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Research Paper

Augmentation of intracellular iron using iron sucrose enhances the toxicity of pharmacological ascorbate in colon cancer cells

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ABSTRACT

Pharmacological doses (> 1 mM) of ascorbate (a.k.a., vitamin C) have been shown to selectively kill cancer cells through a mechanism that is dependent on the generation of H_2O_2 at doses that are safely achievable in humans using intravenous administration. The process by which ascorbate oxidizes to form H_2O_2 is thought to be mediated catalytically by redox active metal ions such as iron (Fe). Because intravenous iron sucrose is often administered to colon cancer patients to help mitigate anemia, the current study assessed the ability of pharmacological ascorbate to kill colon cancer cells in the presence and absence of iron sucrose.

In vitro survival assays showed that 10 mM ascorbate exposure (2 h) clonogenically inactivated 40–80% of exponentially growing colon cancer cell lines (HCT116 and HT29). When the H_2O_2 scavenging enzyme, catalase, was added to the media, or conditionally over-expressed using a doxycycline inducible vector, the toxicity of pharmacological ascorbate was significantly blunted. When colon cancer cells were treated in the presence or absence of 250 μM iron sucrose, then rinsed, and treated with 10 mM ascorbate, the cells demonstrated increased levels of labile iron that resulted in significantly increased clonogenic cell killing, compared to pharmacological ascorbate alone. Interestingly, when colon cancer cells were treated with iron sucrose for 1 h and then 10 mM ascorbate was added to the media in the continued presence of iron sucrose, there was no enhancement of toxicity despite similar increases in intracellular labile iron. The combination of iron chelators, deferoxamine and diethylenetriaminepentaacetic acid, significantly inhibited the toxicity of either ascorbate alone or ascorbate following iron sucrose. These observations support the hypothesis that increasing intracellular labile iron pools, using iron sucrose, can be used to increase the toxicity of pharmacological ascorbate in human colon cancer cells by a mechanism involving increased generation of H_2O_2.

1. Introduction

Although the number of new colorectal cancer (CRC) cases being diagnosed has been declining, in recent years CRC remains the third leading cause of cancer-related death in the United States [1]. Treatment of CRC typically includes a combination of surgical resection and 5- fluorouracil (5FU) based chemotherapy regimens. Complications of 5FU based treatment can include peripheral neuropathy, mucositis, bone marrow toxicity, and hand-foot-mouth syndrome. Due to CRC's high incidence and mortality rates, as well as the toxicities associated with the chemotherapeutic agents used to treat this disease, less toxic, more effective, and easily implemented treatments are urgently needed.

In 1959 it was first postulated that ascorbate, (vitamin C, an essential nutrient that is a cofactor in many enzymatic reactions) might limit the spread of cancer [2]. An early uncontrolled trial of terminal cancer patients of various etiologies using 10 g intravenous ascorbic acid for 2 weeks followed by oral ascorbate treatment showed significant improvement in cancer morbidity and mortality with clinicians agreeing that there was an increase in survival beyond reasonable clinical expectation [3]. In subsequent controlled clinical trials

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performed, patients with advanced cancers of varying origins were treated with oral ascorbic acid therapy vs. placebo and no statistically significant benefit was demonstrated [4,5]. This dampened enthusiasm for studies on the use of high dose ascorbate to treat cancer. However, later pharmacokinetic studies of ascorbate found that blood levels of ascorbate are tightly controlled; to achieve > 1 mM blood levels intravenous administration is required. This information provides an explanation for the discrepancy between the findings of earlier trials [6,7]. Additional trials have since shown that patients easily tolerate peak plasma concentrations of ascorbate > 25 mM, with only mild associated clinical adverse events [8–11].

Ascorbate readily undergoes pH-dependent autoxidation producing hydrogen peroxide (H_2O_2); in the presence of catalytic metals this oxidation can be greatly accelerated [12,13]. Previous studies have demonstrated that pharmacologic ascorbate treatment, which preferentially kills cancer cells, relative to non-malignant cells, is dependent of the production of H_2O_2 through the formation of the ascorbate radical [14,15]. It has been demonstrated that redox active metal ions, such as loosely bound labile iron, significantly contribute to ascorbate-induced H_2O_2-mediated cytotoxicity [14,10,16]. Furthermore, it was shown in neuroblastoma and lung cancer cell lines that susceptibility to ascorbate toxicity was associated with ferritin concentrations [10,15]. Furthermore colon cancer cells have been shown to be susceptible to pharmacological ascorbate-induced cytotoxicity [17], and newly diagnosed cases of colorectal cancer, 60% of the patients, have some evidence of iron deficiency that is often treated with intravenous iron sucrose supplementation [18].

