Combining PARP-1 Inhibition and Radiation in Ewing Sarcoma Results in Lethal DNA Damage

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Abstract

Ewing sarcomas (ES) harbor a chromosomal translocation that fuses the EWS gene to an ETS transcription factor, most commonly Friend leukemia integration 1 (FLI1). The EWS-FLI1 fusion protein acts in a positive feedback loop to maintain the expression of PARP-1, which is involved in repair of DNA damage. Here, we examine the effects of PARP-1 inhibition and radiation therapy on Ewing sarcomas. In proliferation assays, the Ewing sarcoma cell lines RD-ES and SK-N-MC were much more sensitive than non-Ewing sarcoma cell lines to the PARP-1 inhibitor olaparib (Ola; IC50 0.5–1 μmol/L vs. >5 μmol/L) and to radiation (IC50 2–4 Gy vs. >6 Gy). PARP-1 inhibition with short hairpin RNA (shRNA) or Ola sensitized Ewing sarcoma cells, but not non-Ewing sarcoma cells, to radiation therapy in both proliferation and colony formation assays. Using the Comet assay, radiation of Ewing sarcoma cells with Ola, compared to without Ola, resulted in more DNA damage at 1 hour (mean tail moment 36–54 vs. 26–28) and sustained DNA damage at 24 hours (24–29 vs. 6–8). This DNA damage led to a 2.9- to 4.0-fold increase in apoptosis and a 1.6- to 2.4-fold increase in cell death. The effect of PARP-1 inhibition and radiation therapy on Ewing sarcoma cells was lost when EWS-FLI1 was silenced by shRNA. A small dose of radiation therapy (4 Gy), when combined with PARP-1 inhibition, stopped the growth of SK-N-MC flank tumors xenografts. In conclusion, PARP-1 inhibition in Ewing sarcomas amplifies the level and duration of DNA damage caused by radiation therapy, leading to synergistic increases in apoptosis and cell death in a EWS-FLI1–dependent manner. Mol Cancer Ther; 12(11); 2591–600. ©2013 AACR.

Introduction

Ewing sarcoma (ES) was initially described by Dr. James Ewing as a tumor that was sensitive to radiation therapy (1). The Ewing sarcoma family of tumors (ESFT), including Ewing sarcoma and primitive neuroectodermal tumors, are a group of malignant bone and soft tissue tumors generally occurring in children and young adults (2). Nearly all ESFT are defined by a characteristic chromosomal translocation, which fuses the central exons of the Ewing sarcoma breakpoint (EWS) region 1 gene to the central exons of one of the five ETS family genes, with the most frequent fusion occurring with Friend leukemia integration 1 (FLI1; ref. 3). The cell of origin for ESFT has not yet been clearly defined, but recent evidence suggests that it may be the mesenchymal stem cell (4). Aberrant transcription from the EWS/ETS fusion gene products induces transformation through induction or repression of target genes involved in controlling cell growth, signal transduction, and differentiation (5).

It has been known since 1990 that Ewing sarcomas have high levels of PARP-1 mediated via increases in PARP-1 transcription (6). However, there has been significant evidence recently in the fundamental role of PARP-1 in Ewing sarcoma. Garnett and colleagues in a systematic examination of the efficacy of 130 drugs in more than 600 cell lines found that Ewing sarcoma cell lines had marked sensitivity to PARP-1 inhibitors (7). Brenner and colleagues further showed that the EWS-FLI1 and EWS-ERG fusion genes in Ewing sarcoma cells induce DNA damage, and that this DNA damage is potentiated by PARP-1 inhibition (8). Interestingly, the product of the EWS-FLI1 fusion gene acts in a positive feedback loop to maintain expression of PARP-1, and PARP-1 is required for EWS-FLI1–mediated transcription.

