Utilization of Intracellular Ferritin Iron for Hemoglobin Synthesis in Developing Human Erythroid Precursors

By Boris Vaisman, Eitan Fibach, and Abraham M. Konijn

Ferritin (Ft) plays an important role in cellular iron metabolism. It can store substantial amounts of iron in a nontoxic soluble form. However, its ability to donate iron for cellular needs, in particular for hemoglobin (Hb) synthesis in human erythroid cells, is still controversial. We studied the role of intracellular Ft-iron in Hb synthesis and the involvement of lysosomal proteolysis in iron release from Ft. Ft-iron release and its subsequent incorporation into Hb was investigated in normal human erythroid precursors developing in culture. Dual staining flow cytometry with antibody (Ab)-specific for Ft and for Hb showed a decrease in cellular Ft content in erythroid cells during their maturation. Cellular Ft-iron participation in Hb synthesis was studied by labeling cells with $^{59}$Fe. Cells were incubated with $^{59}$Fe-labeled human dimeric transferrin (Tf), then chased, and intracellular radioiron distribution between Ft and Hb was determined on subsequent days by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and/or Ft immunoprecipitation and heme extraction. On day 6, most of the $^{59}$Fe accumulated in Ft. Thereafter, a progressive decrease of radioiron in Ft and a corresponding increase of the label in Hb was observed. Inhibition of Hb synthesis with succinylacetone caused radioiron to remain in Ft and prevented its redistribution. Addition of unlabeled dimeric Tf to the culture medium did not prevent radioiron from appearing in Hb. Chloroquine represses lysosomal function prevented radio-iron redistribution between Ft and Hb. Inhibition of proteolysis by chymostatin and/or leupeptin led to Ft-protein accumulation in the cells and also prevented radioiron transfer from Ft to Hb. The results of the present study suggest that intracellular Ft donates iron for Hb synthesis and that proteolytic Ft degradation in a lysosomal-like compartment is necessary for iron release and its transfer to Hb.

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Using immunofluorescence flow cytometry, we followed the temporal relationship between intracellular Ft content and the developmental stage of erythroid cells. The results demonstrated a decline in intracellular Ft content concomitant with erythroid maturation.

With radioactive iron, we followed the ability of erythroid cells to use Ft-iron for heme and Hb synthesis. The results showed that at the pre-Hb production stage, iron was taken up and accumulated mainly in Ft. Following maturation, it was gradually removed from Ft and appeared in Hb. Succinylacetone inhibition of heme synthesis prevented radioiron transfer from Ft to Hb. Inhibition of lysosomal functions by chloroquine or inhibition of intracellular proteases by chymostatin and/or leupeptin caused the radioiron to remain in Ft and prevented its redistribution. These results suggest that at early stages of erythroid maturation, iron taken up via the Tf pathway, accumulates within the cells, mainly in Ft. Then, during the stage of intense Hb synthesis, intracellular Ft is gradually degraded, in a process that requires lysosomal proteolytic activity, and its iron is used for heme synthesis.

MATERIALS AND METHODS

**Erythroid cell cultures.** Blood was obtained from normal volunteers. The two-phase liquid culture was employed as previously described.20,21 Briefly, mononuclear cells were isolated from peripheral blood samples by Ficoll-Hypaque density gradient centrifugation and seeded in alpha-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS; both from Gibco, Grand Island, NY), 1 μg/mL cyclosporin A (Sandoz, Basel, Switzerland), and 10% conditioned medium from 5637 bladder carcinoma cell cultures, containing different hematopoietic growth factors not including erythropoietin (EPO). The cultures were incubated at 37°C, under an atmosphere of 5% CO2 in air with extra humidity. After a 7-day incubation in this phase-I culture, the nonadherent cells were harvested, washed, and recultured in fresh medium composed of α-MEM, 30% FCS, 1% deionized bovine serum albumin, 10-7 mol/L dexamethasone, and 1 μM human recombinant EPO (Ortho Pharmaceutical Co, Raritan, NJ). This part of the culture is referred to as phase II. After 5 days of incubation, erythroblasts were purified by centrifugation on 45% Percol (Pharmacia, Uppsala, Sweden, density 1.0585 g/mL). The upper layer, containing mainly proerythroblasts and basophilic normoblasts, was collected, washed, and resuspended in the original medium for future incubation. Cell samples were analyzed usually between the sixth and seventh day of phase II. Viability of the cells was determined by trypan blue exclusion and was higher than 95%. Maturation was confirmed by cell morphology, which was assessed microscopically on cytocentrifuge-prepared slides after May-Grunwald-Giemsa staining. The majority of erythroid cells, on day 6 of phase II, were basophilic normoblasts, on days 8 to 10, polychromatophilic normoblasts, and on day 12, orthochromatotic normoblasts.

