Pharmacological Ascorbate Radiosensitizes Pancreatic Cancer

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Abstract

The toxicity of pharmacologic ascorbate is mediated by the generation of H2O2 via the oxidation of ascorbate. Because pancreatic cancer cells are sensitive to H2O2 generated by ascorbate, they would also be expected to become sensitized to agents that increase oxidative damage such as ionizing radiation. The current study demonstrates that pharmacologic ascorbate enhances the cytotoxic effects of ionizing radiation as seen by decreased cell viability and clonogenic survival in all pancreatic cancer cell lines examined, but not in nontumorigenic pancreatic ductal epithelial cells. Ascorbate radiosensitization was associated with an increase in oxidative-stress-induced DNA damage, which was reversed by catalase. In mice with established heterotopic and orthotopic pancreatic tumor xenografts, pharmacologic ascorbate combined with ionizing radiation decreased tumor growth and increased survival, without damaging the gastrointestinal tract or increasing systemic changes in parameters indicative of oxidative stress. Our results demonstrate the potential clinical utility of pharmacologic ascorbate as a radiosensitizer in the treatment of pancreatic cancer.

Introduction

Pharmacologic ascorbate induces cytotoxicity and oxidative stress in pancreatic cancer cells, compared with normal cells (1). In the extracellular environment, pharmacologic concentrations of ascorbate can oxidize to form hydrogen peroxide (H2O2; refs. 1–4). H2O2 will diffuse readily across the cell membrane causing oxidative damage to cellular proteins, lipids, and DNA. The generation of H2O2 correlates with the concentration of ascorbate in both a time- and dose-dependent manners. Ascorbate has been shown to decrease viability in all pancreatic cancer cell lines studied, but has no effect on nontumorigenic pancreatic ductal epithelial cells (1), and cytotoxicity was reversed with scavengers of H2O2. Furthermore, in vivo treatment with pharmacologic ascorbate inhibited tumor growth and prolonged survival. Thus, ascorbate has been hypothesized to be a “prodrug” for formation of H2O2 in pancreatic cancer xenografts (1, 3). Therapeutic interventions designed to increase oxidant stress (such as ionizing radiation, IR) in combination with pharmacologic ascorbate would be predicted to preferentially sensitize tumor cells versus normal cells via metabolic oxidative stress (1, 5).

IR has long been known to induce DNA damage. In addition to direct damage, IR generates reactive oxygen species (ROS) that can damage proteins, lipids, and DNA, inducing both single- and double-strand DNA breaks (6). Formation of double-strand breaks results in the rapid phosphorylation of histone H2AX (7). Mammalian-phosphorylated H2AX (γ-H2AX) is believed to facilitate the recruitment and retention of DNA repair and checkpoint proteins (8, 9). Radiosensitive tumor cells have been shown to retain γ-H2AX for a longer duration after IR than radioresistant cells.

Pharmacologic ascorbate-mediated H2O2 formation also causes DNA damage, which involves transition metal ions such as Fe2+ associated with DNA (10). Fe2+ reacts with H2O2, producing site-specific hydroxyl radical (HO•), damaging DNA bases as well as the sugar/phosphate backbone of DNA (11). The base excision repair pathway is the major system for repair of oxidative-induced DNA damage (12). Thus, DNA damage can be assessed by measuring γ-H2AX, which is upregulated in the presence of double-strand breaks.

Because both IR and pharmacologic ascorbate initiate DNA damage, we hypothesize that pharmacologic ascorbate has potential as a radiosensitizer in pancreatic cancer. Here, we demonstrate that pharmacologic ascorbate is a selective radiosensitizer in pancreatic cancer versus normal nontumorigenic pancreatic ductal epithelial cells. Pharmacologic ascorbate also enhanced IR-induced DNA damage in pancreatic cancer cells as well as enhancing IR-induced inhibition of tumor growth in established human pancreatic xenografts without causing systemic changes in parameters indicative of oxidative stress or enhancing normal tissue injury to the gut epithelium. These results demonstrate the potential utility of pharmacologic ascorbate as an adjuvant to pancreatic cancer radiotherapy.
Materials and Methods

Cell culture

MIA PaCa-2, AsPC-1, and Panc-1 human pancreatic adenocarcinoma cells were obtained from the American Type Culture Collection and passaged for fewer than 6 months after receipt. MIA PaCa-2 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. AsPC-1 cells were maintained in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. Panc-1 cells were cultured in DMEM supplemented with 10% FBS. Also, patient-derived pancreatic cancer cell lines (403 and 339a) from the Medical College of Wisconsin surgical oncology tissue bank (13, 14) were cultured in Dulbecco's modified Eagle's Media Nutrient Mixture F-12 with penicillin/streptomycin, human recombinant EGF, bovine pituitary extract, hydrocortisone, and human recombinant insulin. In addition to the pancreatic cancer cell lines, we also used the nontumorigenic HPV16-E6E7 immortalized cell line derived from normal pancreatic ductal epithelium (H6c7) with near normal genotype and phenotype of pancreatic duct epithelial cells (15). H6c7 cells were maintained in keratinocyte serum-free media supplemented with epidermal growth factor (5 ng/mL) and bovine pituitary extract (50 µg/mL). The H6c7 cells were characterized by IDEXX-RADIL. Profile data were not available for comparison purposes for H6c7 cells. However, the genetic profile for H6c7 cells was compared with the cell line genetic profiles available in the DSMZ STR database and did not match any other reported profiles in the DSMZ database. The H6c7 cells alone do not form colonies so feeder cells were used as described (15).