PARP-1 was the first identified member of the PARP superfamily, which currently has 18 members, and is a multifunctional enzyme involved in DNA repair and other cellular functions (9). In the presence of DNA damage, PARP-1 detects DNA strand breaks, becomes activated, and synthesizes poly(ADP) ribose (PAR) on...
acceptor proteins involved in DNA repair including PARP-1 itself. PARP-1 is a member of the base excision repair (BER) pathway and modulates repair of single-strand breaks (SSB). In the presence of PARP-1 inhibition, SSBs accumulate and are converted to double-strand breaks (DSB) during replication (10). PARP-1 may also be involved in the repair of DSBs via homologous recombination and nonhomologous end joining.

Given that radiation kills cancer cells primarily through DNA damage, it is not surprising that PARP-1 inhibitors are radiosensitizers in a variety of cell lines with enhancement ratios of up to 1.7 (11). Nontoxic doses of PARP-1 inhibitors have been shown to increase the efficacy of radiation in several xenograft tumor models. However, there are currently no published studies examining the combination of radiation and PARP-1 inhibitors in Ewing sarcomas. Surgical resection of certain Ewing sarcomas is not always possible or at times possible only with severe morbidity. Thus we sought to determine whether we could control these tumors by combining genetic or pharmacologic inhibition of PARP-1 with low to moderate radiation doses. We find that PARP-1 inhibition amplifies the quantity and duration of DNA damage caused by radiation therapy, increases apoptosis and cell death synergistically with radiation therapy, and reduces the dose of radiation therapy required to block tumor growth.

Materials and Methods

Cell lines and reagents

Two Ewing sarcoma cell lines, RD-ES and SK-N-MC, and two non-Ewing sarcoma cell lines, HT1080 and SK-LMS-1, were obtained from the America Type Culture Collection (ATCC). Both these Ewing sarcoma cell lines harbor t(11;22), leading to expression of the EWS-FLI1 fusion protein. RD-ES and SK-N-MC cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL of streptomycin. HT1080 and SK-LMS-1 cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with the same supplements. Cancer cell lines were actively passaged for less than 6 months from the time that they were received from ATCC, and United Kingdom Coordinating Committee on Cancer Research guidelines were followed (12). A673 Ewing sarcoma cells with inducible short hairpin RNA (shRNA) for EWS-FLI1 (shA673-1C) was generously provided by Dr. Olivier Delattre (Institut Curie, Paris, France; ref. 13). These cells were cultured on TPP flasks (TPP Techno Plastic Products AG) in DMEM glutamax with 10% FBS, 1% penicillin/streptomycin, 200 μg/mL Zeocin and 20 μg/mL Blasticidin (Invitrogen). Expression of EWS-FLI1 in shA673-1C cells was inhibited with doxycycline (1 μg/mL). Olaparib (Supplementary Fig. S1) was obtained from MedKoo Biosciences, Inc.

Western blot analysis

Samples were collected in radioimmunoprecipitation assay buffer (Sigma) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics), and protein concentration was determined by bovine serum albumin assay (Bio-Rad). Western blot analysis was conducted using the following antibodies: anti–PARP-1 and anti-EGFR were purchased from Santa Cruz Biotechnology, anti-PAR from BD Transduction Laboratories, anti-pATM, anti-pDNA-PKcs, and anti-cleaved caspase-3 from Cell Signaling Technology, anti-γH2AX from Millipore, and anti-β-actin from Sigma.

Irradiation of cells

Cells were plated in tissue culture dishes or 96-well plates and incubated at 37°C in humidified 5% CO2 in culture medium until 70% to 80% confluent. Cells were then exposed to a 137Cs γ-ray source (Atomic Energy of Canada Ltd.) at the specified doses.

shRNA

RD-ES and SK-N-MC cells were transduced with lentiviral vectors carrying an inducible shRNA to PARP-1 (SC-29437V, Santa Cruz Biotechnology). Scrambled control shRNA (SC-108080, Santa Cruz Biotechnology) was used as a control for comparison. Cells with stable integration of shRNA were obtained by adding puromycin (2 μg/mL) to culture media overnight. A colorimetric MTT assay was used to assess cell number by optical density after 3 days. Colonies were fixed with a mixture of 75% methanol and 20% acetic acid, and stained with 0.4% Trypan blue. The number of colonies consisting of 50 or more cells was scored. Data reflect the mean of nine samples. The IC50 represents the concentration of drug or radiation that was required for 50% inhibition.

