THE CLN9 PROTEIN – A REGULATOR OF DIHYDROCERAMIDE SYNTHASE

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Running title: CLN9, a regulator of dihydroceramide synthase

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A new variant of a group of pediatric neurodegenerative diseases known as neuronal ceroid lipofuscinosis (NCL)§ or Batten disease has been identified. It is termed CLN9-deficient. CLN9-deficient fibroblasts have a distinctive phenotype of rapid growth and increased apoptosis, and diminished levels of ceramide, dihydroceramide and sphingomyelin. Transfection with CLN8 but not other NCL genes, corrected growth and apoptosis in CLN9-deficient cells, although the entire CLN8 sequence was normal. CLN8 is one of the TLC§ proteins containing a Lag1 motif. The latter imparts (dihydro)ceramide synthase activity to yeast cells. Transfection with the yeast gene Lag1 Sc and the human homolog LASS1 increased ceramide levels, partially corrected growth and apoptosis in CLN9-deficient cells. LASS2, 4, 5, and 6 also corrected growth and apoptosis. Dihydroceramide levels and dihydroceramide synthase activity were markedly diminished in CLN9-deficient cells. Sequencing of LASS1, LASS2, LASS4, LASS5, and LASS6 genes was normal, and expression levels were increased or normal in CLN9-deficient cells by RT-PCR. 4-HPR, a dihydroceramide synthase activator, corrected growth and apoptosis and increased dihydroceramide synthase activity. Ceramide levels dropped further, and there was no increase in de novo ceramide synthesis, probably due to the effects of 4-HPR as activator of dihydroceramide synthase and inhibitor of dihydroceramide desaturase. Fumonisin B1, a dihydroceramide synthase inhibitor, exaggerated the CLN9-deficient phenotype of accelerated growth, decreased ceramide and increased apoptosis. This was neutralized by 4-HPR. We conclude that the CLN9 protein may be a regulator of dihydroceramide synthase, and that 4-HPR could be developed as a treatment for CLN9-deficient patients.

The neuronal ceroid lipofuscinoses (NCL) are a group of autosomal, recessively inherited storage diseases. They represent the most common cause of neurodegeneration in childhood. Neuronal death is caused by apoptosis (1). Clinical features of these diseases include visual loss, mental and motor deterioration, seizures, and early death (2-3). To date nine forms of NCL have been described. Six genes are known: CLN1 and CLN2 encode for the lysosomal enzymes palmitoyl protein thioesterase 1 (PPT1)§ and tripeptidylpeptidase 1 (TPP1)§, respectively (4-5). CLN5p has been described as a soluble glycoprotein (6-7). CLN3, CLN6 and CLN8 genes code for novel transmembrane proteins (8-11). Increased apoptosis has been documented in CLN1-3, 6- and 8-deficient
Dysregulated sphingolipid metabolism and increased apoptosis have been described in the late infantile CLN2-, juvenile CLN3-, variant late infantile CLN6- and the Northern Epilepsy with Mental Retardation CLN8-deficient variants. CLN3 is a negative modulator of ceramide synthesis resulting in high ceramide levels in CLN3-deficient patient fibroblasts (14). High ceramide levels, caspase-8 activation and engagement of both the extrinsic and intrinsic apoptotic pathway, as well as caspase-dependent autophagy explain the increased apoptosis and slow growth observed in CLN3-deficient cells. Also, CLN3 protein harbors a galactosylceramide binding motif that is involved in CLN3 trafficking from Golgi to lipid rafts via recycling endosomes (14). The ninth form of NCL, CLN9, has recently been described in four patients, two German brothers and two Serbian sisters (15). These patients present with clinical features identical to those seen in juvenile or CLN3-deficient patients. The CLN9 gene is still unknown. Mutations in all known NCL genes, including CLN3, were ruled out in the CLN9-deficient variant. CLN9-deficient patient fibroblasts have a distinctive phenotype. They have small and rounded cell bodies, grow rapidly, are sensitive to apoptosis and manifest a cell adhesion defect. Their gene expression pattern is significantly different from that in other NCL forms. Genes involved in cell cycle, cell adhesion and apoptosis are significantly dysregulated (www.dbsr.duke.edu/pub/cln9/).

Sphingolipid metabolism, similarly to other NCL variants, is also perturbed in CLN9-deficient cells: Ceramide, sphingomyelin (SM)§, lactosylceramide (LacCer)§, gangliosides, ceramide trihexoside (CTH)§, and globoside (GB4)§, galactosylceramide (GalCer)§ and glucosylceramide (GluCer)§ are diminished.

The purpose of this study is to dissect out the specific biochemical defect in CLN9-deficient cells, with the ultimate goal of identifying the CLN9 gene and protein. Low levels of LacCer and its metabolites including gangliosides, CTH and GB4 can implicate the seven genes for beta-1,4 galactosyltransferase (β4GalT)§ and the two for GB3/CD77 synthase as possible candidate genes.

Review of genechip data provided additional information: acid sphingomyelinase activity was normal, but mRNA expression of acid sphingomyelinase was 22-fold decreased in the setting of low baseline sphingomyelin levels. Defects in sphingomyelin synthase 1 and/or 2 (SMS1, SMS2)§ could account for this. A trafficking defect of ceramide from ER to Golgi due to defects in CERT§, the ER-to-Golgi ceramide transporter, could also account for low SM (16).

