mitotic recombination between homologous alleles, or whole chromosome loss [20]. Molecular analysis can distinguish between them and reveal the mechanism and the characteristics of the mutants. In this study, $KBrO_3$ predominantly induced large deletions that resulted in hemizygous LOH (Table 1). The large deletions were mainly terminal deletions in the proximal region of chromosome 17q, which were rarely observed in spontaneously arising *TK* mutants (Fig. 3). The mutational spectrum and LOH pattern induced by $KBrO_3$ were similar to those induced by X-irradiation (Figs. 2 and 3) [20,21]. DSBs induced X-rays cause large deletions [19,20]. When the DSBs are repaired by the non-homologous end-joining pathway, interstitial deletions result. The broken chromosome ends can be also stabilized by the addition of new telomere sequences. Because TK6 cells have high telomerase activity [20], the result is terminal deletions. Thus, the major genotoxicity of $KBrO_3$ may be due to DSBs, but not to 8OHdG converting GC > TA transversion.

Some 8OHdG lesions can convert DSBs through the BER pathway [37]. In the initial step of BER, Ogg1 removes 8OHdG by DNA glycosylase activity and nicks the DNA backbone because of its associated lyase activity. The resulting SSB is processed by an apurinic endonuclease, which generates a single nucleotide gap. The gap is filled in by a DNA polymerase and sealed by a DNA ligase [38]. Clustered 8OHdG lesions induced by $KBrO_3$ may not be appropriately repaired by BER and cause DSB, however, because it is possible that two closely opposed 8OHdGs convert two closely opposed SSBs by BER resulting DSB [39,40]. Yang et al. developed Ogg1 over-expressing TK6 cell (TK6-hOGG1) and examined cytotoxic and mutagenic responses to gamma-irradiation [41]. They demonstrated that TK6-hOGG1 cells are more sensitive than the parental TK6 cells to cytotoxicity and mutagenicity by gamma-irradiation, and most of the induced *TK* mutants in TK6-OGG1 exhibited SG phenotype, which were probably large deletion mutants resulted by DSBs. This result clearly indicates that BER pathway contributes to convert oxidative damages to DSBs. Some clustered 8OHdG induced by $KBrO_3$ may covert to DSBs in TK6 cells, because TK6 is Ogg1 proficient cells [37].

To clarify the genotoxic characteristics of $KBrO_3$, we investigated the gene expression profile using Affymetrix GeneChip® Expression analysis. Many genes were up- or down-regulated by exposure to 2.5 mM $KBrO_3$ (Tables 2 and 3). Akerman et al. investigated the alterations of gene expression profiles in ionizing radiation-exposed TK6 cells [42]. They reported a >50% increase in expression of ATF-3 (stress response), Cyclin G (cell cycle), FAS antigen (apoptosis), GADD45 (repair and apoptosis), PCNA (repair), Rad51 (repair), and p21 (cell cycle) and a 40% decrease in expression of c-Myc (transcription factor), interferon stimulatory gene factor-3 (cell signaling), and p55CDC (cell cycle). We also observed up-regulation of p21 and down-regulation of c-Myc. Up-regulation of p21, however, is observed in TK6 cells exposed to any DNA-damaging chemical [43]. Islaih et al. also demonstrated the relationship between the gene expression profiles and the DNA damaging agents using TK6 cells [43]. They examined six chemicals including H_2O_2 and bleomycin which induce oxidative DNA damage. Although 10 genes were commonly up-regulated between H_2O_2 and bleomycin treatments, these genes except for p21 were not observed in our experiment. Thus, we could not find the similarity of gene expression profile by the treatment with $KBrO_3$ to by the treatment with ionizing radiation as well as oxidative damage inducers. Comparing gene expression profiles across platforms, laboratories, and experiments must be difficult [44]. Although it is difficult to judge from the expression analysis of the single chemical, information on genes which altered their expression gives a clue to understand the mechanism of action. Firstly, predominance of DNA repair and cell cycle related genes in up-regulated genes supports the genotoxic action of $KBrO_3$. Up-regulation of stress genes and apoptosis related genes suggests an involvement of oxidative stress. Up-regulation of catalase may be responsible for the oxidative damage by $KBrO_3$ (Table 2). Unclassified genes for alteration may have a functional relationship with genotoxic mechanism.

In conclusion, $KBrO_3$ predominantly induced large deletions at chromosomal level in human TK6 cells. The major genotoxicity leading to carcinogenesis of $KBrO_3$ may be due to DSBs rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed.

Acknowledgments

The TK6 cell line used in this study was a kind gift of Dr. John B. Little of the Harvard School of Public Health, Boston, MA. This study was supported by Health, Welfare, and Labor Science Research Grants (H15-chem-002, H15-food-004) in Japan.

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Experimental Hematology

Experimental Hematology 35 (2007) 1190–1200

Glycolytic inhibition by mutation of pyruvate kinase gene increases oxidative stress and causes apoptosis of a pyruvate kinase deficient cell line

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(Received 13 November 2006; revised 8 May 2007; accepted 9 May 2007)

Objective. **SLC3 is a Friend erythroleukemic cell line established from the *Pk-1*^{slc} mouse, a mouse model of red blood cell type-pyruvate kinase (R-PK) deficiency. This study was aimed to elucidate the mechanisms attributing to apoptosis induced by R-PK deficiency.**

Materials and Methods. **SLC3 and a control Friend cell line, CBA2, were cultured in a condition of glucose deprivation or supplementation with 2-deoxyglucose, and apoptosis was detected by annexin V. We established two stable transfectants of SLC3 cells with human R-PK cDNA, and examined the effect of R-PK on an apoptotic feature by cell cycle analysis. Intracellular oxidation was measured with 2′,7′-dichlorofluorescin diacetate. DNA microarray analysis was performed to examine gene-expression profiles between the two transfectants and parental SLC3.**

Results. **SLC3 was more susceptible than CBA2 to apoptosis induced by glycolytic inhibition. The forced expression of R-PK significantly decreased cells at the sub G_0/G_1 stage in an expression-level dependent manner. Microarray analysis showed that proapoptotic genes, such as *Bad*, *Bnip3*, and *Bnip3l*, were downregulated in the transfectants. In addition, peroxiredoxin 1 (*Prdx1*) and other antioxidant genes, such as *Cat*, *Txnrd1*, and *Glrx1* were also downregulated. A significant decrease of dichlorofluorescein fluorescence was observed by R-PK expression. Preincubation with a glutathione precursor showed a significant decrease of apoptosis.**

Conclusion. **These results indicated that glycolytic inhibition by R-PK gene mutation augmented oxidative stress in the Friend erythroleukemia cell, leading to activation of hypoxia-inducible factor-1 as well as downstream proapoptotic gene expression. Thus, R-PK plays an important role as an antioxidant during erythroid differentiation.** © **2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Glycolysis is an essential metabolic pathway in all organisms. Pyruvate kinase (PK) is a key glycolytic enzyme, and has four isoenzymes in mammals, designated M_1, M_2, L (liver), and R (red blood cell). In humans, these isozymes are encoded by two structural genes, *PKM* and *PKLR*, respectively [1]. M_2-PK is the only isozyme that is active in early fetal tissues and also almost ubiquitously expressed in adult tissues, including hematopoietic stem cells, progenitors, leukocytes, and platelets. Red blood cell type-pyruvate kinase (R-PK) becomes a major isozyme during erythroid differentiation/maturation [2,3], and in mature red blood cells (RBCs), R-PK is the only detectable PK isozyme. Deficiency of R-PK causes shortened RBC survival, resulting in hemolytic anemia. In humans, PK deficiency is the most prevalent glycolytic enzyme defect, which is responsible for hereditary hemolytic anemia [4,5].

