Pattern of Pyruvate Kinase Isozymes in Erythroleukemia Cell Lines and in Normal Human Erythroblasts

By I. Max-Audit, U. Testa, D. Kechemir, M. Titeux, W. Vainchenker, and R. Rosa

To further investigate the erythroid nature of the two human erythroleukemia cell lines, K562 and HEL-60, and to define the ontogeny of pyruvate kinase (PK) isozymes (R, M2), in developing human erythroid cells, we have studied the isozymic alterations, if any, during differentiation of these cell lines in vitro and normoblasts isolated from fetal liver in vivo. PK activity of erythroleukemic cell lines was intermediate between that observed in leukocytes and in fetal liver erythroblasts. These cell lines contained a high level of M2-PK, but R-PK was always present, albeit at low concentrations, in all the clones or subclones we studied. Erythroblasts from fetal liver were separated according to density on a Strata-pan gradient. R-PK levels were nearly constant in the different fractions, whereas M2-PK levels markedly decreased as the erythroblasts became mature and almost completely disappeared in late erythroid cells. Thus, these results clearly demonstrate the erythroid origin of these cell lines.

Materials and Methods

All substrates and commercial enzymes were purchased from Boehringer-Mannheim Biochemicals, France. Buffer salts were obtained from Merck, Chemical Division, Darmstadt, West Germany. Cell gel strips were purchased from Medical Products, Chemetrom Corp, Milan, Italy. Agarose was from Litex, Denmark; and bovine serum albumin, hemin, sodium butyrate, and tetradeconyl phorbol-13-acetate (TPA) from Sigma Chemical Co, St Louis. Cell culture medium and fetal calf serum were obtained from Boehringer (Boehringer, Mannheim, Germany).

Experiments

Normal leukocytes were prepared according to Kahn and Dreyfus. RBCs were cleared of leukocytes according to Beutler et al. Cell lysates were performed in 75 mmol/L Tris, 4 mmol/L EDTA, citric acid, pH 8, buffer containing 0.5 mmol/L fructose-1-6-diphosphate (FDP). The lysates were stirred, centrifuged at 25,000 g for 30 minutes, and the supernatants used.

Cell Culture

K562 and HEL cells were grown in suspension in RPMI 1640 with 10% fetal calf serum at 37 °C in a fully humidified atmosphere with 5% CO2. The cells were incubated at a concentration of 107/mL. They grew exponentially during the next four days; after that time, they reached a plateau concentration (cell density inhibition) ranging from 8 x 107 to 1 x 108/mL. Serial culture passages and pyruvate kinase determinations usually were performed during the exponential phase of growth.

Three chemical "inducers" were added at the following concentrations: hemin 0.1 mmol/L; sodium butyrate 1 mmol/L; TPA 160 nmol/L. The growth rate remained unchanged with hemin, was reduced by half with sodium butyrate, and was completely inhibited by TPA. The viability of the cells was not modified by hemin and sodium butyrate; in TPA-induced cells, the viability was 90% to 95%. Two nonerythroid leukemic cell lines were also investigated, HL-60 promyelocyte cell line10 and U-937 histiocytes cell line. They were grown under similar conditions.
PATTERN OF PYRUVATE KINASE ISOZYMES

Density Separation of Fetal Liver Erythroblasts

Fetal liver cells were obtained from 8- to 11-week-old normal human embryos. Human embryos were obtained by aspiration from pregnant women undergoing voluntary abortion; consent was obtained for the scientific use of the human material.

Fetal liver cells were dissociated within two hours of delivery by gentle aspiration through a needle (gauge 18G) and washed four times in Hanks' salt solution. The cells were then separated by ultracentrifugation on a discontinuous gradient of Stratagel II, purified and prepared as described by Corash et al.8 with slight modifications.8 Eight different fractions from 1.025 to 1.09 were purified and prepared as described by Corash et al., with bovine serum albumin as the working standard.12 Partially purified M2- and R-PK were prepared as previously reported.13

Immunologic Studies

Anti-R-PK antiserum was obtained by immunization of rabbits with intramuscular injection of purified R-PK. Specificities of anti-R-PK antiserum were tested by double immunodiffusion according to the method of Ouchterlony.16 A line containing a known amount of antigen is applied just in front of the holes containing the samples to be tested. On an 11 x 9 cm glass plate, was poured a first agarose gel of 11 x 2 x 0.2 cm (contact gel, 1% in migration buffer, see below). A second, 1% agarose gel, 0.15 cm thick, containing the anti-R-PK antiserum was poured on the remainder of the plate. In the contact gel, a 0.2-cm wide and 10-cm long strip was cut and 0.4 mL of migration buffer, containing 0.5% agarose and 25 μL of PK from a hemolysate, was poured. Just behind this line were punched application holes in which 10 μL of samples to be tested were applied. Electrophoresis was carried out at 4°C and 250 V for four hours in TRIS-citrate acid 35 mmol/L, EDTA 2 mmol/L, pH 8.