Low ceramide levels also suggest a defect in de novo ceramide synthesis. Activity of serine palmitoyltransferase (SPT), the key enzyme of de novo ceramide synthesis, was four-fold increased expanding the list of candidate genes to those involved in de novo ceramide synthesis beyond the SPT step. Transfection with CLN8 corrected the CLN9 phenotype. The CLN8 protein contains a Lag1 motif similar to other TLC proteins (TRAM-Lag1p-CLN8) (17). Lag1p and Lac1p are homologous, multispanspanning transmembrane proteins that reside in the ER. Both contain a 52 amino acid long Lag1 motif that is necessary for ceramide synthesis in yeast (18-19). Lac1p, Lag1p and Lip1p are components of the enzyme activity in yeast (20). CLN8 sequencing results were normal in CLN9-deficient cells, suggesting human Lag1 homologs or activators of their proteins could be candidate genes for CLN9.

Experimental Procedures

Cell Lines - CLN9-deficient fibroblast lines were derived from skin biopsies of two CLN9 patients. Use of human cell lines in research is covered by an approved Duke institutional review board protocol.

Tissue culture studies - Fibroblasts were grown at 37°C with 5% CO₂ in DMEM (Gibco-Invitrogen, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS) (Gibco-Invitrogen) and 1%
antibiotics/antimycotics.

**Growth curves** - 1 x 10^5 cells/well were plated in 6 well plates, then harvested and counted at 24, 48, 72, and 96 hours in triplicate for each time point using the Trypan blue dye exclusion method.

**Trypan blue dye exclusion** - Harvested cells were centrifuged at 1200 rpm for 5 min and pellets resuspended in 1:1 mixture of 4% trypan blue dye (Gibco-Invitrogen) and 1x PBS, loaded onto a hemocytometer. Viable white and dead blue cells were counted.

**Etoposide treatment** - Cells were treated with 10 µg/ml etoposide (Sigma, St. Louis, MO) for 18 h. Apoptosis was determined by propidium iodide (PI)§, JC-1 or TUNEL staining.

**4-HPR treatment** - Cells were treated with 2.5 µM, 5 µM and 10 µM 4-HPR (Scienclab.com, Houston, TE). 4-HPR was dissolved in Ethanol at a concentration of 10 mM and stored at -20°C. Stock solutions were diluted to the required concentrations with growth medium just before use.

**Fumonisin B1 treatment** - Cells were treated with 1 µM Fumonisin B1 (FB1)§ (Sigma, St. Louis, MO). FB1 was dissolved in DMSO at a concentration of 1 mM and stored at -20°C. Stock solutions were diluted to the required concentration with growth medium just before use.

**PDMP treatment** - Cells were treated with 20 µM PDMP§ (Sigma). PDMP was dissolved in DMSO at a concentration of 20 mM and stored at -20°C. Stock solutions were diluted to the required concentration with growth medium just before use.

**Transfection** - Cells were transfected with different cDNA-vector constructs: **CLN1-pGEM, CLN2-pGEM, CLN3-pGEM, CLN6-pGEM, CLN8-pGEM** (cDNAs amplified and cloned as previously described), **Lag1-pGEM, LASS1-pIRES2-EGFP** (cDNA clones provided by S.M. Jazwinski), **LASS1trv3-pGEM, LASS2trv1/2-pGEM, LASS3-pIRES2-EGFP, LASS5-pGEM** (cDNA clones purchased from Open Biosystems, Huntsville, AL, USA), **LASS2trv3-pGEM** (cDNA clone purchased from OriGene Technologies Inc, Rockville, MD), **LASS4-pBluescript** (cDNA clone purchased from RZPD, Berlin, Germany). The protocols for the Lipofectamine 2000 Transfection Kit (Invitrogen-Life Technologies) were followed. The cells were washed and plated for analysis. Transfection efficiency of pGEM-transfected cells was determined using an YFP-pGEM construct and counting yellow fluorescent cells/total cells/vision field in triplicate under fluorescent microscopy. Transfection efficiency of pIRES2-EGFP-transfected cells was determined by counting green fluorescent cells/total cells/vision field in triplicate under fluorescent microscopy. Transfection efficiency of pcDNA3.1/V5-His-TOPO transfected cells was determined using a pcDNA3.1/V5-His-TOPO-LacZ construct. Transfected cells were stained using a β-Gal Staining Kit (Invitrogen-Life Technologies). Transfection efficiency was determined counting stained cells/total cells/vision field in triplicate under light microscopy.

**Sequencing** - All exons including exon-intron junctions of GB3/CD77 synthase, β4Galt1-7, **LASS1, LASS2, and LASS4**, were amplified from genomic DNA of CLN9-deficient cells. Entire coding regions of **LASS5, LASS6, SMS1** and **SMS2** were amplified from cDNA of CLN9-deficient cells. Products were sequenced using an automated sequencer (377XL Prism DNA Sequencer; PE Biosystems, Foster City, CA, USA).

