

Mechanisms of Ascorbate-Induced Cytotoxicity in Pancreatic Cancer

Juan Du², Sean M. Martin³, Mark Levine⁶, Brett A. Wagner², Garry R. Buettner^{2,4}, Sih-han Wang³, Agshin F. Taghiyev³, Changbin Du², Charles M. Knudson³, and Joseph J. Cullen^{1,2,4,5}

Abstract

Purpose: Pharmacologic concentrations of ascorbate may be effective in cancer therapeutics. We hypothesized that ascorbate concentrations achievable with i.v. dosing would be cytotoxic in pancreatic cancer for which the 5-year survival is <3%.

Experimental Design: Pancreatic cancer cell lines were treated with ascorbate (0, 5, or 10 mmol/L) for 1 hour, then viability and clonogenic survival were determined. Pancreatic tumor cells were delivered s.c. into the flank region of nude mice and allowed to grow at which time they were randomized to receive either ascorbate (4 g/kg) or osmotically equivalent saline (1 mol/L) i.p. for 2 weeks.

Results: There was a time- and dose-dependent increase in measured H₂O₂ production with increased concentrations of ascorbate. Ascorbate decreased viability in all pancreatic cancer cell lines but had no effect on an immortalized pancreatic ductal epithelial cell line. Ascorbate decreased clonogenic survival of the pancreatic cancer cell lines, which was reversed by treatment of cells with scavengers of H₂O₂. Treatment with ascorbate induced a caspase-independent cell death that was associated with autophagy. *In vivo*, treatment with ascorbate inhibited tumor growth and prolonged survival.

Conclusions: These results show that pharmacologic doses of ascorbate, easily achievable in humans, may have potential for therapy in pancreatic cancer. *Clin Cancer Res*; 16(2): 509–20. ©2010 AACR.

Pancreatic cancer is the fourth most common cause of cancer death in the United States with 33,000 fatal cases annually in the United States alone (1). Surgical resection of the primary tumor remains the only potentially curative treatment for pancreatic cancer. However, in population-based studies, the number of patients undergoing resection with curative intent can be less than 3% (2). Even after resection, median survival is only 12 to 18 months and less than 20% of resected patients survive 5 years. The majority of patients die of metastatic cancer recurrence (3). Other adjuvant treatments such as radiation therapy and chemotherapy have not significantly improved long-term survival. The rate of chemotherapeutic response is less than 20% (4), whereas less than 10% of patients benefit from radiation therapy (3). Because of the lack of therapeutic responsiveness of pancreatic cancer to surgery,

chemotherapy, and radiation therapy, survival beyond 5 years is rare, with a median survival of less than 6 months. Thus, novel and effective therapies directed against pancreatic cancer are needed to control progression and metastatic disease.

Pharmacologic ascorbate concentrations have been shown to selectively kill some cancer cell types (5). Cell death was independent of metal chelators and dependent on H₂O₂ formation. H₂O₂ generation was dependent on ascorbate concentration and incubation time and displayed a linear relationship with ascorbate radical formation, ascorbate being the electron donor (6). Thus, pharmacologic ascorbate concentrations producing extracellular H₂O₂ diffuses into cells, causing cell death.

Chen and colleagues have hypothesized that pharmacologic ascorbate may deplete ATP in three mechanisms that would lead to cell death in tumors (6). First, DNA damage induced by H₂O₂ can activate poly(ADP-ribose) (PAR) polymerase (PARP). The activated PARP catabolizes NAD⁺, thereby depleting the substrate for NADH formation and subsequent ATP synthesis (7, 8). Second, H₂O₂ can be catabolized by concurrent oxidation of glutathione to glutathione disulfide. In reducing glutathione disulfide back to glutathione, glutathione reductase uses NADPH, which is provided by the pentose shunt from glucose. Glucose used to reduce NADP⁺ to NADPH cannot be used for glycolysis or NADH production so that ATP generation is decreased (9, 10). Finally, H₂O₂ could directly damage mitochondria, especially ATP synthase, so that ATP production decreases (11–13).

Authors' Affiliations: Departments of ¹Surgery, ²Radiation Oncology, and ³Pathology, University of Iowa College of Medicine; ⁴Holden Comprehensive Cancer Center and ⁵Veterans Affairs Medical Center, Iowa City, Iowa; and ⁶Molecular and Clinical Nutrition Section, Digestive Diseases Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

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Corresponding Author: Joseph J. Cullen, University of Iowa Hospitals and Clinics, 4605 JCP, Iowa City, IA 52242. Phone: 319-353-8297; Fax: 319-356-8378; E-mail: joseph-cullen@uiowa.edu.

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Translational Relevance

Our present study shows that production of H_2O_2 mediates the increased susceptibility of pancreatic cancer cells to ascorbate-induced metabolic oxidative stress. Our results show that ascorbate inhibits pancreatic cancer cell growth *in vitro* and *in vivo*. Treatment with ascorbate induced a caspase-independent cell death that was associated with autophagy. These results show that pharmacologic doses of ascorbate, easily achievable in humans, may have potential for therapy in pancreatic cancer. Ascorbate induces preferential cytotoxicity and oxidative stress in human pancreatic cancer cells and this mechanism provides evidence supporting the rational design of combined-modality pancreatic cancer therapy based on a fundamental difference between the biochemistry of oxidative metabolism in normal versus pancreatic cancer cells.

Recent studies have also suggested that ascorbate may lead to death through a unique caspase-independent autophagy pathway (14). Autophagy is characterized by the activation of a conserved biochemical pathway that results in the formation of double-membrane organelles that initially engulf cellular proteins and cytoplasmic organelles and subsequently fuse with lysosomes to facilitate the degradation of these components. Activation of this pathway is characterized by the biochemical processing of light chain 3 (LC3), which results in altered size of the protein and a redistribution of the protein. Activation of this pathway can either inhibit or promote cell death, depending on the cellular context. Anticancer drugs may induce both apoptosis and autophagy separately or simultaneously (15–17). In a recent study by Ohtani et al. (14), ascorbate in conjunction with the tumor suppressor gene 101F6 initiated autophagy, as determined by induction of acidic vesicular organelles and LC3 formation.

The goal of our study was to determine whether ascorbate is cytotoxic to pancreatic cancer cells and, if so, to characterize the mechanism of ascorbate-induced cytotoxicity. Our results show that ascorbate decreased clonogenic survival and cell viability. Although ascorbate decreased ATP levels in pancreatic cancer cell lines in a dose-dependent manner, this depletion may not play a role in ascorbate-induced cytotoxicity. Overexpression of extracellular and intracellular catalase reversed ascorbate-induced cytotoxicity. Treatment with ascorbate did not induce PARP cleavage and caspase inhibition did not reverse ascorbate-induced cell death. Ascorbate induced the processing and activation of LC3, a hallmark of autophagy. In mice with preestablished pancreatic tumors, systemic treatment with pharmacologic ascorbate treatment inhibited tumor growth and prolonged survival.

Materials and Methods

Cell culture. MIA PaCa-2, AsPC-1, and BxPC-3 human pancreatic adenocarcinoma cells were obtained from the American Type Culture Collection. MIA PaCa-2 was maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 2.5% horse serum. AsPC-1 was maintained in RPMI 1640 with 20% bovine serum and 1% sodium pyruvate. BxPC-3 was maintained in RPMI 1640 with 10% FBS. In addition, we used an immortalized cell line derived from normal pancreatic ductal epithelia with near-normal genotype and phenotype of pancreatic duct epithelial cells HPV16-E6E7 (H6c7), and the isogenic cell line that expresses *K-ras*^{G12V} H6c7eR-Kras+ (18). These cell lines were maintained in keratinocyte serum-free medium and supplemented with epidermal growth factor and bovine pituitary extract as previously described (19). To determine the role of mitochondrial oxidative phosphorylation in ascorbate-induced cytotoxicity, MIA PaCa-2 ρ^0 cells depleted of mitochondrial DNA were generated by incubating wild-type cells (ρ^+) for 6 to 8 wk with 100 ng/mL ethidium bromide. The medium was supplemented with 4.5 mg/mL glucose, 50 μ g/mL uridine, and 100 μ g/mL pyruvate to compensate for the respiratory metabolism deficit as previously described (20). After selection, the MIA PaCa-2 ρ^0 cells were cultured in the above specified medium without ethidium bromide. To verify mitochondrial DNA depletion, PCR showed the absence of mitochondrial DNA in ρ^0 cells as previously described (19). All media were obtained from Invitrogen, and all cell lines were maintained at 37°C in 5% CO_2 . All cells were routinely tested for *Mycoplasma* and only used when found to be negative.