Cancer cell proliferation and colony formation assays

To assay for cancer cell proliferation, 1 × 104 cells were plated onto 96-well flat bottom plates and maintained in media overnight. A colorimetric MTT assay was used to assess cell number by optical density after 3 days as previously described (14). Data reflect the mean of six samples. To assay of colony-forming ability, 200 cells were plated onto 60 mm culture dishes and incubated for 7 to 14 days. Colonies were fixed with a mixture of 75% methanol and 25% acetic acid, and stained with 0.4% Trypan blue. The number of colonies consisting of 50 or more cells was scored. Data reflect the mean of nine samples. The IC50 represents the concentration of drug or radiation that was required for 50% inhibition.

Comet assay

The single cell gel electrophoresis assay, also known as the Comet assay, was carried out using Comet Assay Kit (Trevigen Inc.) following manufacturer’s instructions. Olive tail moment values (equal to the product of the tail length and the fraction of total DNA in the tail) were calculated from at least 50 cells for each group using Komet 5.5 software (Andor Technology).

Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections were deparaffinized by xylene and rehydrated. Immunohistochemistry was conducted with Vectastain Elite ABC kit (Vector Laboratories Inc.) following the manufacturer’s protocol. For antigen retrieval, the sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for 10 minutes. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.3% H2O2 in absolute
methanol for 15 minutes at room temperature. The sections were then incubated overnight at 4°C with primary antibody and washed with PBS containing 0.05% Triton X-100. Incubation with corresponding secondary antibody and the peroxidase–antiperoxidase complex was carried out for 30 minutes at room temperature. Immunoreactive sites were visualized by 3,3’-diaminobenzidine. Afterward, the slices were counterstained by hematoxylin. Antibodies used were anti-γH2AX (Millipore), anti-cleaved caspase-3 (Cell Signaling Technology), anti-TUNEL (ApopTag Peroxidase kit), and anti-PCNA (Santa Cruz Biotechnology).

For the TUNEL/DAPI immunofluorescence, sections were deparaffinized, rehydrated, and applied with Dead-End Fluorometric TUNEL system (Promega) and VECTASHIELD with 4’, 6-diamidino-2-phenylindole (DAPI) following the manufacturer’s protocol.

Analysis of the number of positive cells was conducted using 8 to 10 fields. Random, nonoverlapping ×400 magnification fields from each tumor were examined. The positive-stained cells were counted in each field and normalized for total cell number of each field.

Flow-cytometric analysis for cell death

Cells were cultured and harvested at the indicated times, stained with propidium iodide (1 μg/mL) according to the manufacturer’s protocol, and then analyzed using a FACScan flow cytometer (BD Biosciences).

Animal studies

All mouse protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (Boston, MA). To generate subcutaneous flank tumor, 5 × 10⁶ cells were resuspended in 100 μL of Hank’s balanced salt solution and injected subcutaneously into the right flank of athymic, nude, 6- to 8-week-old male BALB/c nu/nu mice following isoflurane anesthesia. Mice were assigned into treatment groups (6 mice per group) when tumors reached 50 mm³ in volume, designated as day 0. Olaparib (MedKoo Biosciences, Inc.) 50 mg/kg was delivered on day 0. Mice were anesthetized using ketamine (125 mg/kg) and xylazine (10 mg/kg), placed in shielded device to expose only the flank tumor, and irradiated using a Gammacell 40 Exactor Irradiator (Best Theratronics). Tumors were measured three times per week for 2 weeks, and tumor volume (TV) was calculated by using the following formula: TV = length × (width)² × 0.52. After mice were sacrificed, tumors were excised and cut into thirds. Portions of each tumor was fixed in 10% buffered formalin for 24 hours, embedded in paraffin, and processed into 5 μmol/L sections.