After electrophoresis, gels were washed overnight in phosphate-buffered saline, and immunoprecipitates were demonstrated using their PK activity. PK activity was revealed as described by Kahn et al.17

RESULTS

Erythroleukemic Cell Lines

Total PK Activity

Samples from the original cell line, K562 (provided by Dr Lozzio, University of Tennessee, Knoxville), and four clones obtained as previously described20 were tested. Total PK activity was similar in these different K562 cells and corresponded to a level intermediate between that observed in leukocytes and erythroblasts from fetal liver (Table 1). Induction of K562 cells by hemin (100 μmol/L), sodium butyrate (1 mmol/L), and TPA (160 nmol/L) did not modify the total level of PK (data not shown).

Electrophoretic Study

The results of the electrophoretic studies showed the presence of only M2-PK in most samples. In some

<p>| Table 1. Comparison of Total PK Activity of K562 Cells and Cells of Erythroid and Nonerythroid Origin |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Number of samples tested</th>
<th>K562</th>
<th>Leukocytes</th>
<th>HL-60</th>
<th>U-937</th>
<th>Mature Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PK (IU/mg Proteins)</td>
<td>21</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(Mean ± SD)</td>
<td>0.79 ± 0.24</td>
<td>1.98</td>
<td>1.85</td>
<td>2.42</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*U/mg hemoglobin.
Fig 2. Cellulose acetate strip electrophoresis of K562 cells. Buffer was 75 mmol/L, pH 5.5, Tris-maleic-acid-NaOH. Electrophoresis was carried out for three hours at 180 V. PK activity was detected under UV light by coupling with lactate dehydrogenase (LDH) activity and NADH oxidation. (1) RBC lysate; (2) uninduced subclone 63 lysate; (3) hemin-induced subclone 63 lysate; (4) leukocyte lysate; (5) uninduced Lozzio clone lysate; (6) hemin-induced Lozzio clone lysate.

In cases, a band exhibiting an intermediate migration between R-PK and M2-PK was detected (Fig 2). This band may represent a hybrid isozyme composed of R-PK and M2-PK subunits. We have previously shown the possibility of formation of such a hybrid.14

Rocket-Line Immunoelectrophoresis

K562 cells contain high levels of PK activity, and M2-PK isozyme is largely predominant, as seen on the electrophoresis pattern (Fig 2). To detect R-PK in these cells, we had to use a method capable of discriminating between R and M2 isozyme and sufficiently sensitive to detect small quantities of R-PK. Rocket immunoelectrophoresis possesses these two qualities. Using this method, no peak is observed with crude leukocyte extracts or partially purified M2-PK (Fig 3); furthermore, less than $0.5 \times 10^{-3}$ IU of R-PK is detectable. Linearity of the method was tested with RBC lysate samples (Fig 3A) and with a purified R-PK preparation (Fig 3B). Linearity was obtained until $2.5 \times 10^{-3}$ IU or $4 \times 10^{-2}$ µg of R-PK.

Because total PK of K562 cells was not determined as an amount of protein but as PK activity, we chose to standardize rocket-line immunoelectrophoresis with PK from RBC lysate (generally three different dilutions) and to express the results in activity, in spite of the fact that immunologic reactivity is related to protein content rather than to activity. This allowed us to compare R-PK levels with total PK.

R-PK was always found in K562 cells; its level was similar in induced and noninduced cells (Table 2). For each clone, the amount of R-PK in noninduced and induced cells was very similar, but was variable from one clone to another (Fig 4). In all nonerythroid cells (HL-60, U-937, normal leukocytes), R-PK was never found.

Cells from the erythroleukemia cell line, HEL, were also studied. Total PK activity was higher (1.71 IU/mg protein) than in K562 cells, and R-PK was present in amounts comparable to those detected in K562 cells (0.1% total PK). No or very little induction of R-PK was obtained with hemin or NO butyrate incubation of HEL cells.

PK Isozyme Pattern During Erythroid Maturation

In order to investigate the pattern of PK isozymes during erythroid maturation, we initially used erythroblasts from cultured BFU-E, but the material thus obtained was only sufficient to allow measurement of total PK activity and cellulose acetate strip electrophoresis. The results obtained with this material were similar to those described below.

In order to overcome this problem of quantity, we decided to use erythroblasts from fetal liver (8 to 11 weeks old), which is highly erythropoietic at this stage of development.