Clonogenic cell survival experiments. At the beginning of each experiment, cells were rinsed with PBS and placed in medium. Cells were treated with ascorbate (0–20 mmol/L) from a stock solution of 1.0 mol/L ascorbate (pH 7.0), which was made under argon and stored in a volumetric flask with a tight-fitting stopper at 4°C. Ascorbate concentration was checked at 265 nm, $\epsilon = 14,500$ (mol/L)⁻¹ cm⁻¹. The solution can be kept for several weeks without significant loss of ascorbate due to the lack of oxygen (21). Cells were treated with ascorbate for 1 h at 37°C. Cells were then trypsinized, counted, diluted, and plated for clonogenic cell survival assay as previously described (22). Surviving colonies were fixed and stained after 10 to 14 d and counted under a dissecting microscope.

Cell viability. As an indicator of cell metabolic viability, the MTT assay was used. Cells were seeded at 1×10^4 in a 96-well plate in full medium. After 48 h, cells were treated with ascorbate for 1 h. For the cell viability experiments, all cell lines were treated in DMEM for consistency. Then, the cells were washed with PBS and incubated with fresh medium for an additional 24 h. MTT (1 mg/mL; Sigma) was added to the wells and incubated at 37°C for 3 h. At the end of incubation, the medium was removed and 100 μ L of DMSO were added to each well for cell lysis. The

plate was read at 590 nm on a Tecan SpectraFluor Plus plate reader (Tecan).

Measurement of H_2O_2 with oxygen electrode. Ascorbate interferes with many of the standard methods to measure H_2O_2 , especially peroxidase-based methods (23). H_2O_2 was measured using an oxygen electrode (oxygen monitor system, YSI Life Sciences). The oxygen electrode was stabilized for 15 min with 3 mL of air-saturated DMEM and 10% FBS in the chamber. The medium was replaced by 3 mL of medium containing various concentrations of ascorbate, and the recorder pen was adjusted to a position 50% of full-scale deflection. Catalase solution (1,000 units/mL) was injected through the cap, and H_2O_2 concentration was calculated from the spike of O_2 evolution.

Measurement of intracellular ATP levels. Cells were grown in 60-mm tissue culture dishes for 48 h before ascorbate treatment. Intracellular ATP was measured with a luciferase-based somatic ATP assay kit (Sigma). Briefly, cells were twice washed with PBS after exposure to ascorbate, and 400 μ L of somatic cell ATP-releasing agent were added directly to the dishes. After incubating for 5 min, samples were transferred to microcentrifuge tubes and cleared by centrifugation. The supernatant (100 μ L) was added to a 96-well tissue culture plate with equal volumes of ATP assay mix working solution added immediately before measuring luminescence. Luminescence was measured with Tecan Spectra Fluor Plus plate reader (Tecan). ATP levels were expressed as a percentage of control values. In addition to measurement of ATP levels, $NADP^+$ / $NADPH$ concentrations in MIA PaCa-2 cells were determined by using EnzyChrom $NADP^+$ / $NADPH$ assay kit (BioAssay Systems). The kit is based on a glucose dehydrogenase cycling reaction, in which a tetrazolium dye (MT) is reduced by $NADPH$ in the presence of phenazine methosulfate. The intensity of the reduced product color is proportionate to the $NADP^+$ / $NADPH$ concentration in the sample.

Treatment of cells with catalase. To determine whether H_2O_2 was responsible for the cytotoxic effects of ascorbate, cells were treated with various catalase preparations, including bovine catalase in the medium (100 μ g/mL) or catalase-polyethylene glycol (PEG-catalase; 200 units/mL). Catalase and PEG-catalase were purchased from Sigma. Pancreatic cancer cells were also infected with adenovirus constructs containing the catalase enzymes. The adenovirus constructs used were replication-defective, E1- and partial E3-deleted recombinant adenoviruses (20). Inserted into the E1 region of the adenovirus genome are the human catalase genes *CAT* and *mitCAT*, both of which are driven by a cytomegalovirus promoter. The AdCAT construct was originally prepared by John Engelhardt (University of Iowa, Iowa City, IA; ref. 24). The AdmitCAT construct was originally prepared by Dr. Andres Melendez with the full-length catalase cDNA combined with the MnSOD mitochondrial leader sequence added to the construct (25). Approximately 10^6 MIA PaCa-2 cells were plated in 10 mL of complete medium in a 100-mm² plastic dish and allowed to attach for 24 h. Cells were then

washed thrice in serum- and antibiotic-free medium. The adenovirus construct(s), 100 multiplicity of infection (MOI), suspended in 3% sucrose, was then applied to cells suspended in 4 mL of serum- and antibiotic-free medium. Control cells are treated with the adenovirus-empty construct. Cells were incubated with the adenovirus constructs for 24 h. The medium was then replaced with 4 mL of complete medium for an additional 24 h before cells were harvested.

Cell homogenization. Cells were washed thrice in PBS (pH 7.0), scraped from the dishes using a rubber policeman, and then collected in phosphate buffer (pH 7.8). This was followed by sonic disruption on ice for 30 s in 10-s bursts using a VibraCell sonicator (Sonics and Materials, Inc.) at 100% power. Protein concentration was determined using the Bio-Rad Bradford dye-binding protein assay kit according to the manufacturer's instructions.

Western blot analysis. Immunoreactive protein corresponding to catalase was identified and quantified from total cell protein by the specific reaction of the immobilized protein with its antibody. Protein (10-40 μ g) was electrophoresed in a 4% to 20% Bio-Rad ready gel. The proteins were then electrotitrated to Immobilon Transfer Membranes (Millipore). After blocking in 5% nonfat milk for 1 h, the membranes were treated with anti-catalase antibody (1:5,000 dilution, Santa Cruz Biotechnology), anti-PARP antibody (1:1,000, Cell Signaling Technology), polyclonal rabbit anti-PAR antibody (1:3,000 dilution, BD Biosciences), or anti-LC3 antibody (1:1,000, MBL International). Horseradish peroxidase-conjugated goat anti-rabbit (1:50,000 dilution) secondary antibody was from Chemicon International. The washed blots were then treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to Classic Blue Autoradiography Film (MIDSCI). All Western blots were done in duplicate.

Cell death measurements. Phosphatidylserine translocation to the outer leaflet of the plasma membrane was assessed by reaction with Annexin V-FITC apoptosis detection kit (BioVision) and detected by a FACScan flow cytometer. MIA PaCa-2 cells (1×10^6) were treated with varying doses of ascorbate and after 48 h, cells were harvested, washed with PBS, and resuspended in 500 μ L of binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$]. Cells were then incubated with 5 μ L of Annexin V conjugated with FITC plus 5 μ g/mL propidium iodide and incubated for 5 min at room temperature in the dark. Samples were then analyzed by flow cytometry to identify apoptotic and necrotic cells.