**PI staining** - Equal numbers of cells treated/not treated with etoposide, were grown on coverslips and stained with PI (5 µg/ml) for 5 min. Three fields of vision were chosen randomly at 100x magnification. The total number of cells/field of vision (20–60 cells/field) was counted. The number of PI-positive red apoptotic cells was determined under fluorescence (excitation wavelength: 525 nm, emission wavelength: 600 nm). The
percentage of PI-positive cells/total cells/field of vision was calculated. Average and standard deviation were determined, and statistical significance calculated using the Student’s $t$-test.

**JC-1 staining** - JC-1§ stain (5,50-60-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR) was used to assess the decrease in mitochondrial membrane potential following treatment with etoposide. JC-1 is a cationic dye that forms J-aggregates with the cytochrome c-APAF-1 complex. Cells undergoing apoptosis are visualized when a shift in the emission spectrum from green (525 nm) to red (590 nm) occurs. This shift signals the onset of apoptosis. Equal numbers of cells treated/not treated with etoposide, were grown on coverslips. The cells were then washed with 1 x PBS and incubated with 1 mg/ml of JC-1 stain for 15 min at 37°C and in 5% CO$_2$. The cells were again washed in PBS and the mounted on a slide with 1:1 PBS/glycerol mix. Red apoptotic and total cells in three different fields of vision were counted (100-200 cells/field). The percentage of red apoptotic cells/total cells/field of vision was calculated, as well as average and standard deviation. Statistical significance was determined using the Student’s $t$-test.

**Analysis of exogenous ceramide trafficking** - Equal numbers (1 $\times$ 10$^5$) of normal and CLN9-deficient fibroblasts were grown on coverslips and incubated in a solution containing Hank’s balanced salt solution, 10mM HEPES (Gibco), 1% (w/v) fatty acid free BSA (Sigma), and 5 $\mu$m C6-NBD-ceramide at 16°C for 3 hours. Cells were then washed in Heps-HBSS solution followed by back exchange for 30 minutes in Heps-HBSS-BSA. Cells were mounted onto a glass slide with 1 drop of Fluoromount (Southern Biotechnology Associates, Birmingham, AL) and viewed with a Leica DMLB fluorescent microscope.

**Ceramide and dihydroceramide levels** - Ceramide and dihydroceramide levels were quantified by mass spectrometry (Lipidomics Core, Medical University of South Carolina) according to published methods. Ceramide and dihydroceramide levels were also measured by DGK* assay using Diacylglycerol Kinase from E. coli (Sigma) and 1.3 µCi of ATP/sample (Amersham Biosciences, Piscataway, NJ) using standards for C16, C24 and C24:1 ceramides and corresponding dihydroceramides (Avanti, Alabaster, AL).

**Dihydroceramide synthase activity** – Dihydroceramide synthase activity was determined by measuring de novo dihydroceramide generated per hour after labeling cells with C17-dihydrosphingosine (C6-dihydrosphingosine with C17 backbone) (Mol.wt.287.5 g/mole; 5 µm) for 4 hours.

**Sphingomyelin synthase activity** - Cells were washed three times with phosphate-buffered saline, harvested using a rubber policeman, and homogenized in 1 ml of 25 mM KCl and 25 mM Tris-Cl (pH 7.4) (TK buffer) and 0.5 mM EDTA. Cells were homogenized by 20 passages through a 27 gauge needle. Homogenates were used fresh. SM synthesis was assayed as described. The reaction mixture contained 100 µg of protein, 10 µl NBD-6-ceramide (1 mM), 1.7 µl of phosphatidylcholine (10 mg/ml), in total volume of 300 µl of TK buffer. The reaction was terminated after 90 min at 37°C by addition of 1 ml chloroform/1 ml methanol/400 µl water. Lipids were then extracted according to Bligh and Dyer and SM spots separated on TLC using chloroform/methanol/CaCl$_2$ (15 mM) solution (120:70:16 v/v/v) as developing solvent. TLC plates were analyzed using a PhosphorImager and bands were quantified by ImageQuant software (Amersham v. 2003).

**Real-time PCR** - Total RNA was isolated from normal and CLN9-deficient fibroblasts using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription reactions were carried out with Super Script III First-Strand-Synthesis System (Invitrogen). Real-time PCR was performed using total cDNA and Absolute QPCR SYBR Green
Fluorescence Mix (Abgene Inc., Rochester, NY) according to the manufacturer’s protocol in a Biorad I-Cycler. The mRNA levels of Cyclophilin/HPRT/28S ribosomal RNA were used as internal controls.

RESULTS

CLN8 corrects growth and apoptosis in CLN9-deficient cells

CLN9-deficient fibroblasts were transfected with CLN1, CLN2, CLN3, CLN6 and CLN8 genes individually and were assessed for growth and sensitivity to apoptosis. Only transfection with CLN8, out of all NCL genes, partially corrected growth in CLN9-deficient cells (Fig. 1 A, B). CLN9-deficient cells transfected with CLN8 cDNA showed a significant decrease in growth rate compared to CLN9-deficient cells transfected with empty vector. Growth rate still exceeded that of normal fibroblasts transfected with EV (Fig. 1 C). Transfection with CLN8 decreased sensitivity to apoptosis in CLN9-deficient fibroblasts. Cells transfected with CLN8 or empty vector were stained with JC-1 after 16 hours of etoposide treatment. Cells transfected with CLN8 showed significantly less red J-aggregate formation than controls transfected with empty vector (Fig. 1 D).

