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Restoration of arylsulphatase A activity in human-metachromatic-leucodystrophy fibroblasts via retroviral-vector-mediated gene transfer

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Metachromatic leukodystrophy is a lysosomal storage disease caused by the deficiency of arylsulphatase A (ASA). A human ASA cDNA was subcloned into the retroviral vector pXT1. Replication-defective retrovirus was generated by tranfection of the vector into the amphotropic packaging cell line PA317. Human fibroblasts from a patient suffering from metachromatic leucodystrophy was infected with the recombinant retrovirus. Infected fibroblasts expressed ten times more ASA compared with control fibroblasts from a normal individual. The ASA encoded by the integrated provirus was shown to be correctly transported into the lysosomes and to normalize the impaired degradation of cerebroside sulphate.

INTRODUCTION

Metachromatic leucodystrophy (MLD) is an autosomal recessively inherited lysosomal storage disorder caused by the deficiency of arylsulphatase A (ASA) [1]. This deficiency causes the accumulation of cerebroside sulphate (CS), a polar glycolipid which is mainly found in the myelin sheaths of the nervous system. This leads to a progressive demyelination, which causes multiple neurological symptoms and is lethal for the patient. Affected indivduals die within a few years after the onset of symptoms. Clinically the disease is heterogeneous with respect to the age of onset and the severity of the symptoms. A late-infantile, a juvenile and an adult form are distinguished. This clinical variablity can be explained by different levels of residual enzyme activity associated with mutant ASA alleles [2]. The ASA cDNA as well as the gene have been cloned recently [3,4]. Here we demonstrate that retroviral transfer of an ASA cDNA into fibroblasts of a patient suffering from the severe late-infantile form of MLD corrects the metabolic defect in these cells.

MATERIALS AND METHODS

The cell line 2301 from a patient with late-infantile metachromatic leucodystrophy was obtained from the Human Genetic Cell Repository (Lyon, France). The packaging cell line was kindly provided by Dr. K. Pfitzenmeyer (Max-Planck-Institut Göttingen, Germany). The retroviral vector pXT1 was from Dr. E. Wagner (Institut für Molekulare Pathologie, Vienna, Austria). [5]. Cells were maintained at 37 °C, 5% CO_2 in minimal essential medium/15% (v/v) fetal-calf serum. Neomycin (G418 Geneticin) was from BRL, and restriction enzymes were from New England Biolabs.

Production of recombinant retrovirus

A 1828-bp *Xho*I–*Sal*I DNA fragment was isolated from the eukaryotic expression vector pBEH ASA [6]. It contains the entire cDNA from ASA, 62 bp of 5′ untranslated sequence of the ASA cDNA and 65 bp of simian-virus-40 5′ untranslated sequence which is part of the expression vector. This fragment was ligated into the *Xho*I site of the retroviral vector pXT1, and the orientation was checked by a double digest with *Xho*I/*Cla*I, making use of a *Cla*I site 430 bp downstream of the 5′ end of the ASA cDNA. Plasmids having the correctly oriented insert yield a fragment of 557 bp upon digestion with *Xho*I and *Cla*I. For the production of replication-defective infectious retrovirus the vector pXT1 ASA was transfected into the helper virus free-packaging cell line PA 317 [7] by the calcium phosphate precipitation technique. Cells were selected with neomycin (0.4 mg/ml). Resistant cells were recultured to about 50% confluency. Selection was withdrawn for 2 days; the tissue-culture medium was harvested and filtered through a 0.45 μm-pore-size filter; it was then tested on NIH 3T3 TK^- cells and stored at −80 °C. Throughout the present paper the retroviral vectors are designated by a 'p' (e.g. pXT1). The 'p' is omitted to designate the corresponding viruses (e.g. XT1).