Within cancer cells, increased steady-state levels of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) have been shown to disrupt iron metabolism leading to increased labile iron that can enhance the cancer cell specific toxicity of ascorbate [10]·H_2O_2 can then further react with redox active metal ions to generate powerful oxidants, such as the hydroxyl radical, that can damage DNA and kill cancer cells [19,20]. Furthermore, exponentially growing colon cancer cells in culture have been shown to have increased iron content, relative to normal cells, which may account for their increased sensitivity to pharmacological ascorbate [17,21]. Since many colon cancer patients experience iron deficiency, this could limit the therapeutic effectiveness of pharmacological ascorbate.

In the current study, the potential to enhance H_2O_2-mediated colon cancer cell killing by increasing intracellular iron with a clinically available preparation of iron sucrose in the presence of pharmacological ascorbate was tested. Causality related to the mechanistic hypothesis was tested using metal chelation or catalase, both intra- and extracellularly. The results support the hypothesis that anti-tumor responses induced by treatment with pharmacological ascorbate in colon cancer are mediated by H_2O_2 and redox active metals. The anti-tumor responses could be significantly enhanced by increasing labile iron pools using clinically available iron sucrose preparations.

2. Materials and methods

2.1. Cells and culture conditions

HCT 116 and HT-29 human colon carcinoma cells were obtained from ATCC, maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone) or RPMI 1640 (Mediatech) with 10% FBS, respectively, and expanded and frozen at −80 °C. Cells for all experiments were used within 17 passages of the original frozen stock. TSA201 (Sigma-Aldridge) cells were maintained in DMEM with 10% FBS. All cultures were maintained in 5% CO_2, 20% O_2 and humidified in a 37 °C incubator.

2.2. Drug treatment

Drugs were added to cells at final concentrations of 250 μM iron sucrose (Venofer, American Regents Inc.), 2.5–10 mM ascorbate (Macron Chemicals) that was prepared in sodium bicarbonate to maintain a neutral pH, 20–200 μM deferoxamine (Sigma), 1 mM diethylenetriaminepentaacetic acid (DETAPAC) (Sigma), 100 Units/mL bovine catalase (Sigma), and 0.5–2 μg/mL doxycycline (Fisher Scientific).

2.3. Clonogenic cell survival assay

HCT 116 and HT-29 cells ($0.7–2.5 \times 10^5$) were plated in 60-mm dishes and allowed to grow in their respective stock culture media for at least 48 h. Media were changed to DMEM with 10% FBS without pyruvate, and iron, iron chelator, or catalase was added. The dishes were returned to the incubator (to allow for equilibration of pH) for the indicated period of time before 10 mM ascorbate treatment (resulting in 21–34 pmoles or 19–43 pmoles of ascorbate per cell for HT-29 and HCT 116 cells, respectively). After drug treatment, media containing any floating cells was removed from the treatment dish. Attached cells were trypsinized with 0.25% trypsin-EDTA and trypsin was inactivated by recombining cells with the media from the same treatment dish containing 10% FBS. Samples were centrifuged, re-suspended in fresh media and the resulting total cell population counted using a Beckman Coulter Counter. The cells were then plated in 60-mm dishes at a variety of densities ranging from 200 to 50,000 cells per dish. Clones were grown for 10–12 days in complete media with 0.1% gentamycin. Cells were fixed with 70% ethanol, stained with Coomassie blue, and colonies containing ≥ 50 cells were counted. The plating efficiencies of treatment groups for each cell line were normalized to the control group. The survival analysis was done using a minimum of 3 cloning dishes per experimental condition, and the experiments were repeated a minimum of 3 times on separate occasions.