LC3-GFP MIA PaCa-2 cells. The pTZV3 Hygro-LC3-GFP plasmid was generated by cloning the LC3-GFP cDNA (obtained from Ana Marie Cuervo, Albert Einstein College of Medicine, Yeshiva University, New York, NY) into the pTZV3 vector (a kind gift of Dr. Andrei Gudkov, Cleveland Clinic, Cleveland, OH) in which the neomycin cassette was replaced with hygromycin. MIA PaCa-2 LC3-GFP cells were then generated using a HIV-based lentiviral system with the pTZV3 LC3-GFP plasmid. HEK 293T cells were

cultured in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 57 µmol/L 2-mercaptoethanol at 37°C with 5% CO₂. These cells were transfected with both a five-plasmid packaging mix (pTRE-GAG-PRO-PRE-polyA, pCMV-VpR-RT-IN-polyA, pCMV-VSV-G-polyA, pCMV-TEToff-polyA, and pCMV-TAT-REV) and the target plasmid pTZV3 Hygro-LC3-GFP as previously described (26). For each 75-cm² flask used, 3.75 µg of lentiviral packaging plasmids and 1.5 µg of target plasmid were resuspended in 300 µL of EC buffer and 42 µL of Enhancer, vortexed for 1 s, and incubated for 5 min at room temperature. Next, 100 µL of Effectene were added to the plasmid mix, vortexed for 20 s, and incubated at room temperature for 20 min. EC buffer, Enhancer, and Effectene were purchased from Qiagen. This transfection mix was used to transfect HEK 293T cells in fresh medium. At 18 h post-transfection, virus particles were harvested from the HEK 293T cells by collecting the medium and filtering with a 0.22-µm syringe filter (Millipore). This collected medium was then used to infect the MIA PaCa-2 cells by direct addition to these cells. The transduction procedure was then repeated five times over the next 3 days. Cells expressing LC3-GFP were then selected with 400 µg/mL hygromycin (Invitrogen) for 10 d in culture.

Autophagy measurements. MIA PaCa-2 wild-type or MIA PaCa-2 LC3-GFP cells were seeded at least 2 d before the ascorbate autophagy induction experiments to ensure ~400,000 cells per well of a six-well plate. Ascorbate (1, 2, or 5 mmol/L) or H₂O₂ (0.2 mmol/L) was added directly to each respective well and cells were then incubated for 1 h at 37°C in 5% CO₂. Following treatment, the medium was removed, cells were washed with Hyclone DPBS, fresh medium was then added, and the cells were incubated for an additional 24 h at 37°C in 5% CO₂. Following this incubation, the medium from each well was collected and each well was washed once with DPBS. The DPBS wash fraction was also collected and added to each respective medium sample. Next, cells were harvested and centrifuged at ~1,000 × g for 5 min to concentrate the samples. Cells were resuspended and propidium iodide (1 µg/mL) was added before running on a flow cytometer to discriminate live cells. In general 5,000 to 10,000 events were collected to examine for both the percentage of green fluorescent protein (GFP)-positive cells and the mean fluorescence intensity (MFI) of the GFP-positive cells.

Confocal microscopy. Cells were grown on a coverslip that had been autoclaved and then coated with 0.001% poly-L-lysine. Cells were treated with ascorbate or H₂O₂ as described above. After washing, the cells were fixed with 4% fresh paraformaldehyde in DPBS at room temperature for ~25 min. The cells were washed twice with DPBS and then incubated with 75 µL of freshly diluted TO-PRO-3 (1:2,000 in DPBS) for 5 min in the dark. TO-PRO-3 was then removed by wicking away with a paper towel and the coverslips were then mounted onto a slide with 30 µL VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories). Excess mounting

medium were removed and then sealed with finger nail polish. The samples were imaged with a confocal microscope equipped with a Kr/Ar laser. The LC3-GFP fluorescence was excited using the 488-nm laser line, whereas TOPRO-3 was excited with the 647-nm laser. Images were acquired at 1,024 × 1,024 pixels with a 60× 1.4-numerical-aperture oil immersion objective lens. Final images were prepared using Image J Software. All images used identical settings for acquisition and analysis.

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 four to a cage and fed a sterile commercial stock diet and tap water *ad libitum*. Animals were allowed to acclimate in the unit for 1 wk before any manipulations were done. Each experimental group consisted of 13 to 15 mice. MIA PaCa-2 tumor cells (2 × 10⁶) were delivered s.c. 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 and 4 mm in greatest dimension (from 10 d to 2 wk), at which time treatment was initiated.

Mice were divided into two treatment groups that received 1 mol/L NaCl i.p. (controls) twice a day for 14 d, or ascorbate 4 g/kg i.p. twice a day for 14 d. This was defined as day 1 of the experiment. Tumor size was measured every 2 to 3 d by means of a vernier caliper, and tumor volume was estimated according to the following formula: tumor volume = $\pi/6 \times L \times W^2$, where *L* is the greatest dimension of the tumor and *W* is the dimension of the tumor in the perpendicular direction (22). Animals were killed by CO₂ asphyxiation when the tumors reached a predetermined size of 1,000 mm³ and this was considered the time to sacrifice.

Statistical analysis. Statistical analysis was done by means of Systat (Systat, Inc.). A single-factor ANOVA, followed by post hoc Tukey test, was used to determine statistical differences between means. All means were calculated from three experiments, and error bars represent SEM. All Western blots were repeated at least twice. All data are expressed as means ± SEM. For the *in vivo* studies, the statistical analyses focused on the effects of different treatments on cancer progression. The primary outcomes of interest were time to death and tumor growth over time. Once tumors were visible, the mice were then randomly assigned to a treatment group and followed until death or until the experiment was terminated. The log-rank test was used to compare the survival times between treatment groups. Kaplan-Meier survival plots were constructed to estimate survival. Tumor sizes (mm³) were measured throughout the experiments, resulting in repeated measurements across time for each mouse. Linear mixed-effects regression models were used to estimate and compare the group-specific tumor growth curves. All tests were two-sided and carried out at the 5% level of significance. Analyses were done with the SAS and R statistical software packages.

Results

Ascorbate induces cytotoxicity in pancreatic cancer cells.

Previous studies have shown that ascorbate decreases cell viability in many, but not all, cancer cell lines (5). The effect of pharmacologic ascorbate on pancreatic cancer cell lines is unknown. We hypothesized that ascorbate would lead to cytotoxicity and cell death in pancreatic cancer. Figure 1A shows the differential susceptibility of the immortalized pancreatic ductal epithelial cell line H6c7 and its derivative, H6c7er-Kras+ (H6c7 cells expressing K-ras oncogene), and pancreatic cancer cells (MIA PaCa-2, AsPC-1, BxPC-3) to the effects of pharmacologic ascorbate concentrations. All cells were treated with ascorbate (0, 5, or 10 mmol/L) for 1 hour in DMEM. These doses have been shown to be readily achievable with little toxicity when administered to humans (27, 28). Using the MTT assay, there were significant decreases in cell viability in the pancreatic cancer cell lines treated with 5 or 10 mmol/L ascorbate compared with no treatment. In contrast, the immortalized H6c7 cell line had little change in viability with the same doses of ascorbate, whereas its derivative, the Kras+ cell line, had significant decreases in cell viability with ascorbate (5 and 10 mmol/L). In addition to measuring short-term cell viability, clonogenic cell survival assays were done to measure long-term effects of ascorbate on cancer cell growth (Fig. 1B and C). In the human pancreatic cancer cell lines MIA PaCa-2 and AsPC-1, pharmacologic doses of ascorbate (5, 10, or 20 mmol/L for 1 hour) markedly decreased clonogenic survival in a dose-dependent manner (Fig. 1B and C). The clonogenic cell survival assay showed that ascorbate treatment at higher doses resulted in no colony formation from 400 cells plated and treated with ascorbate (20 mmol/L) in the MIA PaCa-2 cell line (Fig. 1B). These results support the hypothesis that numerous pancreatic cancer cell lines are susceptible to ascorbate-induced cytotoxicity.