Statistical analysis

Data represented as mean ± SD. One-way ANOVA followed by Bonferroni’s posthoc comparison tests were conducted in all statistical analyses using GraphPad Prism 5.0 software.

Results

In the presence of DNA damage, PARP-1 detects DNA strand breaks, becomes activated, and synthesizes PAR on acceptor proteins (9). We found significantly higher endogenous protein levels of PARP-1 and PAR in two Ewing sarcoma cell lines, RD-ES and SK-N-MC, compared with two non-Ewing sarcoma cell lines, HT1080 and SK-LMS-1 (Fig. 1A). Following DNA damage with 6 Gy of radiation, levels of PAR were only mildly increased at 1 and 24 hours in non-Ewing sarcoma cell lines but significantly increased in Ewing sarcoma cell lines (Fig. 1B). Ewing sarcomas are generally thought to be more sensitive to radiation than other sarcoma subtypes (1). In an in vitro proliferation assay, we confirmed that Ewing sarcoma cell lines were more sensitive to radiation than non-Ewing sarcoma cell lines following radiation doses between 2 and 8 Gy (Fig 1C). The IC₅₀ for Ewing sarcoma cell lines was 2 to 4 Gy, whereas the IC₅₀ for non-Ewing sarcoma cell lines was 6 to 8 Gy. Sensitivity of Ewing sarcoma cell lines to low doses of radiation was even more pronounced in a colony formation assay, especially for SK-N-MC cells (Fig. 1D). Ewing sarcoma cells were identified in a large screening program as sensitive to the PARP inhibitor olaparib (7), and thus we evaluated the toxicity of olaparib, a PARP-1 inhibitor, and confirmed Ewings sarcoma cell lines to be much more sensitive to the olaparib than non-Ewing sarcoma cell lines (Fig. 1E). The IC₅₀ for RD-ES and SK-N-MC cells was 0.5 to 1.0 μmol/L, whereas the IC₅₀ for HT1080 and SK-LMS-1 cell was more than 5 μmol/L. We then examined the combination of radiation and PARP-1 inhibition with olaparib in our cell lines. This bimodality therapy resulted in an increased inhibition in RD-ES and SK-N-MC cells, whereas HT1080 and SK-LMS-1 cells were insensitive to this combination (Fig. 1F).

To confirm that the effects of olaparib were due to inhibition of PARP-1 and not off-target effects, we knocked down expression of PARP-1 in our two Ewing sarcoma cell lines using shRNA. Knockdown of PARP-1 by shRNA was not conducted in the non-Ewing sarcoma cell lines because baseline levels of PARP-1 in these cell lines were already low or undetectable (Fig. 1A). Efficient PARP-1 knockdown in RD-ES and SK-N-MC cells was confirmed by Western blot analysis (Fig. 2A). When the proliferation of RD-ES and SK-N-MC cells with shRNA silencing of PARP-1 was compared with that of control cells transduced with scrambled shRNA, there was a dramatic reduction in proliferation, which was somewhat greater in magnitude than following treatment with olaparib (500 nmol/L, Fig. 2B). In addition, the same increased effect seen with radiation (4 Gy) and olaparib was seen with radiation and PARP-1 shRNA. We also examined the effect of radiation and PARP-1 inhibition on clonogenic survival. In RD-ES and SK-N-MC cells, inhibition of PARP-1 with olaparib...
(50–100 nmol/L) or PARP-1 shRNA potentiated the ability of radiation in blocking colony formation (Fig. 2C). In HT1080 and SK-LMS-1 cells, PARP-1 inhibition with olaparib had no effect on the ability of radiation to block colony formation even at a dose of 1,000 nmol/L (Fig. 2D).

