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Control of hemoglobin synthesis in erythroid differentiating K562 cells
II. Studies of iron mobilization in erythroid cells by high-performance liquid chromatography–electrochemical detection

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Abstract

We have demonstrated that iron controls hemoglobin (Hb) synthesis in erythroid differentiating K562 cells by enhancing the activity of a key enzyme of the Hb synthesis, δ-aminolevulinate synthase (ALAS). In the present study, we studied iron mobilization and the role of iron in erythroid differentiating cells by measuring the level of iron by means of high-performance liquid chromatography using electrochemical detection (HPLC–ED). After treatment of K562 cells with sodium butyrate, the expression of transferrin receptor (TfR) increased initially, followed by an increase in the levels of both total iron and Hb as well as the ALAS activity. However, no increase could be found in the levels of non-heme iron, low-molecular-mass iron (LMMFe) and ferritin. Addition of diferric transferrin (FeTf) enhanced both δ-aminolevulinic acid (ALA) and Hb synthesis. In contrast, addition of hemin elevated the levels of all iron species as well as the Hb synthesis but reduced the TfR expression and ALA contents in both butyrate treated and untreated cells. These results suggest that Hb synthesis is controlled by TfR expression, and that the ALA synthesis is suppressed by iron released from heme and/or Hb due to lowered expression of TfR. © 1998 Elsevier Science B.V.

Keywords: K562 cells; Transferrin receptor; Hemoglobin; Iron

1. Introduction

We have previously demonstrated that iron is an important factor for the control of hemoglobin (Hb) synthesis in the erythroid differentiating K562 cell line. It was suggested that iron controlled Hb synthesis in butyrate-treated K562 cells by enhancing the activity of δ-aminolevulinate synthase (ALAS), a key enzyme in the erythroid heme synthetic pathway [1].

It is well known that iron is bound to serum transferrin (Tf) and is incorporated into cells as diferric transferrin (FeTf) through Tf receptors (TfR) [2,3]. Most of the incorporated iron migrates to mitochondria to be utilized for heme synthesis, and the rest of the iron is stored in ferritin as non-heme iron. It is also reported that a small amount of iron exists in the cells not bound to proteins, but forms

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low-molecular-mass iron (LMMFe) with biological chelators such as citrate and ADP. Despite this knowledge, the exact mode of mobilization of intracellular iron and iron species which control the Hb synthesis is still unclear.

Although the mobilizations of radiolabeled iron chelates or FeTf as tracers in the erythroid cells were well investigated [4–6], there are relatively few reports concerning the changes in iron distribution or the effect of endogenous iron species on Hb synthesis. In previous reports, we established a method for iron determination in biological systems by high-performance liquid chromatography with electrochemical detection (HPLC–ED) [7,8]. This method is based on the separation of the iron–diethylenetriaminepentaacetic acid (DTPA) complex formed directly on an anion-exchange column followed by ED. The method is capable of determining concentration of iron as low as 0.1 μ*M* of ferric ion. In this paper, we applied this method for the determination of total iron, non-heme iron and LMMFe in K562 cells and studied the control mechanism of Hb synthesis by iron.

2. Experimental

2.1. Materials

Sodium butyrate, hemin, δ-aminolevulinic acid (ALA), FeTf and succinyl acetone (SA) were from Sigma (St. Louis, MO, USA). DTPA and trichloroacetic acid (TCA) were from Dojindo (Kumamoto, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Nitric acid and $FeNH_4(SO_4)_2 \cdot 12H_2O$ were from Wako (Osaka, Japan). All other chemicals used were of the highest purity available. RPMI-1640 was from Gibco, and FCS was from Cytosystems (Australia). EIA test ferritin BMY was from Boehringer Mannheim (Germany). Mouse anti-human TfR, NU-TfR1 was from Nichirei (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin antibody was from Cappel (West Chester, PA, USA). Recombinant human erythropoietin (EPO) from Chinese hamster ovary cells was a kind gift from Kirin Brewery (Tokyo, Japan).

