Exploiting Arginine Auxotrophy with Pegylated Arginine Deiminase (ADI-PEG20) to Sensitize Pancreatic Cancer to Radiotherapy via Metabolic Dysregulation

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Abstract

Distinct metabolic vulnerabilities of cancer cells compared with normal cells can potentially be exploited for therapeutic targeting. Deficiency of argininosuccinate synthetase-1 (ASS1) in pancreatic cancers creates auxotrophy for the semi-essential amino acid arginine. We explored the therapeutic potential of depleting exogenous arginine via pegylated arginine deiminase (ADI-PEG20) treatment as an adjunct to radiotherapy. We evaluated the efficacy of treatment of human pancreatic cancer cell lines and xenografts with ADI-PEG20 and radiation via clonogenic assays and tumor growth delay experiments. We also investigated potential mechanisms of action using reverse-phase protein array, Western blotting, and IHC and immunofluorescence staining. ADI-PEG20 potently radiosensitized ASS1-deficient pancreatic cancer cells (MiaPaCa-2, Panc-1, AsPc-1, HPAC, and CaPan-1), but not ASS1-expressing cell lines (Bxpc3, L3.6pl, and SW1990). Reverse phase protein array studies confirmed increased expression of proteins related to endoplasmic reticulum (ER) stress and apoptosis, which were confirmed by Western blot analysis. Inhibition of ER stress signaling with 4-phenylbutyrate abrogated the expression of ER stress proteins and reversed radiosensitization by ADI-PEG20.Independent in vivo studies in two xenograft models confirmed significant tumor growth delays, which were associated with enhanced expression of ER stress proteins and apoptosis markers and reduced expression of proliferation and angiogenesis markers. ADI-PEG20 augmented the effects of radiation by triggering the ER stress pathway, leading to apoptosis in pancreatic tumor cells.

Introduction

Pancreatic cancer is the second most common gastrointestinal cancer in the United States, with an estimated incidence of 53,670 cases and 43,090 deaths in 2017 (1). Survival outcomes are poor, with estimated 5-year overall survival rates of less than 5% (2). Studies on the tumor biology of pancreatic cancer have given insights into the metabolic perturbations that occur in cancer cells. Briefly, some cancer cells undergo metabolic reprogramming to overcome the extreme energy requirements required for their rapid proliferation. This reprogramming may manifest as a preference for anaerobic glycolysis (Warburg effect) or alterations in fatty acid oxidation and amino acid metabolism, which are distinct from those in normal cells.

Argininosuccinate synthetase-1 (ASS1) is a pivotal enzyme in arginine biosynthesis that catalyzes the synthesis of argininosuccinate from citrulline and aspartate, and argininosuccinate is then metabolized to arginine. ASS1 expression is low or deficient in a wide variety of tumors, including pancreatic cancer, rendering these cells dependent on exogenous arginine for growth (auxotrophy; refs. 3–5). Downregulation of ASS1 is thought to confer a biological advantage to tumor cells, in that any available arginine is preferentially diverted for nucleotide synthesis to support faster growth and for nitric oxide synthesis to enhance angiogenesis and support tumor survival (6). Conversely, depriving arginine-dependent tumor cells of arginine can induce tumor cell death; consequently, arginine deprivation therapy is an important focus of research in tumors that are ASS1-deficient (3). Targeted degradation of extracellular arginine in the absence of ASS1 mainly triggers apoptosis in arginine auxotrophs; indeed, supplementation of arginine-metabolizing enzymes such as arginase and arginine decarboxylase can hasten cell death in several ASS1-deficient cell lines (7, 8). However, early enthusiasm for the use of arginase or arginine decarboxylase did not prevail because of the very high doses of arginase required and the production of toxic metabolites from arginine decarboxylase. Another type of arginine-metabolizing enzyme, arginine deiminase (ADI or E. C.3.5.3.6), isolated from Mycoplasma showed promise in term of restricting cell growth in vitro, but ADI was not effective in vivo because of its strong antigenicity and its rapid plasma clearance (half-life of 4 hours). Formulation of ADI with a 20-kDa polyethylene glycol (PEG) moiety rendered it capable of evading circulating and resident (reticuloendothelial) macrophages and reduced antigenicity. This construct, ADI-PEG20, with an increased circulatory time was shown to retain potent antitumor
efficacy against ASS1-negative cancer cells both in vivo and in vitro (9).

ADI-PEG20 as monotherapy demonstrated encouraging anti-tumor activity in humans with hepatocellular carcinoma or metastatic melanoma in phase II trials (10). However, a phase III monotherapy trial in hepatocellular carcinoma did not meet its goal of improved overall survival, but the adverse effect profile was comparable with placebo (11). More recent trials have demonstrated medical benefit when combined with at least one other agent in a number of cancers, including pancreatic carcinoma (7, 12, 13). Some reports suggest that tumors can develop resistance to ADI-PEG20, possibly by the eventual upregulation of ASS1 after prolonged arginine deprivation. Reduced internalization of ADI-PEG20 or its neutralization by an antibody could also lead to resistance to ADI-PEG20 (14).

