Inhibitors of Cell Division Reversibly Modify Hemoglobin Concentration in Human Erythroleukemia K562 Cells

By Francois Erard, Ann Dean, and Alan N. Schechter

The human leukemia K562 cell line can be induced by 20 μM hemin to reversibly accumulate embryonic and fetal hemoglobins without any change in the rate of cell division. When we reduced the rate of cell division by glutamine starvation or addition of hydroxyurea, the cells increased by tenfold the basal hemoglobin level of 0.3–0.5 pg Hb/cell. The combined effects of hemin and inhibitors of cell division permitted K562 cells to attain levels of hemoglobin (26–34 pg Hb/cell) close to that found in normal red cells. This superinduction was reversible and cells could be recycled indefinitely. Furthermore, electrophoresis experiments show that the three primary hemoglobin species produced by these cells (Hb Gower 1, Hb Portland, and fetal Hb), were induced, or reinduced, synchronously by inhibitors of cell division but asynchronously by hemin. Differing effects of hemin and inhibitors of cell division were observed in the absence of irreversible differentiation and suggest different molecular mechanisms controlling globin gene expression.

The differentiation of murine erythroleukemia cells in culture has been extensively studied, but the role various inducers play in this process is still poorly understood. The human erythroleukemic K562 cell line was originally isolated from a patient with chronic myelogenous leukemia. Recent evidence suggests that these cells possess erythroid features and will accumulate embryonic and fetal hemoglobins when treated with hemin, actinomycin D, and hydroxyurea. We have recently noted that induction by low levels of hemin is reversible, does not affect the rate of cell division, and that K562 cells can be indefinitely subcultured in the presence of hemin.

In the present report we demonstrate that inhibition of the rate of cell division, by glutamine-deficient medium or hydroxyurea, will increase the amount of hemoglobin in hemin-induced K562 cells up to levels found in human red cells. These findings demonstrate that hemoglobin levels in K562 cells can be regulated by in vitro manipulations under conditions where irreversible differentiation does not occur, thus facilitating investigation of the mechanism(s) controlling intracellular hemoglobin levels.

MATERIALS AND METHODS

K562 cells, a kind gift of T.R. Rutherford, were cultured at 37°C in suspension in RPMI 1640 medium containing 10 mM HEPES and 10% fetal calf serum (Flow Laboratories, Detroit, Mich.), in the presence or absence of 0.3 mg/ml of glutamine. Cells were counted in a hemocytometer after dilution in 0.05% trypsin blue (Allied Chemical Co.) in 0.15 M NaCl. All cultures were diluted to 5 x 10^4 cells/ml when they reached a density of 0.8–1 x 10^6 cells/ml. Hemin (Gibco, Grand Island, N.Y.) was used in cultures at 20 μM, and was prepared as described. Hydroxyurea (Sigma, St. Louis, Mo.) was used in cultures at 130 μM and was dissolved in culture medium and filter-sterilized before use.

The hemoglobin concentration within cells was determined from absorption bands at 540, 576, and 414 nm. No free hemin could be detected in lysates under these conditions. Separation of hemoglobins by nonequilibrium isoelectric focusing was performed as recently described. The gels were stained for 2 min in a solution of 0.2% benzidine di-HCl (Sigma) dissolved in 0.5 M acetic acid, with 5130% H2O2/ml. The linearity of the benzidine stain with protein concentrations was determined using purified hemoglobin A and hemoglobins in K562 cell lysates. Isoelectric focusing gels were photographed on 4 x 5 inch Ektapan 4162 black and white film. The negatives were scanned with a Perkin-Elmer 1010G microdensitometer, and computer analysis was performed using a specially designed computer program (Nikodem B, Trus BL, Ral JE: manuscript in preparation).

RESULTS

In standard medium, K562 cells exhibit a doubling time of about 24 hr, which is unaffected by the presence of 20 μM hemin. In glutamine-deficient medium, a gradual inhibition of cell division is observed in the presence or absence of 20 μM hemin (Fig. 1A). When glutamine is replaced, the cells resume growth at the rate of untreated controls indicating that the alteration of growth rate is reversible.

In Fig. 1B we show the intracellular hemoglobin levels obtained by spectroscopic methods under these conditions. In standard medium, K562 cells routinely contained 0.3–0.5 pg of hemoglobin per cell. In glutamine-deficient medium, K562 cells reached intracellular hemoglobin levels of 3–5 pg in 5 days. We show for comparison the accumulation of hemoglobin in K562 cells upon treatment with 20 μM hemin. Our present results demonstrate that when the hemin treatment is performed in glutamine-deficient me-
INHIBITION OF K562 CELL DIVISION

Fig. 1. Effects of glutamine deprivation on K562 cells. (A) Growth rate of K562 cells cultured in glutamine-deficient medium with (°) and without (A) 20μM hemin and in standard medium with (O) and without (△) 20μM hemin. On the right side is shown growth of cells transferred to standard medium after 5 days of growth in glutamine-deficient medium. (B) Accumulation of hemoglobin in K562 cells under the conditions described above. Note that when one open symbol interrupts a series of closed symbols, it means that the cells have been cultured 24 hr in standard medium.

dium, K562 cells accumulated in 5 days an average of between 26 and 34 pg of hemoglobin per cell.

When such “superinduced” cells are subcultured in standard medium, in the absence of hemin, the hemoglobin concentration drops in a few days to the level observed for control cells (Fig. 1B). If these cells are reinduced, the hemoglobin accumulation follows the same kinetics as observed for the first induction. Thus, the unlimited proliferative capacity of K562 cells is not affected by the large accumulation of hemoglobin resulting from deprivation of glutamine and addition of hemin, suggesting that no irreversible differentiation has occurred. The cells can also be kept indefinitely at these very high hemoglobin levels if the culture medium is supplemented with glutamine for 24 hr every 5 days.

