

LONG-TERM RESTORATION OF ALPHA-L-IDURONIDASE ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH MUCOPOLYSACCHARIDOSIS TYPE I AFTER NON-VIRAL GENE TRANSFER

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ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder caused by deficiency of alpha-L-iduronidase (IDUA). Limitations such as the need for weekly injections, high morbidity and mortality, and high cost of current treatments show that new approaches to treat this disease are required. In this study, we aimed to correct fibroblasts from a patient with MPS I using non-viral gene therapy. Using a plasmid encoding the human IDUA cDNA, we achieved stable high IDUA levels in transfected fibroblasts up to 6 months of treatment. These results serve as proof of concept that a non-viral approach can correct the enzyme deficiency in cells of patients with lysosomal storage disorders, which can be used as a research tool for a series of disease aspects. Future studies should focus on showing if this approach can be useful in small animals and clinical trials.

Keywords: *Mucopolysaccharidosis I; gene therapy; iduronidase*

In mucopolysaccharidosis type I (MPS I), the deficiency in alpha-L-iduronidase (IDUA) leads to lysosomal accumulation of the glycosaminoglycans (GAGs) heparan and dermatan sulfates. Abnormal storage of these GAGs results in progressive cellular and multi-organ dysfunction¹.

MPS I has multiple clinical presentations. In its most severe form, also known as Hurler syndrome, the disease, in the first year of age, is characterized by umbilical hernia, hepatosplenomegaly, skeletal abnormalities and developmental delay. The signs are progressive and, when untreated, typically result in death in the first two decades of life. In its mildest form, also known as Scheie syndrome, the disease has its onset in late childhood or puberty with slowly progressive skeletal, heart and eye manifestations. Intermediate forms (i.e., Hurler-Scheie syndrome) also exist².

Treatment approaches include hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT), both with limited effects. Despite recent advances, HSCT still presents a significant morbidity and mortality risk, and finding compatible donors is difficult. HSCT is also unable to halt the progression of skeletal manifestations even in patients with full engraftment and, currently, is indicated only to patients with the severe form of the disease, under the age of 2-2.5 years³. Conversely, ERT does not correct difficult-to-reach organs, such as the brain and the heart valves, and also has limited effects on the skeletal system⁴⁻⁶. Hence, novel strategies are needed to treat MPS I.

Gene transfer is a promising option, but safety concerns over the use of viral vectors are still a major problem, since insertional mutagenesis and development of immune response to the virus have already been described⁷. In the present study, we tested a non-viral gene transfer method aimed to correct the enzyme deficiency in fibroblasts from patients with MPS I.

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METHODS

For experiments, human skin fibroblasts (SF) were obtained from skin biopsies⁸ in a patient biochemically diagnosed with MPS I. The primary SF culture was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (Cultilab, Brazil) at 37 °C in the presence of 5% CO₂. The day before transfection, cells were plated onto 24-well plates at a density of 2×10^5 cells/well to reach 90-95% confluence (N = 4 or 5 wells/group).

We used the mammalian episomal expression vector pREP9, which carries the Epstein-Barr Virus replication origin (EBV oriP), the EBNA-1 gene (EBV nuclear antigen) and also the neomycin resistance (neoR) gene (Invitrogen, San Diego, USA) as backbone for plasmid construction. The human IDUA cDNA was inserted downstream to the Rous sarcoma virus (RSV) constitutive promoter as described elsewhere⁹. The resulting plasmid was named pRIDUA (Figure 1). DH5 α -T1R *Escherichia coli* (Invitrogen, Carlsbad, USA) was transformed with pRIDUA using standard procedures¹⁰ and the plasmid was purified with Qiagen Plasmid Maxi-Prep Kit (Qiagen, Valencia, USA).

Transfections were performed using lipofectamine 2000[®] reagent (Invitrogen, Carlsbad, USA) in serum-free DMEM for 24 h according to Balestrin et al.¹¹ or naked DNA. The amounts of lipofectamine used were 4 μ L for 1.5 μ g plasmid in 95% confluent cells plated onto 24-well plates (approximately 2×10^5 cells/well). Every 4 days medium was changed and supplemented with geneticin (400 mg/mL) for two weeks for cloning selection. After this period, clones were maintained with 200 mg/mL of geneticin.

Alpha-L-iduronidase activity in the cells was assessed with a fluorimetric assay that uses 4-methylumbelliferyl-alpha-L-iduronide (Glycosynth, UK) as substrate. Protein content was measured using the Lowry method. Results were expressed as nmol/h/mg protein¹². Comparisons between groups were performed using one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons. A $p < 0.05$ was considered statistically significant.

Fibroblasts from patients with MPS I were submitted to transfection with pRIDUA alone or combined with lipofectamine, and 24 hours later geneticin selection was initiated. We were able to observe clusters of cells after 2 weeks only in the plates transfected with lipofectamine. No clones were obtained using naked DNA.

