RESEARCH COMMUNICATION

Repression of the heavy ferritin chain increases the labile iron pool of human K562 cells

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The role of ferritin in the modulation of the labile iron pool was examined by repressing the heavy subunit of ferritin in K562 cells transfected with an antisense construct. Repression of the heavy ferritin subunit evoked an increase in the chemical levels and pro-oxidant activity of the labile iron pool and, in turn, caused a reduced expression of transferrin receptors and increased expression of the light ferritin subunit.

Key words: antisense, fluorescence detection of metals, transfection.

INTRODUCTION

The expression of ferritin, the major intracellular iron-storage protein, is thought to have two principal regulatory functions, which involve scavenging of intracellular iron and the attenuation of reactive oxygen species (ROS) formation. In the first, synthesis of ferritin subunits is triggered by a rise in the putative cellular labile iron pool (LIP) that is sensed and transduced by iron-responsive proteins (IRPs). In the second, ferritin transcription is modulated in response to factors that either raise or lower cellular growth-dependence on LIP. Thus oncogene suppression of ferritin expression purportedly raises the LIP and supports proliferation [1–6]. On the other hand, cytokines, by promoting ferritin expression and thereby lowering the LIP, confer upon cells protection from invading pathogens and oxidative damage [7–11].

Thus far, experimental support for the alleged role of ferritin in the control of LIP levels has been provided only by studies of heavy ferritin subunit (H-FT) overexpression. In these studies [12,13], expression of the H-FT gene mutated in the iron-responsive element enabled the iron-independent translation of H-FT. This strategy led to an increased expression of H-FT which, in turn, reduced the steady-state LIP levels and the ensuing ROS production, and increased resistance to ROS toxicity [13]. Furthermore, stable inducible transfection of HeLa cells with wild-type H-FT cDNA, but not with its ferroxidase mutant, led to a substantial reduction in the LIP [14]. This reduction was associated with up-regulation of IRP activity, transferrin-mediated iron uptake and resistance to ROS toxicity.

In the present study, we analysed the opposite situation, i.e. the functional consequences of H-FT repression in terms of its capacity to increase the LIP. For that purpose, we used transient transfection of cells with an antisense sequence against H-FT as a means to down-modulate expression of H-FT. The choice of antisense was made in order to manipulate ferritin in a specific manner, and independently of the iron status of the cells. The specificity of the antisense H-FT was confirmed by its inability to repress light ferritin subunit (L-FT). We used fluorescence detection of metals as a means to assess LIP [15], and the oxidation of carboxydichlorodihydrofluorescein (carboxy-H$_2$DCF) as a means to assess exogenously induced ROS production [16]. The levels of cell-surface expression of the transferrin receptor (TfR) served as a tool for confirming the iron-regulation capacity of LIP. Our results provide direct experimental support for the alleged roles of H-FT as a regulator of LIP and as an attenuating factor in the antioxidative cell response.

EXPERIMENTAL

Materials

Calcine, calcine acetomethoxy, 2'-7'-carboxydichlorodihydro-fluorescein-diacetate diacetoxymethyl ester (H$_2$DCFDA-diAM) and fluorescein-/galactosidase were obtained from Molecular Probes (Eugene, OR, U.S.A.), and Triton X-100 and the DOTAP lipid reagent were from Boehringer Mannheim. The following gifts were received: human H-FT cDNA and rabbit anti-human sera against placental and spleen ferritin from Professor H. Rosen and Professor A. M. Konijn respectively (Hadassah Medical School, Hebrew University), salicylaldehyde hydrazone from Professor Prem Ponka (McGill University, Montreal, Canada), mouse anti-human H-FT and anti-human L-FT monoclonal antibodies and recombinant human H-FT and L-FT from Dr Paolo Santambrogio (Dipartimento di Ricerca Biologica e Tecnologica, San Raffaele Hospital, Milan, Italy), and the pRGB4 vector from Dr Israel Seckler (Faculty of Medicine, Ben Gurion University, Beer Sheva, Israel). Unless specified otherwise, all other chemicals were from Sigma or were of the best available grade.

Cells

Human erythroleukaemia K562 cells were grown as described in [17].

