Hemin-Induced Acceleration of Hemoglobin Production in Immature Cultured Erythroid Cells: Preferential Enhancement of Fetal Hemoglobin

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The effects of heme, when added as the ferric chloride salt, hemin, on human erythroid cells grown in a two-phase liquid culture system were studied. When added together with erythropoietin, on initiation of the second phase of the culture, heme greatly accelerated hemoglobin (Hb) accumulation in these cells. The effect was greater during their early stages of maturation, suggesting that hemoglobin availability is a rate-limiting step for Hb synthesis. Hemin increased preferentially the production of fetal Hb (HbF) compared with adult Hb; this was associated with a selective twofold elevation in γ-mRNA levels. Using succinylacetone, a potent inhibitor of heme synthesis, we showed that exogenously supplied heme could be incorporated into the de novo formed Hb. Therefore, the mechanism of heme action may be several fold, including effects on globin gene transcription and posttranslational events, e.g., supplying the prosthetic group for Hb assembly. Hemin increased HbF of cells derived from patients with sickle cell anemia and β-thalassemia as well as that of cells from normal donors. Moreover, when added in combination with other HbF-augmenting agents such as the cytotoxic drug, hydroxyurea, a synergistic effect was obtained, with considerably less cytotoxicity than with hydroxyurea alone. These results have clinical potential in light of the ameliorating effect that increased HbF has in patients with genetic diseases of the β-globin chain and raise the possibility of combined treatment with hemin and other drugs now being used to treat these diseases. © 1995 by The American Society of Hematology.

MATERIALS AND METHODS

Erythroid cell cultures. Blood was obtained from normal volunteers and from patients with either sickle cell anemia or β-thalassemia. In the case of transfused patients, blood samples were withdrawn before transfusion. Erythroid progenitors were grown according to the two-phase liquid culture protocol.13,14 In short, PB mononucleated cells were isolated by centrifugation on Ficoll-Hypaque and seeded in α-minimal essential medium supplemented with 10% fetal calf serum (FCS; both from GIBCO, Grand Island, NY), 1 μg/mL cyclosporin A (Sandoz, Basel, Switzerland), and 10% 5637 bladder-carcinoma cell-conditioned medium.23 The cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air with extra humidity.

After 7-day incubation in this phase-I culture, the nonadherent cells were harvested, washed, and recultured in phase II, which

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cDNA was synthesized using globin-specific primers as previously described. DNA amplification free hemin was eluted from the column in the first hemin-containing cultures indicated that, under the conditions used, elute only after 22 minutes (data not shown).

Hb-containing cells were determined by using the benzidine-HCl procedure. Mean cellular Hb (MCH) was calculated by dividing the total Hb content of the lysate by the number of benzidine-positive cells. Cell morphology was assessed microscopically on cytocriftuge-prepared slides (Shandon, Cheshire, UK) stained with alkaline benzidine and Giemsa.

Preparation of hemin solution. A stock solution of 5 mmol/L hemin was prepared as follows: 32.5 mg bovine hemin (GIBCO) was dissolved in 0.5 mL of 1N NaOH for about 30 minutes; then, 0.5 mL of 0.5 mol/L Tris base was added, followed by 10 mL of bovine serum albumin (10% in α-medium, Sigma Chemical Co, St Louis, MO). The mixture was neutralized with 0.5 mL of 1N HCl, filtered through a 0.45-μm filter, and stored at 4°C for not more than a week.

HPLC. Cells were washed 3 times with phosphate-buffered saline, and the pellet was lysed in double-distilled water. After spinning for 1 minute in a microcentrifuge, the supernatant was collected and stored at 4°C. Hb proteins in the hemolysates were separated by cation-exchange HPLC (Maxima 820; Waters Chromatography Division, Millipore Co, Milford, MA), using Synchropak CM300 (250-mm×4.6-mm) column (Synchron Inc, Lafayette, IN) and Bis-Tris (30 mmol/L) buffer. Standard Hb solutions (Isolab Inc, Akron, OH) were used for chromatography of supernatant free hemin was eluted from the column in the first 10 minutes and it was well separated from heme-proteins (ie, Hb) that started to elute only after 22 minutes (data not shown).

