solution; Cell Signaling Technology, Beverly, MA) was probed on nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) with a peroxidase-conjugated goat anti-mouse/rabbit antibody and detection of proteins by enhanced chemiluminescence (Amersham Biosciences).

**Apoptosis.** A carboxyfluorescein polycaspase detection kit (APO LOGIX, Cell Technology, Inc., Mountain View, CA) was used to detect active caspases in cells undergoing apoptosis (25). U87-MG cells were seeded in triplicate at a density of 10^5 cells/well in six-well flat-bottomed plates overnight and were mock- or d106-infected for 24 hours and then irradiated. U87-MG cells were irradiated with 0, 5, 10, and 20 Gy. Active caspases, 1, 2, 3, 6, 8, 9, and 10, were detected with a carboxyfluorescein (FAM)-labeled peptide fluoromethyl ketone caspase inhibitor 24 hours after irradiation. Quantitation of cells with active caspases undergoing apoptosis was done with single color flow cytometry (Beckman-Coulter Epics XL, Fullerton, CA) after cell fixation (Fixative Solution, Cell Technology). Data acquisition and analysis was done with the EXPO32 ADC software (Beckman-Coulter).

Staurosporine (A.G. Scientific, San Diego, CA), a potent apoptosis-inducing agent, was used as a positive control for apoptosis in U87-MG cells for Western analysis of cleaved caspase-3 levels. Staurosporine was dissolved in anhydrous DMSO and diluted to a concentration of 0.01 μM/L in medium before treatment of U87-MG cells for 2 hours.

**Indirect immunofluorescence for γH2AX and ICP0.** Confluent monolayers of U87-MG and T98 cells were grown on circular 18 mm coverslips. Cells were mock- or d106-infected (MOI 10) for 6 hours and then irradiated (8 Gy). At 2, 6, and 24 hours after ionizing radiation treatment, cells were fixed and permeabilized in cold methanol (−20°C) for 5 minutes. Cells were then treated with deionized water for 20 minutes and then 10% PBS for 15 minutes. Cells were washed in 1% bovine serum albumin thrice for 10 minutes each. Anti-γH2AX (rabbit polyclonal at a dilution of 1:200 in PBS; Upstate Biotechnology) and anti-ICP0 (mouse monoclonal at a dilution of 1:500 in PBS; Goodwin Institute for Cancer Research) antibodies were added and cells were incubated at room temperature for 1 hour. Cells were washed five times for 10 minutes each before incubating in the dark with FITC-labeled secondary antibodies at a dilution of 1:500 in PBS. Cells were washed again in PBS six times 10 minutes each in the dark and coverslips were mounted with an antifade solution (Gelvatol, Simon C. Watkins Laboratory, University of Pittsburgh). Slides were examined on a Nikon Diaphot 300 photomicroscope (Melville, NY). Images were captured by a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) and imported into the SPOT version 3.5.9 for MacOCS image analysis software package (Diagnostic Instruments) running on a Macintosh G3 computer (Apple, Cupertino, CA). Final image analysis was done with Adobe Photoshop. For each treatment condition, γH2AX foci were determined in at least 50 cells.

**Statistical analysis.** Cell proliferation and viability data represent the average of three independent absorbance values on day 6 of the MTT assay. Statistical proliferation and viability differences were assessed using an unpaired two-sample Student's t test assuming equal variance. A probability value <0.05 was considered significant.