Transient transfection of cells with antisense to ferritin mRNAs

A partial human H-FT cDNA sequence (residues 283–616 in human H-FT mRNA sequence, GenBank* accession number L20941) was PCR-synthesized using the primers 5'-GGAACA-TGCTGAGAAACTG-3' (forward) and 5'-GGTGTTGTCTTGT-CAAAGG-3' (reverse). An EcoRI recognition sequence, 5'-CCG-
GAATTCC-3', was added to the 5' of each primer and used for ligation in an antisense orientation between the promoter and the polyadenylated sequences of the cytomegalovirus in the pRBG4 vector [18]. The antisense orientation was verified by sequencing the insert and junction sequences. Transfection of pRB4 with and without the H-FT antisense insert was done by the DOTAP method (Boehringer Mannheim) and assessed 24 h later.

**Ferritin quantitation by ELISA**

For ferritin determination, samples of approx. 400000 cells were centrifuged, and the pellet was extracted at 4 °C for 15 min with 200 µl of buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.3 % Triton X-100 and 0.25 % NaN₃) containing an antiprotease cocktail (P-8340; Sigma) and 250 µM PMSF. The extract was centrifuged at 8500 g for 2 min at 4 °C, and the supernatant was analysed for protein content (bicinchoninic acid method; Sigma). Immunoplates (Nunc, Roskilde, Denmark) were coated with mouse anti-human H-FT or mouse anti-human L-FT monoclonal antibodies (20 µg/ml in carbonate buffer, pH 9.6) and incubated for 1 h at 37 °C. The plates were then blocked with 3 % BSA in PBS for 2 h at room temperature. Subsequently, samples (2 µg of protein) and ferritin standards (0–1 ng of recombinant H-FT or L-FT) were dissolved in 3 % BSA and added to the plates for incubation of 1 h at 37 °C. Incubation (3 h at room temperature) with rabbit antiserum against human placental ferritin (approx. 50 % anti-H-FT and 50 % anti-L-FT activity) or against human spleen ferritin (95 % anti-L-FT and 5 % anti-H-FT activity) was used for determination of H-FT and L-FT respectively. Rabbit IgG was detected by incubation with goat anti-rabbit antibodies coupled to β-galactosidase (Amersham International) for 1 h at 37 °C and then 1 h at room temperature. Fluorescence (λemission = 488 nm; λexcitation = 525 nm) of the fluorogenic substrate fluorescein-β-galactosidase was followed with time of incubation in a Fluostar II fluorescence plate reader (BMG LabTechnologies, GmBH, Offenburg, Germany) and quantified with the aid of calibration curves of normal rabbit IgG.

**Assessment of RNA levels of H-FT and L7 by reverse transcriptase (RT)-PCR**

Total RNA from K562 cells was isolated using Ultraspec RNA isolation reagent (Biotec Laboratories, Houston, TX, U.S.A.). RNA (1 µg) was reverse-transcribed and PCR amplified using the Titan One Step RT-PCR system according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Total RNA was added to the PCR mix (containing avian myeloblastosis virus RT, Taq and Pwo DNA polymerases and 40 units/µl RNase inhibitor) together with the following primer pairs. For human H-FT (GenBank* accession number L20941): forward, 5'-GCCAATACATTCTTCACCC-3'; reverse, 5'-TTCTATTACGTCTCCACC-3'. These primers spanned a 390 bp sequence between residues 365 and 754. For human L7 (an internal control, GenBank* accession number X57958): forward, 5'-GAAGAAGAAGAAGAAGAGGAG-3'; reverse, 5'-GGTACATAGAAGTGGCCAG-3'. These primers spanned a 239 bp sequence between residues 50 and 288. Following RT at 50 °C for 30 min (terminated by heating for 2 min at 94 °C), touchdown-PCR was performed for 10 cycles using the following temperatures: denaturation for 1 min at 94 °C, annealing for 2 min at 65 °C, with a lowering increment of 2 °C per cycle, and elongation for 3 min at 68 °C. Thermocycling was then continued for up to 21 cycles as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 45 °C, and elongation for 45 s (plus 5 s per cycle) at 68 °C. Samples were collected at cycles 16–21 following the touchdown-PCR. All RT-PCR reactions were performed by the Mastercycler thermocycler (Eppendorf, Hamburg, Germany). The samples were separated on a 1.6 % agarose gel containing the SYBR Gold nucleic acid stain (Molecular Probes) and photographed under UV light. The attenuation of samples taken from cycles 16–21 were within a linear range.