L-ascorbic acid was purchased from Macron Chemicals. Stock solutions of ascorbate (1.0 mol/L, pH 7.0) were made as previously described (1). For ascorbate treatments, cells were placed in fresh media and treated with ascorbate for 1 hour at 37°C. To determine clonogenic survival, cells were treated, trypsinized, counted, diluted, and plated for clonogenic cell survival assay as previously described (1). Surviving colonies were fixed and stained with Coomassie blue after 10 to 14 days and counted under an inverted light microscope. As another indicator of cell viability, an assay monitoring the reduction of MITT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] was used. Cells were seeded in 60 mm² dishes at 3 x 10⁵ cells per well and allowed to attach for 24 hours. Next, cells were irradiated with cesium-137 source, doses ranging from 0 to 10 Gy. Immediately prior and during IR, cells were treated with ascorbate (0.25 mmol/L) in DMEM with 10% FBS for 1 hour. After treatment, the media were changed, and cells were allowed to recover for 72 hours. The media were removed, and cells were then incubated in a solution of MITT, 1 mg/mL (Sigma-Aldrich), dissolved in serum-free DMEM at 37°C for 3 hours in the dark. At the end of the incubation time, the media were removed, and DMSO was added to each plate to dissolve the precipitate. Samples were transferred to a 96-well plate for plate reader analysis and read at 590 nm on a Tecan SpectraFluor Plus plate reader (Tecan).

Catalase treatment

To determine whether H₂O₂ was responsible for the cytotoxic effects of ascorbate and radiation, cells were treated with various forms of catalase, including adenovirus catalase (AdCAT), bovine catalase (100 U/mL), or catalase-polyethylene glycol (PEG-CAT; 200 U/mL). Catalase and PEG-catalase were purchased from Sigma-Aldrich. The AdCAT construct used was a replication-defective, E1- and partial E3-deleted recombinant adenovirus (16). Inserted into the E1 region of the adenovirus genome is the human catalase gene, which is driven by a cytomegalovirus promoter. For the adenovirus experiments, approximately 10⁶ cells were plated in 10 mL of complete media in a 100 mm² tissue culture dish and allowed to attach for 24 hours. Cells were then washed three times in serum- and antibiotic-free media. The adenovirus constructs were applied to cells in 4 mL of serum- and antibiotic-free media. Control cells were treated with the empty adenovirus (AdEmpty) construct. Cells were incubated with the adenovirus constructs for 24 hours. Media were replaced with 4 mL of complete media for an additional 24 hours before cells were harvested for Western blot or treated for clonogenic assay.

Cell cycle analysis

Cell viability was measured by flow cytometry using propidium iodide (PI). After treatment, cells were collected by trypsinization, centrifuged at 500 g, and resuspended in 500 µL of Hank’s Buffered Salt Solution. After addition of 5 µL of PI (50 µg/mL), cells were incubated in the dark at room temperature for 5 minutes. PI fluorescence was analyzed by flow cytometry (excitation at 488 nm, emission at >530 nm). To analyze alterations in cell cycle by quantitation of DNA content, cells were collected and fixed in suspension with 70% ethanol for 4 hours at 4°C. Cells were washed with 1 mL PBS, centrifuged, and resuspended in 100 µL RNase A (1 mg/mL in PBS). After 30-minute incubation at room temperature, 500 µL PI (35 µg/mL in PBS) was added to each sample. After 1-hour incubation in the dark at room temperature, PI fluorescence was analyzed by flow cytometry.

Determination of intracellular hydrogen peroxide

Intracellular H₂O₂ concentrations were determined by analysis of the rate of aminotriazole-mediated inactivation of endogenous catalase activity (17). Catalase is irreversibly inactivated by aminotriazole (3-AT, 3-amino-1,2,4-triazole; Sigma-Aldrich) in the presence of H₂O₂. Cells grown in 150 mm² culture dishes were irradiated at 3 Gy and then treated with ascorbate (20 mmol/L) in the presence of 3-AT (20 mmol/L) for 0, 5, 10, 20, 30, 60, and 120 minutes at 37°C. Cells were washed with ice-cold PBS, harvested, and lysed by freeze/thaw at -80°C followed by room temperature. To determine the amount of fully active cellular catalase, cell lysates (2 mL) were introduced into the reaction chamber of an oxygen monitor (YSI model 5300, YSI Inc.). Then 333 µmol/L of H₂O₂ was injected into the reaction chamber, and the rate of production of oxygen was continuously recorded for 5 minutes or until the curve reached a plateau. The rate of appearance of dioxygen reflects the amount of active catalase in the cell lysate. Cell protein was determined by Bio-Rad DC protein assay. The intracellular steady-state concentration of H₂O₂ was calculated from the equation [H₂O₂]ss = k_activation/k_1, where k_1 is the rate constant for the formation of catalase compound I.

The rate of extracellular production of H₂O₂ was determined with a Clark electrode as in refs. 1 and 5.

