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

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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 ($\Delta\psi_m$), detected by rhodamine 123 (R123) and $diSC_3(5)$ 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 $\Delta\psi_m$ 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 erythrodifferentiation, 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^{slc}/Pk-1^{slc}* 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 isozymes[6, 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^{slc}/Pk-1^{slc}*) 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

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($\Delta\psi_m$) 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-*Pk-1^{slc}/Pk-1^{slc}* 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-*Pk-1^{slc}/Pk-1^{slc}* 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% CO_2 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-*Pk-1^{slc}/Pk-1^{slc}* 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[15] in IMDM medium with 10% FCS and 20 μM 2-mercaptoethanol. T3-K-1 (K-1) and T3-Cl-2-0 (2-0), both derived from DDD/1 mice, were used as normal R-PK Friend leukemia cell lines. K-1 is resistant and 2-0 is sensitive to induced erythrodifferentiation. They were maintained in RPMI-1640 medium (Life Technologies, Inc.) with 10% FCS.

Erythrodifferentiation induction The cells were seeded at 5×10^5 cells/ml in IMDM medium or RPMI-1640 medium containing one of the following erythrodifferentiation 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 mM and recombinant human erythropoietin (EPO) 2 U/ml were used as erythrodifferentiation inducers. Hemoglobin-positive cells were counted after staining with the non-toxic benzidine analog, 2,7-diaminofluorene (DAF).[16]

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*.[5] Briefly, total cellular RNA obtained from the respective cell lines by the acid guanidinium-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-1^{slc}* 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 *Bst*PI to see whether the recognition site was destroyed by the mutation.

Measurement of cellular ATP content A total of 1×10^6 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 $\Delta\psi_m$ by flow cytometry To measure $\Delta\psi_m$, R123 (Sigma) and a cyanine dye, diSC_3(5) (Molecular Probes, Eugene, OR), were used as described by Darzynkiewicz *et al*.[17] and Bammel *et al*.[18] Briefly, 1×10^6 cells were incubated with rhodamine 123 (R123, 10 μg/ml) for 15–30 min in culture medium under 5% CO_2 at 37°C, or were incubated with diSC_3(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 diSC_3(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 1×10^6 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 $CaCl_2$ (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 (1×10^5 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 Ca^{2+} 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 5×10^7 living cells were sonicated in 200 μl of 2.7 m*M* EDTA, 0.7 m*M* 2-mercaptoethanol, and centrifuged at 15,000*g* for 10 min at 4°C. Supernatants were used for PK assay,[19] diphosphoglycerate mutase (DPGM) assay[20] and protein assay.

Western blot analyses of PK isozymes Protein extracts prepared from 5×10^6 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 m*M* Tris, 192 m*M* 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-1*^{slc}/*Pk-1*^{slc} 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 glycophorin-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).

