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Pharmacological Ascorbate as an Adjuvant for Enhancing Radiation-Chemotherapy Responses in Gastric Adenocarcinoma

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Abstract

Gastric adenocarcinoma most often presents at an advanced stage and overall five-year survival of ~30%. Pharmacological ascorbate (high-dose IV ascorbate) has been proposed as a promising nontoxic adjuvant to standard radio-chemotherapies in several cancer types. In the current study, pharmacological ascorbate (0.5–2 mM) caused a dose-dependent decrease (70–85% at 2 mM) in clonogenic survival of gastric adenocarcinoma cells (AGS and MNK-45), but was relatively nontoxic to a small intestinal epithelial non-immortalized human cell isolate (FHs 74 Int). The addition of pharmacological ascorbate (1 mM) to standard radio-chemotherapies [i.e., 5-FU (5 μ M); cisplatin (0.5 μ M); irinotecan (2.5 μ M); carboplatin (5 μ M); paclitaxel (2–4 nM); and X rays (1.8 Gy)] also potentiated gastric cancer clonogenic cell killing [additional decreases were noted with: ascorbate plus 5-FU/radiation (1%); ascorbate plus cisplatin/irinotecan (9–19%); and ascorbate plus paclitaxel/carboplatin (6–7%)]. The gastric cancer cell toxicity and chemosensitization seen with pharmacological ascorbate was dependent on H₂O₂ and the presence of catalytic metal ions. In addition, pharmacological ascorbate dosing resulted in a concentration-dependent decrease (64% at 20 mM, $P = 0.0001$) in cancer cell invasion and migration that was inhibited by catalase. Finally, pharmacological ascorbate significantly increased the overall survival of mice with gastric cancer xenografts when used in combination with paclitaxel, carboplatin and radiation ($P = 0.019$). These results demonstrate that pharmacological ascorbate is selectively cytotoxic to gastric adenocarcinoma cells (relative to normal intestinal epithelial cells) by a mechanism involving H₂O₂ and redox active metal ions. Furthermore, pharmacological ascorbate significantly enhances gastric cancer xenograft responses to radio-chemotherapy as well as inhibiting tumor cell migration and invasiveness. Overall, these results support the hypothesis

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that pharmacological ascorbate can be used as an adjuvant with standard-of-care radio-chemotherapies for the treatment of gastric adenocarcinomas.

INTRODUCTION

Gastric adenocarcinoma (GAC) is a highly lethal disease due to treatment resistance and a propensity for metastasis. GAC is currently the fourth leading cause of cancer-related deaths worldwide and the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) database estimates 28,000 new cases in the U.S. for 2017 (1). The overall five-year relative survival rate of patients with GAC in the U.S. is approximately 30%, with a median survival of only 12 months (1, 2). The reason for this dismal survival rate is that most stomach cancers are diagnosed at an advanced stage or after distant metastases have formed, impacting patient prognosis. Treatment options for GAC vary by stage and include combinations of multimodality treatments, such as surgery, chemotherapy (neoadjuvant and adjuvant), radiation therapy, radio-chemotherapy and potentially targeted therapy.

In 1976, it was suggested by Cameron and Pauling that ascorbate, given both orally and intravenously at a high dose (pharmacological ascorbate), could be used as a potential anti-cancer therapy (3). Shortly after this suggestion, two separate clinical trials failed to show efficacy of ascorbate on patient survival (4, 5). However, these trials used high-dose oral ascorbate without the addition of intravenous ascorbate. At physiologic concentrations (plasma levels 50–90 μM), ascorbate (vitamin C) is well known as an effective reducing agent and antioxidant (6). Interestingly, at pharmacological levels ($\sim 10\text{--}20\text{ mM}$), ascorbate can also act as a pro-oxidant that generates increased levels of hydrogen peroxide (H_2O_2) (7–13). It does so by increasing the steady-state concentration of H_2O_2 [(reactive oxygen species (ROS))] in the presence of intracellular catalytic metal ions. The varying pro-oxidant and antioxidant effects of ascorbate can be attributed to the tightly controlled uptake of oral ascorbate in the intestinal tract, which can be bypassed through intravenous administration, thus producing elevated plasma levels when using pharmacological doses of ascorbate (10). When given intravenously, pharmacological ascorbate can achieve blood concentrations nearly 1,000 times higher than oral ascorbate and deliver levels of ascorbate in the millimolar range to the plasma (14).