2.4. Lentivirus production and transduction

The human catalase coding gene sequence was purchased from ViraQuest. To generate plasmid with doxycycline-inducible expression of catalase, HA tagged human catalase PCR fragment was inserted in between Age I and Mlu I restriction sites of pTRIPZ vector (obtained from Dr. Dana Levasseur's lab, The University of Iowa) to replace RFP-mir30-shRNA cassette (Fig. 1**C**). The vector allowed for puromycin resistance. Lentivirus was produced in TSA201 cell line using pCMV-VSV-G and psPAX2 helper vectors (Addgene). Virus was collected from TSA201 cell cultures, centrifuged to remove cell debris, and filtered using 0.45 μm filters. HCT 116 cells were plated and allowed to grow for 48 h, and then virus was added to cells with 8 μg/mL of polybrene for a total of 48 h, with fresh virus being added after 24 h. Following transduction, cells were selected with 1.5 μg/mL puromycin. For expansion of single transduced clones, cells were re-plated in 150 mm dishes, with 1200 cells per dish. Clones were grown for two weeks, and 12 colonies were picked and expanded. Catalase activity was assessed following induction with 0.5–2 μg/mL doxycycline for 24–72 h in order to determine the best clone and induction conditions for further experiments (Fig. 1).

2.5. Catalase assay

Cells were grown and treated as stated above. Treatment medium was removed; cells were scraped into 200 μL of phosphate buffer pH 7.0, and frozen at −20 °C. Cells were sonicated to break open the cells, and protein concentrations of the samples were determined using the Lowry Assay [22]. Catalase activity was determined at 25 °C according to the method of Beers and Sizer with the analysis of Aebi [23–25]. Briefly, 80 μL of samples were added to 4,0 mL phosphate buffer, mixed, and separated into two quartz cuvettes, blank and active. An H_2O_2 working solution was added to the active cuvette and the rate of disappearance of absorption at 240 nm was measured. Bovine catalase