Intracellular GSH and GSSG measurement

Cells grown in 100 mm² dishes were treated with IR, ascorbate, or in combination. After treatments, cells were washed with PBS and harvested by trypsinization (floating cells in culture media and PBS were collected and combined with trypsinized cells).
After centrifugation, cell pellets were suspended in 100 μL per-chloric acid buffer [5% perchloric acid with 100 μmol/L DETAPAC (diethylentriaminopentaneacetic acid). Sigma-Aldrich Chemical Co.] to precipitate protein. Samples were sonicated and centrifuged at 18,500 g for 5 minutes at 4°C using an Eppendorf Microcentrifuge. Supernatants were collected and stored at −80°C or analyzed immediately using HPLC (ESA CoulArray; Dionex/Thermo Scientific) with electrochemical detection (ECD) following the protocol described in Park and colleagues (18). In addition, in separate groups of treated mice, red blood cells (RBC) were harvested and centrifuged (500 g for 5 minutes). The plasma was removed from the sample, and the remaining pellet of intact RBCs was washed twice with cold isotonic saline. An aliquot was removed for counting by a hemocytometer. From the RBC pellet, 100 μL of RBCs were lysed with 300 μL of perchloric acid buffer and then centrifuged to pellet the protein (500 g, 5 minutes). The clean supernatant was stored at −80°C or immediately analyzed for total glutathione (GSH) using a plate-reader–based assay (19).

**Immunoblot analysis**

Protein (10–40 μg) was electrophoresed in a 4% to 20% Bio-Rad ready gel and then electrotransferred to an Immobilon PVDF membrane (EMD Millipore). Membranes were blocked in 5% nonfat milk for 1 hour, then treated with anti-γ-H2AX (Ser 139) antibody (1:1,000; EMD Millipore), or with anti-catalase antibody (1:5,000 dilution; Cell Signaling Technology). Horseradish peroxidase (HRP)–conjugated goat anti-rabbit or goat antimouse (1:50,000; Chemicon International) was used as a secondary antibody. Anti-GAPDH (1:1,000; EMD Millipore) or anti-actin (1:4,000; Sigma-Aldrich) was used as a loading control. Blots were treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposed to autoradiography film.

**Immunohistochemistry**

MIA PaCa-2 cells were seeded in 8-well (0.8 cm²/well) glass Lab-Tek Chamber slides at a density of 30,000 cells/well. After 48 hours, cells were treated with ascorbate prior to IR treatment. Cells were fixed in 3% paraformaldehyde and rehydrated in Dulbecco’s PBS (pH 7.4) 30 minutes after treatment and then incubated in RNase (0.5 μg/mL) at 37°C for 30 minutes, and 10% normal goat serum was applied overnight for blocking. Cells were incubated in anti-γ-H2AX primary antibody (1:200; Calbiochem) for 1.5 hours. A goat anti-rabbit antibody conjugated to Alexa Fluor 488 (1:500) was used as a secondary antibody to fluorescently tag γ-H2AX. Slides were mounted in Vectashield with DAPI as a nuclear counterstain. Slides were imaged on a Bio-Rad Radiance 2100 confocal multiphoton microscope with laser sharp software. Z-plane images of each treatment were captured, and the intensity of fluorescence was quantified on ImageJ using a maximum intensity Z-projection with identical threshold values to calculate the mean fluorescence intensity for individual cells.

**Animal experiments**

Thirty-day-old athymic nude mice were obtained from Harlan Sprague-Dawley. The nude mice protocol was reviewed and approved by the Animal Care and Use Committee of The University of Iowa. The animals were housed in a cage and were fed a sterile commercial stock diet and tap water, ad libitum. Animals were allowed to acclimate in the unit for 1 week before any manipulations were performed. Each experimental group consisted of 9 to 12 mice. MIA PaCa-2 or PANC-1 human pancreatic tumor cells (2 × 10⁶) were delivered subcutaneously into the flank region of nude mice with a 1-mL tuberculin syringe equipped with a 25-gauge needle. The tumors were allowed to grow until they reached between 3 mm and 4 mm in greatest dimension (2 weeks), at which time the mice were randomized and treatment was initiated. This was defined as day 1 of the experiment. Before IR, the animals were anesthetized with 80 to 100 mg/kg ketamine and 10 mg/kg xylazine i.p. and shielded in a lead block such that only the tumor-bearing right hind flank was irradiated. Tumor size was measured twice a week using a digital caliper, and tumor volume was estimated according to the following formula: tumor volume = π/6 × L × W², where L is the greatest dimension of the tumor, and W is the dimension of the tumor in the perpendicular direction (20). Animals were euthanized by CO₂ asphyxiation when the tumors reached a predetermined size of 1,000 mm³. In the orthotopic pancreatic cancer model, 6-week-old athymic nude mice were injected with the human pancreatic cancer cell line MIA PaCa-2, which had previously been stably transfected with firefly luciferase. Ultrasound guidance was used to identify the pancreas as described (21), and 400,000 cells suspended in 20 μL of a 1:1 mixture of PBS and Matrigel were injected into the pancreas under direct visualization. Bioluminescence-imaging microscopy was used to identify mice with established tumors within the pancreas, and tumor-containing mice were randomly assigned and treatment was initiated. Tumor growth was monitored using bioluminescence-imaging microscopy to determine the average radiance (photons/s/cm²) of the tumors.