Recently, utilization of pharmacological ascorbate has shown potential as an anti-cancer therapy in several preclinical cancer studies. In pancreatic, ovarian, glioblastoma and non-small cell lung cancer *in vitro* and *in vivo* models, pharmacological ascorbate demonstrated concentration-dependent, cancer cell-selective cytotoxicity and radio-chemo sensitization (11–16). Studies by Du *et al.* and others have shown that the addition of pharmacological ascorbate to ionizing radiation potentiates its cytotoxic effects in both cell and animal models; suggesting the use of pharmacological ascorbate as a radiosensitizer (13, 17–19). Additionally, pharmacological ascorbate has been shown to augment the toxicity of several chemotherapy regimens *in vitro* as well as decrease tumor growth and increase survival rates *in vivo* (12, 13, 20). Previously reported clinical trials in pancreatic and ovarian cancers, as well as more recent trials in glioblastoma and non-small cell lung cancers, demonstrated that pharmacological ascorbate is: safe, well tolerated by patients, may reduce toxicity of

chemotherapies, and has the potential to be efficacious in treating a broad range of cancers in conjunction with current therapies (12, 13, 21–23).

These encouraging translational results combined with few apparent safety concerns, make the use of pharmacological ascorbate in cancer therapy a promising avenue of inquiry. Therefore, the current study extended these previously reported studies to investigate the use of pharmacological ascorbate combined with radio-chemotherapies for gastric adenocarcinoma. Similar to results in other disease sites, our results show that pharmacological ascorbate dose-dependently decreased clonogenic survival alone and concurrently with many chemotherapeutics, as well as when combined with irradiation by a mechanism involving the formation of H_2O_2 and interaction with redox active metal ions. We also report data indicating that pharmacological ascorbate decreases *in vitro* gastric adenocarcinoma invasion and migration, which can be reversed with the addition of an H_2O_2 scavenger, catalase. Finally, we demonstrate that the addition of pharmacological ascorbate to radio-chemotherapy *in vivo* prolonged survival in a xenograft mouse model of GAC.

MATERIALS AND METHODS

Cell Culture

Human cell lines (AGS gastric adenocarcinoma and FHs 74 Int normal nonimmortalized small intestinal epithelial cells) were obtained directly from American Type Culture Collection, Gaithersburg, MD (CRL-1739 and CCL-241, respectively) and were used at less than passage 20 for AGS and passage 6 for FHs 74 Int. The human gastric cancer cell line, MKN-45, was a kind gift of Dr. Yoon at Memorial Sloan Kettering Cancer Center and was used at less than passage 20. Once obtained, the cells were verified by IDEXX BioResearch (Columbia, MO) using the CellCheck 9 marker STR profile and interspecies contamination test. Results indicated the cells to be of human origin with no mammalian interspecies contamination. The genetic profile of the sample was concluded to be identical to the genetic profile reported for this cell line. All cells were grown at 21% oxygen. AGS and MKN-45 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 100 μ g/ml penicillin/streptomycin. FHs 74 Int cells were grown according to instructions in Hybri-Care Medium 46-X™ (ATCC) supplemented with 30 ng/ml human epidermal growth factor and 10% FBS.

Clonogenic Survival

Clonogenic survival assays were performed as previously described elsewhere by O'Leary *et al.* (24). Cells were plated at a density of 1×10^5 in 60-mm dishes and allowed to attach for 24 h. After attachment, the cells were incubated with the listed doses of: 5-fluorouracil (NDC: 63323-117-10; Fresenius Kabi USA, Lake Zurich, IL) for 24 h, ascorbate for 1 h, cisplatin (NDC: 0703-5747-11; Teva Pharmaceuticals USA Inc., North Wales, PA) for 24 h, irinotecan (NDC: 61703-349-16; Hospira, Lake Forest, IL) for 24 h, paclitaxel (NDC: 61703-342-50; Hospira) for 24 h, carboplatin (NDC: 61703-339-56; Hospira) for 24 h, catalase (C1345; Sigma-Aldrich LLC, St. Louis, MO) for 1 h, deferoxamine (DFO, D9533; Sigma-Aldrich) for 3 h, diethylenetriamine pentaacetic acid (DTPA, D6518; Sigma-Aldrich) for 3 h, and irradiated once immediately before plating. Radiation experiments were