Dihydroceramide synthase activity, dihydroceramide and ceramide levels are low in CLN9-deficient cells

CLN9-deficient cells have significantly decreased dihydroceramide synthase activity, as reflected by the decreased levels of dihydroceramide and ceramide in CLN9-deficient cells. The most significant decreases were in dhC16-, dhC24- and dhC26-ceramide and C16-, C24-, and C24:1-ceramide species compared to control cells (Fig. 2 A, B). DhC24-Cer, dhC24:1-Cer and dhC26:1-Cer levels were 4.4%, 8.2% and 9.5%, respectively, compared to normal controls.

Dihydroceramide synthase activity was measured by labeling cells with C17 dihydrospingosine and measuring dihydroceramide generated over four hours (Fig. 5 G).

Lagl Sc increases ceramide and corrects growth and apoptosis in CLN9-deficient cells

Transfection with Lagl Sc increased total ceramide levels in CLN9-deficient cells 105% above levels in cells transfected with empty vector. This was determined by DGKS assay (Fig. 3 A). Tandem mass spectrometry showed an increase in the following long chain ceramide species C18-Cer/C20-Cer/C24:1-Cer/C24-Cer*.

Lagl Sc decreased growth rate after transfection (Fig. 3 B). Lagl Sc also decreased sensitivity to apoptosis by PI staining after treatment with etoposide. Two CLN9-deficient cell lines had a 32.4% and 29.6% apoptotic rate after transfection with empty vector compared to a 12.8% and 4.1% apoptotic rate after transfection with Lagl Sc (Fig. 3 C). Normal fibroblasts, on the other hand, had increased apoptosis after transfection with Lagl Sc (22.6% vs. 40.6%, Fig. 3 C). Baseline ceramide levels were higher in normal fibroblasts (Fig. 2 B). Lagl Sc over-expression leads to higher, proapoptotic ceramide levels. In conclusion, yeast Lagl Sc partially corrected ceramide levels, growth, and apoptosis in CLN9-deficient cells.

Human Lagl homologs correct ceramide, growth and apoptosis in CLN9-deficient cells.

LASS1 is the human homolog to Lagl Sc. LASS1 increased total ceramide levels more than 4-fold in CLN9-deficient cells, as determined by DGK assay (Fig. 4 A). 14C-palmitate labeling established an increase in de novo ceramide synthesis as well*. LASS1 increased mass measurements of C18- and C18:1-ceramide species by 100% in normal fibroblasts and 300% in CLN9-deficient fibroblasts following transfection (Fig. 4 B). This is consistent with published reports on LASS1 ceramide species specificity.

LASS1 partially corrected growth rate in CLN9-deficient cells (Fig. 4 C), and decreased cell proliferation as shown by [H3]thymidine incorporation*. LASS1 decreased CLN9-deficient cell sensitivity to apoptosis as observed by PI staining (Fig. 4 D) and JC-1 staining*. LASS1 sequences were normal in CLN9-deficient cells. Other human Lagl homologs LASS1-LASS6 (including multiple
transcript variants) were tested for their ability to correct the CLN9 phenotype. All, except LASS3, partially corrected growth and diminished apoptosis in CLN9-deficient cells*. LASS1, LASS2, LASS4, LASS5, and LASS6 sequences were normal in CLN9-deficient cells. LASS2 expression levels were normal, and LASS1, 3, 4 and 6 expression levels were increased 12.7, 6.2, 9.6 and 4.3-fold excluding the possibility that the CLN9 gene could be one of the known human Lag1 homologs.

4-HPR, increases dihydroceramide synthase activity and dihydroceramide levels, corrects growth and decreases LASS1 expression by RT-PCR

CLN9-deficient cells were treated with Fenretinide or 4-HPR, a dihydroceramide synthase activator (21). Treatment with 4-HPR increased dihydroceramide levels in both normal and CLN9-deficient fibroblasts. Normal fibroblasts showed an increase of all dihydroceramide species, except dhC18:1-Cer. CLN9-deficient cells showed an increase in all dihydroceramide species that was significantly higher than the increase in normal cells. DhC16-Cer, dhC24-Cer and dhC26:1-Cer levels increased the most after 4-HPR treatment and were elevated 244-fold, 359-fold, and 320-fold, respectively, in CLN9-deficient cells (Fig. 5 A). The same dihydroceramide species had the lowest levels in untreated CLN9-deficient cells (Fig. 2 A).

Dihydroceramide synthase activity was also increased by 4-HPR (Fig. 5 G). There was, however, no increase in rate of de novo ceramide synthesis after 4-HPR treatment in CLN9-deficient cells as measured by 14C-Palmitate labeling. Treatment with 4-HPR decreased levels of all ceramide species as measured by tandem mass spectrometry (Fig. 5 B). This effect might be due to a combined action of 4-HPR as activator of dihydroceramide synthase and inhibitor of dihydroceramide desaturase #.

