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SHORT COMMUNICATION

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Adeno-associated virus vectors are able to restore fatty aldehyde dehydrogenase-deficiency. Implications for gene therapy in Sjögren-Larsson syndrome

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Abstract Sjögren-Larsson Syndrome (SLS) is caused by an autosomal recessive defect in the gene coding for fatty aldehyde dehydrogenase (FALDH), an enzyme necessary for the oxidation of long-chain aliphatic aldehydes to fatty acid as one enzyme of the fatty alcohol:nicotinamide-adenine dinucleotide (NAD+)-oxidoreductase complex (FAO). The impaired activity of FALDH leads to the clinical symptom triad of generalized ichthyosis, mental retardation, and spastic diplegia or tetraplegia. Treatment options are primarily symptomatic. Gene therapy by means of genetic reintroduction of the functional FALDH gene into defective cells has so far not been considered as a therapeutic modality. In order to pursue such an approach for SLS, we constructed a recombinant adeno-associated virus-2 vector containing the human cDNA of functional FALDH and evaluated its capability to restore the enzyme-deficiency in a FALDH-deficient cell line resembling the gene defect of SLS. rAAV-2 transduction of FALDH-deficient cells, usually exhibiting less than 10% of normal FALDH activity, resulted in an increase of FALDH activity within the range of unaffected cells. Moreover, FALDH-transduced cells regained resistance over exposure to long chain aldehydes, which are otherwise toxic to FALDH-deficient cells. These results indicated that rAAV-2 vectors are able to restore FALDH-deficiency in a cell system resembling SLS. The findings give the first support to the concept that gene therapy might be a future option for the treatment of SLS.

Keywords AAV · Cutaneous gene therapy · Genodermatosis · Ichthyosis · FALDH

Abbreviations rAAV-2: Recombinant adeno-associated virus type 2 · CHO: Chinese hamster ovary cells · FALDH: Fatty aldehyde dehydrogenase · GFP: Green fluorescent protein · SLS: Sjögren-Larsson syndrome

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Introduction

Sjögren-Larsson syndrome (SLS (MIM 270200)) is an autosomal recessive disorder characterized by generalized ichthyosis, mental retardation, and spastic diplegia or tetraplegia [7, 9]. The ichthyosis is usually apparent at the time of birth and brings the patient to medical attention. The skin changes clinically resemble lamellar ichthyosis or congenital ichthyosiform erythroderma. In most cases, neurological symptoms develop within the first three years of life going along with a developmental delay in motor and cognitive functions. The spasticity leads to progressive contractures, preventing or impairing the ability to walk in most SLS patients. Mental retardation is profound with two thirds of the patients having an IQ of less than 50. Additional clinical features include glistening white dots on the retina, seizures, short stature and speech defects of various kinds.

The major causal feature of SLS is a defect in fatty alcohol oxidation due to deficiency of fatty aldehyde dehydrogenase (FALDH) (E.C. 1.2.1.48; Enzyme Nomenclature Database), a component of the fatty alcohol:nicotinamide-adenine dinucleotide (NAD+)-

oxidoreductase complex (FAO) [8]. FALDH is a microsomal NAD-dependent enzyme that is necessary for the oxidation of long-chain aliphatic aldehydes to fatty acids [6]. In SLS patients, a striking number of different mutations have been found in the FALDH gene on chromosome 17p11.2 that cause the deficient enzyme activity of FALDH and subsequently of FAO [3, 10]. The resulting tissue accumulation of free fatty alcohol, fatty aldehyde, and related lipid metabolites is thought to be responsible for the clinical symptoms, although more detailed information about the pathogenesis is missing today.

Concerning therapy, most interventions are purely symptomatic. Only recently, some beneficial effects of the leucotriene B4 synthesis inhibitor zileuton have been described [14].

One promising alternative to treat this recessive neurocutaneous disease might be gene augmentation by reintroduction of the wild-type gene into defective cells. In order to pursue such an approach, we introduced the cDNA for functional FALDH into recombinant adeno-associated virus type 2 (rAAV-2) vectors and tested their ability to restore the deficient FALDH activity in a FALDH-deficient cell line resembling the gene defect of SLS.

