Induction of Erythroid Differentiation in K562 Cells by Inhibitors of Inosine Monophosphate Dehydrogenase

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ABSTRACT

The effects of three inhibitors of inosine monophosphate (IMP) dehydrogenase on a human erythroleukemic cell line, K562, were studied. Following incubation with these inhibitors, K562 cells underwent differentiation and accumulated hemoglobin. The induction of hemoglobin accumulation was dose dependent; maximum induction was observed at 100, 25, and 3 μM, respectively, for ribavirin, tiazofurin, and mycophenolic acid. The induction was associated with reduction of intracellular GTP content and was blocked by adding guanosine within 24 h after adding inducer. The effective dose for half-maximum induction by ribavirin was 3 times less than that for 50% inhibition of K562 proliferation; however, for tiazofurin and mycophenolic acid, it closely approximated the concentrations which suppressed cellular proliferation. Ribavirin was sequestered preferentially inside the K562 cells, and the induction by ribavirin had a greater than 30-fold increase in hemoglobin. Studies with isoelectric focusing, globin chain analyses, and immunochromatographic assays indicated that adult-type β globin was found, while no γ globin chains were demonstrated. Thus, accumulation of fetal hemoglobin and production of α globin chain in ribavirin-treated cells are different from the pattern of hemoglobins induced by hemanin.

INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), and mycophenolic acid (1–3) were shown to be inhibitors for IMP dehydrogenase and growth of tumor cells. Their primary effect on purine metabolism appeared to be a marked depletion of guanine nucleotide pools (3–6). In vivo studies have indicated that ribavirin and other inhibitors of IMP dehydrogenase might induce hematological changes. Administration of ribavirin in high doses frequently results in a reversible anemia, and the effect is dose and time dependent (7). Tiazofurin was also found to cause myelosuppression in Phase I clinical trials (8). In addition, it was found that several inhibitors of IMP dehydrogenase were potent inducers of myeloid maturation in the HL-60 cell line (9–11). These inhibitors were also reported to promote the terminal maturation and inhibit the self-renewal of normal myeloid progenitors (12, 13). Several previous studies explored the effects of ribavirin on mature erythrocytes (7); so far, there have been few data on its effect on immature erythroid precursor cells in vitro or in vivo. In this study we report the effects of ribavirin, tiazofurin, and mycophenolic acid on the induction of erythroid differentiation of a human erythroleukemic cell line, K562 (14). The types of hemoglobin produced by incubation with ribavirin were also analyzed. Preliminary reports of this investigation have been presented (15, 16).

MATERIALS AND METHODS

Cell Culture. Stock cultures of K562 (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 15% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT), as previously described (17). Cells were caused to differentiate by addition of inducers to final concentrations as specified in the text. Ribavirin (Viratek, Inc., Covina, CA), tiazofurin (gift from Dr. Roland Robins), and mycophenolic acid (Sigma, St. Louis, MO) were diluted to 10 mg/ml in RPMI 1640 medium and stored at 4°C. Experimental cultures were grown for 3 to 4 days to densities less than 2 x 10^6 cells/ml. Cell numbers were counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion. The number of cells containing hemoglobin was assayed by benzidine staining (18). The clonogenic assay was performed using 0.3% agar in RPMI 1640 medium containing 15% fetal calf serum (19, 20). The amount of hemoglobin accumulation was assayed using a benzidine colorimetric method (21).

GTP and ATP Assay. For the determination of intracellular GTP and ATP in K562 cells, samples were prepared as we described previously (22, 23). Briefly, K562 cells were incubated with ribavirin or tiazofurin. After a specified time of incubation, approximately 2 to 5 x 10^6 cells were removed, counted with a hemocytometer, and centrifuged for 5 min at 1000 rpm. The resulting cell pellets were immediately extracted with 100 μl of 1 M ice-cold HClO4 at 4°C. After vortexing, samples were spun at 4°C at 10,000 rpm, and the supernatant was collected. The supernatant was immediately neutralized with 4 M KOH, and the pH was carefully titrated to 7.4 with 1 M KOH. The final volume was recorded and samples were quickly frozen and stored at -20°C until final assay for ATP and GTP concentrations by high-pressure liquid chromatography as previously described (23).