Cells were separated by density centrifugation on a discontinuous gradient of Stractan II. On each of the erythroid cell fractions, we investigated the following parameters: morphological aspect of the cells, total PK activity, R-PK and M2-PK activity, and isozyme pattern as determined by electrophoresis on cellulose acetate strips.

R-PK and M2-PK activity was determined by immunoinactivation of R-PK. This method was used rather than rocket-line immunoelectrophoresis (used in the study of K562 cells) because immunoinactivation is more precise and more convenient for erythroblasts that contain relatively high levels of R-PK. Controls were performed with RBC lysate and leukocyte lysate, having total PK activities similar to those observed in erythroblast lysates. Activity remaining after incubation with anti-R-PK antiserum was,
respectively, 2% in RBC lysate and 99% in leukocyte lysate.

Fractionation of fetal liver cells according to density gives rise to five cell fractions corresponding to progressive stages of maturation from proerythroblasts-basophilic erythroblasts to acidophilic erythroblasts (Fig 5). Fractions 1 and 2 contained predominantly basophilic erythroblasts, fraction 3 polychromatophilic erythroblasts, fractions 4 and 5 acidophilic erythroblasts. Contamination with nonerythroid cells was always less than 5%.

In these fractions, the proportion of R-PK with respect to total PK activity increased with maturation, while the proportion of M2-PK decreased (Fig 5).

Total PK activity was much higher in the early than in the late erythroblasts (Table 3). “Evolution” of R-PK and M2-PK during erythroid maturation was markedly different: M2-PK activity markedly decreased as the cells matured and was almost undetectable in acidophilic erythroblasts; R-PK activity was only slightly modified during the process of erythroid maturation, and in acidophilic erythroblasts, was the only enzyme detectable (Table 3).

These results were confirmed by electrophoretic analysis of the PK isozymes present in the different erythroblast fractions (Fig 6). A band exhibiting an intermediate migration between R-PK and M2-PK was also detected at all stages and probably is a hybrid isozyme.

**DISCUSSION**

Several erythroid features have already been described for the K562 cell line. **R-PK** is a marker characteristic of red cells, and its presence in K562 cells has previously been investigated by two authors. Horton was unable to detect any R-PK

<table>
<thead>
<tr>
<th>Number of tested samples</th>
<th>Uninduced K562 Cells</th>
<th>Hemin-Induced K562 Cells</th>
<th>Na Butyrate-Induced K562 Cells</th>
<th>TPA-Induced K562 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-PK IU x 10^3/mg protein</td>
<td>3.5 ± 2.6</td>
<td>4.1 ± 3.5</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Percent total PK (mean ± SD)</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.4</td>
<td>0.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Fig 4.** Rocket line immunoelectrophoresis of K562 cells. Immunoprecipitate was revealed by its PK activity by coupling ATP production to hexokinase and glucose-6-phosphate dehydrogenase reactions and formazon formation. (1 through 4) RBC lysates containing, respectively, 0.3, 0.6, 1.2, 2.4 IU x 10^3 PK; (5) uninduced Lozio clone lysate: 73 IU x 10^3 PK; (6) hemin-induced Lozio clone lysate: 90 IU x 10^3 PK; (7) uninduced subclone 63 lysate: 155 IU x 10^3 PK; (8) butyrate-induced subclone 63 lysate: 150 IU x 10^3 PK.

**Fig 5.** Cell types (A) and pyruvate kinase isozymes (B) in human fetal liver cells separated according to density. Human fetal liver cells have been separated by centrifugation on a discontinuous gradient of Stractan II. Five fractions were obtained, and the cellular types and pyruvate kinase isozymes contained in each of these fractions were determined. (A) (●) Proerythroblasts; (○) basophilic erythroblasts; (△) polychromatophilic erythroblasts; (○) acidophilic erythroblasts; (□) RBCs; (●) in fractions 1 and 2, 5% and 2%, respectively of nonerythroid cells were present. (B) (□) M2-PK; (■) R-PK.
Table 3. Total PK, R-PK, and M2-PK Activities in Fractions of Increasing Maturity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total PK Activity (IU/mg Protein)</th>
<th>R-PK Activity (IU/mg Protein)</th>
<th>M2-PK Activity (IU/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12</td>
<td>0.041</td>
<td>0.079</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.043</td>
<td>0.037</td>
</tr>
<tr>
<td>3</td>
<td>0.04</td>
<td>0.029</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>0.03</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>0.026</td>
<td>0.024</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*R-PK and M2-PK activities were determined by immunoinactivation of R-PK.
†ND, not determined.

activity in either uninduced or in sodium butyrate and hemin-induced K562 cells. This result may be tentatively attributed either to a low sensitivity of the methodology used or to a variation among different cell clones of K562 cells, as described by Dimery et al. On the other hand, Jansen et al found that M2-PK is largely predominant in K562 cells and that only a small amount of R-PK is present as hybrids with M2-PK. These authors did not find any modification of total PK after hemoglobin synthesis induction.

