Pharmacological ascorbate and ionizing radiation (IR) increase labile iron in pancreatic cancer

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Introduction

Iron is an essential redox active metal used to catalyze many chemical reactions within organisms. The principal catalytic activity of iron is the facilitation of electron transfer between different biomolecules. Within the active site of enzymes, iron catalyzes many essential bio-reactions. In general, iron within these enzymes is sterically, thermodynamically, and kinetically restricted so that electron transfer only occurs between desired reactants. Although the majority of cellular iron is found within proteins, there is a small, transient pool of weakly bound iron within cells that is not restricted in its catalytic activity and can therefore aid in the transfer of electrons to unwanted targets, such as dioxygen, resulting in the formation of oxidative species [1]. This iron is referred to as labile iron or the labile iron pool (LIP) [2,3]. As it is weakly bound, this iron can be chelated by compounds such as deferrioxamine (Desferal®; DFO) and is thus also referred to as the chelatable iron pool [2,4]. Although the properties of having unrestricted redox activity and being chelatable are not necessarily mutually inclusive, we here assume that the magnitude of the labile iron pool is proportional to that of the chelatable iron pool.

The oxidations initiated by labile iron can result in damage to proteins, lipids, and DNA. DNA damage brought about by labile iron is proposed to be involved in the pathogenesis of various cancer [5–7]. This pool of iron has been estimated to be less than 5% of the total iron within cells; in hepatocytes this pool is estimated as 1% [3,8] with intracellular concentrations being 0.2–5 μM [4–11]. Although small, this subset of iron is biologically significant as only very small amounts of catalytic metals are needed to initiate oxidation cascades [12,13]. Along with playing a role in the development of cancer, labile iron also has therapeutic relevance in cancer. Many cancer therapies rely in the catalytic activity of iron to form oxidative species.
For example, bleomycin requires iron to form oxidative species that damage DNA [14]; labile iron is the likely source of this iron.

There is renewed interest in the use of high dose, pharmacological ascorbate (high plasma levels, > ≈ 20 mM in plasma) in the treatment of many different types of cancer [15–19]. Pharmacological ascorbate has been shown to selectively kill cancer cells via the extracellular formation of hydrogen peroxide [15,20–23]. Catalytic metals can accelerate the oxidation of ascorbate [24], leading to a greater flux of H2O2 and thereby increase its toxicity. Catalytic metals, such as labile iron, can also react with hydrogen peroxide, forming the very damaging hydroxyl radical via the Fenton reaction. [25],

Fe2+ + H2O2 → Fe3+ + OH− + HO∗

thereby increasing the oxidative stress initiated by pharmacological ascorbate. In fact, Lipinski et al. showed that in murine lymphoma cell lines the size of the labile iron pool correlated with cellular susceptibility to pharmacological ascorbate [11].

We propose that catalytic iron is important to the cytotoxic effects of pharmacological ascorbate. Therefore, in order to understand the mechanism by which pharmacological ascorbate can be selectively toxic to cancer cells, one must look at the labile iron pool. The labile iron pool can increase in size via the release of iron from ferritin, the main storage protein of iron, in response to oxidative stress [26,27]. Thus, changes in the magnitude of the labile iron pool in oxidatively stressed and oxidatively non-stressed states are of great interest. Since catalytic metals can enhance the oxidation of ascorbate, both the magnitude of the labile iron pool, as well as changes of the labile iron pool could be major determinants of the susceptibility of cells to pharmacological ascorbate. Both AscH− and IR have been known to cause the release of iron from ferritin [28,29]. In fact, chelation of this iron has been shown to be protective against the oxidative stress caused by IR [30].

Pharmacological ascorbate has been shown to sensitize cells to IR [31–33]. As a redox active iron plays a central role in both the mechanisms of pharmacological ascorbate and IR, we hypothesized that the combination of IR and ascorbate will increase the labile iron pool to a greater extent than either modality alone. In this study we measured the labile iron pool of pancreatic tumor xenografts grown in murine models, and compared it to non-neoplastic murine tissue. We also investigated the change in the labile iron pool caused by treatment with pharmacological ascorbate with and without IR.

Materials methods

Materials

Phosphate buffered saline (PBS) was made from laboratory reagents using Nanopure® Type 1 water; all buffers were treated with Chelex™ 100 (Sigma, C7901) to remove adventitious metals [34]. Desferal (DFO, Sigma) 1 M stock solutions were made with Nanopure® Type 1 water. Ferritin from equine spleen was purchased from Sigma Chemical Co., St. Louis, MO (F4503). Before use, horse spleen ferritin was placed in dialysis tubing and suspended overnight in Nanopure® water containing EDTA to remove loosely bound iron.