**Preparation of cell lysates.** Cells were harvested, washed, and lysed on ice in solubilization buffer containing 1% Triton X-100 (Pierce, Rockford, IL), aprotinin 10 μg/mL, leupeptin 10 μg/mL, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) 3.7 μg/mL, N-tosyl-L-lysine chloromethyl ketone (TLCK) 3.7 μg/mL, pepstatin 1 μg/mL, and phenylmethylsulfonyl fluoride (PMSF) 0.25 mmol/L and sodium azide 0.02% (all from Sigma-Israil, Cholon, Israel) in Tris-HCl 10 mmol/L, pH 7.4. The lysates were centrifuged at 10,000g for 10 minutes, and the supernatants were collected and stored at −80°C until use.

**Preparation of rabbit antihuman-Ft serum.** Human Ft was isolated from human term placenta as previously described (subunit ratio of H- and L-subunits is about 1:3).22 The purity of the Ft was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without heating; nonreducing conditions were used. When subunits were separated by SDS-PAGE, reducing conditions and heating were used. Antibodies (Ab) to this Ft were prepared in New Zealand rabbits (body weight 2.5 to 3.0 kg). They were injected with 1.5 to 2.0 mg of the human Ft solubilized in complete Freund's adjuvant. After 30 days, they were given a booster of 1.0 to 1.5 mg Ft in incomplete Freund's adjuvant. The rabbits were bled 7 days after the second injection. The titer of the antiserum obtained was tested by double immunodiffusion in agar. The serum was centrifuged at 10,000xg for 10 minutes and stored at −20°C.

**Ft determination by enzyme-linked immunosorbent assay (ELISA).** The concentration of total cellular Ft was measured by ELISA using antiotot-placental–Ft-Ab containing Ab against L- as well as H-Ft subunits. Placental Ft-Ab were affinity purified from the immune serum on agarose-immobilized total-placental-Ft, and coupled to β-galactosidase (obtained from Boehringer-Manheim) as previously described.23 Optimal working concentrations for the ELISA reagents were found by checkerboard titration.

**Two-color flow cytometric analysis.** Cultured cells were washed, fixed in 3% paraformaldehyde for 1 hour, and the membranes were permeabilized with saponin-containing buffer (phosphate-buffered saline [PBS] containing 0.1% saponin, 1% glycine, both from Polysciences, Warrington, PA) and 2% human albumin (Kamada, Ltd, Israel) in Tris-HCl 10 mmol/L, pH 7.4 for 2 hours at room temperature and then overnight at 4°C. The immunoprecipitate was labeled with 59 Fe by incubating 100 μCi per mL) during the first 6 days in phase II. The cells were then centrifuged, washed three times, and stored at −20°C until use.

**Preparation of 59 Fe-labeled human diferric Tf (hdTf).** 59 Fe-labeled hdTf (7.44 kBq or 0.2 Ci per mL) during the first 6 days in phase II. The cells were then centrifuged, washed three times, and stored at −20°C until use.

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with 30% FCS and 1 μmol/L hdTf. On days 6, 8, and 10, cell aliquots were washed, lysed, and analyzed.

Heme extraction and ⁵⁹Fe-heme determination. Heme was extracted as described by Thunell.²⁴ A total of 0.5 mL of 1 N HCl was added to 0.2 mL of cell lysate and extracted twice with 0.5 mL ethylacetate:acetic acid (3:1 vol/vol) mixture. The organic extracts were pooled and their ⁵⁹Fe-radioactivity was measured.

SDS-PAGE and autoradiography. Cell lysates were submitted to SDS-PAGE on 6% (T) gels under nonreducing conditions and without heating the samples. The gels were subsequently dried and exposed to phosphor-imaging and/or autoradiography.

RESULTS

Changes in cellular Ft content during erythroblast development. Ft content of cells at different stages of the culture was assessed by immunofluorescence flow cytometry. Cells were harvested, washed, fixed, and simultaneously stained with antihuman Ft rabbit serum followed by FITC-conjugated antirabbit IgG and with phycoerythrin-conjugated antihuman HbA monoclonal Ab. Controls were stained with rabbit nonimmune serum. After 6 days in phase II, 93% of Hb-positive (erythroblast) cells were also positive for ferritin (upper right quadrant) and only 7% of Hb-positive cells were ferritin-negative (upper left quadrant). After 12 days in phase II, 34% of the Hb-positive population became ferritin-negative. The plots shown are representative of three separate experiments.

