Delayed Onset of Hemolytic Anemia in CBA-Pk-1\textsuperscript{slc}/Pk-1\textsuperscript{slc} Mice With a Point Mutation of the Gene Encoding Red Blood Cell Type Pyruvate Kinase

By Kumiko Tsujino, Hitoshi Kanno, Koji Hashimoto, Hisaichi Fujii, Tomoko Ippe, Eiichi Morii, Young-Mi Lee, Hidekazu Asai, Shiro Miwa, and Yukihiko Kitamura

PYRUVATE KINASE (PK, EC 2.7.1.40) catalyzes the conversion of phosphoenolpyruvate to pyruvate in the glycolytic pathway. In humans, deficiency of PK activity is the most common cause of hereditary anemias due to deficiency of glycolytic enzymes.\textsuperscript{1,3} PK has four isozymes in mammals;\textsuperscript{4} the red blood cell (RBC) type PK (R-PK) is expressed almost exclusively in mature RBCs.\textsuperscript{5} A unique structural profile of the R-PK is the longer amino-terminal sequence as compared with that of liver-type PK (L-PK), which is transcribed from alternative promoter of the L/R-PK gene.\textsuperscript{6} In mice, L/R-PK is encoded by the Pk-1 gene.\textsuperscript{7} In contrast with mature RBCs, undifferentiated erythroid precursor cells express a different isozyme of PK (M2-PK),\textsuperscript{8,10} which is encoded by the Pk-3 gene of the mouse. The Pk-3 gene encodes both M1-PK and M2-PK; they are produced by alternative RNA splicing,\textsuperscript{11} and the former was expressed chiefly by muscle and the latter by fetus and most adult tissues.\textsuperscript{12,13} The expression of the Pk-3 gene switches to that of the Pk-1 gene during differentiation of RBCs. The persistence of M2-PK in RBCs has been described in some patients of hereditary PK deficiency.\textsuperscript{14,16}

We recently found the mutant Pk-1\textsuperscript{slc} gene in the CBA/N strain of mice (hereafter called CBA mice).\textsuperscript{18,19} Mice of CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} showed severe nonspherocytic hemolytic anemia. The activity of PK in RBCs of the adult mutants decreased to 16.2% of normal (+/+ ) adults. Because CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice showed a remarkable reticulocytosis (41.6%) and PK activity of reticulocytes is much higher than that of mature RBCs, the PK activity in mature RBCs of the CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice was calculated to be 2.8% that of mature RBCs of CBA-+/+ mice.\textsuperscript{19}

The abnormality of CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice was characterized by comparing normal and mutant Pk-1 CDNAs. A missense mutation at nucleotide 1013 G\textsuperscript{T} \rightarrow \textsuperscript{C} GT\textsuperscript{G} was identified in the cDNA sequence of the mutant, causing a single amino acid substitution of 338Gly to Asp. The missense mutant was also confirmed in genomic sequence by the analysis using polymerase chain reaction for restriction fragment length polymorphism.\textsuperscript{18}

Although adult CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice show a severe hemolytic anemia, neonatal CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice are not pale. We cannot distinguish neonatal CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice from neonatal CBA-+/+ mice by appearance. In the present study, we investigated when the significant anemia developed in CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} mice. Moreover, we compared the survival time of RBCs between CBA-Pk-1\textsuperscript{wt}/Pk-1\textsuperscript{slc} and -+/+ mice of various ages.
RESULTS

Delayed onset of anemia. Number of RBCs and proportion of reticulocytes were measured in CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and +/+/+ mice on days 1, 7, 14, 28, and 42 after birth. No significant differences were detectable between CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and +/+/+ mice on day 1 (Fig 1). Although the number of RBCs increased in both mutant and normal mice with ages, the number was significantly greater in normal mice than in mutant mice on day 14 and thereafter. As a result, significant anemia was detectable in CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice from day 14 after birth. The proportion of reticulocytes continuously decreased after birth in CBA-+-/+ mice. On the other hand, it increased until day 14 and then retained the high value in CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice (Fig 1). The spleen weight was comparable between CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and +/+/+ mice on day 1 after birth, but a significant splenomegaly was observed in CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice on day 42 (Table 1). In spite of the severe anemia of CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice, body weight of CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice were comparable with that of CBA-+-/+ mice throughout the observation period.

When RBCs of adult CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and +/+/+ mice were labeled with <sup>51</sup>Cr and injected into adult CBA-+-/+ mice, the disappearance of RBCs derived from CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice was much faster than that of RBCs from CBA-+-/+ mice. Since the number of RBCs of 1-day-old CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice was comparable with that of 1-day-old CBA-+-/+ mice, we compared the survival time of RBCs of CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> mice of various ages to that of RBCs of CBA-+-/+ mice of the same ages. RBCs of 1-day-old, 7-day-old, 42-day-old, and -/- mice were kindly provided by Dr Tamio Noguchi of Fukui Medical School. We used them to characterize the isozyme expression in RBCs of CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and -/- mice of various ages. RBC lysate was mixed with anti-L-PK or anti-M1-PK antibody, followed by overnight incubation at 4°C. The mixture was then centrifuged at 15,000g at 4°C for 15 minutes, and the PK activity in the supernatant was measured.

