Changes in Activities and Isozyme Patterns of Glycolytic Enzymes During Erythroid Differentiation In Vitro

By W. Nijhof, P.K. Wierenga, G.E.J. Staal, and G. Jansen

Late committed progenitor cells of erythropoiesis, CFU-E (colony-forming unit—erythroid), were isolated from mouse spleens to near homogeneity by a three-step enrichment procedure. The procedure included a four-day pretreatment of bled mice with the antibiotic thiamphenicol, a recovery period of 3/2 days, followed by centrifugal elutriation and Percoll density gradient centrifugation of the spleen cells. This practically pure CFU-E population was used to study some aspects of erythroid differentiation in vitro. Colony growth, as well as morphology and glycolytic enzyme activities of cells isolated at selected times of the 48-hour culture period, were determined. Marked declining activities of several enzymes, including hexokinase, phosphofructokinase, aldolase, enolase, pyruvate kinase, and glucose-6-phosphate dehydrogenase, were observed during in vitro differentiation. The activity of diphosphoglycerate mutase was almost absent in the CFU-E, but progressively increased during differentiation. The isozyme distribution of aldolase and enolase did not change during CFU-E in vitro differentiation into the reticulocyte. Hexokinase (HK) in the CFU-E contained mainly a double-banded type I isozyme, in addition to a minor amount of HK II. During differentiation, a shift was noticed within the double-banded HK I region, whereas HK II disappeared after one cell division. Pyruvate kinase in the CFU-E was characterized by the presence of both the K-type and the L-type isozyme and hybrids of these isozyme types. During in vitro differentiation, the production of the K-type isozyme rapidly stops in favor of the L type.

MATURE RED CELL METABOLISM is quite well documented. Efforts to study immature red cell metabolism are mostly restricted to reticulocytes, which can be obtained rather simply from peripheral blood by density gradient centrifugation. Recently, Nijhof et al described a method for the isolation of large quantities of almost pure mouse erythroid progenitor cells (colony-forming units—erythroid, CFU-E), which enabled biochemical characterization. Subsequent culture of erythroid colonies from CFU-Es allows us to study erythropoiesis continuously at different stages of development, as was shown by Nijhof et al.

This article focuses on the changes in the activities and isozyme distribution of some glycolytic enzymes during erythroid cell differentiation.

MATERIALS AND METHODS

Experimental Procedures

Isolation of the CFU-E

Three-month-old RPTV mice (male or female, inbred wild type xC57BL from the Department of Radiopathology, State University, Groningen, The Netherlands) were bled and treated with thiamphenicol (in a dialysis bag subcutaneously implanted in the neck) for four days, as previously described. Three and a half days after the removal of the dialysis bag, the mice were killed by cervical dislocation. The spleens of thiamphenicol-treated bled mice were excellent sources of CFU-E (5% to 10% of total nucleated cells). Further enrichment of CFU-E from spleen cells by fractionation according to cell size (centrifugal elutriation, which removes the bulk of lymphoid, granuloid, and erythroid cells) and cell density (Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden) gave rise to CFU-E populations with a seeding efficiency of 45% and 100%, respectively. Up to 10⁷ CFU-E can be isolated from one spleen.

CFU-E Colony Assay, Growth Characteristics, and Morphology

The in vitro colony assay of Iscove and Sieber was employed. Cells suspended in culture medium were plated at 2 × 10⁵ nucleated cells/mL in a volume of 10 mL in 6-cm Petri dishes (Greiner, Nürtingen, FRG). The medium consisted of 0.8% methylcellulose (Fluka, Bachs S.G., Switzerland), 30% fetal calf serum (GIBCO, Grand Island, NY), 10⁻⁴ mol/L 2-mercaptoethanol (Merck, Darmstadt, FRG), 1% bovine serum albumin (GIBCO), 100 μg streptomycin/mL (Mycofarm, Delft, The Netherlands) in a-medium (GIBCO) with 10 mmol/L HEPES, pH 7.2. Human urinary erythropoietin (CAT-1 (1140 U/mg) prepared by Dr E. Goldwasser, Chicago, as provided by the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, NIH, Bethesda, Md) was added at 0.5 U/mL. The plates were incubated at 37°C in a 5% CO₂ humidified atmosphere. For colony counting, cells were plated at 5 × 10⁴ cells/mL. At regular times, 500 colonies plus undivided cells were counted, and the percentage of colony formation was calculated. At the time of maximal colony formation, cells were isolated and stained with May-Grünwald-Giemsa solution. The distribution among the different erythroid cells was calculated, mainly based on cell size but also considering cytoplasmic staining and nuclear condensation.