We have previously established SLC3 [6], a line of Friend erythroleukemic cells from the *Pk-1*^{slc} mouse [7], which has chronic hemolytic anemia with marked splenomegaly due to a missense mutation of the murine *Pklr* gene [8]. SLC3 showed spontaneous apoptosis during routine passage and in vitro erythroid differentiation by butyrate exacerbated apoptosis of SLC3 [6]. Recently, we examined the spleen of a subject with severe PK deficiency [9], and discovered enhanced extramedullary hematopoiesis as well as apoptotic erythroid cells. Enhanced apoptosis

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0301-472X/07 $–see front matter.
doi: 10.1016/j.exphem.2007.05.005

was also identified in TER119-positive erythroid cells isolated from *Pk-1*^{slc} mice [10]. These results provide evidence that the metabolic disturbances in PK deficiency affect not only the survival of RBCs but also the maturation of erythroid progenitors, which results in apoptosis.

In this study, we examined whether Friend erythroleukemic cell lines showed apoptosis when glycolysis was inhibited. To evaluate whether overexpression of the normal R-PK gene ameliorated apoptosis, we established stable transfectants of SLC3 and compared their apoptotic characteristics and transcriptional profiles with parental SLC3. We present here several pieces of evidence, revealing the biological significance of R-PK to suppress oxidative stress during erythroid differentiation.

Materials and methods

Cell culture and flow cytometric analysis

Friend erythroleukemic cell lines SLC3 and CBA2 have been described previously [6]. Both cell lines are maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 20 μM 2-mercaptoethanol, and a mixture of penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA).

To evaluate the adverse effects of glycolytic inhibition, cells were cultured in either glucose-free RPMI-1640 (Invitrogen) or RPMI-1640 with 2-deoxyglucose (2-DG) at final concentrations of 0.1, 1, and 10 mM. Iscove's modified Dulbecco's medium containing 110 mg/L sodium pyruvate, and RPMI-1640 containing no pyruvate.

Flow cytometric analysis was performed by EPICS XL and analyzed with software, EXPO32 ADC (Beckman-Coulter, Fullerton, CA, USA). Annexin V-Alexa568 and rhodamine 123 were obtained from Roche Diagnostics (Basel, Switzerland) and Sigma, respectively. To examine the effect of N-acetyl-L-cysteine upon apoptosis, we preincubated cells in RPMI-1640 supplemented with 10 mM N-acetyl-L-cysteine for 12 hours, followed by 12- to 24-hour incubation with RPMI-1640.

Establishment of stable transfectants expressing normal R-PK in SLC3 cells

We constructed a human R-PK cDNA expression plasmid vector in erythroid cells. A 1.7-kb fragment covering the entire coding region of human R-PK cDNA [11] was introduced into *KpnI-EcoRV* sites of pcDNA3.1 (Invitrogen). Plasmid DNA was purified with an EndoFree Maxi DNA purification kit (Qiagen, Hilden, Germany). Transfection was done with Effectene Transfection Reagent (Qiagen) as indicated by the manufacturer. Transfected cells were selected using G418 (400 μg/mL).

RT-PCR, Western blotting, and enzyme assay

Total cellular RNA was extracted with an RNeasy purification kit (Qiagen), and 2 μg RNA was reverse-transcribed (RT) at 42°C for 90 minutes with 50 pmole oligo (dT)17 primer, 0.5 U/μL cloned RNase inhibitor (Takara Bio, Shiga, Japan), 10 mM dithiothreitol, 1 mM deoxyribonucleoside triphosphate, and 50 U Expand Reverse Transcriptase (Roche Diagnostics). Aliquots (1/10) were subjected to PCR using primer pairs specifically amplified with human and murine R-PK cDNA, hRPK-F (5′-TGGCCCAGC CTACCCTTGTA-3′)/hRPK-R (5′-CTTAAAGGTGGGGCTTTG GA-3′) and mRPK-F (5′-GCAGATGATGTGGACCGAAG-3′)/ mRPK-R (5′-CTAGATGGCAGATGTGGGACTA-3′), respectively. The reaction mixtures were subjected to 40 cycles of amplification consisting of 94°C for 20 seconds, 60°C for 10 seconds, and 72°C for 10 seconds for hRPK and 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds for mRPK in a GeneAmp PCR system 2400 (Roche Diagnostics, Switzerland), and separated using 2% agarose gel electrophoresis.

For Western blot analysis, cells were harvested, followed by washing with phosphate-buffered saline twice. Following three-times freezing and thawing in extraction buffer (10 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 0.003% 2-mercaptoethanol, 0.02 mM ethylenediamine tetraacetic acid), cell extracts were obtained for Western blot analysis. Protein assays were performed by the method of Bradford using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).Western blot analysis was conducted using anti-rat L-PK (kindly provided Tamio Noguchi, Nagoya University) and ECL advance Western Blotting Detection Kit (Amersham Biosciences, Buckinghamshire, UK).

PK and lactate dehydrogenase (LDH) activity was measured, as described [12].

Microarray analysis

To prepare high-quality total cellular RNA for the GeneChip assay, RNA was extracted with modified protocols using the TRIzol LS (Invitrogen) and RNeasy purification kit (Qiagen). Briefly, cells were harvested with no washing step, and immediately homogenized with the RLT buffer. The lysate was then mixed with 3 volumes of the TRIzol LS. After a 10-minute incubation at room temperature, the sample solution was mixed with an equal volume of chloroform. The sample was centrifuged at 10,000*g* for 15 minutes at 4°C, and then the upper aqueous phase was transferred to a fresh tube. After mixing with an equal volume of 70% ethanol, the sample was incubated for 10 minutes at room temperature. Without any flash step, the sample solution was transferred to the RNeasy column, and then processed by the manufacturer recommended protocol.

