RAPID COMMUNICATION

Metabolic Correction of Congenital Erythropoietic Porphyria by Retrovirus-Mediated Gene Transfer Into Epstein-Barr Virus-Transformed B-Cell Lines

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Congenital erythropoietic porphyria (CEP) is an inherited metabolic disorder resulting from the accumulation of porphyrins because of defective uroporphyrinogen III synthase (UROIIIS). This autosomal recessive disorder is phenotypically heterogeneous with respect to the age of onset and the severity of the symptoms. Different exonic point mutations in the UROIIIS gene have been identified, providing phenotype-genotype correlations in this disease. Severe cases may be treated by bone marrow transplantation and are potential candidates for somatic gene therapy. Epstein-Barr virus-transformed B-cell lines from patients with CEP provide a model system for the disease. We have used retrovirus-mediated expression of UROIIIS to restore enzymatic activity in a B-cell line from a patient. We have also demonstrated the metabolic correction of the disease, i.e., porphyrin accumulation into the deficient transduced cells was reduced to the normal level. These data show the potential of gene therapy for this disease.

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Submitted November 29, 1994; accepted December 28, 1994.

Supported by grants from Association Française Contre les Myopathies, Fondation pour la Recherche Médicale, and Conseil Régional d’Aquitaine.

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0006-4971/95/8506-0039$3.00/0


Barr virus-transformed B-cell lines from patients with CEP provide a model system for the disease. We have used retrovirus-mediated expression of UROIIIS to restore enzymatic activity in a B-cell line from a patient. We have also demonstrated the metabolic correction of the disease, i.e., porphyrin accumulation into the deficient transduced cells was reduced to the normal level. These data show the potential of gene therapy for this disease.

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The vector Sac1 cells in the presence of 8 pg/mL polybrene during 3 hours for 6
tracted from lymphoblastoid cells by the usual methods and analyzed.
transferred onto nylon membrane (Hybond N'; Amersham, mid
tant obtained from producer cells was added to the
origin performed as described above.
of selection in 0.4 mg/mL G418 for 2 weeks and then maintained
For retroviral supernatant infection, 4 mL of the filtered super-

tant obtained from producer cells was added to the 2 x 10^6

target cells in the presence of 8 µg/mL polybrene during 3 hours for 6

consecutive days (2:1 colony-forming units/cell ratio). Selection was
performed as described above.

Southern blot analysis. High molecular weight DNA was ex-
ttracted from lymphoblastoid cells by the usual methods and analyzed
by gel blot hybridization. Ten micrograms of genomic DNA was
digested with SacI; this enzyme cuts in both LTR regions in our
construct and produces a 3.6-kb band from the LUSN vector (Fig 2).
The resulting fragments were separated on a 1% agarose gel and
transferred onto nylon membrane (Hybond N'; Amersham, Les Ulis,
France). Known amounts of digested plasmid DNA (20, 10, and 5
pg) mixed with control LB DNA (10 µg) were run on the same
gel. Filters were hybridized with a 780-bp human UROIIIS cDNA
fragment that was labeled with ^32P)dCTP by random priming
(Multilprime DNA labeling system; Amersham). The vector copy
number per genome was estimated by comparison of the amount
of radioactivity (Phosphor Imager; Molecular Dynamics, Sunnyvale,
CA). The radioactivity obtained with 10 pg of plasmid DNA corre-
sponds to the presence of one copy of the human genome when 10
µg of genomic DNA is used for the blot.

Determination of UROIIIS enzyme activity. A total of 10^6
to 10^9 LB cells (depending on the specific enzymatic activity) were
microfuged for 30 seconds and the pellets were washed with phos-
phate-buffered saline (PBS). The cells were resuspended in 250 µL
of UROIIIS lysis buffer (100 mMol/L Tris-Cl, pH 8.0, containing 1
mmol/L EDTA and 1% Tween-20), disrupted by sonication on ice,
and microfuged for 5 minutes. Supernatants were assayed for URO-
IIIS activity as described elsewhere.12 One unit of UROIIIS activity
was defined as the amount of enzyme that formed 1 nanomole of
urophyrinogen III per hour at 37°C.