Because CFU-E preparations may have variable amounts of contaminated cells or inactivated CFU-E, growth characteristics of plated CFU-E were followed for each experiment. A specific example is given in Fig 1A. After seven hours of culture, 87% of the inoculated cells gave rise to two-cell (66%) and four-cell (21%) colonies.
clusters. Subsequently, the number of two-cell clusters decreased in favor of the four-cell clusters. The maximum numbers of 4-, 8-, and 16-cell colonies were found at 14, 21, and 28 hours, respectively.

At 48 hours, 80% of the colonies consisted of 16 or more cells. The morphology of cells isolated from these colonies is shown in Fig 1B. Basophilic cells were found up to 14 hours of culture. After seven hours, cells gradually decreased in size. Hemoglobinization of the cytoplasm was evident at 21 hours, as was nuclear condensation. The process of enucleation started at 28 hours of incubation. Table 1 shows the percentages of the different nucleated erythroid cells in the colonies isolated at the indicated time points. Until 14 hours, the cells were mainly early erythroblasts. At 28 hours, the cell mixture was predominantly late erythroid. After two days of culture, the majority of the cells was reticulocytes.

Cell Preparation, Enzyme Assays, Electrophoresis, and Other Procedures

The cells were harvested after culture times of 7, 14, 21, 28, and 48 hours. The collected cells (20 to 30 × 10⁶) were freed of serum and methylcellulose by three washes with α-medium. Cell counts were performed on a Coulter Counter, model ZF (Coulter Electronics, Hialeah, Fla). Cells for enzymologic analysis were stored as a pellet in liquid nitrogen. Blood samples from mice were collected in heparin and filtered through a mixture of α-cellulose/microcrystalline cellulose in order to remove platelets and leukocytes. Cells, stored in liquid nitrogen, were thawed and lysed by sonication for 30 seconds at 4°C with a 150-W ultrasonic disintegrator MK2 in a 10-mmol/L Tris-HCl buffer, pH 8.0, which contained 1 mmol/L...
EDTA, 1 mmol/L dithiothreitol, and 1 mmol/L diisopropylfluorophosphate. Mitochondrial, nuclear, and membrane fragments were discarded after centrifugation at 48,000 g for 20 minutes. The resulting cytoplasmic fractions were tested for enzyme activities by the methods of Beuther.

Electrophoreses of hexokinase, pyruvate kinase, and aldolase were carried out on cellulose acetate gel strips as described before. Enolase electrophoresis was carried out on cellulose acetate gel strips as described before. Enolase electrophoreses were carried out on cellulose acetate strips as described before. Enolase electrophoreses were carried out on cellulose acetate strips as described before.

Immunoprecipitation with anti-L type pyruvate kinase (PK-L) was performed as described by Kahn. Rabbit anti-human PK-L antisera were a generous gift from Dr A. Kahn (Institute de Pathologie Moléculaire, Paris). Substrates, coenzymes, and auxiliary enzymes for the determination of glycolytic enzymes were obtained from Boehringer (Mannheim, FRG). Cellogel strips were purchased from Medical Products, Chemetron Corp. Milan, Italy. Dithiothreitol, phenazine methosulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 0.17 mmol/L phenazine methosulfate, 0.5 IU pyruvate kinase, 0.14 IU hexokinase, and 0.07 IU glucose-6-phosphate dehydrogenase.

