Apoptotic Changes Precede Mitochondrial Dysfunction in Red Cell-type Pyruvate Kinase Mutant Mouse Erythroleukemia Cell Lines

Ken-ichi Aisaki,1 Hitoshi Kanno,2 Naoki Oyaizu,1 Yukichi Hara,1 Shiro Miwa2 and Yoji Ikawa1, 3

1Department of Retroviral Regulation, Tokyo Medical and Dental University Medical Research Division, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519 and 2Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470

Two erythroleukemia cell lines have been established from the splenic lesions of red blood cell-type pyruvate kinase (R-PK) activity-deficient mice of CBA/N origin infected with a polycythemic strain of Friend leukemia virus complex (FVp). Ten to 30 % of the cells of these cell lines undergo apoptotic changes in routine passage, as shown by nuclear fragmentation, DNA laddering, DNA content (propidium iodide (PI) staining), and annexin V binding assay. In these cells, however, although adenosine 5′-triphosphate (ATP) levels were lower than in the control cells, the mitochondrial inner transmembrane potential (Δψm), detected by rhodamine 123 (R123) and diSC3(5) staining, remained unchanged until the final stage of apoptosis. No evidence was obtained to relate this finding to R-PK mutation due to difficulty in cloning stable, conditionally inducible R-PK gene transfectants. However, low Δψm in the apoptotic cell population of the control T3-K-1 (K-1) and T3-Cl-2-0 (2-0) Friend erythroleukemia cells supports a possible relationship, as do results obtained in two Friend erythroleukemia cells recently isolated from normal CBA/N mice. These cell lines are expected to be useful for clarifying both the primary apoptotic changes independent of mitochondrial dysfunction and the PK-isozyme changes during erythroiddifferentiation, for example, the decreased muscle type 2 (M2) PK level. Modification of growth signals in these cell lines may modulate differentiation and/or apoptosis and allow further elucidation of the signaling networks.

Key words: Apoptosis — Mitochondrial dysfunction — Pyruvate kinases — Friend erythroleukemia cell lines

Pyruvate kinase (PK, EC 2.7.1.40) catalyzes phosphoenolpyruvate-pyruvate conversion in the glycolytic pathway. Among the diseases caused by glycolytic enzyme defects, PK deficiency is the most common cause of hereditary nonspherocytic anemia in humans. Since the first report by Valentine et al. in 1961,1) several hundred cases of PK deficiency have been reported.2) Gene therapy by transferring the normal PK gene into the hematopoietic stem cells of the patients has been considered,3) and a mouse model for the corresponding disease was described.4) CBA/N-Pk-1+/−/Pk-1−/− animals, possessing a Gly-Asp mutation at the 338th amino acid of the red blood cell-type pyruvate kinase (R-PK) protein within the substrate-binding region,5) were segregated from an inbred colony of the CBA/N strain by Mr. Hideaki Asai of the Japan SLC Harumi Farm, Shizuoka.

Mammalian PK has four isozymes6-7; R-PK is expressed almost exclusively in mature red blood cells (RBCs),8) and the liver-type PK (L-PK) is transcribed from an alternative promoter of the PKLR gene.9, 10) In contrast with mature RBCs, erythroid precursors as well as other hematopoietic cells express a different isozyme of PK (muscle type 2 (M2)-PK),11-13) which is encoded by the PKM gene. The PKM gene encodes both M1-PK and M2-PK, which are produced from a common primary transcript by alternative splicing.14) M2-PK is considered a prototype enzyme, since it is almost ubiquitously expressed in fetal and most adult tissues. The shift of isozyme expression from M2 to R-PK might be attributable to both transcriptional and post-transcriptional mechanisms.

In order to develop an in vitro model for R-PK deficiency, we attempted to infect R-PK-mutant mice (CBA/N-Pk-1+/−/Pk-1−/−) with Friend leukemia virus complex (FVp), and successfully established two erythroleukemia cell lines from their splenic lesions. We confirmed their R-PK gene mutation by a reverse transcript-polymerase chain reaction (RT-PCR) method. These cell lines behaved like other Friend erythroleukemia cell lines except for the remarkable spontaneous apoptotic changes during routine passage.