**Immunofluorescence**

Cells fixed in methanol and treated with 3 % BSA were incubated first with mouse monoclonal antibodies against human H-FT (20 µg/ml) and subsequently with FITC-conjugated antimouse IgG and 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Samples of 10 µl were loaded on to a glass slide and examined on an Olympus IX70 (Tokyo, Japan) fluorescence microscope (λexcitation = 490 nm; λemission = 520 nm). DAPI-labelling assisted us in confirming that the FITC staining was cell-associated. Images were processed by EPIX’s (Buffalo Grove, IL, U.S.A.) XCAP Image Acquisition, Display, Processing and Analysis Software. Quantitation of the data was performed by the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, U.S.A.).

**LIP measurement and cell ROS production**

LIP was measured by the calcine-based fluorescence method, as described previously [15]. ROS was determined in cells (10⁶/ml) loaded with 10 µM H₂DCFDA-diAM for 20 min at 37 °C (based on the method described in [16]), centrifuged, and resuspended in Heps-buffered saline supplemented with hydroxyethyl-starch-deferoxamine (BioMedical Frontiers, Minneapolis, MN, U.S.A.) in order to prevent extracellular-metal-catalysed substrate oxidation. Tracing of H₂O₂ (5 µM)-induced oxidation of the non-fluorescent carboxy-H₂DCF to the fluorescent carboxy-DCF was carried out fluorimetrically (λexcitation = 490 nm; λemission = 520 nm) (PTI, South Brunswick, NJ, U.S.A.). Maximum fluorescence attained in the sample (Fₘₐₓ) was obtained by cell permeabilization with 0.5 % Triton X-100 and addition of fresh 1 mM ferrous ammonium sulphate and 1 mM H₂O₂. The rate of ROS formation was assessed by the amount of carboxy-DCF produced per second. The concentration of carboxy-DCF is given by:

\[
(Carboxy-DCF)_c = \left(\frac{F}{F_{max}}\right) \times (carboxy-DCF)_{max}
\]

Where F and Fₘₐₓ are the fluorescence at any given time and the maximal fluorescence (both in relative units) respectively, and (carboxy-DCF)ₘₐₓ is the concentration factor obtained by calibration with standard carboxy-DCF. The concentration of carboxy-DCF in the cells (carboxy-DCF)ₜₐₛ was obtained from:

\[
(Carboxy-DCF)_t = \frac{(carboxy-DCF)_c}{(N_c \times V_c)}
\]

Where Nₖ is the cell concentration in the cuvette (cells/ml) and Vₖ is the volume of a single cell (ml/cell). Cells were counted in a Coulter counter and their volume obtained as described in [19].

**Cellular iron content.**

Total cell iron was measured as described in [12].

[56Fe]Transferrin uptake and TIR surface expression

K562 cells (10⁶/ml in Dulbecco’s modified Eagle’s medium supplemented with 20 mM Heps and 1 mg/ml BSA) were exposed to 15 µg/ml of [56Fe]transferrin alone (total) or with 1 mg/ml unlabelled transferrin-Fe (non-specific) at 37 °C and 5 % CO₂. After 0 min, 30 min, 60 min and 120 min, the cells were transferred to ice, washed twice with cold PBS, stripped with...
that the cells were incubated at 4 °C and were not stripped.

RESULTS

Repression of ferritin subunits

Transfection of K562 cells with an antisense fragment reduced the cellular H-FT levels by approx. 50% as compared with mock-transfected or untreated cells (Figure 1A and Table 1). The reduction in H-FT level was comparable with that attained by treatment with the iron chelator deferoxamine (50 µM for 24 h) (Figure 1A). However, unlike deferoxamine, which reduces the protein levels of both H-FT and L-FT (results not shown), H-FT antisense transfections evoked an increase in L-FT (Table 1), whereas H-FT levels were decreased. This indicates that the action of the antisense was not the result of a non-specific effect on the synthetic machinery of the cell. The blockage of H-FT expression by the H-FT antisense treatment was also assessed at the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. Expression by the H-FT antisense treatment was also assessed at the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level. According to the RT-PCR analysis shown in Figure 1(B), the transfection significantly reduced the mRNA level.