Globin mRNA analysis. Cells were homogenized in guanidinium thiocyanate, RNA was extracted, and polyadenylated RNA was isolated on an oligo (dT) cellulose column using the QuickPrep mRNA purification kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). cDNA was synthesized using 1 μg RNA, reverse transcriptase, and globin-specific primers as previously described. DNA amplification was performed by the PCR using specific synthetic oligonucleotide primers (Applied Biosystems 380B DNA synthesizer, Foster City, CA) for either the y- or β-globin genes on a Perkin-Elmer Cetus DNA Thermal Cycler (Foster City, CA) for 35 cycles, melting at 94°C for 1 minute, annealing at 55°C for 1.5 minutes, and extending at 72°C for 1.5 minutes, and then maintained at 4°C. For quantitation, specific DNA standards were prepared so that the 5' and 3' ends were compatible with a globin-specific primer pair used to amplify the cDNA sample but that produced a PCR product with a different size. Multiple reaction tubes were prepared for each cDNA sample, and serial dilutions of the globin specific standard DNA was added to each tube with 0.5 μL of α32P dCTP. The cycle number (25 cycles) was chosen to ensure that the reaction was within the exponential phase as determined by preliminary experiments. PCR products were analyzed by agarose gel electrophoresis, and the amount of 32P incorporated into each PCR product was determined by liquid scintillation counting (LKB Mallac 1209). The amount of standard necessary to provide PCR products of equal intensity for the standard and cDNA sample was used to determine the amount of cDNA present. The determination for control samples were within twofold of the actual amount over a range of several log units.

RESULTS
The effect of hemin on HbF production was determined by growing human erythroid precursors in phase II of the two-phase liquid culture in the presence of 1 U/mL Epo and different concentrations of hemin. Cells were harvested on day 13 and were lysed, and their Hb content was then analyzed by HPLC. The results indicated that total Hb (Fig 1A), which was because of some increase in cell number (Fig 1B), but a more significant increase in the percentage of HbF (mean, threefold; range, twofold to fivefold) in each of these individuals (Fig 3).

Figure 2 depicts the HPLC chromatograms of lysates of normal cells from 6-day cultures in the absence or presence of hemin. The results indicated that (1) cells did not accumulate substantial amounts of intracellular free hemin, (2) hemin-treated cultures had about fivefold more total Hb content, and (3) hemin-treated cultures showed a threefold increase in the percentage of HbF, which was associated with a decrease in the percentage of HbA as compared with that for untreated cells.

Adding hemin (100 μmol/L) to cells derived from patients with β-thalassemia or sickle cell anemia, who usually have higher-than-normal HbF levels, caused a similar proportional increase in the percentage of HbF (mean, threefold; range, twofold to fivefold) in each of these individuals (Fig 3).
HEMIN EFFECTS FETAL HEMOGLOBIN PRODUCTION

5.0

Without Hemin

HbA

79.0%

With Hemin

HbA

60.1%

HbF

33.7%

HbA2

3.8%

$10^5$ Volts

x $10^1$ Minutes

Fig 2. HPLC chromatography of Hb content of erythroid cells cultured in phase II with or without hemin. Cells were harvested and lysed on day 6.

We then determined the optimal time of addition of hemin to the cultures. The results (Fig 4) indicated that adding hemin, together with Epo, at the onset of phase II of normal cell cultures produced a maximal effect. When added at later times, the effect diminished, and after day 7, when all erythroid cells were already engaged in Hb accumulation as evidenced by benzidine staining, there was no effect at all. Adding hemin to phase I of the culture was ineffective, unless it was also added to phase II. These results indicate that, to enhance HbF production, hemin has to be present during early stages of erythroid maturation, when Hb production is initiated.

