With the advent of recent molecular studies, nonspherocytic hemolytic anemia caused by red blood cell pyruvate kinase (PK) deficiency is now considered to be caused by a structural mutation of the PK-LR gene. Because PK deficiency is a monogenic disorder, the introduction of the normal PK gene into a patient's bone marrow stem cells should cure the disorder. To study the feasibility of gene therapy for PK deficiency, we first constructed the PK retrovirus pMNSMHPK using human liver-type PK (LPK) cDNA and obtained a producer cell line of E86/amPK. By using the supernatant of this virus-producer cell, we transduced NIH/3T3 cells, mouse leukemic cells (MEL), and human leukemic cells (K562, HEL). The expression of human LPK enzyme activity was ascertained from the retrovirally transduced NIH/3T3 cells. Northern blot analysis demonstrated the expression of the human LPK mRNA in each transduced cell line. Furthermore, bone marrow stem cells (c-kit+, Lin-, Thy-1+) sorted by fluorescence-activated cell sorting were also transduced by the producer cells in the presence of interleukin-3 and interleukin-6, and were transplanted into lethally irradiated C57BL/6 mice. Polymerase chain reaction analysis demonstrated the expression of human LPK mRNA in both the peripheral blood and hematopoietic organs on day 30 and on day 135 of bone marrow transplantation.

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Materials and Methods

Reagents. Restriction enzymes and other enzymes were purchased from Takara Shuzo Co Ltd, Amersham (Arlington Heights, IL), and Bethesda Research Laboratories (Gaithersburg, MD). [α-32P]dCTP was purchased from Amersham. All other reagents were of analytical grades.

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Submitted May 12, 1993; accepted December 8, 1993.

Supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science and Culture in Japan, and Japan Cell Science Research Foundation.

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0006-4971/94/8308-0015$3.00/0

Retrovirus-Mediated Gene Transfer of Human Pyruvate Kinase (PK) cDNA Into Murine Hematopoietic Cells: Implications for Gene Therapy of Human PK Deficiency

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PYRUVATE KINASE (PK; ATP:pyruvate O2-phosphotransferase, EC 2.7.1.40) deficiency, the most common disorder of the glycolytic erythroenzymopathy responsible for chronic hemolysis, is inherited as an autosomal recessive trait and was first reported by Valentine et al in 1961. In PK deficiency, in addition to splenomegaly and occasional hepatomegaly, the clinical symptoms are highly variable, ranging from pronounced neonatal jaundice requiring multiple exchange transfusions to a fully compensated hereditary anemia. Clinical symptoms are observed either in true homozygotes or compound heterozygotes. The most symptomatic patients suffer from anemia, chronic jaundice, splenomegaly, and gallstones. Furthermore, PK deficiency can sometimes be fatal in early childhood. Although splenectomy is effective in most patients, some patients suffer from persisting hemolytic anemia resulting in gallstones or hemochromatosis even after splenectomy. Recently, several groups have reported that a point mutation of the exon of PK-LR gene is the cause of hemolytic anemia in PK deficiency. Although there have been no reported cases of bone marrow transplantation (BMT) for PK-deficient patients, BMT was successfully performed for a PK-deficient dog.

The treatment of human disease by gene transfer has recently progressed from speculation to reality. The first human gene therapy trial was begun in 1990 for correction of adenosine deaminase (ADA) deficiency. The best disease targets for somatic cell gene therapy are currently considered to be disorders caused by single-gene defects and those of recessive inheritance. Furthermore, the genetic defect should be expressed in a tissue that is easily manipulated in vitro and that is capable of long-term survival in vivo after transplantation back into the recipient. Many investigators consider hematopoietic stem cells to be one of the best targets for gene therapy because of their accessibility, as well as their replicating abilities. However, recent studies have shown the difficulties of obtaining long-term expression of introduced genes in BM cells particularly of large outbred animals because most harvested pluripotent stem cells are in the dormant phase and are thus resistant to retroviral infection. Enrichment of the hematopoietic stem cell (HSC) population by using monoclonal antibodies as well as various cytokines during the course of retroviral infection to raise the infection efficiency has been tried by a few investigators.

