Differential Expression of the Globin Genes in Human Leukemia K562(S) Cells Induced to Differentiate by Hemin or Butyric Acid

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ABSTRACT

Human leukemia K562(S) cells were induced to differentiate by 50 μM hemin or 1.4 mM butyric acid, and the types of hemoglobins synthesized were compared. In both cases, embryonal hemoglobins [Portland, Gower 1, Hb X, and fetal hemoglobin (Hb F)] were detected. Butyric acid-treated K562(S) cells contained mostly Hb Gower 1 (γεγε) and a hemoglobin with the electrophoretic characteristics of Portland (γεγε). For hemin-treated K562(S), the most abundant hemoglobin synthesized was Hb X (εεγγ), and the second most abundant was Bart’s (γγ). Traces of Gower 1 were observed in nontreated K562(S) cells.

The kinetics of hemoglobin induction as a result of the two treatments differed; increased hemoglobin synthesis was detected after only 24 hr of hemin treatment, whereas 4 days were required in butyric acid-treated cells. Both hemin and butyric acid were able to induce their respective patterns of hemoglobin synthesis independent of the presence of serum in the K562(S) growth medium. Analysis of the globin chains in induced K562(S) cells induced to differentiate indicated that, with both inducers, adult α- but not β-globin chains were present. Karyotype analysis of K562(S) cells revealed a nearly triploid chromosome complement with a modal number of 68 chromosomes. Three copies of chromosome 11 and four copies of chromosome 16 (coding for the β-like and α-like globin genes, respectively) were present. A large marker chromosome, involving chromosome 7, and a Philadelphia chromosome were also seen. These data characterize the K562(S) subline and also indicate that hemin and butyric acid differ in their effects on the expression of embryonal globin genes.

INTRODUCTION

A cell line (K562) was established by Lozzio and Lozzio (21) from a patient with chronic myeloid leukemia in the acute phase. These cells were thought to represent stem cell precursors of the myeloid lineage. Andersson et al. (5), using specific antibodies and the benzidine reaction, have since shown that these cells could be induced to synthesize hemoglobin by butyric acid treatment. They also showed that glycophorin, a protein present in cells of the erythroid lineage, could be detected on the surface membranes of uninduced K562 cells (6). Rutherford et al. (30, 31) have reported that hemin treatment of a particular cell subline [K562(S)] with hemin or butyric acid. Karyotype analysis of K562(S) cells indicated the presence of several copies of chromosomes 11 and 16 which code for the globin genes.

MATERIALS AND METHODS

Cell Line and Inducibility. Three sublines of human leukemia K562 cells obtained from different laboratories were examined for inducibility. Only the K562(S) responded with increased hemoglobin synthesis to both hemin and butyric acid induction, the other cultures being poorly inducible by butyric acid. Dimethyl sulfoxide, hypoxanthine, and hexamethylene bisacetamide did not induce increased hemoglobin synthesis in K562(S) cells. The K562(S) cells were not recloned for the experiments reported here.

Cell Culture. The K562(S) cells were grown in either Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal bovine serum (Flow Laboratories) in 5% CO2 humidified atmosphere or in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing insulin (5 μg/ml), transferrin (5 μg/ml), and selenium dioxide (3 x 10^-8 M) (8).

Hemoglobin Determination. After growth in the presence of inducing agents, cells were chilled on ice, pelleted by low-speed centrifugation, and washed twice with PBS.4 The washed cell pellets were then resuspended in an equal volume of distilled water. Cells were lysed by 3 cycles of freeze-thawing and centrifuged once at 2,000 rpm for 10 min and again at 15,000 rpm for 45 min.

The visible absorbance spectrum of the supernatant and the

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absorbance at 414 nm were determined. Readings were taken at 403 and 425 nm to detect any nonspecific absorbance due to light scattering. Absorbance at 414, 540, and 576 nm, shifting to 417, 537, and 568 nm, respectively, after carbon monoxide monoxide treatment, positively identified the material as hemoglobin (30). Hemoglobin was then quantitated at 414 nm, assuming an absorbance of 1.0 at 414 nm is equivalent to 0.094 mg/ml (17).

