Intracellular Heme Coordinately Modulates Globin Chain Synthesis, Transferrin Receptor Number, and Ferritin Content in Differentiating Friend Erythroleukemia Cells

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The effect of succinylacetone (SA), a highly specific inhibitor of ALA-dehydratase and heme synthesis, on hemoglobin (Hb) production, transferrin receptor (TfR), and ferritin expression was analyzed in differentiating Friend leukemia cells (FLC). This compound exerted a pronounced inhibitory effect not only on heme and Hb synthesis, but also on all the remaining above-mentioned parameters. In particular, SA induced: (1) a reduction of the level of α-globin mRNA; (2) a decreased number of exposed TfR molecules, without modification of their affinity for the ligand; and (3) a reduced level of TfR RNA.

L.EUKEMIC, AND ESPECIALLY erythroleukemic, cells express a large number of transferrin receptors (TfRs), thus providing a unique model to investigate the mechanism(s) controlling TfR expression. Friend leukemia cells (FLC) are erythroid precursors blocked in their differentiation pathway at the proerythroblast stage. Treatment of FLC with dimethylsulfoxide (MeSO) and other inducers leads to a sequence of differentiative events that mimic normal differentiation of erythroid cells.

The TfR system is involved in the control of cell growth and differentiation. Several factors contribute to the regulation of the number of TfRs on the cell surface. Hemin, iron, and protoporphyrin IX represent the main molecules involved in the control of TfR expression. Studies of fibroblasts, leukemia lines, and mitogen-activated T lymphocytes indicate that TfRs are specifically modulated by the intracellular iron concentration via a negative feedback. Thus, iron treatment induces a decrease in TfR synthesis, whereas addition of an iron chelator enhances its production. However, different regulatory mechanisms have been observed in cells involved in iron storage: in monocytes maturing to macrophages iron induces an upregulation of TfR expression, in contrast to the negative feedback observed in other cell types.

Heme has long been known as the prosthetic group of hemoproteins, eg, hemoglobin (Hb), catalase, and the cytochromes. As a prosthetic group, heme regulates both the structure and the activity of hemoproteins. Heme is also involved in the regulation of the biosynthesis of hemoproteins at different levels, ie, transcription, translation, transport, assembly, and/or protein degradation.

In erythropoietic cells heme has been shown to exert a regulatory effect on Hb synthesis and other related events, eg, iron uptake and heme synthesis.

In FLC exogenous hemin increases the accumulation of globin mRNA and protein without blocking cell proliferation. In human erythroleukemia cells, heme treatment similarly increases the synthesis of globin mRNAs.

The effect of heme on the synthesis of ferritin, the major eukaryotic iron-storage protein, has been investigated in several cell lines. The synthesis of ferritin is positively regulated at the translational level in response to extracellular iron or heme. Translational control of ferritin synthesis involves the binding of a repressor protein, the iron regulatory element-binding protein (IRE-BP), to a highly conserved sequence, the IRE, within the 5′ untranslated region of ferritin mRNAs. On iron administration, the IRE-BP is released, and translation of the stored mRNAs provides more ferritin to sequester the uptaken iron. Recent studies on the derepression of ferritin translation in vitro suggest that heme, through the modulation of IRE-BP activity, acts as the physiologic inducer of ferritin synthesis.

We have recently shown that heme modulates TfR synthesis in differentiating FLC. In this report we indicate that blockade of heme synthesis in differentiating FLC leads to a significant decrease not only of globin chains synthesis, but also of both TfR number and ferritin content. These findings suggest that in erythropoietic differentiation optimal heme synthesis is required to coordinately modulate globin chains synthesis, iron uptake, and its intracellular localization, ie, the TfR/ferritin system.

MATERIALS AND METHODS

Cells. FLC 745E were grown in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics. Induction of FLC differentiation. FLC were seeded at a concentration of 10^4 cells/mL in the presence of MeSO (stock solution undiluted, at room temperature; Merck, Darmstadt, Germany).
Inhibition of differentiation was induced by 1 mmol/L succinylacetone (SA), a specific inhibitor of heme synthesis (4,6-dioxoheptanoic acid; Sigma, St Louis, MO). Inducer and/or inhibitor were added to the culture medium before cell seeding. Cells were counted daily with a Coulter Counter or a hemocytometer. Cell mortality, evaluated by the trypan blue dye exclusion method, never exceeded 2%. The degree of differentiation was determined by evaluating the percentage of benzidine-positive (B⁺) cells according to Orkin et al.19