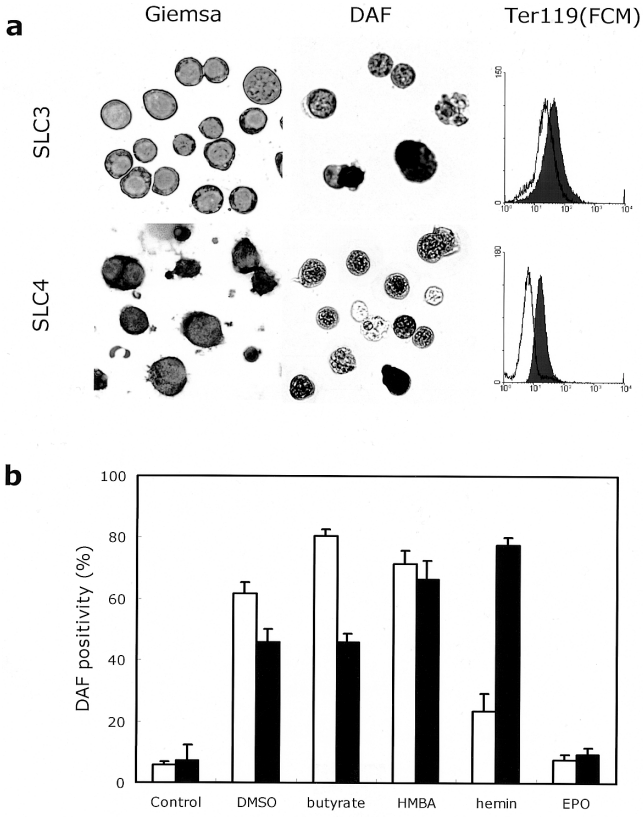

Fig. 1. a) Erythroid nature of SLC cells visualized by DAF staining and flow cytometry using Ter119 on the cells 3 days after DMSO treatment. ■ Ter119, □ isotype. b) Erythrodifferentiation by several inducers. DAF positivity of the cells 3 days after inducer treatment. □ SLC3, ■ SLC4.

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-1*^{slc}/*Pk-1*^{slc} 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.[5, 21]

Apoptotic changes during passage Both SLC3 and SLC4 lines undergo spontaneous apoptosis during routine

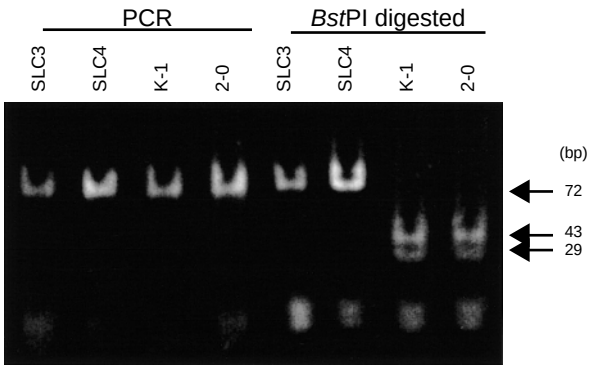

Fig. 2. Polyacrylamide gel electrophoresis showing uncleaved bands of the 72 bp DNA fragment by *Bst*PI.

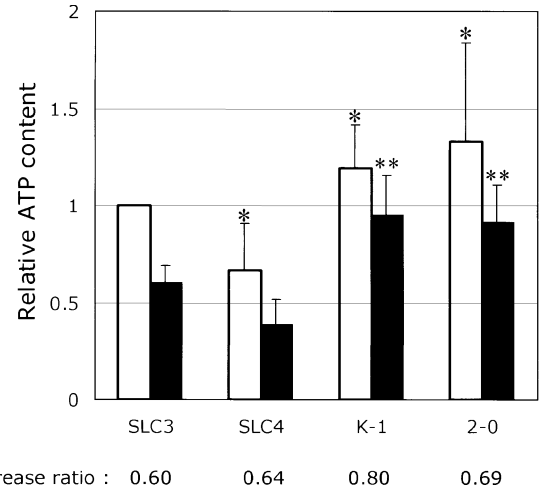

Fig. 4. ATP assay by a bioluminescence method. The cells were harvested 2 days after treatment with 2 % butyrate. Values are mean±SD. ∗ $P<0.05$ compared with non-differentiated SLC3 and ∗∗ $P<0.05$ compared with differentiated SLC3. □ non-differentiated, ■ differentiated.

