Redox Biology 2 (2014) 22–27

Contents lists available at ScienceDirect

Redox Biology

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

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

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ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form
13 November 2013
Accepted 14 November 2013
Available online 26 November 2013

Keywords:
EPR
Electron paramagnetic resonance
Ionizing radiation
Labile iron
Ascorbate
Ferritin

ABSTRACT

Labile iron, *i.e.* iron that is weakly bound and is relatively unrestricted in its redox activity, has been implicated in both the pathogenesis as well as treatment of cancer. Two cancer treatments where labile iron may contribute to their mechanism of action are pharmacological ascorbate and ionizing radiation (IR). Pharmacological ascorbate has been shown to have tumor-specific toxic effects due to the formation of hydrogen peroxide. By catalyzing the oxidation of ascorbate, labile iron can enhance the rate of formation of hydrogen peroxide; labile iron can also react with hydrogen peroxide. Here we have investigated the magnitude of the labile iron pool in tumor and normal tissue. We also examined the ability of pharmacological ascorbate and IR to change the size of the labile iron pool. Although a significant amount of labile iron was seen in tumors (MIA PaCa-2 cells in athymic nude mice), higher levels were seen in murine tissues that were not susceptible to pharmacological ascorbate. Pharmacological ascorbate and irradiation were shown to increase the labile iron in tumor homogenates from this murine model of pancreatic cancer. As both IR and pharmacological ascorbate may rely on labile iron for their effects on tumor tissues, our data suggest that pharmacological ascorbate could be used as a radiosensitizing agent for some radio-resistant tumors.

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, here we 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 has been 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.

Abbreviations: DFO, deferrioxamine or Desferal®; EPR, electron paramagnetic resonance; IR, ionizing radiation; LIP, labile iron pool; PBS, phosphate-buffered saline

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http://dx.doi.org/10.1016/j.redox.2013.11.005

For example, bleomycin requires iron to form oxidative species that damage DNA [14]; labile iron is the likely the source of this iron.

There is renewed interest in the use of high dose, pharmacological ascorbate (high plasma levels, $> \approx 20$ 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 H_2O_2 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],

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

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 the change 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 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% CO_2.

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 (Fe^{3+}–DFO) at $g=4.3$ at 100 K (using the Bruker ER4111VT 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 (Fe^{3+} Desferal®) standards were made using ferrous sulfate and phosphate-buffered saline, pH 6.5. DFO (1 mM) was added and then 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 ^{137}Cs 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.