In this study, PK isozyme-shift (M2 to R type), intracellular adenosine 5′-triphosphate (ATP) levels, and apoptosis-related changes including cationic lipophilic dye-assayed mitochondrial inner transmembrane potential

3To whom correspondence should be addressed.
E-mail: y.ikawa.mbc@med.tmd.ac.jp
(ΔΨₐ) were investigated in the above R-PK-mutant erythroblastoid cells and their normal counterparts. We discuss here the possible involvement of the R-PK mutation in the occurrence of apoptosis preceding mitochondrial dysfunction in the above cell lines.

MATERIALS AND METHODS

**Mice**  
CBA/N-{}^{ΔPK} /{}^{ΔPK} mice and CBA/N+ +/- mice were raised in the Japan SLC Harumi Farm (Shizuoka). Male and female mice, 6–15 weeks of age, were used.

**Establishment of R-PK-mutant Friend erythroleukemia cell lines, SLC3 and SLC4**  
CBA/N-{}^{ΔPK} /{}^{ΔPK} mice were injected intravenously with FVP. Seven to 9 weeks after infection the mice were killed and a single cell suspension of spleen cells was plated in 1.4% methylcellulose (4000 centipoises, Sigma, St. Louis, MO) in Iscove’s modified Dulbecco’s medium (IMDM, Life Technologies, Inc., Gaithersburg, MD) containing 30% heat-inactivated fetal calf serum (FCS, Moregate, Australia) and 20 µM 2-mercaptoethanol (Nacalai, Kyoto). After incubation at 37°C and 5% CO₂ for 10 to 30 days, large colonies were picked up and cultured in IMDM liquid culture medium containing 30% FCS and 20 µM 2-mercaptoethanol for 1 month or longer.

The cell lines SLC3 and SLC4 were obtained from male and female CBA/N-{}^{ΔPK} /{}^{ΔPK} mice, respectively and were maintained in suspension for over 18 months.

**Cell lines**  
The R-PK-mutant cell lines SLC3 and SLC4 were maintained like other Friend leukemia cell lines in IMDM medium with 10% FCS and 20 µM 2-mercaptoethanol. T3-K-1 (K-1) and T3-C1-2-0 (2-0), both derived from DDD/1 mice, were used as normal R-PK Friend leukaemia cell lines, SLC3 and SLC4 or were incubated with diSC3(5) (Molecular Probes, Eugene, OR), were used as described by Darzynkiewicz et al. and Bammel et al. Briefly, 1×10⁶ cells were incubated with rhodamine 123 (R123, 10 µg/ml) for 15–30 min in culture medium under 5% CO₂ at 37°C, or were incubated with diSC3(5) (1 µM) for 1 min in culture medium at room temperature. The cells were washed once with PBS, and stored in the dark at 4°C until analysis by flow cytometry, using a FACScan “Calibur” (Becton Dickinson, San Jose, CA). Analysis was completed within 1 h to prevent cell damage. The data on R123 staining were obtained using 488 nm excitation and green fluorescence detection at 515 to 545 nm, and the data on diSC3(5) were obtained using 598 nm excitation and red fluorescence detection at 650 to 670 nm.

**Erythroiddifferentiation induction**  
The cells were seeded at 5×10⁵ cells/ml in IMDM medium or RPMI-1640 medium containing one of the following erythroiddifferentiation inducers and were incubated for 2–4 days. Dimethyl sulfoxide (DMSO) 1.0% (v/v), sodium butyrate 1.0 or 2.0 mM, hexamethylene bis-acetamide (HMBA) 3 mM, hemin 0.1 µM and recombinant human erythropoietin (EPO) 2 U/ml were used as erythroiddifferentiation inducers. Hemo-globin-positive cells were counted after staining with the non-toxic benzidine analog, 2,7-diaminofluorene (DAF).