To normalize the variation in data based on the cell count, we used *Bacillus subtilis* RNA for an external standard signal, which was added to the cell lysate in proportion to the sample's DNA contents [13]. Ten microliters of cell lysate was provided for DNA quantification using Picogreen (Invitrogen). GeneChip (Affymetrix, Santa Clara, CA, USA) analysis was carried out according to the Affymetrix-recommended protocols. Processed RNA was hybridized to the Affymetrix Murine Genome 430A arrays (22960 probe sets). Signal values were calculated from scanned images by the Affymetrix Microarray Operation System (GCOS). The cell sample was pooled from six culture dishes at each condition and one GeneChip was used per one pooled sample.

Data analysis

Data were normalized by an original program (SCal), which processes data in proportional conversion based on the DNA content of each biosample [13]. This DNA content-based normalization method improves the measurement accuracy of GeneChip. For example, a series of samples was measured by quantitative PCR and Affymetrix GeneChip microarrays using this method, and the results showed up to 90% concordance [13].

To identify differentially expressed genes, we used an empirical threshold calculated by an original algorithm (Fx). The Fx threshold is based on the signal intensity level and is calculated as follows: $Y = X \cdot (1 + RC^{(w-\log X)})$ and $Y = X \cdot (1 + C^{(w-\log X)})^{-1}$ (Fx1 and Fx2 respectively; C and w are constant parameters reflecting actual measurement data by GeneChip hybridized with the standard sample). C and w were set to 3.0 and 2.5, respectively, which was equivalent to $p < 0.02$. In the scatter plot, the spots above the Fx1 line were evaluated as upregulated, and the spots below the Fx2 line were evaluated as downregulated.

Results

SLC3 is more susceptible than the control to apoptosis due to glycolytic inactivation

Figure 1 shows flow cytometric analysis using annexin V (horizontal axis) and rhodamine 123 (vertical axis) to examine the effects of glycolysis inhibition on Friend leukemic cells with or without R-PK mutation. SLC3 showed spontaneous apoptosis during routine passage, and apoptosis preceded mitochondrial dysfunction in the R-PK–deficient erythroleukemia cells as reported previously [6]. The result showed that a part of apoptotic cells kept similar mitochondrial transmembrane potentials and that SLC3 were much more susceptible to glucose deprivation as well as 2-DG.

Overexpression of wild-type R-PK decreases apoptosis of SLC3

In order to evaluate how wild-type R-PK rescues apoptotic phenotypes, we established two stable transfectants of SLC3 with overexpression of the human R-PK cDNA. Figure 2 shows RT-PCR and Western blot analysis of a parental SLC3 and SLC3-hRPK.Hi (hRPK.Hi) and SLC3-hRPK.Lo (hRPK.Lo). As shown in Figure 2A, the expression level of the transgene was higher in hRPK.Hi than hRPK.Lo. Overexpression of human R-PK suppressed endogenous R-PK expression as observed in the lane of hRPK.Hi.

Enzymatic analysis of transfectants revealed that PK activities of hRPK.Lo and Hi were 17.2 and 24.2 IU/mg protein, respectively. The PK activity of hRPK.Hi was almost comparable to parental SLC3, 23.5 IU/mg protein. It should be noted that endogenous LDH activity was decreased by transgene expression, leading to a PK/LDH ratio increase from 0.4 (SLC3) to 0.48 (hRPK.Lo) and 0.6 (hRPK.Hi).

We evaluated apoptosis of the two transfectants by cell cycle analysis. Figure 2C shows that the expression of wild-type R-PK decreased the number of cells at the sub-G_0/G_1 stage. While hRPK.Lo showed almost the same number of sub-G_0/G_1 cells (55.5%) as SLC3 (57.4%), only 19.3% of hRPK.Hi were arrested at the sub G_1-stage. Because apoptotic cells were rescued from apoptosis in an R-PK expression level-dependent manner, it is most likely that R-PK activity is required to suppress apoptosis of erythroid cells.

Microarray analysis elucidates the differential expression of genes involved in reactive oxygen species removal, cell cycle, and apoptosis

Gene expression profiles between the two transfectants and the parental SLC3 cell line were analyzed by DNA microarray analysis. After exchanging culture medium, SLC3, hRPK.Lo, and Hi were sampled at 24 and 67 hours, which were the phase of reentry into cell cycling and of subconfluence, respectively. Transgene expression upregulated only about 2% (469 probe sets) of genes, whereas approximately 25% (5754 probe sets) of genes were downregulated both in hRPK.Hi and hRPK.Lo at 24 and/or 67hours. As shown in Figure 3B, major categories of the downregulated genes involved the cell cycle, development, and apoptosis. Proapoptotic genes including *Bad*, *Bnip3*, and *Bnip3l*, as well as *Casp 2, 6, 7,* and *8* were downregulated (Figs. 3A and 4).

Genes of key glycolytic enzymes such as hexokinase-2 (*Hk2*), phosphofructokinase (*Pfkl*), phosphoglycerate kinase (*Pgk1*), and PK (*Pklr*) were downregulated, and expression levels were characteristically decreased after 67 hours of transfection, suggesting that suppression requires protein synthesis.

