The Adenoviral E4orf6 Protein Induces Atypical Apoptosis in Response to DNA Damage*

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Adenoviral proteins interact with host-cell proteins to either exploit or inhibit cellular functions for the purpose of viral propagation. E4orf6, the 34-kDa gene product of the E4 gene, interacts with the double-strand break repair (DSBR) protein DNA-dependent protein kinase and cooperates with binding partner E1B-55K to degrade MRE11, preventing viral DNA concatemer formation. We previously demonstrated that E4orf6 radiosensitizes human tumor cells through the inhibition of DSBR, notably in the absence of E1B-55K. Here, we report that E4orf6 prolongs the signaling of DNA damage by inhibiting the activity of protein phosphate 2A (PP2A), the phosphatase responsible for dephosphorylating γH2AX. The inhibition of PP2A occurs without significant disruption of the DNA re-ligation rate. Prolonged signaling of DNA damage in the presence of E4orf6 initiates caspase-dependent and independent cell death. This is accompanied by poly(ADP-ribose) polymerase (PARP) hyperactivation and the translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus. Knockdown of AIF by shRNA rescues the radiosensitization induced by E4orf6. Taken together, these data suggest that E4orf6 disrupts cellular DSBR signaling by inhibiting PP2A, leading to prolonged H2AX phosphorylation, hyperactivation of PARP, and AIF translocation to the nucleus. The function of E4orf6 as an inhibitor of PP2A and activator of PARP in the absence of other adenoviral gene products is of importance in delineating the adenovirus-host cell interplay.

Adenovirus has evolved ways to commandeer host cell machinery for successful entry, viral DNA replication, and propagation of progeny virions. For example, the adenovirus E1A gene product binds Rb to release E2F and promote entry into S phase resulting in efficient viral DNA replication. The 34-kDa gene product of the E4 gene, E4orf6, cooperates with the 55-kDa gene product of the E1B gene to degrade p53, preventing an early cellular apoptotic response to infection (1, 2). E4orf6 and E1B-55K inhibit both adenoviral DNA concatemer formation, which has been viewed as a cellular defense to viral replication imposed by cellular DNA repair mechanisms (3), and V(DJ) recombination, a cellular mechanism employed for the generation of antigenic diversity within the immunoglobulin genes. Both concatemer formation and V(DJ) recombination depend on the non-homologous end-joining pathway (4–8) of DNA double-strand break repair (DSBR), and require proteins such as DNA-dependent protein-kinase (DNA-PK), DNA ligase IV, and MRE11. These same DSBR proteins are also required for the repair of DNA double-strand breaks (DSBs) following exposure to radiation. The primary mechanism of cytotoxicity from ionizing radiation (IR) is the induction of DNA damage and DSBs, which are known to be the most lethal of DNA lesions (9–12). In addition, an increased capacity of DSBR has been identified as contributing significantly to the radiosensitivity of gliomas (13). Due to these observations, the inhibition of DNA DSBR is an attractive method for radiosensitization; therefore, we recognized the ability of E4orf6 to interfere with cellular DSBR as a great opportunity to exploit E4orf6 for the purpose of radiosensitizing radioresistant tumor cells.

We have previously demonstrated that E4orf6, independent of E1B-55K and therefore independent of p53 and MRE11 degradation, is capable of significantly radiosensitizing tumor cells by inhibiting cellular DSBR in response to radiation-induced DSBs (14). In E4orf6-expressing cells, we found prolonged levels of H2AX phosphorylation at Ser-139 (γH2AX) and DNA-PK autophosphorylation at Thr-2609 at 360 min post-irradiation, a time when DNA repair should be complete and the repair proteins should be dephosphorylated. However, it was unclear as to whether E4orf6 inhibited the physical re-ligation of the dsDNA breaks or inhibited a step downstream of re-ligation and how this DNA damage signal was translated into increased radiosensitivity. Therefore, we sought to determine the reason for prolonged signaling of damage, as well as the cell death pathway responsible for the radiosensitization. Here, we report our findings on the atypical mechanism of cell death induced by E4orf6 in the presence of DNA damage. In irradiated E4orf6-expressing cells, poly(ADP-ribose) polymerase (PARP) becomes hyperactivated and apoptosis-inducing factor (AIF) translocates from the mitochondria to the nucleus, inducing cell death. The ability to radiosensitize tumor cells

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‡ The abbreviations used are: DSBR, double-strand break repair; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; IR, ionizing radiation; γH2AX, Ser-139-phosphorylated histone 2AX; PARP, poly(ADP-ribose) polymerase; GBM, glioblastoma multiforme; PFGE, pulsed-field gel electrophoresis; PP2A, protein phosphatase 2A; PAR, poly(ADP-ribose); AIF, apoptosis-inducing factor; STS, staurosporine; Gy, gray.
through expression of a single gene makes E4orf6 a promising genetic tool to be used in conjunction with standard radiation therapy in the treatment of radioresistant tumors, such as glioblastoma multiforme (GBM).