2.2. Cell culture

K562 cells were donated by the Japan Cancer Research Resource Bank (Tokyo, Japan). K562 cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 30 mg/l kanamycin at 37°C in an atmosphere of 5% CO_2 in air. For use in the experiments, the cells were plated at $0.4 \cdot 10^5$/ml on RPMI 1640 medium containing 10% FCS. To examine the effect of FeTf on differentiation, the cells were suspended in medium containing 1% FCS and FeTf at a cell density of $1 \cdot 10^5$/ml. The Hb levels of the butyrate-treated cells in 1% FCS were less than those observed in 10% FCS. Hemin was dissolved in 0.15 *M* NaOH, and mixed with an equal volume of PBS. The pH of the solution was adjusted to 7.4 with 1 *M* HCl.

2.3. Chromatographic conditions

Chromatography was carried out using a metal-free system equipped by a Tosoh (Tokyo, Japan) CCPD pump and a Rheodyne Model 7125 injector (Tosoh). The column was a TSK gel IC-Anion-PW column (50×4.6 mm I.D., 10 μm particle size) (Tosoh). A Tosoh Model EC-8010 electrochemical detector with a glassy carbon working electrode was used. The detector potential was set at −0.3 V versus an Ag/AgCl reference electrode. The detector output was recorded on a Hitachi (Tokyo, Japan) Model D-2500 chromatointegrator.

The eluent was 2 m*M* DTPA solution adjusted to pH 2.0 with hydrochloric acid. The flow-rate was 1.0 ml/min. Samples were injected into a 10-μl PTEE sample loop with a syringe.

A standard solution was prepared by dissolving $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 50 m*M* hydrochloric acid just prior to use.

2.4. Sample preparation

2.4.1. Total iron

To determine total iron, $1–2 \cdot 10^7$ cells were washed with PBS twice and lysed by heating in 61% nitric acid at 90°C for 2 h. After cooling, the lysate was diluted 100-fold with 50 m*M* hydrochloric acid,

and 10 μl of the diluted sample was injected into the HPLC system.

2.4.2. Non-heme iron

The sample was prepared by the method of Percival [6]. $1–2\cdot10^7$ washed cells were washed and suspended in four times as much volume of 0.25 *M* sucrose solution and were frozen and thawed twice. After centrifugation at 8000 *g* for 20 min, 75 μl of the supernatant was incubated with an equal volume of 10% TCA solution at 90°C for 15 min, and then the precipitate was removed by centrifugation. The supernatant was diluted 10-fold with 50 m*M* hydrochloric acid, and iron in the diluted supernatant was determined as non-heme iron by using a 10-μl sample.

2.4.3. LMMFe

$1–2\cdot10^7$ cells suspended in nine equivalent volumes of 0.25 *M* sucrose solution containing 20 μ*M* DTPA were frozen and thawed twice, and the precipitate was removed by centrifugation. The supernatant was ultrafiltered using a 10 000 molecular mass cut-off membrane filter, and the LMMFe in the ultrafiltrate was determined.

2.5. Accuracy and precision

For accuracy and precision of the method for the total iron, the lysate of K562 cells was spiked with $FeNH_4(SO_4)_2\cdot12H_2O$ at a final concentration of 500 μ*M* before heating with nitric acid. For non-heme iron, the reaction mixture of the supernatant and TCA was spiked with $FeNH_4(SO_4)_2\cdot12H_2O$ at a final concentration of 50 μ*M* before incubation. To calculate accuracy and precision for LMMFe, iron standard at a final concentration of 5 μ*M* was added to the ultrafiltrate.

2.6. Hemoglobin determination

Hemoglobin content was measured by the method of Cioe et al. [9] from the absorbance at 414 nm, correcting for nonspecific absorption due to light scattering using absorbances at 403 and 425 nm. The absorbance of 1.0 at 414 nm corresponded to a Hb concentration of 0.13 mg/ml.

2.7. Measurement for TfR expression

All procedures were performed at 4°C. Cells were washed with PBS and incubated with mouse anti-human TfR IgG in PBS containing 0.5% bovine serum albumin for 30 min. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse immunoglobulin antibody for 30 min. Unbound antibody was removed by washing with PBS twice. Flow cytometric analysis was performed using Cyto ACE (Jasco, Tokyo, Japan). The excitation wavelength was 488 nm by argon-ion laser, and the green fluorescence from FITC collected through a 530 nm band-pass filter was measured on a log scale.