In this communication, we report transduction of human LPK cDNA using a retroviral vector into mouse and human leukemic cell lines, and into mouse BM cells. As far as we know, this is the first report of a feasibility study for gene therapy of hereditary hemolytic anemia caused by PK deficiency.
Retroviral vectors. The construction of retroviral vector (pMNSM-LPK) encoding human LPK and neomycin phosphotransferase is described in Fig 1. Vector names are based on the order of the genetic elements within the vector: LTR, long terminal repeat; SV, simian virus 40 promoter; Neo, neomycin phosphotransferase gene; LPK, 2.3-kb BamHI-EcoRI fragment of the full-length human LPK gene; LPK, 2.3-kb BamHI-EcoRI fragment of the full-length human LPK cDNA plasmid of hLPK-F. The neomycin phosphotransferase gene was transcribed from the viral LTR, and the LPK cDNA was transcribed from an internal simian virus (SV) 40 early promoter with a viral LTR. Ecotropic and amphotropic vector-producing cell lines were selected from the GP+E-86/pMNSM-LPK and GPfenvAm-12/pMNSM-LPK cell clones and named E86PK and AmPK, respectively. The E86PK virus titer was 4 × 10⁶ CFU/mL and the AmPK virus titer was 5 × 10⁷ CFU/mL. By coculture of E86PK and AmPK at a ratio of 1:3, the E86/Am PK cell line with a virus titer of 5 × 10⁶ CFU/mL was obtained and was used for the following experiment.  

Cell culture. All cell culture in the following studies was performed in humidified 5% CO₂/95% air at 37°C. Ecotropic GP+E-86 cells were maintained in Dulbecco’s modification of Eagle’s medium (DMEM; Flow, McLean, VA) supplemented with 10% (vol/vol) newborn calf serum, 15 µg/mL hypoxanthine, 250 µg/mL xanthine, 25 µg/mL mycophenolic acid, and 1× antibiotic-antimycotic (Sigma, St Louis, MO). Amphotropic GP+envAm-12 cells were also maintained in the same medium as for GP+E-86 except that 69°C was selected as anneal-extend temperature. The primer Ipk-l, 5’-GGGAACGCGAACACCGTG-3’; the primer Ipk-2, 5’-CAGGTCTCGGACGTCCTG-3’ and Ipk-2 spanned from 706 to 723 of the nucleotide sequence of human LPK cDNA and 490 to 507 of the nucleotide sequence of human LPK cDNA. The former sequence differed from the corresponding sequence of rat LPK cDNA by 233-bp band. Detection of transduced LPK enzymatic activity was made for the LPK retrovirus-transduced NIH/3T3 cells. Because most of the cells express human M₂ type PK, the removal of M₂-type PK was necessary to specifically detect the small amount of transduced LPK activity by polyacrylamide gel electrophoresis. To remove M₂-type PK, fractionation using ammonium sulfate was used as previously described. The PK activity of the cell lysate was determined by the methods of Beutler. Polyacrylamide gel electrophoresis was performed as briefly described here. For comparison, lysates of human liver and human erythrocytes were also applied. The liver and erythrocytes samples were kept at −70°C until use. The samples were sonicated for 20 seconds at 4°C. After sonication, one-fifth vol of toluene was added to each sample, vortex mixed for 1 minute, and centrifuged at 13,000g for 5 minutes at 4°C. The water phase was centrifuged at 13,000g for 5 minutes at 4°C and the supernatant was used for further studies. Approximately 0.01 PK activity unit of the samples was applied to each lane. After electrophoresis, the visualization of protein PK expression was performed as previously described. For the detection of LPK mRNA expression, total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method and used for the following experiments. Twenty micrograms of total RNA from cells with or without retroviral transduction was denatured and electrophoresed on a 1% agarose/formamide gel and transferred