performed at the Radiation and Free Radical Research (RFRR) Core Facility of the University of Iowa on the Pantak Therapax DXT 300 X-ray unit with maximum X-ray energy of 300 kVp. Regardless of varying cell type and media components, all cells were treated in fresh 10% RPMI media with ascorbate for 1 h at 37°C. Ascorbate came from a stock solution of 1 M (pH 7) made under argon and stored with a tight-fitting stopper at 4°C. Ascorbate concentration was checked at 265 nm, $\epsilon = 14,500 \text{ (mol/l)}^{-1} \text{ cm}^{-1}$. After treatment, cells were trypsinized, counted and plated in 6-well dishes at 500–3,000 cells/well. Normal FHs 74 Int cells were plated on top of Chinese hamster fibroblast (B1 designation) feeder cells (1×10^5 per well) that had been irradiated at 30 Gy. Dishes were incubated for 7–18 days, depending on cell type, to allow for colony formation. Colonies were fixed in methanol and stained. Colonies containing ≥ 50 cells were scored as survivors.

Matrigel Invasion and Migration Assays

Matrigel invasion and migration assays were performed as previously described elsewhere by O'Leary *et al.* (24). The effects of ascorbate (11 and 20 mM or 25 and 45 pmole cell⁻¹) and catalase (15 U/ml) on invasion and migration were examined after incubation with cells for 1 h before being trypsinized, counted and added to the chambers. Live AGS cells were seeded at a density of 2×10^5 in serum-free media into Matrigel chambers (8 μm pore size) from the BD BioCoat™ Matrigel Invasion Assay System (354480; BD Biosciences, San Jose, CA). Invading AGS cells were stained using 0.5 mg/ml of a Thiazolyl Blue Tetrazolium Bromide (MTT) solution (M2128; Sigma-Aldrich) in RPMI 1640 media without FBS or phenol red. For transwell migration, cells were seeded similarly and placed into BD Falcon™ PET track-etched membrane chambers (8 μm pore size, 353097; BD Biosciences).

In Vivo Studies

Female 6–8-week-old athymic-nu/nu mice were purchased from Envigo (previously Harlan Laboratories, Indianapolis IN) and handled in accordance with the University of Iowa Animal Care and Use Committee (IACUC). No known pathogens existed during time of *in vivo* experiments. MKN-45 cells (5×10^5) were injected subcutaneously into mice to allow for xenograft formation. Mice were randomly assigned to experimental groups (20 per group) when tumor size reached 2–3 mm in diameter. Treatment of paclitaxel (10 mg/kg) and carboplatin (15 mg/kg) were given weekly (IP) for the duration of the experiment. Animals received 2 Gy doses for a total of 8 fractions while ascorbate (4 g/kg) was given daily (IP) for the duration of the experiment. For exposures, animals were anesthetized with 80–100 mg/kg ketamine and 10 mg/kg xylazine and shielded in a lead block so that only the leg containing a tumor was irradiated. Animals were sacrificed when tumor volumes reached 15 mm in diameter of either length or width or when an animal lost more than 20% of its initial body weight.

Statistical Analysis

Statistical analysis was performed primarily using analysis of variance (ANOVA) followed by a Bonferroni's multiple comparisons unless otherwise stated. Irradiation curves (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14978.1.S1>) were generated using Igor Pro version 6.36 (WaveMetrics Inc., Lake Oswego, OR). All *in vitro* studies were

performed in triplicate. *In vivo* studies were analyzed with Kaplan-Meier survival plots using Mantel-Cox log-rank comparisons. All statistical tests were two-sided and assessed for significance at the 0.05 level using GraphPad Prism version 7.0 (San Diego, CA).

RESULTS

Determination of Dosing Ranges to be Used in Combined Modality Experiments with Ascorbate, Chemotherapeutics and Radiation Ascorbate

Clonogenic survival assays are widely used to determine the effects of xenobiotic agents on potential, clinically relevant drug therapies. Historically employed to investigate the effects of radiation on cells, these assays are now largely performed in the preclinical setting to examine the ability of a cell to survive treatment insults from interventions, such as drugs, antioxidants, chemotherapy, etc. (25). To determine the effective dose of ascorbate that clonogenically inactivated 30–50% of gastric adenocarcinoma cell lines, AGS and MKN-45 cells were treated with increasing concentrations of ascorbate (0.5 mM–2 mM or 7–48 pmole cell⁻¹) for 1 h before plating. To account for variation in media concentration and cellular metabolism among cell lines, final concentrations were calculated in units of moles-per-cell as described elsewhere (26, 27). Both gastric adenocarcinoma cell lines exhibited a serial decline in normalized surviving fraction at increasing doses of ascorbate compared to control cells (Fig. 1). For the AGS cell line, 1 mM (25 pmole cell⁻¹) ascorbate yielded a 30% reduction in clonogenic survival (Fig. 1). For the MKN-45 cell line a concentration of 1 mM (14.5 pmole cell⁻¹) ascorbate resulted in a 42% statistically significant reduction in normalized surviving fraction (Fig. 1). In contrast, the normal intestinal epithelial cell line, FHs 74 Int, displayed only a 6% decrease in normalized surviving fraction at 1 mM ascorbate (10 pmole cell⁻¹), compared to control (Fig. 1). The selectivity of pharmacological ascorbate toxicity between normal and cancer cells is even more evident at 2 mM ascorbate, where the normal epithelial cells undergo a 26% decrease in clonogenic survival compared to the 72% and 86% seen in the AGS and MKN-45 cell lines, respectively (Fig. 1). These results indicate that normal intestinal cells are more resistant to the cytotoxic effects of ascorbate compared to cancer cells.