Retroviral-gene transfer

Human MLD fibroblasts were seeded at 30% confluency into a 75 cm^2 culture flask 16 h before infection. Cells were incubated for 16 h with 5 ml of the filtered culture supernatant of PA 317 cells producing XT1 ASA retrovirus (5×10^4–10^5 colony-forming units/ml) in the presence of Polybrene (10 μg/ml). After 48 h, infected cells were selected by the addition of G418 (gentamycin; 2 mg/ml). Selection efficiency was checked by comparison to a mock infected control.

Enzyme assay, metabolic labelling and ASA immunoprecipitation

ASA activity was determined as described [7]. Metabolic labelling conditions, immunoprecipitation of ASA [8] and subcellular fractionation [9] was done as described.

[^{35}S]CS preparation

[^{35}S]CS was prepared by injection of $H_2{}^{35}SO_4$ into the brain of developing rat pups as described [10].

Cerebroside sulphate loading of fibroblasts

^{35}S-labelled rat brain CS (700000 c.p.m.) and 400 μg of bovine CS (Sigma) were combined in chloroform/methanol. Solvent

Abbreviations used: ASA, arylsulphatase A; CS, cerebroside sulphate; LTR, long terminal repeat; MLD, metachromatic leucodystrophy.

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was removed by a stream of nitrogen, 20 ml of minimal essential cell-culture medium was added, and the mixture was stirred for several hours until a stable suspension was achieved. This medium was warmed to 37 °C, and 3 ml was added to nearly confluent fibroblasts cultures in 25 cm^2 flasks. Aliquots (200 μl) of medium were removed on alternate days and assayed for inorganic sulphate in the aqueous phase after partioning with chloroform/methanol [11]. On the final day cells were harvested by trypsin treatments and the washed cell pellets were extracted with chloroform/methanol. Protein was determined in cell residues, and intracellular CS was determined by counting the radioactivity of the extract. Total CS uptake was the sum of intracellular CS and media inorganic sulphate derived from intracellular hydrolysis.

RESULTS

A cDNA containing the coding sequence of human ASA was cloned into the retroviral expression vector pXT1 [5], yielding the pXT ASA construct (Fig. 1). The retroviral vector contains an internal herpes-simplex-virus thymidine kinase promotor for the transcription of the exogeneous DNA. The neomycin-resistance gene is expressed from the Moloney-murine-leukaemia-virus 5′ long terminal repeat (LTR) of the vector.

The cell line 2301 was from a patient suffering from late-infantile MLD. The patient was homozygous for an ASA allele, having a splice-donor-site defect at the 3′ end of exon 2, and the fibroblasts lack ASA mRNA and cross-reacting material [2]. Infection of these fibroblasts with the XT ASA retrovirus and subsequent selection with neomycin resulted in cells expressing about 10-fold higher ASA activity when compared with normal cells (see Table 1).

To investigate the synthesis of ASA polypeptides in the MLD fibroblasts after retroviral-gene transfer, cells were labelled with [^{35}S]methionine and ASA was immunoprecipitated from cell homogenates. Compared with normal fibroblasts, about 8 times more cross-reacting material was found in the infected cells, which correlates well with the increase in catalytic activity (see Fig. 2). The size of the ASA polypeptides in cells infected with the XT ASA retrovirus was identical with that found in control fibroblasts. Correct lysosomal localization of the virus-coded ASA was demonstrated by subcellular fractionation on Percoll density gradients. Cells were labelled for 16 h and chased for 24 h. A postnuclear supernatant was prepared and subjected to Percoll-density-gradient centrifugation [9]. Enzyme activity determinations and immunoprecipitation showed the presence of newly synthesized metabolically labelled ASA polypeptides in the fractions containing dense lysosomes (see Fig. 3).