Ascorbate-induced cytotoxicity is due to H₂O₂. Previous studies have suggested that ascorbate-induced cell death is dependent on the generation of H₂O₂ through the ascorbate radical; ascorbate serves as the electron donor to O₂ to form H₂O₂ (5). We have previously observed a positive linear correlation for [H₂O₂] versus [AscH⁻] and a quadratic correlation with the ascorbate radical; that is, [H₂O₂] = function of ([Asc^{•-}]²). When ascorbate is infused i.v., the resulting pharmacologic concentration will distribute rapidly into the extracellular water space (6). Thus, pharmacologic ascorbate concentrations in the medium, as a surrogate for extracellular fluid, should generate the ascorbate radical and H₂O₂. Because AscH⁻ interferes with most peroxidase-based detection methods, we measured the formation of H₂O₂ upon addition of AscH⁻ using a Clark-type oxygen electrode (21). Oxygen evolution was measured upon introduction by catalase: 2H₂O₂ → 2H₂O + O₂. Ascorbate-mediated generation of H₂O₂ in cell culture medium was both time- and dose-dependent (Supplemental Fig. S1A and B). In Supplemental Fig. S1A, ascorbate in the medium for 1 hour increases H₂O₂

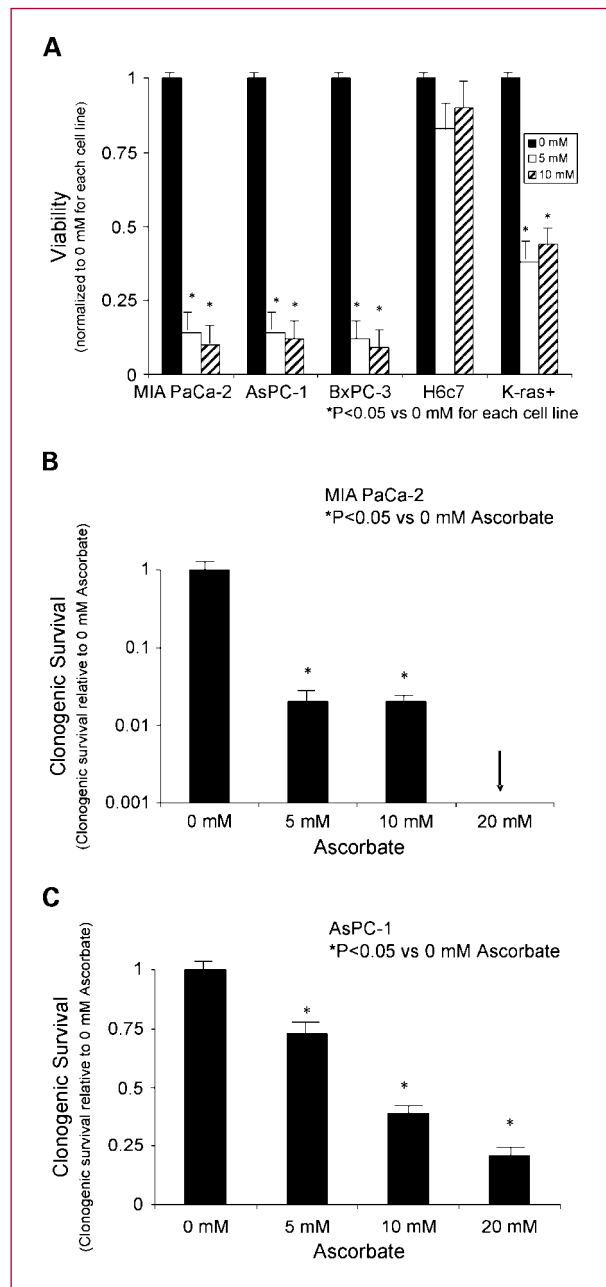


Fig. 1. A, effects of pharmacologic ascorbic acid concentrations on pancreatic cancer and pancreatic ductal epithelial cells. All cells were treated with ascorbate (0, 5, or 10 mmol/L) for 1 h. Cell viability was determined by the MTT assay. MIA PaCa-2, AsPC-1, and BxPC-3 are pancreatic cancer cell lines. The immortalized pancreatic ductal epithelial cell line H6c7 and its derivative, H6c7er-Kras (H6c7 cells expressing the K-ras oncogene), also received ascorbate (0, 5, or 10 mmol/L) for 1 h. Ascorbate decreased cell viability in all pancreatic cancer cell lines and in the H6c7 cell lines that express K-ras. B, MIA PaCa-2 pancreatic cancer cells were treated with ascorbate (0–20 mmol/L) for 1 h and clonogenic survival was determined. Ascorbate caused a dose-dependent decrease in clonogenic survival. Arrow indicates that no colonies were formed when 20 mmol/L ascorbate was given for 1 h. C, AsPC-1 pancreatic cancer cells were treated with ascorbate (0–20 mmol/L) for 1 h and clonogenic survival was determined. Ascorbate again caused a dose-dependent decrease in clonogenic survival.

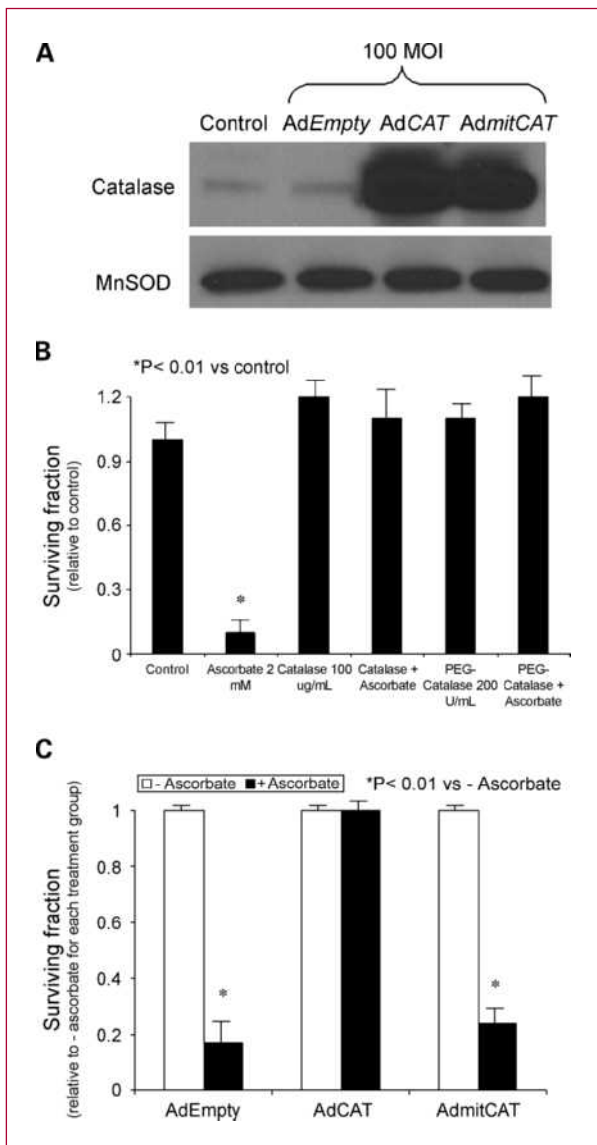


Fig. 2. Overexpression of extracellular and cytosolic catalase reverses ascorbate-induced cytotoxicity. **A**, catalase is overexpressed in MIA PaCa-2 cells transfected with adenoviruses containing human catalase cDNA. Transfection with AdCAT (100 MOI) targeted catalase expression to the cytosol whereas AdmitCAT (100 MOI) directed catalase to the mitochondria through an 80-bp MnSOD leader sequence. Western blotting confirmed the overexpression of intracellular catalase in cells transfected with AdCAT and AdmitCAT. MnSOD was used as a loading control. **B**, catalase pretreatment reverses ascorbate-induced decreases in clonogenic survival. Ascorbate (2 mmol/L) treatment for 1 h decreased clonogenic survival in MIA PaCa-2 human pancreatic cancer cells. Catalase (100 µg/mL) and PEG-catalase (200 units/mL) alone had little effect on clonogenic survival. Pretreatment of cells with catalase or PEG-catalase followed by ascorbate (2 mmol/L) reversed ascorbate-induced cytotoxicity. *, $P < 0.05$ versus control, means \pm SEM, $n = 3$. **C**, cytosolic-directed catalase, but not mitochondrial-directed catalase, blocked ascorbate-induced cytotoxicity. MIA PaCa-2 cells were treated with AdEmpty (100 MOI), AdCAT (100 MOI), or AdmitCAT (100 MOI) and treated with ascorbate (2 mmol/L) for 1 h. Ascorbate (2 mmol/L) decreases clonogenic survival in cells infected with the AdEmpty and AdmitCAT vectors, but has no effect on cells infected with AdCAT. *, $P < 0.05$ versus -ascorbate, means \pm SEM, $n = 3$.

concentration in a dose-dependent manner. In addition, 1 mmol/L ascorbate results in a steady increase in the concentration of H_2O_2 for up to 60 minutes after ascorbate is added to the medium (Supplemental Fig. S1B).