RESULTS

Enzyme activity in pRIDUA transfected cells reached high levels, even higher than reference values for normal fibroblasts. The activity observed at 30 days was statistically significant compared to untreated MPS I fibroblasts ($p < 0.05$). This increase in enzyme activity was maintained along 60, 120 and 180 days of experiment (Figure 2). As an additional control to verify if the high IDUA levels observed were not due to geneticin or the pREP9 plasmid, we determined enzyme activity in cells transfected with pREP9 alone (without IDUA cDNA) and selected with antibiotics, and no increase in IDUA activity was observed (data not shown).

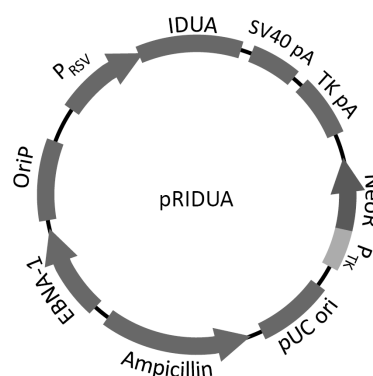


Figure 1: Structure of the pREP9-derived plasmid, pRIDUA. Ampicillin = ampicillin resistance gene (complementary strand); EBNA-1 = Epstein-Barr virus nuclear antigen gene; IDUA = human alpha-L-iduronidase gene; Neor = neomycin resistance gene (complementary strand); OriP = Epstein-Barr virus replication origin; P_{SRV} = Rous sarcoma virus long terminal repeat promoter; pUC ori = pUC origin of replication; P_{TK} = thymidine kinase promoter; SV40pA = simian virus 40 polyadenylation signal; TKpA = thymidine kinase polyadenylation signal.

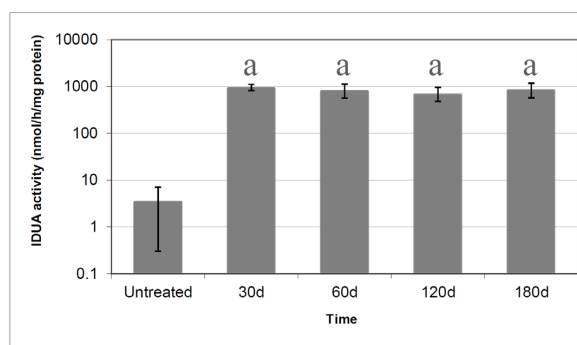


Figure 2: IDUA levels in MPS I fibroblasts before and after gene transfer. a) Statistically significant compared to untreated cells. IDUA reference values for normal fibroblasts are 73-150 nmol/h/mg protein. IDUA = alpha-L-iduronidase; d = days.

DISCUSSION

The inability of current therapeutic options to correct some aspects of MPS I has led researchers to develop several new approaches, including new delivery routes for ERT¹³, fusion proteins¹⁴, read-through compounds¹⁵⁻¹⁷, and inhibitors of GAG synthesis¹⁸.

The administration of the enzyme by the intrathecal route allows it to reach the cerebrospinal fluid directly, from where it can go to the brain parenchyma through the ependymal layer. Fusion proteins allow a therapeutic enzyme to cross the blood-brain barrier through receptor-mediated transcytosis. Read-through are compounds that allow the translation of a full protein in the presence of a premature stop codon and are being investigated in the subset of patients with MPS I with at least one nonsense mutation (e.g., p.Trp402Ter and p.Gln70Ter). Genistein is a soy isoflavone used to decrease the GAG synthesis and is currently being studied in patients with MPS III. It is still not clear if any of these approaches will benefit patients with MPS.

Gene therapy is a promising approach to treat lysosomal storage disorders such as MPS I. Attempts to correct IDUA deficiency have shown interesting results in mice and dogs with MPS I^{19,20}. However, most of the studies have used viral vectors to deliver cDNA, and safety issues such as insertional mutagenesis and development of an immune response have been raised for different types of viral vectors^{7,21}.

Non-viral gene delivery approaches are a safer option; however, they were ignored for a long time, until it was demonstrated that they are also able to provide long-term transgene expression²².

Here, a single transfection procedure followed by antibiotics selection was able to produce stable high

enzyme levels for up to 6 months. This approach, using a pREP9-derived construction, which is an episomal vector capable of autonomous replication independent of the genome of recipient cells²³, could be used to transfect other types of cells, such as mesenchymal or hematopoietic stem cells, which could then be re-infused into the patient, allowing a new therapeutic approach for MPS I. However, it is important to note that it is still uncertain whether gene expression would be maintained *in vivo*, since loss of plasmid and gene silencing can occur^{24,25}. Besides, as shown by previous studies on non-viral gene therapy in MPS I animal models^{25,26}, immune responses are still expected to occur, and an immunomodulatory strategy is likely to be required *in vivo*. Still, non-viral gene therapy protocols such as the one tested here are cheaper and safer than viral vectors, which favors our approach.

In the present study, we showed that non-viral *ex-vivo* gene therapy in human MPS I cells is feasible and long-term correction can be obtained. Future studies on the clinical application of such approach shall be conducted to obtain data on safety and efficacy of this procedure.

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Conflicts of interest

The authors declare no conflicts of interest.

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