We next examined the effects of PARP-1 inhibition on radiation-induced DNA damage in our sarcoma cell lines. DNA strand breaks were measured using the Comet assay. In all four cell lines, 6 Gy of radiation led to significant DNA strand breaks at 1 hour (mean tail moment ≥20) and near complete resolution of DNA strand breaks by 24 hours (mean tail moment <8; Fig. 3A). The degree of DNA strand breaks at 1 hour and the resolution of DNA strand breaks at 24 hours were unaffected by the addition of olaparib (500 nmol/L) in HT1080 and SK-LMS-1 cells. However, in RD-ES and SK-N-MC cells, the addition of olaparib led to increased DNA strand breaks at 1 hour (mean tail moment 36–54) and persistent DNA strand breaks at 24 hours (mean tail moment 25–29). After 48 hours, mean tail moments in Ewing sarcoma cells in the presence of olaparib declined to 15 to 17 (data not shown). Given γH2AX is elevated in response to DNA DSBs (15), we measured γH2AX levels in RD-ES and SK-N-MC cells and found elevation in γH2AX expression in both Ewing sarcoma cell lines 1 hour following radiation exposure (Fig. 3B). The addition of olaparib (500 nmol/L) or PARP-1 shRNA led to higher levels of γH2AX following radiation, with a greater effect seen with olaparib than PARP-1 shRNA. Activation of PARP-1 leads to

Figure 1. A, Western blot analysis of endogenous PARP-1 and PAR in Ewing’s sarcoma (RD-ES and SK-N-MC) and non-Ewing sarcoma (HT1080 and SK-LMS-1) cell lines. Actin blot served as loading control. B, Western blot analysis of PAR in Ewing and non-Ewing sarcoma cell lines at 0, 1, and 24 hours after 6 Gy of radiation (IR). C, proliferation assay of Ewing and non-Ewing sarcoma cell lines at 72 hours following treatment with different doses of radiation. D, colony formation assay in cell lines treated with varying doses of radiation. E, proliferation assay of cell lines at 72 hours following treatment with olaparib. F, proliferation at 72 hours following a combination of radiation and olaparib. Bars, SD. DMSO, dimethyl sulfoxide.
a number of DNA damage responses including activation of ataxia telangiectasia mutated (ATM), which is involved in DSB repair by homologous recombination and activation of DNA-protein kinase catalytic subunit (DNA-PKcs), which is involved in DSB repair by nonhomologous end-joining (9). Following radiation, we found increases in ATM phosphorylation in RD-ES cells and of SK-N-MC cells (Fig. 3B). Both RD-ES and SK-N-MC cells also showed significant phosphorylation of DNA-PKcs following radiation.

Cellular responses to DNA damage following radiation include the initiation of apoptosis within hours or cell death through abnormal chromosomal segregation during mitosis (16). We examined apoptosis in our Ewing sarcoma cell lines following radiation combined with PARP-1 inhibition. Of note, 6 Gy of radiation led to mild elevations in cleaved caspase-3 in Ewing sarcoma cell lines, which were further elevated following inhibition of PARP-1 with either olaparib or PARP-1 shRNA (Fig. 4A and Supplementary Fig. S2). Olaparib (500 nmol/L) had no effect on cleaved caspase-3 levels following irradiation of non-Ewing sarcoma cell lines. Apoptosis was also examined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) immunofluorescence, and both olaparib (500 nmol/L) and PARP-1 shRNA increased TUNEL reactivity following radiation in Ewing sarcoma cells, whereas olaparib had no effect on TUNEL reactivity following radiation in non-Ewing sarcoma cells (Fig. 4B). We next measured cell death in our cell lines using propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis. Following 72 hours of treatment, olaparib (500 nmol/L) alone caused cell death in 19% of RD-ES, 24% of SK-N-MC cells, and less than 8% of cell of HT1080 and SKLMS-1 cells (Fig. 4C). Of note, 6 Gy of radiation to cell death in 19% to 29% of Ewing sarcoma cells and 13% to 16% of non-Ewing sarcoma cells. The combination of olaparib (500 nmol/L) and radiation led to a synergistic increase in cell death in Ewing sarcoma cell lines, with 46% of RD-ES and 49% of SK-N-MC cells dying from this combination, whereas cell death in non-Ewing sarcoma cells increased only to 16% to 22%.