In separate groups of treated animals, mouse tumors were excised and processed as described to determine 4-hydroxy-2-nonalen-(4HNE)–modified proteins (22). Briefly, tissues were homogenized and 25 μg of protein was blotted onto PVDF membranes. Blots were incubated with the primary antibody recognizing the Michael addition product of 4HNE-modified cellular proteins (23) diluted 1:2,000 overnight at 4°C, followed by 2 hours in secondary antibody, HRP-conjugated goat anti-rabbit polyclonal antibody (1:20,000), and chemiluminescence detection with X-ray film. Immunoreactive protein on the dot blot was analyzed using integrated densities determined with ImageJ software.

In separate groups of mice, a crypt cell assay was performed. Mice were exposed to 10 Gy or 13 Gy of total abdominal radiation with and without ascorbate (4 g/kg/d). After 48 hours, each animal was sacrificed and sections were made of the jejunum and then viewed under light microscopy and expressed as surviving cells/circumference. In addition, blood samples for TNFα were collected into 4.0 mL heparinized vial from control mice and mice treated with radiation (13 Gy) and/or ascorbate (4g/kg). Samples were centrifuged at 200 g for 20 minutes. Serum samples were collected from each tube and stored at −80°C. TNFα levels in the serum were measured using a Quantikine ELISA Kit (R&D Systems).

**Statistical analysis**

A single factor ANOVA followed by the Tukey post hoc test was used to determine statistical differences between mean for multiple comparisons, or a Student t test was used when only two comparisons. All mean were calculated from three experiments, with error bars representing the SEM. All Western blots were repeated at least twice. All data are expressed as mean ± SEM. A dose-modifying factor (DMF) was calculated for each cell line.
Figure 1. Ascorbate radiosensitizes pancreatic cancer cells. A, MIA PaCa-2 pancreatic cancer cells were irradiated (0–3 Gy) with and without ascorbate (0.25 mmol/L) and clonogenic survival determined. The resulting dose modification factor at 10% iso-survival was 2.5, indicating enhanced radiosensitivity (mean ± SEM, n = 3). Pharmacologic ascorbate (0.25 mmol/L) produced a flux of H$_2$O$_2$ of 550 amol cell$^{-1}$ s$^{-1}$ under our experimental conditions. This rate is considerably greater than the rate of cellular oxygen consumption by these cells, 57 amol cell$^{-1}$ s$^{-1}$ (29). B, PANC-1 pancreatic cancer cells were irradiated (0–10 Gy) and treated with ascorbate (0.25 mmol/L) in a similar fashion. Clonogenic survival yielded a DMF of 2.2, indicating enhanced radiosensitivity (mean ± SEM, n = 3). C, AsPC-1 human pancreatic cancer cells were irradiated (0–10 Gy) with and without ascorbate (0.25 mmol/L) and clonogenic survival determined. The resulting dose modification factor at 10% iso-survival was 1.25, indicating enhanced radiosensitivity (mean ± SEM, n = 3). D, H6c7 immortalized human pancreatic ductal epithelial cells were irradiated (0–6 Gy) and treated with ascorbate (0.25 mmol/L) to determine clonogenic survival. The clonogenic survival assay demonstrates no radiosensitization after IR with or without ascorbate (mean ± SEM; n = 3). E, MIA PaCa-2 pancreatic cancer cells were irradiated (0–4 Gy) and treated with ascorbate (0.25 mmol/L) to determine viability using the MTT assay and demonstrated radiosensitization. Data were normalized to drug or vehicle control (mean ± SEM; n = 3). F, H6c7 immortalized human pancreatic ductal epithelial cells were irradiated (0–10 Gy) and treated with ascorbate (0.25 mmol/L) to determine cell viability. The MTT assay demonstrates minimal changes in cell viability after IR with or without ascorbate (mean ± SEM; n = 3). G, patient-derived pancreatic cancer cells (339) in 4% O$_2$ were irradiated (0–6 Gy) with and without ascorbate (0.5 mmol/L) and clonogenic survival determined. The resulting dose modification factor at 40% iso-survival was 1.4, indicating enhanced radiosensitivity (mean ± SEM; n = 3). H, patient-derived pancreatic cancer cells (403) in 4% O$_2$ were irradiated (0–6 Gy) with and without ascorbate (0.5 mmol/L) and clonogenic survival determined. The resulting dose modification factor at 40% iso-survival was 1.6, indicating enhanced radiosensitivity (mean ± SEM; n = 3).
based on clonogenic surviving fraction. For the in vivo studies, the statistical analyses focused on the effects of different treatments on tumor progression. The primary outcomes of interest were time to death and tumor growth over time. The log-rank test was used to compare the survival times between treatment groups. Kaplan–Meier survival plots were constructed to estimate survival. Linear mixed effects regression models were used to estimate and compare group-specific tumor growth curves. Tests of statistical significance were two-sided and performed using the Systat and SAS software.