Our results are very similar to those of Jansen et al, but the method we used allows us to measure R-PK present in the cells and to compare R-PK content of uninduced and induced cells. It is interesting to note that R-PK was always present, albeit at low concentrations, in all the K562 subclones tested, as well as in HEL cells, which also possess erythroid features. However, R-PK was never detected in nonerythroid cell lines, such as HL-60 and U-937, or in normal leukocytes. This result suggests that R-PK is specific to erythroid cells and that its synthesis occurs early in erythroid differentiation.

In contrast to hemoglobin synthesis, R-PK was not increased by hemin or sodium butyrate induction. This result may suggest that, in erythroleukemia cells, erythroid-specific proteins are not simultaneously expressed when the cells are induced to differentiate. Similar differential responses of erythroid markers to induction have been previously described. Alternatively, the two chemical inducers used in this study were unable to induce a complete differentiation of both erythroleukemia cell lines.

TPA has been shown to induce an inhibition of differentiation in K562 cells, as indicated by change in surface glycoprotein profile or hemoglobin synthesis. In contrast, no effect of TPA was seen on R-PK.

Takegawa et al, using immunofluorescence techniques, have recently shown the presence of both R-PK and M2-PK in erythroblasts obtained from adult bone marrow, the decline of M2-PK, and the increase of R-PK with cell maturation. In this study, we have used fetal liver because, until 18 weeks, fetal liver contains mostly erythroid cells. Identical results were also obtained when purified populations of mature or immature erythroblasts from adult blood BFU-E colonies were studied. Indeed, these results do not appear to be a fetal-specific form of PK in humans.

The results obtained with erythroblasts show the early onset of R-PK synthesis during maturation of erythroblasts. Complete onset of R-PK synthesis is probably prior to the basophilic erythroblast stage. This result is also supported by the presence of R-PK in K562 cells, which can be considered similar to very immature erythroblasts (proerythroblasts).

The erythroblast electrophoretic pattern shows a band of intermediate mobility in all fractions. Probably, this band represents a R-M2-PK hybrid, which indicates that some erythroblasts may contain both types of PK concomitantly. The maturation of erythroblasts is characterized by a progressive decrease in M2-PK until its complete absence in mature red blood cells, whereas the activity of R-PK remains almost constant during all stages of erythroid maturation. Our results are partially in accordance with those of Takegawa et al because we found, like them, a marked decrease of M2-PK levels, but we did not observe an increase of R-PK levels during erythroblast maturation. This discrepancy could be ascribed to the differences both in the methods used to investigate the expression of pyruvate kinase isozymes and in the erythroid populations present in bone marrow and fetal liver.

This system may represent an interesting model of gene regulation in which two genes responsible for the synthesis of two enzymes are both expressed during the early stages of erythroid maturation, but only one...
remains active during the late stages of erythroid maturation. The mechanism(s) responsible for this gene inactivation remain(s) to be investigated.

The persistence of M2-PK in erythrocytes has previously been described in different pathologic circumstances. On one hand, M2-PK was found in erythrocytes of certain patients with PK deficiency. The abnormal presence of M2-PK in these R-PK-deficient erythrocytes was considered to be a compensatory mechanism by the authors, and some arguments have recently been put forth by Takegawa et al. On the other hand, we have described a family with PK hyperactivity related to the persistence of M2-PK in red blood cells. In this case, the presence of M2-PK in erythrocytes leads to a disturbed metabolism of RBCs. Because we have now shown the disappearance of M2-PK during erythroid maturation, we can tentatively consider the pathologic persistence of M2-PK in RBCs as a disorder of the mechanism(s) responsible for the disappearance of M2-PK during erythroid maturation.

In conclusion, the results we have obtained with K562 cells, HEL cells in vitro, and erythroblasts in vivo corroborate the erythroid origin of both malignant cell lines and indicate that synthesis of R-PK occurs very early during the maturation of erythroblasts. However, we could not observe any modification of PK expression in K562 and HEL cells, despite induction and concomitant differentiation in vitro, whereas decrease of M2-PK was clearly displayed in the course of the erythroid maturation in vivo.

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REFERENCES

26. Dimery IW, Ross DD, Testa JR, Gupta SK, Felsted RL,


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