Materials and methods

Cells and culture conditions

Two different strains of Chinese hamster ovary cells, CHO-K1A and FAA.K1A, were generously provided by Dr. R. A. Zoeller, Boston University School of Medicine, USA [4]. Both cells strains were maintained in Ham's F12 medium supplemented with 10% newborn calf serum (NCS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all purchased from Biochrom KG, Berlin, Germany). Cells were routinely grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Media were renewed every 2-3 days. Cells were passaged at 80%–90% confluence with a split-ratio of 1:10.

Reagents

C-18 aldehyde octadecanal was synthesized with 97% purity at the Biochemical Institute for Environmental Carcinogenes, University of Hamburg, Germany. All other biochemicals were purchased from Sigma, Munich, Germany, unless otherwise noted.

Plasmid construction

pTR-UF/C-FALDH was constructed by inserting the *Not*I-*Nhe*I-fragment of pCIneo-FALDH (Stratagene, La Jolla, USA) into the *Not*I-*Nhe*I sites of pTR-UF/C via sticky end ligation. pCIneo-FALDH was a kind gift of Dr. W. B. Rizzo, Medical College of Virginia, USA. The excised fragment contained an open reading frame of 1455 bp encoding the functional human FALDH (GenBank-NM000382) [10]. pTR-UF/C is a derivative of pTR-UF (kindly provided by Dr. R. J. Samulski, University of North Carolina, USA), which was digested with *BamH*I and *Kpn*I, blunt ended, and ligated to the blunt-ended *Sal*I-fragment of pCep4 (Invitrogen, Carlsbad, USA), the former containing the inverted terminal repeats (ITR) of AAV-2, the minimal viral DNA sequences necessary in cis for the production of recombinant vector particles, the later containing an expression cassette with a cytomegalovirus immediate-early promoter and a SV40 polyadenylation signal. Vector plasmid pTR-UF/C-GFP containing green fluorescent protein instead of FALDH within the same construct has been described in detail previously [2] and was used for the preparation of rAAV-2/C-GFP vectors, which functioned as a negative control throughout the study. The packaging plasmid pRC containing the genes for AAV-2 replication (Rep) and -capsid (Cap) proteins, has been described previously [1]. The pXX6 adenovirus helper plasmid was also provided by Dr. Samulski [15].

Packaging of rAAV-2 vector particles

Preparation of rAAV-2 vector particles were performed using a standard protocol described by Xiao and colleagues [15]: 15 plates of 293 cells at 80% confluence were cotransfected by calcium-phosphate precipitation with a total 37.5 µg plasmid-DNA/plate of pTR-UF/C-FALDH or pTR-UF/C-GFP, pRC and pXX6 at a 1:1:1 molar ratio. After 48 h, cells were collected and pelleted by low-speed centrifugation. Cells were resuspended in 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), freeze-thawed at least three times, and treated with Benzonase (50 U/ml) for 30 min at 37°C. Cell debris was removed by centrifugation, and the supernatant was further purified by ammonium sulfate precipitation. The pellet was resuspended in PBS-MK buffer (1 × PBS, 1 mM $MgCl_2$, and 2.5 mM KCl), loaded onto an iodixanol gradient as described and subjected to centrifugation at 70,000 rpm for 75 min at 18°C [16]. The resulting 40% iodixanol phase containing rAAV-2/C-FALDH vector particles was harvested, aliquoted and stored at −80°C until being used. The genomic titer usually revealed up to 5×10^{11} particles/ml.

AAV-2 transduction

FAA-K1A cells (4×10^5) were plated into wells of 6-well plates (Nunc, Roskilde, Denmark) and allowed to adhere and grow overnight. When the cultures reached approximately 70%–80% confluence, the medium was

removed and cells were washed with PBS. For transduction, cells were incubated for 24 h in 2 ml of fresh Ham's F12 medium containing 50 µl rAAV-2/C-FALDH or rAAV-2/C-GFP lysate. Afterwards, the virus containing medium was removed and the cells were washed twice in PBS, passaged and expanded under normal circumstance onto fresh culture dishes.