**Detection of mutant R-PK expression by RT-PCR**  
To confirm the expression of mutant R-PK gene in SLC cells, RT-PCR assay was conducted as described by Kanno et al. Briefly, total cellular RNA obtained from the respective cell lines by the acid guanidium-phenol-chloroform (AGPC) extraction method was employed for RT reaction. A portion of the reaction mixture was subjected directly to PCR. To confirm the nucleotide change detected in the PK-1⁰ locus, the murine R-PK gene was amplified by using the primers ML-5 (5’-AAG TGA GCG ATG GCA TCA TGA T-3’) and ML-6 (5’-CCA AGA AAA CCT TCT CTG CT-3’). The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 s, at 60°C for 10 s, and at 72°C for 10 s in a GeneAmp PCR System 9700 (Perkin Elmer-Cetus, Emeryville, CA).

The amplified DNA was digested with BstPI to see whether the recognition site was destroyed by the mutation.

**Measurement of cellular ATP content**  
A total of 1×10⁶ cells were washed once with cold phosphate-buffered saline (PBS), pH 7.4, collected by centrifugation at 200g, resuspended in 500 µl of boiling 100 mM Tris-HCl, 4 mM EDTA (pH 7.75) and incubated for 2 min at 100°C. Samples were then centrifuged at 10,000g for 1 min and the supernatants were kept in ice or stored at −80°C until ATP measurement. ATP levels were measured with a Boehringer Mannheim (Mannheim, Germany) ATP Bioluminescence Assay Kit CLS II. A 100 µl sample was mixed gently with 100 µl of the luciferase reagent and luciferase activity was measured for 10 s using a luminescence reader (Lumicounter ATP-237, Toyo Kagaku Sangyo, Yamanashi).

**Analysis of ΔΨₐ by flow cytometry**  
To measure ΔΨₐ, R123 (Sigma) and a cyanine dye, diSC3(5) (Molecular Probes, Eugene, OR), were used as described by Darzynkiewicz et al. and Bammel et al. Briefly, 1×10⁶ cells were incubated with rhodamine 123 (R123, 10 µg/ml) for 15–30 min in culture medium under 5% CO₂ at 37°C, or were incubated with diSC3(5) (1 µM) for 1 min in culture medium at room temperature. The cells were washed once with PBS, and stored in the dark at 4°C until analysis by flow cytometry, using a FACScan “Calibur” (Becton Dickinson, San Jose, CA). Analysis was completed within 1 h to prevent cell damage. The data on R123 staining were obtained using 488 nm excitation and green fluorescence detection at 515 to 545 nm, and the data on diSC3(5) were obtained using 598 nm excitation and red fluorescence detection at 650 to 670 nm.

**Annexin V binding assay**  
Apoptotic cells were analyzed by using an apoptosis detection kit (R&D Systems, Minneapolis, MN). A total of 1×10⁶ cells were washed once with cold PBS, pH 7.4, collected by centrifugation at 200g, resuspended in 1 ml of HEPES-buffered saline solution supplemented with 2.5 mM CaCl₂ (annexin V binding buffer). Then 100 ng of annexin V-fluorescein isothiocyanate (FITC) and 500 ng of propidium iodide (PI) were added to 100 µl of the cell suspension (1×10⁵ cells) and the mixture was incubated for 15 min at room temperature. The cells, without being washed, were placed in 400 µl of annexin V binding buffer and kept in ice until flow cytometry assay.
In the presence of a high Ca²⁺ concentration, the annexin V binding buffer is quite toxic and induces apoptotic and/or necrotic cell death. We finished the assay within 1 h to avoid artifacts due to the binding buffer.

**Enzymatic assay of Friend cells** Cells were harvested and washed twice with physiological saline and the cell pellets were stored at −80°C before enzymatic analysis. About 5×10⁷ living cells were sonicated in 200 µl of 2.7 mM EDTA, 0.7 mM 2-mercaptoethanol, and centrifuged at 15,000g for 10 min at 4°C. Supernatants were used for PK assay, diphosphoglycerate mutase (DPGM) assay and protein assay.

