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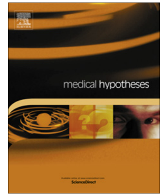

Iron accumulation, glutathione depletion, and lipid peroxidation must occur simultaneously during ferroptosis and are mutually amplifying events

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ABSTRACT

Ferroptosis is a recently discovered form of regulated necrosis that involves iron-dependent lipid peroxidation. How cells die once ferroptosis is triggered remains unclear. Ferroptosis is hypothesized to require three critical events: (1) accumulation of redox-active iron, (2) glutathione depletion, and (3) lipid peroxidation. It is proposed that these three events must unfold simultaneously because stopping any critical event also stops ferroptosis. These events are hypothesized to amplify in severity through positive feedback loops. The cause of death in ferroptosis is therefore the synergistic combination of antioxidant depletion, iron toxicity, and membrane denaturation. The relevance of these feedback loops for cancer and neurodegenerative therapies is discussed.

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Introduction

Ferroptosis is an iron-dependent mode of regulated necrosis that is biochemically, genetically, and morphologically distinct from apoptosis, autophagy, and other forms of necrosis, and is therefore a new way that cells can die [1,2]. The glutamate/cystine antiporter (named X_C^-) supplies extracellular cystine in exchange for intracellular glutamate, a process required for the biosynthesis of the endogenous antioxidant glutathione. The antitumor molecules erastin and sorafenib trigger ferroptosis by inhibiting X_C^-, resulting in glutathione depletion and oxidative damage [3,4]. Ferroptosis may also be induced by small molecules RSL3 and ML162 by inhibiting glutathione peroxidase 4 (Gpx4), a lipid repair enzyme essential for life [5]. Iron is suggested to be involved in ferroptosis because death is prevented by co-treatment with the iron chelator deferoxamine [1]. Ferroptosis is regulated by several genes, for example, iron metabolism genes *TFRC* and *IREB2* [1], glutaminolysis-regulating genes *SLC38A1* and *GLS2* [6], the pentose phosphate pathway gene *G6PD* [1], and autophagy-regulating genes *ULK1* and *BECN1* [7]. Interest in ferroptosis as a natural tumor-suppressing process has been spurred by the discovery that tumor-suppressor proteins RB1 and p53 can activate ferroptosis [8–11]. Ferroptosis is observed in both *in vitro* and *in vivo* models, and research is underway expanding the repertoire of ferroptosis-inducing and -suppressing molecules [12].

How death occurs once ferroptosis is triggered remains unclear. Firstly, it is hypothesized that for ferroptosis to occur three critical events are required: (1) Accumulation of ‘free’ iron, which causes oxidative stress through Fenton catalysis, (2) depletion of the antioxidant glutathione, resulting in oxidative stress, and (3) accumulation of lipid oxidative damage, leading to cell membrane denaturation. Secondly, it is hypothesized that each event must unfold simultaneously for ferroptosis to occur because experimental evidence suggests that stopping any of these events also stops ferroptosis. Thirdly, it is hypothesized that these three critical events, once triggered, can be mutually exacerbated through positive feedback loops and are therefore amplifiable events. Death from ferroptosis is therefore proposed to be the synergistically lethal combination of iron toxicity, antioxidant depletion, and membrane damage. These three hypotheses are summarized in Fig. 1.

Hypotheses

Hypothesis 1A – Relevance of glutathione depletion to ferroptosis

Glutathione is a tripeptide (Glu-Cyc-Gly) and an important cellular antioxidant that protects lipids, proteins, and DNA from oxidative damage [13]. Glutathione donates electrons via

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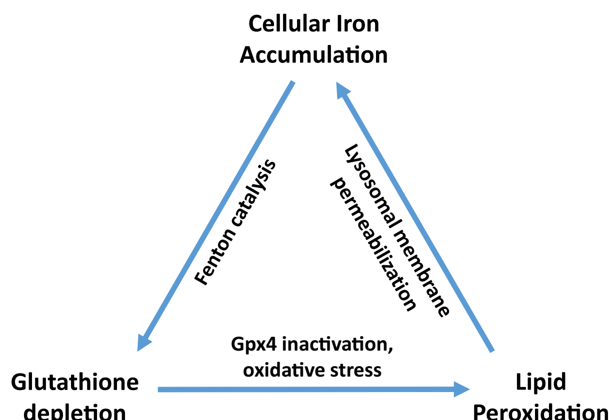

Fig. 1. Summary of hypotheses No. 1–3. Glutathione depletion, cellular iron accumulation, and membrane lipid peroxidation are proposed to be three requisite, mutually required, and amplifiable events causing ferroptosis.

glutathione peroxidase by dimerizing to glutathione disulfide. Enzymatic reduction of glutathione disulfide restores glutathione. Glutathione depletion is associated with Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Friedreich's ataxia [14]. Glutathione depletion is a well-established feature of ferroptosis because one trigger of ferroptosis includes inhibition of X_C^-, the antiporter required for glutathione biosynthesis [1].