Several strategies have been proposed to overcome the limitations of ADI-PEG20. Combining ADI-PEG20 with other therapeutic agents [e.g., 5-fluorouracil, cytarabine (15), doce-taxel, paclitaxel, gemcitabine, P53 inhibitor] yielded additive antitumor effects as compared with either agent alone, both in preclinical models and in clinical trials (7, 12, 13, 16–21). Thus, arginine deprivation by ADI-PEG20 and simultaneous inhibition of the subsequent metabolic adaptations has also been shown to improve treatment outcome. Furthermore, selective intracellular delivery of ADI-PEG20 using pH-sensitive cell penetrating peptide (CPP) has been shown to overcome hypoxia-induced resistance to ADI (22). However, to the best of our knowledge, the radiosensitizing effect of arginine deprivation by ADI-PEG20 has never been investigated in pancreatic cancer.

Recent data suggest that local recurrence is a significant contributor to pancreatic cancer mortality (23). Local treatment intensification with radiotherapy dose escalation may reduce local recurrences and improve overall survival but has to contend with prohibitively high toxicity to surrounding gastrointestinal mucosa (24, 25). Alternatively, enhanced antitumor efficacy of standard-dose radiotherapy can be achieved using exogenous radiosensitizing drugs that exploit the unique vulnerabilities of pancreatic cancer (26). We hypothesized that, by inhibiting metabolic adaptations of pancreatic cancers, ADI-PEG20 can sensitize arginine auxotrophic ASS1-negative cancers to radiotherapy and should be elucidate the mechanisms underlying the potential therapeutic effects.

Materials and Methods

Cell lines and reagents

We tested eight human pancreatic cancer cell lines with various degrees of expression of ASS1: MiaPaCa-2, AsPC-1, BxPC-3, Capan-1, HPAC, and SW1990 cells were obtained from the ATCC, and L3.6pl and Panc-1 cells were obtained from the Characterized Cell Line Core Facility at MD Anderson Cancer Center (Houston, TX). All cells were cultured in media and under conditions recommended by the manufacturer. The ADI-PEG 20 was provided by Polaris Pharmaceuticals, Inc. The ADI is a recombinant protein of approximately 46,173 mw having the following sequence: VGVSERTDLDITLAKNIAKANEVKFRIAVNVKWTNLHMDTWTLMKDNKLFLYSPANDVFFKYWDYDLVNGGAEPQQQNLGLPLDKILLASIINKEPVLLIPGAGATEMEIERTNFDTNYLAIKPLGLVIGDRNTEAKNLAALKAVGTYLPHGNQLSGLMGAN-ARCMSMLPSRKDVKW. ADI is modified by covalent attachment to PEG of 20,000 MW. The resulting PEGylated ADI drug substance is referred to as ADI-PEG 20 (9). All reagents were of anadequate grade.

Clonogenic assay

Pancreatic cells were seeded in 60-mm Petri dishes (PD60) at 0.5 × 10^5 cells/5 mL and incubated overnight. Attached cells were treated with 0, 0.04, or 0.08 μg/mL ADI, incubated for another 48 hours, and then irradiated with 0, 2, 4, or 6 Gy using an XRAD 320 orthovoltage irradiator (Precision X-Ray), followed by continued incubation with fresh ADI for an additional 24 hours. Trypsinized cells were plated in sextuplicates in 6-well plates and resulting colonies were counted on a high resolution Oxford optotronics gel counter around 12 days after staining with 0.5% crystal violet diluted in 95% ethanol. The number of surviving colonies (>50 cells per colony) were plotted against radiation dose, and dose enhancement factors at surviving fractions of 10% (DEF10) were calculated using Sigma plot (27). Experimental conditions were untreated or treated with radiation only, ADI-PEG20, or radiation+ADI-PEG20, and each experiment was performed in triplicate.

Reverse phase protein array assay

Panc-1 cells were treated with 0, 0.4, 0.8, or 1.6 μg/mL of ADI-PEG20 for 48 hours and then exposed to 0, 2, or 4 Gy of ionizing radiation (IR). Cell lysates were prepared 24 hours later, denatured by 1% SDS with βmercaptoethanol, and five 2fold serial dilutions of samples were arrayed on nitrocellulose-coated slides (Grace Bio Lab) by an Aushon 2470 Arrayer (Aushon BioSystems). Each slide was probed with 305 primary antibodies and a biotinconjugated secondary antibody, with signals amplified with a Catalyzed Signal Amplification System (DakoCytomation). Samples were stained and precipitated with 3.3’ diaminobenzidine tetrahydrochloride (DAB), and the slides were then scanned, analyzed, and quantified for spot intensity by using customized software. Target antibodies with a Pearson correlation coefficient (RPPA: Western blotting) greater than 0.7 were used for RPPA analysis. Each dilution curve was fitted with a logistic model (“Supercurve Fitting,” developed by the Department of Bioinformatics and Computational Biology at MD Anderson Cancer Center). A heatmap was visualized using R software and the package Ggplot2. Spot Intensity higher expression is depicted in red gradient and lower expression in green gradient.