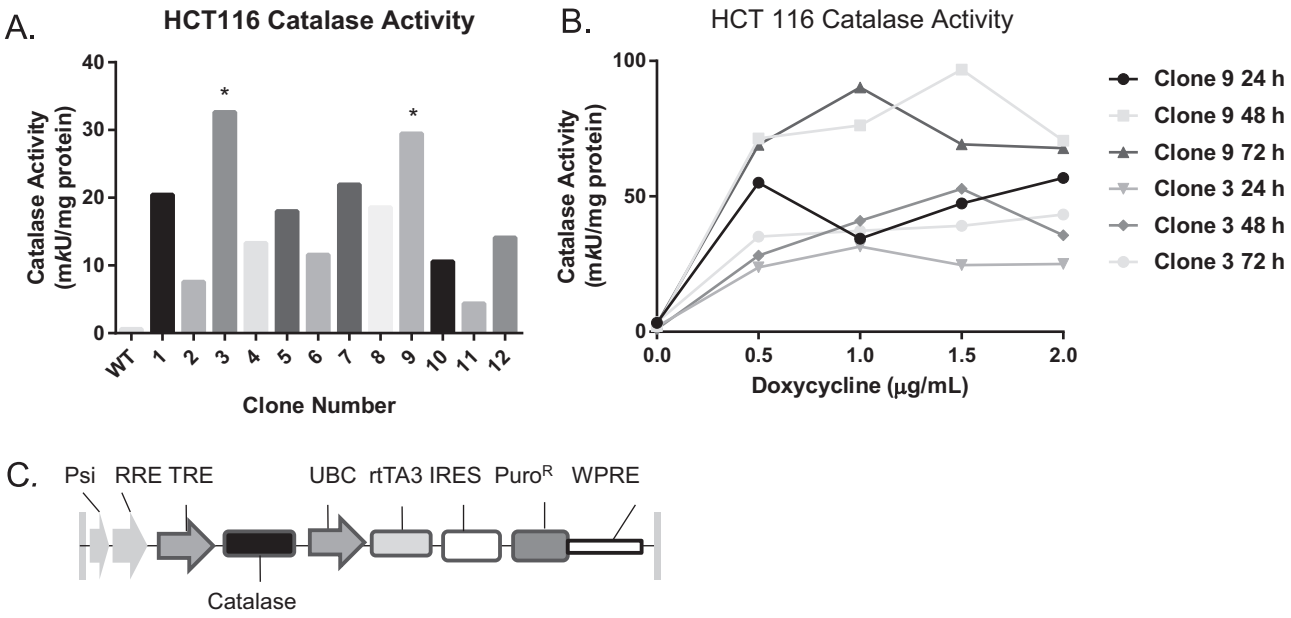

Fig. 1. HCT 116 cat clone 9 has highest catalase activity with 1.5 μg/mL doxycycline induction for 48 h. (A) 12 clones of and wildtype HCT 116 catalase overexpression cells transduced with a pTRIPZ catalase overexpression vector were isolated, and catalase activity was measured following 0.5 μg/mL doxycycline induction for 48 h. **(B)** Clones 3 and 9 were induced with 0.5–2.0 μg/mL doxycycline for 24–72 h, and catalase activity was measured. **(C)** Catalase expressing vector map.

was used for the standard. All activity assays were normalized per mg cellular protein.

2.6. Calcein flow cytometry assay of labile iron

Cells were grown and treated as described above and assayed for labile iron using a variation of an assay described previously [26,27]. Ferrous ammonium sulfate (80 μM) was used as a positive control. Following treatments, media were removed and cells were washed in PBS. Cells were collected with trypsin-EDTA and washed again with PBS. Cell pellets were then re-suspended in 1.5 mL PBS, and 500 nM Calcein-AM (Molecular Probes) was added. Cells were incubated at 37 °C and 21% O_2 for 15 min. Cells were then pelleted and resuspended in 1 mL PBS, and filtered through 70 μm mesh. The samples were then split into two aliquots of 500 μL each and one was treated with 2,2′-bipyridyl (BIP; Sigma). BIP -treated samples were run at least 15 min after the addition to ensure adequate time for iron chelation. Samples were analyzed using a FACScan flow cytometer on LSR Violet with 515/15 emission filter. The mean fluorescence intensity (MFI) of 10,000 cells was analyzed in each sample and the MFI of non-BIP samples was subtracted from the MFI of BIP-treated samples to obtain the iron specific signal for each treatment group. MFI data were normalized to corresponding control group levels for each cell type.

3. Results

3.1. Pharmacological ascorbate-induced clonogenic cell killing is mediated by H_2O_2 in colon cancer cells

To test the hypothesis that treatment with pharmacological ascorbate decreases clonogenic survival in colon cancer cells, HCT 116 and HT-29 were treated with 10 mM ascorbate for 2 h. In both cell lines, pharmacological ascorbate treatment resulted in significant decreases in clonogenic cell survival compared to untreated controls (Fig. 2**AB**). When comparing the two colon cancer cell lines, HCT 116 cells initially appeared to be more sensitive than HT-29 cells to pharmacological ascorbate treatment (Fig. 2**AB**). However, these two cell lines had different growth media (HCT 116; DMEM 10% FBS with 110 mg/L of pyruvate and HT-29; RPMI1640 10% FBS with no pyruvate) as well as cellular confluency at the time of treatment and these variables have been reported to alter ascorbate-induced killing, so clonogenic survival cannot be compared directly in these experiments [10,28].

As expected, the addition of bovine catalase (100 units/mL) to the cell culture media 20 min prior to ascorbate treatment completely protected cells against killing mediated by 10 mM ascorbate (Fig. 2AB). These results indicate, parallel to data reported previously [10,29,30], that the toxicity of ascorbate in this model system is mediated by H_2O_2. Although H_2O_2 can freely transfuse across cellular membranes [31,32], catalase does not cross the membranes of most cells. To demonstrate that ascorbate toxicity is dependent on intracellular H_2O_2, HCT 116 cells were stably transfected with a doxycycline inducible catalase overexpression vector (HCT 116-cat 9; Fig. 1**AB**). Induction of these cells with 1.5 μg/mL doxycycline for 48 h resulted in 58 m*k*U of catalase activity per mg of protein compared to 2.8 m*k*U/mg of protein in cells not treated with doxycycline (Fig. 2**C**). These cells were then treated with 1–5 mM ascorbate for 2 h. As expected, overexpression of catalase significantly inhibited the toxicity of ascorbate (Fig. 2**D**), consistent with ascorbate toxicity being mediated by intracellular H_2O_2.