Chemotherapies—Both MKN-45 and AGS cell lines were treated with 5-FU (0.1–50 μM), cisplatin (0.1–2.5 μM), irinotecan (1–5 μM), paclitaxel (1–8 nM) and carboplatin (1–10 μM) for 24 h, then allowed to grow for 7–10 days. Results were normalized to control cloning efficiency (Supplementary Fig. S2A–J; <http://dx.doi.org/10.1667/RR14978.1.S1>). 5-FU titrations showed a significant decline in surviving fraction in both the AGS and MKN-45 cell lines at the 10 μM concentration with a 60% and 68% reduction, respectively (Supplementary Fig. S2A and B). Because there was approximately a 30% reduction in both cell lines at the 1-μM concentration and greater than 50% reduction at the 10-μM concentration, a mid-point of 5 μM 5-FU was selected as the treatment dose for 5-FU. Cisplatin titration revealed a significant reduction in clonogenic surviving fraction starting at the 0.1 μM concentration for the MKN-45 cell line with a 33% reduction in survival of clones (Supplementary Fig. S2D). However, in the AGS cell line, significance did not occur until a cisplatin concentration of 0.5 μM was reached where a 29% decrease was observed (Supplementary Fig. S2C). In this case, the concentration at which both cell lines

reached ~30% cell killing was chosen as the dose for cisplatin for further study. Irinotecan showed a similar dosing pattern in the MKN-45 cell line compared to the AGS cell line where the MKN-45 cells appeared to have greater sensitivity at lower concentrations (Supplementary Fig. S2E and F). Both cell lines had a significant drop in survival of approximately 30% at the 2.5 μM concentration; therefore, this concentration was chosen as the dose of irinotecan for further study. The doses of paclitaxel chosen for further studies were 4 nM (39% killing) for the AGS cell line (Supplementary Fig. S2G) and 2 nM (53% killing) for the MKN-45 cell line (Supplementary Fig. S2H). Both cell lines displayed a similar decrease (AGS = 39%, MKN-45 = 41%) in clonogenic cell survival after 5 μM carboplatin (Supplementary Fig. S2I and J), and this concentration was chosen for further study.

Radiation dose—To determine radiation dosing, clonogenic survival was calculated as a function of radiation dose titration up to 3 Gy (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14978.1.S1>). A significant drop in survival was noted early at 0.5 Gy for the AGS cell line and 1 Gy at the MKN-45 cell line and the dose chosen for future study was 1.8 Gy (35–65% reduction) (Supplementary Fig. S1).

Ascorbate Enhances Cytotoxicity of First-Line Chemotherapeutics Used for Gastric Cancer

At pharmacological plasma levels (0.3–20 mM), ascorbate has been shown to be toxic to several different cancer cell lines (7, 11–13). Similarly, it has shown promise as a radiosensitizer (17–19) and demonstrated the ability to enhance the efficacy of several types of chemotherapeutics (12, 13, 20). We hypothesized that this phenomenon would hold true in human gastric adenocarcinoma cell lines, MKN-45 and AGS. Current treatment regimens include neoadjuvant and adjuvant chemoradiation, which consist of compounds such as 5-FU, cisplatin, irinotecan, carboplatin and paclitaxel (28). Clonogenic survival was compared against treatments of chemotherapeutics and radiation both alone and in combination with pharmacological ascorbate.