Immunofluorescence assay

To evaluate the effect of the chemical chaperone protein 4-phenylbutyrate (PBA) on ADI-PEG20 and IR-mediated endoplasmic reticulum (ER) stress protein expression, we performed an immunofluorescence assay. Panc-1 cells were seeded in 8-well chamber slides. Attached cells were treated with PBS or ADI-PEG20 (0.08 μg/mL) for 48 hours. Slides received PBA (0 or 10 mmol/L) 4 hours before 0 or 6 Gy of radiation exposure. Six hours after the radiation exposure, chambers were washed in PBS, and cells fixed in 4% paraformaldehyde. Cells were permeabilized with 100% methanol and 0.5% triton X-100 (1 × PBS) and
blocked for 1 hour in blocking solution (3% BSA + 1% FBS). Slides were incubated overnight at 4°C in a primary antibody cocktail: mouse anti-calreticulin antibody (catalog no. ab22683; Abcam), rat anti-CD44 (catalog no. MA4405; Invitrogen), and rabbit anti-BIP (catalog no. 3177S; Cell Signaling Technology) diluted in blocking solution. Chambers were washed in PBS and incubated for 1 hour at room temperature in secondary antibodies (goat anti-mouse Alexa Fluor 647, goat anti-rat Alexa Fluor 488, and goat anti-rabbit Alexa Fluor 594) diluted in blocking solution. After washing, counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI), with Vectashield mounting medium (Vector Laboratories) used to prevent rapid photo bleaching. The expression and localization of probes was captured using an Olympus IX81/3/E microscope with appropriate filters and analyzed using Fluoview software (Olympus America) and Slidebook 3.0 (Intelligent Imaging Innovations, Inc.).

Flow cytometry

Flow cytometry was used to evaluate the early apoptotic cell death and cell-cycle distribution of Panc-1 cells treated with ADIPEG20 (0.08 μg/mL) for 48 hours and/or PBA (0 or 10 mmol/L) 4 hours before 4 Gy of radiation exposure. Cells were harvested 24 hours after irradiation for early apoptosis analysis and after 24, 48, and 72 hours for cell-cycle analysis. Cells were stained according to manufacturer’s protocol (Annexin V: FITC Apoptosis Detection Kit, BD Biosciences). Early apoptotic cells (Annexin V+ and PI−) were scored in different treatment groups. For cell-cycle analysis, Panc-1 cells were fixed in 70% ethanol and stained with propidium iodide and analyzed for sub-G1, G1, S, and G2–M phases using FlowJo software.

Evaluation of intracellular reactive oxygen species

Radiation induces damage by generating reactive oxygen species in a cellular milieu. We evaluated the reactive oxygen species (ROS) using the CellROX kit (Life Technologies). Briefly, 15,000 Panc-1 cells were seeded in 96-well plates, incubated overnight in an incubator with 5% CO2 at 37°C, and treated with ADI-PEG20 (0.08 μg/mL) for 48 hours. Panc-1 cells received PBA (0 or 10 mmol/L) and 5 μmol/L CellROX suspension in culture media 4 hours and 30 minutes before radiation exposure (4 Gy). Later, the cells were washed with PBS and absorbance was measured at 520 nm within 15 minutes and averaged across four wells. All data were normalized to irradiated control.

ER01-α knockdown by siRNA silencing

Panc-1 cells were seeded in 30-mm Petri dishes at 0.5 × 10^3 cells/2 mL and incubated overnight. They were treated with 0 μg/mL or 0.08 μg/mL ADI-PEG20 for 48 hours and exposed to graded doses of radiation of 0, 2, 4, or 6 Gy using an XRAD 320 orthovoltage irradiator (Precision X-Ray). Postirradiation, the media were replaced with fresh media containing 0 or 0.08 μg/mL ADI-PEG20. One set of cells was transfected with human ERO1-α siRNA (Thermo Fisher Scientific, HSS1211196) at a final mass of 50 pg siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions while another set was transfected with a scrambled siRNA control at the same concentration. All dishes were further incubated for 24 hours at 37°C in a 5% CO2 incubator. Cells were trypsinized and defined numbers of cells were freshly seeded in plates for the clonogenic assay. Plates were incubated for around 12 days at 37°C in a 5% CO2 incubator until colonies formed. Colonies were stained with 0.5% crystal violet diluted in 95% ethanol as noted before and counted. Clonogenic survival was plotted as described above.

Mice

All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals, prepared by the Institute for Laboratory Animal Resources, National Research Council, and Institute of Laboratory Animal Resources, National Research Council, and US National Academy of Sciences, and the MD Anderson Cancer Center Institutional Animal Care and Use Committee. Mice were purchased from MD Anderson’s Experimental Radiation Oncology Animal Facility and housed in an air-conditioned facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were housed (5 per cage) in individually ventilated Tecniplast cages in a room with a HEPA-filtered air supply at relative humidity of 60% ± 10% on a 12-hour light/dark cycle. All mice were quarantined for 1 week before any experiments were begun. Food (Purina PicoLab Rodent Diet 5053, Harlan Teklad) and water (reverse osmosis chlorinated or acidified water, pH 2.5–2.8) were provided ad libitum.

Irradiation

Cell irradiations with X-rays (320 kVp, 12.5 mA) were performed on X-RAD 320 from Precision X-Ray (06471). Effective beam-energy, measured in narrow-beam geometry, in terms of Half-Value Layer is 1.015 mm copper. Irradiation geometry consisted of Source-Skin-Distance 50 cm, field-size 20 cm × 20 cm, and back-scatter 1 cm acrylic. Dose output in cell irradiation geometry is 4.541 cGy/sec. Quality assurance checks (Beam parameters, irradiation geometry, and dose output) are performed on a yearly basis employing a 0.6cc Farmer-type Ion-Chamber calibrated by Accredited-Dosimetry-Calibration Laboratory traceable to NIST (National Institute of Standards and Technology) standards. Annual checks for recent 4 years indicate beam output to be reproducible within 4%.

