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RNAi-mediated inhibition of the glucosylceramide synthase (*GCS*) gene: A preliminary study towards a therapeutic strategy for Gaucher disease and other glycosphingolipid storage diseases

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Abstract

Small interference RNAs (siRNAs) have recently been used in various experimental settings to silence gene expression. In some of them, chemically synthesized or *in vitro* transcribed siRNAs have been transfected into cells. In others, siRNAs have been expressed endogenously from siRNA expression vectors. Enzyme replacement and substrate deprivation therapies are currently used to treat Gaucher disease. Although good results have been reported, there are several limitations and side effects that make necessary to search for new alternatives. We present a new approach based on the inhibition of the *GCS* gene using siRNAs as a potential therapeutic strategy for Gaucher disease. We have designed four siRNAs for the human *GCS* gene and transfected them into HeLa cells. A clear reduction of *GCS* RNA levels and enzyme activity was obtained using two of the four siRNAs. Furthermore, a reduction in glucosylceramide synthesis was also observed. Similar results were obtained when plasmids expressing shRNAs (targeting the same sequences) were transfected into the cells. The inhibition of the mouse homolog *Ugcg* gene was also achieved, using a siRNA that targeted both human and mouse sequences.

Keywords: Gaucher disease; GCS/UGCG gene; siRNA; Therapeutic approach

Introduction

Glycosphingolipid (GSL) lysosomal storage disorders are a group of inherited genetic diseases caused by mutations in genes encoding enzymes involved in GSL catabolism. These diseases are characterized by the accumulation of the GSL substrates within lysosomes of cells, which results in cellular dysfunction and damage. The most common glycosphingolipidoses is Gaucher disease (GD), an autosomal recessive trait due to the accumulation of glucosylceramide, which is caused by deficient activity of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45). The main clinical symptoms are: progressive anaemia, thrombocytopenia, hepatosplenomegaly and skeletal disease in the presence (types II and III) or absence (type I) of central nervous system involvement.

Several disease-specific therapies have been developed. These therapies include: bone marrow transplantation, enzyme replacement therapy (ERT) or gene therapy, all of which focus on the partial recovery of the enzyme activity. ERT has proved successful for the treatment of most type I GD patients [1]. However, some limitations and disadvantages of ERT, such as the lack of effect on neurological symptoms, the high cost or the lifelong dependence on frequent infusions, have prompted the search for new therapeutic strategies. Recently, a new treatment for Gaucher disease, substrate reduction therapy (SRT), proved to be efficient [2] and was approved for particular cases. SRT is based on partial inhibition of synthesis of the substrate (glucosylceramide) by *N*-alkylated iminosugar analogues to a level that can be effectively cleared by the affected enzyme with residual hydrolytic activity. These compounds act on glucosylceramide synthase (GCS), also known as UDP-glucose ceramide glucosyltransferase (UGCG), EC 2.4.1.80, which catalyses the first step in GSL synthesis. In particular, the iminosugar

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N-butyldeoxynojirimycin (*N*B-DNJ; OGT 918, referred to as miglustat) has been through clinical trials [2,3] and has been approved for treatment [4]. However, some side effects have also been reported.

Other experimental approaches have also been tested. On one hand, some of the aforementioned inhibitors seem to act as chaperons assisting protein folding and stability of mutant GBA enzymes [5]. On the other hand, gene therapy reached the clinical trials with disappointing results [6]. Nevertheless, research in this field is still actively trying to find methodological improvements that could make gene therapy feasible [7]. Other approaches, such as the use of chimeraplasts for gene repair, have proven unsuccessful in correcting GD mutations [8].

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs), a type of posttranscriptional gene silencing, has been used to mediate the down-regulation of gene expression. This methodology has a wide range of possible applications including those with a potential therapeutic effect. Research projects focused on the treatment of viral, oncological, or neurological diseases have been reported (for a review see [9]).

This technology is applied in our study, to inhibit *GCS* gene expression. We analyzed the effect of different siRNAs and, with some, a clear reduction in *GCS* mRNA level was achieved. We also observed a decrease in the activity of the enzyme and in the GlcCer formation. Finally, we analyzed the effect of siRNAs on the mouse *Ugcg* gene, ortologue of the human *GCS* gene, and also found a reduction in mRNA levels.