3.2. Intracellular labile iron facilitates toxicity of ascorbate

The importance of redox active iron as the critical mediator of the cellular pathology of H_2O_2 has been demonstrated [20]. Likewise, the addition of a metal chelator that inhibits the redox cycling of Fe^{2+}/Fe^{3+} (20 μM deferoxamine) partially inhibited the toxicity of ascorbate in HCT 116 cells but not in HT-29 cells (Fig. 2AB). This suggests that redox cycling of iron is necessary for ascorbate toxicity in HCT 116 cells.

To further elucidate the role of intracellular *vs*. extracellular iron on ascorbate toxicity, HCT 116 (0.8–1.7 × 10^6 cells/60 mm plates) and HT-29 (1.2–2.2 × 10^6 cells/plate) cells were pre-treated with a clinically relevant preparation of iron sucrose (250 μM) for 2 h, and either washed with PBS or not washed, prior to treatment with 10 mM ascorbate for 2 additional hours. In both cell lines, ascorbate treatment alone resulted in significantly decreased clonogenic cell survival compared to control (Fig. 3**AB**; $p < 0.0001$). The presence of iron sucrose during ascorbate treatment led to increased survival in HCT 116 cells but not HT-29 cells (Fig. 3**AB**). However, when iron sucrose was

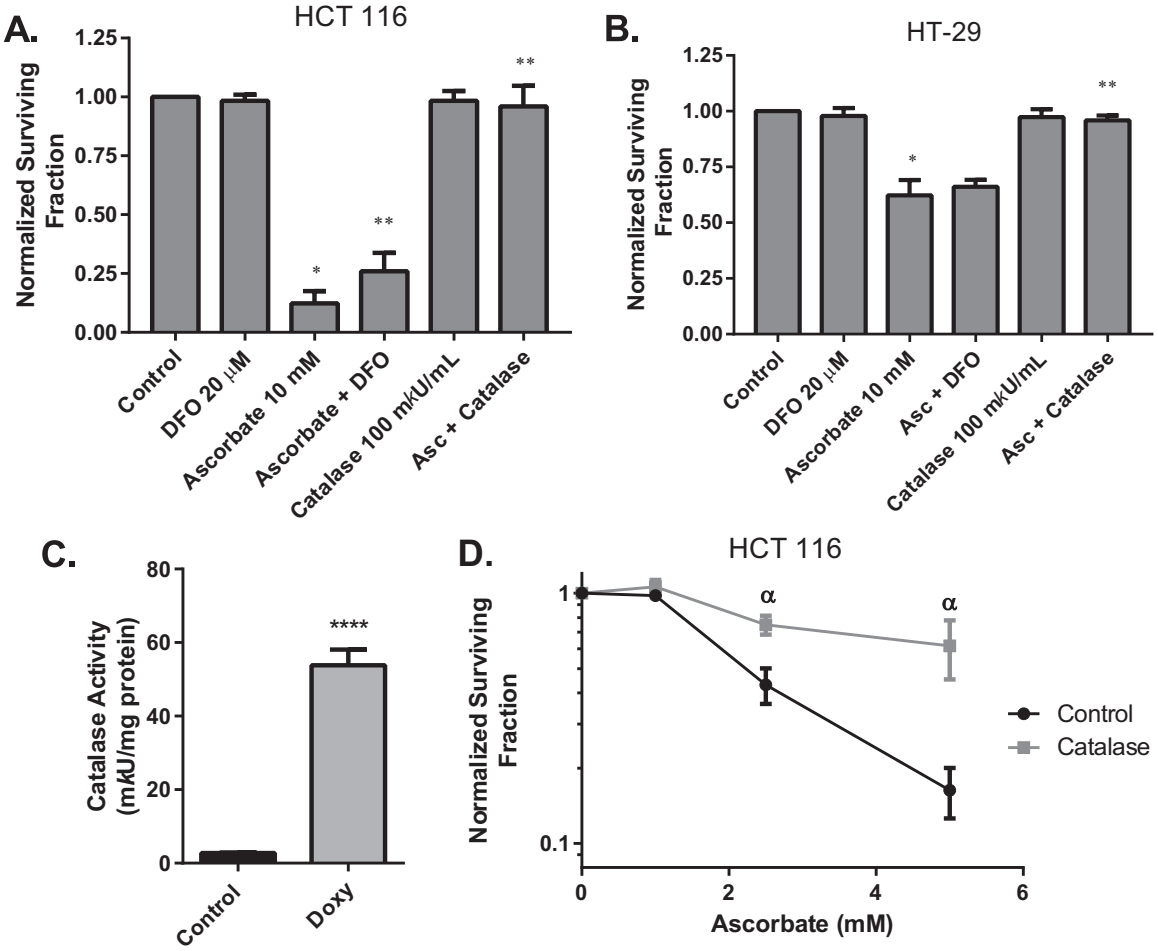

Fig. 2. Ascorbate causes HCT 116 and HT-29 clonogenic cell killing that is inhibited by both inter and extracellular catalase. (A) HCT 116 and **(B)** HT-29 cells were pre-treated with 250 μM iron sucrose, 20 μM deferoxamine, or 100 U/mL bovine catalase for 20 min followed by 10 mM ascorbate for 2 h. Toxicity was assessed via clonogenic survival assays. Errors represent ± SEM, $n = 3$ with at least 3 cloning dishes per treatment. * = $p < 0.05$ vs control by one way ANOVA, ** = $p < 0.05$ *vs.* ascorbate by paired *t*-test. HCT 116 cells were transduced with lentivirus containing a doxycycline inducible catalase overexpression vector. **(C)** A single transduced clone (HCT 116-cat 9) was induced with 1.5 μg/mL doxycycline for 48 h and catalase activity was assessed compared to cat 9 cells not treated with doxycycline in clonogenic survival assays **(D)**, HCT 116 cat-9 cells were induced with 1.5 μg/mL doxycycline for 48 h. Cell culture media was changed to DMEM with no pyruvate 1 h prior to treatment, followed by 2-h treatment with 1–5 mM ascorbate. Toxicity was assessed via clonogenic survival assays. Errors represent ± SEM, $n = 3$, with at least 3 cloning dishes per treatment. α = $p < 0.05$ vs. control by two way ANOVA.