Fig 1. Flow cytometric analysis of ferritin content in cultured erythroid cells. Erythroid precursors were grown according to the two-phase liquid culture procedure. On different days of phase II (EPO-dependent phase, see Materials and Methods), cells were stained for ferritin with antihuman placental ferritin rabbit serum and antirabbit IgG labeled with FITC and for Hb with phycoerythrin-labeled antihuman HbA monoclonal Ab. Controls were stained with rabbit nonimmune serum. After 6 days in phase II, 93% of Hb-positive (erythroblast) cells were also positive for ferritin (upper right quadrant) and only 7% of Hb-positive cells were ferritin-negative (upper left quadrant). After 12 days in phase II, 34% of the Hb-positive population became ferritin-negative. The plots shown are representative of three separate experiments.

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trations of 5 to 20 μmol/L. At these concentrations, chloroquine showed little or no toxic effects and the cells survived and proliferated. Three days after addition of chloroquine, cells were harvested, lysed, and the radioactivity in heme and in Ft was determined after Ft immunoprecipitation and heme extraction. Other cultures grown under the same experimental conditions were analyzed by SDS-PAGE followed by phosphor-imaging. Iron transfer from Ft to heme was inversely related to the concentration of chloroquine: the higher the concentration of chloroquine the greater the fraction of intracellular $^{59}$Fe that remained in Ft and, thus, less was incorporated in heme (Fig 4A and B).

The inhibition by chloroquine of radioiron transfer from
led to a 2.3-fold increase, and both inhibitors together caused a 3.9-fold increase in cellular Ft content (Fig 5B). These results suggest that inhibition of lysosomal proteases leads to a decrease in Ft-protein degradation.

In another experiment, $^{59}$Fe-labeled cells were chased and then reincubated at day 6 of phase II in fresh, radioactive-free medium, supplemented with 1 $\mu$mol/L hdTF and the protease inhibitors chymostatin (200 $\mu$g/mL) and leupeptin (20 $\mu$g/mL). Samples of the cultures were harvested after 2 and 4 days and analyzed by SDS-PAGE. The results (Fig 6) demonstrate that in treated cells $^{59}$Fe remained in Ft and its transport to Hb was inhibited.

**DISCUSSION**

Erythroid precursors have an extremely high iron requirement, especially during the period of Hb synthesis. Their developmental program involves, therefore, a drastic increase in transferrin receptor (TfR) expression, which suggests that lysosomal-like organelles are involved in the release of iron from Ft and eventually its transfer to heme. The question remains: is iron released from intact Ft or is Ft degraded by lysosomal proteases and as a result iron is released and becomes available for heme synthesis? The protease inhibitors chymostatin and leupeptin were used to resolve this question. Chymostatin is a reversible serine and cysteine protease inhibitor, and leupeptin is a reversible inhibitor of trypsin-like and cysteine proteases. Chymostatin was dissolved in dimethylsulfoxide (DMSO) and added to cell cultures at a concentration of 200 $\mu$g/mL on day 7 or 8 of phase II. The following day, the same quantity of chymostatin was added again. DMSO was added to cultures in parallel flasks, which served as controls. On day 10 or 11, cells were harvested, lysed, and Ft content was determined by ELISA. The results (Fig 5A) showed that there was a 2.1-fold increase in cellular Ft content after treatment with chymostatin. In experiments presented in Fig 5B, the cells were treated with 10 $\mu$g/mL leupeptin, 200 $\mu$g/mL chymostatin, or both inhibitors together. Leupeptin alone caused a 2.2-fold increase in cellular Ft content, chymostatin
allows accelerated uptake of iron. Excessive iron, especially before production of Hb, is stored in Ft. Another potential source of cellular iron is extracellular Ft, whose uptake by early erythroid cells is regulated and whose iron can be used for heme synthesis. The fate of the iron of intracellularly synthesized Ft during erythroid cell maturation, the potential of Ft to supply iron for Hb synthesis, and the mechanisms involved are still poorly understood.

To answer these questions, we followed changes in intracellular Ft content as a function of erythroid maturation. For this purpose, we developed and used an immunofluorescence flow cytometry method. Cells were double stained with specific Ab for Ft and Hb. The procedure allowed us to avoid contaminating nonerythroid (Hb-negative) cells (eg, myeloid and lymphoid cells), which could bias the results. Analysis of cultures at different stages of development, demonstrated a decline in intracellular Ft content concomitant with erythroid maturation. Our results are in agreement with previous studies in which erythroblasts from human bone marrow were used. In these studies Ft was measured by radioimmunoassay or assessed by microscopic immunocytochemical analysis.