2.8. Measurement for ALA

ALA was converted to a pyrrole derivative with acetylacetone, and the absorbance of the derivative formed from ALA pyrrole, which was reacted with Ehrlich reagent to form a chromogen, was measured. Suspensions of the washed cells were sonicated (3× 10 s). After addition of 10% TCA to the suspension, the supernatant (0.2 ml) was mixed with 0.1 ml of 1 *M* sodium acetate buffer, pH 5.5, containing 10% acetylacetone, and heated at 80°C for 15 min. After cooling, the reaction mixture was neutralized with 1 *M* Na_2HPO_4. The pyrrole formed from aminoacetone with acetylacetone was separated from ALA pyrrole by extraction of the neutralized solution with an equal volume of dichloromethane. A modified Ehrlich reagent containing $HgCl_2$ was added to an equal volume of the aqueous phase and the absorbance was measured at 553 nm. An absorbance value of 1.0 corresponded to an ALA concentration of 17.2 m*M*.

2.9. Assay for ALAS activity

ALAS activity was determined by a minor modification of the method of Sinclair and Granick [10]. $1–2\cdot10^7$ cells were suspended in 0.15 ml of assay medium containing 100 m*M* glycine, 10 m*M* succinic acid, 10 m*M* $MgCl_2$, 5 m*M* EDTA, 30 m*M* levulinic acid, 0.1 m*M* pyridoxal phosphate, 0.1 m*M* coenzyme A, 10 m*M* GTP and 0.75 units of succinyl CoA synthetase in 50 m*M* Tris–HCl, pH 7.4. The

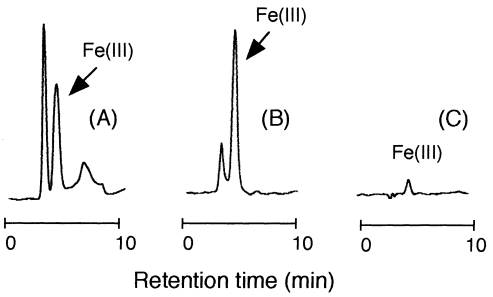

Fig. 1. Chromatograms of total iron (A), non-heme iron (B) and LMMFe (C) in K562 cells. Ten μl of each sample was injected.

mixture was sonicated (3×10 s), and incubated at 37°C for 15 min. After addition of 0.1 ml of 10% TCA to the mixture, the levels of ALA in the supernatant were measured by the method described in Section 2.8 above.

2.10. Assay for ferritin

A suspension of washed cells in 0.25 m*M* sucrose solution was frozen and thawed twice, and then centrifuged at 8000 *g* for 20 min. An aliquot of the supernatant and 40 m*M* phosphate buffer, pH 6.8, were added into a test tube coated with anti-human ferritin antibody and incubated at room temperature for 1 h. After washing the tube, 40 m*M* phosphate buffer containing peroxidase labeled sheep anti-ferritin antibody was added into the tube and incubated at room temperature for 1 h. Again the tube was washed with PBS and 2,2′-azino-bis(3-ethylbenzothiazolin-6-sulphonic acid ammonium) was incubated in the tube at room temperature for 1 h. Absorbance of the reaction mixture was measured at 420 nm and ferritin concentration was determined from a standard curve prepared by human serum containing standard ferritin.

3. Results

3.1. Determination of total iron, non-heme iron and LMMFe

HPLC–ED was used to determine the levels of total iron, non-heme iron and LMMFe in K562 cells. As shown in Fig. 1A, total iron was detected as ferric iron by HPLC–ED. The peak shape of the total iron tended to be broad, and that was possibly due to the biological chelators in the sample, which interfere the formation of the DTPA–iron complex. However, peak-area calibration indicated sufficient accuracy and precision (Table 1).