The effect of catalase overexpression on ascorbate-induced cytotoxicity. To determine if H_2O_2 mediated ascorbate-induced cytotoxicity, MIA PaCa-2 cells were pretreated with various forms of catalase. In the first set of experiments, we pretreated cells with 100 µg/mL catalase or 200 units/mL PEG-catalase, added 2 mmol/L ascorbate for 1 hour, and performed a clonogenic survival assay. Western blots determined that catalase in the medium and PEG-catalase increased the catalase protein in the medium (data not shown) but did not increase the intracellular catalase protein (Fig. 2A). To determine if overexpression of intracellular catalase could protect from ascorbate-induced cytotoxicity, MIA PaCa-2 pancreatic cancer cells were infected with adenoviral vectors, including AdEmpty (empty vector, 100 MOI), an adenovirus containing human catalase cDNA (AdCAT, 100 MOI), and an adenovirus containing human catalase cDNA with an 80-bp MnSOD mitochondrial leader sequence (AdmitCAT, 100 MOI). Figure 2A shows the expression of catalase in MIA PaCa-2 cells infected with the AdEmpty, AdCAT, AdmitCAT, PEG-catalase, or catalase. Infection with the AdCAT or the AdmitCAT increased intracellular catalase immunoreactivity.

Pretreatment of MIA PaCa-2 cells with catalase or PEG-catalase in the medium prevented ascorbate-induced cytotoxicity (Fig. 2B). Ascorbate decreased clonogenic survival to $10 \pm 4\%$ of control values; pretreatment with catalase or PEG-catalase maintained clonogenic survival at control values. Catalase alone or PEG-catalase alone had no effect on clonogenic survival. To determine if intracellular catalase would prevent ascorbate-induced cytotoxicity, MIA PaCa-2 cells were infected with the adenoviral vectors and clonogenic survival assays were done. The clonogenic survival of cells infected with the AdCAT vector maintained clonogenic survival at control when treated with ascorbate 2 mmol/L for 1 hour (Fig. 2C). However, cells infected with the AdmitCAT vector had a significant decrease in clonogenic survival when treated with ascorbate. Ascorbate (2 mmol/L) resulted in a 4-fold decrease in clonogenic survival in cells infected with the AdmitCAT vector. These results indicate that ascorbate-induced cytotoxicity is due to extracellular generation of H_2O_2 that diffuses intracellularly into the cytosolic compartment.

Ascorbate decreases intracellular ATP levels. Chen and colleagues have hypothesized that ascorbate depletes ATP, leading to cell death (6). To examine this possibility, MIA PaCa-2 cells were treated with ascorbate (0, 1, 2, or 5 mmol/L) for 1 hour, with and without catalase (100 µg/mL) pretreatment and ATP levels were determined (Fig. 3A). ATP levels showed a dose-dependent decline with ascorbate treatment, whereas pretreatment of catalase prevented the decrease in ATP seen with 2 or 5 mmol/L ascorbate. To determine if intracellular overexpression of catalase would reverse ascorbate-induced decreases in ATP levels, cells were infected with the adenoviral vectors

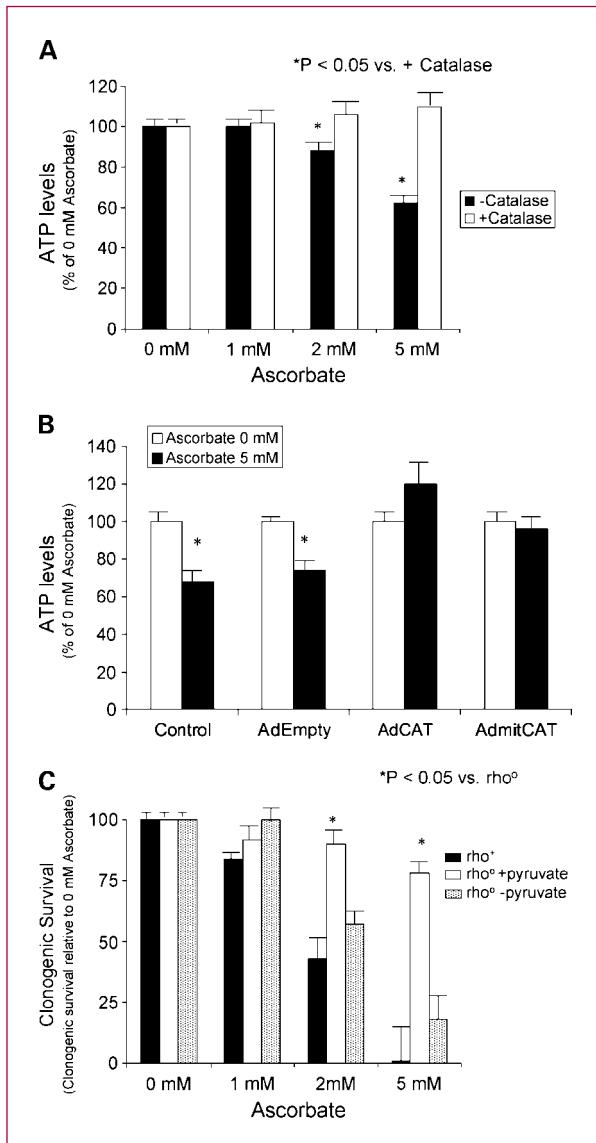


Fig. 3. Ascorbate decreases intracellular ATP levels. **A**, catalase pretreatment blocks the ascorbate-induced dose-dependent decrease in ATP. ATP levels were significantly lower in MIA PaCa-2 cells treated with ascorbate (2 and 5 mmol/L) when compared with the same cells pretreated with catalase (100 μ g/mL). A luciferase-based somatic ATP assay kit was used in the measurement of intracellular ATP. *, $P < 0.05$ versus +catalase, means \pm SEM, $n = 3$. **B**, MIA PaCa-2 cells transfected with AdCAT or AdmitCAT restore ATP following treatment with ascorbate (5 mmol/L for 1 h) relative to their controls. Ascorbate (5 mmol/L) decreases ATP levels in control and AdEmpty-treated pancreatic cancer cells. However, the decreased ATP levels are reversed in MIA PaCa-2 cells with infection of the adenoviral vectors containing human catalase cDNA (AdCAT and AdmitCAT). Although AdCAT had reversed ascorbate-induced cytotoxicity, AdmitCAT did not, suggesting that mitochondrial depletion of ATP may not play a role in ascorbate-induced cytotoxicity. *, $P < 0.05$ versus ascorbate 0 mmol/L, means \pm SEM, $n = 3$. **C**, ascorbate (0–5 mmol/L) showed significant decreases in clonogenic survival in both MIA PaCa-2 ρ^+ and ρ^- cells without pyruvate. However, ascorbate-induced cytotoxicity was reversed when pyruvate is added to the media of ρ^- cells to compensate for the respiratory metabolism deficit induced by the generation of mitochondria-deficient cells.

AdEmpty, AdCAT, and AdmitCAT (100 MOI). Control cells and cells infected with the AdEmpty vector had significant decreases in ATP levels after ascorbate (5 mmol/L for 1 hour). Parallel to clonogenic survival, ascorbate-induced decreases in ATP were prevented in cells treated with the AdCAT vector. However, in contrast to clonogenic survival (Fig. 2C), AdmitCAT blocked depletion of ATP (Fig. 3B), suggesting that loss of ATP may not have a role in ascorbate-induced cytotoxicity. In addition, measurement of NADP⁺/NADPH levels revealed only a very minor change ($6 \pm 1\%$ at 6 hours) after ascorbate (2 mmol/L) treatment. Also, the ratio of NADP⁺/NADPH was 1.0 ± 0.1 before ascorbate and remained unchanged (at 1.0 ± 0.1) 6 hours after ascorbate treatment.