Cell culture

The human pancreatic cancer cell lines MIA PaCa-2 were used in this study. They were purchased from American type culture collection (ATCC) and passaged for fewer than six months after receipt. DMEM was supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin to make 500 mL medium for growing MIA PaCa-2 cells at 37 °C humidified atmosphere containing 5% CO2.

Tumor xenografts and treatments

Athymic nude mice were obtained from Harlan Laboratories (Indianapolis, IN). The nude mice protocol was reviewed and approved by the Animal Care and Use Committee of The University of Iowa and was in compliance with The U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH). MIA PaCa-2 cells (2 × 10^6) were injected s.c. with a 1–mL tuberculin syringe equipped with a 25-gauge needle into the hind legs of 30-day old mice. Tumors and other tissues were harvested from the mice when the tumors reached 1000 mm^3. Harvested tumors and murine tissues were stored at −80 °C or immediately processed.

Tumors or tissues were homogenized using a mortar and pestle, with 2 μL of pH 6.5 PBS per mg of wet tissue weight. DFO (10 mM stock solution) was then added to achieve a final concentration of 1 mM DFO. Tumor homogenates were incubated with DFO on ice for 1 h, unless otherwise noted.

Prepared samples in 4 mm O.D. EPR tubes (Wilmad-LabGlass, Vineland, NJ, 707-SQ-250M; for this analytical work a set of tubes was selected such that each had the same characteristics and i.d.) were flash frozen in liquid nitrogen. The samples were analyzed for labile iron using electron paramagnetic resonance (EPR; Bruker EMX EPR spectrometer), monitoring the signal of the high spin ferrioxamine (Fe3+–DFO) at g = 4.3 at 100 K (using the Bruker ER411VT variable temperature accessory) with the following EPR instrument parameters: center field 1575 G, sweep width 500 G, typical microwave frequency 9.766 GHz, power 20 mW, receiver gain 2 × 10^5, modulation frequency 100 kHz, modulation amplitude 2 G, time constant 163.84 ms, conversion time 20.48 ms, resolution 1024 points, and number of scans 5. Use of the variable temperature accessory allowed signal-averaging with the relatively weak signals from samples with low levels of ferrioxamine. All samples were analyzed three times; each time the sample was removed from the EPR cavity and then repositioned within the cavity before initiating spectral scans. The median signal intensity (A.U.) from the triplicate measures was used to determine the labile iron concentration of the samples in conjunction with a standard curve.

Ferrioxamine (Fe3+– Desferal®) standards were made using ferrous sulfate and phosphate-buffered saline, pH 6.5. DFO (1 mM) was added and standard samples were incubated overnight at room temperature to ensure that all of the iron was chelated by DFO. Standards were flash frozen in liquid nitrogen and analyzed by EPR using identical procedures and instrument settings as the tissue samples. Multiple standards of the same concentration were made and analyzed for quality control. Final concentrations of ferrioxamine in the standards ranged from 1.0 to 15.0 μM. A linear response of signal intensity vs. [ferrioxamine] was obtained with correlation coefficients of ≥ 0.98.

IR was delivered from a 137Cs source in the Ionizing Radiation Services Facility at The University of Iowa. Freshly harvested tumors were cut into halves with one half to receive IR and the other serving as control. Tumor halves were then homogenized and analyzed for labile iron as above.

To determine how ascorbate would modulate the labile iron pool, tumors were grown, harvested and homogenized as above without incubation on ice. Each homogenate was split into two aliquots with one treated with AscH− (10 mM) and the other with saline. Both aliquots were incubated at 37 °C for 4 h and analyzed for labile iron using EPR as above.
To determine how ascorbate with IR would modulate the labile iron pool in tumors homogenates were split into 4 aliquots: one received no treatment, the second received 10 mM AscH−, the third received 15 Gy IR, and the last received 10 mM AscH− and 15 Gy IR. Samples were incubated on ice for 1 h and analyzed for labile iron as above.

For the experiments with ferritin, samples of 50 μM ferritin; 50 μM ferritin with 1 mM DFO; and 50 μM ferritin with 1 mM DFO and 10 mM AscH− were incubated at room temperature for 24 h and then analyzed using EPR for labile iron as above.

