Hydroxyurea Increases Fetal Hemoglobin in Cultured Erythroid Cells Derived From Normal Individuals and Patients With Sickle Cell Anemia or β-Thalassemia

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Hydroxyurea (HU), an inhibitor of DNA synthesis, has been shown to increase fetal hemoglobin (HbF) levels in patients with sickle cell anemia and in some patients with β-thalassemia. However, until now there have not been good in vitro model systems that simulate this effect for study of the molecular and cellular mechanism(s) involved in perturbing the normal ontogeny of the globin genes. We analyzed the cellular effects of HU using a two-phase liquid culture procedure (Fibach et al: Blood 73:100, 1989) in which human peripheral blood-derived progenitor cells undergo proliferation and differentiation. HU was found to have multiple effects on these cultured cells: (1) an increase in the proportion of HbF produced; (2) a decrease in cell number due to inhibition of cell proliferation; (3) an increase in hemoglobin content per cell (mean corpuscular hemoglobin [MCH]); and (4) an increase in cell size (mean corpuscular volume). The extent of these effects was related to the HU dose and time of addition. When added to cell cultures from normal individuals 4 days following their exposure to erythropoietin (EPO), 100 μmol/L HU caused a 1.3- to 3.5-fold increase in the proportion of HbF, from 0.4% to 5.2% (mean 1.6) in untreated to 1.5% to 8.2% (mean 3.1) in HU-treated cultures and a 45% ± 10% increase in MCH but only a 25% ± 7% decrease in cell number on day 13. Cultures of cells derived from five patients with sickle cell anemia have shown a twofold to fivefold increase in the percentage of Hb F following addition of HU while four patients with β-thalassemia showed a 1.3- to 6.2-fold increase. We believe that this primary cell culture procedure should prove useful in studying the cellular and molecular mechanisms of pharmacologic induction of HbF and might provide a valuable predictive assay system for evaluation of the response of individual patients with hemoglobinopathies to HU and similar agents.

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Submitted July 10, 1992; accepted November 9, 1992.

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Erythroid cell cultures. The two-phase liquid culture procedure has been previously described in detail. In short, peripheral blood mononuclear cells obtained from normal volunteers or patients with hemoglobinopathies were isolated by centrifugation on a gradient of Ficoll-Hypaque and seeded at a density of $5 \times 10^6$ cells/mL 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% conditioned medium collected from cultures of the 5637 bladder-carcinoma cell line. The cultures were incubated at 37°C in an atmosphere of 5% CO$_2$ in air with extra humidity.

After a 5-day incubation in this phase I culture, the nonadherent cells were harvested, washed, and recultured in fresh medium composed of α-medium, 30% FCS, 1% deionized bovine serum albumin, 1 × 10$^{-5}$ mol/L 2-mercaptoethanol, 1.5 mmol/L glutamine, 1 × 10$^{-6}$ mol/L dexamethasone, and 1 U/mL human recombinant EPO (Cilag AG, Schaffhausen, Switzerland).

After a 4-day incubation in phase II, lymphocytes were removed as follows: the cells were harvested, spun down, the medium saved, and the cells layered on a Percoll (Sigma, St Louis, MO) solution (density = 1.0585 g/mL) and centrifuged at 1,000g for 20 minutes at room temperature. The upper layer, containing the proerythroblasts, was collected, resuspended in the saved medium, and incubation continued. These cultures yielded up to $4 \times 10^6$ erythroid cells (normoblasts) per milliliter of blood.

Viable cell count was performed using the Trypan Blue exclusion technique. The number of Hb-containing cells was determined using the benzidine-HCl procedure. Erythroid cell maturation was assessed morphologically by preparing cytocentrifuge slides (Shandon, Cheshire, UK) stained with alkaline benzidine and Giemsa (Fluka, Buchs, Switzerland).

Preparation of lysates. Peripheral blood or cultured cells were washed three times with phosphate-buffered saline and the pellet was lysed by resuspending the cells in double distilled water and incubating on ice for 5 to 10 minutes. After spinning for 1 minute in a microcentrifuge, the supernatant was collected and stored at 4°C.