The same state can be reached when cells already completely induced by treatment with 20μM hemin for more than 10 days are transferred to glutamine-deficient medium containing hemin (data not shown). Inversely, “superinduced” cells, when subcultured in standard medium in the presence of 20μM hemin, drop to hemoglobin levels observed for cells induced with hemin in normal medium (data not shown). These observations suggest that the effect of glutamine deprivation, with respect to the steady-state levels of hemoglobin accumulation, is independent of that of hemin.

Another inhibitor of cell division, hydroxyurea, has an effect similar to that of glutamine deprivation: gradual inhibition of cell division produced by 130μM hydroxyurea leads to a marked increase in the accumulation of hemoglobin. The cells accumulate in 4 days, 22 pg of hemoglobin/cell in the presence of 20μM hemin and 7 pg in the absence of hemin. This effect is reversible and the kinetics of induction and of cell growth are analogous to those obtained by glutamine deprivation (data not shown).

Using isoelectrofocusing techniques, we have compared the major hemoglobins produced by K562 cells and their sequence of appearance. In untreated cells embryonic hemoglobins Gower 1 (α2ε2) and Portland (ε2γ2), and fetal hemoglobin (α2γ2) were the only detectable hemoglobins (Fig. 2). Their relative proportions were 16% Hb Gower 1, 69% Hb Portland, and 15% HbF, as determined by microdensitometry (Table 1). Growth of cells in glutamine deficient medium resulted in a tenfold increase in the levels of intracellular hemoglobin over the basal level, but the relative proportions of the three hemoglobins remained the same.

In contrast, when the cells were grown in standard medium in the presence of hemin, the induction of accumulation of the three hemoglobins was asynchronous: Hb Gower 1 increasing last (Fig. 2). Table 1 shows that when this accumulation is maximum, the proportion of Hb Gower 1 as compared to fetal hemoglobin is larger than in uninduced cells. If hemin induction is performed in glutamine-deficient me-

Fig. 2. Isoelectrofocusing pattern of hemoglobin. Lysate obtained from untreated K562 cells (A). K562 cells cultured 5 days in glutamine-deficient medium (B), and K562 cells treated with hemin 1 day (C) and 9 days (D). Approximately 2μg of hemoglobin per sample were applied to the gels.
Table 1. Relative Proportions of Hemoglobins Produced by K562 Cells

<table>
<thead>
<tr>
<th>Culture Day</th>
<th>pg Hb/Cell</th>
<th>Percentage of Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard medium</td>
<td>0.4</td>
<td>16%</td>
</tr>
<tr>
<td>Glutamine-deficient medium</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Standard medium containing 20 μM hemin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Glutamine-deficient medium containing 20 μM hemin</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>20 μM hemin</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

dium, the same asynchrony and final relative proportions of the three hemoglobin species are observed but over a shorter time period. We have also found these results when cells are reinduced a second time.

DISCUSSION

Our results show that decreasing the growth rate of K562 cells results in hemoglobin accumulation. Two inhibitors of cell division, glutamine-deficient medium and hydroxyurea, markedly increase intracellular hemoglobin in a completely reversible manner. Furthermore, when each was used simultaneously with hemin, the cells reached the hemoglobin level observed in red cells without permanent loss of proliferative capacity or loss of the ability to be repeatedly superinduced. The isoelectric focusing experiments show that hemin induces, in an asynchronous manner, the accumulation of the three most prominent hemoglobin species produced by these cells, Hb Portland, Hb Gower I, and fetal Hb. Further, the final ratios of these hemoglobins are different after hemin induction as compared to the basal state. When glutamine deprivation alone is used for induction, hemoglobin accumulation is synchronous and the ratios of the hemoglobins do not change.

We believe for these reasons, and also because of the multiplicative nature of the response to hemin and inhibitors of cell division, that inhibitors of cell division act differently from hemin in K562 cells. The two inhibitors of cell division block cells in the G1 phase, a phenomenon that has been associated with the process of differentiation and accumulation of globin mRNA in murine erythroleukemia cells. Accumulation of globin mRNA, but not differentiation, appears to be significant in the mechanism of hemin induction in K562 cells.

If an agent affects the cell cycle, it is possible that diminution of the dilutional effects that occur at mitosis contributes to the modification of the intracellular protein concentration. Our preliminary calculations suggest that the effect of glutamine-deficient medium or hydroxyurea on the intracellular hemoglobin levels of K562 cells may be explained by such a mechanism, but other factors may be operative as well. This effect should be considered when assessing studies using murine erythroleukemia cells or K562 cells where putative inducers of hemoglobin accumulation affect cell growth rate.

The combined effects of hemin and inhibitors of cell division permit K562 cells to attain levels of intracellular hemoglobin close to that found in circulating red cells. The ability to reversibly control hemoglobin levels in K562 cells should permit studies aimed at understanding the mechanism of control of hemoglobin levels in erythroid cells. These mechanisms may be relevant to understanding diseases such as thalassemia, where control of globin synthesis is abnormal or sickle cell disease where mean corpuscular hemoglobin concentration is an important pathophysiologic variable.

ACKNOWLEDGMENT

We thank Dr. Benes L. Trus for performing the microdensitometry studies and Drs. W. French Anderson, Samuel K. Ackerman, and Philip D. Noguchi for critical reading of the manuscript. We thank also Laura Smith for preparation of the manuscript.

REFERENCES

Inhibitors of cell division reversibly modify hemoglobin concentration in human erythroleukemia K562 cells

F Erard, A Dean and AN Schechter