Ferritin, like other cellular proteins, has its own intracellular rate of turnover, during which it is likely to release its iron. This iron can join the cytoplasmic labile iron pool and thus can supply iron for Hb synthesis in developing erythroid cells. However, the question of the participation of Ft-iron in Hb synthesis in human erythroid cells remains unresolved. Human diferric Tf can be taken up via the TFR-Tf pathway during the entire period of intense Hb synthesis and thus, may provide all the iron needs of the cell. It was shown that iron taken up via the TFR-Tf pathway may pass directly to heme without using Ft as intermediate. In murine erythroleukemia cells, labeled with $^{59}$Fe and induced to synthesize Hb with DMSO, some of the $^{59}$Fe in the Hb originated from Ft. Similarly, radio-iron from amphibian larval red cell Ft was found in adult Hb. These results indicate that, potentially, Ft can donate iron to Hb. However, attempts to demonstrate the participation of cellular Ft-iron in heme synthesis in rabbit or rat reticulocytes have failed. Reticulocytes represent the penultimate stage of erythroid development, when the rate of iron uptake and heme synthesis are already diminished. In addition, in these experiments short observation periods were employed making detection of iron transfer from Ft to Hb extremely difficult.

In our studies, we used a two-phase liquid culture procedure, which allowed us to follow the fate of Ft-iron at early stages of developing erythroid cells. The cultured cells were labeled with radioiron at a pre-Hb stage (day 0 to 6 of phase II), and the distribution of their radioiron in Ft and heme was followed. The results showed that in early erythroid precursors (in day-6 cultures), iron was taken up and it accumulated mainly in Ft. Following maturation, this iron was redistributed; it was gradually removed from Ft and appeared in Hb (Fig 2). This redistribution of the radioiron strongly supports the possibility that the iron, which has accumulated in Ft before Hb production is released from this Ft at later stages and subsequently used for heme synthesis. Human diferric Tf did not affect the redistribution of $^{59}$Fe from Ft to Hb, indicating that Ft donates iron for heme synthesis also when the cells are supplemented with sufficient or even excessive iron.

To prove that the decrease in intracellular $^{59}$Fe-Ft was caused by the translocation of the $^{59}$Fe from Ft to heme rather than by leakage from the cells, cells were treated with succinylacetone, an inhibitor of δ-aminolevulinic acid dehydratase. Under these conditions, the radioiron was retained in Ft, thus indicating that Ft-iron in erythroid cells serves as a repository of iron for heme synthesis.

Mechanisms involved in the process of iron release from intracellular Ft are still an enigma. In vitro, this process is triggered by acidic pH and various reducing agents and it is enhanced by free radicals, which alter the Ft protein shell and accelerate its degradation. However, such agents are either unphysiological or are not present in normal cells at sufficient concentrations necessary for iron release. In vitro studies suggested that iron release from Ft may be triggered by intermediates of the heme synthetic pathway. It was shown, in a cell free system, that δ-aminolevulinic acid was able to release iron directly from Ft.

Our present study suggests that at least part of the process of iron release from Ft in normal, Hb synthesizing, cells takes place in an acid intracellular compartment and involves proteolytic activity. Thus, treatment of erythroid precursors with chloroquine inhibits iron redistribution between Ft and Hb in a dose-dependent manner, suggesting that increasing the compartmental pH leads to inhibition of the process. After treating the cells with the protease inhibitors, chymostatin and leupeptin, an increase in the Ft protein content was observed suggesting that lysosomal proteases are involved in Ft protein degradation. As a result, much of the iron re-
mained in Ft and was not used for heme synthesis. These results are in agreement with previous studies in non-Hb synthesizing cells, which have shown that chymostatin leads to Ft accumulation in HepG2 hepatoma cells and inhibits the restoration of the deferoxamine (DFO) chelatable iron pool in cultured rat hepatocytes following iron depletion by DFO, presumably by inhibiting iron release from Ft. Leupeptin and the lysosomal inhibitor, methylamine, inhibited Ft degradation in uninduced K562 cells and decreases Ft-iron release and Ft-iron mobilization by DFO.

The results of the present study suggest that at early stages of erythroid maturation, preceding intensive Hb synthesis, erythroid cells actively take up extracellular iron in Ft-bound form and accumulate it in Ft. Then, with the increase in the rate of Hb synthesis, iron is released from Ft and is used by the cells for heme synthesis. Iron release from Ft is placed in an acid compartment and requires protein shell degradation by proteases. It should be noted that these results do not rule out the possibility that iron can also pass directly from Ft to heme without using Ft as an intermediate, nor do they determine the relative contribution of Ft nor the Ft-TfR pathway to Hb synthesis at different stages of erythroid maturation.

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