Fluorometric assay of heme. Heme concentration was determined fluorometrically by a modification of the method described by Sassa.17 This method is based on the conversion of the heme moiety of Hb to its fluorescent porphyrin derivative by the removal of iron from heme under acidic reducing conditions. Cell suspensions containing 10⁶ cells were centrifuged at 500g for 10 minutes. After removal of the supernatants, 3 mL of 2 mol/L oxalic acid was added to the pellet. The mixture was shaken vigorously and immediately heated for 30 minutes at 120°C in a preheated autoclave. A tissue blank containing cells with the oxalic acid was run without heating to check for the presence of endogenous porphyrins in the cell. Standards were made by adding 10 µL of hemin solution prepared in 1% (wt/vol) bovine serum albumin, 0.01 N KOH, 50% (vol/vol) methanol, to 2 mol/L oxalic acid solution, and then heating as previously described. After cooling, fluorescence was determined in a Perkin Elmer LS-5B luminescence spectrophotometer. The exciting light was 400 nm and the fluorescence emission 662 nm.

Cytoplasmic RNA extraction and analysis. FLC, 10⁶, were harvested, centrifuged, and washed twice with phosphate-buffered saline (PBS). Cell pellets were resuspended in 2 mL Nonidet P-40 (NP-40; Sigma) lysis buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.5% [vol/vol] NP-40), incubated for 5 minutes on ice, and centrifuged at 500g for 5 minutes.

Cytoplasmic RNA present in the supernatant was extracted and purified by the guanidine/cesium chloride method.20,21 The RNA (10 µg) was run on denaturing (1.2%) agarose gels containing formamide, transferred onto a Hybond-N nylon membrane (Amersham, Buckinghamshire, UK), and hybridized to random-primed 32P-labeled (1.5 x 10⁶ cp/m/L) cDNA probes. The hybridizations were performed with the α-globin cDNA clone designated pCR, αα G1072 (generous gifts of M.G. Farace, University of Catanzaro, Catanzaro, Italy) and with the TfR cDNA clone termed pTFR-207 (generous gift of J. Goding, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

The hybridization conditions were: 50 mmol/L sodium phosphate, pH 7.0, 50% formamide, 5X sodium chloride and citrate (SSC), 4X Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 200 µg/mL of sonicated salmon sperm DNA at 42°C for 20 hours. The filters were washed two times in 1X SSC and 0.1% SDS for 30 minutes at room temperature and two times in 0.1X SSC and 0.1% SDS for 20 minutes at 42°C, then exposed to Fuji X-ray film using intensifying screens.

Radioiodinated transferrin (Tf) binding assays. Purified human Tf (Sigma) was conjugated with 125I by the solid-phase lactoperoxidase method (New England Nuclear [NEN] DuPont, Bad Homberg, Germany; radioiodination system).

The binding conditions were performed as previously reported.22,24

Nuclear run-on transcription assay. Cells collected and washed with ice-cold PBS were centrifuged at 1,600g for 5 minutes. The cell pellet was suspended in an NP-40 lysis buffer (10 mmol/L Tris pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.5% NP-40), incubated for 5 minutes on ice, and sheared gently through a Gilson tip. Nuclei were isolated on a 0.7 mol/L sucrose cushion. RNA was labeled with 32P-UTP and purified as described.25 Labeled RNA was hybridized to specific cDNA probes immobilized on nitrocellulose membranes. Hybridizations were performed in a solution containing 50% formamide, 0.5% SDS, 5X SSC, and 5X Denhardt's solution at 42°C for 3 days. Filters were then washed for 15 minutes twice in 0.1X SSC, 0.1% SDS at room temperature, and then for another 30 minutes at 65°C.

Evaluation of ferritin content. The level of ferritin in the cellular extracts of FLC was evaluated by immunoblotting.

Proteins (50 to 100 µg) from postmitochondrial supernatant fraction were heated, electrophoresed on 15% SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose paper.

Ferritin subunits were then shown by using antiferritin antibody (Boehringer Mannheim, Germany) and then 125I-labeled protein A (NEN). An LKB 2202 ultrasound laser microdensitometer was used to scan the density of individual polypeptide bands.

RESULTS

Inhibition of erythroid differentiation and globin chains synthesis by SA. SA is known as a specific inhibitor of heme synthesis. Heme content and the percentage of B⁺ cells in FLC treated with MeSO (1.5%) are dramatically reduced by the combined addition of MeSO (1.5%) + SA (1 mmol/L) (Table 1). Differentiation of MeSO-treated FLC is associated with an increase of globin mRNA, globin chain synthesis, and Hb accumulation.26 To investigate the effect of heme synthesis inhibition in this pathway, we analyzed globin mRNA steady-state levels. Northern blot analysis of mRNA encoding α-globin chains indicates that in cells treated with MeSO and SA the level of α-globin mRNA is 40% to 80% lower than in control cells treated with MeSO alone (Fig 1).