**Western blot analyses of PK isozymes** Protein extracts prepared from 5×10⁶ living cells were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Polyclonal antibodies against rat L- and M1-PK were kindly provided by Prof. Tamio Noguchi, Nagoya University, and were used to characterize the PK isozyme expression of the cells. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Germany) in buffer containing 20% (v/v) methanol, 25 mM Tris, 192 mM glycine, pH 8.3, at 20 volts, 4°C, overnight in a mini trans-blot electrophoretic transfer cell (BioRad, Hercules, CA). The membrane was incubated with anti-rat L- and M1-PK antibodies, followed by chemiluminescence detection using a Western-light Protein Detection Kit (Tropix, Bedford, MA). Signals detected on X-ray films (Reflection NEF, Dupon) were semi-quantified by densitometric analysis using a BioRad GS-700 Imaging Densitometer.

**RESULTS**

**Establishment of R-PK-mutant Friend erythroleukemia cell lines** The enlarged spleens of CBA/N-Pk-1slc/Pk-1slc mice 7 weeks after FVp infection were minced and seeded on methylcellulose plates. Cells of colonies were cultured as described in “Materials and Methods,” and after a number of trials, two Friend erythroleukemia cell lines (SLC3 and SLC4) were established. It took several months to obtain stable cell lines because of the remarkable apoptotic changes and difficulty in the recovery of the materials frozen in the presence of DMSO, which is one of the erythrodifferentiation inducers of Friend erythroleukemia cells, and was thus not applicable for freezing the cells. We use a “Cellbanker” (Jyujikagaku Inc., Tokyo) for cell freezing.

The biological characteristics of the stabilized cell lines, SLC3 and SLC4, of male and female origin, respectively, are quite similar to those of other Friend erythroleukemia cell lines. They respond to most of the erythrodifferentiation inducers except erythropoietin (Fig. 1b), but show remarkable apoptosis during passage. The cells of both lines, 2 to 3 days after inducer treatment, became smaller and showed some increase of apoptotic changes. Ter119, a monoclonal antibody directed to a glycophrin-like molecule of murine erythroid cells, was used as a marker of erythrodifferentiation. The cells of both cell lines were Ter119-positive, showing their erythroid nature (Fig. 1a).

**Confirmation of the R-PK mutation in SLC cell lines** The point mutation of the R-PK gene causing the Gly-Asp conversion at the 338th amino acid in CBA/N-Pk-1slc/Pk-1slc mice abolishes the Bst PI-restriction site of the gene. Bst PI could not cleave the RT-PCR-amplified 72 bp band within the R-PK transcripts of the cell lines (Fig. 2), showing the presence of such point mutation in their R-PK genes. This mutation lies within the substrate (phosphoenolpyruvate) binding region. The mutant R-PK had indeed lost its response to the substrate, as described in previous reports by one of the present authors (Kanno) and others.

**Apoptotic changes during passage** Both SLC3 and SLC4 lines undergo spontaneous apoptosis during routine
passage. The apoptotic cell population ranges from 10 to 30%. The apoptotic changes are evidenced by 1) the presence of DNA laddering (Fig. 3a), 2) fragmentation or particularization of the nuclei revealed by Hoechst 33258 staining (Fig. 3b), 3) DNA content analysis in PI-stained samples by flow cytometry (Fig. 3c), and 4) annexin V binding assay by flow cytometry (Fig. 3d). The transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method was not applicable because of high DNA nick induction during erythroidifferentiation of erythroblastoid cells.22)
Low ATP concentration in SLC3 and SLC4

ATP levels of both SLC3 and SLC4 erythroleukemia cell lines were measured by a bioluminescence method based on ATP activation of luciferase, and the levels were lower than those of ordinary Friend erythroleukemia cell lines without R-PK mutation, K-1 and 2-0. The levels became even lower after induction of erythrodifferentiation (Fig. 4).