to NitroPlus (Micron Separations Inc, Westboro, MA). Total RNA was determined to be degraded by the detection of the 28S and 18S ribosomal RNA bands after ethidium bromide staining of the electrophoresed gels. The filters were hybridized with 3²P-labeled hlpk-A (human LPK cDNA) probe as previously described. Autoradiography was performed for 7 days. To detect low-level LPK mRNA expression, the LPK mRNA sequence was specifically amplified as previously described. Briefly, using 1 µg of total RNA samples obtained from mouse peripheral blood cells and mouse hematopoietic organs (BM, spleen, and thymus), human LPK mRNA expression was detected after the amplification of reverse-transcribed RNA using GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer’s instructions. The oligonucleotides were synthesized by DNA synthesizer (model 381A; Applied Biosystems, Foster City, CA). Sequences of the oligonucleotides used in this study were as follows: primer Ipk-1, 5’-GGAACGCGAACACCGTG-3’; primer Ipk-2, 5’-CAGGTCTCGGACGTCCTG-3’. The primer Ipk-1 spanned from 490 to 507 of the nucleotide sequence of human LPK cDNA and the primer Ipk-2 spanned from 706 to 723 of the nucleotide sequence of human LPK cDNA. The former sequence differed from the corresponding sequence of rat LPK cDNA by 6 of 18 nucleotides and the latter sequence differed by 7 of 18 nucleotides. The experimental procedure was essentially the same as the manufacturer’s protocol except that 69°C was selected as anneal-extend temperature. The amplified DNA samples were electrophoresed on 4% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, MD). After electrophoresis, the DNA was transferred to NitroPlus and hybridized with 3²P-labeled hlpk-A cDNA probe as described previously following the usual Southern blotting procedure as described before. Autoradiography was performed for 7 days. Under our polymerase chain reaction (PCR) conditions, only human L-PK mRNA was amplified as a 233-bp band.
Retroviral infection and BMT. NIH/3T3 cells, NFS-60 cells, FDCP-2 cells, K562 cells, and HEL cells were transduced with virus supernatant in the presence of 12 μg/mL polybrene at multiplicities of infection of 50 for 48 hours and then cultured in virus-free IMDM medium as described above in the presence of 400 ng/mL of G418 for 7 days. Female BDF1 (C57BL6×DBA2) mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). C57BL6 (Thy1.2, Ly5.1) mice were obtained from Dr T. Takahashi (Aichi Cancer Center, Aichi, Japan) and C57BL6 (Thy1.2, Ly5.2) mice were obtained from Clea Japan Inc (Tokyo, Japan).21 BM cells were harvested from the femurs of BDF1 or C57BL6 mice as described elsewhere.21 BDF1 mice (7 weeks old, female) were killed 48 hours after treatment with 150 mg of 5-fluorouracil (5-FU) per kilogram of body weight. From one mouse we could obtain 6 × 10^6 of femoral BM cells. The BM cells were transduced with the viral supernatant in the presence of 12 μg/mL of polybrene and 200 U/mL of mouse interleukin-3 (IL-3; kindly provided by Dr A. Miyajima, DNAX Research Institute, Palo Alto, CA), with or without 100 ng/mL of human IL-6 (kindly provided by Ajinomoto Co, Tokyo, Japan) and with or without 10 ng/mL of rat stem cell factor (SCF; kindly provided by Amgen Inc, Thousand Oaks, CA) at multiplicities of infection of 100 for 48 hours. As controls, the BM cells were cultured in the presence of IL-3, SCF, and polybrene at the concentrations described above in virus-free IMDM medium. Approximately 2 × 10^6 BM cells were transplanted into each mouse after irradiation of 950 rad. Three mice were used for each respective experiment. Fifty microliters of peripheral blood was withdrawn from each mouse on days 30, 60, and 90 of BMT.