removed from the media by washing with PBS immediately prior to ascorbate exposure, a significant decrease in clonogenic survival was noted in both colon cancer cell lines (Fig. 3**AB**; $p < 0.05$). Use of Calcein AM dye to measure intracellular labile iron demonstrated that iron sucrose significantly elevated the intracellular iron in the two cell lines, both when removed from and when left in the media (Fig. 3**C**). It therefore appears that the location of the iron (extracellular *vs.* intracellular) may cause the differential toxicity when the Fe-sucrose is washed off the cells. This is consistent with previous results showing that if excess iron is in the media when H_2O_2 is formed, this iron can react with the H_2O_2 in the medium, removing it, and thereby resulting in reduced toxicity to the cells [33–35], Interestingly, after Fe-sucrose treatment, HCT 116 had more labile iron than HT-29, and also demonstrated greater toxicity when combined with ascorbate (Fig. 3**C**) suggesting that intracellular iron content was a critical determinant of the anti-cancer effects of ascorbate [10]. To further show that intracellular labile iron is necessary for ascorbate toxicity, Fe chelators that inhibit redox cycling of metal ions (DFO and DETAPAC) were added during exposure to ascorbate following the wash off of iron sucrose; results showed that ascorbate toxicity was significantly inhibited (Fig. 3**D**). Overall, these data support the hypothesis that intracellular redox cycling of iron is necessary for ascorbate toxicity.

4. Discussion

It has been hypothesized that cancer cells demonstrate increased steady-state levels of mitochondrial reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These ROS react with Fe^{2+}/Fe^{3+} bound to proteins (*i.e.*, certain FeS-proteins and ferritin) to mobilize small molecular weight chelates of Fe^{2+}/Fe^{3+} that are capable of redox cycling with ascorbate to generate even greater steady-state levels of H_2O_2[10]. It has further been hypothesized that reducing equivalents from NADPH regenerated by glucose-6-phosphate dehydrogenase in the Pentose Phosphate Cycle are then utilized to reduce cellular disulfides back to their thiol form [GSH and $Grx(SH)_2$] via glutathione disulfide reductase (GR). These thiols then regenerate $AscH^-$ from dehydroascorbate (DHA) setting up a vicious cycle for selectively generating H_2O_2 in cancer cells.[10] Thus, due to the presence of pharmacological ascorbate, excessive amounts of H_2O_2 are generated in tumors, which in the presence of the larger pool of intracellular labile iron, can cause DNA damage and cancer cell death.