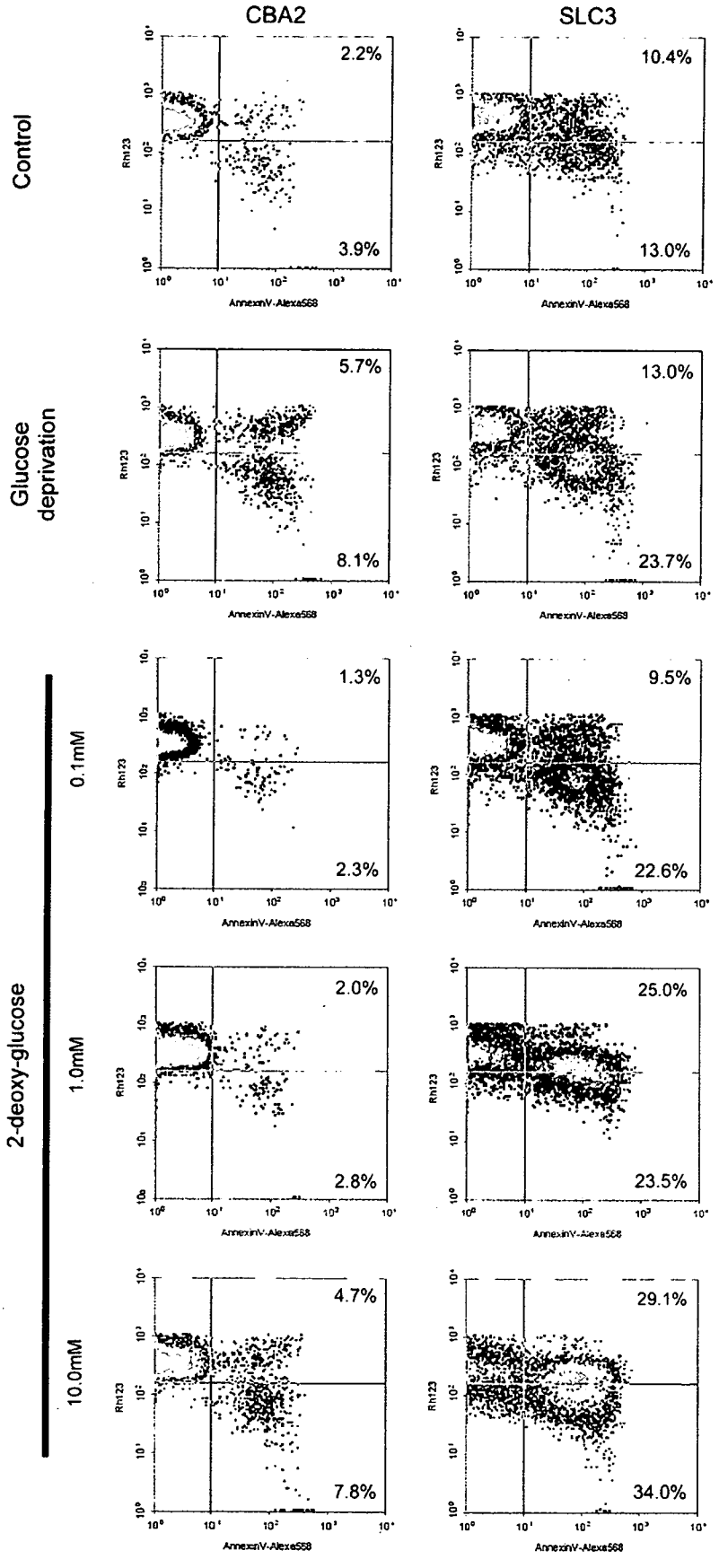
It should be noted that genes for antioxidant protein, such as peroxiredoxin 1 (*Prdx1*) and related genes, such as catalase (*Cat*), thioredoxin reductase 1 (*Txnrd1*), and glutaredoxin 1 (*Glrx1*), which have a role in the modulation of oxidative stress, are also downregulated. As for *Prdx2*, expression change by the transgene was not evident. Intracellular reactive oxygen species (ROS) are known to cause DNA damage, inducing the expression of DNA repair genes. In this experiment, expressions of genes involved in DNA repair were decreased, including *Brca1*, *Brca2*, and *Rad51*.

PK gene mutation and glycolytic inhibition by 2-DG augment intracellular ROS

We examined intracellular ROS in SLC cells and control CBA2 cells by 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), an indicator of the intracellular formation of hydrogen peroxide and free radicals. Nonfluorescent DCFH-DA turns into DCFH (2′,7′-dichlorofluorescin) in the presence of hydrogen peroxide, and then DCFH is quickly photo-oxidized to fluorescent DCF (2′,7′-dichlorofluorescein).

Figure 5A shows that SLC3 is hypersensitive to a glycolytic inhibitor, 2-DG, producing intracellular DCF by adding 1 mM 2-DG. In contrast, control CBA2 cells do not produce DCF even at 10 mM 2-DG for 30 minutes.

Reduced glutathione (GSH) is an important antioxidant in erythrocytes. GSH is produced by a two-step enzymatic reaction involving γ-glutamylcystein synthetase and glutathione synthetase (GSH-S). Apoptosis induced either by the glycolytic gene mutation (SLC3) or the glycolytic inhibitor (CBA with 2-DG) was suppressed by preincubation with the glutathione precursor, NAC (Fig. 5B). Finally, the


CBA2
SLC3
Control
Glucose deprivation
2-deoxy-glucose
0.1mM
1.0mM
10.0mM
2.2%
3.9%
10.4%
13.0%
5.7%
8.1%
13.0%
23.7%
1.3%
2.3%
9.5%
22.6%
2.0%
2.8%
25.0%
23.5%
4.7%
7.8%
29.1%
34.0%
Rh123
AnnexinV-Alexa568

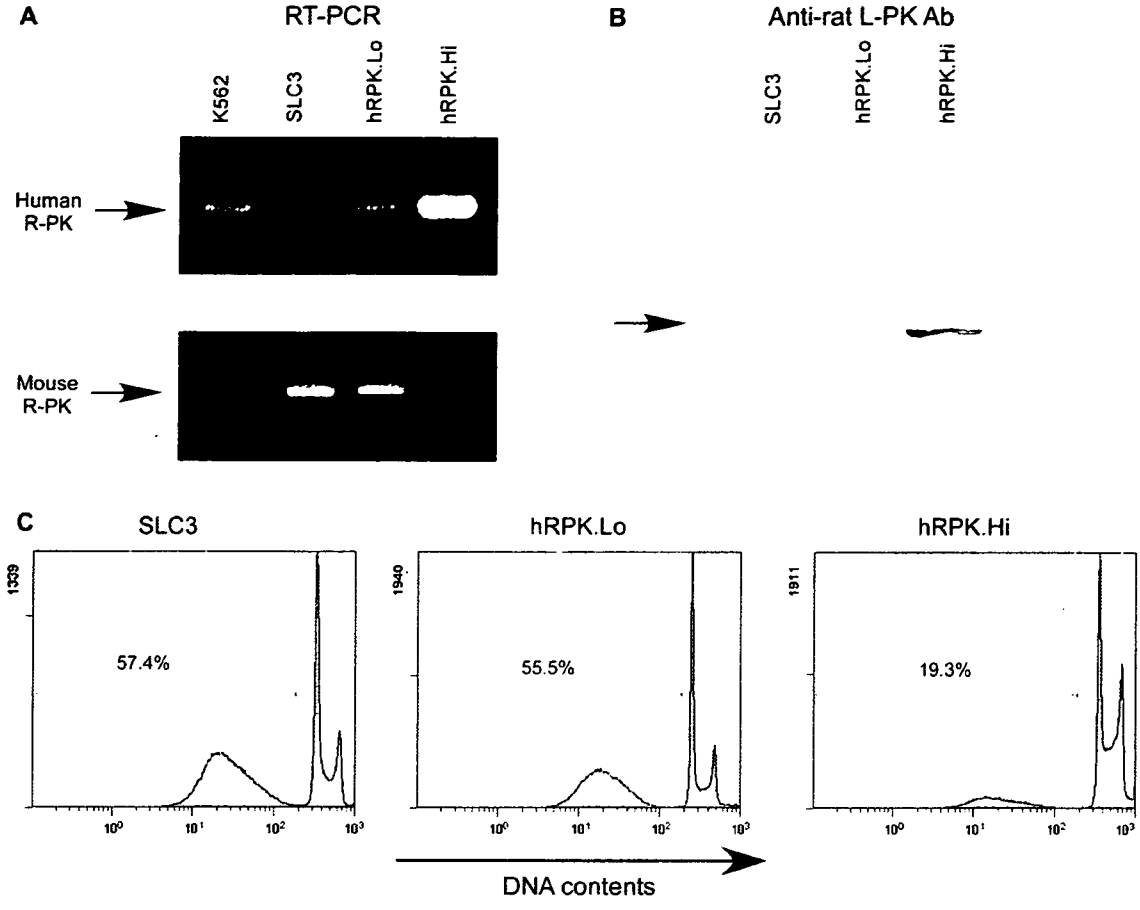


Figure 2. Establishment of the transfectants, SLC3-hRPK.Hi (hRPK.Hi) and SLC3.hRPK.Lo (hRPK.Lo), by introducing the human red blood cell type-pyruvate kinase (R-PK) gene into murine R-PK–deficient cells. Transgene-expression was confirmed by reverse transcriptase polymerase chain reaction (**A**) and Western blotting (**B**). The expression level of hRPK.Hi was higher than that of hRPK.Lo. (**C**) Apoptosis induction in the PK-deficient cells and transfectants. Transfected human R-PK recovered the glycolytic function and showed reduced spontaneous apoptotic changes. The numbers in figures represent the apoptotic change ratio.