EXPERIMENTAL PROCEDURES

Cell Lines, Irradiations, Infections, and Reagents—U251 cells (American Type Culture Collection, Manassas, VA) were incubated at 37 °C with 5% CO2 and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin. All irradiations were carried out with a 137Cs irradiator at a dose rate of 4.0 Gy (gray)/min. All chemicals were purchased from Sigma unless otherwise noted. Infections were performed as previously described (14).

Pulsed-field Gel Electrophoresis—PFGE was performed using the CHEF-DR II system (Bio-Rad). U251 cells, infected with either null or E4orf6 virus, were irradiated at 40 Gy and harvested by trypsinization. Cells (1.5 × 10^6) were resuspended in phosphate-buffered saline, mixed with an equal volume of 2.0% agarose, and embedded in plugs. Solidified plugs were immersed in lysis buffer (100 mM EDTA, pH 8.0, 10 mM Tris-Cl, pH 8.0, 1% w/v N-lauroylsarcosine sodium salt) with freshly added 100 μg/ml proteinase K and incubated at 55 °C overnight. Lysed plugs were rinsed with storage buffer (10 mM Tris/borate, 1 mM EDTA, pH 8.0, 1% w/v l-arginine) and stored in storage buffer at 4 °C. For electrophoresis, plugs were cut into thirds and loaded into the wells of a 1.0% agarose gel (in 0.5 TBE) and imaged and quantified on a Typhoon 9210 variable mode imager (GE Healthcare). All values were normalized to their respective samples at 40 Gy without repair time (total induced damage) and shown as fold decrease in damage over time.

Protein Phosphatase 2A (PP2A) Activity Assay—PP2A activity was measured according to manufacturer’s protocol (catalog 17-313, Upstate Biotechnology, Lake Placid, NY). Approximately 3 × 10^6 cells were directly lysed by sonication in imidazole buffer containing complete mixture mini (Roche Applied Science), pepstatin, aprotinin, leupeptin and phenylmethylsulfonyl fluoride. Each sample (500 μg) was subjected to immunoprecipitation with PP2A antibody (catalog 05-421). As outlined in the protocol, beads containing precipitated PP2A were added to a phosphatase reaction with threonine phosphopeptide in a shaking incubator. Samples were then aliquoted into three wells of a 96-well plate, into which malachite green detection solution was added. Plates were incubated for 15 min at room temperature and then read at 650 nm on an automated plate reader (Molecular Devices, Sunnyvale, CA).

Immunoblotting—Immunoblotting was performed as described previously (14). Membranes were incubated with primary antibodies in TBS-Tween with 1% milk at the following dilutions: PP2A at 1:3000, cleaved caspase-3 at 1:1000 overnight at 4 °C (Cell Signaling Technology, Danvers, MA), PAR at 1:2000 (Trevigen, Gaithersburg, MD), AIF at 1:3000 (Santa Cruz Biotechnology, Santa Cruz, CA), lamin a/c at 1:3000 (BD Biosciences), and β-actin at 1:50,000. All membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology), and immunoreactive bands were detected using ECL Plus chemiluminescence (Amersham Biosciences). Optical density of reactive bands was measured by image analysis software (Scion Image; Scion Corp., Frederick, MD) as described previously (15).