Non-heme iron and LMMFe were detected without any interference of contaminants in the post-mitochondrial fraction (Fig. 1B, Fig. 1C). No decomposition of heme by incubation with TCA was confirmed by the addition of hemin to the post-mitochondrial fraction before incubation with TCA. Accuracy and precision of the determination of non-heme iron and LMMFe calculated from their peak-

Table 1
Accuracy and precision of the iron determination method by HPLC–ED

Iron	Spiked concentration (μ*M*)	Accuracy (recovery, %)	Precision ($n=4$) (R.S.D., %)
Total iron[a]	500	88.0	4.3
Non-heme iron[b]	50	92.8	5.7
Non-protein bound iron[c]	5	82.2	7.7

[a] $1–2\cdot10^7$ cells were washed with PBS and then lysed by heating with iron standard at a final concentration of 500 μ*M* in 61% nitric acid at 90°C for 2 h. The lysate was diluted 100-fold with 50 m*M* hydrochloric acid.

[b] The cells suspended in 0.25 *M* sucrose solution were frozen and thawed twice. After centrifugation, 75 μl of the supernatant was incubated with an equal volume of 10% TCA and an appropraite amount of iron standard at 90°C for 15 min. The precipitate formed was removed by centrifugation. The supernatant was diluted 10-times with 50 m*M* hydrochloric acid.

[c] The cells suspended in 0.25 *M* sucrose solution containing 20 μ*M* DTPA were frozen and thawed twice, and the precipitate was removed by centrifugation. The supernatant was ultrafiltered using a 10 000 molecular mass cut-off filter, and an iron standard was added to the ultrafiltrate.

heights proved to be satisfactory for the study on iron mobilization in K562 cells (Table 1).

3.2. Changes in the total iron in erythroid differentiating cells

Sodium butyrate, hemin and EPO are known to cause some biological changes in K562 cells [11–13]. K562 cells were incubated with 1 m*M* sodium butyrate, 10 μ*M* hemin or 2 mU EPO for four days, and Hb contents and total iron in those cells were measured. As shown in Fig. 2, both the levels of Hb and total iron were increased in the butyrate- and hemin-treated cells, whereas EPO affected neither total iron nor Hb contents in the cells. To investigate whether the increase in the total iron is responsible for the enhancement of Hb synthesis, ALA and SA, which are known to modify heme synthesis were added to butyrate-, hemin- and EPO-treated cells and untreated cells. In butyrate-treated cells, the Hb synthesis was enhanced by the addition of ALA, but ALA caused no significant changes in the total iron. SA reduced the Hb contents but did not change the level of total iron in butyrate-treated cells. The changes caused by ALA and SA in the levels of Hb and total iron in hemin- and EPO-treated cells were almost the same as those in untreated cells.

3.3. Changes in TfR expression in erythroid differentiating cells

Iron is mostly incorporated into cells as FeTf through TfR. Fig. 3 shows changes in the expression of surface TfR, the level of total iron, the Hb content and the ALAS activity after treatment with butyrate. The TfR expression was the first to rise after butyrate treatment, followed by ALAS activity and Hb con-

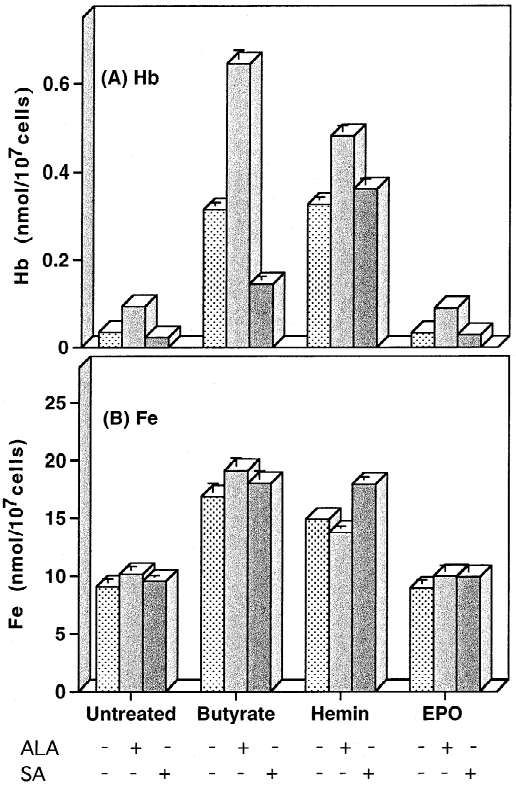

Fig. 2. The levels of Hb (A) and total iron (B) in K562 cells. The cells were treated with 1 m*M* sodium butyrate, 10 μ*M* hemin or 2 mU EPO in the presence of 0.5 m*M* ALA or 0.5 m*M* SA for four days. Values are the mean±S.D. of results from three experiments.