The blood was kept at -70°C until used for the detection of human LPK mRNA expression as described above. In vitro colony studies were also performed for each group as previously described.22 Briefly, 2.5 × 10^4 cells/dish were cultured in α-medium (Sigma) supplemented with 1.2% 1,500 centipoise methylcellulose (Wako Pure Chemical Ind, Osaka, Japan), 30% fetal calf serum (Biocell Lab, Carson, CA), 1% denizolized bovine serum albumin (Sigma), 1 × 10^−4 mol/L mercaptoethanol (Sigma), 200 U/mL of mouse IL-3, 100 ng/mL of human IL-6, 10 ng/mL of rat SCF, and 2 U/mL of human erythropoietin (EPO; kindly provided by Chugai Co, Tokyo, Japan) with or without 800 μg/mL of G418. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO2. The numbers of colony-forming units of granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM) were counted for each dish at day 14. Colony numbers were obtained by averaging counts for each of quadruplicated dishes.

A C57BL6/Ly5.1 mouse (7 weeks old, female) was killed and BM cells were obtained from the femur. Approximately 1 × 10^7 BM mononuclear cells were obtained after a Lympho-paque (Nyggaard & Co, Oslo, Norway) gradient. By using FACStar™ (Becton Dickinson, Mountain View, CA), 7 × 10^4 BM cells characterized by the surface markers Thy-1, Lin−, c-kit+ were analyzed and sorted from the BM mononuclear cells as previously described.23 The sorted BM cells were cultured with mouse IL-3 (200 U/mL), human IL-6 (20 ng/mL), and polybrene (12 μg/mL) for 48 hours in virus supernatant at multiplicities of infection of 100 in virus-free IMDM medium. After irradiation of 950 rad, 5 C57BL6/Ly5.2 mice (7 weeks old, female) were transplanted with 5,000 LPK retrovirustransduced sorted BM cells through the tail vein. As a control, one C57BL6/Ly5.2 mouse was also injected with 5,000 nontransduced sorted BM cells through the tail vein. About 50 μL of peripheral blood was withdrawn from each mouse on days 30, 60, and 90 of BMT and was kept at −70°C until use. At day 135 of BMT, all mice were killed and their BM, spleens, and thymuses were removed and kept at −70°C until use.

RESULTS

Expression of human LPK cDNA in retrovirally transduced cells. Analysis of the lysates by polyacrylamide gel electrophoresis showed that NIH/3T3 cells transduced with E86/AmPK had a PK band corresponding to the LPK seen in human liver lysate. On the other hand, lysates of untransduced NIH/3T3 cells showed no LPK bands (data not shown).

Northern blot analysis showed the expression of human LPK mRNA in E86/AmPK-transduced NF-60, FDCP-2, K562, and HEL cells as shown in Fig 2. Three species of mRNA were mainly visualized, namely 5.1 kb, 4.6 kb, and 3.0 kb. Among these transcripts, the 3.0-kb mRNA transcript was considered to be promoted by the SV40 early promoter and to be functional for translation of human LPK cDNA.10 As K562 and HEL are human erythroid leukemia cells, they constitutively expressed human RPK mRNA, which hybridized with the human LPK cDNA probe, without the transduction of pMNSM-LPK. However, the intensity of RPK mRNA expression was increased in cells after the transduction of pMNSM-LPK because of the expressed LPK mRNA.

In vitro colony assays for the retrovirus-transduced mouse BM cells. The results of in vitro colony assays are shown in Table 1. In the CFU-GM assays, the number of colonies in each series was almost the same as that obtained from pretransduced or nontransduced BM samples in the absence of G418 selection. The G418-resistant colonies were only obtained from retrovirus-transduced BM cells. On the other hand, the CFU-GEMM numbers were generally decreased during in vitro culture. In our studies, the number of G418-resistant CFU-GEMM colonies increased after the virus transduction. The transduction efficiency was 54% to 62% in the former group and was 23% to 38% in the latter group.

One representative G418-resistant CFU-GEMM colony from each combination was picked and analyzed for the detection of human LPK mRNA expression; positive human LPK mRNA expression by RT-PCR method was seen in all transduced samples (data not shown).

Expression of human LPK cDNA in mouse after BMT. At days 30, 60, and 90 after BMT, peripheral blood from the transplanted BDF1 mice was withdrawn to obtain total RNA for the detection of human LPK mRNA expression by RT-PCR. However, our results did not demonstrate the expression of human LPK mRNA in the peripheral blood cells of recipient mice transplanted with retrovirus-transduced BM cells.

We then decided to use C57 BL/6 mice so that we could preliminarily check the engraftment after BMT simply by checking the immunologic markers Ly5.1 and Ly5.2 on leukocytes as previously described.21 The engraftment in the five mice and control was confirmed by fluorescence-activated cell sorter (FACS) analysis of the peripheral blood leukocytes using Ly5.1 and Ly5.2 monoclonal antibodies. At day 30 of BMT, 78.6%, 23.2%, 80.1%, 75.6%, 34.5%, and 77.6% of the peripheral blood leukocytes were donor derived (Ly5.1) in those five mice (no. 1 through 5) and the control, respectively. The degree of chimerism in the
Peripheral blood in these mice remained relatively constant throughout the course of study. When these mice were killed at day 135, the same degree of chimerism was found in thymus, spleen, and BM in each individual mouse, suggesting a stable engraftment of the hematopoietic stem cells. At day 30 of BMT, two (no. 3 and 4) of five mice transplanted with the retrovirus-transduced FACS-sorted BM cells were demonstrated to have human LPK mRNA expression in the peripheral blood by using RT-PCR (Fig 3A). However, at day 40 of BMT, one (no. 5) of the five mice transplanted with E86/AmPK-transduced FACS-sorted BM cells died accidentally. Additionally, mRNA expression in the peripheral blood in the rest of the mice was not detected at day 60 nor at day 90. At day 135 after BMT, these four mice were killed and their hematopoietic organs were studied. As shown in Fig 3B, human LPK mRNA expression was demonstrated in the thymus of mouse no. 1, in the BM and the thymus of mouse no. 3, and in the BM and the spleen of mouse no. 4. No human LPK mRNA expression was shown in mouse no. 2.