The total cell lysate was subjected to electrophoresis in order to separate hemoglobins. Total cellular proteins from 106 cells were obtained by resuspending the cell pellet in an equal volume of distilled water, lysing the cells by 3 cycles of freeze-thawing, and centrifuging them at 2,000 rpm for 10 min followed by 15,000 rpm for 45 min. The supernatant was electrophoresed on Cellogel (Chemetron) in borate buffer (pH 8.6) (34) at 5 ma for 2 hr. Hemoglobin bands were visualized by benzidine staining using o-dianisidine (24).

**Determination of Globin Chains.** Hemin- or butyric acid-induced cultures were maintained in the log phase of growth and labeled for 16 hr with 14C-amino acid mixture (0.5 µCi/ml) at the height of hemoglobin synthesis as determined by benzidine staining and absorbance at 414, 540, and 576 nm of cell lysates.

Cells were harvested, washed twice in ice-cold PBS, and lysed in an equal volume of hemoglobin lysis buffer (50 mM Tris, pH 7.0:25 mM KCl:5 mM MgCl2:1 mM mercaptoethanol:0.3% Triton X-100) (17). A total of 0.2 to 0.5 × 106 cpm of total cell lysate contained 2 absorbance units/ml at 280 nm. Carrier hemoglobin (cord blood lysate) in a final sample volume of 75 µl containing 4% ampholines (pH 6.0 to 8.0) was added, and the samples were subjected to isoelectric focusing on 11-× 0.3-cm disc gels at 500 V for 4 to 5 hr at 4° (25).

The main hemoglobin bands of both adult and cord blood were cut from the isoelectric focusing gels and placed in hemoglobin lysis buffer for 16 hr at 4°. The eluted proteins were separated on 2% Triton X-100:urea gels (27, 28). The material (4 to 7 × 105 cpm) present in each adult or cord hemoglobin band was resuspended in a final volume of 50 µl of denaturation buffer (4 µM urea:5% acetic acid:5% 2-mercaptoethanol:0.02% pyronin Y) (27).

Gels were electrophoresed for a period of 8 hr at 120 V (27, 28); stained with a mixture of Coomassie blue, acetic acid, and methanol (0.1% w/v, 10% v/v, 20% v/v, respectively); and fluorographed (9) for 2 to 3 weeks at -70°.

**RNA Extraction.** Cells were centrifuged at low speed and washed twice in ice-cold PBS. The washed cell pellet was resuspended in 1 ml of hemoglobin lysis buffer. After 15 min on ice, the pellet was centrifuged at 15,000 rpm for 10 min. SDS was added to the supernatant to a final concentration of 0.5% and then extracted at room temperature with an equal volume of phenol:chloroform (1:1, v/v). Material in the aqueous phase was separated by centrifugation at 10,000 rpm for 10 min. Extraction was repeated until no protein was visible in the interphase. The RNA was then precipitated by the addition of sodium chloride to a final concentration of 3% followed by 3 volumes of ethanol. RNA was refrigerated overnight at -20°, pelleted from ethanol by centrifugation at 10,000 rpm for 15 min, and washed in ice-cold 70% ethanol. Finally, RNA was dissolved in distilled water and stored at -20°.

**Isolation of Poly(A)+ RNA.** Total cytoplasmic RNA, prepared as described above, was resuspended in an equal volume of 2 × “binding buffer” (40 mM Tris-HCl, pH 7.4:0.3 mM NaCl:2 mM EDTA:0.1% SDS) and subjected to oligodeoxythymidylic acid-cellulose chromatography (oligodeoxythymidylic acid-cellulose type 7, P-L Biochemicals) (7). Poly(A)+-bound RNA was eluted with a buffer of 10 mM Tris-HCl, pH 7.4:1 mM EDTA:0.1% SDS and precipitated in ethanol at -20° as described above.

**Determination of Globin mRNA.** The plasmid containing human γ-globin cDNA sequences, pHyG1 (19), was grown in _Escherichia coli_ HB101 under P2/EK1 conditions. The plasmid was purified by the hydroxyapatite method (11). After digestion with Pst I, the plasmids were denatured by 0.2 n NaOH, loaded onto nitrocellulose filters (Millipore HAWP, 0.45 µm, 25-mm diameter) at approximately 50 µg/filter, and then heated at 80° for 2 hr (16).