The effects of radiation and PARP-1 inhibition with olaparib were next examined in a xenograft mouse model using SK-N-MC cells. SK-N-MC tumors treated with vehicle alone grew rapidly over a 14-day period (Fig. 5A). Radiation (4 Gy) alone or olaparib (50 mg/kg daily) alone slowed tumor growth, but the combination of radiation and olaparib essentially stopped tumor growth beyond 9 days of treatment. After 14 days of treatment, the size of tumors treated with radiation and olaparib was only 18% that of control tumors and 30% to...
40% that of tumors treated with olaparib or radiation alone (Fig. 5A). The combination of olaparib and radiation led to increases in levels of γH2AX, cleaved caspase-3, and TUNEL staining (Fig. 5B). Olaparib alone or radiation alone had no effect on proliferation as measured by proliferating cell nuclear antigen (PCNA), but the combination of olaparib and radiation led to significantly decreased proliferation. We also examined and repeated this experiment using EWS-FLI1-negative HT1080 xenografts (Supplementary Fig. S3). Radiation had a modest effect in inhibiting HT1080 xenograft growth. Olaparib had no effect on tumor growth, and the addition of olaparib to radiation did not provide any additive effect over radiation alone.

Brenner and colleagues found that the EWS-FLI1 fusion protein induces DNA damage and drives PARP-1 transcription (8). PARP-1 inhibition in EWS-FLI1-driven Ewing cells potentiates DNA damage and, in combination with chemotherapy, synergistically inhibits Ewing sarcoma cell growth. We thus examined PARP-1 inhibition and radiation using shA673-1C cells, which are A673 Ewing sarcoma cells that have inducible expression of EWS-FLI1 shRNA following the administration of doxycycline (13). We confirmed that in the presence of doxycycline, expression of the EWS-FLI1 protein is dramatically diminished (Fig. 6A). We examined proliferation and clonogenic survival of shA673-1C cells in response to olaparib and found decreased proliferation with increasing doses of olaparib, but this effect was lost in the presence of doxycycline and knockdown of EWS-FLI1 (Fig. 6B). We also examined the effect of combining olaparib (500 nmol/L) and radiation (4 Gy) on the proliferation and clonogenic survival of shA673-1C cells. In the absence of doxycycline, the combination of olaparib and radiation had a synergistic effect in decreasing shA673-1C cell proliferation (Fig. 6C). After decreasing EWS-FLI1 expression with doxycycline, the effect of combining olaparib and radiation on cell proliferation was dramatically reduced. Similar results were obtained when examining clonogenic survival (Fig. 6D and Supplementary Fig. S4). Of note, the silencing of EWS-FLI alone in shA673-1C cells reduced proliferation by 24% and colony formation by 27% (data not shown). Levels of DNA damage, as measured by γH2AX levels, in response to radiation and olaparib, were maximized in the presence of EWS-FLI1 (Fig. 6E).
Discussion

In the present study, we found that Ewing sarcomas, in contrast with non-Ewing sarcomas, are uniquely sensitive to the DNA-damaging effects of ionizing radiation and to PARP-1 inhibition. The combination of radiation and PARP-1 inhibition in Ewing sarcoma cells led to sustained DNA damage, apoptosis, and cell death. In a xenograft flank tumor model, olaparib combined with only 4 Gy of radiation synergistically blocked tumor growth. Using RNA interference of EWS-FLI1 in an Ewing sarcoma cell line, we also found that expression of EWS-FLI1 is necessary for the synergistic effect of radiation and PARP-1 inhibition to occur. These findings may have significant implications for the treatment of patients with Ewing sarcoma with tumors that are unresectable or difficult to resect.