Table 1  H-FT expression, LIP, pro-oxidant-induced ROS production and cellular iron content in K562 cells transiently transfected with H-FT AS

<table>
<thead>
<tr>
<th>Transfection vector</th>
<th>H-FT†</th>
<th>L-FT†</th>
<th>Normalized LIP†</th>
<th>ROS production†</th>
<th>Iron content (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100±10</td>
<td>100±19</td>
<td>100±0.14</td>
<td>100±0.14</td>
<td>1088±407</td>
</tr>
<tr>
<td>pRBG4</td>
<td>106±5</td>
<td>119±3</td>
<td>102±0.14</td>
<td>123±7</td>
<td>799±372</td>
</tr>
<tr>
<td>pRBG4 + H-FT antisense</td>
<td>52±15</td>
<td>220±32</td>
<td>149±0.28*</td>
<td>646±68*</td>
<td>735±225</td>
</tr>
</tbody>
</table>

*Values for the ferritin antisense-transfected cells were significantly different from the values for untreated cells (P < 0.01), as determined by Student’s t test. Only cells in (D) showed a bimodal distribution of intensity (Gaussian 2 Peak fit, P < 0.05): in 7 out of 23 cells the average intensity was 122±25 and in the remaining 16 out of 23 cells it was 186±13. Magn. ×1000.
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**Figure 3** LIP levels and ROS production in H-FT-repressed cells

(A) Determination of LIP in K562 cells. Following quenching of non-cell-associated calcein (CA) fluorescence by anti-CA antibodies (Abs), the permeable chelator salicylaldehyde isonicotinoyl hydrazone (SIH) was added, and LIP was determined as described in [15] from the stabilized signal attained after SIH addition. Cell incubations prior to LIP determination: dotted line, 24 h, no treatment; solid line, mock-transfected cells; broken line, cells transfected with an H-FT cDNA fragment in an antisense (AS) orientation. The bars represent concentrations of calcein (\(\mu M\)) in the cells following the addition of the chelator SIH. 

(B) Determination of pro-oxidant-induced ROS formation in K562 cells. ROS production was determined based on the rise in fluorescence in H2DCFDA-diAM-loaded cells, as described in the Experimental section. H2O2 (5 \(\mu M\)) was used as the pro-oxidant, and was added to the cells where indicated. Treatment annotations are the same as in (A).

Although substantial, the level of antisense repression of H-FT expression was not complete in the experimental conditions used. It was, therefore, of interest to ascertain whether the antisense affected the cells differentially. In order to address this issue, we used indirect immunofluorescence staining of cells with H-FT antibodies. All staining was confirmed to be cell-associated, by both phase-contrast and immunofluorescent microscopy, which showed co-localization of the FITC stain with the nuclear stain DAPI (results not shown). A quantitative analysis of the fluorescent intensity associated with transfected cells revealed a bimodal distribution: in two-thirds of the cells, the average intensity (in 0–256 scale) was 123 ± 25, whereas in the remaining third it was 186 ± 13. The latter corresponded to the average intensity obtained in non-transfected cells, whereas the former corresponded to the value obtained in transfected cells in which treatment with the primary anti-H-Ft antibody was omitted. The results agree with the possibility that H-FT was repressed only in that fraction of cells that was successfully transfected (Figure 2). Therefore the cellular parameters affected by the antisense transfection should be regarded as underestimates when expressed as means of the whole cell population.