AIF Knockdown—Non-selectable pKD-NegCon-v1 (negative control shRNA) and pKD-AIF-v3 (AIF shRNA) plasmids were purchased from Upstate Biotechnology. The plasmids were digested with NotI and EcoRI restriction enzymes (Promega, Madison, WI) for the shRNA to be subcloned into pSilencer 4.1-CMV puro vector (Ambion, Austin, TX) to obtain the puro-resistance gene while maintaining the original H1 RNAP III promoter from the Upstate vectors. The reengineered plasmids were used in transfections with U251 cells and Lipo- fectamine PLUS reagent (Invitrogen). At 48 h post-transfection the U251 cells were treated with 3 μg/ml puromycin for 48 h, a time point when mock transfected cells were dead. The surviving NegCon and AIF cells were then plated at limited dilution for harvesting of individual clones. Selected cells were maintained in 0.5 μg/ml puromycin, and the addition of 100 μM non-essential amino acids and 55 μM 2-mercaptoethanol aided in their growth.

Clonogenic Survival Assay—To analyze clonogenicity in infected and irradiated cells, a modified clonogenic assay was used, due to the sensitivity of the infected cells to the combination of irradiation and replating. U251 clones transfected with negative control or AIF-targeting shRNA were plated in 35-mm plates in triplicate at equal density, infected the next day at an MOI predetermined to yield maximal infectivity, and irradiated 48 h later with a 137Cs source (dose rate of 4.0 Gy/min) at a range of IR doses. When unirradiated control plates were near confluence (or at near day 7 post-IR), the cells were fixed and stained with crystal violet as previously described (15). Crystal violet was solubilized in 33% acetic acid and the absorbance at 540 nm was measured in triplicate for each well as described by Bernardi et al. (16).

RESULTS

E4orf6 Does Not Inhibit the Re-ligation of DSBs—We previously demonstrated that E4orf6 radiosensitizes human tumor cells by inhibiting DSBR as measured by prolonged γH2AX and Thr-2609-phosphorylated DNA-PK levels and by sublethal damage repair assay in U251 and RKO tumor cells, respectively (14). Because detection of DSBs (H2AX phosphorylation) and DSBR complex formation (DNA-PK autophosphorylation) were not inhibited by E4orf6, we hypothesized that E4orf6 was interfering with the late stages of repair resulting in the prolonged signaling of damage at times when complete repair would result in dephosphorylation of H2AX and DNA-PK. However, it remained possible that the physical re-ligation of DSBs is inhibited by E4orf6, perhaps through interference with DNA Ligase IV, the enzyme responsible for ligating breaks during NHEJ. To determine whether E4orf6 inhibits re-ligation of the DSBs, we employed the PFGE method to quantify the DNA DSBs over time based on the ability of damaged DNA to
migrate through an agarose gel. U251 cells were infected with null (control) or E4orf6-expressing adenoviral vectors (previously optimized (14)). The infected cells were irradiated at 48 h post-infection and harvested at varying times post-irradiation. As measured by PFGE, U251 cells expressing E4orf6 repaired DSBs at a rate similar to those infected with control virus (Fig. 1). There was a trend of E4orf6 attenuating repair at early times post-irradiation; however, this trend was not significant and levels of damage for E4orf6 virus- and null virus-infected U251 cells were overlapping at 360 min post-irradiation (data not shown).


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E4orf6 inhibits the activity of PP2A—Based on the similar rates of re-ligation in null virus- and E4orf6 virus-infected U251 cells, we hypothesized that E4orf6 must inhibit a post-ligation step involving γH2AX dephosphorylation. It was recently demonstrated that PP2A is responsible for the dephosphorylation of γH2AX following repair of radiation-induced DSBs (17). Therefore, we sought to determine whether the activity of PP2A was altered by E4orf6, resulting in the prolonged phosphorylation of H2AX, by performing in vitro phosphatase reactions with lysates from infected U251 cells. Whereas radiation treatment led to an increase in PP2A activity in null virus-infected U251 cells, PP2A activity in response to IR was significantly inhibited in cells expressing E4orf6 (Fig. 2). The well known inhibitor of PP2A okadaic acid completely inhibited PP2A activity, and no PP2A activity was recovered by a negative control IgG antibody. These data are consistent with the prolonged H2AX phosphorylation in the presence of E4orf6 (14) and suggest that the inhibition of PP2A activity contributed to the increased sensitivity of E4orf6-expressing cells to IR.