Nearly all Ewing sarcomas and ESFT have a characteristic chromosomal translocation, which fuses the EWS gene to an ETS family member such as FLI1 or ERG (2), and the transcriptional program initiated by EWS-ETS translocations results in a high sensitivity to PARP-1 inhibition. Garnett and colleagues in a screen of several hundred cancer cell lines with 130 drugs unexpectedly found marked sensitivity of Ewing sarcoma cell lines to PARP-1 inhibitors (7). Brenner and colleagues found that the product of another ETS gene fusion TMPRSS2-ERG, which is present in about half of prostate cancers, interacts with PARP-1 as well as the catalytic subunit of DNA-PKcs, and PARP-1 and DNA-PKcs activity are required for ETS gene-mediated transcription (17). Overexpression of the TMPRSS2-ERG gene product induces DNA damage, which is potentiated by PARP-1 inhibition. The same group also found that PARP-1 inhibition can potentiate the DNA-damaging effects of chemotherapy in EWS-FLI1 or EWS-ERG–driven Ewing sarcoma cells (8). In this study, we confirmed that Ewing sarcoma cell lines are uniquely sensitive to PARP-1 inhibition compared with sarcoma cell lines not harboring EWS-ETS translocations. We further showed that EWS-FLI1 is not only required for sensitivity to PARP-1 inhibition but also is required for the synergistic effect seen with the combination of PARP-1 inhibition and radiation.

Following exposure of cancer cell DNA to ionizing radiation, potential consequences include normal cell death. PARP-1, which is involved in DNA repair, can become activated by DNA damage and can inhibit the DNA repair process, leading to cell death. Olaparib, a PARP-1 inhibitor, can potentiate the DNA-damaging effects of radiation by inducing cell death in Ewing sarcoma cells.

Figure 4. A, Western blot analysis of cleaved caspase-3 in cell lines following treatment with 6 Gy of radiation (IR), olaparib (500 nmol/L), and/or PARP-1 shRNA (sh.PARP-1). Actin blots serve as loading control. B, photos of immunofluorescence staining for TUNEL-positive cells (top) and graph of TUNEL-positive cells (bottom) 24 hours after treatment with 6 Gy of radiation (IR) and dimethyl sulfoxide (DMSO), olaparib (500 nmol/L), or sh.PARP-1. Magnification, ×400. *, P < 0.05 compared with DMSO + IR. C, FACS analysis after propidium iodine staining for cell death in cell lines following treatment with 6 Gy of radiation (IR) or olaparib (500 nmol/L). Bars, SD. **, P < 0.05 compared with DMSO group; ***, P < 0.05 compared with DMSO, olaparib, and IR groups.
division, DNA damage-induced apoptosis, or mitotic-linked cell death (18). The various manifestations of DNA damage following radiation can occur rapidly or manifest after many cell divisions. Cells may respond to DNA damage by initiating apoptosis within hours of radiation injury, or DNA damage may lead to death through abnormal chromosomal segregation during mitosis. Mitotic-linked cell death may be more important in cancer cells than in normal tissues, as the p53 pathway is commonly mutated in solid tumors (16). Dysfunction of p53-related machinery prevents cells from initiating rapid apoptotic death in response to radiation, and predisposes to premature entry into M phase, before DNA damage is repaired (19). Acute cell death by this mechanism is delayed in comparison with apoptosis, with histological stigmata of mitotic catastrophe occurring 2 to 6 days after radiation (20). In this study, non-Ewing sarcoma cell lines, HT1080 and SK-LMS-1, were not reliant on PARP-1 to repair radiation-induced DNA damage, and most of the DNA damage resolved after 24 hours as measured by the Comet assay. The Ewing sarcoma cell lines, RD-ES and SK-N-MC, still had significant DNA damage 24 hours after radiation when PARP-1 was inhibited, and this DNA damage led to apoptosis and cell death. PARP-1 is most closely associated with the BER pathway (9), but the sensitivity of Ewing sarcomas to the combination of radiation and PARP-1 inhibition would suggest a defect in other mechanisms of DNA repair such that PARP-1 inhibition leads to unrepaired and lethal DNA damage. Genetic screens have identified a host of homologous recombination-related genes that when deleted cause cells to be highly sensitive to PARP-1 inhibition (21). We are currently examining alternative DNA repair pathways in Ewing sarcoma including homologous recombination and nonhomologous end joining.