FALDH activity assay

FALDH activity was assayed fluorometrically by measuring the fatty aldehyde-dependent production of NADH using octadecanal as substrate according to a standard protocol adapted from Rizzo and colleagues [6, 8]. Cells, either transduced with FALDH or GFP, were grown to near confluence in a 10 cm diameter tissue culture dish. Media was removed and cells were washed twice with ice-cold phosphate-buffered saline (PBS, Biochrom) and trypsinized with 0.05% trypsine (w/v) and 0.02% (w/v) EDTA in PBS until they became detached. After stopping the trypsine action with NCS, cells were pelleted by centrifugation at 300 g for 10 min, washed once with ice-cold PBS, and resuspended in 1 ml of homogenization buffer (25 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 0.1% Triton X-100). Cell suspension was homogenized with 40 up and down strokes in a manual glass Teflon homogenizer after Fenbroeck. An aliquot of each homogenate was removed for determination of protein content by a modification of the Lowry method using a commercial available kit (BCA-Protein Assay, Pierce, Rockford, USA). The rest of the homogenates were either immediately further processed or stored at −70°C. Frozen cell homogenates were thawed on ice and sonicated for 30 sec in a sonic water bath (Sonorex super RK103H, Bandelin, Berlin, Germany) prior to assay.

Reaction was performed in 50 mM glycine-NaOH buffer, pH 9.0, 10 mM pyrazole, 0.2 mg/ml fatty acid-free BSA and 1.5 mM NAD^+. 150 µg protein was assayed in a final volume of 800 µl. The reactions were initiated by the addition of the aldehyde substrate (160 µM octadecanal dissolved in ethanol) and monitored for 40 min using a Varian Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, USA) at an excitation wavelength of 365 nm and emission wavelength of 460 nm. The aldehyde-dependent activity was calculated by subtracting the change in fluorescence measured in the absence of aldehyde (control reactions contained ethanol as substrate) from that measured in the presence of aldehyde.

Aldehyde-cytotoxicity assay

600 FAA.K1A cells transfected either with FALDH or GFP were plated onto 96-well plates and allowed to grow for 24 h, before they were incubated with 60 µM octadecanal in fully supplemented media for 20 hrs. Thereafter cells were washed twice with PBS and culture under optimal conditions for another 5–7 days. Cytotoxicity was measured by a colorimetric assay using sulforhodamin B (SRB) for the detection of living cells [13]. Cells were fixed in 25% trichloroacetic acid for 30 min at 4°C, washed in distilled water and stained with 0.4% (w/v) in SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound dye was removed by four washes with 1% acetic acid. The 96-well plate was air-dried overnight. Protein-bound dye was extracted with 10 mM unbuffered TRIS. The optical density was determined in a computer-interfaced 96-well microtiter plate reader at 564 nm.

Results

In order to estimate the capability of rAAV-2 vectors to restore FALDH-deficiency in defective cells, we used a mutant strain of the Chinese hamster ovary cell line CHO-K1, designated FAA.K1A. FAA.K1A is specifically defective in long-chain FALDH similar to the enzymatic deficiency displayed by keratinocytes or fibroblasts from patients with SLS [4].

FAA.K1A cells were transduced with rAAV-2/C-FALDH vector particles and further expanded. As a negative control, FAA.K1A cells were transduced in parallel with rAAV-2/C-GFP to eliminate any influence of the vector per se on FALDH activity. In addition, untransduced FAA.K1A cells served as another set of negative and maternal CHO-K1, cells as a positive control. Figure 1 summarizes several independent transduction experiments: Homogenates derived from CHO-K1 cells displayed an almost constant, linear increase of NADH overtime resembling highly active FALDH, whereas untransduced (FAA.K1A) and GFP-transduced FAA.K1A (FAA.K1A-GFP) homogenates were severely FALDH-deficient, with a reduction below 10% of wild-type cells [8, 11]. Following FALDH gene transfer, FAA.K1A cells (FAA.K1A-FALDH) completely regained their ability to convert C18-aldehydes to fatty acids.

Another possibility to evaluate the enzyme function of FALDH is the assessment of cell cytotoxicity due to exogenous long chain fatty aldehydes [4]. In the CHO model, exposure of FAA.K1A cells to 60 µM octadecanal was highly toxic for the FALDH-deficient cells resulting in 99% dead cells. In contrary, 96% of the maternal CHO-K1 cells survived the incubation with the same amount of aldehydes (Fig. 2). Following FALDH transduction, FAA.K1A cells regained resistance to octadecanal with an increase in surviving cells of up to 82%.