The results of the current study demonstrate that pharmacological ascorbate is capable of inhibiting the reproductive integrity of colon cancer cell lines (HCT 116 and HT-29) by a mechanism that is mediated by H_2O_2 (Fig. 2**A-D**) using concentrations that are easily achievable with the IV administration of ascorbate in clinical trials with pancreatic

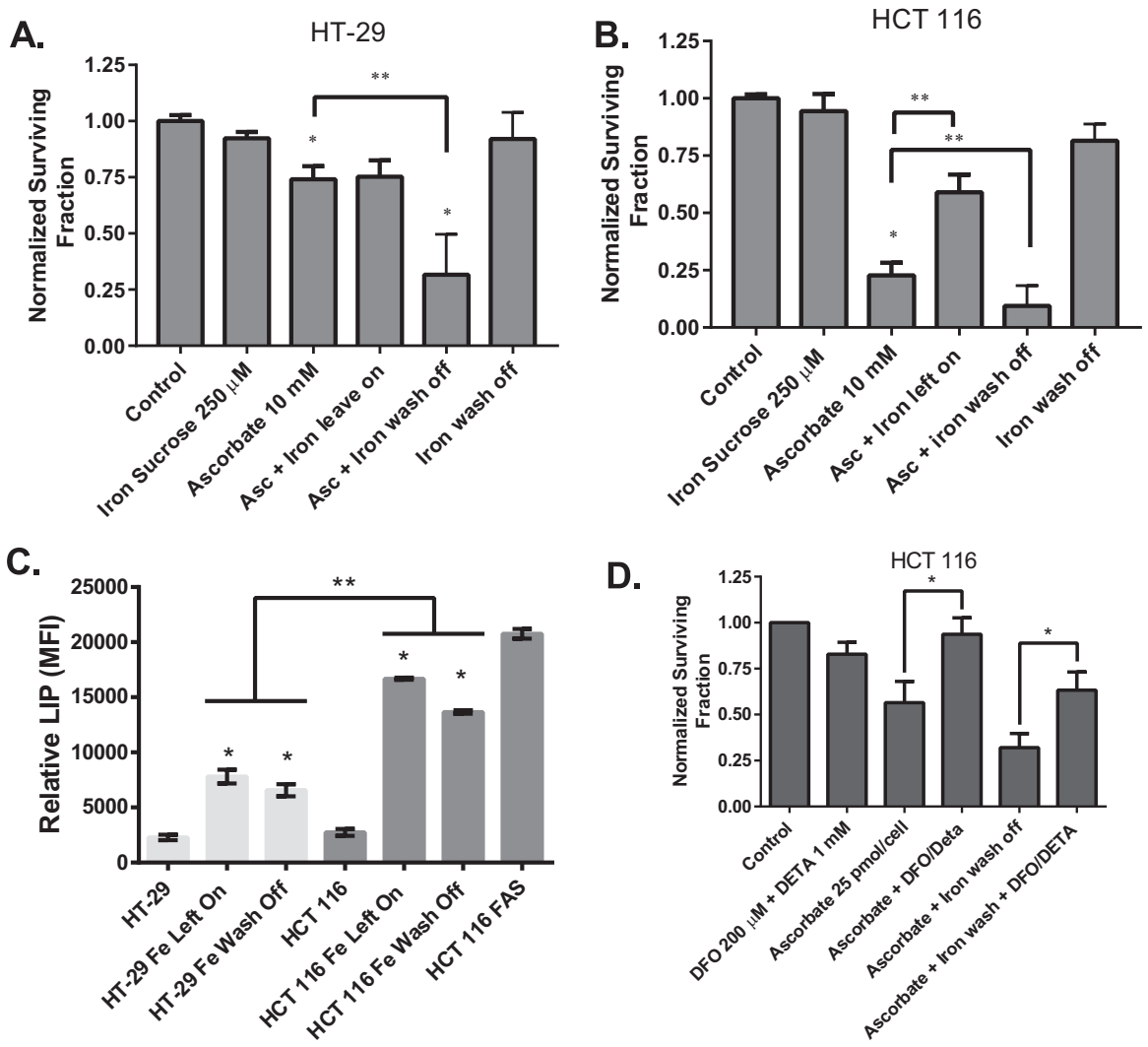

Fig. 3. Intracellular iron sucrose increases ascorbate-induced HCT 116 and HT-29 clonogenic cell death. (A) HCT 116, and **(B)** HT-29 cells were pre-treated with 250 μM iron sucrose for 2 h, washed with PBS, and then treated with 10 mM ascorbate for 2 h. Toxicity was assessed via clonogenic survival assays. **(C)** Calcein assay demonstrates that treatment with iron sucrose significantly increased intracellular iron levels in both HCT 116 and HT-29 cells. Treatment with 80 μM ferrous ammonium sulfate for 3 h was used as a positive control. **(D)** HCT 116 cells were also pre-treated with iron sucrose, washed with PBS, and treated with the iron chelators deferoxamine (DFO) and diethylenetriaminepentaacetic acid (DETAPAC) during treatment with 25 pmol/cell ascorbate. Errors represent ± SEM, $n = 3$ with at least 3 cloning dishes per treatment. * = $p < 0.05$ *vs.* control, ** = $p < 0.05$ *vs.* ascorbate by paired *t*-test.

cancer, lung cancer, and brain cancer patients done at The University of Iowa Hospitals and Clinics [9,10]. Previous publications have shown that in general pharmacologic concentrations of ascorbate also selectively kill cancer cells *vs.* normal cells [29,30]. Therefore the current results continue to support the potential clinical utility of pharmacological ascorbate as an adjuvant in the treatment of colon cancer.

This study also demonstrates that ascorbate toxicity in colon cancer cell lines could be enhanced by increasing the intracellular levels of iron using treatment with iron sucrose, which is a clinically relevant source of iron supplementation in colon cancer patients. These results continue to support the previously proposed hypothesis that increasing the levels of redox active transition metals (such as labile iron) intracellularly in human cancer cells can enhance the oxidation of ascorbate and facilitate the oxidation of critical biomolecules, which can ultimately result in enhanced cancer cell killing and therapeutic responses via H_2O_2-mediated toxicity.