Redox Biology 14 (2018) 82–87

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

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

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ARTICLE INFO

Keywords:
Oxidative stress
Redox active iron
Iron sucrose
Catalase
Deferoxamine
Chelators

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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http://dx.doi.org/10.1016/j.redox.2017.08.017
Received 12 July 2017; Received in revised form 17 August 2017; Accepted 21 August 2017
Available online 26 August 2017

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