Discussion

This study was undertaken to evaluate the capability of rAAV-2 vectors to restore FALDH activity in a mutant

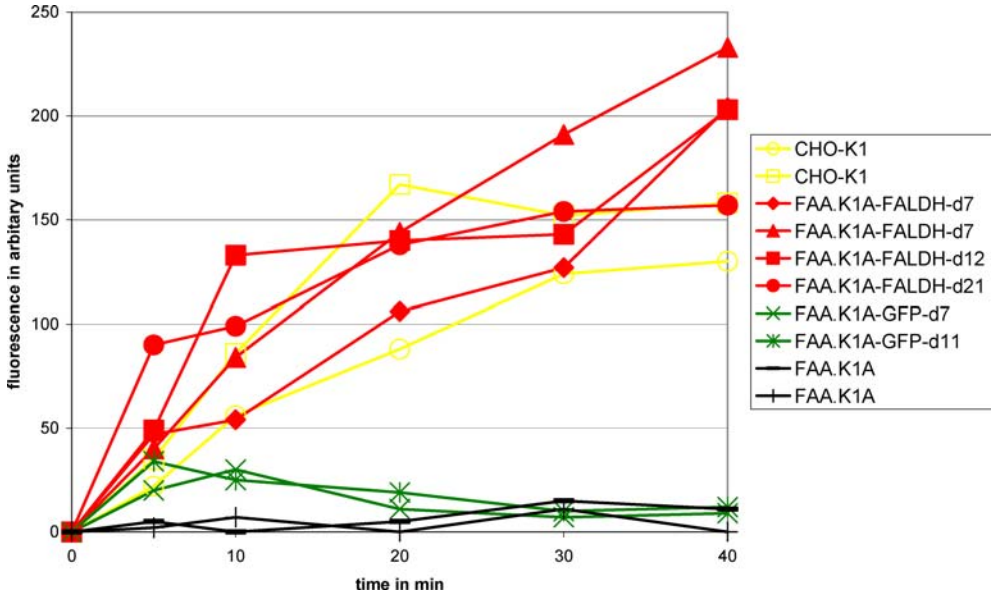

Fig. 1 Augmentation of FALDH activity in FALDH-deficient cells after rAAV-2-mediated gene transfer of functional FALDH. CHO-K1 wild type cells naturally expressing FALDH revealed a constant increase in NADH accumulation over a 40 min period reflecting highly active FALDH (yellow open symbols). FALDH-deficient FAA.K1A cells, either untransduced (black lines) or GFP-transduced (green crossed symbols) sdemonstrated severe FALDH-deficiency with less than 10% of normal activity. FAA.K1A cells transduced with rAAV-2/C-FALDH (red closed symbols) regained their ability to convert long-chain aldehydes to fatty acids. Shown are graphs from three independent transduction experiments

CHO model resembling the gene defect of SLS. Whereas the FALDH-deficient mutant FAA.K1A cells revealed a FALDH activity below 10% of the maternal CHO-K1A cells, they completely regained their ability to convert C18-aldehydes to fatty acids after rAAV-2-mediated gene transfer of the functional FALDH gene. Moreover, their resistance against exogenous long-chain aldehydes could be increased substantially to a level of almost 80% of the normal.

So far, gene transfer has not been considered as a therapeutic modality in the treatment of SLS. Our results give a first impression for the accessibility of this approach. The goal of the genetic reintroduction of functional FALDH genes would be a restoration of enzyme function to a level of up to 50–60% of the normal. When transferred to the human situation, this level of enzyme activity should be theoretically beneficial since heterozygous carriers of FALDH mutations display a partial enzyme deficiency with an activity between 50%–60% of the normal without the manifestation of an altered phenotype [5, 8].