Changes of PK activity and isozymes during erythrodifferentiation

To evaluate the effects of the R-PK gene mutation on PK activity and the ontogenic shift of the PK isozymes of the SLC cells, PK activity was assayed using diphosphoglycerate mutase (DPGM) as an internal control. DPGM catalyzes the synthesis of 2,3-diphosphoglycerate (2,3-DPG), which controls the oxygen affinity of hemoglobin. DPGM activity has been shown to be increased by induced erythroid differentiation of Friend erythroleukemia cells23, 24) as well as CFUe isolated from murine spleens.12) As shown in Table I, all examined Friend cells showed increased DPGM activity (up to 2-fold) when compared to undifferentiated cells on the 3rd day. The DPGM induction rate seemed similar in cells with and without the R-PK mutation. In contrast, the PK activities of the SLC cells were reduced to approximately 70% at the 3rd day of induction, whereas the control Friend cells showed increased PK activity (to nearly 150%) after 3 days of induction. On the 3rd and 4th days of differentiation-inducer treatment, total protein levels decreased in parallel with the degree of differentiation.
Fig. 7. Annexin V binding assay of those diSC₃(5)-stained cells which retain dye-exclusion in PI-staining. The upper left population is live cells and the lower right population is completely apoptotic cells. The upper right population existed only in the case of SLC cells.
Western blot analyses were performed to examine the expression of the M2- and R-PK isozymes during erythroid differentiation. In K-1 and 2-0 cells, both M2-PK and R-PK showed little change, while in SLC3 cells, M2-PK and R-PK levels decreased to 47% and 83% respectively, and in SLC4 cells, M2-PK and R-PK levels decreased more markedly to 27% and 53% respectively (Table I and Fig. 5). The retained \( \Delta \psi_m \) in apoptotic SLC3 and SLC4 cells As previously described, the ATP levels of R-PK-mutant SLC3 and SLC4 cells were lower than those of the control R-PK normal cells and this tendency increased during induced differentiation (Fig. 4). It is generally accepted that ATP biosynthesis takes place mainly in the mitochondria in eukaryotic cells under aerobic conditions, and low ATP levels in the R-PK-mutant cells may possibly be due to a decreased supply of pyruvic acid in the mitochondrial cytoplasm. Accordingly, to investigate the change of the electron transport system of the mitochondrial inner membrane, \( \Delta \psi_m \) was evaluated by using membrane potential-sensitive, cationic, lipophilic dyes, such as R123 and diSC3(5). Unexpectedly, \( \Delta \psi_m \) of both R-PK-mutant erythroleukemia cell lines remained the same as that of the control K-1 and 2-0 cells.

When R123 staining was subjected to three-dimensional analysis (x, y, z; FSC, SSC, R123), the apoptotic cell population (R1 and R2) was clearly separated into two groups; high R123 and low R123. A representative three-dimensional analysis is shown in Fig. 6a. The two distinct high R123 and low R123 cell populations are marked as R1 and R2. In both SLC3 and SLC4 cells, the R1 population, which retaining almost normal mitochondrial function as assayed with R123, showed remarkable apoptotic changes morphologically, as detected by Hoechst 33258 staining (Fig. 6b). In the corresponding condition, only 1 to 10% of the control K-1 and 2-0 cells underwent apoptotic nuclear fragmentation.

Annexin V binds to the cell surface in the early stage of apoptosis in the presence of Ca\(^{2+}\). Because the emission wavelengths of FITC and R123 overlap each other, FITC-labeled annexin V (the most common modification in commercial kits) is not available for R123-stained cells. To analyze the mitochondrial state in the early phase of apoptosis, we used annexin V-FITC and a cyanine dye, diSC3(5), which shows the same staining pattern as R123. In Fig. 7, many annexin V-positive SLC cells retained \( \Delta \psi_m \) (upper right quadrant), but most of the annexin V-positive K-1 and 2-0 cells were already associated with mitochondrial dysfunction.