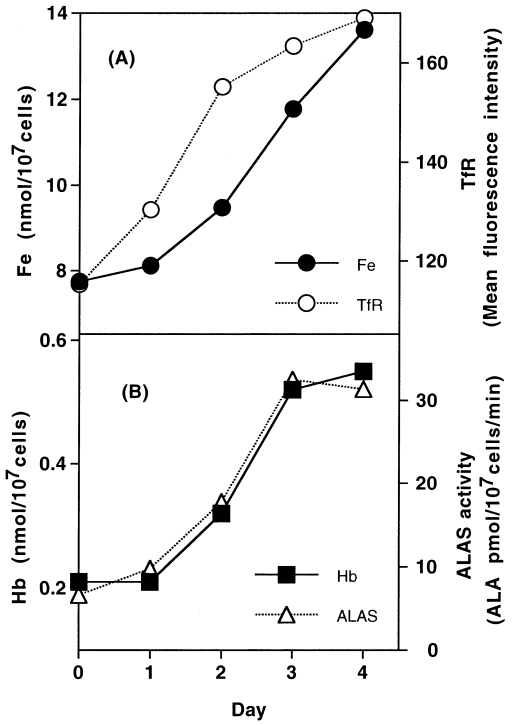

Fig. 3. Changes in the TfR expression and the levels of total iron (A), and in the levels of Hb and ALAS activity (B) in butyrate-treated cells. Values are the mean of results from two experiments.

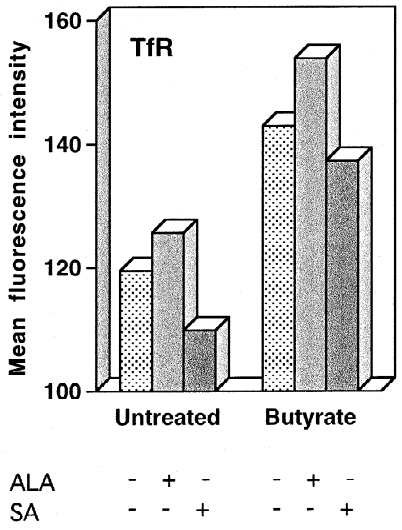

Fig. 4. Effect of ALA and SA on the expression of FeTf on the surface of butyrate-treated cells and untreated cells. Values are the mean of results from two experiments.

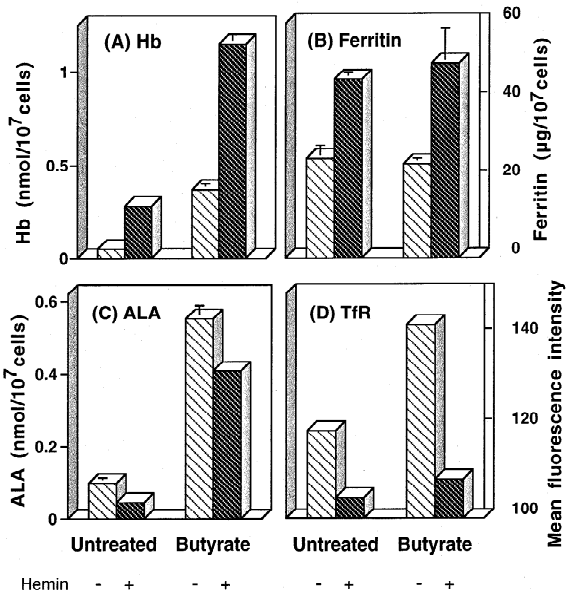

Fig. 5. Hb content (A), ferritin content (B), ALA content (C) and TfR expression (D) in butyrate-treated and untreated cells cultured with or without hemin. Values are the mean±S.D. of results from three experiments.

tent and total iron, which follow a similar time course. Fig. 4 indicates the effects of ALA and SA on the expression of the TfR on butyrate-treated and untreated cells. The TfR expression was enhanced by ALA and suppressed by SA. These results suggest that Hb synthesis is associated with the TfR expression.