Isoelectric Focusing. Approximately 1 x 10^6 cells of ribavirin-induced K562 were washed twice in phosphate-buffered saline (pH 7.4). Then one volume of packed cells was mixed with one volume of deionized water and one-half volume of CCl4. After mixing for 10 min and centrifugation for another 10 min at 4°C, KCN was added to the supernatant collected to a final concentration of 100 μg/ml, and the sample was stored at -20°C. Isoelectric focusing of the samples was performed using 1% agarose containing ampholites at pH 6 to 8 in LKB Multiphor II apparatus at a constant 15 watts and 4°C for 20 to 30 min. The agarose gel was then fixed in 10% trichloroacetic acid and stained with benzidine for the presence of hemoglobin.

Globin Chain Analysis. After isoelectric focusing of cell lysates, individual gel slices were excised from gel without prior fixation and then stored in 20 μl of β-mercaptoethanol at 4°C. These samples were denatured with 100 μl of buffer containing 6.7 M urea, 8.3% acetic acid, 8.3% β-mercaptoethanol, and 0.3 mg/ml of pyronin Y and boiled for 2 min prior to subsequent electrophoresis in 12% polyacrylamide gel containing 6 M urea, 2% Triton, and 5% acetic acid (24). Electrophoresis was carried out in 5% acetic acid for 17 h at a constant current of 10 mA. Gels were stained using Coomassie blue. In some cases, the globin chain composition of the induced K562 cells was further analyzed by a combination of Western-blotting and immunochromatographic assay as follows. The urea-Triton-acid polyacrylamide gel prepared as described was electroblotted to nitrocellulose paper (25) and probed sequentially with a 1:2000 dilution of rabbit anti-human hemoglobin F or hemoglobin A serum and biotin-conjugated anti-rabbit antibodies.
(Vector Laboratories, Burlingame, CA). The respective globin chains on the nitrocellulose paper were then analyzed with an avidin-biotin-glucose oxidase assay (26).

**Quantitation of Ribavirin.** Concentrations of ribavirin in cell extracts and culture media were quantitated with a competitive binding radioimmunoassay as described previously (27). The antibody is specific for both ribavirin and its phosphorylated nucleotides and can detect concentrations as low as 0.01 \( \mu M \). The cell number was counted with a hemocytometer. The intracellular concentrations were calculated on the basis that 1 ml of packed K562 cells contains \( 1.85 \times 10^6 \) cells, which translates to a volume of \( 5.4 \times 10^{-9} \) ml/cell.

**RESULTS**

**Effects of Ribavirin and Other IMP Dehydrogenase Inhibitors on K562 Cells.** Exposure of K562 cells to a wide range of concentrations of ribavirin resulted in a concentration-related decrease in cellular proliferation (Fig. 1A). The concentration of ribavirin which caused 50% inhibition of cell proliferation after 3-day incubation was approximately 10 \( \mu g/ml \) or 40 \( \mu M \) (Fig. 1B). The viability of these ribavirin-treated cells remained greater than 95% after 3 days of incubation (Fig. 1B). In order to ascertain whether growth restriction by high concentrations of ribavirin reflects a cytotoxic effect on proliferation of K562 cells and/or a commitment to differentiation, clonogenic assay (19, 20) was performed after various periods of preincubation with ribavirin (Table 1). The colonies derived from K562 cells reached an average size of 9 to 16 cells per colony, after 3 days of cultures. With prior exposure to ribavirin for 1 day, the size of colonies was significantly smaller (Table 1), although the total number of colonies per plate remained similar to control cultures. However, when K562 cells were preincubated with ribavirin for more than 2 days before plating, the total number of colonies decreased significantly, and most of the colonies consisted of less than 4 cells/colony. These results indicated that exposure to ribavirin led to growth restriction of K562 cells.

As also shown in Fig. 1B was the response of K562 cells after incubation with ribavirin in liquid culture for 3 days, when they became benzidine positive in a dose-dependent manner. Maximum induction was observed with ribavirin at 25 \( \mu g/ml \) (\( \approx 100 \mu M \)). The effective dose for half-maximum induction by ribavirin was approximately 4 \( \mu g/ml \) (\( \approx 16 \mu M \)).

The effects of other inhibitors of IMP dehydrogenases on K562 cells were also investigated. The addition of tiazofurin and mycophenolic acid for 3 days induced K562 cells to become benzidine positive in a dose-dependent manner (Fig. 2). Maximum induction was observed at 25 \( \mu M \) for tiazofurin and 3 \( \mu M \) for mycophenolic acid. The effective doses for half-maximum induction by tiazofurin and mycophenolic were approximately 2 and 0.3 \( \mu M \), respectively. Both tiazofurin and mycophenolic acid restricted the growth of K562 cells in a dose-dependent manner; the concentrations for 50% inhibition of K562 proliferation were 2 \( \mu M \) for tiazofurin and 0.3 \( \mu M \) for mycophenolic acid (Fig. 2).