## Results

### Labile iron is present in tumors

Labile iron is a subset of the total pool of intracellular iron. In general it is iron that is weakly bound and redox active. Its concentration is highly variable between different cells and tissues. It has been demonstrated that a higher level of labile iron correlates with susceptibility to H2O2, and a possible increased susceptibility to pharmacological ascorbate [11]. Therefore, we examined the magnitude of the labile iron pool in tumor tissue and compared to normal murine tissues. While a significant amount of labile iron was found in tumors of the MIA PaCa-2 xenograft model for pancreatic cancer (Avg. $\pm$ 1 μmol kg⁻¹ ≈ 17 μM, $n$ = 37), larger amounts of labile iron were found in normal murine tissues, Table 1 and Fig. 1, with the highest levels detected in the pancreas (62 μmol kg⁻¹ ≈ 62 μM). In general, these levels in whole tissue homogenates are higher than previous reports of the level of the intracellular LIP, ≈ 0.2–10 μM [4,9,11]. Since our study examines whole tissue, the mechanical processing of tissues may affect this pool. In addition, the release of proteases could lead to some additional release of iron.

As expected, we also observed that murine liver had high levels of chelatable iron. Liver is known to have a high iron content, ≈ 400 μmol kg⁻¹ [35]. The level of chelatable iron in liver determined here is roughly 6% of the total liver iron content. The level of the intracellular LIP in hepatocytes has been estimated to be on the order of 1% of total iron [4].

### Ascorbate induces the release of iron from ferritin in vitro

Pharmacological ascorbate has been shown to release iron from ferritin [36]. Thus, to fully understand the role of labile iron in the cytotoxic actions of pharmacological ascorbate, one must look at potential sources of labile iron, such as ferritin. Solutions of 50 μM ferritin, 50 μM ferritin with 1 mM DFO, and 50 μM ferritin with 1 mM DFO and 10 mM AscH− were incubated at room temperature for 24 h and then analyzed for labile iron by EPR. Pharmacological ascorbate caused a significant release of iron from ferritin, Table 2 and Fig. 2. Under these experimental conditions, it is estimated that approximately 10 iron were released from each ferritin molecule, i.e. 0.3% of the iron stored in ferritin, assuming the ferritin was saturated containing ≈ 4500 ions of iron per molecule of ferritin [27]. Cell lines have been shown to be 8–15 μM ferritin [37]; this would indicate that the amount of iron released into the intracellular labile iron pool would be on the order of 100 μM. Ferritin is only one of the possible sources for labile iron, but could be the principal source under these conditions.

### Ascorbate increases the labile iron pool in tumor homogenates

Since pharmacological levels of ascorbate may increase the labile iron pool due to release of iron from sources such as ferritin, we next examined tumor homogenates to see if a parallel change in the labile iron pool could be observed. Tumor homogenates incubated with AscH− (10 mM), a plasma level easily achieved with pharmacological AscH− therapy [19,38], had a larger labile iron pool, Fig. 3. The average labile iron concentration of the AscH−-treated group was 32 μM, compared to that of the control

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### Table 1

| Tissue     | Mean  | Median | Std error | n  |
|------------|-------|--------|-----------|----|
| Tumor      | 17    | 15     | 1         | 37 |
| Lung       | 21    | 17     | 4         | 6  |
| Muscle     | 24    | 24     | 2         | 6  |
| Spleen     | 30    | 23     | 9         | 3  |
| Heart      | 33    | 36     | 5         | 6  |
| Kidney     | 38    | 34     | 4         | 6  |
| Liver      | 50    | 41     | 20        | 6  |
| Pancreas   | 62    | 62     | 3         | 4  |

### Table 2

| Ferritin (50 μM) | Ferritin (50 μM) + DFO (1 mM) | Ferritin (50 μM) + DFO (1 mM) + AscH− (10 mM) |
|------------------|-------------------------------|---------------------------------|
| [Fe²⁺−DFO] (μM)  | 15                            | 250                            | 760                            |
| Moles of chelatable iron in sample | $7.3 \times 10^{-5}$ | $1.3 \times 10^{-7}$ | $3.8 \times 10^{-7}$ |
| Percent of iron released | NA<sup>a</sup> | NA<sup>b</sup> | 0.3<sup>c</sup> |

<sup>a</sup> All solutions were incubated at room temp for 24 h. The solution was then flash frozen in liquid nitrogen and analyzed for labile iron using EPR at g = 4.3 at 100 K. Concentrations were determined using a standard curve. Kinetically and thermodynamically, DFO will chelate all of the iron released from ferritin. The reverse process was assumed to be negligible.

<sup>b</sup> Not applicable.

<sup>c</sup> The percent of iron released assumed that a saturated ferritin molecule holds 4500 atoms of iron.
Labile iron in these tumor halves was 3.8 μM ferritin; 50 μM ferritin and 1 mM DFO; or 50 μM ferritin, 1 mM DFO with 10 mM ascorbate were made using pH 6.5 PBS. Samples were incubated at room temperature for 24 h and analyzed for labile iron using EPR at g=4.3 and 100 K.

