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

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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 Stractan 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.

PYRUVATE KINASE (adenosine triphosphate [ATP]: pyruvate phosphotransferase, 2.7.1.40; PK) is present in humans in four different isozymic forms (L, R, M1, M2), expression of which differs from one tissue to another.1 R-type isozyme (R-PK) is present in mature red blood cells and is not present in other tissues; however, L-type PK (L-PK), present in liver cells, exhibits immunologic identity with R-PK.2 These two isozymes seem to be encoded by the same gene.3 M2-type PK (M2-PK) is expressed in leukocytes. This isozyme is considered as "prototype" PK, whereas the other isozymes are regarded as differentiated forms.1 The pattern of PK isozymes in erythroblasts has been investigated by Takegawa et al.4 These authors have recently shown the presence of both M2-PK and R-PK in erythroblasts using immunofluorescence techniques.4,5

The purpose of our study was to investigate the expression of PK isozymes during the process of erythroid maturation in normal and malignant erythroblasts. Thus, we investigated the isozyme pattern of PK in erythroblasts from human fetal liver, separated according to density, and in two human erythroleukemic cell lines, K5626 and HEL.7 Our results provide direct evidence that the isozyme pattern of PK is markedly modified in vivo during the process of erythroid maturation in fetal liver erythroblasts with a preferential expression of the M2 isoenzyme during the early stages of maturation, and also show the nearly constant expression of the R isozyme during the different stages of maturation. On the other hand, we found very little R-PK in K562 and HEL cell lines, in spite of induction and concomitant differentiation in vitro. This fact confirms the erythroid feature 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. Cellgel strips were purchased from Medical Products, Chemetrom Corp, Milan, Italy. Agarose was from Litex, Denmark; and bovine serum albumin, hemin, sodium butyrate, and tetradecanol 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.2 RBCs were cleared of leukocytes according to Beutler et al.9 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 105/mL. They grew exponentially during the next four days; after that time, they reached a plateau concentration (cell density inhibition) ranging from 8 x 106 to 1 x 107/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 histiocytic lymphoma cell line.11 They were grown under similar conditions.
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 Stracan II, purified and prepared as described by Corash et al., with slight modifications. Eight different fractions from 1.025 to 1.09 were purified and prepared as described by Corash et al., with slight modifications. The tubes were then centrifuged at 35,000 g in an SW 41 Beckman rotor in an L-50 Beckman ultracentrifuge for 50 minutes at 4 °C. At the end of centrifugation, the cells were carefully removed by aspiration with Pasteur pipettes and then extensively washed in Hanks' salt solution. An aliquot of the cells was used for morphological identification of the cells after May-Grünewald-Giemsa staining. The remainder was used for pyruvate kinase studies.

Cellulose acetate strip electrophoresis was performed as previously described. PK activity was assayed by the classical method, using the reaction coupled to lactate dehydrogenase activity, as previously described. Protein concentration was determined by the method of Lowry et al., with bovine serum albumin as the working standard. Partially purified M₂- and R-PK were prepared as previously reported.

Immunologic Studies

Anti-R-PK antiserum was obtained by immunization of rabbits by intramuscular injection of purified R-PK. Specificities of anti-R-PK antiserum were tested by double immundiffusion according to the method of Ouchterlony. A simple precipitation line was obtained between the anti-R-PK serum and hemolysate of purified PK (Fig 1).

Immunoinactivation was performed according to Kahn et al. Erythroblast lysates, hemolysates, or leukocyte lysates, adjusted at the same activity level, were mixed with an amount of anti-R-PK antiserum sufficient to inactivate all R-PK in 100 mmol/L Tris-HCl buffer, pH 8, containing 100 mmol/L KCl, 0.1 mmol/L fructose-1,6-diphosphate (FDP), 1 mmol/L EDTA, 1 mmol/L e-amino-capric acid, 0.1 mmol/L dithiorthreitol, 2 mg/mL bovine albumin, and 500 mmol/L sucrose. After incubation for one hour at 37 °C and three hours at 4 °C, the tubes were centrifuged, and the residual PK activity was assayed in the supernatant.

Rocket-Line Immunoelectrophoresis

This method allows the detection of very small quantities of antigen by a modification of rocket immunoelectrophoresis, as described by Kroll. 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 mL 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-citric 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.

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 described 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 mmol/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

Table 1. Comparison of Total PK Activity of K562 Cells and Cells of Erythroid and Nonerythroid Origin

<table>
<thead>
<tr>
<th></th>
<th>K562</th>
<th>Leukocytes</th>
<th>HL-60</th>
<th>U-937</th>
<th>From Fetal Liver</th>
<th>Mature Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples tested</td>
<td>21</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Total PK (IU/mg) Proteins (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>
<td>0.006 ± 0.001*</td>
</tr>
</tbody>
</table>

*IU/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.

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 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 Stratann 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,
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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 formazan formation. (1 through 4) RBC lysates containing, respectively. 0.3, 0.6, 1.2, 2.4 IU x 10^3 PK; (5) uninduced Lozzio clone lysate: 73 IU x 10^3 PK; (6) hemin-induced Lozzio 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.

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.21-23 R-PK is a marker characteristic of red cells, and its presence in K562 cells has previously been investigated by two authors.24,25 Horton was unable to detect any R-PK.

Table 2. R-PK in Uninduced and Induced K562 Cells Determined by Rocket-Line Immunoelectrophoresis

<table>
<thead>
<tr>
<th></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>Number of tested samples</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>R-PK IU x 10^3/mg protein (mean ± SD)</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>
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</table>

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 Stratagel 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.
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, there does 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 an 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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