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Iron for proliferation of cell lines and hematopoietic progenitors: Nailing down the intracellular functional iron concentration

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ABSTRACT

Iron is an essential nutrient which must be provided in sufficient amounts to support growth of eukaryotic cells. All organisms devote specialized pathways to ensure proper delivery. Yet, a quantitative assessment of the intra-cellular iron concentration needed to allow the cell cycle to proceed in mammalian cells is missing. Starting from iron-depleted cell lines or primary hematopoietic progenitors prepared with clinically implemented iron chelators, replenishment via transferrin and other iron sources has been quantitatively monitored through the main endogenous markers of the cellular iron status, namely proteins involved in the uptake (transferrin receptor), the storage (ferritin), and the sensing (Iron Regulatory Proteins) of iron. When correlated with measurements of iron concentrations and indicators of growth, this minimally intrusive approach provided an unprecedented estimate of the intracellular iron concentration acting upon iron-centered regulatory pathways. The data were analyzed with the help of a previously developed theoretical treatment of cellular iron regulation. The minimal cellular iron concentration required for cell division was named *functional iron concentration* (FIC) to distinguish it from previous estimates of the cellular labile iron. The FIC falls in the low nanomolar range for all studied cells, including hematopoietic progenitors. These data shed new light on basic aspects of cellular iron homeostasis by demonstrating that sensing and regulation of iron occur well below the concentrations requiring storage or becoming noxious in pathological conditions. The quantitative assessment provided here is relevant for monitoring treatments of conditions in which iron provision must be controlled to avoid unwanted cellular proliferation.

1. Introduction

Iron is required to maintain viability and to support growth of almost all kinds of cells. In eukaryotes, it is abundantly present in the electron-transfer centers of the mitochondrial respiratory chain, for instance, and, more generally, it participates at the active sites of a myriad of proteins and enzymes. Consequently, iron is mandatory for cellular proliferation [1], and iron removal from the growth medium generally stops the cell cycle at the G1/S transition. One contributing factor to this blockade is the iron-dependent enzyme ribonucleotide reductase, which relies on a subunit containing a tyrosyl-di-iron cofactor [2]. The arrest of the cell cycle induced by iron chelators justifies considering these compounds as anti-neoplastic agents [3].

In metazoans, the most iron demanding pathway is erythropoiesis since iron is the anchoring site of oxygen in hemoglobin, a protein supplying oxygen to tissues. Iron-deficient individuals suffer from anemia, in which not enough iron is incorporated into nascent hemoglobin and which is the most widespread single nutrient deficiency worldwide [4,5]. Iron provision for hematopoiesis is delivered by transferrin (Tf), a circulating glycoprotein which can be easily measured together with its iron load to gauge the iron status. Although the critical levels of this

Abbreviations: AML, acute myeloid leukemia; BSA, bovine serum albumin; DPF, deferiprone (3-hydroxy-1,2-dimethyl-4-pyridinone); DFO, deferoxamine; ESA, erythropoietic stimulating agents; EDTA, ethylene-diamine tetracetate; FAC, ferric ammonium citrate; FIC, functional iron concentration; HO-1, inducible heme oxygenase; IRP, Iron Regulatory Proteins; LIP, labile iron pool; MDS, myelodysplastic syndrome; NDRG1, N-myc downstream regulated 1; PBS, Phosphate buffered saline solution; PCBP, poly(rC) binding proteins; ROS, reactive oxygen species; Tf, transferrin

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useful biomarker have been set a long time ago, it remains unclear how much Tf-delivered iron is needed to support proliferation of cells that must divide to maintain homeostasis or to fulfill specific functions. Throughout hematopoiesis, cells must properly handle iron according to the specific requirements of the different stages, from renewal of stem cells to full differentiation of specialized blood components [6].

Cells need to maintain the proper level of iron availability, and this requirement is challenged in transfusion-induced iron overload or, conversely, by neoplasm-triggered large iron consumption. The former situation occurs in cases of thalassemia [7] and myelodysplastic syndromes [8], for instance. The latter applies to cancer cells in general, acute myeloid leukemia clones in particular, for which ample iron provision is needed to support growth, particularly in the blast crisis. The pivotal role of iron in such circumstances has recently been clearly evidenced by the redirection of proliferating blasts into the monocyte lineage with a treatment including iron chelation [9–11].

To clarify conditions under which cell proliferation occurs, the quantitative iron needs of model cell lines and of hematological progenitors for amplification have been evaluated herein. The implemented integrative approach minimally perturbed cells, and it allowed us to show that proliferation is supported by smaller amounts of iron than assumed before. With the help of a recently developed theoretical model of cellular iron homeostasis, these data should help understand the role of iron deregulation in a wealth of pathological conditions, and they will have to be considered for a better implementation of the therapeutic strategies targeting iron-dependent pathways.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma-Aldrich unless stated otherwise. Human holo-transferrin (i.e. iron-loaded transferrin, Sigma T4132) was iron saturated with 2 Fe atoms/molecule. The iron content of human apo-transferrin (apo-Tf), i.e. the protein devoid of iron, was measured by inductively coupled plasma-mass spectrometry at less than 0.025 iron atom/transferrin molecule. Recombinant human Iron Regulatory Proteins (IRP) 1 and 2 were obtained as previously detailed [12,13].