Results
Ascorbate enhances IR-induced cytotoxicity
Previous studies from our laboratory have demonstrated that pharmacologic ascorbate is cytotoxic to pancreatic cancer cells while normal cells are resistant (1). To test the effect of ascorbate on radiation response in cancer versus noncancerous cell lines, we performed clonogenic (Fig. 1A–D) and cell viability assays (Fig. 1E and F) using three pancreatic ductal adenocarcinomas [MIA PaCa-2, PANC-1 (Fig. 1A and B), and AsPC-1 (Fig. 1C)], compared with normal nontumorigenic H6c7 pancreatic ductal epithelial cells (Fig. 1D). The radiation survival curves were normalized to ascorbate- or sham-treated controls and fit with a linear quadratic model. Pharmacologic ascorbate (0.25 mmol/L) enhanced IR-induced decreases in clonogenic survival. The pancreatic cancer cell lines showed dose modification factors at 10% iso-survival of 2.5 (MIA PaCa-2), 2.2 (PANC-1; Fig. 1A and B), and 1.25 (AsPC-1; Fig. 1C). In contrast, no radiosensitization was noted with ascorbate in the normal nontumorigenic H6c7 pancreatic ductal epithelial cells (Fig. 1D). Similar results were seen using cell viability where there was radiosensitization in the MIA PaCa-2 cell line (Fig. 1E) but no radiosensitization in the H6c7 cell line (Fig. 1F). To determine if ascorbate radiosensitization occurs in the physiologically relevant environment seen in pancreatic cancer, we treated patient-derived pancreatic cancer cell lines, 339 (Fig. 1G) and 403 (Fig. 1H; refs. 13, 14) in 4% O2. Again, we demonstrated ascorbate radiosensitization in the 339 line with a
DMF of 1.4 at 40% iso-survival and a DMF of 1.6 in the 403 cell line at 40% iso-survival. The timing for the administration of pharmacologic ascorbate is also important. Treating cells with ascorbate for 1 hour prior to IR or 1 hour immediately after IR demonstrated a similar decrease in clonogenic survival when compared with IR or ascorbate alone. However, when ascorbate was administered 6 hours after IR, the decrease in clonogenic survival was decreased (Supplementary Fig. S1). These data strongly support the hypothesis that pharmacologic ascorbate is a selective radiosensitizer in pancreatic cancer cells versus normal nontumorigenic pancreatic ductal epithelial cells.

Cell cycle analysis of ascorbate-induced radiosensitization

Hydrogen peroxide generation has been reported to induce cell cycle arrest at G1 and G2 (24). IR-induced DNA damage also triggers G1 or G2 arrest, allowing time for cells to repair DNA damage (25). In MIA PaCa-2 cells treated with ascorbate (2 mmol/L) or 2 Gy alone or combination for 6, 24, 48, and 72 hours, there was no significant change in cell cycle distribution. However, the sub-G1 population increased in cells treated with ascorbate (2 mmol/L) or combination of 2 Gy IR and ascorbate (2 mmol/L) at 6 and 24 hours. At 24 hours, there was a significant sub-G1 fraction in the combination group compared with the control, IR alone, and ascorbate alone groups (Supplementary Fig. S2). In addition, the PI viability assay at 24 hours after treatment demonstrated that there was no significant change in viability with IR (2 Gy) alone while pharmacologic ascorbate alone induced an 89% sub-G1 fraction that was increased to 96% ± 2% with the combination treatment. These data, taken together with the clonogenic survival and cell viability assays, strongly support the hypothesis that pharmacologic ascorbate is a radiosensitizer in pancreatic cancer cells.

Ascorbate radiosensitization is mediated by H2O2

Ascorbate-induced cytotoxicity is mediated by the formation of H2O2 during the oxidation of ascorbate. Similarly, IR generates numerous ROS, including H2O2 and hydroxyl radical from the radiolysis of water. We hypothesized that ascorbate-induced radiosensitization is mediated by H2O2. To determine if intracellular levels of H2O2 change upon exposure to pharmacologic...
Figure 4.
Pharmacologic ascorbate radiosensitization in vivo. A, linear mixed effects regression models were used to estimate and compare group-specific tumor growth curves. Tumor growth was significantly inhibited with the ascorbate + IR treatment compared with control animals or animals that received ascorbate alone. Control was saline (1 mol/L NaCl i.p. daily, 22.7 μL/g); IR (7.5 Gy on days 5 and 8 and 1 mol/L NaCl i.p. daily); ascorbate (4 g/kg i.p. daily); or ascorbate + IR (mean ± SEM, n = 9–11 animals/group). B, Kaplan-Meier survival plots demonstrating survival as a function of time. The log-rank test was used for pairwise treatment group comparisons of survival between treatment groups, demonstrating significantly increased overall survival of animals receiving pharmacologic ascorbate and IR. C, pharmacologic ascorbate alters the status of the GSH redox buffer of RBCs. Blood was collected from separate groups of mice after treatments and assayed for the intracellular concentration of GSH in the RBCs. (Continued on the following page.)
Ascorbate radiosensitization

Ascorbate, intracellular concentrations of H$_2$O$_2$ were determined by the rate of aminotriazole-mediated inactivation of endogenous catalase activity. Cells were treated with IR (3 Gy), ascorbate (20 mmol/L), or in combination, and catalase activity was assayed in cells treated with 3-aminotriazole (3-AT). Using the stoichiometric inactivation of catalase in the presence of H$_2$O$_2$, and excess 3-AT, baseline intracellular concentration of H$_2$O$_2$ 6 hours after treatment was 53 ± 6 pmol/L in pancreas cancer cells; this increased to 71 ± 9 pmol/L with exposure to pharmacologic ascorbate, and 79 ± 14 pmol/L following IR. However, the combination of IR + ascorbate increased the concentration of intracellular H$_2$O$_2$ to 105 ± 2 pmol/L (Fig. 2A).