To determine the percentage of [3H]uridine incorporation into globin RNA, a filter containing 5 µg globin DNA was hybridized with [3H]uridine-labeled poly(A)+ RNA in 50% formamide:0.75 M NaCl:50 mM Tris-HCl, pH 7.5:1 mM EDTA:carrier yeast RNA (100 µg/ml) at 45° for 16 hr. The filters were washed 3 times with 0.5 M NaCl:10 mM Tris-HCl, pH 7.5, followed by treatment with 5 µg RNase A at 37° for 30 min. After further washing in the same buffer, filters were dried and counted in Aquasolve 2.

**Chromosome Analysis.** Log-phase cells were treated with Colcemid (0.02 µg/ml) for 15 min prior to harvesting. The cells were then centrifuged and resuspended in 0.075 M KCl hypotonic solution for 30 min. The cells were fixed in Carnoy’s solution (methanol:acetic acid, 3:1), and air-dried slides were prepared. Giemsa banding was carried out by the method of Seabright (33). A test for the translocation of mouse genetic material to the K562(S) chromosomes was performed using the G-11 banding procedure of Friend et al. (15) under conditions in which human-mouse somatic cell hybrids consistently gave differential staining. The chromosomes from 52 metaphase spreads were counted. G-11 staining of these cells, which have been previously passaged through nude mice (20) for mouse chromatin translocated to the human chromosomes, revealed that no mouse sequences were present.

**RESULTS**

**Hemoglobin Patterns of K562(S).** K562(S) cells were treated with 50 µM hemin or with 1.4 mM butyric acid for 4 or 5 days. The cell lysates and appropriate amounts of marker cord blood were electrophoretically separated on Cellogel gels. The separation of different benzidine-positive bands is shown in Fig. 1. In both cases, the 5 bands, Gower 1, X, F, Portland, and Bart’s, as described by Rutherford _et al._ (30, 31), could be detected. The 2 major bands that resulted from hemin treatment were Hb X and Hb Bart’s. After butyric acid treatment, the most abundant band appeared at a position corresponding to Gower 1; the second most abundant band appeared at the position characteristic of either Portland or the adult Hb A; however, the lack of detectable β chains in induced K562(S) cells ruled out Hb A. A faint band comigrating with the Hb F marker was seen both in hemin-treated and butyric acid-treated cells. In nontreated cells, only a band with the migration characteristics of the Hb Gower 1 could be detected. Essentially, the electrophoretic pattern of hemin- and butyric acid-treated cells corresponded to the electrophoretic pattern reported by Rutherford _et al._ (30, 31). The relative amount of the different hemoglobin bands (evaluated by intensity of benzidine
staining) appeared to be different, however, when hemin induction of K562(S) cells was compared to hemin induction of K562 cells used by Rutherford et al. (30).

Globin Chains of K562(S) Cells. If the total radioactive cytoplasmic lysate was separated on Triton-Urea gels, many polypeptides were present which interfered with the identification of the globin chains (Fig. 2). Nontreated cells and both hemin- and butyric acid-treated K562 cell lysates contained a prominent band (corresponding to the partially unresolved ε and ζ chains) (4, 8). The band was present in untreated cells and appeared broad and more prominent in hemin-treated cells (Fig. 2A) than in butyric acid-treated cells (Fig. 2B).

In order to detect γ-, α-, and β-globin chains, cells were labeled with the ¹⁵C-amino acid mixture, and the hemoglobins were separated by isoelectric focusing using cord blood hemoglobin (containing Hb A and Hb F) as a carrier. The adult and fetal hemoglobin bands were cut from the gel, and the globin chains were separated by electrophoresis on Triton-Urea gels (27, 28). Radioactive bands comigrating with nonlabeled carrier β chains could not be detected after butyric acid treatment (Fig. 3). The relative migration of the Gγ and Aγ globin chains was identified on Triton-Urea gels as described by Alter (1).

Time Sequence of Induction. The kinetics of induction of hemoglobin by hemin or butyric acid were different. After treatment of K562(S) cells with 0.5 × 10⁻⁵ M hemin, hemoglobin could be detected after only 24 hr and reached a peak between Days 3 and 4 (Chart 1). The same type of globin chains were seen throughout (data not shown). By contrast, hemoglobin could be detected only after 4 days and reached a peak after approximately 5 days in butyric acid-treated cells (Chart 1).

A base line of 3 to 10% of cells was benzidine-positive in nontreated K562(S) cells. The percentage of benzidine-positive cells after butyric acid treatment reached a peak of 60% on Day 5 under optimal conditions; whereas, after hemin treatment, it was approximately 80% after 3 days. It is possible that benzidine positivity after hemin treatment could be due to the presence of free hemin in the cells. However, presence of hemoglobin in hemin-treated cells was confirmed by scanning at 414 nm and following the shift after carbon monoxide treatment (Chart 2) as described in "Materials and Methods."