Recently we have established another two Friend erythroleukemia cell lines, CBA2 and CBA3, from FVp-infected CBA/N+/? mice without R-PK deficiency. Preliminary annexin V binding assay of diSC3(5)-stained cells of these lines indicated that \( \Delta \psi_m \) of annexin V-positive cells was already decreasing (Fig. 7). These data suggest that high \( \Delta \psi_m \) of apoptotic SLC cells was possibly associated with the loss of R-PK.

**DISCUSSION**

The SLC cell lines were well maintained in vitro, but the recovery rate from frozen materials was quite low, and this caused a population-selection problem in early passages of the cell lines. Therefore we had to use the SLC cell lines after stabilization by routine passaging for 12 months, during which time gradual loss of mutant R-PK expression occurred. This also caused variability in the experimental results with cells from earlier passages to later passages. The recently established cell lines from normal CBA/N mice without R-PK deficiency, CBA2 and CBA3, were easily kept frozen and their recovery rates were quite high.

Several studies have been carried out to investigate changes in PK activities and PK isozyme composition during erythroid differentiation. Takagawa et al. showed the presence of the M2-PK isozyme in proerythroblasts by immunofluorescence microscopy. Nijhof et al. reported that the major PK isozyme in murine CFUe was R-PK, and that M2-PK and R-M2 hybrids were also detectable by zymograms. During erythroid differentiation induced by EPO, the production of M2-PK decreased after one cell division, and the R-PK expression persisted, although the total PK activity per cell decreased dramatically. Max-Audit et al. used erythroblasts isolated from human fetal liver for erythroid differentiation studies. They demonstrated that total PK activity was much higher in basophilic than orthochromatid erythroblasts, and that the M2-PK activity markedly decreased, whereas the R-PK activity was only slightly modified during differentiation. These observations seemed similar to our present findings, and imply that R-PK gene mutation is unlikely to affect the expression of other PK isozyme genes in erythroid precursors, and that the SLC cells may develop decreased PK activity and decreased ATP content after R-PK becomes predominant owing to erythrodifferentiation.

In the differentiation-induction experiments on the SLC cell lines, differentiation or apoptotic change occurred unevenly and further study is necessary to confirm the relationship between mutant R-PK expression and apoptosis-induction among sorted cell populations. The apoptotic rate of the SLC cell lines during routine passage is much greater than that of ordinary Friend erythroleukemia cell lines, including the CBA2 and CBA3 cell lines discussed in “Results.” This might be caused in part by the cell populations with failed induction of erythrodifferentiation.

It is generally believed that mitochondrial change is essential in the induction of apoptotic phenomena, and we investigated the relationship between the occurrence of apoptotic change and the change of mitochondrial trans...
membrane potential. Unexpectedly, both R-PK-mutant erythroleukemic cell lines retained similar levels of mitochondrial membrane potential to those of the control erythroleukemic cells. The bioluminescence method is only applicable for the mass detection of ATP levels (Fig. 4). Flow cytometry enables the investigation of the relationship between the apoptotic change and Δψ_m change at the single cell level in defined cell populations. The latter method also showed that the R-PK-mutant cells retained normal Δψ_m levels until the final stage of apoptosis, as shown in Figs. 6 and 7. Actually, some fractions of apoptotic cell populations (R1 in Fig. 6a, for example) had almost normal levels of mitochondrial membrane potential (comparable to that of the live cell population, R3 in Fig. 6a). This suggests that apoptosis precedes mitochondrial dysfunction in our R-PK-mutant SLC cells.

If the levels of mitochondrial ATP biosynthesis decrease, Δψ_m should also decrease. However, if apoptosis is induced by other mechanisms than lowered ATP, apoptosis would not proceed in parallel with a decrease of Δψ_m. Then there should be a new category of apoptosis unrelated to mitochondrial changes.

Whether this apoptotic phenomenon without apparent mitochondrial change is directly due to the loss of R-PK activity requires further study. Normal murine R-PK gene transfection to these cell lines has not been successful due to marked cell death during the selection procedure of the transfectants, although this phenomenon was not observed in their normal counterparts without R-PK deficiency.