Mouse irradiations of thigh tumors were performed with 6 MV X-rays from Varian-2300CD linear accelerator (Varian Medical Systems, 94304). Two mice at a time were irradiated in this geometry: source-skin-distance 100 cm, field-size 15 cm × 15 cm, Cerrobend-Block casting two circular-fields of 3 cm diameter each, and a back-scatter of 15 cm “Solid Water” plastic block. Dose output in this geometry is 0.958 cGy per Monitor Unit. Quality assurance check of beam output and energy are performed on a monthly basis employing a 0.6cc Farmer-type Ion-Chamber calibrated by Accredited-Dosimetry-Calibration Laboratory traceable to NIST (National Institute of Standards and Technology) standards. Monthly checks typically indicate beam output to be reproducible within 2%.

Tumor growth delay assay

ADI-PEG20–mediated radiosensitization of Panc-1 and MiaPaCa-2 pancreatic cancer cells was assessed in a xenograft mouse model as follows. Swiss nude mice (nu/nu) were injected sub-cutaneously with 5 × 10^6 MiaPaCa-2 cells or 2 × 10^6 Panc-1 cells. When the resulting tumors reached 5–8 mm in diameter, the mice were randomly assigned to one of the following treatment groups (at least 6 mice per group): control; ADI-PEG20 only; radiation only; or radiation + ADI-PEG20. ADI-PEG20 (5 IU per mouse) was injected intraperitoneally twice a week. Radiation was...
delivered to the mouse tumors as 2-Gy fractions delivered once a day on 5 consecutive days with a 6-MV photon beam from a Varian 2300CD linear accelerator (Varian Medical Systems; Supplementary Fig. S1). Mice were monitored and tumors measured three times a week until the tumors reached 2 cm or up to 30 days. At the end of the experiment all mice were euthanized humanely. Tumor volume was measured as $\frac{1}{2}a \times \frac{1}{2}b \times \frac{1}{4}h$ (where $a =$ long axis, $b =$ short axis dimension). Normalized tumor volumes were calculated by dividing current tumor volume by tumor volume at the start of treatment and plotted with GraphPad. The mean tumor doubling time (in days) was calculated for each treatment group.

The protocol used for Western blot analysis, DNA damage assessment by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and tumor cell proliferation assay has been provided in the Supplementary Data.

Statistical analysis

All experiments were carried out in triplicate unless otherwise specified. Results are presented as means and SDs. Statistically significant differences were calculated by using two-tailed unpaired Student t tests or by one-way ANOVA. Results with a $P$ value of <0.05 were considered significant.

Results

Evaluation of ADI-PEG20–mediated radiosensitization effect in ASS1-positive or -deficient pancreatic cancer (PaCa) cells

We began by identifying the stand-alone toxicity of ADI-PEG20 using an MTS assay as described previously (28). Various concentrations of ADI-PEG20 were tested in Panc-1, MiaPaCa-2, and L3.6pl cells; plots of the normalized percentage viability values are shown in Supplementary Fig. S2. We chose two ADI-PEG20 doses (0.04 μg/mL and 0.08 μg/mL), which were nontoxic in the ASS1-expressing cell line L3.6pl, to evaluate the radiosensitization effects of ADI-PEG20 in clonogenic assays of five ASS1-deficient cell lines (Panc-1, MiaPaCa-2, AsPc-1, HPAC, and CaPan-1) and three ASS1-expressing cell lines (L3.6pl, BxPC-3, and SW1990; ref. 4). We found that ADI-PEG20 augmented the effect of radiation in the ASS1-deficient cell lines MiaPaCa-2 and Panc-1 in a dose-dependent manner; ADI-PEG20 did not affect the response of the ASS1-positive cell line L3.6pl. For the MiaPaCa-2 cells, the DEF10 values were 1.16 at 0.04 μg/mL and 1.57 at 0.08 μg/mL; the DEF10 values for the Panc-1 cells were slightly higher at 1.34 and 1.79, respectively (Supplementary Fig. S2), leading us to choose the 0.08 μg/mL concentration for further testing. Survival curves of ASS1-positive and ASS1-deficient pancreatic cancer cell lines at different doses of radiation and ADI-PEG20 (0.08 μg/mL) are shown in Fig. 1. For the ASS1-deficient cell lines, the DEF10 values were 1.11 for AsPc-1, 1.4 for HPAC, and 1.12 for CaPan-1; the ASS1-expressing cells did not show evidence of ADI-PEG20–mediated radiosensitization (DEF10 values were 1.02 for BxPC-3 cells, 0.91 for L3.6pl cells, and 1.03 for SW1990 cells).