5-FU and Radiation—As a single agent, ascorbate in both cells lines had a reduction in clonogenic survival comparable to 5-FU and radiation, which resulted in a 53% decrease in the AGS cell line ($P = 0.0001$) and 38% decrease in the MKN-45 cell line ($P = 0.0001$) (Fig. 2A and B). Ascorbate combined with 5-FU or radiation further reduced clonogenic survival over the single agent treatments by 50% in the AGS cell line ($P = 0.0001$) and 37% in the MKN cell line ($P = 0.0001$) (Fig. 2A and B). When ascorbate was added to 5-FU and radiation, a 49–54% additional decline in cell killing was observed ($P = 0.01$, $P = 0.001$) (Fig. 2A and B).

Cisplatin and irinotecan—As expected, cisplatin and irinotecan in both cell lines exhibited a significant reduction in clonogenic survival ($P = 0.0001$) (Fig. 2C and D). In the AGS cell line, the cisplatin-irinotecan combination caused a significant drop in survival over either monotherapies, which was comparable to the combinations of cisplatin-ascorbate and irinotecan-ascorbate, resulting in an additional 24% reduction over the traditional first-line monotherapies ($P = 0.0001$) (Fig. 2C). Similar results were observed for the MKN-45 cell

line (Fig. 2D). In addition, for the AGS cell line, the irinotecan-ascorbate combination reduced cell killing by an additional 14% compared to traditional cisplatin-irinotecan treatment ($P = 0.05$) (Fig. 2C). Interestingly, when ascorbate was combined with cisplatin and irinotecan, an additional 19% decrease in cell killing was observed over the cisplatin-irinotecan traditional first-line treatment ($P = 0.0001$) (Fig. 2C).

Paclitaxel and carboplatin—Treatment with paclitaxel or carboplatin with pharmacological ascorbate was more effective at decreasing clonogenic survival of the AGS cell line compared to either single treatment alone ($P = 0.0001$) (Fig. 2E). Similarly, the triple combination of paclitaxel, carboplatin and ascorbate significantly decreased clonogenic cell survival by an additional 18% and 34%, in both AGS and MKN-45 cell lines, respectively, compared to the combination of carboplatin and ascorbate ($P = 0.0001$) (Fig. 2E and F). For the MKN-45 cell line, the combination of paclitaxel, carboplatin and pharmacological ascorbate decreased cell killing by an additional 14% compared to paclitaxel combined with ascorbate ($P = 0.01$) (Fig. 2F).

Ascorbate Reduces the Capacity for Invasion and Migration in Gastric Adenocarcinoma

The aggressive nature of tumor cells is often attributed to their ability to mobilize through environmental boundaries. Cell migration is defined as the movement of individual cells, cell sheets and clusters from one location to another (29). Cell invasion refers to the 3-dimensional migration of cells as they penetrate an extracellular matrix (ECM) and is a process typically associated with cancer cell metastasis (30). To investigate the impact on tumor cell aggressiveness we performed cell migration and cell invasion assays. AGS invasion and migration showed a 30% decrease with ascorbate at a concentration of 11 mM (25 pmole cell⁻¹) (Fig. 3A and B) and a ~64% decrease in migration and invasion after treatment with 20 mM ascorbate (45 pmole cell⁻¹) ($P = 0.0001$) (Fig. 3A and B). Importantly, catalase reversed the effects of ascorbate on invasion and migration consistent with H₂O₂ mediating the effects of ascorbate on cancer cell aggressiveness. These data support the hypothesis that ascorbate treatment can attenuate the malignant potential in this gastric adenocarcinoma cell line *in vitro*.

Ascorbate-Mediated Clonogenic Cell Killing is Mediated by Hydrogen Peroxide and the Presence of Catalytic Metal Ions

Platinum-based chemotherapies are a common clinical adjuvant for the treatment of gastric adenocarcinoma (28). Of these, cisplatin exhibits its cytotoxicity primarily through DNA cross-links, and the mechanism has been hypothesized to involve the production of ROS (30). There is also ample evidence in the literature that supports ascorbate oxidation to form hydrogen peroxide in several cell lines, which causes cytotoxicity (7, 9, 31). To investigate this potential mechanism of cytotoxicity in gastric adenocarcinoma, ascorbate along with cisplatin and catalase (both alone and in combination) were applied to the tumor cell lines AGS and MKN-45. In these cell lines, ascorbate and cisplatin as single treatments significantly reduced clonogenic survival ($P = 0.0001$) (Fig. 4A and B). However, there was no difference in the magnitude of cell killing with the addition of catalase to cisplatin (Fig. 4A and B). Furthermore, the cell killing induced by ascorbate combined with cisplatin was restored to levels similar to that seen with cisplatin alone in the presence of catalase,

demonstrating that hydrogen peroxide plays a major role in the cytotoxic effects of ascorbate (Fig. 4A and B) when combined with cisplatin.