Porphyrin accumulation in lymphoblastoid cells and in the med-

uum. Experiments were performed in polystyrene multiwell trays
seeded with 5 x 10^3 cells/well. LB were exposed to 0.3 mmol/L
and 0.6 mmol/L δ aminolevulinic acid (ALA; Sigma) for 24 and 48
hours. The medium was then kept, the cells were washed with PBS,
and the porphyrins were extracted from the cells and the culture
medium with 1 mol/L HClO4/CH3OH (1:1, vol/vol). The total por-

phyrin concentrations of the extracts were determined by spectroflu-
orimetry (excitation, 404 nm; emission, 595 nm) using an uropor-
phyrin I standard solution (10 nmol/L). The porphyrins accumulated
in the cells and in the medium were identified after separation by
high performance liquid chromatography (HPLC).13 Uroporphyrin
isomers were also separated by C18 reversed HPLC.13

RESULTS

Transduction of target cells. Lymphoblastoid cell lines
from patients with CEP were transduced with LXSN (control
virus) and LUSN particles at a multiplicity of infection
(MOI) of 2:1.

A Southern blot analysis showed that LB-deficient cell
lines transduced with LUSN and selected by G418 carried 1
copy per genome. We observed the same gene transfer

efficiency for the cells transduced by cocultivation and for those
transduced by retroviral filtered supernatant (Fig 2).

UROIIIS activity in normal cells as well as in transduced
and nontransduced deficient cell lines. LBN, nontrans-
duced LB-, and transduced LB- cells were collected before
and after G418 selection for UROIIIS assay. UROIIIS activity
was not detectable in nontransduced LB- or in LXSN-
transduced LB- cells (Table 1 and data not shown). In con-
trast, after transduction of the LB- cells by LUSN, UROIIIS
activity was increased to 20% and 24% of normal levels for
the transduced selection and the cocultivation techniques,
respectively. The same result was observed during 12 weeks of
culture, suggesting a stable integration and expression of the
retroviral construct. A higher level of UROIIIS activity was

Fig 1. General organization of the pLUSN retroviral vector in plas-
mid form. The UROIIIS cDNA is driven by the MoMLV/MoMSV LTR.
The vector also contains the neomycin phosphotransferase gene
(NEO) driven by the SV40 early promoter (SV40 ep) as well as the
origin of replication (ori) and ampicillin resistance gene (amp) from
PBR 322 for bacterial growth.

Fig 2. Identification of LUSN proviruses by Southern blot analysis.
Genomic DNAs were prepared from normal (lanes 5 and 6), untrans-
duced LB C73R/T228M (lane 4), untransduced LB C73R/C73R (lane
3), and transduced and G418-selected LB C73R/T228M (lane 1, cocul-
tivation technique; lane 2, supernatant infection) lymphoblastoid
cells. The DNA (10 µg/lane) was digested with SacI, separated on a
1% agarose gel, transferred to Hybond N' membrane (Amersham),
and hybridized with a 780-bp UROIIIS cDNA ^32P-radiolabeled
probe. Twenty, 10, and 5 pg of pLUSN was used with 10 µg of normal DNA
for the estimation of copy number per cell of the transgene. Note
that, for the 5 pg lane, the intensity of the endogenous bands are
lower because of a partial loss of the DNA carrier.
observed when the transduced cell lines were selected in medium containing G418 (115% and 127% of normal value, respectively; Table 1). Note that the level of UROHIS activity of LB- cells transduced by coculture was not significantly different compared with LB- cells transduced by viral supernatant.

Porphyrin accumulation. To evaluate the ability of LUSN transduction to correct the metabolic defect, the profile of porphyrin accumulation was first studied in normal and deficient LB cells in the presence or absence of ALA, the natural precursor of tetrapyrrole biosynthesis. In the normal cell lines studied, a very low amount of porphyrin could be found when they were incubated in the absence of ALA. The level of porphyrin generated by normal cells in the presence of ALA was also very low and identified to be the natural precursor of tetrapyrrole biosynthesis.

In the case of deficient cells, a significant amount of porphyrin was even found in the absence of ALA (4 to 8 fmol/10^3 cells). When the two different deficient LB cell lines were incubated with ALA for 24 and 48 hours at concentrations of 0.3 and 0.6 mmol/L, the level of accumulated porphyrins was very high (from 28 to 175 fmol/10^3 cells for the different conditions; Fig 3). These porphyrins were identified after separation by HPLC as uroporphyrin I (80%) and coproporphyrin I (20%).