**Results**

Effect of ICP0 and ionizing radiation on human glioblastoma multiforme cell survival and proliferation: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. To determine the effect of ICP0 on human GBM cell survival and proliferation after ionizing radiation treatment, U87-MG and T98 cells were infected at a MOI of 10 pfu/cell with two replication-defective HSV-1 viruses, d106 or d109, 24 hours before single-dose irradiation (0, 5, 10, or 20 Gy). The MTT assay was done at 0, 2, 4, and 6 days after cell irradiation (Fig. 1). Both cell lines used are radioresistant and the U87-MG cell line contains wild-type p53, whereas T98 contains mutant p53 protein. Both cell lines, when irradiated but not infected with virus, showed a dose-dependent decrease in cell survival and proliferation compared with cells that received no ionizing radiation (P < 0.005). The U87-MG cell line seems more radioresistant than the T98 cell line in this study (Fig. 1). Infection of U87-MG and T98 cells by the ICP0-producing virus, d106, followed by ionizing radiation treatment, resulted in a dose-dependent decrease in cell proliferation and survival after 6 days compared with cells receiving only ionizing radiation (P < 0.005; Fig. 1). The greatest decrease in cell survival and...
proliferation occurred at the highest ionizing radiation dose of 20 Gy ($P < 0.005$). The largest decline in cell survival occurred between 2 and 4 days after ionizing radiation treatment of U87-MG cells and infection with d106. T98 cells showed a decline in cell survival immediately after ionizing radiation. Infection of both cell lines by the immediate-early–deficient virus, d109, did not result in a dose-dependent decrease in cell proliferation and survival after ionizing radiation treatment. Cell proliferation occurred in both cell lines after d109 infection in the absence of irradiation consistent with the documented lack of toxicity upon d109 infection (21). Infection of both cell lines with d106 resulted in cell toxicity as proliferation and cell viability decreased soon after infection ($P < 0.05$; ref. 26). Cell toxicity with d106 infection was greatest in the T98 cell line (Fig. 1B).

**Adenovirus expression of ICP0.** To confirm the effect of ICP0 on cell survival and proliferation in GBM cells after irradiation, U87-MG cells were infected with 104 particles per cell with two replication-defective adenoviruses, AdS.11E4(ICP0) or AdS.11D, 24 hours before a single-dose of ionizing radiation (20 Gy). The MTT assay was done at 0, 2, 4, and 6 days after irradiation.

**Figure 1.** Effect of HSV mutant viruses and escalating doses of ionizing radiation on U87-MG and T98 cell proliferation and survival using the MTT assay. U87-MG or T98 cells were mock infected or infected with HSV mutant viruses, d106 or d109, at 10 pfu/cell. The MTT assay was done 0, 2, 4, 6 days after irradiation. A, effect of 5, 10, and 20 Gy ionizing radiation exposure 24 hours postinfection with mock-, d106-, and d109-infected U87-MG cells. B, effect of 5, 10, and 20 Gy ionizing radiation exposure 24 hours postinfection with mock-, d106-, and d109-infected T98 cells. (○, mock ionizing radiation; •, d109 ionizing radiation; ■, d106 ionizing radiation; ○, mock; ⊙, d109; □, d106).
AdS.11E4(ICP0) is an E1 E3 E4+ adenovirus vector with ICP0 expression controlled from the endogenous adenoviral E4 promoter. From this construct, 1,000-fold less ICP0 is expressed relative to the level of expression from d106 (22). AdS.11D is an empty E1 E3 E4+ adenovirus vector that does not express ICP0. U87-MG cells infected with the ICP0-producing adenovirus, AdS.11E4(ICP0), showed a decrease in cell survival and proliferation after irradiation compared with cells that were only irradiated (P < 0.005; Fig. 2).

**Effect of ICP0 and ionizing radiation on human glioblastoma multiforme cell survival: clonogenic survival assay.** Clonogenic survival assays were done to confirm the results of the MTT assay with d106 infection of U87-MG cells and subsequent irradiation. Cells were serially diluted and plated after d106 infection and 10 Gy of ionizing radiation treatment. After 2 weeks of growth, cell survival was estimated from the number of colonies formed and expressed as a fraction of colonies resulting from uninfected, unirradiated cells (Fig. 3). The survival fraction was lowest among cells that were infected by d106 and irradiated (0.001). U87-MG cells that only received ionizing radiation had an impaired ability to form colonies with a low survival fraction (0.01). Cells that did not receive ionizing radiation but were infected with d106 had a higher survival fraction (0.2). These results support the data from the MTT assay showing an enhanced decrease in GBM cell survival with ICP0 production and subsequent irradiation.