Effect of ADI-PEG20 and ionizing radiation on functional protein expression

To discern the possible mechanism of ADI-PEG20–mediated radiation sensitization, we used high-throughput RPPA assays to analyze the effect of different concentrations of ADI-PEG20 (0.00, 0.04, 0.08, and 0.16 μg/mL) and radiation (0, 2, or 4 Gy) on the expression of 308 proteins in various biological pathways in Panc-1 cells (29). Twenty-four of the 308 analyzed proteins showed a dose-dependent relationship with ADI-PEG20, radiation, or both (Supplementary Fig. S3). ADI-PEG20 plus radiation enhanced the expression of proteins related to ER stress and apoptosis (Akt, Akt_pS473, Akt_pT308, 53BP1, p53, Bax, BID, caspase 3, caspase 7) relative to the radiation-only control; the only differences noted in cell-cycle–related proteins were a decrease in CDK1 and an increase in cyclin B1 and cyclin D1 levels. The expression of activated focal adhesion kinase (FAK_pY397) was lower in the ADI-PEG20–only or radiation-only treatment groups relative to the untreated control; on the other hand, combining ADI-PEG20 with radiation did not affect FAK_pY397 expression (Fig. 2A and B). ADI-PEG20 in combination with radiation inhibited the expression of axonal glycoprotein CD171, a mediator of cell adhesion and migration; AXL receptor tyrosine kinase (Axl), a mediator of cell proliferation and metastasis; proliferating cell nuclear antigen (PCNA), a protein involved in DNA replication and repair; and EZF1, a mediator of cell proliferation. The combination treatment also inhibited polo-like kinase 1 (PLK1) expression leading to activation of proapoptotic pathways; monocarboxylate transporter 4 (MCT4) leading to lactic acidosis, and Cox-IV (cytochrome c oxidase IV), which indicates a transmission of ER stress to the mitochondrial inner membrane causing alteration in mitochondrial oxidative dynamics (Fig. 2A and B).

ADI-PEG20 mediates radiosensitization via ER stress signaling in ASS1-deficient pancreatic cells

Given the dominant effect of ADI-PEG20–mediated radiosensitization on ER stress pathways, we explored this mechanism further by analyzing the expression of ER stress proteins by Western blotting as follows: Panc-1 and MiaPaCa-2 cells were treated with ADI-PEG20 (0.08 μg/mL), radiation (6 Gy), or both; total proteins were extracted 6 hours later and analyzed for expression of binding immunoglobulin protein (BIP), protein disulfide isomerase (PDI), endoplasmic reticulum oxidoressudoxin-1 (ERO-1), and C/EBP homologous protein (CHOP, also known as GADD153). In Panc-1 cells, all four proteins (BIP, PDI, ERO-1, and CHOP) were expressed at significantly ($P < 0.05$) higher levels in the ADI-PEG20 + radiation group compared with the untreated, radiation-only, or ADI-PEG20–only groups; similar results were noted (except for CHOP) in the MiaPaCa-2 cells (Fig. 2C). These results implicate a role for ER stress proteins in ADI-PEG20–mediated radiosensitization.

To confirm these results, we next evaluated the effect of the chemical chaperone protein 4-phenylbutyrate (PBA), a known inhibitor of ER stress signaling (30), on ER stress protein expression. As expected, ADI-PEG20 plus radiation significantly enhanced the expression of the ER stress protein BIP compared with the irradiated control ($P < 0.001$), and addition of the ER stress inhibitor PBA abrogated this effect ($P < 0.005$; Fig. 3A and B). The BIP expression in the ADI-PEG20 + radiation group was validated to be localized in the ER by a colocalization experiment with calreticulin, an ER protein marker. BIP expression was confirmed to colocalize with calreticulin (Supplementary Fig. S4). We quantified the early apoptosis using flow cytometry. As expected, the combination of ADI-PEG20 with radiation showed significantly ($P < 0.005$) higher percentage of early apoptotic cells as compared with radiation alone or ADI-PEG20 + PBA + IR (Fig. 3C) Finally, PBA was shown to abrogate the...
radiosensitizing effect of ADI-PEG20 + radiation in a clonogenic assay (Fig. 3D), confirming the crucial role of ER stress protein in ADI-PEG20–mediated radiosensitization.

Effect of ADI-PEG20 and radiation on ER stress protein expression, apoptosis, and proliferation

Next, we evaluated the effects of ADI-PEG20 (5 IU/mouse × 2), radiation (6 Gy), or both on ER stress protein production, apoptosis, and proliferation in an ASS1-deficient Panc-1 cell xenograft model. In the first set of experiments, Panc-1 tumors were resected 2 hours after the last intervention (ADI-PEG20 or radiation), fixed, and processed for immunofluorescence staining to detect the expression of BIP and PDI in tissue sections. Notably, the numbers of BIP-positive and PDI-positive cells per high-power field were highest in the ADI-PEG20 + radiation group (P < 0.005; Fig. 4A and B), strengthening our in vitro findings that the combination of ADI-PEG20 and radiation enhances ER stress signaling.

In the next set of experiments, we evaluated xenograft tumors for markers of apoptosis in mice treated with ADI-PEG20, radiation, or both. We found that the ADI-PEG20 + radiation group had significantly higher numbers of TUNEL-positive cells at
2 hours (P < 0.01) and 24 hours (P < 0.0005) after irradiation relative to the radiation-only control (Fig. 4C). Moreover, even though the number of TUNEL-positive cells had decreased at 24 hours in the radiation-only control (P < 0.03), no such decrease was observed in the irradiated group pretreated with ADI-PEG20 (P > 0.47; Fig. 4C). We also tested for active caspase-3, another marker of apoptosis, and found significantly higher numbers of caspase-3-positive cells in the ADI-PEG20 + radiation group compared with ADI-PEG20 alone (P < 0.005) or radiation alone (P < 0.01) (Fig. 4D), further confirming that ADI-PEG20 potentiates the effect of radiation (Fig. 4D).