Treatment with 4-HPR corrected growth of CLN9-deficient cells in a time- and dose-dependent manner. Continuous treatment with 4-HPR at the same concentration showed significant decrease of growth in CLN9-deficient cells after 48 hours (Fig. 5 C). Treatment with 2.5 µM 4-HPR did not have a significant effect on growth, but 5 µM and 10 µM 4-HPR decreased growth in a dose-dependent manner (Fig. 5 C). There was no effect of 4-HPR on growth of normal cells when used at the same concentration*. 4-HPR decreased LASS1 expression to 0.22-fold in mRNA from untreated CLN9-deficient cells. In normal cells 4-HPR decreased LASS1 expression to 0.5-fold expression from normal fibroblasts.

4-HPR or PDMP protect CLN9-deficient cells from apoptosis

Treatment with 4-HPR protected CLN9-deficient cells from apoptosis. Cells treated with 4-HPR showed significantly less apoptosis by PI staining (20.4% untreated vs. 5.4% treated with 5 µM 4-HPR). This effect was dose-dependent. 10 µM 4-HPR decreased apoptosis even further (4.1%; Fig. 5 D). These results were confirmed by JC-1 and TUNEL staining.

4-HPR normally increases apoptosis (22). CLN9-deficient cells were protected from apoptosis by 4-HPR. Treating CLN9-deficient cells with PDMP, which inhibits glucosylceramide synthase, results in an increase in ceramide mass measurements (23). PDMP diminished apoptosis in CLN9-deficient cells. Treatment with PDMP and 4-HPR, however, increased apoptosis above the level of untreated CLN9-deficient cells. This was determined by PI staining (Fig. 5 E). This effect may be due to the combined effect of an increase in ceramide due to PDMP, and an increase in dihydroceramide due to 4-HPR (Fig. 5 F). These drugs act at different steps of the pathway.

Fumonisin B₁, a dihydroceramide synthase inhibitor, exaggerates the CLN9 phenotype

To confirm that the CLN9-phenotype could be due to a defect in activation of dihydroceramide synthase, fumonisin B₁ (FB₁) was used. It inhibits dihydroceramide synthase and should, therefore, exaggerate the CLN9-phenotype. Pretreatment with FB₁ decreased baseline low ceramide levels in CLN9-deficient cells even further. 4-HPR neutralized the effect of FB₁ on ceramide
levels. Ceramide levels were measured by DGK assay after 34 hours of 4-HPR treatment (Fig. 6 A). FB1 treatment accelerated growth of CLN9-deficient cells exaggerating the CLN9 phenotype. Treatment with 4-HPR opposed this increase in growth (Fig. 6 B). CLN9-deficient cells pretreated with FB1 also had a higher apoptotic rate than untreated cells. FB1 opposed the protective effect of 4-HPR. The effects on apoptosis were amplified in the presence of etoposide as visualized by PI staining and confirmed by JC-1 staining (Fig. 6 C).

Low sphingomyelin in CLN9-deficient cells is not due to a ceramide trafficking defect

Ceramide produced at the endoplasmic reticulum (ER) is transported via CERT or ceramide transporter protein to the lumen of the Golgi for conversion to sphingomyelin (SM) by sphingomyelin synthase 1 (16). Should a ceramide trafficking defect be present, sphingomyelin synthase activity would be elevated. Sphingomyelin synthase activity was 40% of normal negating the possibility of a ceramide trafficking defect in CLN9-deficient cells. Also, CLN9-deficient cells when exposed to fluorescent NBD-C6-ceramide, demonstrated an intensely fluorescent Golgi reflecting normal trafficking and uptake of ceramide with conversion to SM in the Golgi lumen (Fig. 7).

The CLN9-phenotype is best explained by the extremely low dihydroceramide and ceramide levels. Increasing dihydroceramide by 4-HPR, or ceramide by PDMP corrected the phenotype.

DISCUSSION

CLN9-deficient cells manifest increased apoptosis, but accelerated growth combined with very low ceramide levels (15). The marked decrease in de novo LacCer synthesis and glycosphingolipids and gangliosides derived from it begged the question of whether one of the seven β-1,4 galactosyltransferases (β4GalT 1-7)§ or GB3/CD77 synthase could be candidates for CLN9. All seven β4GalT proteins are membrane proteins that reside in the Golgi, and the latter is also the seat of SM synthesis from ceramide, and LacCer synthesis from GluCer (24-25). A defect in β4GalT7 had already been described in a patient with a progeroid, Ehlers-Danlos-like syndrome (26). We ruled out mutations in these genes in CLN9 patients by sequence analysis. Low sphingomyelin suggested a possible defect in sphingomyelin synthesis. SM synthase proteins SMS1 and SMS2 reside in the Golgi and the plasma membrane, respectively (27-28). Sphingomyelin synthase activities were 40 % of normal and sequences of both of SMS1 and SMS2 were intact in CLN9-deficient cells. Also, SMS1 and SMS2 gene expression in CLN9-deficient cells was comparable to normal. Moreover, overexpression of SMS1 or SMS2 in CLN9-deficient cells had no effect on growth, essentially ruling them out as candidate genes for CLN9.

Transfection with CLN8 corrected growth and decreased apoptosis in CLN9-deficient cells. Human dihydroceramide synthase is still poorly characterized. Overexpression of the yeast gene Lag1 Sc and LASS1, the human Lag1 homolog for Lag1 Sc, complemented the defect in CLN9 cells with correction of ceramide levels, growth and apoptosis.