**Sequestration of Ribavirin Inside K562 Cells.** To determine the pharmacokinetics of ribavirin during the incubation of K562 cells, the intracellular concentrations of ribavirin were determined. After incubation of cells with 20 \( \mu M \) ribavirin, the intracellular concentration of ribavirin was approximately 1.5 \( \mu M \); this amounts to a 75-fold increase in concentration inside the cells over the ribavirin concentration in the medium (Fig. 3). The elevated intracellular concentration of ribavirin remained relatively constant for up to 96-h incubation. When K562 cells were incubated with 100 \( \mu M \) ribavirin for 24 h, the intracellular concentration increased to 11.5 \( \mu M \), which reflects a 115-fold increase in intracellular concentration. After longer incubation with ribavirin, the intracellular concentration gradually declined to approximately 50-fold of the extracellular concentration by 96 h. These experiments indicate that ribavirin was sequestered preferentially inside the K562 cells to at least 50-fold.

**Effect on Intracellular Concentrations of Purine Nucleotides.** When K562 cells were incubated with ribavirin or tiazofurin, consistent alterations in purine metabolism were observed; the GTP level decreased within 24 h of incubation, and this decrease persisted throughout the 4-day culture periods (Table 2). In the treated cells, the decrease of intracellular GTP content became more apparent when the intracellular GTP concentrations were normalized against the measured concentrations of intracellular ATP (Table 2).

To further evaluate the role of GTP depletion in the induction of hemoglobin accumulation in K562 cells, the effect of guanosine, which should prevent the depletion of GTP, was studied. In the presence of 25 \( \mu M \) guanosine, the number of benzidine-positive cells in the culture after incubation with ribavirin or tiazofurin decreased remarkably as compared with the cultures of each inducer and in the absence of guanosine (Table 3). A
K562 cells were preincubated in the RPMI 1640 medium in the presence or absence of 100 μM ribavirin for a period of 5 h, 1 day, or 2 days. After preincubation, cells were washed extensively and plated, in triplicates, at 500 cells per ml of 0.3% agar in the culture medium without the addition of ribavirin. After 3 days of incubation, the number of colonies per plate and the cell number per colony were counted.

Table 1 Clonogenic analysis of K562 cells after preincubation with ribavirin

<table>
<thead>
<tr>
<th>Treatments</th>
<th>3–4</th>
<th>5–8</th>
<th>9–16</th>
<th>&gt;17</th>
<th>Total no. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>27.0 ± 3.0 (11.4)*</td>
<td>77.0 ± 5.0 (32.6)</td>
<td>131.0 ± 3.0 (55.4)</td>
<td>1.3 ± 2.3 (0.6)</td>
<td>236.3 (100)</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>31.7 ± 4.7 (14.6)</td>
<td>103.6 ± 7.5 (47.7)</td>
<td>82.0 ± 12.0 (37.7)</td>
<td>0</td>
<td>217.3 (100)</td>
</tr>
<tr>
<td>One day of preincubation</td>
<td>28.0 ± 13.3 (11.2)</td>
<td>58.3 ± 14.2 (23.4)</td>
<td>154.5 ± 19.7 (61.9)</td>
<td>8.8 ± 6.1 (3.5)</td>
<td>249.6 (100)</td>
</tr>
<tr>
<td>Control medium</td>
<td>88.3 ± 5.8 (36.3)</td>
<td>130.3 ± 34.9 (53.6)</td>
<td>24.7 ± 3.0 (10.1)</td>
<td>0</td>
<td>243.3 (100)</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>26.7 ± 2.5 (11.5)</td>
<td>64.0 ± 4.6 (27.5)</td>
<td>127.0 ± 5.7 (54.7)</td>
<td>14.6 ± 8.3 (6.3)</td>
<td>232.3 (100)</td>
</tr>
<tr>
<td>Two days of preincubation</td>
<td>42.0 ± 3.6 (61.2)</td>
<td>26.0 ± 4.0 (37.8)</td>
<td>0.7 ± 1.2 (1.0)</td>
<td>0</td>
<td>68.7 (100)</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Numbers in parentheses, percentage of distribution of various sizes of colonies within each sample.