forced overexpression of the PK gene reduced intracellular ROS in an expression-level dependent manner (Fig. 5C).

Discussion

Overexpression of human R-PK in SLC3 results in the reduction of apoptotic cells (Fig. 2C), and DNA microarray analysis showed that genes involved in the cell cycle, DNA repair, and antioxidants were downregulated. In general, gene expression levels of transfectants were lower than that of SLC3 (Fig. 3). However, aberrant apoptosis and invalid cell proliferation were restrained in the transfectants. These observations suggested that the cellular activity was not suppressed but was reverted to the normal level by the transgene. It is most likely that the candidate genes suppressed in transfectants were induced in R-PK mutant cells.

Although there were several candidate genes attributing to apoptosis-induction in SLC3, it was still unclear whether these genes were associated with each other or independent. However, there was a possibility that a signal cross-talk phenomenon occurred [14]. *Bad*, a gene encoding a member of the Bcl2-family proapoptotic molecules in mitochondria was significantly downregulated by the transgene (Figs. 3A and 4). Danial et al. [15] reported that *Bad*, BCL2-antagonist of cell death, formed a functional holoenzyme complex together with several molecules, such as glucokinase (hexokinase-4) in liver mitochondria, and contributed to apoptosis induction by glucose deprivation. Our observation suggested that *Bad*

Figure 1. Apoptosis induced by glycolytic inhibition in erythroid cell lines. Glucose deprivation or exposure to 2-deoxyglucose inhibits glycolysis and finally causes apoptosis. The red blood cell type-pyruvate kinase (R-PK)–deficient erythroid cell line (SLC3) is more susceptible than wild-type cells (CBA2) in these conditions. The horizontal axis shows AnnexinV-Arexa568 (= apoptotic change) and the vertical axis shows Rhodamin123 fluorescence (= mitochondrial membrane potential).

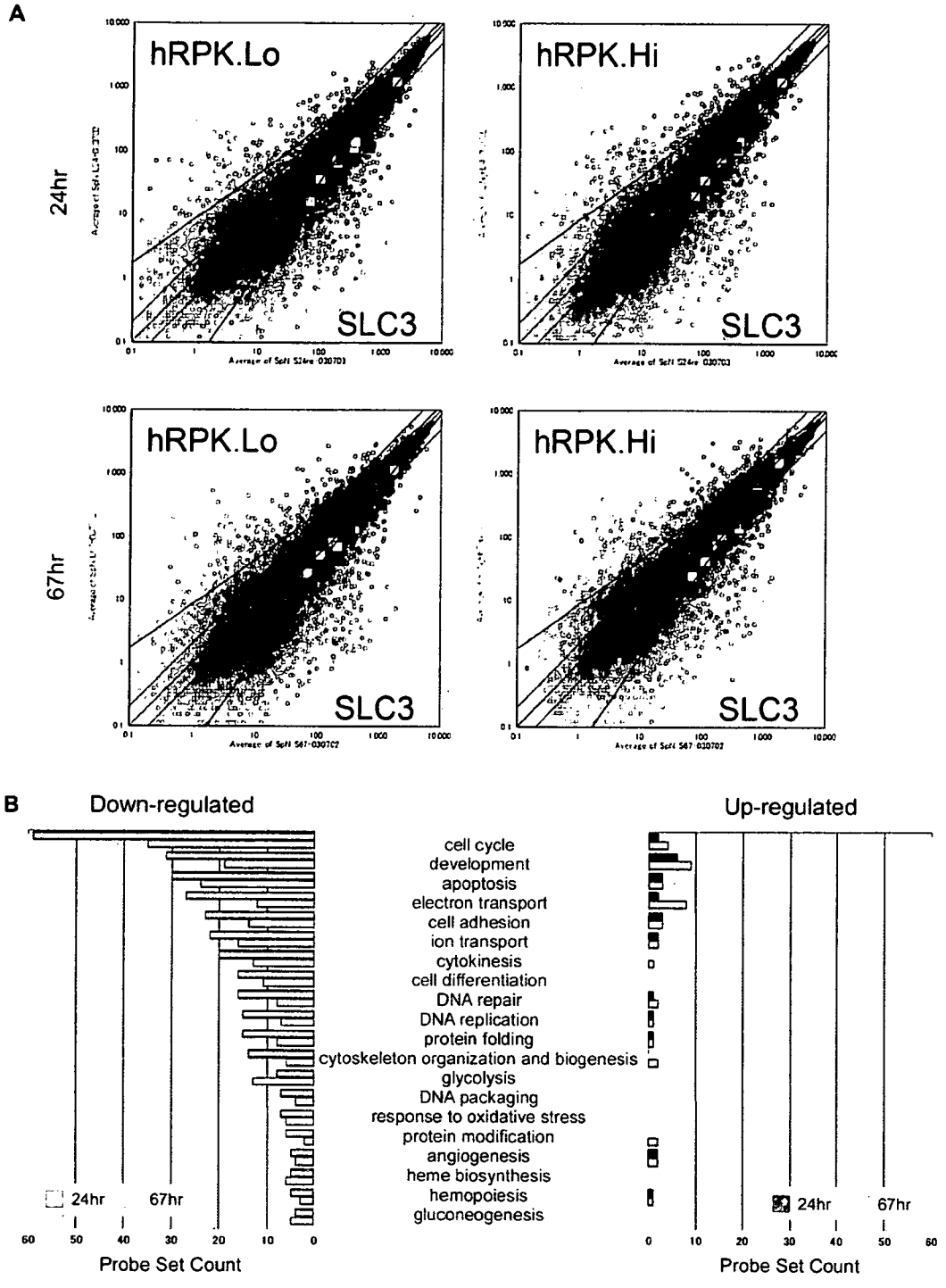
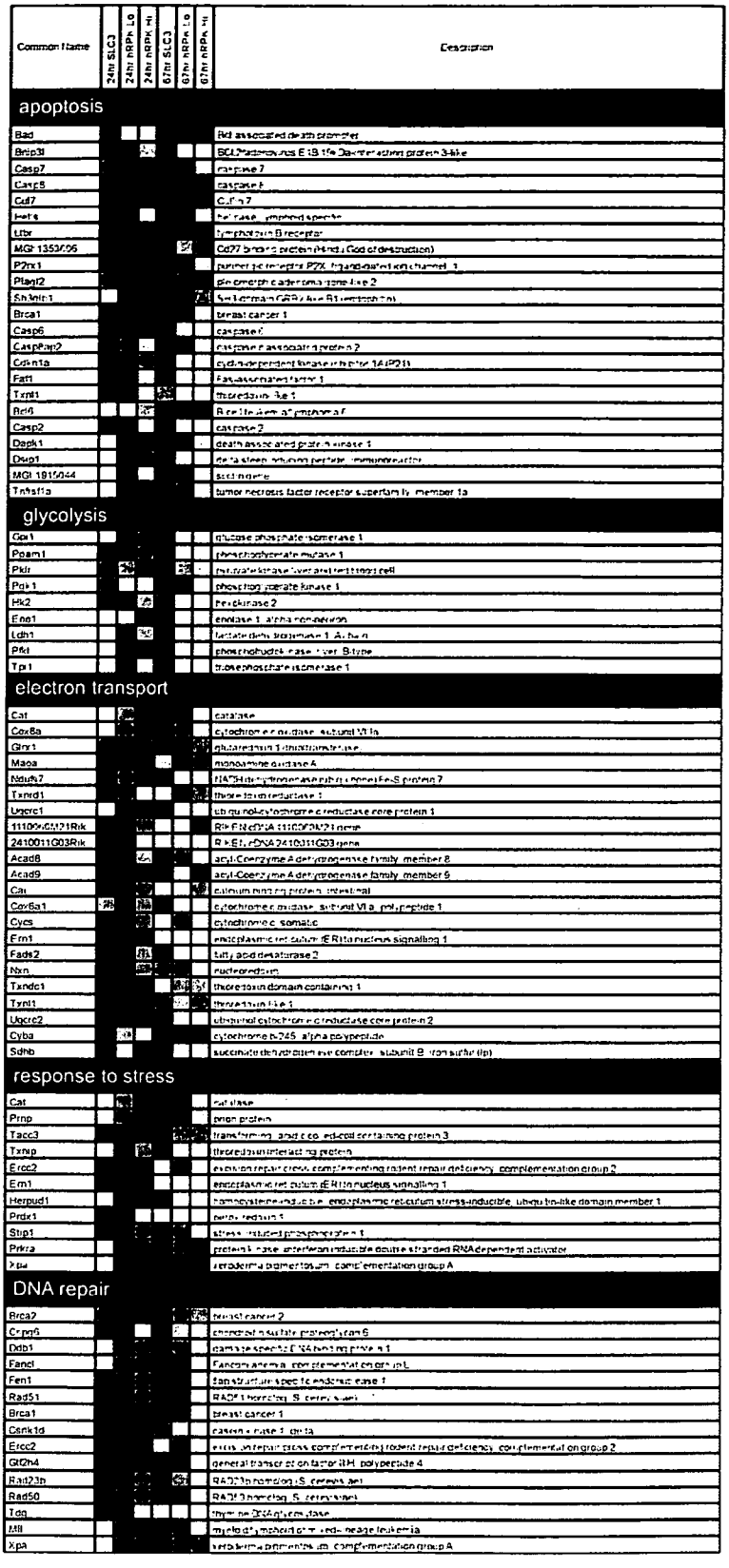