[10,12,19,36,37] These novel observations are potentially significant to the development of combined modality approaches using pharmacological ascorbate as an adjuvant for colon cancer therapy, since many of these patients are regularly treated for iron deficiency using iron sucrose [38]. These results suggest that Fe-sucrose should not be given simultaneously with ascorbate, but given far enough in advance of ascorbate administration to allow for Fe uptake into tumor tissue.

Furthermore, when comparing the two different colon cancer cell lines (HCT 116 and HT-29), it appeared that HCT 116 cells were more susceptible to 10 mM ascorbate toxicity, relative to HT-29 in both the presence and absence of pretreatment with iron sucrose (Fig. 3AB). Consistent with this observation, HCT 116 cells treated with iron sucrose had greater increases in labile iron, relative to HT-29 cells treated in a similar fashion (Fig. 3C). These observations provide further support for the hypothesis that increased levels of intracellular iron in the presence of pharmacological ascorbate enhance cancer cell killing.

Overall, these results demonstrate that pharmacological ascorbate shows great promise as a selective cancer cell cytotoxin that can be utilized for the development of combined modality approaches for treating colon cancer. The tumor cell toxicity of pharmacological ascorbate is significantly increased with increasing levels of intracellular labile iron, which may be manipulated with Fe-sucrose to enhance cancer cell killing.

Since many colon cancer patients demonstrate iron deficiency anemia or anemia of chronic disease as well as being treated with Fe-sucrose to bolster erythropoiesis, future studies of the efficacy of

pharmacological ascorbate as an adjuvant to colon cancer therapy should consider the sequencing of treatment with Fe-sucrose and pharmacological ascorbate in the context of systemic iron metabolism and tumor Iron content [39]. Given our in vitro finding that extracellular Fe-sucrose can inhibit ascorbate toxicity, further pre-clinical in vivo research is needed to determine the optimal strategy and pharmacokinetics of iron sucrose administration, relative to administration of ascorbate. This could be important to maximizing the sensitization of cancer cells by increasing intracellular labile iron without significantly inhibiting ascorbate toxicity by increasing the extracellular labile iron.

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