To further investigate the role of mitochondrial ATP generation in ascorbate-induced cytotoxicity, human pancreatic cancer cells deficient in oxidative phosphorylation due to the absence of mitochondrial DNA were used (20, 29). Figure 3C shows clonogenic survival in mitochondria-deficient ρ^- cells and the parental ρ^+ cells following ascorbate treatment (0, 1, 2, or 5 mmol/L for 1 hour). In the parental cell line, there is a significant decrease in clonogenic survival with doses of ascorbate of 2 or 5 mmol/L, whereas in the ρ^- cells, there is no significant decrease in clonogenic survival. However, the ρ^- medium was supplemented with 100 μ g/mL pyruvate, a known scavenger of peroxide, to compensate for the respiratory metabolism deficit as described (20, 29). When pyruvate was removed from the ρ^- medium, clonogenic survival decreased following ascorbate treatment (Fig. 3C). This result suggests that the direct scavenging of H₂O₂ by pyruvate may explain the resistance of the ρ^- cells to ascorbate. Consistent with this idea, the addition of pyruvate to ρ^+ cells enhanced clonogenic survival and protected the cells from ATP depletion following ascorbate treatment (data not shown). Thus, ascorbate-induced cytotoxicity and ATP depletion were similar in cells deficient in functional mitochondria when compared with cells with functional mitochondria. This suggests that ascorbate-induced cytotoxicity is not due to inhibition of mitochondria oxidative phosphorylation and subsequent ATP generation.

Mechanism of ascorbate-induced cell death. DNA damage induced by H₂O₂ can activate PARP (7, 8). Activated PARP catabolizes NAD⁺, thereby depleting the substrate for NADH formation and subsequent ATP synthesis. To determine if PARP is activated, MIA PaCa-2 cells were treated with 5 mmol/L ascorbate for 1 hour and then cell homogenates were assayed for PARP cleavage. Supplemental Fig. S2A shows minimal PARP cleavage at 6 and 24 hours after ascorbate treatment. In addition, the mechanism of ascorbate-induced cell death was studied by flow cytometry using Annexin/propidium iodide staining. Treatment of MIA PaCa-2 cells with ascorbate (5 mmol/L) resulted in an initial peak in the percentage of apoptotic cells 1 hour after ascorbate treatment and a steadily increasing percentage of necrotic cells that was time dependent (Supplemental Fig. S2B). In addition, treatment of MIA PaCa-2 cells with ascorbate (1, 2, 5, or 10 mmol/L) resulted in significant

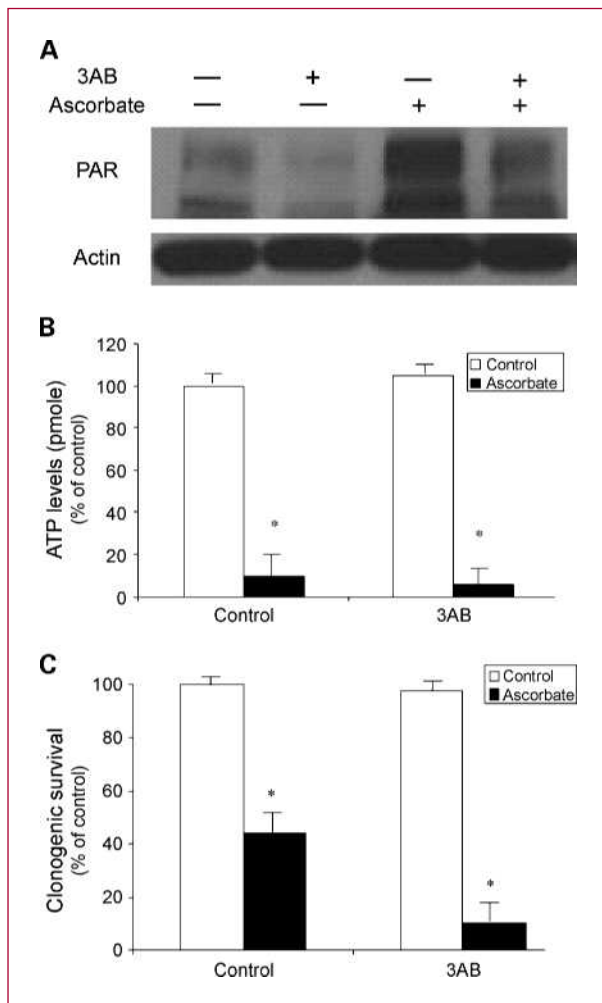


Fig. 4. PARP-1 activation is not associated with ascorbate-induced cell death. Effect of 3AB (10 mmol/L) on ascorbate-induced PARP activation, intracellular ATP depletion, and cell death. MIA PaCa-2 cells were treated with 5 mmol/L ascorbate for 30 min with or without 1 h pretreatment of 10 mmol/L 3AB (Tocris Bioscience), an inhibitor of PARP. **A**, cells were collected and subjected to Western blot analysis. Cell lysate (40 μ g) was probed with polyclonal rabbit anti-PAR antibody (BD Biosciences) at a dilution of 1:3,000. PAR is identified at 116 to 200 kDa. Ascorbate (5 mmol/L) induces PAR formation, which is reversed with 3AB. **B**, ATP levels were measured as described. ATP decreases with ascorbate treatment, which is not reversed with 3AB pretreatment. There was a significant decrease in ATP levels with ascorbate (5 mmol/L) treatment. 3AB alone had little effect on ATP, whereas 3AB did not reverse the ascorbate-induced ATP depletion. Means \pm SEM, $n = 3$. *, $P < 0.05$ versus controls. **C**, clonogenic survival was measured after ascorbate (5 mmol/L for 1 h) with and without 3AB (10 mmol/L). The ascorbate-induced decrease in clonogenic survival is not reversed with 3AB. Means \pm SEM, $n = 3$. *, $P < 0.05$ versus control.

dose-dependent increases in the percentage of necrotic cells with only minor increases in the apoptotic fraction (data not shown). Moreover, treatment of cells with QVD, a known inhibitor of caspase-mediated cell death, did not reverse the percentage of necrotic or apoptotic cells with ascorbate (5 mmol/L). Likewise, treatment of MIA PaCa-2 cells with H_2O_2 (80-220 μ mol/L) also increased

the percentage of necrotic cells with little change in the apoptotic fraction and QVD did not reverse the percentage of necrotic cell death (data not shown). These findings suggest that ascorbate induces caspase-independent necrotic cell death. PARP-1 is readily activated in response to DNA damage and associated with necrotic cell death (30). As shown in Fig. 4A, there was an increase in the formation of the PAR polymer, a direct result of PARP-1 activation within 1 hour of ascorbate (5 mmol/L) treatment. As seen above, intracellular ATP levels were also decreased after ascorbate treatment. Ascorbate (5 mmol/L for 1 hour) significantly decreased ATP levels 6 hours after treatment (Fig. 4B). To verify whether PARP-1 activation contributes to the cell death induced by ascorbate, cells were treated with ascorbate (5 mmol/L for 1 hour), with and without 3-aminobenzamide (3AB, 10 mmol/L), a specific PARP-1 inhibitor. Pretreatment with 3AB inhibited ascorbate-induced PAR formation (Fig. 4A) but did not reverse the decrease in intracellular ATP depletion (Fig. 4B) and the ascorbate-induced decrease in clonogenic survival (Fig. 4C). Together, these results suggest that PARP-1 activation and ATP depletion may not contribute to ascorbate-induced cell death.