Induction of PARP Activation and PARP-induced Cell Death in Irradiated E4orf6-expressing U251 Cells—Despite the almost complete re-ligation of DSBs, E4orf6 significantly sensitizes cells to die in response to IR. Thus, we hypothesized that the prolonged signaling of damage initiates a cell death program in E4orf6-expressing cells. Time-lapse microscopy suggested that a significant portion of infected cells undergo apoptotic death, observed by the formation of membrane blebs and spikes in the days following irradiation (data not shown). Representative phase microscopy images shown in Fig. 3A demonstrate similar amounts of rounded cells between null virus- and E4orf6 virus-infected U251 populations 48 h following IR. We decided to further characterize apoptosis in these U251 cells. Since caspase-3 is a terminal caspase activated by various apoptotic stimuli, we analyzed caspase-3 cleavage in response to infection and irradiation. The level of cleaved caspase-3 beginning at 48 h post-irradiation were similar to that induced by treatment with staurosporine but did not differ significantly between null virus-infected and E4orf6 virus-infected U251 cells (Fig. 3B); therefore, caspase-dependent cell death is unlikely to be responsible for the significant radiosensitization induced by E4orf6. One well characterized caspase-independent mechanism of DNA damage-induced cell death is via PARP hyperactivation. PARP is activated by nicked DNA and responds by covalently modifying signaling and repair proteins with the addition of poly(ADP-ribose) (PAR) in polymers of varying length. PARP is necessary for cell survival under conditions of mild DNA damage (18, 19); however, in the presence of excessive DNA damage (or unrepaired DNA damage) hyperactivation of PARP leads to induction of a caspase-independent cell death program (20). To determine the PARP response in null...
virus-infected and E4orf6 virus-infected cells, we analyzed the presence of PAR-modified proteins with an antibody recognizing PAR polymers. We observed PAR modified proteins under control conditions and an induction in newly ribosylated proteins within 30 min of radiation treatment. In null virus-infected U251 cells this increase in ribosylation returned to baseline levels within 2 h post-IR (black arrows, Fig. 4A), a time when the majority of dsDNA breaks have been repaired (Fig. 1). However, in irradiated E4orf6-expressing cells, there was a significant and continual increase in PAR-modified proteins (black asterisks, Fig. 4A). Similar results were obtained in three independent experiments. These results mirror the effects of E4orf6 on prolonged H2AX phosphorylation and DNA-PK autophosphorylation following IR and suggest that this difference in the PARP-dependent cellular response to IR between null virus-infected and E4orf6 virus-infected cells could be responsible for the radiosensitization by E4orf6. To determine whether the PARP activation plays a role in cell death induction in the presence of E4orf6, we decided to analyze AIF, a key effector molecule downstream of PARP hyperactivation.

AIF is a mitochondrial resident protein and has been identified as a downstream modulator of PARP activity (21). PARP hyperactivation in the presence of massive DNA damage leads to disruption of the mitochondrial membrane potential, resulting in the release of AIF from the outer mitochondrial membrane (22). Once released from the mitochondria, AIF translocates to the nucleus and aids in events characteristic of apoptosis, including chromatin condensation, high molecular weight DNA fragmentation requiring endonuclease G, and phosphatidylserine exposure (23). We measured AIF translocation to the nucleus by subcellular fractionation of nuclei and immunoblotting. The nuclear levels of AIF were elevated in E4orf6-expressing U251 cells, compared with null virus-infected cells in response to IR (Fig. 4B). Staurosporine (STS), a chemical inducer of apoptosis by both AIF-dependent and caspase-3 dependent means, also induced substantial AIF nuclear translocation. Therefore, the higher levels of AIF released from the mitochondria in response to IR in the E4orf6-expressing cells could mediate all, or part of, the observed radiosensitization induced by E4orf6.