To demonstrate restoration of normal CS metabolism in the mutant fibroblasts that had been corrected by retroviral-gene transfer, the cells were incubated with CS that had been ^{35}S-

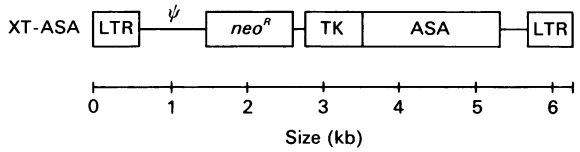

Fig. 1. Organization of the genome of the recombinant retrovirus XT ASA

The ASA cDNA was inserted in the retroviral expression vector pXT1 as described in the Materials and methods section. The ASA cDNA (ASA) is expressed from the internal herpes-simplex thymidine kinase promotor (TK) and the neomycin-resistance gene (*neo*^{R}) from the Moloney-murine-leukaemia-virus 5′ LTR. The location of the viral packaging signal is indicated (ψ).

Table 1. ASA activities in retrovirally infected cells

The Table summarizes the ASA activities measured in normal (control) or MLD fibroblasts (2301) infected with different retroviruses. S.D. values are given in parentheses. XT 1, retrovirus without cDNA insert; XT ASA, retrovirus with ASA cDNA insert.

Cell line	Retrovirus used for infection	ASA activity (munits/mg)	No. of infections
Control	XT 1	12.8±3.0	2
2301	XT	1.1	1
2301	XT ASA	126.6±9.6	3

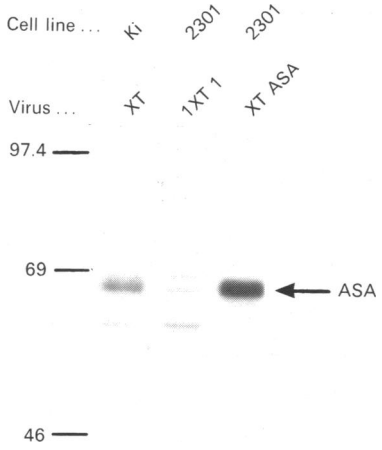

Fig. 2. Metabolic labelling and ASA immunoprecipitation of infected fibroblasts

Normal (Ki) and mutant (2301) fibroblasts infected with different retroviruses were labelled for 16 h with [^{35}S]methionine, and ASA was immunoprecipitated from the cells. Fibroblasts were infected with retrovirus XT 1 or XT ASA. Values on the left show the molecular masses of standards (in kDa). ASA-specific polypeptides are indicated by an arrow.

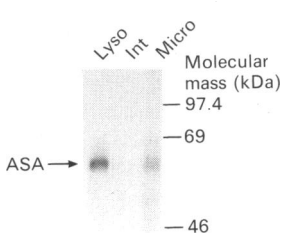

Fig. 3. Subcellular fractionation of infected fibroblasts

MLD fibroblasts (2301) infected with the retrovirus XT ASA were labelled for 16 h with [^{35}S]methionine and chased for 24 h. A postnuclear supernatant was subjected to Percoll-density-gradient centrifugation, and six fractions were collected. To confirm the identity of the fractions the distribution of ASA and β-hexosamidase activity (in parentheses) was determined in each fraction: 1, 53% (49%); 2, 10% (11%); 3, 4% (5%); 4, 0% (4%); 5, 7% (12%); 6, 25% (19%). Fractions 1+2, 3+4, and 5+6 were combined to give pools Lyso (dense lysosomes), Int (intermediate fraction) and Micro (microsomal fraction) respectively. ASA was immunoprecipitated from these pools and subjected to SDS/PAGE and fluororadiography.