Ascorbate may lead to death through a unique caspase-independent autophagy pathway (14) and is characterized by accumulation of autophagosomes that fuse with lysosomes to form autophagolysosomes. Activation of this pathway can be detected by the processing of LC3 to the lipidated form referred to as LC3-II (14). To determine if ascorbate induced autophagy, MIA PaCa-2 cells were treated with ascorbate (5 mmol/L for 1 hour), then Western blotting of the cells for LC3 was done. Figure 5A shows an increase in LC3-II from 4 and 6 hours after ascorbate treatment. To determine if the increase in LC3-II was mediated by H_2O_2 , cells were pretreated with 100 μ g/mL catalase and AdCAT (100 MOI) before treatment with ascorbate (5 mmol/L). Figure 5A shows a decrease in LC3-II protein at 6 hours following ascorbate treatment in cells treated with catalase in the medium. To confirm this finding, cells were infected with the AdCAT vector and then treated with ascorbate (5 mmol/L). Once again, the increase in LC3-II protein induced by ascorbate at 6 hours was reversed with overexpression of the catalase gene targeted to the cytosol. Also, high levels of LC3-II are present upon pretreatment with catalase. Preliminary studies in our laboratory have shown that pancreatic cancer cells that express the *K-ras* oncogene have increased levels of reactive oxygen species (31). Perhaps the increase in LC3-II with catalase pretreatment may be due to the activity of catalase scavenging these high levels of endogenous H_2O_2 .

To confirm whether ascorbate induces functional autophagy, we generated cells (MIA PaCa-2 LC3-GFP) expressing a fusion product of LC3 and GFP that is used to detect the induction of autophagy (32). Cells were treated with ascorbate (0, 1, 2, or 5 mmol/L) and H_2O_2 (200 μ mol/L as a positive control). Both ascorbate and H_2O_2 significantly increased the GFP MFI in these cells (Fig. 5B). In

addition, both ascorbate and H_2O_2 significantly increased the percentage of LC3-GFP cells (*, $P < 0.05$ versus no treatment, means \pm SEM, $n = 3$; Fig. 5C). To further confirm these findings, the MIA PaCa-2 LC3-GFP cells were examined under confocal microscopy. As shown in Fig. 5D, both ascorbate and H_2O_2 markedly increased the punctate GFP fluorescence in these cells. The percentage of punctated cells increased from 25% with no treatment to 56% and 59% in cells treated with 1 and 2 mmol/L ascorbate, respectively. The percentage of punctated cells

increased to 67% in cells treated with H_2O_2 (200 μ mol/L). In addition, there was a significant increase in the MFI of LC3-GFP-positive cells with ascorbate (5 mmol/L), and this increase was blocked by catalase pretreatment (Fig. 5E).

Ascorbate inhibits tumor growth and increases survival. To test the hypothesis that pharmacologic ascorbate could reduce tumor growth *in vivo*, we injected 2×10^6 MIA PaCa-2 cells s.c. into the flank region of nude mice and allowed tumors to reach 4 mm \times 5 mm in greatest dimension. Mice were then treated with 4 g/kg ascorbate i.p., b.i.d., for 14

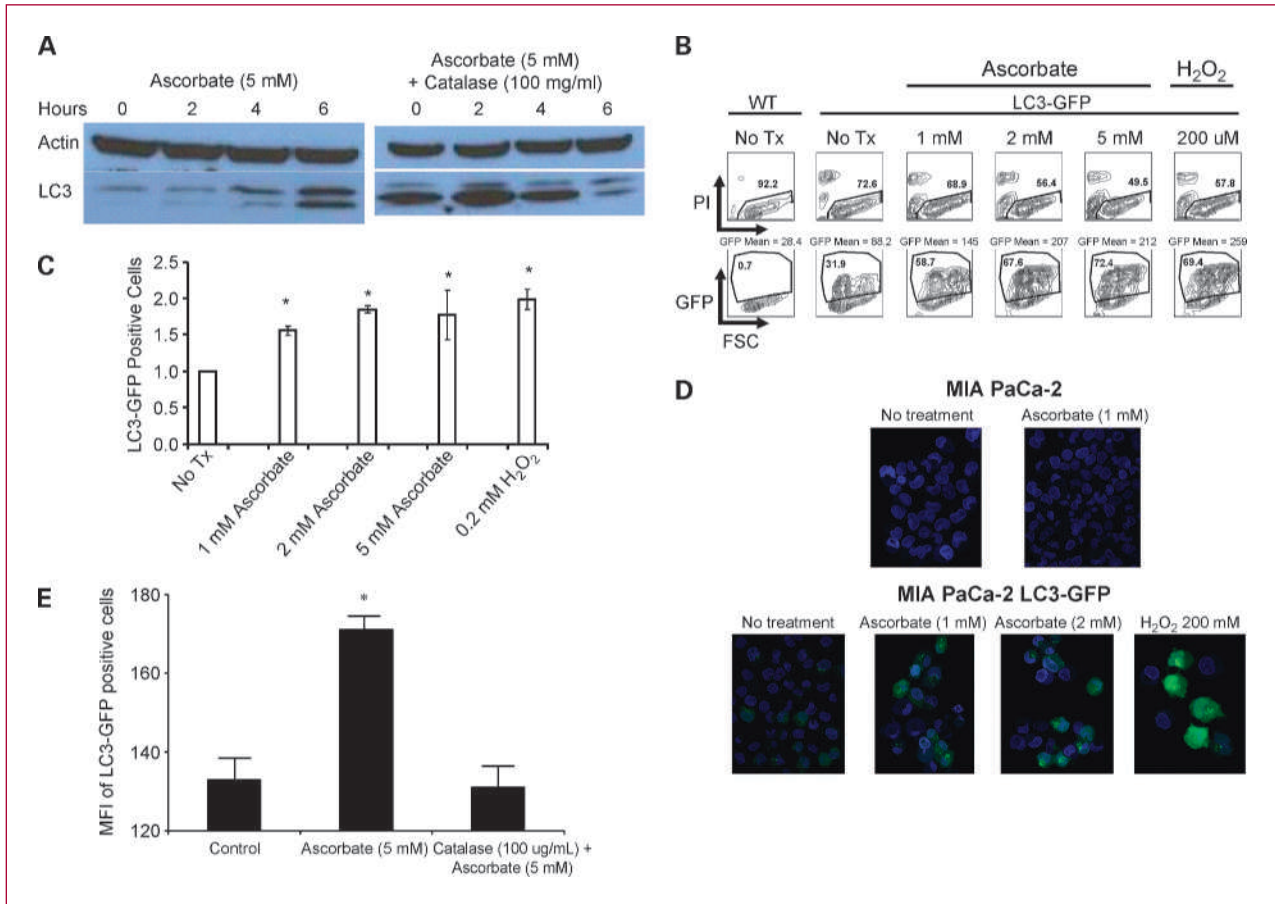


Fig. 5. Ascorbate induces autophagy in MIA PaCa-2 cells. **A**, MIA PaCa-2 cells were treated with ascorbate (5 mmol/L), harvested at various time points, and Western blot analysis was done. Forty micrograms of protein per well were loaded onto a 4% to 20% Tris-HCl gel. Polyclonal rabbit anti-LC3 primary antibody (1:1,000 dilution) was followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:50,000 dilution). The increase in LC3-I and LC3-II proteins observed 6 h following ascorbate treatment is inhibited by pretreatment with catalase. **B**, MIA PaCa-2 cells transduced with LC3-GFP were treated with the indicated doses of ascorbate or H_2O_2 for 1 h and allowed to recover in fresh medium for 24 h. Cells were harvested, stained with propidium iodide (PI, 1 μ g/mL) to discriminate live cells, and then run on a BD Calibur flow cytometer. Top row, gating strategy for the live cells (propidium iodide negative). The percentage of live cells is shown in each plot with decreasing viability shown for both treatments (Tx). Bottom row, GFP fluorescence of the live cells. The GFP-negative cells (WT) were used to adjust the gate for the LC3-GFP-positive cells. The percentage of LC3-GFP-positive cells is indicated within each plot and the GFP MFI of this population is indicated above each plot. Both ascorbate and H_2O_2 increase the percentage of positive cells as well as the MFI. **C**, the relative increase in the percentage of LC3-GFP cells (No Tx = 1.0) is shown for three independent experiments (mean \pm SD). A one-way ANOVA with Tukey's post hoc test shows that all four treatment groups were significantly different from the control (*, $P < 0.05$). **D**, ascorbate and H_2O_2 induced punctated distribution of LC3-GFP and autophagosome maturation detected by selective increase in GFP fluorescence in cells expressing the LC3-GFP fusion protein. Cells were fixed and stained, and digitized images were analyzed by confocal microscopy; representative cells were then selected and photographed. The percentage of LC3-GFP-positive cells as evaluated by digital image analysis was increased in pancreatic cancer cells treated with ascorbate (1 and 2 mmol/L) and H_2O_2 (200 μ mol/L) compared with no treatment. Cells were fixed and stained, and the digitized images were analyzed. **E**, pretreatment of MIA PaCa-2 LC3-GFP cells with 100 μ g/mL catalase reverses the increase in MFI induced by ascorbate. Means \pm SEM, $n = 3$. *, $P < 0.05$.