Table 1. Cell Classification

<table>
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<th>Incubation Time (h)</th>
<th>Pronormoblasts</th>
<th>Basophil Erythroblasts</th>
<th>Polychromatic Erythroblasts</th>
<th>Orthochromatic Erythroblasts</th>
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The average values of cell classification are presented in Nijhof and Wierenga. This is a specific example of this classification. Classification was done by one person and checked by another; the variation was less than 5%.

RESULTS

The activities of seven glycolytic enzymes in the CFU-E and in erythroid cells derived from cultured CFU-E are shown in Fig 2. Three kinds of enzyme behavior can be distinguished: first, a group of enzymes with a rapid decline in enzyme activity after one cell division (hexokinase, pyruvate kinase, phosphofructokinase, and enolase, Fig 2A), a second group of enzymes with no decline in enzyme activity after one cell division (aldolase and glucose-6-phosphate dehydrogenase, Fig 2B), and third, an enzyme with increasing enzyme activity (diphosphoglycerate mutase, Fig 2C).

In the first group, enzyme activities decline after two cell divisions (14 hours) to 25% to 35% of the original CFU-E values. Prolonged culture times demonstrate a gradual decline in activities to 2% to 5% of the CFU-E values in the mature red cell. The activities of the enzymes of the second group progressively decline after the initially constant level, although aldolase activity is again rather constant between 14 and 28 hours of culture. The activity of 2,3-diphosphoglycerate mutase was almost absent in the CFU-E, but progressively increased during in vitro differentiation.

The isozyme distribution of four enzymes (aldolase, enolase, hexokinase, and pyruvate kinase) was studied at different culture times. Aldolase is a tetrameric enzyme of which three main isozymes are known. The homomeric A₄ is predominant in skeletal muscle and in most other tissues. Aldolase B₄ is confined to kidney and liver, whereas aldolase C₄ is the principal form in nervous tissue. Only the A₄ type isozyme could be demonstrated in the CFU-E, CFU-E-derived cells, and mature mouse red cells (results not shown).

Enolase is a dimeric enzyme, and three loci are known to produce the subunits designated as α, β, and γ. The αα type is usually referred to as liver type, although it occurs in most tissues; ββ enolase is found predominantly in muscle; and γγ enolase is neuron specific. During mouse erythroid differentiation, only the αα type was demonstrated (results not shown).

Four main isozymes are known of hexokinase and are designated I to IV in order of increasing anodal electrophoretic mobility. Hexokinase I can be com-

![Fig 2. Activities of glycolytic enzymes at different stages of erythroid differentiation in vitro. (A) Hexokinase (34.4) — — — — pyruvate kinase (30.7) — — — —. (B) Phosphofructokinase (39.0) — — aldolase (41.7) — —. (C) 2,3-Diphosphoglycerate mutase (3.7) — —. One hundred percent values are indicated between brackets and expressed as IU/10⁶ cells. The results are the mean of duplicate experiments.](www.bloodjournal.org)
posed of two or more subtypes. Hexokinase in the CFU-E is characterized by the presence of the type I isozyme (mainly IA subtype), besides a minor hexokinase type II band, which has disappeared after one cell division. Prolonged culture time shows a shift within the IA/IB subtype ratio toward the IB subtype (Fig 3).

There are at least three isozymes of pyruvate kinase, each of which is composed of four identical or nearly identical subunits. According to the nomenclature proposed by Ibsen, these isozymes are designated as the liver, or L type, the muscle, or M type, and the kidney, or K type (M and K isozymes are also frequently designated M1 and M2, respectively). Pyruvate kinase in red cells is synthesized as L' subunits. In vitro mild tryptic treatment can convert the L' tetramers into a form with properties similar to the liver L4 pyruvate kinase. L' tetramers are predominant in erythroblasts and reticulocytes. L and L1L2 have a slightly reduced anodal mobility electrophoretically.