Alkali denaturation procedure for determination of Hb F. Ninety microliters of the tested hemolysate was added to 10 μL of human immune globulin (5 g/dL; Miles Inc, Cutter Biological, Elkhart, IN). Then, 7 μL of 1.2 N NaOH was added, and the mixture vortexed. After exactly 2 minutes, 70 μL of a saturated solution of ammonium sulfate was added, mixed, incubated at room temperature for 5 minutes, and spun for 1 minute in a microcentrifuge. The supernatant, which contains Hb F only, was collected and saved for spectrophotometric Hb determination. For total Hb, 10 μL of lysate was diluted in water to give a 90-μL volume that was treated as in the previous procedure except for water instead of NaOH.

Quantitation of total Hb. Fifty microliters of the supernatant was added to 0.5 mL of a 3,3',5,5'-tetramethyl-benzidine (TMB) solution followed by the addition of 0.5 mL 0.3% (vol/vol) hydrogen peroxide

Fig 1. HU effect on cultured erythroid cells. Cells derived from the peripheral blood of normal donors were cultured in the two-phase liquid culture system. HU, at the indicated concentrations, was added on day 4 (□) or 7 (○) of the secondary cultures. Cells were harvested on day 13 and analyzed for the number of Hb-containing cells (A), Hb content per cell (MCH) (B), and the proportion of HbF out of total Hb (C) as detailed in Materials and Methods. The data points represent the mean of four determinations. The deviation of the results of each determination from the mean did not exceed 10%.
solution (Plasma Hemoglobin Kit, Cat. No. 527; Sigma). After 10 minutes, the mixture was read at 600 nm using Beckman's DU-65 spectrophotometer (Beckman, Fullerton, CA). Blank solutions, containing all the components except for Hb, were used for calibration. A standard curve was prepared using Hemoglobin Standards Set (Cat. No. 527-11; Sigma).

**High performance liquid chromatography (HPLC).** Hb proteins in the hemolysates were separated by cation exchange HPLC using Synchropak CM 300, 250  \times  4.6 mm (Synchro Inc, Lafayette, IN), and Bis-Tris (30 mmol/L) buffer and the Maxima 820 (Waters Chromatography Division, Millipore Co, Milford, MA). Standard Hb solutions (Isolab Inc, Akron, OH) were used for reference.

**RESULTS**

Addition of HU, at concentrations of 25 to 400 \( \mu \text{mol/L} \), to cultures of cells derived from the peripheral blood of normal donors as well as from patients with either sickle cell anemia or \( \beta \)-thalassemia during the first phase of the culture or during the first 4 days of the second phase (phase II) completely ablated erythroid cell development (data not shown). Adding HU at later stages was compatible with cell survival and differentiation. Figure 1 depicts the effects of addition of HU on days 4, 7, and 10 of phase II, following exposure to EPO, on erythroid cell numbers (Fig 1A), Hb per cell (Fig 1B), and the proportion of HbF (Fig 1C). The results indicate that all these parameters were dependent on the HU dose and time of addition to the cultures. At concentrations of 400 \( \mu \text{mol/L} \), HU decreased cell yield by 90% when added on day 4 of phase II, but had only a moderate effect when added on days 7 or 10. At concentrations of 200 \( \mu \text{mol/L} \) and below, although cell number decreased, no apparent toxicity was evident by the Trypan Blue exclusion test. These results suggest that HU affects cell yield in these cultures mainly through its cytostatic effect by blocking cell proliferation.

HU at concentrations of 100 \( \mu \text{mol/L} \) and higher significantly increased both the total amount of Hb synthesized per cell (mean corpuscular hemoglobin [MCH]), which increased from 20 pg to 33 pg/cell, and the proportion of HbF, which increased from 1.2% in untreated cells up to 4.8% in cells treated on day 4 of phase II with 400 \( \mu \text{mol/L} \) HU. The increase in the percent HbF could not by itself account for the increase in MCH. Thus, treatment of the cells with HU was also associated with increased levels of adult Hb. Similar results were obtained in more than six independent experiments using cells obtained from different donors. With 100 \( \mu \text{mol/L} \) HU, the MCH increased by 38% to 65% (mean 45%) and the proportion of HbF increased 1.3- to 3.5-fold (mean 2.7) (Fig 2). The results were similar (±10%) whether the percent HbF was measured by the alkali-denaturation-TMB assay or by cation exchange HPLC.