Figure 5 shows the effects of hemin on the kinetics of erythroid cell proliferation and accumulation of Hb. The patients with either sickle cell anemia or β° thalassemia. The latter patients were from the Israeli-Jewish-Kurdish population whose molecular characterization has been previously reported. PB derived cells were cultured in phase II either in the presence (100 μmol/L) or absence of hemin. On day 13, cultures were harvested and analyzed.

Fig 3. Effect of hemin on HbF production by cultured erythroid precursors derived from normal donors, and patients with either sickle cell anemia or β° thalassemia. The latter patients were from the Israeli-Jewish-Kurdish population whose molecular characterization has been previously reported. PB derived cells were cultured in phase II either in the presence (100 μmol/L) or absence of hemin. On day 13, cultures were harvested and analyzed.

Fig 4. Effect of time of addition of hemin on HbF production by cultured erythroid precursors. Hemin (100 μmol/L) was added at the indicated days of phase II. Cells were harvested on day 13, and their Hb was analyzed. The data points indicate the mean ± SE obtained in four experiments using cells from different normal donors.

Fig 5. Effect of hemin on HbF production by cultured erythroid precursors derived from normal donors, and patients with either sickle cell anemia or β° thalassemia. The latter patients were from the Israeli-Jewish-Kurdish population whose molecular characterization has been previously reported. PB derived cells were cultured in phase II either in the presence (100 μmol/L) or absence of hemin. On day 13, cultures were harvested and analyzed.
changes in cell number (Fig 5A), percentage of Hb-containing (benzidine-reactive) cells (Fig 5B), total Hb content per culture (Fig 5C), and Hb content per cell (MCH; Fig 5D) were followed up in phase II cultures initiated either with or without hemin. The results indicated that hemin had some effect on the kinetics of cell proliferation (20% increase on day 6), but that it had a major effect on the kinetics of Hb accumulation. With respect to all these parameters, the hemin effect was most pronounced at early stages of maturation (days 2 to 6).

Figure 6 shows the temporal changes in proportion of HbF (%HbF) when hemin was added on onset of phase II. The results indicated that both hemin-treated and untreated cultures had high %HbF levels at their early stages. But in hemin-treated cultures, Hb production was initiated earlier (benzidine-positive cells were already detected on day 3, as compared with day 5 in the control cultures) and was associated with higher %HbF. Although %HbF decreased with time in both cultures, it remained considerably higher throughout the period in hemin-treated cultures. On day 13, when the cells reached the stage of orthochromatic normoblasts, hemin-treated cultures contained a threefold higher %HbF than control cultures.

The effect of hemin, added on the first day of phase II, on β- and γ-globin mRNA contents was next determined. The results showed 2.2-fold more cytoplasmic γ-mRNA on day 6 and 1.8-fold more on day 12 in hemin-treated cultures as compared with that for control cultures. No significant difference was detected between the cultures on either dates in the level of the β-mRNA (Fig 7).

We next determined the ability of exogenously supplied hemin to serve as heme in maturing erythroid precursors. In the experiment presented in Fig 8, cells were treated in phase II with either succinylacetone, an inhibitor of δ-aminolevulinic acid dehydratase (the second enzyme in the heme-synthetic pathway), hemin, or hemin with succinylacetone. On day 13, cells were harvested, and their Hb was measured. The results indicated that the Hb content of cultures treated with succinylacetone was only 10% of the control (untreated).
HEMIN EFFECTS FETAL HEMOGLOBIN PRODUCTION

![Graph 1](image1)

**Fig 7.** The effect of hemin on γ- and β-globin mRNA content. PB-derived normal cells were grown in phase II either in the absence (□) or presence (■) of hemin. Cells were harvested on day 6; RNA was extracted and analyzed as described in Materials and Methods.

cultures. When hemin was added together with succinylacetone, Hb content was 120% that of the control. Hemin by itself increased the Hb content by 50% over the control. These results confirmed that succinylacetone effectively inhibits heme biosynthesis and that exogenously supplied hemin could replace endogenously produced heme for Hb formation.