In the third set of experiments, we evaluated the effects of ADI-PEG20, radiation, or both on markers of DNA replication, cellular proliferation, and angiogenesis. As predicted, the untreated control and the ADI-PEG20-only groups had significantly higher numbers of BrdU-positive (proliferating) cells relative to the radiation-only group; the combination of ADI-PEG20 + radiation produced the lowest numbers of such cells (P < 0.005; Fig. 4E). Furthermore, ADI-PEG20 + radiation resulted in the greatest suppression of Ki-67, a proliferation marker, and VEGF, an angiogenesis marker, of the various treatment groups (Supplementary Fig. S5). In the fourth set of experiments, we evaluated the effects of ADI-PEG20, radiation, or both on ROS generation. ROS was measured in the presence and absence of ER stress inhibitor (PBA). Significantly higher ROS (P < 0.005) was observed in ADI-PEG20 + IR treatment group as compared with IR group. Treatment with ER stress inhibitor (PBA) 4 hours before irradiation significantly (P < 0.05) inhibited ROS generation in Panc-1 cells (Fig. 4F). To validate the result observed with ER stress protein PBA, we used siRNA to silence the expression of ERO-1α. Panc-1 cells were treated with 0 or 0.08 mg/mL ADI for 48 hours and exposed to radiation (0, 2, 4, and 6 Gy). As expected, ADI enhanced the effect of radiation (DEF 1.56); however, siRNA knockdown of...
ERO-1α partially reversed the ADI-PEG20–mediated radiosensitizing effect (DEF 1.3), whereas scrambled siRNA did not (Supplementary Fig. S6). Taken together, these findings suggest that the combination of ADI-PEG20 + radiation increases ER stress protein expression and apoptosis while reducing proliferation and angiogenesis, collectively diminishing the likelihood of tumor regrowth.

ADI-PEG20 profoundly radiosensitizes pancreatic xenograft tumors

Finally, we evaluated the effects of ADI-PEG20, radiation, or both on the in vivo growth of two ASS1-negative xenograft cell lines in mouse models. Panc-1 or MiaPaCa-2 cells were implanted subcutaneously in the right thighs of Swiss nu/nu mice, which were treated with ADI-PEG20 (5 IU i.p. × 2), radiation (2 Gy × 5), or both (Supplementary Fig. S1). The tumor doubling times for the control (untreated) groups were 13 days and 9 days for Panc-1 and MiaPaCa-2 tumors, respectively; 16 days and 9 days for the ADI-PEG20–only group; and 22 days and 13 days for the radiation-only group. The combination of ADI-PEG20 and radiation led to the greatest delays in tumor doubling time, namely 30 days for Panc-1 tumors ($P < 0.01$) and 28 days for MiaPaCa-2 tumors ($P < 0.001$; Fig. 5).
Discussion

Metabolic reprogramming is considered one of the hallmarks of carcinogenesis and each metabolic phenotype has a distinct molecular signature that portends a different prognosis (31). This is seen quite notably in pancreatic cancer where treatment-resistant cancer cells reside and thrive within a hostile environment characterized by sparse vascularity, chronic hypoxia, and an exuberant stroma. Distinctive adaptations for survival

Figure 4.
Effect of ADI-PEG20 and radiation on the expression of ER stress proteins, DNA damage, apoptosis, and proliferation. Mice received ADI-PEG20 (5 IU/mouse × 2), radiation (6 Gy), or both. To quantify the cells expressing ER stress protein per high power field (HPF), tumor was excised 2 hours after radiation exposure. A, Graph shows the number of cells expressing binding immunoglobulin protein (BIP). **, ADI vs. ADI + IR (P < 0.003); *, IR vs. ADI + IR (P < 0.005). B, Graph shows the number of cells expressing protein disulfide isomerase (PDI). **, ADI vs. ADI + IR (P < 0.003); *, IR vs. ADI + IR (P < 0.005). C, To quantify the number of TUNEL-positive cells per HPF, tumor tissue was taken out at 2 and 24 hours after IR exposure. There was a significantly higher (P < 0.01) number of TUNEL-positive cells in the ADI + IR treatment group at 2 or 24 hours compared with IR or ADI alone. The decrease in the number of TUNEL-positive cells at 24 hours is larger in the IR alone-treated group (**, IR (2 hours) vs. IR (24 hours; P < 0.03)), and it is considerably lower in the ADI + IR-treated group (**, ADI + IR (2 hours) vs. ADI + IR (24 hours; P > 0.47)). Active caspase-3 and BrdU-positive cells per HPF were quantified on 4 days after the radiation treatment. D, The number of caspase-3-positive cells per HPF is significantly higher in the ADI + IR treatment group compared with ADI or IR alone. **, ADI vs. ADI + IR (P < 0.005); *, IR vs. ADI + IR (P < 0.01). E, The number of BrdU-positive cells per HPF is significantly (P < 0.005) lower in the ADI + IR treatment group compared with ADI or IR alone. F, The reactive oxygen species optical density normalized to radiation control is significantly higher in the ADI + IR vs. ADI + PBA + IR (P < 0.01).
include the Warburg effect, autophagy, micropinocytosis, glutamine addiction, and increased expression of enzymes of fatty acid synthesis. Among these metabolic adaptations designed to confer a survival advantage to cancer cells, a unique feature is the increased need for nutrients and raw materials to facilitate rapid proliferation and dissemination of cells. ASS1 deficiency creates an arginine auxotrophy by forcing cells to outsource their arginine requirements. In turn, it serves as a biomarker of pancreatic cancer aggressiveness predicting a higher chance of local recurrence (5). Nonetheless, viewed from a therapeutic perspective, this is a chink in the metabolic armor of pancreatic cancer which can be exploited by targeted arginine deprivation through a PEGylated arginine deiminase, ADI-PEG20. We hypothesized that arginine deprivation with ADI-PEG20 in conjunction with radiotherapy, a known mediator of reduced local recurrence risk and an integral component of multimodality treatment of cancer (32, 33), could have additive or synergistic effects in experimental models of pancreatic cancer, much like that described in experimental neuroblastoma models (34).