Figure 5. A, growth of SK-N-MC flank tumors in athymic nude mice treated with 4 Gy of radiation, olaparib (50 mg/kg i.p. daily), or both. Six mice were used for each group. * P < 0.05 compared with control group; ** P < 0.05 compared with control, olaparib, and radiation groups. B, photos of SK-N-MC xenograft tumors stained for γH2AX, cleaved caspase-3, TUNEL, and PCNA. Quantification of the number of positive cells shown in graphs to right of photos (control group value normalized to 1.0). All graphs give relative number of positive cells compared with control except TUNEL graph, which gives absolute number of positive cells. Bars, SD. *, P < 0.05 compared with control group; ** P < 0.05 compared with control, olaparib, and radiation groups.
We found that PARP-1 silencing with shRNA was not as effective as PARP-1 inhibition with olaparib in enhancing the effects of radiation on DNA damage (as measured by levels of γH2AX) and on apoptosis (as measured by levels of cleaved caspase-3). We suspect that PARP-2 may partially compensate for PARP-1 activity when PARP-1 is silenced, while olaparib can inhibit both PARP-1 and PARP-2.

The clinical development of PARP-1 inhibitors have followed two primary strategies: targeting cancer cells that are predisposed to die when PARP-1 is inhibited and combining PARP inhibition with DNA-damaging agents (9). An example of the first strategy is the use of PARP-1 inhibitors in BRCA-mutated cancers where defects or inhibition of BRCA 1/2 or PARP-1 have little or no effect, but deletion of both genes is cytotoxic (a.k.a synthetic lethality; refs. 22;23). PARP-1 inhibition leads to conversion of single-strand DNA breaks to DSBs, and BRCA mutation leads to defects in homologous recombination and the ability to repair DSBs. An example of the second strategy is the combination of PARP-1 inhibitors with DNA-damaging chemotherapeutics or radiation (9). In this study, the combination of PARP-1 inhibition and radiation in Ewing sarcoma may take advantage of both of these strategies. Ewing sarcoma cells seem to be similar to BRCA-mutated cancers in their sensitivity to PARP-1 inhibition alone, and adding radiation takes further advantage of this inability to repair DNA damage.

The effect of olaparib and radiation in inhibiting SK-N-MC flank tumor growth in athymic nude mice was quite dramatic. We used radiation at higher doses (12–15 Gy) without olaparib and were unable to completely inhibit tumor growth (Supplementary Fig. S5), despite the marked sensitivity of SK-N-MC cells to radiation in vitro (Fig. 1C and D). However the addition of olaparib and only 4 Gy of radiation was enough to stop SK-N-MC tumor growth (Fig. 5A), and examination of treated tumors showed profound effects on DNA damage, apoptosis, and proliferation (Fig. 5B). It is quite possible that this combination therapy had effects on non-cancer cells within the tumor. The examination of effects of PARP-1 inhibition and radiation on the tumor microenvironment was beyond the scope of this study, but there may be effects of PARP-1 inhibition and radiation on stromal cells and endothelial cells. Other investigators have found that PARP-1 inhibition and radiation inhibits tumor vasculature (24).

To our knowledge, this study is the first examine the combination of PARP-1 inhibition and radiation in Ewing sarcoma. Local control of Ewing sarcoma is about 85% with surgery alone or radiation alone (25). However, these tumors can occur in difficult central...
locations such as the skull, spine, and pelvis where local control rates are reduced and surgery or high-dose radiation can be accompanied by severe morbidity. Thus the ability to eradicate these tumors with modest doses of radiation combined with targeted agents, which enhance radiation efficacy, may significantly benefit some patients. On the basis of this study, we are currently developing a clinical trial of radiation and PARP-1 inhibition in patients with ESFT with tumors that are either unresectable or difficult to resect without significant morbidity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.-J. Lee, S.S. Yoon

Development of methodology: H.-J. Lee, S.S. Yoon

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Grant Support

This work was supported by NIH grant R01 CA158301-01 (S.S. Yoon). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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