Tsuji-moto and his colleagues originally reported on the importance of the mitochondrial condition in the initiation of apoptotic change, and the ATP level in the mitochondrial membrane was found to differentiate apoptosis from necrosis. In this regard, there may be two types of apoptosis: in the first type, mitochondrial dysfunction precedes apoptotic change in association with the release of cytochrome c, while in the second type, apoptotic change somehow occurs and mitochondrial change comes later, in the final stage of apoptosis. In our preliminary experiments using our R-PK mutant cells, we could not detect caspase 8, which had been reported to be involved in mitochondrial dysfunction-independent apoptosis.

Our R-PK-mutant erythroleukemic cell lines as well as their normal counterparts thus provide suitable experimental systems for studying the role of mitochondrial change in apoptosis, particularly the relationship between the cellular ATP loss and such mitochondrial dysfunctions as lowered mitochondrial inner transmembrane potential and release of cytochrome c, as well as for studying PK isozyme changes during erythrodifferentiation.

ACKNOWLEDGMENTS

This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan. The authors are grateful for technical assistance by Ms. M. Watanabe, Mr. T. Kojima, Ms. J. Oka and Mr. Y. Tomimori. We also thank Mr. H. Asai who generously supplied CBA/N-\textit{Pk-1}^{lo}/\textit{Pk-1}^{lo} mice. (Received October 8, 1998/Revised November 11, 1998/ Accepted November 13, 1998)

REFERENCES

1) Valentine, W. N., Tanaka, K. R. and Miwa, S. A specific erythrocyte glycolytic enzyme defect (pyruvate kinase) in three subjects with congenital non-spherocytic hemolytic anemia. Trans. Assoc. Am. Physicians, 74, 100–110 (1961).

2) Miwa, S., Kanno, H. and Fujii, H. Concise review: pyruvate kinase deficiency: historical perspective and recent progress of molecular genetics. Am. J. Hematol., 42, 31–35 (1993).

3) Tani, K., Yoshikubo, T., Ikebuchi, K., Takahashi, K., Tsuchiya, T., Takahashi, S., Shimane, M., Ogura, H., Tojo, A., Ozawa, K. and Asano, S. Retrovirus-mediated gene transfer of human pyruvate kinase (PK) cDNA into murine hematopoietic cells: implications for gene therapy of human PK deficiency. Blood, 83, 2305–2310 (1994).

4) Morimoto, M., Kanno, H., Asai, H., Tsujimura, T., Fujii, H., Moriyama, Y., Kasugai, T., Hirono, A., Ohiba, Y. and Miwa, S. Pyruvate kinase deficiency of mice associated with nonspherocytic hemolytic anemia and cure of the anemia by marrow transplantation without host irradiation. Blood, 86, 4323–4330 (1995).

5) Kanno, H., Morimoto, M., Fujii, H., Tsujimura, T., Asai, H., Noguchi, T., Kitamura, Y. and Miwa, S. Primary structure of murine red blood cell-type pyruvate kinase (PK) and molecular characterization of PK deficiency identified in the CBA strain. Blood, 86, 3205–3210 (1995).

6) Imamura, K. and Tanaka, T. Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. J. Biochem., 71, 1043–1051 (1972).

7) Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K. and Nishima, T. Electrophoretic and kinetic studies of mutant erythrocyte pyruvate kinase. Blood, 43, 537–548 (1974).

8) Imamura, K., Tanaka, T., Nishina, T., Nakashima, K. and
Miwa, S. Studies of pyruvate kinase (PK) deficiency. II. Electrophoretic, kinetic, and immunological studies of pyruvate kinase of erythrocytes and other tissues. *J. Biochem.*, **74**, 1165–1175 (1973).

9) Noguchi, T., Yamada, K., Inoue, H., Matsuda, T., and Tanaka, T. The L- and R-isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J. Biol. Chem.*, **262**, 14366–14371 (1987).