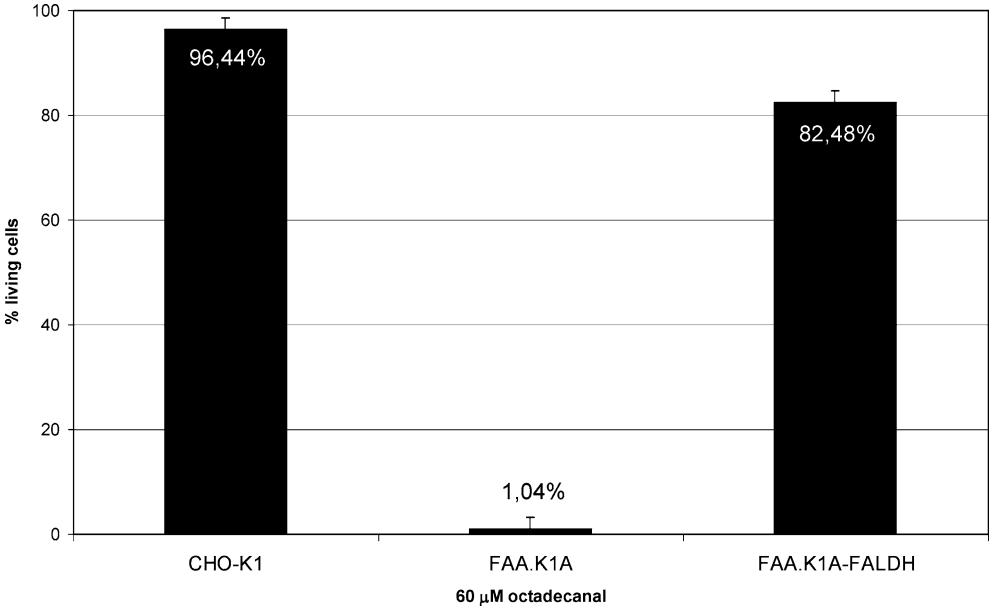

Fig. 2 Reduction of aldehyde cytotoxicity in FALDH-deficient cells after rAAV-2-mediated gene transfer of functional FALDH. Long chain aldehydes are toxic to FALDH-deficient cells, as indicated by 1% survival of FAA.K1A cells after exposure to 60 µM octadecanal, whereas normal CHO-K1 cells were almost unaffected. FALDH-transduced FAA.K1A-FALDH cells regained resistance to octadecanal with 82% surviving cells. Shown is one out of three independent experiments (SD ± of triplets)

Nevertheless, it will be a matter for future research to decide, which tissue will be the ideal target for gene transfer in order to treat this neurocutaneous disease.

We used AAV-2 vectors, because they combine many advantages as gene transfer vehicles, e.g. the apparent lack of pathogenicity, the low immunogenicity, and the potential to integrate the transgene into the human chromosome; a prerequisite to achieve long-term gene expression necessary for the treatment of inherited diseases [2, 12]. AAV-2 is able to infect a variety of cell types either in the proliferating or quiescent state with particular susceptibility for brain, liver and muscle tissue. Moreover, AAV-2 allows efficient gene transfer into human keratinocytes [1, 2].

Since skin and brain are the predominantly affected organs in SLS, rAAV-2 with the ability to target both tissues, may be promising as a vector for this indication. The skin would be advantageous because most studies concerning the pathophysiology of SLS have been performed on human keratinocytes and fibroblasts, thus facilitating the monitoring of any biochemical benefit after gene transfer. Moreover, the skin is an attractive tissue for gene transfer because it is easily accessible, its methods for cultivation and transplantation are well established in clinical routine, and it contains stem cells which are ideal targets for achieving long-term persisting genetic correction.

In SLS, there is a great theoretical chance that genetic modification of keratinocyte function might lead to clinical improvement of ichthyosis. On the other hand, it is unclear whether genetically modified keratinocytes are able to serve as a metabolic sink, thus reducing accumulated fatty alcohols in the plasma and helping to ameliorate neurological symptoms. Successful targeting of the central nervous system with rAAV-2 vectors has been demonstrated in several animal models. Unfortunately, the lack of an appropriate animal model for SLS, and missing insights in characteristic biochemical changes within the nervous system currently hamper experiments, which are focusing on the neurological symptoms in SLS. If a reduction of free plasma fatty alcohols might be beneficial for SLS patients, then targeting the liver, might be another possibility [6].

In conclusion, this study provides first evidence that therapeutic intervention on a genetic level may have potential benefits for SLS patients. While rAAV-2 vectors can target the pathophysiologically relevant tissues in SLS, namely skin, brain and liver, it should be considered as a vector of first choice for gene therapy of SLS. Nevertheless, it remains a matter for future research to determine the best applicable route to pursue such a strategy.

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