3.4. Changes in iron mobilization in Hb synthesizing cells

To investigate the changes in iron mobilization during Hb synthesis, the levels of total iron, non-heme iron and LMMFe were measured using HPLC–ED. Although total iron was increased after butyrate treatment, there was a slight decrease in non-heme iron and no change in LMMFe (Table 2). The addition of hemin enhanced all the iron species in both butyrate-treated and untreated cells. Further effects of butyrate and hemin on proteins relating with iron mobilization were investigated. Fig. 5 indicates the contents of Hb (A), ferritin (B) and ALA (C) and the TfR expressions (D) in butyrate-treated and untreated cells cultured with or without hemin. Butyrate treatment enhanced Hb synthesis, and an addition of hemin caused a further increase in the Hb contents in the butyrate-treated cells. The addition of hemin resulted in an increase in ferritin contents although the butyrate treatment caused no changes in the ferritin content in cells. Hemin

Table 2
Levels of iron species in K562 cells

Cells	Fe (nmol/10^7 cells)		
	Total	Non-heme	LMM
Untreated cells	6.8±0.6	2.8±0.0	0.09±0.03
Butyrate-treated cells	10.8±0.6	1.9±0.2	0.11±0.02
Hemin-treated cells	14.2±0.4	4.9±0.1	0.18±0.01
Butyrate and hemin-treated cells	18.0±0.8	5.2±0.2	0.19±0.01

Values are the mean±S.D. from the results of three experiments.

suppressed both ALA contents and TfR expression in butyrate-treated cells in which ALA synthesis and TfR expression were enhanced. Both butyrate and hemin enhanced the Hb synthesis but induced different biological changes in K562 cells.

3.5. Effects of FeTf on iron mobilization and Hb synthesis

Previously, we suggested that ALA and Hb synthesis were dependent on concentrations of FeTf. A change in iron mobilization in the cells supplemented with different concentrations of FeTf were compared with the changes in ALA and Hb contents. The ALA contents in butyrate-treated cells were dependent on the concentrations of FeTf added to the culture medium, whereas the Hb contents tended to saturate at higher concentrations of FeTf (Fig. 6A). Neither the ALA contents nor the Hb contents in untreated cells were influenced by the addition of FeTf (data not shown). The total iron in butyrate-treated cells increased by addition of FeTf in a similar manner as that in untreated cells (Fig. 6B). In contrast, non-heme iron levels in butyrate treated cells were increased more noticeably in the presence of higher concentrations of FeTf, which caused saturation of the Hb synthesis (Fig. 6C).

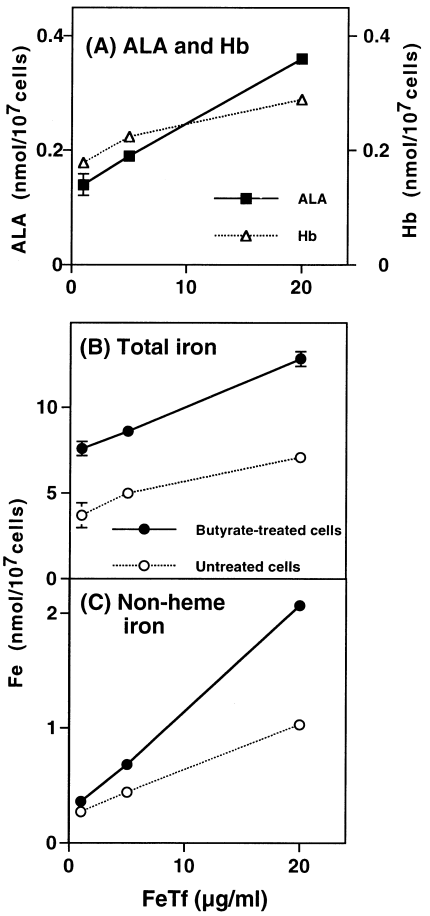

Fig. 6. Effect of FeTf on ALA and Hb content in butyrate-treated cells (A), the total iron (B) and the non-heme iron (C) in untreated and butyrate-treated cells. Untreated and butyrate-treated cells were cultured with 1% FCS in the presence of different concentrations of FeTf for four days. Values are the mean±S.D. of results from three experiments.