We therefore evaluated the effects of ADI-PEG20 and ionizing radiation, singly and in combination, on functional proteins expression by using RPPA analysis. We observed higher expression of Akt, Akt_pS473, Akt_pT308, 53BP1, p53, Bax, BID, CoxIV, caspase 3, and caspase 7 with ADI-PEG20 + radiation compared with either one alone. Akt, Akt_pS473, and Akt_pT308 proteins have been found to mediate ER stress through the PI3K/Akt pathway (35) and increased expression of 53BP1, p53, Bax, BID, caspase 3, and caspase 7 indicates increased apoptosis. Radiation, as well as several anticancer agents, are known to induce ER stress. ADI-PEG20 monotherapy has been shown to inhibit leukemia cell proliferation by arresting cell cycling (36); however, no clear evidence of cell-cycle arrest was present in the expression patterns of cell-cycle–related proteins in pancreatic cancer cell lines. Flow cytometric cell-cycle analysis further indicated that ADI-PEG20 (0.08 μg/mL) did not induce cell-cycle...
modulation in pancreatic cells (Supplementary Fig. S7). This might be due to radiation-induced cell-cycle changes dominating over those of ADI-PEG20 and/or due to cell-cycle dynamics varying considerably based on context (cell line, dose of drug or radiation, sequencing of the two).

ADI has also been shown to have antiangiogenic effects (34, 37). We also observed that either ADI-PEG20 or radiation suppressed the expression of FAK_pY397 and PKM2 relative to the untreated control condition, but combining ADI-PEG20 with radiation did not further inhibit expression of FAK_pY397 or PKM2 in Panc-1 cells. FAK_pY397 is a cytoplasmic tyrosine kinase that mediates cytoskeletal integrity, cell motility, and regulates integrins (38). PKM2 is an isoenzyme of pyruvate kinase that catalyzes the final step of glycolysis and is a multi-dimensional protein that has a role in angiogenesis, nuclear transport and protein–protein interactions (39). Additional research, beyond the scope of this article, is needed to clarify the mechanisms by which angiogenesis is inhibited by ADI-PEG20 in ASS1-deficient pancreatic cancer cells and if FAK_pY397 and PKM2 contribute to this or not.

In this study, the combination of ADI-PEG20 and radiation significantly enhanced the expression of proteins related to apoptosis, ER stress, and the unfolded protein response (UPR). Briefly, as unfolded proteins accumulate in the lumen of the ER, the resulting stress activates a cascade of adaptive responses known as the UPR (40). UPR promotes protein folding and clearance and thereby reduces the amount of misfolded proteins in the ER lumen (40, 41). However, prolonged ER stress triggers apoptotic pathways that eliminate irreversibly damaged cells (42). Therefore, ER stress resulting from treatment acts as a double-edged sword in cancer; moderate ER stress tends to have prosurvival effects, but severe ER stress mediates cell death via apoptosis. When misfolded proteins enter the ER, they bind to BIP and activate BIP signaling. BIP expression correlates not only with cancer cell proliferation but also with therapeutic response (43).

We found that treatment with ADI-PEG20 and radiation enhanced the expression of BIP and PDH in MiaPaCa-2 and Panc-1 cells. PDH, a thiol-oxidoreductase chaperone protein, helps in the isomerization, reduction, and oxidation of nonnative disulfide bonds in unfolded proteins entering the ER of normal