**Apoptosis as a mode of glioblastoma multiforme cell death with ICP0 production and ionizing radiation.** To determine whether apoptosis is a mode of GBM cell death with ICP0 production and ionizing radiation, Western blot analysis was done on U87-MG cells after d106 infection and irradiation (10 Gy). The blot was probed with an antibody specific to cleaved caspase-3. Caspase-3, a key executor of apoptosis, is activated by cleavage into two subunits (p17 and p12; ref. 27). Levels of cleaved caspase-3 (p17) were detected in U87-MG cells that were infected by d106 and irradiated (Fig. 4A). Lower levels of cleaved caspase-3 were found in cells that were d106 infected but did not undergo irradiation. Both groups of cells that were mock infected and did or did not receive ionizing radiation had minimal detectable levels of cleaved caspase-3.

Carboxyfluorescein polycaspase detection was done with flow cytometry to quantify the number of U87-MG cells undergoing apoptosis after d106 infection and ionizing radiation treatment (Fig. 4B). Flow cytometry was done 24 hours after cell irradiation. Minimal apoptosis was detected in mock-infected cells that did or did not undergo irradiation, confirming the results of our Western analysis of cleaved caspase-3 levels (Fig. 4A). U87-MG cells that were d106 infected and received subsequent ionizing radiation treatment underwent apoptosis in greater numbers than d106-infected cells that were not irradiated. The percentage of d106-infected cells undergoing apoptosis were very similar 24 hours after irradiation at 5, 10, or 20 Gy (Fig. 4B).

Our results confirm apoptosis as a mode of cell death with cell ICP0 production and irradiation. At 24 hours after irradiation, a large portion of U87-MG cells infected with d106 underwent apoptosis. Other modes of cell death, such as necrosis or autophagy, may also occur (5). Our results support other studies showing irradiation alone causes a poor apoptotic response in GBM cells. Most importantly, these results show enhanced GBM cell apoptosis with d106 infection and subsequent irradiation.

**ICP0 degradation of catalytic subunit of DNA-dependent protein kinase in human glioblastoma multiforme cells.** To determine whether degradation of DNA-PKcs by ICP0 occurs in human GBM cells, Western blot analysis was done on U87-MG and T98 cells after d106 or d109 infection. The blot was probed with anti-DNA-PKcs and degradation was found to occur between 6 and 24 hours postinfection with d106 in U87-MG cells (Fig. 5). Degradation of DNA-PKcs in T98 cells occurred at 6 hours postinfection with d106 and complete degradation was found at 24 hours postinfection (Fig. 5). No degradation of DNA-PKcs occurred with d109 infection of the U87-MG or T98 cell lines.

**Persistent DNA double-strand breaks with ICP0 production and ionizing radiation in human glioblastoma multiforme cells.** To determine whether DNA double-strand break repair is inhibited by ICP0, indirect immunofluorescence was done on U87-MG and T98 cells infected with d106 for 6 hours, irradiated, and incubated for 2, 6, and 24 hours (0 or 8 Gy; Fig. 6). Cells were labeled with antibodies to ICP0 and γH2AX, the phosphorylated form of a histone H2A variant (H2AX) found at sites of DNA double-strand breaks.
double-strand breaks (28, 29). Phosphorylation of H2AX seems to play a critical role in the recruitment of repair or damage-signaling factors to sites of DNA damage in the nucleus (30). The repair of DNA double-strand breaks after ionizing radiation correlates with the loss of γ-H2AX foci (31). Prior reports have shown the loss of γ-H2AX foci in cells within 6 hours after irradiation (31).