Polycrylamide gel electrophoresis (PAGE). Erythrocytes were lysed by sonication in the PK sample buffer containing 10 mmol/L Tris/HCl, pH 7.5, 100 mmol/L KCl, 2 mmol/L 2-mercaptoethanol, 10 mmol/L L-aminocaproic acid, and 10 mmol/L EDTA. Protein extracts prepared from fetal liver were also homogenized in the PK sample buffer. PK activity assay, PAGE, and staining for PK activity were performed as described previously.

Antibody neutralization of PK. Rabbit antiserums against L-PK and M1-PK of the rat were kindly provided by Dr Tamio Noguchi of Fukui Medical School. We used them to characterize the isozyme expression in RBCs of CBA-Pk-1<sup>1slc</sup>/Pk-1<sup>1slc</sup> and -/- mice of various ages. RBC lysate was mixed with anti-L-PK or anti-M1-PK antibody, followed by overnight incubation at 4°C. The mixture was then centrifuged at 15,000g at 4°C for 15 minutes, and the PK activity in the supernatant was measured.

Table 1. Hematologic and Anatomical Parameters in CBA-+-/+ and -/- Mice of 1 and 42 Days of Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>Age (d)</th>
<th>Values (×10&lt;sup&gt;12&lt;/sup&gt;/L)</th>
<th>Mean ± SE of 10 mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of RBCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>1</td>
<td>3.6 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>42</td>
<td>8.9 ± 0.1</td>
<td>5.2 ± 0.2†</td>
</tr>
<tr>
<td>Proportion of reticulocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>1</td>
<td>35 ± 1</td>
<td>35 ± 1</td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>42</td>
<td>4.4 ± 0.3</td>
<td>54 ± 2†</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>1</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>42</td>
<td>20 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>1</td>
<td>2.0 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CBA-+-/+</td>
<td>42</td>
<td>31 ± 1</td>
<td>255 ± 20†</td>
</tr>
</tbody>
</table>

*Mean ± SE of 10 mice.
†P < .01 when compared with values of CBA-+-/+ mice of the same age by t test.

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and 90-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> and -/+/+ mice were labeled with <sup>51</sup>Cr and injected into 90-day-old CBA-/+/+ mice. Radioactivity retained in RBCs was measured at various times after the injection. In contrast with the large difference between 90-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> and -/+/+ mice, the difference between 1-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> and -/+/+ mice was slight (Fig 2). When the radioactivity retained in the RBCs was plotted semi-logarithmically, two-component curves were observed after the injection of labeled RBCs obtained from 1-day-old and 7-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> and -/+/+ mice. Two-component curves were also observed after the injection of labeled RBCs from 42-day-old and 90-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice, whereas one-component curves were observed after the injection of labeled RBCs from 42-day-old and 90-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice. The difference became greater in parallel with the increase of age (Table 2).

**Age-dependent changes of PK activity.** In either CBA-/+/+ or CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice, the PK activity was much higher in neonatal mice than in 90-day-old mice (Table 3). In spite of the high activity of PK, examination of smears detected no nucleated erythroblasts. The PK activity gradually decreased with ages in both CBA-/+/+ and CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice. Although age-dependent decrease in PK activity of RBCs was observed in both CBA-/+/+ and CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice, the absolute value of PK activity was much greater in CBA-/+/+ mice than in CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice. Moreover, different isozyme pattern was observed by electrophoresis between CBA-/+/+ and CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice of 1 and 7 days of age. Only R-PK was detectable in RBCs of 1-day-old and 7-day-old CBA-/+/+ mice, whereas only M2-PK was detectable in RBCs of 1-day-old and 7-day-old CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice (Fig 3). In the fetal liver of CBA-/+/+ mice, both R-PK and M2-PK were detectable, but only M2-PK was detectable in the fetal liver of CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice (Fig 3). Moreover, anti-L-PK antibody neutralized the PK activity in the RBC lysate obtained from CBA-/+/+ mice but did not neutralize the PK activity in the RBC lysate of CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice (Table 4). On the other hand, anti-M1-PK antibody neutralized the PK activity in the RBC lysate of CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice but did not neutralize the PK activity in the RBC lysate of CBA-/+/+ mice (Table 4).