Hypothesis 1B – Relevance of iron toxicity to ferroptosis

The role of iron in ferroptosis is unclear. It is herein hypothesized that the principle role of iron in ferroptosis is free radical production. Iron is a pro-oxidant because it converts hydrogen peroxide to hydroxyl radicals via the Fenton reaction [15]. Iron exacerbates the relatively benign cytotoxicity of hydrogen peroxide and potentiates nucleic, proteomic, and membrane damage [16]. Iron-using organisms regulate iron to supply enough iron for essential metabolism while mitigating iron toxicity [17,18]. Iron is detoxified by sequestering the metal inside ferritin or by binding iron to metallo-enzymes. Nonetheless, both the cytosol and the mitochondrial matrix have small pools of loosely coordinated redox-active iron that may participate in Fenton chemistry [19]. Maintaining this labile iron pool at a tolerably low level protects the cell from oxidative stress. If the concentration of the labile iron pool is increased beyond homeostatic limits, profound oxidative damage occurs. An example of this is seen when the iron regulatory gene (*fur*) is deleted in *E. coli* [20]. An inverse relationship between glutathione and iron loads is well documented through iron-dosing experiments [21,22] as well as the pathology of neurodegenerative diseases [23]. It is likely that Fenton radicals contribute to glutathione depletion during ferroptosis.

It is hypothesized that free radical generation by iron is a pivotal event during ferroptosis and that iron toxicity could be facilitated by disrupting the labile iron pool. Specifically, it is hypothesized that ferroptosis involves cellular accumulation of redox-active iron. This iron can originate from both extracellular and intracellular sources. Under amino acid starvation, ferroptosis can be triggered by incubating mouse embryonic fibroblasts in serum containing transferrin, an extracellular iron scavenger [6]. The rate of cell death was reduced when transferrin receptor expression was inhibited with RNA interference or when cells were incubated in the presence of iron-free transferrin, demonstrating that iron metabolism is relevant to ferroptosis [6]. Recent experiments with a fluorescence-based probe have also indicated that changes in the labile iron content occur during ferroptosis [24].

One way of activating extracellular iron scavenging could include disruption of the iron regulatory protein 1 (IRP1). This post-transcriptional regulator possesses a [4S-3Fe] cluster when iron concentration is low, allowing IRP1 to bind to the mRNA of ferritin and transferrin receptor genes. When IRP1 is bound to transferrin receptor mRNA, the mRNA is stabilized and translation is facilitated. When IRP1 is bound to ferritin mRNA, translation is inhibited [25]. When iron levels rise, IRP1 is charged to [4S-4Fe], releasing IRP1 from mRNA [26]. Hence IRP1, when possessing a [4S-3Fe] cluster, facilitates iron scavenging. The [4S-4Fe] cluster is also converted to [4S-3Fe] through free radical damage, resulting in adventitious accumulation of labile iron [27,28]. Fenton catalysis by labile iron creates a positive feedback loop with IRP1 denaturation [29]. Glutathione depletion, germane to ferroptosis, likely sensitizes IRP1 to oxidation causing iron to accumulate inside the cell [6]. The liberation of iron from [4Fe-4S] clusters in IRP1 as well as many other proteins by free radical damage is an example of an intracellular source of 'free' iron [30]. In summary, it is hypothesized that ferroptosis involves disruption of iron metabolism and the intracellular accumulation of redox-active iron.

Hypothesis 1C: Relevance of lipid peroxidation to ferroptosis

Lipid peroxidation is an extensively investigated event that is often associated with cell death because it compromises membrane structural integrity, has downstream cytotoxic effects, and is involved in suicide signaling cascades [31–33]. Fatty acids are vulnerable to oxidation from Fenton radicals, devoted enzymes such as lipoxygenases and cyclooxygenases, and the lipid peroxidation chain reaction. Endogenous free radicals that damage lipid membranes at sub-lethal rates are repaired by constitutive antioxidant defences. When repair mechanisms are overwhelmed, necrotic or apoptotic death follows.

Deletion experiments on lipid repair enzymes suggest that membrane damage is a pivotal event during ferroptosis. Glutathione peroxidase 4 (Gpx4) is an enzyme that repairs lipid peroxides [34]. Gpx4 deletion in mice triggers ferroptosis [35,36]. Death is mitigated with lipoxygenase inhibitors or with lipophilic antioxidants such as α-tocopherol and ferrostatin-1 [37]. The relevance of lipid membrane damage to ferroptosis is exemplified by the observation that the lipophilic antioxidant decylubiquinone requires 100-fold lower concentration to stop ferroptosis than the mitochondria-targeting antioxidant mitoquinone [35]. Lipid membrane peroxidation is therefore hypothesized to be a requisite event during ferroptosis.

Hypothesis 2 – Critical events must operate simultaneously for ferroptosis to occur

Mouse cells with inactivated glutathione peroxidase 4 (Gpx4) undergo ferroptosis. Notably, Δ*gpx4*-T cells can be rescued by treatment with the iron chelator deferoxamine [35]. This demonstrates that although lipid oxidation is a pivotal event during ferroptosis, ferroptosis simultaneously requires iron. Ferritin is an iron storage protein important for detoxifying pro-oxidant iron. Knockout of ferritin in mice results in higher cellular 'free' iron concentration and heightened oxidative stress. However, ferroptosis is only observed when X_C^- was also inhibited [35]. This demonstrates that iron is required for ferroptosis to occur but can only induce ferroptosis when glutathione reserves are simultaneously depleted. Conversely, X_C^- -inhibited cells treated with iron chelators are protected from ferroptosis [1], demonstrating that glutathione depletion is necessary to trigger ferroptosis but can only do so when iron is simultaneously available. These experiments suggest that iron accumulation, glutathione depletion, and lipid oxidation are distinct but mutually required events.

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