**E4orf6-induced Radiosensitization Is AIF-dependent**—To determine whether radiosensitization by E4orf6 is dependent upon AIF translocation, we established U251 clones stably expressing shRNA against AIF or a negative control shRNA that does not target any known proteins. Fig. 5A shows the level of AIF knockdown in a mixed population and an individual clone, as well as the normal levels of AIF in control shRNA-expressing cells. AIF shRNA induced a decrease in AIF protein of ~83% in clone AIF.1 compared with the control clone. When the individual clones were analyzed for clonogenic survival following IR, E4orf6 radiosensitized the control shRNA clone to a similar degree as untransfected U251 cells. However, the cells expressing AIF shRNA were significantly more radioresistant (Fig. 5B). This indicates that AIF-dependent cell death significantly contributes to the increase in radiation-induced cell death in the presence of E4orf6. However, AIF knockdown cells...
We have previously identified E4orf6 as a potent radiosensitizer of glioblastoma cells in vitro (14). Here, we further define the action of E4orf6 in the presence of IR and report an atypical mechanism of cell death by which E4orf6 induces tumor cell radiosensitization. We demonstrate that E4orf6 has the ability to inhibit DSBR by prolonging signaling from DSBs, despite the physical repair of DNA damage. These data suggest that E4orf6 does not significantly inhibit the recruitment or activity of DNA Ligase IV. Because our previous data showed that E4orf6 inhibits the dephosphorylation of DSBR complex proteins H2AX and DNA-PK, we conclude that E4orf6 interferes with the very late stages of repair, after re-ligation of the break but before complex dissociation and dephosphorylation. These late, post-ligation events of repair have been established to be as important as physical re-ligation in conferring radiosensitivity. The repair complex proteins must undergo a conformational change and dissociate to signal complete repair (25, 26). This finding highlights the sensitive nature with which cells react to prolonged signaling of damage, despite the physical repair of damage. In the case of E4orf6, the DSBs resulting from IR are repaired at a similar rate to control cells, but PP2A activity is compromised by E4orf6 rendering γH2AX unable to be dephosphorylated, and therefore, the cells respond as if significant levels of unrepaired damage remain by inducing cell death.

We also report that PARP-induced cell death is a consequence of the prolonged DSBR signaling. To our knowledge, this is the first demonstration that an adenoviral protein elicits PARP activation in response to cellular stress, such as DNA damage. As a well defined downstream mediator of PARP activation, AIF translocation contributes a significant amount of cell death in E4orf6-expressing cells exposed to radiation. The rescue of radiosensitization in AIF knockdown cells was incomplete (93%). Although not significant, this trend could be attributed to the incomplete AIF knockdown or the possibility of an AIF-independent pathway contributing to the cell death induced by E4orf6. AIF-independent caspase-3 cleavage is a likely component responsible for this alternate pathway. Caspase-3 cleavage induced by IR is decreased but still present in AIF knockdown cells, suggesting that caspase-3 cleavage is not a significant contributor to radiation-induced cell death in the absence of E4orf6.

**DISCUSSION**

Tumor therapy regimens of the most aggressive cancers, such as GBM have become multi-modality by including surgery, chemotherapy, and radiation therapy. Clinically, it is possible to deliver localized and very large doses of IR by stereotactic gamma knife radiosurgery; however, a proportion of GBM tumor cells will survive these high doses of IR and continue to proliferate. There is a need for novel therapeutics, and it is ideal to explore radiosensitizers, as the survival time for patients with GBMs and other radioresistant tumors increases significantly from the addition of therapeutics that potentiate the radiation treatments (24).
from the large scale addition of PAR polymers to cellular proteins (27) leads to a collapse of the mitochondrial membrane potential and AIF release. We attempted to determine whether addition of pyruvate or NAD$^+$, having been shown to restore cellular energy pools (28), could inhibit AIF translocation in E4orf6-expressing and irradiated cells. However, these attempts were not successful. This is likely due to the fact that in the studies in which pyruvate and NAD$^+$ were used, the stress was acute, while in our experimental system, E4orf6 causes a prolonged, modest level of sustained PARP hyperactivation. Therefore, a discrepancy exists between the DSB re-ligation rate to the one log of sensitization at 8 Gy achieved by E4orf6 (14); however, it is the cells expressing E4orf6 and prolonged $\gamma$H2AX signaling that propagate the DNA damage response resulting in a significant increase in death, in contrast to control, repair-proficient cells that proliferate without amplifying DNA damage. Another explanation for a delay in the onset of death is the possibility that the E4orf6-expressing cells require more than 360 min for the PARP activation to translate into a death signal, such as from the depletion of cellular energy. This is supported by published data confirming the role of PARP in DNA repair and survival at low doses of IR but inducing cell death at higher levels of damage (18–20) and our data revealing the translocation of AIF to the nucleus at 48 h post-radiation. This is consistent with reports suggesting the almost immediate onset of death following AIF translocation. We therefore propose a model by which aberrant $\gamma$H2AX signaling is sufficient to induce PARP hyperactivation and eventual AIF translocation to the nucleus, resulting in cell death in the presence of E4orf6 (Fig. 6). Collectively, our results point to an atypical apoptotic response as a significant contributor to E4orf6-induced radiosensitization.

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