**DISCUSSION**

Although PK encoded by the mutant Pk-1<sup>+/</sup> gene hardly possesses the enzymatic activity, the numbers of RBCs were comparable between 1-day-old CBA-/+/+ and CBA-Pk-1<sup>+/</sup>Pk-1<sup>+/</sup> mice. The comparable RBC values in 1-day-old mice was chiefly attributable to the low RBC counts of 1-day-old CBA-/+/+ mice. The RBC number increased continuously...
slc mice were calculated using the first component of the two-component curves. Chapman and Schaumburg,25 Nathan et al, 26 Paglia and Valentine/CBA-11, the high PK activity in neonatal CBA-1 mice paralleled with the proportion of reticulocytes in mice of the same genotype. In fact, the PK activity in CBA-1 mice was attributable to the high proportion of reticulocytes. This is consistent with the result reported by Chapman and Schaumburg,25 Nathan et al,26 Paglia and Valentine27 and Lakomek et al28 that PK activity was higher in reticulocytes than in mature RBCs. Although the PK activity was higher in 1-day-old and 7-day-old CBA-Pk-1slc/Pk-1slc mice than in the older mice of the same genotype, the PK activity did not parallel with the proportion of reticulocytes in mice of the Pk-1slc/Pk-1slc genotype. In fact, the PK activity significantly decreased on day 14 after birth whereas the proportion of reticulocytes significantly increased on the same day (Fig 1 and Table 3). Therefore, the relatively high PK activity in neonatal CBA-Pk-1slc/Pk-1slc mice cannot be explained by the high proportion of reticulocytes. When isozyme pattern of PK was compared between 1-day-old and 7-day-old CBA-+/+ mice and CBA-Pk-1slc/Pk-1slc mice by zymograms as well as the antibody neutralization, R-PK was exclusively observed in +/+ RBCs, whereas M2-PK in Pk-1slc/Pk-1slc RBCs (Fig 3, Table 4). Therefore, the relatively high PK activity in RBCs of 1-day-old and 7-day-old CBA-Pk-1slc/Pk-1slc mice may be attributable to the delayed switching from M2-PK to R-PK.

Switching of isozymes from R-PK to M2-PK during erythrocyte differentiation was investigated by zymography (Fig 3). The PK activity of 1-day-old CBA-+/+ mice was over six times higher than adults, and it decreased with age (Table 3). Since the PK activity in CBA-+/+ mice paralleled with the proportion of reticulocytes, the high PK activity in neonatal CBA-+/+ mice was attributable to the high proportion of reticulocytes. This is consistent with the result reported by Chapman and Schaumburg,25 Nathan et al,26 Paglia and Valentine27 and Lakomek et al28 that PK activity was higher in reticulocytes than in mature RBCs. Although the PK activity was higher in 1-day-old and 7-day-old CBA-Pk-1slc/Pk-1slc mice than in the older mice of the same genotype, the PK activity did not parallel with the proportion of reticulocytes in mice of the Pk-1slc/Pk-1slc genotype. In fact, the PK activity

| Table 2. Half Life of RBCs Obtained From CBA-+/+ and -Pk-1slc/Pk-1slc Mice of Various Ages After Transfusion Into 90-Day-Old CBA-+/+
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of RBC Donors (d)</td>
<td>Half Life (d, mean ± SE)*</td>
<td>+/+</td>
<td>Pk-1slc/Pk-1slc</td>
</tr>
<tr>
<td>1</td>
<td>2.4 ± 0.1</td>
<td>1.8 ± 0.1t</td>
<td>1.8 ± 0.1t</td>
</tr>
<tr>
<td>7</td>
<td>3.3 ± 0.2t</td>
<td>1.8 ± 0.1t</td>
<td>1.8 ± 0.1t</td>
</tr>
<tr>
<td>42</td>
<td>6.0 ± 0.2t</td>
<td>1.4 ± 0.1t§</td>
<td>1.4 ± 0.1t§</td>
</tr>
<tr>
<td>90</td>
<td>6.3 ± 0.1t</td>
<td>1.4 ± 0.1t§</td>
<td>1.4 ± 0.1t§</td>
</tr>
</tbody>
</table>

*Calculated from the same data shown in Fig 2. Half lives of RBCs obtained from 1-day-old and 7-day-old CBA-Pk-1slc/Pk-1slc and -+/+ mice and those of RBCs from 42-day-old and 90-day-old CBA-Pk-1slc/Pk-1slc mice were calculated using the first component of the two-component curves.

†P < .01 when compared with values of CBA-+/+ mice of the same age by t-test.
‡P < .01 when compared with values of 1-day-old CBA-+/+ mice.
§P < .01 when compared with values of 1-day-old CBA-Pk-1slc/Pk-1slc mice.

from day 1 to day 42 after birth in both CBA-+/+ and -Pk-1slc/Pk-1slc mice, but the magnitude of the increase was much greater in CBA-+/+ mice than in CBA-Pk-1slc/Pk-1slc mice.