**DISCUSSION**

To date, only palliative treatments are available for PK deficiency. In almost all cases, splenectomy reduces the number of transfusions for patients; however, some cases still require repeated transfusions and become fatal, eg, we had a case of PK "Kyoto" who recently succumbed to repeated atrial fibrillation and ventricular tachycardia caused by myocardial hemochromatosis and cerebral embolism (manuscript in preparation). An approach to PK deficiency through gene therapy could be curative.

In this report, we described the introduction of human LPK cDNA into murine hematopoietic cells in vitro and in vivo. We first tried 5-FU-treated BDF1 mouse BM cells as targets for PK retrovirus. Although the in vitro colony studies showed some favorable results of the introduction of human LPK cDNA into mouse hematopoietic progenitors, we discontinued use of this system because our in vivo studies showed no PK mRNA expression in the peripheral blood at

**Table 1. Effects of Combination of Synergistic Factors on Colony Formation by PK-Retrovirus-Transduced BM Cells From 5-FU-Treated Mouse**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>G418 Selection</th>
<th>GM</th>
<th>GEMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre*</td>
<td>(+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>31 ± 4</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>IL-3</td>
<td>(+)</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>38 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IL-3 + SCF</td>
<td>(+)</td>
<td>3 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>45 ± 5</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IL-3 + IL-6</td>
<td>(+)</td>
<td>1 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>35 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>IL-3 + IL-6 + SCF</td>
<td>(+)</td>
<td>2 ± 1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>42 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IL-3 + SCF (untransduced)</td>
<td>(+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>34 ± 6</td>
<td>13 ± 5</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of the number of colonies in quadruplicate cultures, each containing 2.5 x 10⁵ cells from 5-FU-treated mice. Experiments were performed in the presence of 2 U/mL erythropoietin to allow erythroid expression.

Abbreviations: GM, CFU-GM; GEMM, CFU-GEMM.

* Assay was performed on BM sample before transduction with PK-retrovirus.
30, 60, or 90 days after BMT. To easily ensure successful grafting and to elevate the transduction efficiency into BM stem cells, we then used the C57BL/6 Ly5 mouse system using an FACS-sorted stem cell population.8,9 Our in vivo results showed human LPK mRNA expression in the peripheral blood at 30 days, but not at 60 or 90 days after BMT. At 135 days after BMT, mice hematopoietic organs were examined and variably expressed human LPK mRNA in each organ. Our results of human LPK mRNA expression studies were compatible with those of chimemer studies using Ly5.1 and Ly5.2 monoclonal antibodies. We considered that the human LPK mRNA expression in peripheral blood on day 30 might originate from virally transduced late progenitor cells. Because we did not perform secondary transplantation assays,24,25 we could not conclude whether LPK mRNA expression in the hematopoietic organ on day 135 originated from virally transduced pluripotent stem cells.

The insufficient LPK mRNA expression in hematopoietic cells in our studies was considered because of low viral transduction efficiency as well as the use of N2-SV-based vector as reported previously.24 Although we did not know the true transduction efficiency into hematopoietic cells in this study, coculture of the hematopoietic cells with virus-producing cells and the use of IL-3, IL-6, and SCF during the transduction step would increase the transduction efficiency.25 Furthermore, the use of new retroviral vector system would also help obtain sustained and higher expression of transferred gene.25,26 As our goal is the lineage-specific expression of human PK gene in the erythroid series, the development of an erythroid-specific enhancer or promoter in the retroviral construct,27 or an erythroid-specific virus vector system, eg, B19 parvovirus,28 might be helpful for the establishment of ideal gene therapy for PK deficiency.

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Retrovirus-mediated gene transfer of human pyruvate kinase (PK) cDNA into murine hematopoietic cells: implications for gene therapy of human PK deficiency

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