Under steady-state conditions, intracellular GSH is maintained at millimolar concentrations, which keeps cells in a reduced environment and serves as the principal intracellular redox buffer when cells are subjected to an oxidative stressor including H$_2$O$_2$ (26). Glutathione peroxidase (GPx) activity reduces the reduction of H$_2$O$_2$ to water with the conversion of GSH to glutathione disulfide (GSSG). Under steady-state conditions, GSSG is recycled back to GSH by glutathione disulfide reductase using reducing equivalents from NADPH. However, under conditions of increased H$_2$O$_2$ flux, this recycling mechanism may be overwhelmed, leading to a depletion of intracellular GSH (27, 28).

Because ascorbate combined with radiation significantly increases the intracellular H$_2$O$_2$, we next examined the effects of the combination of ascorbate and IR on the GSH/GSSG intracellular redox couple. MIA PaCa-2 cells were treated with IR (2 Gy) at ascorbate (2 mmol/L) and harvested at various time points (1–24 hours). Cells treated with IR + ascorbate showed a significant decrease in intracellular GSH concentration at 6 hours compared with untreated cells (2.5 ± 0.1 mmol/L vs. 2.1 ± 0.1 mmol/L, respectively, mean ± S.D., n = 3, P < 0.05) and failed to recover after 24 hours (Fig. 2B). The half-cell reduction potential ($E_{\text{PC}}$) of the GSSG/2GSH couple, which is a marker for overall redox environment of the cell (26), was calculated using the Nernst equation. $E_{\text{PC}}$ increased (more positive), indicating a more oxidized intracellular redox environment. This increase mirrored the depletion of GSH (Fig. 2B). MIA PaCa-2 cells have a baseline oxygen consumption rate of 57 amol cell$^{-1}$ s$^{-1}$ (29); assuming 1% efficiency, this corresponds to a metabolic rate for the production of H$_2$O$_2$ of less than 1 amol cell$^{-1}$ s$^{-1}$. The flux of H$_2$O$_2$ in these experiments was 1,400 amol cell$^{-1}$ s$^{-1}$, considerably greater than the metabolic flux, thus recycling of GSSG to GSH is rate-limiting and depletion of GSH and an increase (more positive) in $E_{\text{PC}}$ would be expected (28). Changes in $E_{\text{PC}}$ have been shown to correlate with biologic status of the cell with a change from −240 mV to −170 mV as cells shift from the state of proliferation to the onset of cell death (26). These results indicate that ascorbate radiosensitization can create an overwhelming oxidative stress to pancreatic cancer cells, resulting in oxidation/depletion of the GSH intracellular redox buffer, resulting in cell death.

To further determine whether H$_2$O$_2$ was responsible for the cytotoxic effects of ascorbate + radiation, cells were pretreated with either AdEmpty (50 MOI) or AdCAT (50 MOI) adenoviral vectors. Figure 2C demonstrates that there was robust expression of immunoreactive intracellular catalase in MIA PaCa-2 cells treated with the AdCAT vector when compared with control- or AdEmpty-pretreated cells. IR decreased clonogenic survival to 50% of AdEmpty alone values, whereas ascorbate (2 mmol/L) decreased clonogenic survival to 26% ± 2% (Fig. 2D). The combination of 2 Gy IR and ascorbate (2 mmol/L) further decreased clonogenic survival to 6% ± 1%. This decrease in clonogenic survival was significantly reversed, with overexpression of intracellular catalase resulting in clonogenic survival of 41% ± 1%, which was not significantly different from IR, suggesting that H$_2$O$_2$ mediates ascorbate radiosensitization (Fig. 2D).

**Ascorbate radiosensitization induces DNA damage**

γ-H2AX is a sensor of DNA strand breaks (30) and promotes efficient double-strand break repair (31). As shown in Fig. 3A, 2 Gy IR and ascorbate (2 mmol/L) induced formation of γ-H2AX in MIA PaCa-2 cells. However, the combination of IR and ascorbate increased formation of γ-H2AX, indicating a greater number of double-strand breaks. Previous studies have demonstrated that ascorbate-induced cytotoxicity is mediated by generation of H$_2$O$_2$ (1–3). To determine if H$_2$O$_2$ mediates the increase in γ-H2AX during ascorbate-induced radiosensitization, cells were treated with extracellular catalase. Figure 3B demonstrates that the combination treatment of IR and ascorbate induced increased formation of γ-H2AX in AsPC-1 cells compared with either treatment alone. The increase in γ-H2AX with the combination treatment was inhibited with catalase pretreatment, suggesting that H$_2$O$_2$ mediates the increase in γ-H2AX during ascorbate-induced radio-sensitization. In addition to Western blot, we demonstrated similar increases in double-strand DNA breaks as seen by γ-H2AX on immunohistochemistry (Fig. 3C). In cells treated with ascorbate (1 mmol/L), IR alone, or the combination of the two treatments, the increase in fluorescence was reversed with catalase pretreatment. Once again, quantification of these images demonstrated significant increases in fluorescence after ascorbate treatment and after IR + ascorbate combination treatment that was ameliorated with catalase pretreatment (Fig. 3D). These experiments were repeated with varying doses of pharmacologic ascorbate and varying doses of IR with similar results (Supplementary Fig. S3). Thus, H$_2$O$_2$ significantly contributes to the DNA damage observed upon exposure to pharmacologic ascorbate and IR, as seen by γ-H2AX.