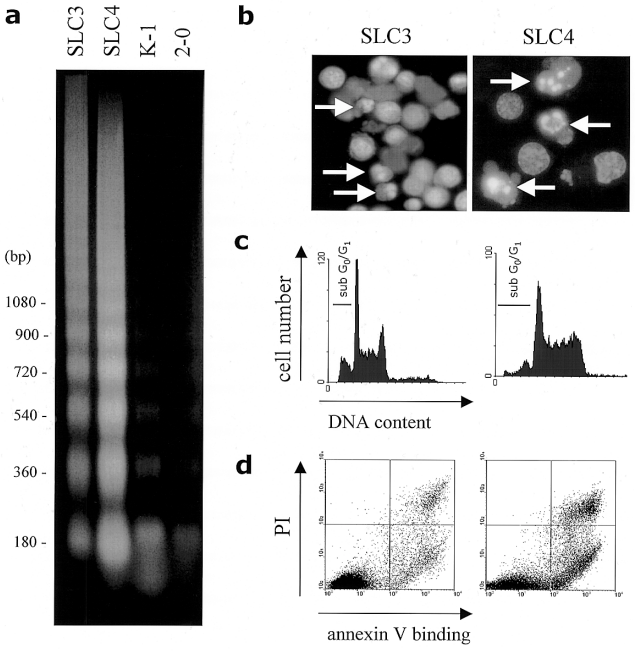

Fig. 3. Spontaneous apoptosis of SLC cell lines, day 3 after subculture. a) DNA laddering of the cell samples at confluent stages. b) Nuclear fragmentation visualized with Hoechst 33258. c) Flow cytometric analysis of PI-stained cells. Each peak shows subG_0/G_1, G_0/G_1, S, G_2/M (from left to right). SubG_0/G_1 peaks reflect apoptotic cells. d) Annexin V binding flow cytometry. Populations in the right lower quadrant are in apoptosis; 10–30 % in routine passage.

Table I. DPGM and Pyruvate Kinase Activities with or without Induction of Erythrodifferentiation

	Erythrodifferentiation induction		(+)/(−)
	(−)	(+)	
DPGM activity			
SLC3	4.76±1.20	6.64±0.49	1.4*
SLC4	7.62±2.42	14.3±2.11	1.88*
K-1	5.34±1.03	6.94±0.64	1.3*
2-0	4.01±0.32	8.18±0.51	2.04*
PK activity			
SLC3	4.2±0.78	3.0±1.08	0.72
SLC4	2.7±0.50	1.9±0.16	0.71*
K-1	3.5±0.95	6.4±0.64	1.81*
2-0	3.9±0.71	5.0±0.73	1.27*

DPGM activity increased upon induction of erythrodifferentiation in the respective cell lines. IU/mg protein (mean ±SD), $n=4$. In contrast, PK activity of SLC cells was reduced, whereas that of the control Friend cells was increased in parallel with DPGM activity. IU/mg protein (mean±SD), $n=5$. ∗ $P<0.05$.

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 erythrodifferentiation of erythroblastoid cells.[22)]

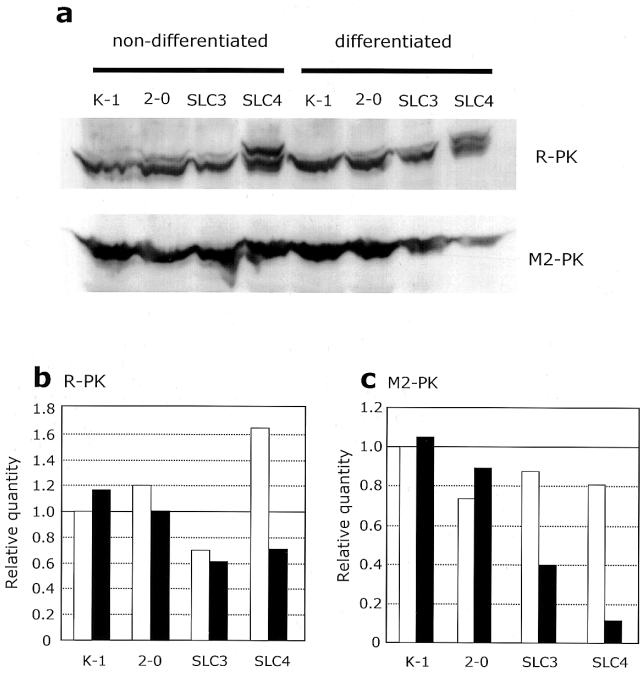

Fig. 5. Western blot analysis of PK isozymes. a) Western blot images, and b, c) densitometric band analysis of R-PK and M2-PK, respectively. The cells were harvested 3 days after usual passages and erythrodifferentiation induced by 2% butyrate. □ non-differentiated, ■ differentiated.