U87-MG and T98 cells that were mock infected and did not receive ionizing radiation had undetectable γ-H2AX foci or DNA double-strand break (Fig. 6). Mock-infected cells, which underwent ionizing radiation treatment, had detectable γ-H2AX foci at 2 hours postirradiation but reduced foci at 6 and 24 hours. The loss of γ-H2AX foci at 6 and 24 hours postirradiation indicates the repair of DNA double-strand breaks. Both T98 and U87-MG cells infected with d106, which did not receive ionizing radiation, had detectable γ-H2AX foci at 6 and 24 hours postirradiation. These results suggest linear HSV-1 genomes present after infection may be treated as DNA double-strand break with formation of γ-H2AX foci (19, 32). Abundant γ-H2AX foci, or DNA double-strand breaks, were found in d106-infected cells at 2, 6, and 24 hours after ionizing radiation treatment (Fig. 6). Persistent γ-H2AX foci at 24 hours suggests ICP0 inhibits DNA repair.

Discussion

Both primary and secondary GBMs are very resistant to the effects of ionizing radiation. GBM cells have a poor to absent apoptotic response to ionizing radiation (3–5). Glioma cells show an absence of either significant induction of bax or repression of bcl-2 and bcl-XI after irradiation (33, 34). Strategies reported to enhance apoptosis after irradiation of malignant glioma cells include exogenous transfer of p53, APAF-1, and caspase-9 (35–37). Radiation-induced apoptosis in most cell types other than glial cells has been shown to depend on the presence of wild-type p53 (38). The presence or absence of wild-type p53 has not been shown to have a significant impact on the radiosensitivity of GBM cells matrix. Radiotherapy, an effective treatment for GBM patients, is able to induce apoptosis in GBM cells only in an effort to decrease toxicity of HSV to the central nervous system and provide a condition for viral replication only in human GBMs. A number of different genetically engineered viruses have been constructed with deletions or mutations in one or more HSV genes in an effort to decrease toxicity of HSV to the central nervous system and provide a condition for viral replication only in models of malignant glioma.
replicating cells that can provide cellular homologues (44, 45). Other HSV mutants have been constructed that are defective for immediate-early gene expression, resulting in replication-incompetent viruses. Various genes have been inserted in these modified viruses for expression in infected cells (46, 47). In our study, we found the HSV-1 virus alone can enhance the radiosensitivity of GBM.

Advani et al. (48) have shown that the conditionally replicative HSV-1 mutant, R3616, has greater oncolytic effects and increased replication when exposed to ionizing radiation. In their study, human U87-MG xenografts in mice underwent significantly greater reduction in tumor volume or total regression when tumors were inoculated with the R3616 mutant and irradiated. Increased spread of the virus was seen with in situ hybridization with DNA probes to the virus. Other studies have confirmed the enhanced tumoricidal effect of HSV when combined with ionizing radiation (49, 50).

We believe targeting the repair of DNA double-strand breaks in malignant glioma cells may be an important method to enhance the tumoricidal effect of ionizing radiation. Inhibition of DNA repair may also play a role in the chemotherapeutic treatment of GBM. Use of an HSV-1 vector, which solely produces ICP0, may form the basis of future gene therapy strategies against human GBM.

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Figure 6. Indirect immunofluorescence for γH2AX and ICP0 in U87-MG and T98 cells. Cells were mock or d106 infected for 6 hours and then irradiated (0 or 8 Gy). At 2, 6, and 24 hours after ionizing radiation, cells were permanently fixed and labeled with anti-γH2AX (rabbit polyclonal) and anti-ICP0 (mouse monoclonal). FITC-labeled secondary antibodies (red for γH2AX and green for ICP0) were then added. The γH2AX foci, or DNA double-strand breaks, are more numerous and persistent with d106 infection and ionizing radiation treatment of cells at 24 hours after irradiation. A, U87-MG cells. B, T98 cells.

References


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