Figure 3. Genome-wide expression analysis of the glycolysis defect. Analysis was performed using the Affymetrix GeneChip Mouse Expression Array 430A, which contains about 20,000 genes. (**A**) Scatter plot between SLC3 and hRPK transfectants at 24 or 67 hours. The open circle shows the expression level of every probe set. The color shows these probabilities provided by the Affymetrix GeneChip Operation System: red means good and green means poor. The colored squares show *Bad* (red), *Bnip3* and *Bnip3l* (blue), *hif1a* (green), *Brca1* and *Brca2* (aqua), *Prdx1* (pink) and *Txnl1* (yellow), respectively. The black lines show twofold, onefold, and 0.5-fold, respectively, and the blue lines show the empirical threshold level. (**B**) The categorized aggregate graph. All probe sets were categorized by the Biological Process Ontology keywords provided by the Gene Ontology project (http://www.geneontology.org/). Up- or downregulation was determined by the spot location in the scatter plotting. Compared with the empirical threshold lines, the upper spots show upregulated genes and the lower spots show downregulated genes.

A Down-regulated Genes



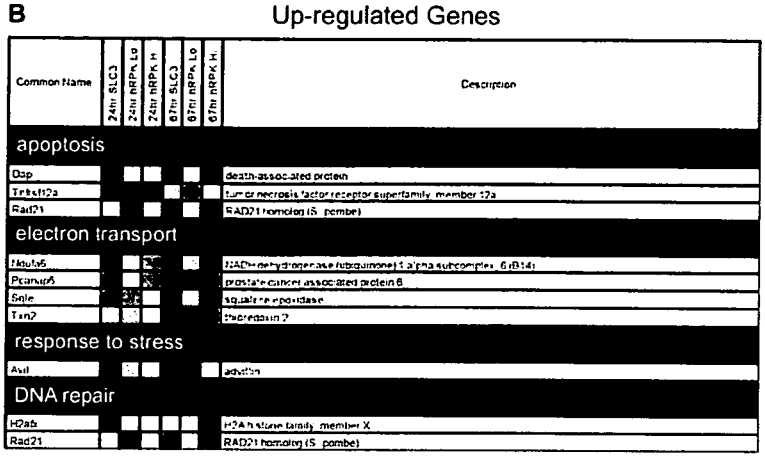


Figure 4. *Continued*

could be involved in the apoptosis induced by glycolysis defect in erythroid cells as well as in the liver.

The genes of apoptosis-inducers related to hypoxia such as *Bnip3* and *Bnip3l*, which are known as inducible genes by hypoxia-inducible factor-1α, were inactivated markedly by the forced expression of the wild-type R-PK gene. Although the extent of downregulation was smaller than for *Bnip3*, *Bnip3l* showed a significant decrease of expression by the transgene (Fig. 3A). Moreover, the downregulation was more obvious at 24 hours, suggesting that these genes may contribute to the initial response caused by a glycolytic defect. These observations strongly suggested that the apoptosis induction by the glycolysis disorder was executed by the *Bnip3-Bnip3l* signal.