Table 2. Half Life of RBCs Obtained From CBA-+/+ and -Pk-1slc/Pk-1slc Mice of Various Ages After Transfusion Into 90-Day-Old CBA-+/+

Table 3. Pyruvate Kinase Activity in RBCs of CBA-+/+ and -Pk-1slc/Pk-1slc Mice of Various Ages

Table 4. Effect of Anti-L-PK or Anti-M1-PK Antibody Treatment on PK Activity of Various RBC Lysates

<table>
<thead>
<tr>
<th>Source of RBC Lysate</th>
<th>Genotype</th>
<th>Day After Birth</th>
<th>Effect of Antibody Treatment on PK Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA-+/+</td>
<td>1</td>
<td>Abolished</td>
<td>No effect</td>
</tr>
<tr>
<td>7</td>
<td>Abolished</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Abolished</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Abolished</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Abolished</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Abolished</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>CBA-Pk-1slc/Pk-1slc</td>
<td>1</td>
<td>No effect</td>
<td>Abolished</td>
</tr>
<tr>
<td>7</td>
<td>No effect</td>
<td>Abolished</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>No effect</td>
<td>Abolished</td>
<td></td>
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<td>42</td>
<td>No effect</td>
<td>Abolished</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>No effect</td>
<td>Abolished</td>
<td></td>
</tr>
</tbody>
</table>

RBCs lysate was mixed with anti-L-PK or anti-M1-PK antibody, followed by overnight incubation at 4°C. The mixture was then centrifuged, and the PK activity in the supernatant was measured. The antibody was diluted sequentially, and the effect of antibody treatment was evaluated at the most effective dilution. When PK activity dropped to 20% of original value after antibody treatment, the PK activity was considered to be abolished. When 70% of original PK activity was retained after antibody treatment, the treatment was considered to be of no effect. The experiments were repeated twice. Each RBC lysate for an assay was collected from the following numbers of mice: sample for day 1 or day 7 from 8 to 10 mice; sample for day 14 from 3 mice; sample for day 28, day 42, or day 90 from 2 mice.

Table 4. Effect of Anti-L-PK or Anti-M1-PK Antibody Treatment on PK Activity of Various RBC Lysates

Fig 3. PAGE and staining for PK activity in extracts of fetal livers or RBCs. Fetal livers were obtained from 16-day embryos of CBA-+/+ or -Pk-1slc/Pk-1slc mice; RBCs from 1-day-old and 7-day-old CBA-+/+ or -Pk-1slc/Pk-1slc mice. M2, M2-PK; R/L, R- or L-PK.
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throid differentiation have been demonstrated by several investiga-
tors. Takegawa et al. examined the transition of PK isozyme expres-
sion by immunologic methods; the M2-PK predomin-
antly expressed in proerythroblasts, and R-PK expression be-
came detectable at the stage of basophilic erythroblasts. Nijhof et al. demonstrated that both R-PK and M2-PK activity was detected in murine erythroid precursor cells (CFU-E) and that M2-PK activity rapidly disappeared during the erythropoi-
etin-induced differentiation. Max-Audit et al. reported that the synthetic rates of PK in human erythroid progenitors were highest in proerythroblasts. They also described that R-PK synthesis remained at the same level during differentiation and that M2-PK activity decreased due to rapid decline of synthesis as well as accelerated degradation.

Present study showed that the half life of 51Cr-labeled RBCs of 1-day-old and 7-day-old CBA-Pk-1+/Pk-1−/ mice was longer than the half life of 42-day-old and 90-day-old CBA-Pk-1+/Pk-1−/ mice. The difference was slight but significant (Table 2). Since the PK activity was greater in the former mice than in the latter mice and since the PK activity in RBCs was attributed to M2-PK not only in sucking CBA-Pk-1+/Pk-1−/ mice but also in adult CBA-Pk-1+/Pk-1−/ mice, the relatively large amount of M2-PK expressed by RBCs of the younger CBA-Pk-1+/Pk-1−/ mice may play a significant role for the survival of the RBCs. It is most likely that the M2-PK activity beyond day 7 after birth may not be sufficient to sustain survival of RBCs in CBA-Pk-1+/Pk-1−/ mice.

There are numbers of globin gene abnormalities that influence switching of active globin gene locus during hematopoietic development in humans. Hereditary persistence of fetal hemoglobin (HPFH) is one of the genetic abnormalities that increase hemoglobin F production in RBCs. Clinical significance of HPFH has been recognized in its relationship to thalassemia and sickle cell anemia. Phenotype of β-thalassemia or the sickle cell anemia was found to be alleviated or even cure of the anemia by marrow transplantation without host irradiation. Blood 86:4323, 1995

We thank Professor Tamio Noguchi of Fukui Medical School for supplying us with rabbit antibodies against rat L-PK and M1-PK.

REFERENCES

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