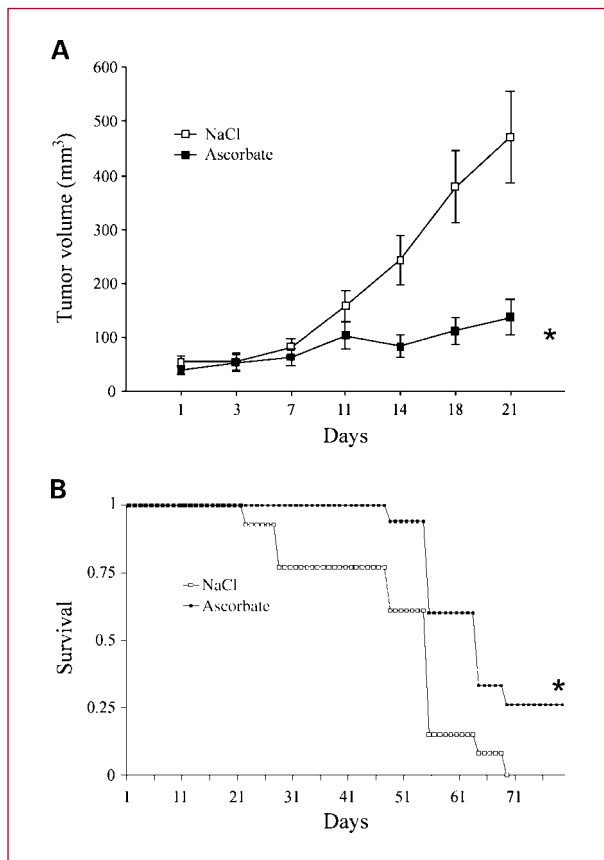


Fig. 6. A, ascorbate treatment decreased MIA PaCa-2 tumor growth in nude mice. Animals that received ascorbate (4 g/kg i.p., b.i.d., for 14 d) had significantly slower tumor growth when compared with animals that received saline (1 mol/L i.p., twice a day, for 14 d; means \pm SEM; *, $P < 0.001$, $n = 13$ control animals and 15 ascorbate-treated animals). On day 21, there was a greater than 3-fold decrease in tumor growth in animals receiving ascorbate when compared to those treated with saline. B, Kaplan-Meier plots of estimated survival in nude mice. There was a significant difference in survival between saline- and ascorbate-treated groups of mice (*, $P < 0.001$).

days or 1 mol/L NaCl i.p., twice a day, for 14 days. For the controls, 1 mol/L NaCl was used at an equivalent osmolarity to the dose of ascorbate needed to give 4 g/kg. Tumor volume was followed over time until they reached 1,000 mm³, at which time the mice were sacrificed. Both controls and ascorbate-treated mice had slight but not significant decreases in weight during the treatment period. All mice completed the treatment period and there were no deaths during the treatment. None of the animals during the study had to be sacrificed for continued weight loss or cachexia. In animals treated with ascorbate, a slower rate of growth in tumor was observed compared with the group of animals that received NaCl (Fig. 6A). On day 21 of the experiment, the control group had a mean tumor volume of 472 mm³, whereas the ascorbate group had a mean tumor volume of 138 mm³. The mixed linear regression analysis of the tumor growth curves showed

that their rate of growth differed significantly between the groups ($P < 0.01$). Estimated tumor growth curves are displayed in Fig. 6A. The estimated survival curves for each treatment group are given in the Kaplan-Meier plots of Fig. 6B. The log-rank analyses of survival showed that the animals that received ascorbate had increased survival compared with controls (68 days versus 78 days). The global test of equality indicates that there is a significant difference in the survival times ($P < 0.0001$) between groups.

Discussion

Ascorbate is one of the early unorthodox therapies for cancer (33–35). This approach was subsequently promoted by Cameron and Pauling (36, 37). Cameron and Campbell initially published case reports of 50 patients, some of whom seemed to have benefited from high-dose ascorbate treatment (38). Cameron and Pauling then published results of 100 patients with terminal cancer, in whom conventional therapy was no longer considered useful and were given i.v. ascorbate (39). The ascorbate-treated patients were compared to 1,000 retrospective controls with similar disease but had not received ascorbate; the comparison showed that patients who received ascorbate survived 300 days longer than controls (39).

To test whether ascorbate was effective, Moertel conducted two randomized placebo-controlled studies using ascorbate given orally; neither study showed any benefit (40, 41). However, oral and i.v. ascorbate have strikingly different pharmacokinetic properties (27). Cameron gave patients ascorbate i.v. as well as orally, whereas Moertel's patients received only oral ascorbate. Our study suggests that the role of ascorbate in pancreatic cancer treatment should be further examined.

Clinical data show that when ascorbate is given orally, fasting plasma concentrations are tightly controlled at <100 μ mol/L (42). As doses exceed 200 mg, absorption decreases, urine excretion increases, and ascorbate bioavailability is reduced (42, 43). In contrast, when 1.25 g of ascorbate are administered i.v., plasma concentrations as high as 1 mmol/L are achieved. Some clinicians have infused more than 10 g of ascorbate in cancer patients and achieved plasma concentrations of 1 to 5 mmol/L (27, 28). Both i.v. and i.p. administration of ascorbate achieved serum ascorbate concentrations up to 20 mmol/L (44). Thus, it is clear that i.v. administration of ascorbate can yield very high plasma levels, whereas oral treatment does not. Our study shows that ascorbate induces cytotoxicity in pancreatic cancer cells at levels achievable with i.v. infusions. Additionally, systemic administration of ascorbate inhibited pancreatic tumor growth in mice.

Our study suggests oxidative stress, mediated by hydrogen peroxide, as a mechanism of ascorbate-induced cytotoxicity because catalase, either extracellularly or over-expressed in the cytoplasmic compartment, reversed the

decrease in clonogenic survival induced by ascorbate. In addition, an autophagic mechanism may also be involved as an increase in LC3 protein was observed in cells treated with ascorbate. The role of autophagy in cellular responses to oxidative stress is as yet unclear. Reactive oxygen species (ROS) can induce autophagy, which contributes to caspase-independent cell death in a variety of cell types (45). In contrast, a number of studies show a protective role of autophagy against ROS-mediated necrosis (46). Additional studies have shown a role of PARP-1 in regulation of autophagy, which enhances cell survival in cells under oxidative stress (31). Both the peroxide-mediated and autophagic-mediated mechanisms could potentially be enhanced to induce more cancer cell killing with peroxide.

In summary, ascorbate in doses achievable in humans decreased viability in all pancreatic cancer cell lines through a H₂O₂-mediated mechanism. Treatment with pharmacologic ascorbate induced a noncaspase-mediated cell death consistent with autophagy. In mice with pre-established pancreatic tumors, treatment with ascorbate inhibited tumor growth and prolonged survival. Pharmacologic doses of ascorbate achievable in humans may have potential for therapy in pancreatic cancer.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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