Ascorbate releases iron from ferritin in vitro. Solutions of 50 μM ferritin; 50 μM ferritin w/ 1 mM DFO; or 50 μM ferritin, 1 mM DFO with 10 mM ascorbate were immediately added. Each tumor homogenate was split into two samples: one to serve as the control and one to receive 10 mM ascorbate. Samples were incubated at 37°C for 4 h and analyzed three separate times for labile iron using EPR at g=4.3 and 100 K. Values represent the median in units of μmol (kg wet weight)−1. Error bars represent the standard error. p=0.008 for control vs. treated with ascorbate.

Group of having 23 μM (p=0.008), a 40% increase under these experimental conditions.

Post mortem IR has no effect on the magnitude of the labile iron pool

IR is another cancer treatment modality that is dependent on labile iron for some portion of its cytotoxic actions [30]. IR of tissues produces free radicals; the primary radicals produced by IR can lead to secondary radical formation that can be enhanced via labile iron; free radical-mediated oxidations can increase the labile iron pool [28]. Therefore, we looked at the ability of IR to modulate the labile iron pool in harvested tumors. MIA PaCa-2 tumors grown in mice were harvested and bisected. Post mortem IR of tumors had no effect on the labile iron pool, Fig. 4. Of the 11 tumors that were irradiated, five irradiated halves had a larger labile iron pool than their non-irradiated counter parts and six did not. The average labile iron concentration for all irradiated tumor halves was 16 ± 0.7 μM and for non-irradiated halves it was 17 ± 1.2 μM, essentially identical (p=0.28). In order to understand these results, tumor halves, both of which received no IR, were analyzed via the same method to look at the homogeneity of labile iron within tumors, Fig. 5. The labile iron concentration of the tumors varied. The average difference in the concentration of labile iron in these tumor halves was 3.8 ± 1.1 μM. This is similar to the average difference of the tumor halves of which one received IR and one did not, 3.1 ± 0.7 μM. Thus, there is no apparent increase in labile iron from IR under these experimental conditions.

Ascorbate and IR increase labile iron

Pharmacological ascorbate has been shown to sensitize tissues to IR [32,33]. Since iron can both potentiate the formation of free radicals and the free radicals formed via IR can increase the labile iron pool, the radio-sensitizing effects could in part be due to an increase in labile iron. The ability of IR to alter the labile iron pool in an oxygenated tumor homogenate was investigated with and without co-treatment with pharmacological ascorbate. Both IR or pharmacological ascorbate alone show a possible trend to an increase in labile iron pool in tumor homogenates, p=0.23 and 0.19, respectively, Fig. 6. However, our data suggest an apparent additive effect with the combined treatment of ascorbate and irradiation, p=0.06.
Acknowledgments

many different types of tumors. These results also suggest that iron- partially oxygenated, contributing to the ability of IR to release labile undoubtedly be hypoxic, whereas the homogenized tissue will be
IR will be greatly diminished in hypoxic tissue reducing the potential in part to the availability of oxygen; secondary radical formation from changes in the labile iron pool. This may be due to the mechanical
disruption of the tissues.

Discussion

Labile iron may play an important role in the mechanism of action of pharmacological ascorbate, both in catalyzing the oxidation of ascorbate to generate a flux of \( \text{H}_2\text{O}_2 \) and in the potentiation of oxidative damage through activation of \( \text{H}_2\text{O}_2 \). The release of labile iron from sources such as ferritin in vivo by high levels of ascorbate could contribute to the effectiveness of pharmacological ascorbate as an adjuvant in cancer therapy. In certain cells, the size of the labile iron pool has been correlated with the sensitivity of cells to pharmacological ascorbate \[11\]. However, our data show normal murine tissues have a larger labile iron pool than ascorbate-sensitive tumors. Thus, the varying ability of cells to handle the flux of \( \text{H}_2\text{O}_2 \) produced by pharmacological ascorbate is also a prime consideration \[22,39\]. Our results on whole tissue show higher levels of labile iron than previously reported intra- cellular levels, which could be due in part to the mechanical disruption of the tissues.

We examined the ability of IR to increase the labile iron pool in tumors. Irradiation of post mortem whole tumor tissue showed no change in the labile iron pool. However, irradiation of tumor homogenates resulted in an increase in the labile iron pool. This may be due in part to the availability of oxygen; secondary radical formation from IR will be greatly diminished in hypoxic tissue reducing the potential for the release of labile iron. The tumor tissue post mortem will undoubtedly be hypoxic, whereas the homogenized tissue will be partially oxygenated, contributing to the ability of IR to release labile iron. If the increase in labile iron contributes to the toxicity of both ascorbate and radiation alone as well as in combination, pharmacological ascorbate could be used to increase the radio-sensitivity of many different types of tumors. These results also suggest that iron- targeted anti-neoplastic agents may be more effective when combined with pharmacological ascorbate.

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