2.2. Cell lines

The cell lines used in the present study originated from the ATCC biological resource. The human myeloid leukemia KG1 and K562 cells were grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum (Biowest, origin: South America, batch containing 1.7 mg of iron/l), 1% L-glutamine, 100 U of penicillin/ml and 0.1 mg steptomycin/ml at 37 °C with 5% CO_2. As a rapidly growing cell model significantly different from the above cell lines, HeLa cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM) supplemented as above for leukemic cell lines. The iron-containing DMEM was replaced by RPMI after iron depletion for direct comparison of the iron-requirements of the HeLa cells as compared to the other cells used in this work.

2.3. Purification of $CD34^+$ progenitors

$CD34^+$ cells were obtained from cord blood after Ficoll-Hypaque (Abcys - Eurobio) density gradient separation and they were isolated by two steps of immunomagnetic separation (Miltenyi Biotech), on large then medium size columns, successively. Cord blood procedures were approved by the French Blood Service's Institutional Review Board, and samples were obtained from healthy donors who gave informed consent.

2.4. Iron depletion and proliferation assay for cell lines

After routine maintenance in rich media, cell lines were handled in a minimal medium composed of RPMI-1640, 1% treated (see below) bovine serum albumin (BSA), 30 nM sodium selenite, 1% L-glutamine, 100 U of penicillin/ml and 0.1 mg steptomycin/ml at 37 °C in 5% CO_2. In the case of HeLa cells, the minimal medium was used after adhesion in the conventional rich medium. To remove as much iron as possible, the BSA powder was dissolved in 10 mM HEPES buffer pH 7.3 containing 5 mM EDTA before dialysis with successive baths of distilled water until reaching an EDTA concentration below 1 nM. Beforehand, distilled water was filtered through Chelex cation exchange resin (BioRad). The BSA concentration was estimated by spectrophotometry at 280 nm ($\varepsilon = 39{,}600\ M^{-1}\ cm^{-1}$).

Cells were seeded at 5×10^5 cell/ml in minimal medium without added iron or Tf, and the chelating agent was added for 24 h. Deferoxamine mesylate salt was used at 200 μM and deferiprone at 500 μM. The depletion medium was replaced by the minimal medium supplemented with the wanted source of iron. Cells were further kept for 24 h, which is the optimum duration to measure growth at low iron concentrations before cell death, and the cells were then processed. Viable cells were determined by Trypan blue staining and quantified with a Luna™ counter (Logos).

2.5. Amplification, iron depletion, and proliferation assay for CD34 cells

A synthetic minimal medium was used to control iron supply. It was composed of Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies), 1% iron-depleted albumin (as described above), 200 μg/ml insulin, 0.1 mM β-mercaptoethanol and the StemMACS HSC expansion cocktail (TPO/FLT3/SCF - Miltenyi Biotech) at 37 °C in 5% CO_2. Freshly isolated $CD34^+$ cells were seeded at 1×10^5 cells/ml and amplified for three days in the above minimal medium supplemented with 1.25 μM holo-Tf. Iron depletion and further experiments were carried out in the same medium in which the iron source was precisely monitored. Viable cells were determined by Trypan blue staining in Neubauer slides.

2.6. Cell cycle assays

Cells were rinsed and suspended at 3×10^6 cells/ml in Phosphate Buffered Saline (PBS) solution. They were fixed by slowly adding cold ethanol up to 50% (v:v) with thorough mixing. The suspension was left at 4 °C for one hour, the cells were centrifuged, washed with cold PBS, and suspended in PBS (4×10^6 cells/ml). RNA were degraded by 0.5 mg/ml RNAse A (Thermo Scientific) at 37 °C for 1 h, and cells were labeled by 10 μg/ml propidium iodide. The fluorescence was measured by flow cytometry with the LSR Fortessa™ cell analyzer (Becton Dickinson) using the 488 nm sapphire laser. The data were analyzed with the Modfit LT v3.2 software (Verity Software House).

2.7. Iron measurements

Iron concentrations were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a XSERIES 2 analyzer (Thermo Scientific). Cellular pellets were suspended at 4000–8000 cells/μl of water. The volume corresponding to 8×10^5 cells was diluted (1:25) and mixed (1:1 v/v) with 1% nitric acid before analysis. Raw results were converted to cell associated concentrations by considering the estimated volumes of KG1 and K562 cells, 0.8 and 2.5 pL, respectively, in agreement with previously published values for the latter [14]. Gallium was used as internal standard. For iron measurements in the growth media, 100 μl were directly treated with acid before analysis.

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