Du *et al.* have shown that the rate of ascorbate oxidation to hydrogen peroxide is dependent on the presence of catalytic metal ions in pancreatic cancer cells, principally through the availability of labile iron (32). DFO and DTPA are iron chelators with an affinity for Fe^{3+} and Fe^{2+} , respectively (33, 34), which inhibit metal-ion-catalyzed redox cycling (13) and have been shown to inhibit the anticancer effects of ascorbate. To test this hypothesis in gastric adenocarcinoma cell lines, DFO and DTPA were added to AGS and MKN-45 cell cultures for 3 h prior to treatment with ascorbate for 1 h followed by clonogenic survival assay. Both catalase and DFO/DTPA added to ascorbate completely inhibited ascorbate-induced cell killing, demonstrating that both hydrogen peroxide production as well as the availability of redox active iron are integrally involved with ascorbate toxicity (Fig. 4C and D).

Ascorbate Enhances the Overall Survival of Gastric Cancer Xenografts Treated with Radiation and Chemo

A common standard of care for gastric adenocarcinoma includes a combination of carboplatin, paclitaxel and radiation. When this treatment combination was used in animals implanted with MKN-45 xenografts, the results demonstrated that pharmacological ascorbate given daily in combination with weekly chemo treatment (paclitaxel at 10 mg/kg and carboplatin at 15 mg/kg) and fractionated irradiation (2 Gy fractions given in a total of 8 fractions) significantly increased overall survival compared to radio-chemo treatments alone ($P = 0.019$) (Fig. 5A). To determine if daily ascorbate administration was tolerable, mouse weight was also measured 3 times per week. No significant difference in mouse weight was observed between the group given ascorbate in conjunction with radio-chemo treatment and the group given radio-chemo treatment alone (Fig. 5B).

DISCUSSION

Gastric cancer has a high mortality rate and is often diagnosed at an advanced stage after becoming metastatic. Patients that present with advanced disease have a poor prognosis and a low overall survival rate (1, 2). Fortunately, over the last few decades, many advances have been made in regards to understanding the biology of gastric cancer and a decline in the incidence of distal gastric cancer has occurred. Unfortunately, increases in the incidence of adenocarcinoma of the proximal stomach and distal esophagus have been observed (35–37), and are likely influenced by dietary factors, obesity and *Helicobacter pylori* (*H. pylori*) infection incidence (38). Systemic chemotherapy is currently one of the most effective treatments for gastric adenocarcinoma, despite the toxicity caused by most regimens. Therefore, the goal of many treatments is to effectively enhance efficacy of therapy in cancer cells while simultaneously reducing the cytotoxicity in normal tissues.

The results of the current study demonstrate that pharmacological ascorbate effectively increases cell killing in gastric adenocarcinoma cell lines in a dose-dependent manner. The effect was also shown to be cancer cell specific, since normal intestinal epithelial cells exhibited increased resistance to pharmacological ascorbate. Furthermore, Du *et al.* showed

that pharmacological ascorbate given *in vivo* did not increase systemic changes and markers of oxidative stress, and protected jejunal crypts from radiation-induced injury (17). These results support the concept that pharmacological ascorbate acts as an antioxidant in normal tissues while selectively acting as a pro-oxidant in cancer tissues.

In the treatment of gastric adenocarcinoma, no one standard regimen exists for standard-of-care therapies (2, 39). Therefore, the current study examined the use of pharmacological ascorbate in conjunction with many common radio-chemotherapies used to treat GAC. Clonogenic survival assays were employed to investigate the potential for additive or greater than additive effects on the toxicity of pharmacological doses of ascorbate combined with radiation, 5-fluorouracil, cisplatin, irinotecan, paclitaxel and carboplatin. In all instances, the addition of pharmacological ascorbate to single-agent or dual-agent therapies caused an increase in cell killing compared to any single or dual radio-chemotherapeutic. These highly encouraging results allow gastric adenocarcinoma to join the list of cancers (12, 13, 20) for which conventional therapies may benefit from the addition of pharmacological ascorbate.