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 cells[23, 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 uninduced 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.

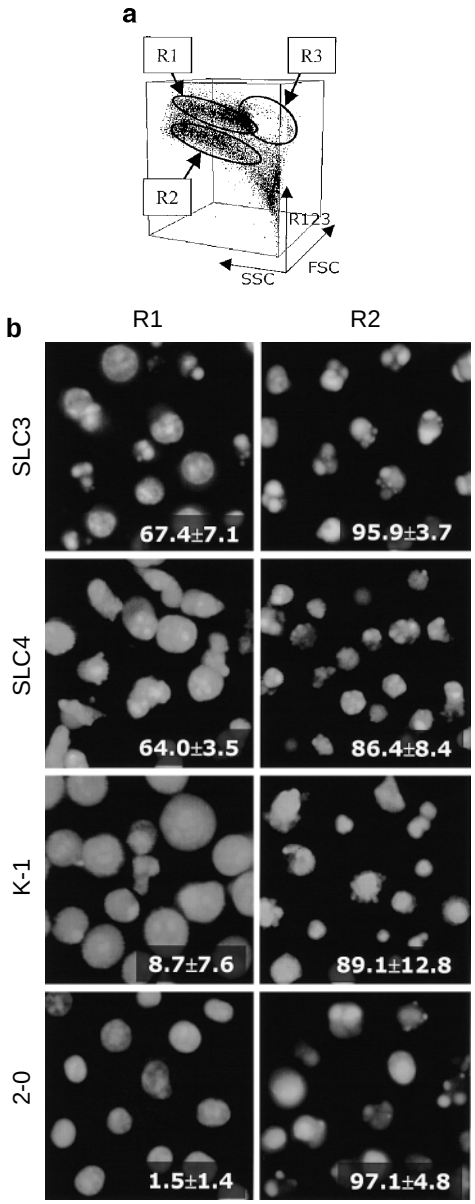

Fig. 6. a) Representative three-dimensional analysis of SLC3 cells, showing two distinct apoptotic populations in R1 and R2 (R1, high rhodamine 123; R2, low rhodamine 123). Live population in R3. b) Hoechst 33258-stained morphology and the percentage of cells with fragmented nuclei of R1 and R2 populations in SLC3, SLC4, K-1 and 2-0 cells (3 days after subcultures) are shown. Two-thirds of R1 populations in SLC3 and SLC4 lines are apoptotic.