Materials and methods

Design and synthesis of siRNAs

Four siRNAs (siRNA11, siRNA34, siRNA48 and siRNA68) designed to inhibit the human *GCS* mRNA (GenBank accession no. NM_003358) were selected according to previously described guidelines [10]: a sequence of the type AA(N19)UU, with a G/C content between 30 and 50%, not found in other genes, or with the shortest homology region with other genes searched in *Blast* (http://www.ncbi.nlm.nih.gov/BLAST/). The situation of the siRNAs on the predicted secondary structure of the *GCS* mRNA was determined using the Michael Zucker Mfold program (http://bioinfo.rpi.edu/applications/mfold/old/rna). The 21-nucleotide double stranded RNAs were synthesized by *in vitro* transcription (*Silencer siRNA Construction Kit*; Ambion). The oligonucleotides used are listed in Table 1. The siRNAs for human *GAPDH*, used as a control, were synthesized using primers provided in the *Silencer siRNA Construction Kit* (Ambion). siRNAs were quantified by measuring the absorbance at 260 nm and their double-stranded nature was tested in 1% agarose gel by ethidium bromide staining.

shRNA expression vectors

Short hairpin RNA (shRNA) expression vectors were constructed using the oligonucleotides GCS11_F, GCS11_R, GCS68_F and GCS68_R described in Table 1. They were annealed to form double-stranded DNA fragments and inserted into the *Acc65*I and *Hin*dIII sites of the psiRNA-hH1-Zeo plasmid (Invivogen San Diego, CA, USA). This plasmid contained a GFP:Zeo fusion gene which allowed GFP detection and Zeocin™ (Invitrogen) selection.

Cell culture and transfection

HeLa cells, and mouse WEHI-3B and RAW264.7 cells (donated by Dr. Celada) were cultured in DMEM medium (Gibco-BRL) containing 100 U/ml penicillin/streptomycin (Gibco-BRL), and 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Cells were seeded in 6-well culture plates at a density of 1.3×10^5 cells per well (30% confluency), 18 h prior to

Table 1

si/shRNA name	Oligonucleotide name	Oligonucleotide sequence (target sequence)	Position in cDNA	Exon	G/C content %
siRNA11	AsiRNA11	5′-***AAAGGGGTAGATCCTAACTTA***CCTGTCTC-3′	468	2	38.1
	SsiRNA11	5′-AATAAGTTAGGATCTACCCCTCCTGTCTC-3′			
siRNA34	AsiRNA34	5′-***AATGCCAGGATATGAAGTTGC***CCTGTCTC-3′	674	4	42.9
	SsiRNA34	5′-AAGCAACTTCATATCCTGGCACCTGTCTC-3′			
siRNA48	AsiRNA48	5′-***AAATGTGTGACAGGAATGTCT***CCTGTCTC-3′	906	6	38.1
	SsiRNA48	5′-AAAGACATTCCTGTCACACATCCTGTCTC-3′			
siRNA68	AsiRNA68	5′-***AATTTGTGAGCCAATTTCAGA***CCTGTCTC-3′	1154	8	33.3
	SsiRNA68	5′-AATCTGAAATTGGCTCACAAACCTGTCTC-3′			
shRNA11	GCS11_F	5′-GTACCTC***AAAGGGGTAGATCCTAACTTA***TCAAG AGTAAGTTAGGATCTACCCCTTTTTTTTGGAAA-3′	468	2	
	GCS11_R	5′-AGCTTTTCCAAAAAAAAGGGGTAGATCCTAAC TTACTCTTGATAAGTTAGGATCTACCCCTTTGAG-3′			
shRNA68	GCS68_F	5′-GTACCTC***AATTTGTGAGCCAATTTCAGA***TCAAG AGTCTGAAATTGGCTCACAAATTTTTTTGGAAA-3′	1154	8	
	GCS68_R	5′-AGCTTTTCCAAAAAAATTTGTGAGCCAATTTCA GACTCTTGATCTGAAATTGGCTCACAAATTGAG-3′			
	GCS_exon5F	5′-CTTTGCTGCCACCTTAGAGC-3′			
	GCS_3′ UTR	5′-GCAAGTGCCATGCAAAAATA-3′			
	GAPDH_F	5′-GTCAGTGGTGGACCTGACCT-3′			
	GAPDH_R	5′-AGGGGTCTACATGGCAACTG-3′			