As previously discussed, gastric adenocarcinoma typically presents at a local advanced stage with distant metastatic sites. Consequently, investigating ways to attenuate the metastatic potential of GAC may be advantageous in the process of slowing disease progression and increasing patient survival. In the current study, we investigated the effects of pharmacological ascorbate on migration and invasion of gastric adenocarcinoma, both of which are implicated in the process of metastasis and cancer progression (30). Results showed a concentration-dependent decrease in invasion and migration with increasing doses of pharmacological ascorbate. Also, the addition of catalase, an H_2O_2 scavenger, was able to reverse effects of ascorbate on both migration and invasion in gastric cancer cells. This suggests that pharmacological ascorbate could conceivably be utilized to reduce the aggressive disease profile in gastric adenocarcinoma. These results also confirm previously reported studies establishing the mechanism of pharmacological ascorbate as a pro-oxidant in cancer cells that generates H_2O_2 to produce cytotoxicity (7–9).

The cancer cell specificity of pharmacological ascorbate is thought to result from increased H_2O_2 production, coupled with the increased redox active labile metal ions in cancer cells driving Fenton chemistry that mediates the toxicity of ascorbate (13, 32, 40, 41). Recently, Schoenfeld *et al.* further confirmed that alterations in mitochondrial oxidative metabolism in non-small cell lung cancer and glioblastoma cells selectively sensitized these cells to pharmacological ascorbate through increased levels of superoxide ($O_2^{\bullet-}$) and H_2O_2 (13). Through the use of iron chelators DFO and DTPA, the current study also demonstrated that reduced clonogenic survival of gastric adenocarcinoma cells in the presence of pharmacological ascorbate can be fully reversed when redox active iron availability is diminished. Incubation of ascorbate in the presence of the H_2O_2 -specific scavenging enzyme, catalase, also reversed the cytotoxicity seen with pharmacological ascorbate. These results continue to support the generality of the previously reported results (13, 32, 41, 42) that the cancer cell specific effects of pharmacological ascorbate are dependent on redox active metal ions and H_2O_2 in gastric adenocarcinoma.

Xenograft studies for gastric adenocarcinoma revealed that pharmacological ascorbate given daily alongside radio-chemotherapy significantly increased overall survival compared to radio-chemotherapy alone. These results are similar to reports in glioblastoma, pancreas and non-small-cell lung cancers, where addition of pharmacological ascorbate to radio-chemotherapy also increased survival of *in vivo* preclinical models (13, 17). Ma *et al.* used ascorbate in preclinical models in addition to the standard chemotherapy regimen (paclitaxel and carboplatin) for ovarian cancer and found that the triple therapy combination reduced tumor weight compared to controls with no discernable adverse effects of ascorbate being noted (12). Similarly, the current study tracked mouse weight as a way to determine toxicity of pharmacological ascorbate, and no difference in weight was found between controls and ascorbate-treated animals. Taken together, pharmacological ascorbate shows promise as an adjuvant treatment that enhances standard radio-chemotherapies while potentially reducing their adverse effects. The data reported here support the use of pharmacological ascorbate as a possible efficacious adjuvant to current therapies for gastric adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Ascorbate attenuates clonogenic cell survival of human gastric adenocarcinoma cell lines. Gastric adenocarcinoma cell lines AGS and MKN-45 along with the normal small intestinal epithelial nonimmortalized human cell isolate FHs 74 Int incubated with 0.5–2 mM pharmacological ascorbate (5–48 pmole cell⁻¹) for 1 h were assessed for clonogenic cell survival. Colony formation was determined after 7–18 days. Survival decreased statistically in both gastric adenocarcinoma cell lines with increasing doses of ascorbate compared to controls. Comparisons of all cells at 2 mM ascorbate showed significant differences between normal and gastric cancer cells. Data represent normalized surviving fractions compared to controls $\pm$ SE (** P 0.01, **** P 0.0001; two-way ANOVA with Tukey's multiple comparisons).

FIG. 2.

Ascorbate (AscH⁻) enhances cytotoxicity of first-line chemotherapeutics. For all graphs colony formation was determined after 7–10 days of incubation. Data represent normalized surviving fractions compared to controls $\pm$ SE. AGS and MKN-45 cells (panels A and B, respectively) were incubated with various combinations of 1 mM/14.5 (MKN-45) or 25 (AGS) pmole cell⁻¹ ascorbate for 1 h, 5 μ M 5-fluorouracil for 24 h and then 1.8 Gy irradiated immediately before plating. Single treatment of radiation or 5-FU with ascorbate was as effective at decreasing clonogenic survival as the combination of 5-FU/radiation. Clonogenic survival of all three therapies showed a statistical decrease compared to any