The inhibiting effect of ceramide on cell growth is well known. The mouse homologue of Lag1 increases C18-ceramide and decrease cell growth in HNSCC (Head and Neck Squamous Cell Carcinoma) cells derived from head and neck tumors (29). LASS1 Hs is primarily expressed in brain, with very low levels of expression in skeletal muscle and testis. Genes involved in neurodegenerative diseases have a distribution pattern similar to that of human LASS1 or LASS1 Hs (30). Analysis of tissue distribution of mouse LASS1, LASS2, LASS4, LASS5, and LASS6 confirmed that only LASS1 expression was mostly brain specific (31). A normal LASS1 sequence, as well as elevated levels of gene expression in CLN9-deficient cells excludes LASS1 from being a candidate for CLN9. All other human Lag1 homologs, except LASS3, could partially correct growth and apoptosis in CLN9-deficient cells. LASS3 contains both a Lag1 motif and a Hox domain. Studies in yeast cells led to the hypothesis that both
motifs are important for ceramide synthesis (32). Our results indicate that in mammalian cells this is not necessarily true. Transfection with either \textit{LASS1, LASS2} transcript variant 1 or 2, or \textit{LASS2} transcript variant 3 corrected growth and apoptosis in \textit{CLN9}-deficient cells. \textit{LASS2} transcript variants 1 and 2 contain both a Lag1 motif and a Hox domain. \textit{LASS2} transcript variant 3 lacks the Hox domain, but was still able to complement \textit{CLN9}. Only the Lag1 motif, and not the Hox domain, is sufficient and necessary for ceramide synthesis in human fibroblasts. Low dihydroceramide level, and low \textit{de novo} dihydroceramide synthesis and increases in \textit{LASS1, LASS3, LASS4} and \textit{LASS6} expression by RT-PCR, suggest that \textit{CLN9} may be a modulator of dihydroceramide synthase at the protein level. 4-HPR, an activator of dihydroceramide synthase, increased dihydroceramide levels in \textit{CLN9}-deficient cells. Dihydroceramide levels were markedly increased in both \textit{CLN9}-deficient and normal fibroblasts by the drug. 4-HPR increased \textit{de novo} dihydroceramide synthesis, but did not increase \textit{de novo} synthesis or mass levels of ceramide. This was not surprising as 4-HPR also inhibits dihydroceramide desaturase.

Increases in dihydroceramide levels in \textit{CLN9}-deficient cells corrected growth and decreased apoptosis. Dihydroceramide has been found to be biologically inactive in some cell systems (33). The data from \textit{CLN9}-deficient cells presented here suggest that dihydroceramide may be biologically active when ceramide levels are extremely low, resulting in modulation of growth and apoptosis.

4-HPR is used as a ceramide-modulating chemotherapeutic agent. A dose and time dependent increase in ceramide after 4-HPR treatment has been described in neuroblastoma cell lines, in prostate and breast cancer cells, lymphoblastic leukemia cells and Ewing family tumors as well (34-38). Maurer et al. showed that 4-HPR did not increase ceramide in non-malignant fibroblasts and EBV-transformed lymphoblastoid cell lines (39). Our data also suggest that 4-HPR has different biologic effects depending on baseline ceramide and dihydroceramide levels of the specific cell type: there is protection from apoptosis in \textit{CLN9}-deficient cells and an increase in apoptosis in normal fibroblasts. We hypothesize that base line dihydroceramide and ceramide levels in cells determine the effect of 4-HPR on apoptosis. Dihydroceramide levels in \textit{CLN9}-deficient cells are increased by treatment with 4-HPR, but ceramide levels are lowered even further. PDMP increases ceramide but not dihydroceramide levels. Each of the treatments alone results in protection from apoptosis. Treatment with both 4-HPR and PDMP increased apoptosis in \textit{CLN9}-deficient cells. This is most likely due to a simultaneous increase of dihydroceramide and ceramide levels. 4-HPR can cause both apoptosis and p53- and caspase-independent cell loss without increases in ceramide (22). Lovat et al. describe that inhibitors of acid sphingomyelinase, but not dihydroceramide synthase, block 4-HPR induced apoptosis in neuroblastoma cells (40). Treatment with both 4-HPR and Desipramine, an inhibitor of acid sphingomyelinase, did not change the protective effect of 4-HPR on apoptosis in \textit{CLN9}-deficient fibroblasts*. There are two paradoxes here: 4-HPR protects \textit{CLN9}-deficient cells from apoptosis, and this occurs in the setting of an increase in dihydroceramide and a further decrease in ceramide levels. PDMP, which increases ceramide levels, but has no effect on dihydroceramide levels, also protects these cells from apoptosis. This can be explained in multiple ways: 1) a threshold lower level of ceramide is necessary for the well-being of cells, below which apoptosis is activated; 2) when ceramide levels are very low, dihydroceramide assumes some of the biologic functions of ceramide, and the sum total of ceramide and dihydroceramide determines biologic function; or, 3) variations in levels of other biologically active sphingolipid metabolite(s) can explain these findings.