Fig. 2. Effect of tiazofurin and mycophenolic acid on proliferation and induction of K562 cells. K562 cells were incubated, in triplicates, with the specified concentrations of tiazofurin (A) and mycophenolic acid (B) for 3 days. Aliquots were then analyzed for percentage of cells staining for hemoglobin with benzidine (●) and for cell density (○). The variation of the data was within 10% among triplicate samples. Cell viability was more than 95% of the control.

Fig. 3. Accumulation of ribavirin in K562 cells upon incubation with ribavirin. K562 cells were incubated at 1 to 2 × 10⁶ cells/ml with ribavirin at concentrations of 20 μM (△) and 100 μM (●). At the specific time interval of incubation, aliquots of approximately 2 to 5 × 10⁶ cells were taken, and the intracellular concentration of ribavirin was determined as described in “Materials and Methods.” The intracellular “ribavirin” includes phosphorylated ribavirins, because the antibody used detects both ribavirin and its phosphorylated nucleotides (27). The results were expressed as nmol/g of cell extract (assuming that 1 ml of packed cells yields 1 g of cell extract). The ribavirin concentrations in the culture medium to which 100 μM (●) were initially added are also indicated in units of μM.

characterization of hemoglobin accumulation. In ribavirin or hemin-induced K562 cells, the amount of hemoglobin accumulation and the extent of induction were found to be comparable with a greater than 30-fold increase in hemoglobin contents (Table 4). The lysates of these cells were analyzed by isoelectric focusing in order to identify the types of hemoglobins produced. The individual hemoglobins produced in the ribavirin-induced K562 cells were designated as hemoglobins 1 to 5 in Fig. 4A. Approximately 60% of hemoglobins, which accumulated in ribavirin-treated cells, was found to be hemoglobin F (HbF) (Band 3 in Fig. 4A) and its acetylated form (Band 2) by comparison with hemoglobin standards (Fig. 4B). The hemoglobin Band 2 in Fig. 4A was more acidic than hemoglobin A (as demonstrated in the experiment of mixing samples with standards) and had the same pI as the acetylated hemoglobin F.
One volume of packed cells was then mixed with one volume of deionized water and 0.5 volume of CCl₄ and centrifuged at 4°C. and the amount of saline, pH 7.4, and aliquots were taken for cell counts and benzidine staining (HbF) in the cord blood (picture not shown). As also indicated in Fig. 4A, the remaining hemoglobins consisted of 21% hemoglobin Portland (Band 1), 8% Gower-1, and 11% Gower-2 (Bands 4 and 5). On the other hand, similar experiments using hemin-induced K562 cells (Fig. 4C) showed that only 14% of accumulated hemoglobins was the hemoglobin F, and the remaining 86% was hemoglobin Portland, Barts, and other unidentified hemoglobins (24, 28–30).

Analysis of Globin Chain Composition. The phenotypes of the hemoglobins that accumulated in the ribavirin-induced K562 cells were confirmed by globin chain analysis. The individual hemoglobin bands in the isoelectric focusing gel (Fig. 5A) were excised. After elution and denaturation, the globin chains were analyzed in urea-Triton-acid polyacrylamide gel (24). As shown in Fig. 5B, the composition of globin chains for hemoglobin Band 1 in Fig. 5A is γ globin chains (i.e., Gγ and Aγ) and α chain, confirming the presence of hemoglobin Portland (ζ; γ). Hemoglobin Band 3, which is the major hemoglobin species accumulated in the lysates, is composed of the γ globin chains along with α globin, confirming the accumulation of hemoglobin F (α₂γ₂). Band 2 showed characteristic patterns of acetylated γ chains (Fig. 5B, sample 6), confirming that this hemoglobin is hemoglobin F₁. Hemoglobin Band 4 is mainly composed of two embryonic globin chains, ζ and ξ, while Band 5 consists of α and ξ chains. They are thus shown to be the hemoglobins Gower-1 and 2, respectively. Therefore, the major species of globin chains which accumulated in cells after ribavirin induction were the γ globin chains and the α chains. In no instances was hemoglobin A (α₂ζ₂) detected in globin chain analysis. These results were also confirmed by immunoblotting of the gels in Fig. 5B with appropriate antibodies. Antibodies directed against hemoglobin F (α₂γ₂) detected the presence of
with benzidine. These results indicate that K562 cells may not be assayed, not all the cells within the colonies are clearly stained. Differentiation prior to plating. While most of the colonies in these differentiating cells exhibited restricted potential for proliferation. The clonogenic assays also showed that cell proliferation closely approximated the concentrations that suppressed cell proliferation (i.e., terminal differentiation). The longer the exposure to the inducer prior to plating, the lower was the cloning efficiency in the clonogenic assay. In the studies of HL-60 cells (37), it was proposed that the observed differentiation in HL-60 might result from an adaptive cellular response to a toxic stress imposed by inducers. However, data on ribavirin showed that the concentration for half-maximum induction by ribavirin was about 3 times less than that for 50% growth inhibition. This would suggest that inhibition of cellular proliferation may not be a necessary prerequisite of cellular differentiation.