other combination (* P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001). AGS and MKN-45 cells (panels C and D, respectively) were incubated with various combinations of 1 mM/14.5 (MKN-45) or 25 (AGS) pmole cell⁻¹ ascorbate for 1 h, 0.5 μ M cisplatin (Cis) for 24 h and 2.5 μ M irinotecan (Iri) for 24 h before plating. Single drug treatment of cisplatin or irinotecan with pharmacological ascorbate was as effective or more effective at decreasing clonogenic survival of the AGS cell line. Combination of all three therapies in the AGS cell line showed a statistical decrease compared to standard chemotherapies combined. In MKN-45 cells, pharmacological ascorbate in combination with cisplatin significantly decreased plating efficiency compared to cisplatin or ascorbate treatment alone (* P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001). AGS and MKN-45 cells (panels E and F, respectively) were incubated with various combinations of 1 mM/14.5 (MKN-45) or 25 (AGS) pmole cell⁻¹ ascorbate for 1 h, 4 nM (AGS) or 2 nM (MKN-45) paclitaxel (Taxel) for 24 h and 5 μ M carboplatin (Carbo) for 24 h before plating. Single drug treatment of paclitaxel or carboplatin with pharmacological ascorbate was more effective at decreasing clonogenic survival of the AGS cell line. Combination of paclitaxel, carboplatin and ascorbate statistically decreased clonogenic cell survival compared to the combination of carboplatin and ascorbate (AGS and MKN-45) as well as paclitaxel and ascorbate (MKN-45) (** P 0.01, **** P 0.0001; one-way ANOVA with Tukey's multiple comparisons).

FIG. 3.

Ascorbate (AsCH⁻) attenuates the malignant phenotype of gastric adenocarcinoma cell lines and is dependent on hydrogen peroxide. Panels A and B: Invasion and migration, respectively, of AGS incubated with 11 and 20 mM (25 and 45 pmole cell⁻¹) ascorbate alone and in combination with 15 U/ml catalase. Invasion at 20 mM decreased significantly by 57% while migration statistically decreased by 61%. The addition of catalase completely reversed the decrease observed with ascorbate. AGS cells were seeded at 2×10^5 and allowed to incubate for 22–24 h before analysis. Data represent relative invasion or migration compared to controls $\pm$ SE (** P 0.01, *** P 0.001, **** P 0.0001).

FIG. 4.

Ascorbate (AscH⁻) toxicity is largely dependent on hydrogen peroxide and the presence of catalytic metal ions. AGS and MKN-45 cells (panels A and B, respectively) were treated with cisplatin (Cis) (0.5 μ M), and ascorbate (25 pmole cell⁻¹), both alone and in combination with catalase (cat) (50 U/ml) in clonogenic survival assays. Colony formation was determined after 7–10 days. The combination of ascorbate and catalase resulted in a full rescue compared to ascorbate treatment alone. Cisplatin combined with catalase showed no significant difference over cisplatin treatment alone, suggesting a negligible influence on clonogenic survival through the production of ROS via hydrogen peroxide. Data represent normalized surviving fractions compared to controls $\pm$ SE (**** P 0.0001). AGS and MKN-45 cells (panels C and D, respectively) were treated with ascorbate (25 pmole cell⁻¹), catalase (50 U/ml), and DFO (200 μ M)/DTPA (1 mM) both alone and in combination in clonogenic survival assays. Colony formation was determined after 7–10 days. A full rescue from the cytotoxic effects of ascorbate is observed when ascorbate is combined with either

catalase or DFO/DTPA. Data represent normalized surviving fractions compared to controls $\pm$ SE (**** P 0.0001).

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FIG. 5.

Administration of ascorbate (AscH⁻) increases median survival of athymic nude mice. Panel A: MKN-45 xenografts were grown in female 6–8-week-old athymic-nu/nu mice (20 per group). Cells (5×10^5) were injected subcutaneously into mice and tumor formation was monitored. Upon tumor formation of 2–3 mm, ascorbate (4 g/kg) was given daily, as well as paclitaxel (Taxel; 10 mg/kg) and carboplatin (Carbo; 15 mg/kg) weekly and received 2 Gy fractions for a total of 8 fractions. Animals were sacrificed when the tumors reached 15 mm in diameter of either length or width or when an animal lost more than 20% of its initial body weight. Group comparisons of Kaplan-Meier survival plots showed that the addition of

ascorbate significantly increased the median survival ($P = 0.019$, Mantel-Cox log-ranked) compared to standard-of-care therapies. Panel B: Average weight of mice over the duration 20 days in MKN-45 xenografts. No difference in weight was observed between groups given daily ascorbate and radio-chemo treatment compared to radio-chemo treatment alone.