The effect of many HbF-augmenting agents such as 5-azacytidine and hydroxyurea is associated with cytotoxicity. In our experience, a combination of two such agents usually does not produce HbF levels higher than those obtained by each agent alone, mainly because of added toxicity (data not shown). Because hemin at moderate concentrations (up to 200 μmol/L) is not cytotoxic in this system and in some experiments even enhances erythroid cell proliferation and maturation, we tested the effect of a combination of hydroxyurea and hemin on the cultured cells. Hemin (100 μmol/L) was added on the first day of phase II, and different concentrations of hydroxyurea (25 to 200 μmol/L) were added on day 5, and cells were harvested on day 13. The results show very large potentiation (up to 14-fold) of HbF production by the combined treatment, compared with treatment with each agent alone (Fig 9A). This effect was obtained at much lower cytotoxicity, as evidenced by the higher cell yield, with the combination of hemin and hydroxyurea as compared with treatment with hydroxyurea alone (Fig 9B).

**DISCUSSION**

Erythroid maturation requires the coordinate expression of multiple genes. For example, the hallmark of erythroid maturation, Hb production, involves the synthesis of several types of globin polypeptide chains and of heme, an iron-containing protoporphyrin that serves as the prosthetic group of the Hb molecule. Heme is synthesized from glycine by mitochondrial enzymes, and its production depends on the activities of these enzymes and on a sufficient supply of iron. The heme synthetic enzymes are upregulated at the transcriptional level on initiation of erythroid differen-
tion. Cellular iron content, which is usually supplied by transferrin, depends on the number and affinity of transferrin receptors. Although these receptors are relatively few in number on other cell types, including erythroid cells before the stage of Hb synthesis, their number increases dramatically after the initiation of erythroid maturation. One mechanism of regulation and coordination seems to be through the GATA-1 binding motifs that many of these genes share.

The results of the present study indicate that exogenously supplied hemin (heme-ferric chloride) accelerated Hb accumulation at early stages of maturation, thereby suggesting that heme availability is a rate-limiting step in this process at these stages. This condition may be caused by either deficient cellular iron uptake because of lack of transferrin receptors or insufficient activity of one or more of the heme-pathway enzymes, with ferrochelatase being considered the limiting enzyme in erythroid cells. Using Fe and Mossbauer spectroscopy, we tested the effect of a combination of hydroxyurea and heme on cultured cells. The results (Fig 7) show a large potentiation of HbF production by the combined treatment as compared with that of each agent alone. This effect was obtained with lower toxicity, as evidenced by the much higher cell yield when the combination was used. Protease effects of heme were also observed in vitro with other cytotoxic drugs, eg, anthracyclins, and in vivo, in conjunction with Epo, in mice treated with azidathymidine.

Our results suggest that heme, either alone or in combination with other drugs, might provide a potentially useful treatment for patients with β-hemoglobinopathies. Hemin has been proven to be well tolerated when used therapeutically in patients with acute intermittent porphyria. New derivatives with a longer shelf life and in vivo half-life (eg, heme arginate) have recently been prepared. These derivatives have recently been used for treatment of patients with myelodysplastic syndrome. A major concern in using heme in polytransfused patients is the risk of aggravating their iron overload. However, recently it has been found that increased erythropoiesis in such patients (when treated with Epo) requires iron supplementation. As an efficient donor of iron (or heme), hemin may serve this function. Therefore, the beneficial effect of hemin in these patients might be fourfold: (1) increasing erythroid cell mass through stimulation of progenitor cell growth; (2) accelerating globin synthesis by affecting the rate of transcription and the efficiency of processing of globin RNAs; (3) serving as an iron (and heme) source; and (4) elevating preferentially the proportion of HbF.

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Hemin-induced acceleration of hemoglobin production in immature cultured erythroid cells: preferential enhancement of fetal hemoglobin

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