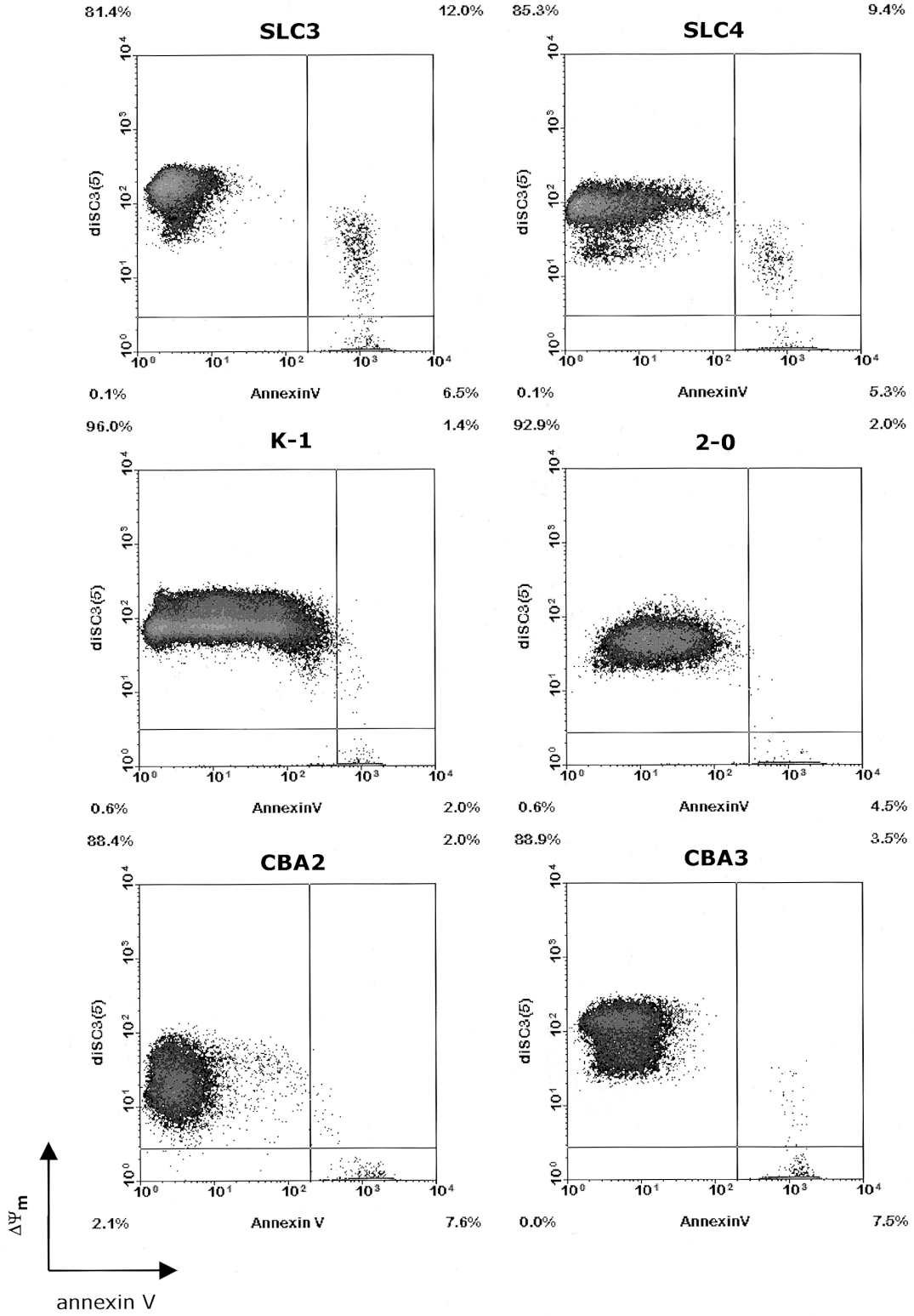

Fig. 7. Annexin V binding assay of those $diSC_3(5)$-stained cells which retain dye-exclusion in PI-staining. The upper left population is live cells and the lower right population is completely apoptotic cells. The upper right population existed only in the case of SLC cells.

Western blot analyses were performed to examine the expression of the M2- and R-PK isozymes during erythrodifferentiation. 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 matrix. 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 $diSC_3(5)$. 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, $diSC_3(5)$, 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 $diSC_3(5)$-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.[11] 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.[12] 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.[13] They demonstrated that total PK activity was much higher in basophilic than orthochromatic 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,[25] 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 $\Delta\psi_m$ change at the single cell level in defined cell populations. The latter method also showed that the R-PK-mutant cells retained normal $\Delta\psi_m$ 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, $\Delta\psi_m$ should also decrease. However, if apoptosis is induced by other mechanisms than lowered ATP, apoptosis would not proceed in parallel with a decrease of $\Delta\psi_m$. Then there should be a new category of apoptosis unrelated to mitochondrial changes. If this is the case, it would be interesting to investigate the signaling cascade in these R-PK-mutant erythroleukemia cell lines.

In this regard, there is a report by Kluck et al. that cytochrome *c* release takes place without $\Delta\psi_m$ change,[26] and there is another report by Scaffidi *et al*. that caspase 8 accumulation induces apoptosis independently of mitochondrial change.[27]

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.

Tsujimoto 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.[28] 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.[27]

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.

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