TfR expression after MeSO and MeSO + SA treatment: Tf binding studies. The kinetics of 125I-Tf binding in control FLC at different days of culture show a marked induction of TfR due to increased cell growth in fresh medium, which peaks at 48 hours and progressively decreases until day 4, when the cultures reach a saturating cell density.27 In MeSO-treated cells the increase in Tf binding in the first 2 days is similar to that observed in control FLC, but remains at high levels in the following 2 days of culture (days 3 and 4) when the cells become mature erythroblast.27

The addition of 1 mmol/L SA to the cultures treated with MeSO induces a drastic reduction of 125I-Tf binding, and is particularly marked on day 4 of culture (Fig 2). In control cultures the addition of SA alone has no effect on the Tf binding (data not shown).

Table 1. Heme Concentration in MeSO-Induced FLC Treated With SA

<table>
<thead>
<tr>
<th>Cells and Treatment</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B⁺ (%)</td>
<td>Heme (nmol/10⁶ cells)</td>
</tr>
<tr>
<td>745A</td>
<td>ND</td>
<td>0.02</td>
</tr>
<tr>
<td>745A + MeSO (1.5%)</td>
<td>65%</td>
<td>0.35</td>
</tr>
<tr>
<td>745A + MeSO (1.5%) + SA (1 mmol/L)</td>
<td>2%</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The percentage of B⁺ cells and heme concentration were evaluated as described in Materials and Methods. Each experimental point was performed in duplicate. Cell mortality never exceeded 2%. Abbreviation: ND, not detectable.
The affinity of the ligand for its receptor was examined by competitive binding experiments. Results shown as Scatchard plots indicate that the affinity of TfRs was similar for MeSO, and MeSO + SA-treated cells (Fig 2).

**TfR expression after MeSO and MeSO + SA treatment: RNA and transcription studies.** To investigate if the decreased number of TfRs correlates with a reduced mRNA steady-state level, we analyzed the TfR RNA by Northern blot. In control cultures, the amount of TfR RNA peaks at 24 hours of culture and later markedly decreases in agreement with 125I-Tf binding data. In MeSO-treated cells a sustained level of TfR RNA was observed at all culture days. In contrast, in MeSO + SA treated cells the amount of RNA was always markedly lower than in cells grown in the presence of MeSO alone (Fig 3). This result clearly indicates that the heme synthesis inhibitor regulates the number of TfRs by modulating the level of TfR mRNA.

A modulation in the level of mRNAs may be mediated by alteration(s) in the transcriptional rate and/or posttranscriptional events. To determine if the effect of the heme synthesis inhibitor on TfR mRNA is exerted at transcriptional or posttranscriptional level, run-on experiments were performed. Labeled RNAs extracted from the nuclei of FLC treated with MeSO + SA were hybridized to a panel of cDNA probes immobilized on nitrocellulose filters and subjected to autoradiography (Fig 4). Similar levels of TfR transcripts are observed both in FLC treated with either MeSO alone or MeSO + SA. This finding suggests that the reduced level of TfR RNA observed in FLC grown in the presence of MeSO + SA is largely due to posttranscriptional mechanism(s).

**Effect of heme addition on TfR expression in MeSO-induced FLC.** On the basis of the previous data, it was postulated that the addition of hemin to MeSO-induced FLC leads to an opposite effect than that elicited by SA. In keeping with this hypothesis, a higher Tf binding capacity was observed in cells grown for 4 days in the presence of MeSO + hemin than in those grown in the presence of MeSO alone (Fig 5). Furthermore, the addition of hemin to control FLC, not induced to differentiate by MeSO, elicited a marked downregulation of TfR expression, as observed in several other cell types.

**Effect of heme addition on ferritin content in MeSO-induced FLC.** We evaluated the effect of hemin addition on ferritin content in both control and MeSO-induced FLC (Table 2). These experiments showed that: (1) MeSO-induced FLC exhibited a higher ferritin content than control FLC, and (2) both untreated FLC and MeSO-induced FLC showed a pronounced increase of their ferritin content after hemin addition. The addition of SA to
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control FLC elicited a slight reduction of their ferritin content starting from day 1 onward. In MeSO-induced FLC, SA similarly induced a marked inhibition of the increase of ferritin content (Table 2). The addition of hemin to SA-treated FLC overrides the inhibitory effect of the heme synthesis inhibitor on ferritin synthesis (Table 2).

Moreover, in both control and MeSO-induced FLC hemin induced an increase of ferritin content more pronounced than iron salts (Table 2). This finding further supports the hypothesis that the increase in heme content occurring during the maturation of erythroid cells is responsible for the increase in ferritin content and TFR expression.