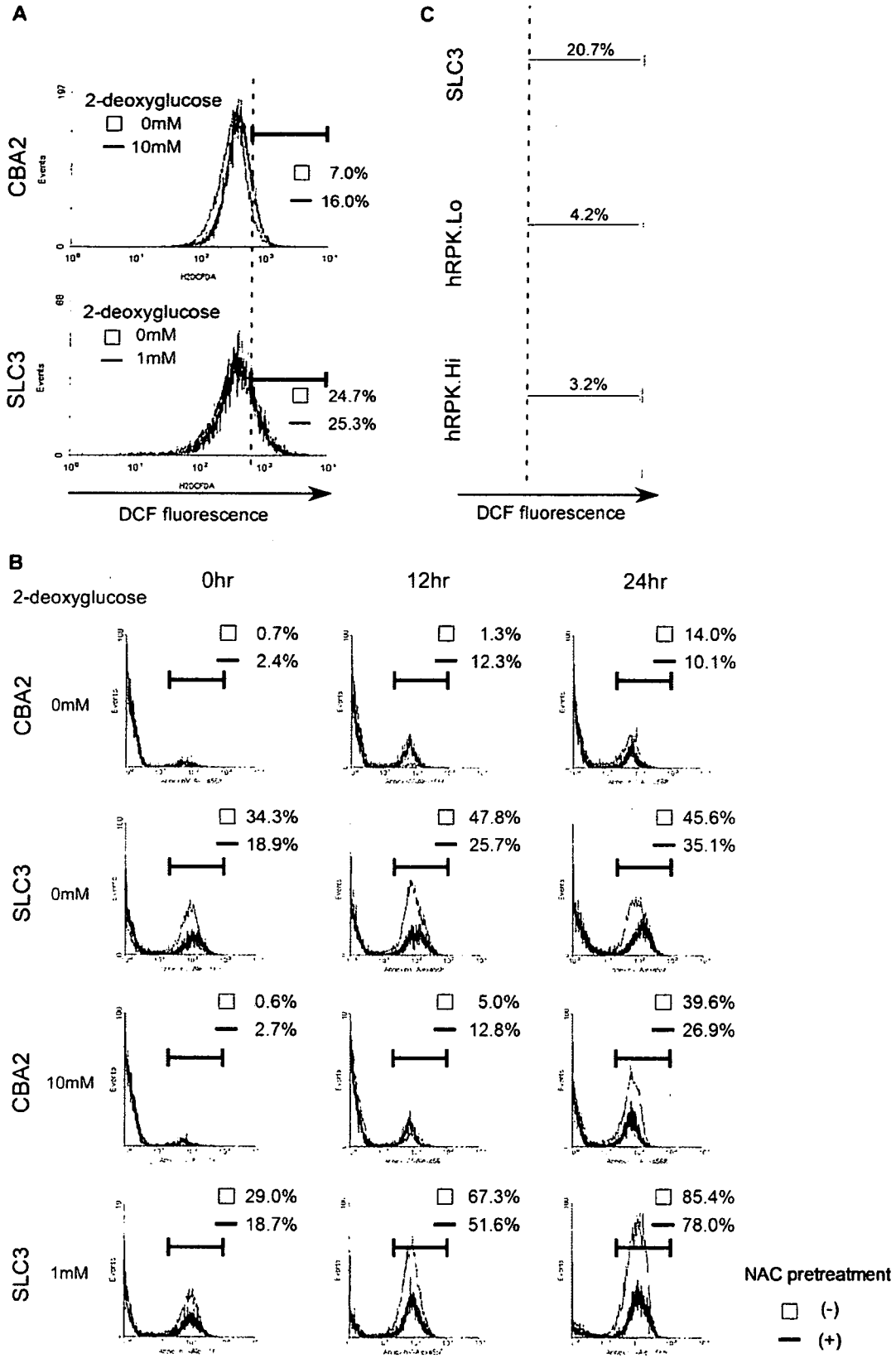
It is noticeable that several genes important for responding to oxidative stress are upregulated, suggesting that R-PK deficiency might account for intracellular ROS production. This speculation is supported by the following experimental observations: Firstly, SLC3 cells were more sensitive to glycolytic inhibitions such as glucose deprivation and supplementation with 2-DG (Fig. 1), and these conditions induced ROS production detected by DCFH-DA (Fig. 5A). Apoptotic changes induced by 2-DG were partly rescued by preincubation with the glutathione precursor (Fig. 5B). Finally, transgene expression reduced intracellular ROS in an expression-level–dependent manner (Fig. 5C).

Glycolytic disorders may cause cellular conditions similar to those of hypoxia. Shim et al. [16] reported that induction of the LDH-A gene by c-Myc was advantageous to transformed cells that exist under hypoxic conditions [15]. However, glucose deprivation induces the extensive apoptosis of cells overexpressing c-Myc. Overexpression of LDH-A alone in fibroblasts is sufficient to sensitize cells to this glucose deprivation-induced apoptosis. They proposed a hypothesis that LDH-A was a downstream target of c-Myc that mediates this unique apoptotic phenotype. We noticed that pyruvate was the final product as well as the substrate of the PK and LDH reaction, respectively. Both LDH hyperactivity and PK deficiency may cause the depletion of intracellular pyruvate, suggesting that pyruvate has an important role in preventing apoptosis.

Several studies have revealed that pyruvate acts as an antioxidant and that PK has a protective role against oxidative stress in this respect. Brand et al. [17] reported that proliferating thymocytes mainly depend on energy derived from aerobic glycolysis, and that their sensitivity to 12-myristate 13-acetate–induced ROS production is much lower than that of resting thymocytes, which produce ATP mainly through oxidative phosphorylation. They suggested that pyruvate functions as an ROS scavenger, because the incubation of proliferating thymocytes with pyruvate reduced ROS formation.

The PK-overexpressing neuronal cells could attenuate oxidative stress and maintain cell viability [18]. Lee et al. [19] showed that hydrogen peroxide depleted intracellular GSH in human umbilical vein endothelial cells, and that was prevented by pyruvate but not by L-lactate or aminooxyacetate. The activation of caspases was strongly inhibited by pyruvate, but markedly enhanced by L-lactate and aminooxyacetate, implicating the redox-related antiapoptotic mechanisms of pyruvate. Myocardial ischemia-reperfusion

Figure 4. Representative list of the genes affected by the functional recovery of glycolysis. Genome-wide expression analysis was performed using Affymetrix GeneChip Mouse Expression Array 430A, which contains about 20,000 genes. In the comparison among hRPK.Hi, hRPK.Lo, and SLC3, about 6000 genes were downregulated and about 500 genes were upregulated by the functional recovery of glycolysis at 24 and/or 67 hours after regular passage. These lists contain the affected genes related to apoptosis and/or the oxidative stress response.