Pyruvate kinase of CFU-E is composed of the K- and L-type isozymes (Fig 4A), as based on the two main bands corresponding to the K- and L-type in the liver control. The additional bands between the homotetramers K4 and L4 are precursor L type (L') and L type containing hybrids, as shown by immunoprecipitation of CFU-E pyruvate kinase (PK) by anti-L-type PK, resulting in a single band at the K4 position (Fig 4B). During in vitro differentiation, the production of the K-type isozymes stops after one cell division in favor of the L type, the only isozyme type present in mature red cells.

DISCUSSION

Biochemical studies of erythroid cell differentiation require techniques which, first of all, separate erythroid cells from other cell types, and secondly, separate erythroid cells in different stages of development. The first requisite is largely met by using bone marrow cells from anemic animals, although contamination of nonerythroid cells is still about 5%. The separation of erythroid cells in different stages of development is still problematic. Attempts at separation of erythroid cells according to density and/or sizes do not result in optimal resolutions of cells in every developmental stage. For this reason, several laboratories are using the murine Friend leukemia or human K562 cell line for studying erythroid differentiation aspects. These cell lines have great proliferation capacities; however, the main disadvantages of these systems is that differentiation is erythropoietin insensitive and does not give rise to nonnucleated erythroid cells.

To overcome these problems, a new technique was used as described by Nijhof. Pure erythroid progenitor cells (CFU-E) were isolated. The subsequent differentiation stages of the erythroid cell line up to the reticulocyte stage were observed after 48 hours of in vitro culture.

The present study describes changes in activities and characteristics of glycolytic enzymes during the development of erythroid cells, which may reflect some changes in metabolic capacities. Although nucleated erythroid cells and reticulocytes have an intact Krebs cycle at their disposal, higher glycolytic enzyme activities, as found in reticulocytes as compared with mature red cells, correspond with higher glycolytic rates, thus enabling reticulocytes to fulfill their higher metabolic needs. In this respect, it seems likely that very active and highly generative cells, such as CFU-E and dividing erythroblasts, require at least the same or an even higher metabolic capacity. It is an interesting phenomenon that the three key enzymes of glycolysis—hexokinase, phosphofructokinase, and pyruvate kinase—have comparable disappearance kinetics (Fig 2A). In the first 14 hours of culture, enzyme activities decrease to 30% of the CFU-E value, showing a large overcapacity of enzymatic potential. This decline obviously does not influence cell division. The rapid
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Fig 4. (A) Pyruvate kinase electrophoresis of CFU-E and CFU-E-derived erythroid cells on cellulose acetate gels at pH 7.5 at culture times t = 0 (CFU-E), 7, 14, 21, 28, and 48 hours. L, mouse liver reference; MR, mature mouse red cells. (B) Pyruvate kinase electrophoresis of CFU-E and mature mouse red cell pyruvate kinase before and after anti-L treatment. L, mouse liver reference.

Decline in activity after one cell division suggests the cessation of synthesis of these enzymes, at least when we exclude selective degradation of enzymes by processes like ATP-dependent proteolysis or action by lipoxygenases. The enzyme activities are relatively constant between the basophilic erythroblast and orthochromatic erythroblast (19 to 28 hours), which is in agreement with data from Arnstein.24 Our system, however, also shows the situation in cell stages of earlier development. After 28 hours of culture, enzyme activities decline, reaching final values that are comparable with data obtained for reticulocyte-enriched cell fractions after density separation of mature red cells.34 After an initial decline, an increase in phosphofructokinase activity in late stages of rabbit erythroid differentiation, as found by Narita et al,19 could not be demonstrated in our mouse system. The activities of aldolase and glucose-6-phosphate dehydrogenase do not decline after one cell division, indicating active synthesis of these enzymes.

Denton et al23 demonstrated constant glucose-6-phosphate dehydrogenase activities (expressed per milligram of protein) up to the polychromatic erythroblast. Then, as a result of a rapidly increasing hemoglobin content within erythroblasts at the transition from the dividing to the nondividing cell compartment, the activity/protein ratio decreased, leading to lower glucose-6-phosphate dehydrogenase activities in later stages of differentiation. However, in our mouse system, the glucose-6-phosphate dehydrogenase activity (expressed per cell) declines after the second cell division (basophilic erythroblast).