Presence of β-like Globin mRNA in Induced K562. Several of the hemoglobins synthesized by K562 cells contained the β-like chains γ and ε. It was therefore of interest to determine the levels of β-like mRNA's before and after treatment. β-like mRNA's were quantitated by RNA-DNA hybridization using plasmid containing γ-cDNA. β-like mRNA's were found to be present in nontreated K562 cells (0.12% of cytoplasmic mRNA). After hemin treatment, β-like mRNA's increased to at least 0.3% of the total mRNA. After butyric acid treatment, a less marked increase was observed.

Growth and Inducibility of K562(S) in Synthetic Medium. K562(S) cells grew well in synthetic medium without serum. The composition of the medium was similar to that described by Breitman et al. (10) for the growth of HL60 human promyelocytic leukemia cells. The cells adapted to growth in synthetic medium without a period of adjustment, but they grew with a longer doubling time and reached a lower saturation density
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Table 1

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Treatment</th>
<th>% of benzidine-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic medium</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Hemin</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Butyric acid</td>
<td>31</td>
</tr>
<tr>
<td>Complete medium</td>
<td>None</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Hemin</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Butyric acid</td>
<td>50</td>
</tr>
</tbody>
</table>

We have characterized the hemoglobin synthesis of a particular subline of K562 cells that can be induced to differentiate by hemin and butyric acid treatment. We confirm the previous reports (4, 8, 30) that hemin treatment of K562 cells induces synthesis of embryonal and fetal hemoglobin, and we report that the induction of K562(S) cells with different inducers of hemoglobin synthesis causes differential synthesis of embryonal hemoglobins. Nontreated K562(S) growing in medium with serum contained a fraction of spontaneously differentiating cells synthesizing Hb Gower 1. Butyric acid treatment of K562(S) favored the synthesis of α- and ε-globin chains (components of Hb Gower 1 and Hb Portland), whereas hemin treatment caused the preferential synthesis of the γ-globin chains. Hb X, the predominant hemoglobin in hemin-treated K562(S) cells, has in fact recently been characterized using K562(S) cells by Rutherford et al. (29) as a γcε2 hemoglobin. After hemin treatment, an excess of the β-like chains over the α-like chains was observed. This finding has some analogies to the observation reported by Rovera et al. (26) in Friend erythroleukemia cells; when Friend cells were induced by hemin, an excess of β minor chain over α was observed.

The kinetics of hemoglobin synthesis after induction with hemin was different and more rapid than after induction with butyric acid. Benzidine-positive cells after hemin treatment could be the result of hemin uptake rather than increased hemoglobin. However, we believe that, in the case of K562(S) cells treated with hemin, the increased percentage of benzidine-positive cells reflects an increase in hemoglobin synthesis because: (a) K562(S) cells treated with hemin show a bright red pellet 24 hr after treatment, while hemin alone would give a brown color; (b) as shown in Chart 2, the visible absorbance spectrum of the supernatant of K562(S) cells is shifted after carbon monoxide treatment with characteristics indicating the presence of hemoglobin; (c) there is a correlation between the increased percentage of benzidine-positive cells after hemin treatment and the amount of hemoglobin measured by the visible absorbance spectrum; and (d) separation of hemoglobin by cellulose-acetate gel electrophoresis indicates that the intensity of the various hemoglobin bands increases after 1 day of treatment with hemin. The delay in appearance of hemoglobin synthesis after butyric acid treatment may suggest that, like Friend erythroleukemia cells, a hemin-synthesizing pathway must be activated (31, 32), and this may require several days. However, when hemin is supplied exogenously, there is a rapid synthesis of hemoglobin suggesting that the rate-limiting step for hemoglobin synthesis is at the level of translation.