**Effects of H-FT repression on LIP and ROS formation**

The effects of the H-FT antisense on the expression of H-FT and L-FT could be attributed to two possible mechanisms: one involving a direct, but opposing, action of antisense on each subunit, and the other based on the specific repression of the H-FT subunit, indirectly leading to increased L-FT synthesis by increasing the LIP, and thereby decreasing the IRP activity. Our results (Table 1) support the second mechanism. The experimental steady-state levels of LIP were significantly higher in the H-FT antisense-transfected cells as compared with either untreated cells or mock-transfected cells (Table 1 and Figure 3A). These effects are consistent with the reduction in L-FT observed in cells overexpressing H-FT in which LIP was reduced [13,20]. In parallel, higher ROS production was observed after the H-FT antisense-transfected cells were challenged with H2O2 (5 \(\mu M\)) (Figure 3B). This result indicates that the up-modulation of LIP by the H-FT antisense transfection is associated with a catalytic (labile) reservoir. Although the LIP levels in cells with different H-FT levels were significantly different, their total iron content was apparently similar (Table 1). It should be emphasized that LIP is the metabolically active cellular iron, even though it constitutes only a minor fraction (< 1%) of the total cellular iron (results not shown and [12]).
Effects of H-FT repression on TIR

The possibility that the rise in LIP evoked by H-FT repression led to IRP inactivation [21,22], and thereby increased L-FT expression, was also assessed in terms of TIR expression, i.e. TIR-mediated iron uptake (Figure 4A) and TIR surface expression (Figure 4B). H-FT repression, like addition of an iron salt (ferric ammonium citrate, 20 μM for 24 h), repressed both transferrin-Fe uptake and TIR expression. This supports the notion that the repressive effect of the H-FT antisense on TIR was via up-modulation of the LIP.

DISCUSSION

We have used transient transfection of an H-FT antisense fragment in order to directly assess H-FT’s role as a regulator of LIP and ROS formation. It has been generally assumed that ferritin protein expression is regulated translationally by IRP activity, which is controlled by the LIP levels [21,22]. However, ferritin has also been proposed to function as an ‘active’ modulator of the LIP by IRP-independent mechanisms associated with transcriptional regulation [5,23,24]. In order to ascertain that ferritin expression can actively affect the LIP, we aimed to set up experimental conditions that allowed ferritin levels to be monitored independently of the LIP. We achieved that goal by specifically reducing H-FT expression, using transient transfection with an H-FT antisense fragment (Figure 1A and Table 1). The reduction in the average cellular H-FT levels was substantial (approx. 50%). However, only about two-thirds of the cells underwent transfection (Figure 2). Thus the actual changes evoked in the transfected sub-population are 25% higher than the average value for the entire population, representing 75% reduction in H-FT in the transfected sub-population.

The reduction in H-FT levels evoked a concomitant increase in the LIP, whether measured directly with a fluorescent sensor or indirectly by its capacity to promote ROS formation or induce synthesis of the L-FT subunit (Table 1). The mechanism by which repression of H-FT affected the LIP differed from that of chelators which act on the LIP–IRP feedback loop. Thus unlike iron chelation by deferoxamine, which reduces both ferritin subunit levels via reduction in the LIP and activation of IRP, the H-FT antisense transfection led to an increase in the steady-state LIP and associated parameters. Moreover, the fact that the transfected cells expressed reduced H-FT content, despite their demonstrably higher LIP levels, indicates that repression of H-FT by antisense-mediated transfection overcame the cell IRP regulatory capacity [21,22].

These results complement studies in which ferritin was over-expressed independently of LIP, using cells stably transfected with H-FT mutated in its iron-responsive element [12–14]. In those studies, overexpression of H-FT led to a concomitant decrease in LIP, supporting the presumed role of ferritin in controlling LIP levels. Taken as a whole, the modulation of H-FT expression by transfections with H-FT constructs provides experimental support for the active role of ferritin in both up- and down-regulation of LIP and ROS generation. This active role of ferritin has been implied in different aspects of cell growth [25], such as protection against oxidative stress [26–28], intracellular pathogens [29–31], and in various inflammatory conditions [30–32]. An active role of H-FT in the control of LIP levels provides an experimental framework for rationalizing the observed oncogene-mediated transcriptional repression of H-FT [1–6,33]. Such a repression has been hypothesized to support cell proliferation, presumably by making more LIP available for accelerated cell growth. However, this study provides the first experimental evidence linking the presumed rise in LIP to H-FT repression. Unfortunately the delayed expression of the ferritin antisense in transfected cells hampered the assessment of the role of ferritin in accelerated cell growth. This subject is currently under investigation with the aid of antisense oligodeoxynucleotides that allow long-term studies in culture (O. Kakhlon, Y. Gruenbaum and Z. I. Cabantchik, unpublished work).

REFERENCES


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