It has been shown that ribavirin 5'-monophosphate is a potent inhibitor of IMP dehydrogenase (4), which is involved in the de novo synthesis of GMP. In agreement with previous reports (5), present studies showed that K562 cells exposed to ribavirin displayed significant decline in the level of GTP. Mycophenolic acid and tiazofurin also promote similar alterations in intracellular levels of GTP (3, 6). The fact that all these inhibitors of IMP dehydrogenase induced cellular differentiation suggests a possible role of GTP depletion in K562 differentiation. It is of interest to note that the induced maturation of myeloid leukemia cell line HL-60 is also accompanied by an intracellular depletion of guanosine ribonucleotides (10, 11), which reflects a down-regulation of guanylate synthase from IMP at the rate-limiting step mediated by IMP dehydrogenase (9, 38, 39). The role of GTP depletion in ribavirin-induced differentiation in K562 is further strengthened by the findings that guanosine, which could replenish the effect of GTP depletion, was able to block the differentiation of K562 induced by ribavirin or tiazofurin but not by an unrelated inducer, hemin. Similar results were also reported in the blockage of induced maturation of HL-60 cells by guanosine (39). On the other hand, the accumulation of hemoglobins in K562 cells was preceded by a decrease in GTP concentration which occurred within 24 h of ribavirin treatment. Whether or not these findings suggest any association between depletion of GTP and "commitment" of K562 cells into differentiation (20) remains to be investigated.

Present studies indicate that ribavirin was sequestered preferentially inside the K562 cells. The results confirm previous reports that there is a selective accumulation of ribavirin in mature human erythrocytes in vitro (40). They also agree with the in vivo observations that approximately 10 to 15% of the total administered ribavirin was incorporated into mature red cells, while red cells accounted for only 3% of the total body mass and that the ratio between plasma trough levels of ribavirin and red cell ribavirin was approximately 1 to 90 (40). Conceivably, ribavirin is entrapped within the mature erythrocytes because it is phosphorylated upon entry into these cells. Ribavirin was shown to be rapidly phosphorylated in mature red cells and mainly exists as the 5'-triphosphate (41).

Present studies using the same subclone of K562 cells showed that hemoglobin F constitutes most of the accumulated hemoglobin in ribavirin-treated cells, in contrast to the production of mainly embryonic hemoglobins in hemin-treated cells (28-31). In ribavirin-treated cells, Gγ represents about 75% of the γ globin chains, which is similar to the value found in normal fetuses and newborns (42). The induction of hemoglobin accumulation with ribavirin was also marked by the accumulation of α globin chains and the lack of β globin production. The α/γ ratio was estimated to be 0.77, and no hemoglobin Bart's was...

DISCUSSION

Many studies have suggested that alterations in the regulation of IMP dehydrogenase may modulate the capacity of cells to undergo proliferation (32, 33). Malignant transformation was found to associate with an increase in the activity of IMP dehydrogenase (34, 35) and various drugs which inhibit this enzyme displayed significant antitumor activities (32, 33). Studies of fetal and regenerating hepatic tissues had demonstrated a direct relation between levels of this enzyme and proliferation (32, 33). Malignant transformation was specific to hemoglobin A (α2β2) and hemoglobin F, respectively, demonstrated the production of α chains (in Samples 2, 3, and 5) in the ribavirin-treated K562. In addition, the presence of hemoglobin F in the ribavirin- or tiazofurin-treated K562 cells was independently confirmed by immunofluorescence studies of the cytospin preparations of the induced cells using monoclonal antibody specific for γ chains (31) (picture not shown).
identified in these cells. In hemin-treated K562 cells, the α/γ ratio was 0.11 in our studies and was reported to be in the range of 0.08 and 0.30 by others (24, 28). Therefore, the pattern of hemoglobin accumulation in ribavirin-treated cells was different from the αthalassemic imbalance observed in hemin-treated K562. These findings raise the possibility of using ribavirin to increase fetal hemoglobin production in patients with sickle cell disease and β-thalassemia syndromes, if similar results could be demonstrated in vivo.

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REFERENCES


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