The findings of low dihydroceramide levels, and partial correction of the \textit{CLN9}-deficient phenotype by human \textit{LAG1} homologs, except \textit{LASS3}, suggested that \textit{CLN9} could be a human Lag1 homolog or a regulator of dihydroceramide synthase.
Normal Lag1 homolog sequences, elevated levels of expression of human LASS genes by RT-PCR, increases in dihydroceramide levels and a drop in LASS1 expression in response to 4-HPR, however, indicate that CLN9 could be an activator of dihydroceramide synthase, should the mutation in CLN9 result in loss of function. Likewise, a gain of mutation function such as in a duplication of a gene, or a CLN9 mutation resulting in tighter binding of the inhibitor to dihydroceramide synthase may suggest that CLN9 or mutated CLN9 could be an inhibitor of dihydroceramide synthase activity at the protein level. This work also has diagnostic and therapeutic implications: determination of dihydroceramide levels by tandem mass spectrometry can be developed as a diagnostic test for ascertainment of new CLN9-deficient cases. Moreover, the oral drug 4-HPR, a dihydroceramide synthase activator, may also have potential as a treatment option for CLN9-deficient patients.

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Footnotes:* data not shown; # Alfred Merrill, personal communication, 2004

§ Abbreviations:
NCL = neuronal ceroid lipofuscinosis, TLC = TRAM-Lag1-CLN8, 4-HPR = N-(4-hydroxyphenyl)retinamide, PPT1 = palmitoyl protein thioesterase 1, TPP1 = tripeptidylpeptidase 1, Cer = ceramide, SM = sphingomyelin, GluCer = glucosylceramide, GalCer = galactosylceramide, LacCer = lactosylceramide, CTH = ceramide trihexoside, GB4 = globoside, B4GalT = beta-1,4-galactosyltransferase, GB3/CD77 = globoside synthase, SMS1/SMS2 = sphingomyelin synthase, CERT = ceramide transporter protein, SPT = serine palmitoyltransferase, TRAM = translocating chain-associated membrane protein, PI = propidium iodide, JC-1 = 5,5′,6′,6′-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide, FB1 = fumonisin B1, PDMP = phenyl-2-decanoulamino-3-morpholino-1-propranolol, EV = empty vector, DGK = diacylglycerol kinase

TLC = thin layer chromatography, CS = dihydroceramide synthase

Legends

Fig. 1. Transfection with CLN8 corrects growth and decreases apoptosis in CLN9-deficient cells. (A) Growth curve of CLN9-deficient fibroblasts transfected with CLN1 (diamond), CLN2 (square) and CLN8 (triangle). Live cells were counted in triplicate at different timepoints. (B) Growth curve of CLN9-deficient fibroblasts transfected with CLN3 (diamond), CLN6 (square), empty vector (EV, circle) and CLN8 (triangle). Live cells were counted in triplicate at different timepoints. A significant decrease in growth rate is seen in cells transfected with CLN8. (C) Growth curve of CLN9-deficient fibroblasts transfected with empty vector (EV, square) or CLN8 (triangle) and normal fibroblasts transfected with empty vector (EV, diamond). A significant decrease in growth rate is seen in cells transfected with CLN8. Growth rate of empty vector transfected normal fibroblasts is even lower. (D) JC-1-staining of CLN9-deficient fibroblasts transfected with EV or CLN8 after etoposide treatment. Apoptotic cells are red, healthy cells are green. CLN8 decreases apoptosis (number of green healthy cells).

Fig. 2. Low dihydroceramide and ceramide levels in CLN9-deficient cells. (A) Dihydroceramide species levels are significantly decreased in CLN9-deficient cells compared to normal fibroblasts (NF). Dihydroceramide levels were measured by tandem mass spectrometry (values from single measures are shown). (B) Ceramide species levels in CLN9-deficient cells are significantly decreased in CLN9-deficient cells compared to normal fibroblasts (NF). Ceramide levels were measured by tandem mass spectrometry (values from single measures are shown).

Fig. 3. Lag1 Sc corrects ceramide levels and growth rate and decreases apoptosis in CLN9-deficient cells. (A) Transfection with Lag1 Sc increases ceramide levels compared to transfection with empty vector (EV) in CLN9-deficient cells (DGK-assay). (B) Growth curve of CLN9-deficient fibroblasts transfected with Lag1 Sc (triangle) or empty vector (EV, square). Live cells were counted in triplicates at different timepoints. A significant decrease in growth rate is seen in cells transfected with Lag1 Sc. (C) Propidium iodide staining of normal (NF) and two CLN9-deficient cell lines [CLN9(1) and CLN9(2)] after treatment with etoposide. Transfection with Lag1 Sc increases the number of PI positive apoptotic cells in normal fibroblasts (22.6% vs. 40.6%, p-Value 0.04) and decreases the number of PI-positive apoptotic
cells in CLN9-deficient fibroblasts compared to empty vector transfection (32.4% / 29.6% vs. 12.8% / 4.1%, p-Values 0.006 / 0.001).

**Fig. 4. LASS1 corrects ceramide levels, growth rate and decreases apoptosis in CLN9-deficient cells.** (A) Transfection of CLN9-deficient cells with LASS1 increases ceramide levels compared to transfection with empty vector (EV) (DGK-assay). (B) Transfection with LASS1 increases levels of C18-Cer and C18:1-Cer species in both normal (NF) and CLN9-deficient fibroblasts (values from single measures by tandem mass spectrometry are shown). (C) CLN9-deficient fibroblasts transfected with LASS1 (triangle) or empty vector (EV, square). Live cells were counted in triplicates at different timepoints. A significant decrease in growth rate is seen in cells transfected with LASS1. (D) Propidium iodide staining of two CLN9-deficient fibroblast cell lines [CLN9(1) and CLN9(2)] after treatment with etoposide. Transfection with LASS1 decreases the number of PI-positive apoptotic cells in CLN9-deficient fibroblasts compared to empty vector transfection.