A
CBA2
2-deoxyglucose
0mM
10mM
7.0%
16.0%
SLC3
2-deoxyglucose
0mM
1mM
24.7%
25.3%
DCF fluorescence
C
SLC3
20.7%
hRPK.Lo
4.2%
hRPK.Hi
3.2%
DCF fluorescence
B
2-deoxyglucose
0hr
12hr
24hr
CBA2
0mM
0.7%
2.4%
1.3%
12.3%
14.0%
10.1%
SLC3
0mM
34.3%
18.9%
47.8%
25.7%
45.6%
35.1%
CBA2
10mM
0.6%
2.7%
5.0%
12.8%
39.6%
26.9%
SLC3
1mM
29.0%
18.7%
67.3%
51.6%
85.4%
78.0%
NAC pretreatment
(-)
(+)

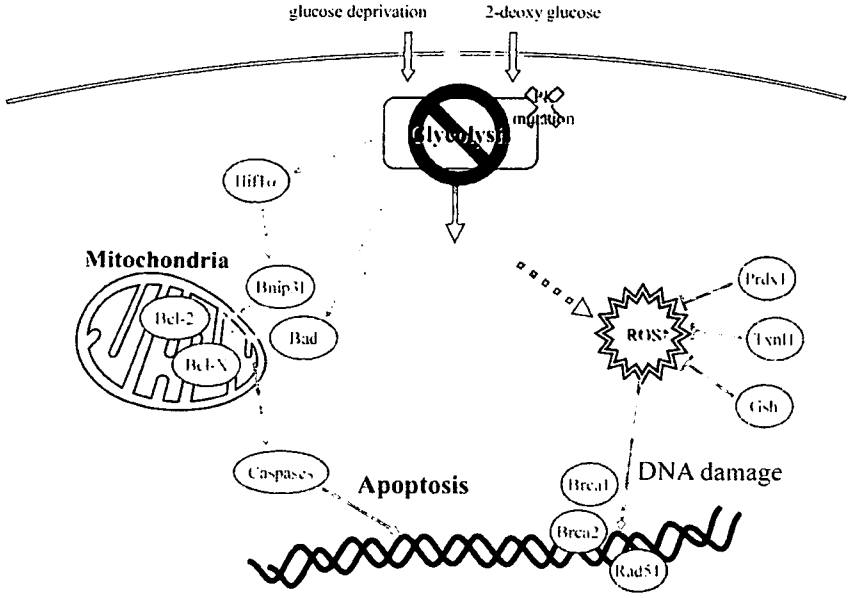


Figure 6. Glycolytic defect causes oxidative stress and hypoxia-like signal activation. Pyruvate, which is final metabolic product of the glycolytic pathway, acts as an antioxidant. Therefore, glycolytic defect elevates intracellular reactive oxygen species (ROS) and causes cellular damage, such as DNA damage and lipid oxidation. At the same time, glycolytic defect is most likely to activate signal transduction through hypoxia-inducible factor-1α (HIF-1α). These cellular responses could be accountable for the apoptosis induced by glycolytic defect.

is reported to be associated with bursts of ROS, such as superoxide radicals, and cardiac superoxide formation can be inhibited by pyruvate [20]. Thus cytotoxicities due to cardiac ischemia-reperfusion ROS can be alleviated by redox reactants such as pyruvate. These results support our present data, which showed that a mutation of the PK gene as well as inhibition of glycolysis by 2-DG augmented intracellular ROS of erythroid cells, leading to apoptosis. Introduction of the wild-type PK gene into SLC3 cells partly reduced ROS and apoptosis (Figs. 2C and 6C).

In human RBC, the most important antioxidant is GSH. Mutations of enzymes involving the synthesis and reduction of GSH, such as γ-glutamylcystein synthetase, GSH-S, glutathione reductase, and glucose-6-phosphate dehydrogenase account for the shortened RBC survival [1,21]. Recently, Neumann et al. [22] and Lee et al. [23] reported the essential roles of both peroxiredoxin (Prdx) 1 and 2 in RBC protection from oxidative stress. The hemolytic anemia of mice with targeted inactivation of *Prdx1* is characterized by an increase in erythrocyte reactive oxygen species, leading to protein oxidation and Heinz body formation. Similarly, the *Prdx2* knockout mice had Heinz body-positive hemolytic anemia with splenomegaly. The dense RBC fractions contained markedly higher levels of ROS. These studies highlighted a pivotal role of *Prdx* as a scavenger of hydrogen peroxide in RBC. *Prdx1* may be concerned with the initial response to glycolytic deficiency, because the gene expression in SLC3 was higher than that in transfectants only at 24 hours (Fig. 3A). The mechanisms responsible for upregulation of *Prdx1* and similar antioxidant enzymes in SLC3 remain to be elucidated.

It is most likely that the main pathogenesis of PK deficiency is decreased ATP production due to impaired glycolysis, resulting in the premature destruction of RBC in the reticuloendothelial system, i.e., extravascular hemolysis. In most cases, hemolysis is partly compensated by enhanced erythropoiesis. We have previously shown that the numbers of hematopoietic progenitors including colony-forming unit (CFU)-erythroid, CFU-granulocyte macrophage, burst-forming unit-erythroid, and CFU-granulocyte-erythrocyte monocyte-megakaryocyte were increased in *Pk-1*^{slc} mice [10]. The proliferation of erythroid progenitors might require

Figure 5. The oxidative stress pathway might play some role in the apoptosis induced by glycolytic disorder. (**A**) The SLC3 cells produce 2′,7′-dichlorofluorescein (DCF) continuously with and without 2-deoxyglucose (2-DG) due to the red blood cell type-pyruvate kinase (R-PK) defect. The control CBA2 cells produce DCF with 10 mM 2-DG for 30 minutes. The gray area shows the nontreated group and the red line shows the treated group with 2-DG. The horizontal axis shows the fluorescence intensity of the DCF. (**B**) The apoptosis induced by glycolytic defect or by glycolysis inhibitor was suppressed by the preincubation with the glutathione precursor, N-acetyl-cysteine (NAC). The gray area shows the nonpretreated group and the blue line shows the pretreated group with NAC. The horizontal axis shows the fluorescence intensity of the Annexin V-Alexa568.

activation of glycolysis in order to suppress intracellular ROS. Therefore, R-PK deficiency becomes a serious problem for erythroid cells to avoid apoptosis. In summary, we concluded that the premature destruction of RBC as well as apoptosis of erythroid progenitors accounts for the pathogenesis of R-PK deficiency.

Although most severe cases die either in utero or during the neonatal period [24,25], there is no curative therapy of PK deficiency except hematopoietic stem cell transplantation [26] at present. Because hematopoietic stem cell transplantation may accompany life-threatening complications, a safer treatment should be considered. Studies on the apoptotic induction of erythroid progenitors in R-PK deficiency may be useful for the identification of molecular targets of causal treatment.

Acknowledgments

We are indebted to Takako Hamada and Miyuki Yuda for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society of the Promotion of Science (project nos. 14570131 and 16590254), and also by a Scientific Research Grant from the Ministry of Health, Labor and Welfare (H15-kagaku-002, H18-kagaku-ippan-001), Japan.

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