Evidence from hybridization studies indicates that β-like mRNA’s are already abundant in nontreated K562(S) cells. At the present time, we have been unable to quantitate the relative amount of β-, γ-, and ε-mRNA because, under the conditions of filter hybridization used, there is some cross-hybridization between the γ-cDNA probe and the ε- and β-mRNA. This cross-hybridization was shown to exist by hybridizing to the filter-bound γ-cDNA probe a radioactive β-complementary RNA made by transcribing the β-cDNA plasmid with E. coli RNA polymerase. When a β-cDNA probe was used for hybridization of the K562 poly(A)-containing RNA, hybridized mRNA was detected. Since no β-globin chains were detected, this confirmed that the conditions of hybridization used were unable to discriminate between β- and γ-mRNA’s. Translation of the hybridized mRNA’s may in the future partially answer these questions but will not rule out the existence of nontranslatable mRNA in noninduced K562 cells. The studies using synthetic medium indicate that both butyric acid and hemin induce synthesis of hemoglobins by direct interaction with the target cells and without the need of serum factors. However, the fact that shifting K562(S) cells from synthetic medium to medium with serum causes an increased level of spontaneous differentiation suggests that serum factors could also be responsible for erythroid differentiation and that K562(S) cells growing in synthetic medium could be used as sensitive indicator cells in the identification of such factors.

Karyotype analysis of K562(S) cells was done to confirm their identity as human leukemic cells, inasmuch as Lozzio et al. (22) have suggested that some of the K562 cell lines available in different laboratories could actually be mouse cells or human cells altered by in vivo passage in immunodeficient mice. We were unable to detect the presence of mouse chromosomes or chromosomal fragments by G-11 staining.

The karyotype of the K562(S) subline shows further rearrangement than the lines studied by both Lozzio et al. (20) and Klein et al. (18). None of the markers reported by Klein et al., including a ring Philadelphia chromosome, is present in K562(S) cells. A marker involving chromosome 7, a Philadel-
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phia chromosome, and more than 3 No. 16 chromosomes is seen by Lozzio et al. (20), but their 15/17 translocation is not present in our line. Their rearranged chromosome 7 is also different from the one observed in K562(S) cells. Our analysis leads us to believe that K562(S) cells are related to the K562 cells of Lozzio et al. (20) and that they have further evolved.

Different lines of K562 apparently behave differently, probably because of variability in karyotype. We did not observe, for example, the atypical morphological patterns of erythroid differentiation reported by Andersson et al. (5) after butyric acid treatment. On the other hand, the K562 cells used by Rutherford et al. (30) were inducible by hemin but not by butyric acid.

The possibility remains that K562 cells can also differentiate along other than erythroid lineages because myeloid markers were found to be present on these cells (14), and their treatment with phorbol diesters induced partial differentiation to myelomonocytic cells.5

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Separation of hemoglobins synthesized by K562(S) cells by electrophoresis on Cellogel using Tris-borate buffer (pH 8.6). Markers for Hb F and Hb A were obtained by parallel electrophoresis of cord blood. The identification of the other hemoglobins was based on their relative migration at alkaline pH as described previously (30). a, hemin treated for 4 days; b, butyric acid treated for 5 days; c, nontreated.

Fig. 2. Separation of globin chains by electrophoresis on Triton:urea gels. Total cytoplasmic lysates of K562(S) cells labeled with 14C-amino acid mixture (1 μCi/ml) were lysed and separated by electrophoresis. Positions of α and β chains were determined by coelectrophoresis of nonlabeled human adult blood. The putative α-γ complex was tentatively identified on the basis of its relative migration rate in the 2% Triton-Urea gel system (4, 8). A, hemin-treated cells for 0, 1, 2, 3, and 4 days; B, butyric acid-induced for 0, 1, 2, 3, and 4 days.

Fig. 3. Separation of globin chains by electrophoresis on Triton gels. Total cytoplasmic lysates of K562(S) cells labeled with 14C-amino acid mixture (1 μCi/ml) were lysed and mixed with unlabeled cord blood. The cord blood was separated by isoelectric focusing on disc acrylamide gels. The proteins with the same isoelectric point of adult and fetal hemoglobin bands were excised and eluted, and the proteins were run on Triton:urea gels. The positions of α, β, Ay, and Gy bands were identified by Coomassie blue. The radioactive peptides were identified by fluorography. 1, polypeptide pattern of proteins with the same isoelectric point as adult hemoglobin; 2, polypeptide pattern of proteins with the same isoelectric point as that of fetal hemoglobin.

Fig. 4. G-banded karyotype of K562(S) cells. Note the large marker chromosome (M1) derived by a translocation involving chromosome 7. Other marker chromosomes (M2 to M7) are present. Philadelphia chromosome (arrow) is also present.
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