Figure 6. Schematic diagram showing the effect of ADI-PEG20 and ionizing radiation on ER stress proteins. ADI-PEG20 and IR, in ASS1-deficient cells, increases the unfolded proteins in ER lumen and enhances the expression of ER stress protein-BIP, PDH, ERO-1, and CHOP proteins. The expression of BIP dictates whether cells choose unfolded protein response pathways leading to repair or apoptotic pathways leading to death. Persistent and irreversible ER stress leads to apoptosis via several pathways. ER stress activates PERK signaling leading to phosphorylation of eIF2α, which causes overall translational arrest; however, it selectively allows the translation of ATF4 protein that enhances the expression of transcription factor CHOP. CHOP enhances the expression of several proapoptotic proteins, including ERO1, PUMA/NOXA, and BIM/BH3-only proteins. Increased ERO1 expression in ER lumen increases the ROS load as well as releases calcium ions by regulating IP3R. Higher ROS load itself is cytotoxic, but cytosolic calcium activates PTP to induce apoptosis as well. The induction of PUMA/NOXA and BIM/BH3-only proteins inhibits the expression of the antiapoptotic protein Bc2 and activates BAX/BAK to release cytochrome c from mitochondria, leading to apoptosis. CHOP also induces the expression of GADD34 protein that may further be a determinant of cell fate via choice of survival (repair signaling) versus demise (death signaling). ADI-PEG20 treatment before radiation exposure causes persistent ER stress in ASS1-deficient pancreatic cancers augmenting the effect of IR or ADI-PEG20 treatment alone. PBA, a well-known ER stress inhibitor, minimizes the ER stress signaling caused by ADI-PEG20 and IR, and thereby reduces the ADI-PEG20-mediated radiosensitization effects.
cells. However, recent evidence indicates that PDI also triggers apoptosis under ER stress in the presence of accumulated misfolded proteins (44). Our observation that ADI-PEG20 plus radiation led to higher expression of PDI supports these findings.

Prolonged ER stress also triggers cell death by activating PERK signaling (45). Death signals from the PERK pathway involve activation of CHOP by ATF4, which further regulates the expression of several Bcl-2 family members. We observed higher expression of CHOP in ADI-PEG20 plus radiation in Panc-1 cells, but not in MiaPaCa-2 cells, by 6 hours after irradiation. As ER stress signaling is an early event, we could have missed its peak expression time in the MiaPaCa-2 cells. Under ER stress, CHOP also downregulates the expression of Bcl-2 and induces apoptosis through activation of Bcl-2-like1 (BIM), p53 upregulated modulator of apoptosis (PUMA), NADPH oxidase activator (NOXA), and BH3-only protein, culminating in the release of cytochrome c and apoptosis through caspase-dependent pathways, as well as inducing expression of ERO-1. Our finding that ADI-PEG20 + radiation increased the expression of ERO-1 in both Panc-1 and MiaPaCa-2 cell lines offers further support for our contention that ADI-PEG20 augments the radiosensitization effect by inducing ER stress. CHOP-induced ERO-1 activates the inositol-1,4,5-triphosphate receptor (IP3R), releasing calcium from ER to cytoplasm (Fig. 6). Higher calcium concentrations in the cytoplasm induce apoptosis by affecting the mitochondrial permeability transition pore (46).

We also tested our hypothesis by using PBA to inhibit ER stress signaling (30) and confirmed that PBA counteracted ER stress-mediated radiosensitization; further, ADI-PEG20 plus radiation did not restore radiosensitization to the baseline relative to the radiation-only condition, suggesting that ADI-PEG20-mediated radiosensitization occurs through several mechanisms, of which ER stress-mediated apoptosis may be the major driver (Fig. 6).

We further assessed whether tumors could overcome the growth delay induced by ADI-PEG20 plus radiation by evaluating the expression of proteins related to cell proliferation. We showed that ADI-PEG20 treatment before radiation exposure significantly inhibited proliferation, as indicated by BrdU and Ki-67. Specifically, we saw significantly higher numbers of TUNEL-positive cells in the ADI-PEG20 + radiation group at all time points tested; indeed, the lack of a drop in the number of TUNEL-positive cells at 24 hours after this treatment suggests that ADI-PEG20 also may suppress DNA repair after irradiation. These results are in corroboration with earlier findings showing that ER stress suppresses DNA repair and sensitizes tumor to ionizing radiation (47). We also demonstrated that ADI-PEG20 + radiation led to downregulation of VEGF, a finding in agreement with previous studies showing that ADI-PEG20–mediated arginine deprivation inhibits the hypoxia-induced activation of HIF-1α and HIF-2α, leading to decreased levels of inducible-nitric oxide synthase (iNOS), nitric oxide, and VEGF (48).

Finally, we validated our hypothesis in a pancreatic xenograft model, where we found that ADI-PEG20 + radiation not only delayed tumor doubling time but also increased the expression of ER stress proteins; increased the number of apoptotic cells; and reduced the number of proliferating cells. Indeed, these effects were noted in two ASS1-negative PaCa cells (Panc-1 and MiaPaCa-2) and were more profound in vivo than in vitro.

In conclusion, we found that ADI-PEG20, a compound combining the arginine-degrading enzyme ADI with PEG, selectively inhibited the proliferation of ASS1-deficient pancreatic cancer cells. ADI-PEG20 augmented radiation-mediated apoptosis and sensitized pancreatic cancer cells to radiation. ADI-PEG20 enhanced the effect of radiation by modulating ER stress signaling. These effects were more pronounced in vivo than in vitro, possibly due to inhibition of angiogenesis by ADI-PEG20. Further studies are warranted to evaluate the magnitude and mechanism of radiation-induced cell death after arginine deprivation via other approaches and whether overwhelming ER stress pathways is a class solution to the vexing challenge of radiation resistance of cancer cells. On the basis of the findings of our study, clinical translation could be contemplated wherein a novel combination of radiation + ADI-PEG20 could be administered selectively to patients with ASS1-deficient pancreatic cancer and the enhancement of ER stress may be used as a surrogate biomarker of response.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Exploiting Arginine Auxotrophy with Pegylated Arginine Deiminase (ADI-PEG20) to Sensitize Pancreatic Cancer to Radiotherapy via Metabolic Dysregulation


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