4. Discussion

In the previous paper [1], we suggested that iron controlled Hb synthesis by enhancing expression of ALAS, which is a key enzyme in heme synthesis. To clarify the iron species which controls Hb synthesis, we investigated iron mobilization in K562 cells during erythroid differentiation by measuring total iron, non-heme iron and LMMFe by using HPLC–ED. Although total iron was increased in butyrate-treated cells, modifiers of Hb synthesis, ALA and SA, failed to change the level of total iron (Fig. 2). In spite of an increase in Hb contents, there were no changes in the levels of ferritin and LMMFe and a slight decrease in non-heme iron after treatment with butyrate (Table 2, Fig. 5). In contrast, ALA and Hb contents were dependent on the concentration of FeTf in the culture medium (Fig. 6A). In addition, once erythroid differentiation in K562 cells was induced by treatment with butyrate, the expression of TfR on the cell surface was enhanced before increases in ALAS activity, Hb contents and total iron (Fig. 3). It is reported that expression of TfR is enhanced during erythroid differentiation [14,15]. Our results suggest that the increase in TfR expression is an important factor in Hb synthesis, and that the FeTf but not the endogenous iron, controls Hb synthesis. Conrad et al. [16] and Richardson et al. [17] have proposed some intermediates for transfer of iron from FeTf to Hb. Our results also suggest the

existence of intermediates from FeTf to Hb other than ferritin and LMMFe.

Hb synthesis was amplified by hemin as well as by butyrate. However, hemin caused some other changes in the cells. Not only the level of total iron but also the levels of non-heme iron, LMMFe and ferritin were increased in hemin-treated cells (Table 2, Fig. 5B). It is well known that heme is decomposed by heme oxygenase, and the enzyme is induced by heme [18]. It is also reported that heme oxygenase is induced in heme-treated K562 cells [19]. Therefore it is considered that the origin of non-heme iron in K562 cells could be iron released from heme decomposed by heme oxygenase. In addition, the differences of non-heme iron between butyrate-treated and untreated cells were increased when the Hb synthesis was saturated in spite of the increase in ALA synthesis (Fig. 6). It could be that heme which has not been utilized for Hb formation is decomposed, and iron released from the heme is stored as non-heme iron.

We previously observed no changes in Hb contents in the hemin-treated cells after additions of FeTf, ALA and SA, and suggested that the effects of hemin to facilitate Hb synthesis were by incorporating hemin into Hb as a heme but not by induction of the ALA synthesis. Although it is well known that a feedback inhibition of heme synthesis exists in hepatocytes, the effect of the heme on erythroid heme synthesis is unclear. Concerning this subject, Gardner et al. [20] reported that hemin reduced the ALAS activity in immature erythroid cells. Here we showed that the ALA contents and TfR expression which controlled Hb synthesis were suppressed in the hemin-treated cells, leading to the conclusion that hemin caused a reduction of ALA synthesis (Fig. 5C,D).

It is well known that expressions of ferritin and TfR are regulated by an iron responsible element (IRE) together with a specific binding protein against IRE [21,22]. The IRE system is supposed to induced both an increase in TfR expression and a decrease in ferritin expression in the iron depleted cells. Therefore, it can be considered that iron released from the decomposed heme enhances the expression of ferritin and also suppresses TfR expression. Consequently, an incorporation of FeTf is decreased, and ALA synthesis is suppressed in hemin-treated cells. In this study, we suggest that TfR expression in erythroid differentiating K562 cells plays an important role in the control of Hb synthesis, and the erythroid heme/Hb synthesis is controlled by the final product, heme/Hb, with a negative feedback mechanism.

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