Analyzing the kinetics of accumulation of Hb-containing cells in the cultures by staining with the benzidine reagent indicated that 4, 7, 10, and 13 days after exposure to EPO, 37%, 75%, 94%, and 96% of the cells, respectively, were benzidine reactive, similar to those obtained in a previous study. Treatment with HU, at any concentration used in this study, did not significantly change the kinetics (data not shown).
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Because HU may be labile under the culture conditions used, we analyzed the effect of multiple additions of HU on the percent HbF. Adding HU only on day 7 of phase II resulted in a 2.4-fold increase in the percent HbF when the cells were harvested and analyzed on day 13. If HU was added repeatedly on days 7 and 9, or on days 7, 9, and 11, the percent HbF increased 3- and 3.4-fold, respectively. Thus, multiple additions result in a greater increment in HbF, suggesting that HU stability may influence results of such experiments.

Exposure to HU was associated with a significant increase in cell size. This effect was evident as early as the first day after its addition to 4-day phase II cultures, and was most prominent on the second day. Erythroid cell maturation in vivo is associated with a decrease in cell size. This was recapitulated in this system in vitro, both in untreated and HU-treated cultures. Nevertheless, when analyzed on day 13, when most of the cells reached the orthochromatic normoblast stage, HU-treated cells were still larger than control cells. This is shown in Fig 3, where normal erythroid cells were exposed to 100 \( \mu \text{mol/L} \) HU on day 7 of phase II and analyzed on day 13 for size by measuring forward light scattering (FS) by flow cytometry. While the untreated population showed a unimodal distribution with a mean at channel 341 (on a linear scale of 1,024 channels), HU-treated cells showed a bimodal distribution caused by the presence of two distinctive subpopulations. The subpopulation with the larger cells comprised about 70% of the total population, and had a mean FS at channel 549, as compared with a mean channel of 305 of the small cell subpopulation. Only 10% of the untreated cells were within the boundaries of the large cell subpopulation of the HU-treated cells. The forward light-scattering property of cells is mainly a function of cell size; therefore, the results indicate a 1.6-fold difference in size between the large cell population in the treated as compared with the bulk of cells in the untreated cultures, but also show the existence of two populations with 1.8-fold difference in size, in the treated culture. These differences were easily discerned by observing unstained cells using an inverted microscope or Giemsa-stained smears using a regular light microscope.

In addition to normal cells, the effect of HU was studied in cultures derived from patients with either sickle cell anemia or \( \beta \)-thalassemia. The HPLC chromatograph of one sickle cell anemia patient, homozygous for the SS genotype, is demonstrated in Fig 4. In this patient, the HbF increased from 5.2% in the untreated to 11.3% in the HU-treated cultures. Analysis of four additional patients indicated a 2.2- to 5.1-fold increase in the proportion of HbF, with a mean of 3.2-fold increase (Fig 2).

![Fig 3. HU effect on size of cultured erythroid cells. HU (100 \( \mu \text{mol/L} \)) was added to day 7 phase II cultures of normal erythroid cells. Cells were harvested on day 13 and analyzed for size by measuring forward light scattering using the EPICS flow cytometer (Coulter Electronics, Hialeah, FL) as described previously.](image)

![HPLC analysis of Hb in cultures from a patient with sickle cell anemia. Untreated cultures or cultures treated by adding 100 \( \mu \text{mol/L} \) HU on day 7 of phase II were harvested on day 13, and lysates were prepared and analyzed on cation-exchange HPLC. The chromatographs show the peaks of the major Hb species and their retention times. The percentage of Hb F in each culture is also indicated.](image)
The results in cultures of cells derived from patients with β-thalassemia are summarized in Table 1. As noted with normal and sickle cells, cells from the β-thalassemia patients, when incubated in HU, also showed a decreased cell number due to inhibition of cell proliferation, but increased MCH, as well as elevation in the proportion of Hb F (varying from 1.3- to 6.2-fold) (Fig 2).

**DISCUSSION**

In the present study, we report that several HU-mediated effects on erythroid cells, previously demonstrated in vivo after treatment of patients with sickle cell anemia,13-16 could be recapitulated in cultures of cells derived from patients with hemoglobinopathies as well as from normal donors. HU was found to cause: (1) an increase in the proportion of HbF; (2) an increase in cell size (mean cell volume [MCV]); (3) an increase in the cellular Hb content (MCH), and (4) an inhibition of cell proliferation.