10) Kanno, H., Fujii, H. and Miwa, S. Structural analysis of human pyruvate kinase L-gene and identification of the promoter activity in erythroid cells. *Biochem. Biophys. Res. Commun.*, **188**, 516–523 (1992).

11) Takagawa, S., Fujii, H. and Miwa, S. Change of pyruvate kinase isozymes from M2- to L-type during development of the red cell. *Br. J. Haematol.*, **54**, 467–474 (1983).

12) Nijhof, W., Wierenga, P., Staal, G. and Jansen, G. Changes in activities and isozyme patterns of glycolytic enzymes during erythroid differentiation in vitro. *Blood*, **64**, 607–613 (1984).

13) Max-Audit, I., Testa, U., Kechemir, D., Mithacila, M., Vainchenker, W. and Rosa, R. Pattern of pyruvate kinase isozymes in erythroleukemia cells lines and in normal human erythroblasts. *Blood*, **64**, 930–936 (1984).

14) Noguchi, T., Inoue, H. and Tanaka, T. The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J. Biol. Chem.*, **261**, 13807–13812 (1986).

15) Ikawa, Y., Aida, M. and Inoue, Y. Isolation and characterization of high and low differentiation-inducible Friend leukemia lines. *Gann*, **67**, 767–770 (1976).

16) Johnson, P., Chung, S. and Benchimol, S. Growth suppression of Friend virus-transformed erythroleukemia cells by p53 protein is accompanied by hemoglobin production and is sensitive to erythropoietin. *Mol. Cell. Biol.*, **13**, 1456–1463 (1993).

17) Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J. and Melamed, M. R. Interactions of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.*, **42**, 799–806 (1982).

18) Bammel, B. P., Brand, J. A., Germon, W. and Smith, J. C. Interaction of the extrinsic potential-sensitive molecular probe diS-C3-(5) with pigeon heart mitochondria under equilibrium and time-resolved conditions. *Arch. Biochem. Biophys.*, **244**, 67–84 (1986).

19) Beutler, E., Blume, K., Kaplan, J., Löhr, G., Ramot, B. and Valentine, W. International committee for standardization in Haematology: recommended methods for red cell enzyme analyses. *Br. J. Haematol.*, **35**, 331–340 (1977).

20) Schröter, W. and Kalinowsky, W. Erythrocyte 2,3-diphosphoglycerate mutase: an optical test in hemolysates. *Clin. Chim. Acta.*, **26**, 283–285 (1969).

21) Tsujino, K., Kanno, H., Hashimoto, K., Fujii, H., Iippo, T., Morii, E., Lee, Y. M., Asai, H., Miwa, S. and Kitamura, Y. Delayed onset of hemolytic anemia in CBA-Pk-1slc/Pk-1slc mice with a point mutation of the gene encoding red blood cell type pyruvate kinase. *Blood*, **91**, 2169–2174 (1998).

22) Moriya, K., Tone, S., Mukaida, M. and Takano-Ohmuro, H. The apoptotic and nonapoptotic nature of the terminal differentiation of erythroid cells. *Exp. Cell Res.*, **240**, 206–217 (1998).

23) Narita, H., Yanagawa, S., Sasaki, R. and Chiba, H. Induction of 2,3-bisphosphoglycerate synthase in Friend leukemia cells. *Biochem. Biophys. Res. Commun.*, **103**, 90–96 (1981).

24) Kim, H., Koury, M., Lee, S., Im, J. and Sawyer, S. Metabolic adaptation during erythropoietin-mediated terminal differentiation by mouse erythroid cells. *Blood*, **77**, 387–392 (1991).

25) Vander-Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T. and Thompson, C. B. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell*, **91**, 627–637 (1997).

26) Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132–1136 (1997).

27) Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Kramer, P. H. and Peter, M. E. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.*, **17**, 1675–1687 (1998).

28) Eguchi, Y., Shimizu, S. and Tsujimoto, Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res.*, **57**, 1835–1840 (1997).