DISCUSSION

The present studies focus on the effects of the inhibition of heme synthesis on FLC erythroid differentiation.

In erythroid cells heme is a key component in various metabolic pathways because it: (1) decreases the proteolysis of abnormal proteins, (2) represents a structural component of Hb, (3) regulates the expression of mRNA at the translational level, and (4) affects the rate of globin mRNA transcription. Indeed, activation of iron transport and heme biosynthesis occur before cell hemoglobinization and seemingly represent a prerequisite for the latter phenomenon during erythroid maturation.

Several studies suggest that in terminal erythroid cells (reticulocytes), free heme negatively modulates the rate of iron uptake because the two parameters are inversely correlated in the cytoplasm. On the other hand, inhibition of heme synthesis by specific inhibitors such as SA is accompanied by enhanced iron uptake. Therefore, heme seems to control the cellular supply of iron for Hb synthesis by a negative feedback mechanism. However, in K562 erythroleukemic cells, heme excess does not inhibit the removal of iron from Tf, thereby suggesting that erythroid cells acquire this mechanism only during the late steps of erythroid differentiation.

Our results indicate that in FLC differentiating to mature erythroblasts the blockade of heme synthesis induces a decrease in the number of TFRs on the cell membrane, whereas an opposite effect is induced by hemin addition. This finding clearly suggests that in mature erythroid cells an optimal level of intracellular heme is required to sustain TFR synthesis. The effect of SA is related to a specific inhibitory effect on heme synthesis, as clearly indicated by the observation that hemin is able to overcome the inhibitory effects mediated by SA, in agreement with previous studies.

As previously mentioned, experiments in a variety of cell types indicate that the expression of TFRs is modu-
lated by the addition of iron and/or heme through a negative feedback, via the intracellular iron and/or heme level. Particularly, experiments performed on Hela cells, human leukemic and erythroleukemic lines,15 mitogen-activated human T lymphocytes,1 cultured hepatocytes,5 and uninduced FLC (this report) show a decrease in the number of TfR on addition of hemin. On the other hand, recent data indicate that in in vitro grown monocyte-macrophages, iron modulates TfR expression through a positive feedback mechanism.5 Similarly, the data presented here show that in differentiating FLC an optimal intracellular heme level is required to sustain TfR expression, indicating that a coordinate regulation of TfR expression, iron uptake, heme synthesis, and Hb production occurs in mature erythroid cells.

Our results also indicate that hemin plays a key role in the control of ferritin accumulation in FLC. This conclusion is directly supported by three observations: (1) hemin treatment of both uninduced or Me2SO-induced FLC elicited a marked increase of ferritin content; (2) the addition of a heme synthesis inhibitor, SA, caused a marked decrease in the ferritin content of both control and Me2SO-induced FLC; and (3) a peak concentration of heme exerted a more marked stimulation of ferritin synthesis than a peak concentration of iron salts. Our results are in line with previous studies indicating a stimulation of ferritin expression in Me2SO-treated FLC57,48 and in hemin-treated K562 erythroleukemia cells.19,20 In our hands, hemin was more active than iron salts in stimulating ferritin accumulation in FLC. This result is particularly interesting in view of recent studies24-26 suggesting that hemin may represent the molecule physiologically involved in the control of ferritin biosynthesis through translational mechanisms, which are dependent on the binding of this molecule to the cytoplasmic repressor IRE-BP.40

However, further studies are required to assess whether the effect of hemin on ferritin synthesis is related to a direct effect of this molecule on the iron-specific cytoplasmic repressor and/or its capacity to donate iron to the chelatable iron pool through the action of heme oxygenase.

In conclusion, our studies support the hypothesis that the increase in heme content occurring during the maturation of FLC plays a key role not only in stimulating globin-chain synthesis, but also in mediating optimal expression of ferritin and TfR at the protein level, which is in turn necessary to sustain an optimal synthesis of heme itself. Thus, the coordinate regulation of these events by intracellular heme may represent a key mechanism in the control of Hb synthesis.

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REFERENCES

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18. Rogers J, Munro H: Translation of ferritin light and heavy subunit mRNAs is regulated by intracellular chelatable iron levels in rat hepatoma cells. Proc Natl Acad Sci USA 84:2277, 1987


37. Scatchard G: The attraction of proteins for small molecules and ions. Ann NY Acad Sci 51:600, 1949


43. Trowbridge IS, Omray MB: Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 78:3039, 1981


49. Leibold EA, Munro HN: Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy and light-subunit mRNAs. Proc Natl Acad Sci USA 85:2171, 1988

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