All of these effects were dose and time dependent; no change was found in cell size and hemoglobin phenotype at concentrations that did not affect cell proliferation (25 μmol/L and lower). Maximal effect was noted at concentrations of 400 μmol/L, when proliferation was completely blocked, although significant changes were also observed at 100 to 200 μmol/L, when cell proliferation was only partially affected. The latter concentrations did not affect cell viability, as determined by staining with Trypan Blue.

The failure of previous studies to show HU-stimulation of HbF production in primary cultures21-22 may be related to the fact that by using the standard cell cloning method in semisolid medium, cells with abnormally high levels of HbF develop. Such cells may have already used their full potential for HbF production and therefore do not respond to additional stimulation, eg, treatment with HU. In contrast, the Hb pattern produced in the two-phase liquid culture system used in the present study (Fig 1C, Fig 2, Fig 4, and Table 1) was similar to that found in vivo, either in normal individuals or in patients with elevated HbF levels. In addition, the two-phase liquid culture system yielded pure and large populations of erythroid cells that allowed direct study of the cells with respect to maturation and Hb accumulation under conditions where cell proliferation was inhibited. This is technically impossible in the colony method that depends on indirect analysis.

Our results suggest that HU affects Hb phenotype by direct interaction with relatively late erythroid precursors that are already engaged in Hb production. The results also suggest that the effect required neither the mediation of accessory cells (eg, macrophages, lymphocytes, or stromal cells), nor specific growth factors, eg, granulocyte-macrophage colony-stimulating factor (GM-CSF), that has been reported to enhance HbF production in the progeny of early erythroid progenitors.23

The mechanisms involved in the HU-mediated changes are still unclear. They may involve a selection of a minor, pre-existing, subpopulation of F cells that has a growth and/or survival advantage. This mechanism may be particularly effective for cells derived from patients with β-hemoglobinopathies, where F cells may be resistant to “ineffective erythropoiesis,” ie, premature death of the erythroid precursors due to their abnormal Hb content.24 An alternative mechanism could involve stimulation of HbF in all or the majority of the population by direct induction, eg, by removing DNA-binding, negative trans-acting proteins from the γ-promoter region, or indirectly, eg, by modifying the cell cycle or growth kinetics. We believe that the two-phase liquid culture system will be a valuable tool for studying these mechanisms on the cellular, biochemical, and molecular levels. Understanding of the mechanism will provide the rationale for a logical search for agents with HbF-stimulating activity and limit the trial and error approach.

The effects of HU were reproduced in cultures of cells derived from normal individuals who had low levels of HbF, as well as from patients with hemoglobinopathies who had elevated levels of HbF. Heterogeneity was found in the response among the normal individuals and patients studied. For example, one thalassemic patient responded with almost no increase in HbF, whereas three other patients showed a 2.7- to 6.2-fold increase. Increased HbF production in vivo was found in about 75% of the HU-treated sickle cell anemia patients.25-27 No molecular, biochemical, cellular, or clinical parameter was found to predict the outcome of the treatment. In an ongoing study, we are comparing the response to HU of cultured cells derived from patients with sickle cell anemia.

**Table 1. Effects of HU on Erythroid Cells Cultured From β-Thalassemia Patients**

<table>
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<tr>
<th>Patient</th>
<th>Benzidine* Cells&lt;sup&gt;†&lt;/sup&gt; (x10&lt;sup&gt;6&lt;/sup&gt;/mL)</th>
<th>HbF&lt;sup&gt;†&lt;/sup&gt; (μg/mL)</th>
<th>MCH&lt;sup&gt;‡&lt;/sup&gt; (pg/cell)</th>
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<tr>
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<td>0.7</td>
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</table>

Cells derived from four patients with β-thalassemia major were cultured in the two-phase liquid culture system. HU (100 mmol/L) was added to day 7 of phase II and cells were harvested on day 13. The patients were from the Israeli Kurdish population whose molecular characterization has been previously reported.28-30

* Benzidine-positive cells.
† Hb content per milliliter of culture.
‡ Mean Hb content per benzidine positive cell.
§ Fold increase of HbF in HU-treated as compared with untreated cultures.

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to the patient’s response to treatment to see if it is possible by testing cultures derived from the patient’s peripheral blood to predict response in vivo.

ACKNOWLEDGMENT

We thank A. Treves for culturing cells from thalassemic patients and Prof E.A. Rachmilewitz for his interest in these studies.

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