**Fig. 5. 4-HPR activates dihydroceramide synthase, corrects dihydroceramide levels, growth and decreases apoptosis in CLN9-deficient cells.** (A) Treatment with 4-HPR increases dihydroceramide species levels more significantly in CLN9-deficient cells than in normal fibroblasts (NF) as measured by tandem mass spectrometry. Fold changes compared to untreated control are shown (values from single measures). (B) Treatment with 4-HPR decreases ceramide species levels in CLN9-deficient and normal fibroblasts (NF) as measured by tandem mass spectrometry (values from single measures). (C) Growth curve of CLN9-deficient fibroblasts untreated (diamond), treated with 2.5 µM 4-HPR (square), 5 µM 4-HPR (triangle), and 10 µM 4-HPR (circle). Live cells were counted in triplicates at different timepoints. A significant dose-dependent decrease in growth rate is seen in cells treated with 4-HPR. (D-E) Propidium iodide staining of CLN9-deficient fibroblasts after treatment with 4-HPR and/or PDMP. Treatment with 4-HPR decreases the number of PI-positive apoptotic cells in a dose-dependent manner (20.4% untreated vs. 5.4%/4.1% treated with 5 µM/10 µM 4-HPR, p-Value 0.009). Treatment with PDMP decreases the number of PI-positive apoptotic cells. Treatment with both, 4-HPR and PDMP, increases the number of PI-positive apoptotic cells. (F) DGK assay measuring ceramide and dihydroceramide in CLN9-deficient cells treated with 4-HPR alone, PDMP alone, or both. Note that the combined total of ceramide and dihydroceramide is very low in untreated cells, somewhat higher in 4-HPR (~2-fold) and PDMP (~1.5 fold) treated cells, but much higher in 4 HPR+PDMP treated cells (~5-fold). (G) Dihydroceramide synthase activity: de novo C17 dihydroceramide generation over 4 hours before and after treatment with 4-HPR (values from single measures by tandem mass spectrometry). Treatment with 4-HPR increases de novo dihydroceramide generation (reflecting increased dihydroceramide synthase activity).

**Fig. 6. Opposing effects of 4-HPR and FB1 on ceramide levels, growth and apoptosis.** (A) Treatment with FB1 decreases ceramide and dihydroceramide levels in CLN9-deficient cells measured at 34 hours by DGK assay. Treatment with 4-HPR neutralizes the effect of FB1 on ceramide and dihydroceramide levels. (B) Growth curve of CLN9-deficient fibroblasts untreated (diamond), pretreated with FB1 (square), treated with 4-HPR (triangle), and treated with both, FB1 and 4-HPR (circle). Live cells were counted in triplicate at different timepoints. FB1 treatment increases the growth rate, exaggerating the CLN9-deficient phenotype. Treatment with 4-HPR neutralizes the increase in growth after FB1. (C) Propidium iodide staining of CLN9-deficient fibroblasts treated with FB1 and/or 4-HPR. Treatment with FB1 increases apoptosis in CLN9-deficient cells even more, and opposes the protective effect of 4-HPR on apoptosis. This is shown in cells without and with etoposide treatment.

**Fig. 7. Cer traffics correctly from ER to Golgi lumen in normal and CLN9-deficient cells.** Treatment of normal (lower panel) and CLN9-deficient fibroblasts (upper panel) with
fluorescently labeled NBD C-6 ceramide at 16°C for 3 hours results in uptake of ceramide by the Golgi with conversion to sphingomyelin within the Golgi lumen by sphingomyelin synthase 1. White arrows point to eccentric intensely fluorescent Golgi next to an oval shaped dark nucleus. Note much smaller CLN9-deficient cells (magnification X 100).

Fig. 8. Schematic of the sphingolipid synthetic pathway. The sites of action of the various sphingolipid activators and inhibitors used in this study and the location of the hypothesized CLN9 action are indicated. Green arrows represent activating action, red lines represent inhibiting action.
Fig. 3 A

Fig. 3 B
Fig. 3 C

Fig. 4 A
Fig. 4 B

Fig. 4 C
Fig. 4 D

Fig. 5 A
Fig. 5 B

- CLN9(1) untreated
- CLN9(1) + 4-HPR
- NF untreated
- NF + 4-HPR

Fig. 5 C

- CLN9(1)
- CLN9(1) + 2.5 µM 4-HPR
- CLN9(1) + 5 µM 4-HPR
- CLN9(1) + 10 µM 4-HPR
Graph 5F: Concentration of Cer + dhCer / Phos [pmol/nmol]

Graph 5G: Dihydroceramide(pmol)/Protein(ug)/hour
(Ceramide+Dihydroceramide)/Phosphate [nmol/pmol]

CLN9(1) UT
CLN9(1) + 4HPR
CLN9(1) + FB1
CLN9(1)+4-HPR+FB1

6A
Fig. 6 B
Fig. 6 C

Fig. 7
Fig. 8