High activities in early stages of erythroid development of glucose-6-phosphate-dehydrogenase, the first enzyme of the hexose monophosphate shunt, might be involved in the purine biosynthesis de novo, which is necessary for DNA synthesis. With respect to the latter, Denton et al23 also reported high activities of some purine metabolism enzymes. In agreement with Narita et al,19 we were able to demonstrate a gradual increase in 2,3-diphosphoglycerate mutase activity. We also showed7 that the synthesis of hemoglobin started after one cell division (predominantly basophilic erythroblasts); this coincides with the production of 2,3-diphosphoglycerate mutase (2,3-DPGM), which is the enzyme responsible for the presence of the allosteric regulator of hemoglobin function: 2,3-DPG. This is in agreement with the 2,3-DPG–hemoglobin relationship shown by Narita et al19 in bone marrow cells of phenylhydrazine-treated rabbits.

As Setchenska and Arnstein22 showed changes in the lactate dehydrogenase isozyme pattern, we also show changes in the isozyme patterns of hexokinase and pyruvate kinase during erythroid differentiation. In the CFU-E, the IA subtype hexokinase is predominant. During differentiation, a shift is noticed in favor
of the IB subtype. At 48 hours of culture, the HK I subtype distribution closely resembles that of rabbit reticulocyte hexokinase. Hexokinase type II is known to be adaptive to the different physiologic states created by nutritional, hormonal, or culture conditions. HK II is easily induced under poor culture conditions and rapidly disappears again when proper culture conditions are restored. With the transfer of CFU-E from a thiamphenicol recovering mouse spleen to a nutrient-rich CFU-E culture medium, the disappearance of the minor amount of HK II in CFU-E after seven hours of culture could be ascribed to this phenomenon.

Pyruvate kinase has at least three isozymes, namely liver (or L type), muscle (or M type), and kidney (or K type). Only the L type is present in mature red cells, reticulocytes, and, according to Kahn et al., in erythroblasts, although the stage of differentiation of the erythroblasts was not documented. The presence of K-type PK in mature red cells has only been described as a compensating mechanism in case of a severe L-type PK deficiency or as an inherited red cell anomaly associated with polycythemia and PK hyperactivity. Recently, Takegawa et al. showed the presence of K-type pyruvate kinase in proerythroblasts by immunofluorescence techniques. The K type decreased gradually with development of the erythroblasts in favor of the L type. In this article, we demonstrate electrophoretically the presence of K-type pyruvate kinase predominantly in the CFU-E and doublet cells after seven hours of culture. This finding confirms the hypothesis of Miwa et al. that erythroid precursor cells contain K-type pyruvate kinase. The L type is present in all differentiation stages, which is in contrast with data from Takegawa.

Considering a common stem cell (CFU-S) for erythrocytes, platelets, and granulocytes (the last two cell types are known to contain K-type pyruvate kinase and erythrocyte L-type pyruvate kinase), it was of interest to see whether or not K-type pyruvate kinase is produced at the erythroid stem cell level. In K562 cells, classified in a developmental stage before the CFU-E, we found predominantly K-type pyruvate kinase and just minor amounts of L-type pyruvate kinase. In CFU-E, however, we now show that the production of K type has almost stopped, and L type becomes predominant.

In conclusion, these results demonstrate the importance of the system used. The CFU-E obviously has a different enzyme pattern than its progeny, which have so far been used in the study of erythroid differentiation. We show that the CFU-E is subject to positive (increase in 2,3-DPGM activity) as well as negative (decrease in HK, PFK, PK activity) genetic regulatory influences with respect to the production of glycolytic enzymes. Differentiation of CFU-E in vitro can thus become an important tool in the study of many aspects of red cell metabolism.

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