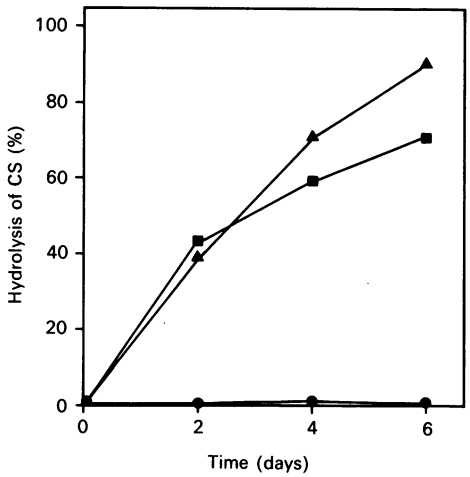

Fig. 4. CS hydrolysis by cultured human fibroblasts

Cultured fibroblasts were incubated with [^{35}S]CS, and the free sulphate released from the substrate was determined in the medium on alternate days. CS hydrolysis indicates the amount of free radioactivity in the media compared with the total amount taken up by the cells during the experimental period. Between 15 and 20% of the administered sulpholipid was assimilated by various cell types over the course of this experiment. ▲, Normal human fibroblasts; ■, 2301 MLD fibroblasts infected with the XT ASA retrovirus; ●, 2301 MLD fibroblasts infected with XT1 retrovirus without the ASA cDNA.

labelled. The lipid is taken up by the cells, and the enzyme releases radioactive sulphate, which then appears in the medium. The amount of free [^{35}S]sulphate in the medium reflects the activity of the ASA. As shown in Fig. 4, 2301 fibroblasts are unable to degrade CS. After retroviral-vector-mediated ASA cDNA transfer the CS-degrading activity was restored to normal levels, showing a correction of the metabolic defect in the mutant cells. A similar metabolic correction was observed in cells infected with a modified ASA cDNA in which the glycosylation in site in exon 6 had been deleted. The correction experiment was also repeated using a load-chase procedure. Comparable results were obtained (not shown).

DISCUSSION

We have demonstrated that a human ASA cDNA inserted into a retroviral vector can be efficiently transferred into human fibroblasts from patients suffering from MLD.

Cells expressing the retrovirus-encoded ASA had about 10-fold more enzyme activity when compared with normal fibroblasts. ASA polypeptides in corrected cells were shown to have the same size as endogeneously synthesized ASA and to be sorted correctly in the lysosomes. When fibroblasts were loaded with CS, the natural substrate of ASA, the cells infected with the ASA cDNA containing retrovirus were able to degrade the substrate at a rate comparable with that shown by normal fibroblasts. This demonstrates correction of the metabolic defect in these cells. The 10-fold difference in ASA enzyme activity, determined with the artificial substrate *p*-nitrocatechol sulphate, is not reflected by a similar difference in the CS loading test. The rate-limiting step in the latter is not the enzyme activity, rather the uptake of sulphatide by the cells. It has been demonstrated that even cells having only 10–20% of normal cell ASA activity will still metabolize CS at the normal rate in this type of assay [11].

Correction of lysosomal enzyme deficiencies by retroviral-gene transfer has so far been reported for three lysosomal storage diseases: β-glucocerebrosidase deficiency (Gaucher's disease) [12], arylsulphatase B deficiency (Mareautoux–Lamy syndrome) [13] and β-glucuronidase deficiency (Sly disease) [14]. The combination of retroviral-gene transfer and autologous bone-marrow transplantation is thought to be a feasible therapeutic approach for these diseases.

Bone-marrow transplantation has also been employed for the treatment of MLD [15]. Although final conclusions about the benefits of this approach cannot be drawn yet, it appears that it can mitigate the course of the disease. The ASA that is provided by the donor's bone-marrow cells must be delivered through the blood/brain barrier, since it is the nervous system which is mainly affected by the deficiency [1]. It appears that macrophages from the donor's bone marrow invade the patients brain and become so-called 'microglial cells' [16]. The ASA secreted by these cells and endocytosed by the patient cells may be sufficient to allow normal catabolism of CS. In fact very low levels of residual ASA activity (≈ 5%) have been shown to be sufficient to sustain a normal life [2]. The biochemical correction of the defect by retroviral-gene transfer that we demonstrated in cultured fibroblasts would most likely be applicable to haematopoetic cells as well, so that MLD may be another genetic disorder in which somatic-cell gene therapy may be a promising approach.

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