Under these conditions, porphyrin accumulation provided an indirect estimation of UROHIS activity. The highest ratio of porphyrin accumulation between normal and deficient cells was found when cells were incubated with 0.3 mmol/L ALA; these conditions were kept for further studies. LB-deficient cells transduced by LUSN, selected by G418, and then exposed for 24 and 48 hours to 0.3 mmol/L ALA displayed very low levels of porphyrin accumulation, similar to the level obtained with normal cells (respectively, 10- and 15-fold lower than for the same nontransduced cells). This result showed that the transduction of deficient cells by LUSN corrected the porphyrin phenotype.

To determine the ability of normal LB and transduced deficient LB cells to effect a metabolic cross-correction of nontransduced deficient LB cells, normal LB and transduced deficient cells were cocultured for 24 hours with a variable percentage of nontransduced deficient LB cells. Porphyrin accumulation was then studied as described above. The level of porphyrin accumulation in cocultured cells corresponded closely to that expected from the proportion of the different cells studied (Fig 4). For instance, when 50% of LB cells were cocultured with 50% of LB transduced cells or 50% of LBN cells, porphyrin accumulation was approximately half that of the accumulation corresponding to 100% of LB cells. These results demonstrate that there is no metabolic cross-correction of deficient cells by normal cells or by LB-transduced cells in vitro.

**DISCUSSION**

Congenital erythropoietic porphyria is the most severe form of porphyria in humans. Forms of therapy that have
been described include hypertransfusion, splenectomy, the administration of haematin, oral activated charcoal, hydroxyurea, and low doses of chloroquine. A single patient has been treated by bone marrow transplantation from an HLA-identical sibling. There was a remarkable amelioration of the porphyria, but the patient died 11 months later from cytomegalovirus infection. Gene therapy by retrovirus-mediated insertion of the normal UROIIIS gene into hematopoietic cells seems to be an appropriate treatment of the disease in the future. We constructed a recombinant retroviral vector LUSN to mediate insertion and expression of human UROIIIS cDNA in a lymphopoietic target cell population. Lymphoblastoid cell lines from a patient with CEP were targeted for LUSN transduction.

A full restoration of enzymatic activity was found in transduced and G418-selected deficient cells. Also, a complete metabolic correction of the porphyrin phenotype was accomplished, as indicated by a total reversion of porphyrin accumulation in the cells. Because we know that the efficiency of retroviral transduction of hematopoietic cells in vitro and in vivo is low, we wanted to look at the possibility of metabolic cross-correction between nontransduced and transduced deficient or normal cell lines. This metabolic correction in neighboring nontransduced cells is well-established for human lysosomal storage diseases such as mucopolysaccharidosis type II (Hunter syndrome) or type VII (Sly disease). Our experiments show the absence of cross-correction between nontransduced and transduced cells. This result is not surprising because we could expect an absence of transfer of the enzyme from one cell to the other and also because uroporphyrin I and coproporphyrin I, which are the accumulated porphyrins, correspond to a metabolic dead-end (Fig 5). Except for the case of a selective advantage of corrected cells, these results suggest that gene replacement need to be performed on a high percentage of cells for a substantial improvement of the biochemical abnormalities and a subsequent amelioration of the clinical manifestations. Because the accumulation of porphyrins predominates in the erythropoietic lineage, the ideal target cell for gene therapy of CEP should be the myeloid stem cell or the pluripotent stem cell for long-term therapy. Recent studies using stromal cell support or cytokines have shown an enhanced efficiency of viral gene transfer (including retrovirus and adeno-associated virus) into such cell populations selected for expression of the CD34 antigen and thus enriched for the reconstituting stem cell compartment. More studies with other enhancers (ie, the locus control region) and the use of hematopoietic progenitor cells are required for the achievement of high expression of the UROIIIS cDNA in the erythropoietic lineage. However, we have documented sufficient gene transfer rate and metabolic correction in lymphopoietic cell lines to indicate that CEP should be a good candidate for treatment by gene therapy of hematopoietic stem cells.

ACKNOWLEDGMENT

We thank D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) for the LXSIN vector. We thank Françoise Denis for technical support and Maïté Sanchez for preparing the manuscript.

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