**Ascorbate-induced radiosensitization in vivo**

We have previously demonstrated that ascorbate alone inhibits tumor growth in a mouse xenograft model with intraperitoneal administration of 4 g/kg twice daily (1). Pre-established MIA PaCa-2 tumors in nude mice were treated with either: saline (1 mol/L NaCl i.p. daily); IR (7.5 Gy on days 5 and 8 and...
1 mol/L NaCl i.p. daily); ascorbate (4 g/kg i.p. daily); or ascorbate + IR. Supplementary Table S1 provides statistical summaries of tumor volumes used in the mixed linear regression analysis of growth curves. The sample sizes (N) given in the table are the total number of measurements available within each group. Pairwise comparisons were carried out to assess group differences. Treatment with the combination of ascorbate + IR significantly delayed tumor growth compared with controls or ascorbate alone (Fig. 4A). Summary statistics for the survival analysis are presented in Supplementary Table S2. The estimated survival curve for the treatment groups is shown in Fig. 4B. Ascorbate + IR also significantly increased overall survival compared with controls, IR alone, or ascorbate alone (Fig. 4B). Most notably, 54% of mice treated with the combination of IR + ascorbate had no measurable tumors (Supplementary Table S2). Treatment was well tolerated as evidenced by similar weight gain patterns in all groups of mice (Supplementary Fig. S4).

**Oxidative stress indicators**

Glutathione is a measurable marker indicative of the oxidative state of the thiol redox buffer in cells. In severe systemic oxidative stress, the GSSG/2GSH couple may become oxidized, i.e., the concentration of GSH decreases and GSSG may increase because the capacity to recycle GSSG to GSH becomes rate-limiting. As seen in Fig. 4C, intracellular concentrations of GSH in red blood cells of mice after ascorbate, IR, and ascorbate + IR treatments decreased. However, the addition of pharmacologic ascorbate with IR did not significantly reduce GSH levels compared with 5 Gy IR alone. This suggests that the very high levels of pharmacologic ascorbate in these experiments may have a pro-oxidant effect on red blood cells as seen by a decrease in the capacity of the intracellular redox buffer. To further determine the effect of these treatments on hematological parameters, complete blood counts were determined in separate groups of mice. Although ascorbate and IR decreased intracellular GSH in red blood cells, there were no significant changes in hemoglobin and/or hematocrits between groups of mice. As seen in Fig. 4D, mice that received IR alone or IR + ascorbate had a significant decrease in white blood cells, suggesting some immunosuppression with IR. However, ascorbate did not add to further decreases in white blood cells, and the percentage of neutrophils and lymphocyte counts were unchanged between groups.

Oxidation of lipids generates highly reactive aldehydes, such as 4-hydroxynonenal (4-HNE), which can be used as a marker of oxidative damage (32, 33). To further examine systemic oxidative stress, we harvested heart, kidney, and liver from mice treated with the various combinations to determine 4-HNE-modified proteins. As shown in Fig. 4E, there were no differences in 4-HNE-modified proteins in heart tissue in mice treated with the various combinations. In addition, dot blot analysis of the other organs demonstrated no differences in 4-HNE proteins after the various treatments (Fig. 4F). These data support the hypothesis that ascorbate radiosensitization does not cause an increase in oxidative damage from lipid-derived aldehydes to other organs.

Orthotopic tumors were established using ultrasound guidance as described (Fig. 5A; ref. 21). After bioluminescence-imaging microscopy identified mice with established tumors within the pancreas (Fig. 5B), mice were randomly assigned to one of four treatment groups: controls received NaCl (1 mol/L) i.p. daily; ascorbate (4 g/kg i.p. daily); NaCl i.p. daily and two separate fractions of 5 Gy IR one week apart on days 4 and 11; a group that received both IR and ascorbate. Tumor sizes were measured weekly throughout the experiments, resulting in repeated measurements for each mouse. Linear mixed effects regression models were used to estimate and compare group-specific tumor growth curves. Supplementary Table S3 provides statistical summaries of tumor volumes used in the linear mixed effects regression analysis of growth curves. The sample sizes (N) given in the table are the total number of measurements available within each group. Pairwise comparisons were carried out to assess group differences. P values for the comparisons are listed in Supplementary Table S3. Tumor growth as determined by bioluminescence-imaging
microscopy (mean radiance photons/s/cm²) demonstrated a decrease in tumor growth of animals receiving both IR and ascorbate compared with controls and compared with IR alone (\( \text{C}3 \), \( P < 0.05 \) control vs. Ascorbate + IR; \( \text{C}3 \), \( P < 0.05 \) Ascorbate + IR vs. IR alone; Fig. 5C; Supplementary Fig. S5). As seen in the previous heterotopic study, weights of the animals did not change during the study (data not shown).

Radiation-induced gastrointestinal toxicity is highly relevant to the treatment of pancreatic cancer with radiation. To determine if pharmacologic ascorbate changes the response of the gastrointestinal tract following radiation in a clinically meaningful way, a crypt cell assay was performed (Fig. 6). Control animals and those treated with pharmacologic ascorbate (4 g/kg) had similar degrees of crypt cell generation. IR alone (10 Gy) decreased crypts to 28% of control values, while 13 Gy decreased crypts to 44% of control values. Addition of pharmacologic ascorbate partially reversed the decreases in both the 10 Gy and 13 Gy groups of mice, suggesting that ascorbate may protect the gastrointestinal tract from the damaging effects of IR. In addition, previous studies have demonstrated that radiation-induced jejunal toxicity was accompanied by increases in serum TNFα (34). TNFα was 7.5 ± 2.2 pg/mL in controls and was increased to 25.0 ± 1.5 pg/mL after 13 Gy. Treatment with pharmacologic ascorbate combined with IR decreased TNFα to 12.5 ± 0.7 pg/mL (mean ± SEM, \( n = 3, P < \)
Treatment was well tolerated as evident by similar weight gain and tumor growth was significantly inhibited in mice with pancreatic tumor xenografts treated with IR/gemcitabine compared with controls and ascorbate alone. Tumor growth was further inhibited in mice that received the ascorbate + IR/gemcitabine treatment compared with control animals or animals that received ascorbate alone (mean ± SEM, n = 11-12 mice/group). B, Kaplan–Meier survival plots demonstrating survival as a function of time. The log-rank test was used for pairwise treatment group comparisons of survival between treatment groups, demonstrating significantly increased overall survival for animals receiving pharmacologic ascorbate and IR/gemcitabine treated on day 43 after the initiation of treatment. C, weight changes of mice during treatment periods in mice during treatments from day 1 through day 15.

Discussion

Our current study demonstrates the potential for pharmacologic ascorbate as a radiosensitizer in the treatment of pancreatic cancer. IR is the standard of care in pancreatic cancer in a variety of clinical scenarios, including (i) locally advanced pancreatic cancer; (ii) positive margins or positive lymph nodes after pancreatic resection; or (iii) large tumors that obstruct the duodenum (36). Chemotherapy combined with radiotherapy has also shown to improve survival in pancreatic cancer when compared with single-modality therapy (37). In addition, radiotherapy can also provide palliation to patients with locally advanced disease (38). Thus, pharmacologic ascorbate-induced radiosensitization may have clinical benefits.

In our present study, we have shown that pharmacologic ascorbate significantly decreases clonogenic survival and inhibits the growth of all pancreatic cancer cell lines as a single agent, as well as sensitizes cancer cells to IR. This corresponds well with other reports demonstrating that pharmacologic ascorbate enhances IR-induced cell killing and DNA fragmentation, leading to induction of apoptosis in HL60 leukemia cells (39). In addition, Hurst and colleagues demonstrated that pharmacologic ascorbate combined with IR leads to increased numbers of double-strand DNA breaks and cell cycle arrest when compared with either treatment alone (40). Our previous studies demonstrated that pharmacologic ascorbate could serve as a "prodrug" for the delivery of H2O2 to tumors (1-4). There was both a time- and dose-dependent increase in measured H2O2 production with increased concentrations of ascorbate. Others have demonstrated increased levels of double-strand breaks with pharmacologic ascorbate and H2O2 treatment to tumor cells (41, 42). In addition, the double-strand breaks induced by H2O2 were more slowly repaired.

Previously, we demonstrated that the immortalized, nontumorigenic pancreatic ductal epithelial cells H6c7 are resistant to...
ascorbate-induced toxicity (1). Consistent with this study, our current study demonstrates that H6c7 cells are also resistant to the combination treatment of IR and ascorbate. The selective ascorbate-induced cytotoxicity may be due to the combination of low levels of antioxidant enzymes and high endogenous levels of ROS in cancer cells (43–45). Moreover, tumor cells are often defective in DNA repair, whereas normal cells are proficient (46). The combination of ascorbate and IR provides two distinct mechanisms of action: ascorbate-induced toxicity due to extracellular production of H$_2$O$_2$ that then diffuses into cells and causes damage to DNA, protein, and lipids; and radiation-induced toxicity as a result of ROS-induced damage to DNA. In addition, reduct metals like Fe$^{2+}$ may play an important role in ascorbate-induced cytotoxicity. By catalyzing the oxidation of ascorbate, labile iron can enhance the rate of formation of H$_2$O$_2$. Labile iron can also react with H$_2$O$_2$. Recently, our group has demonstrated that pharmacologic ascorbate and IR increase the labile iron in tumor homogenates from this murine model of pancreatic cancer (47). In our present study using a pancreatic cancer xenograft model, we demonstrated that ascorbate or IR alone decreased tumor growth, but the combination treatment further inhibited tumor growth, indicating that pharmacologic ascorbate is an effective radiosensitizer in vivo. In addition, all groups of mice had similar weight gain during treatment, suggesting lack of normal tissue toxicity with the combination treatment. That approximately 30% of pancreatic patients receive a diagnosis of advanced locoregional disease underscores the importance of radiotherapy for local disease control (48). Our preclinical model shows that the combination of IR and ascorbate enhances toxicity in pancreatic cancer cells compared with either treatment alone. In a phase 1 clinical trial that investigated the use of pharmacologic ascorbate as an adjuvant to gemcitabine, the standard of care, in the treatment of pancreatic cancer, it was well-tolerated with few dose-limiting toxicities (49). The data reported here support testing of pharmacologic ascorbate as an adjuvant treatment for radiotherapy in pancreatic cancer patients after surgical resection or with locally advanced disease. Indeed, the preclinical translational studies in this current study have led us to develop and implement a phase 1 clinical trial (www.clinicaltrials.gov; NCT01852890, Cullen PI) with FDA approval (IND 105715, Cullen sponsor-investigator).

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

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References


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