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Over-expression of CLN3P, the Batten disease protein, inhibits PANDER-induced apoptosis in neuroblastoma cells: Further evidence that CLN3P has anti-apoptotic properties

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Abstract

Juvenile neuronal ceroid-lipofuscinosis (JNCL) or Batten/Spielmeyer–Vogt–Sjogren disease (OMIM #204200) is one of a group of nine clinically related inherited neurodegenerative disorders (CLN1–9). JNCL results from mutations in *CLN3* on chromosome 16p12.1. The neuronal loss in Batten disease has been shown to be due to a combination of apoptosis and autophagy suggesting that CLN3P, the defective protein, may have an anti-neuronal death function. PANDER (PANcreatic-DERived factor) is a novel cytokine that was recently cloned from pancreatic islet cells. PANDER is specifically expressed in the pancreatic islets, small intestine, testis, prostate, and neurons of the central nervous system, and has been demonstrated to induce apoptosis. In this study, we over-expressed CLN3P in SH-SY5Y neuroblastoma cells and monitored the effects on PANDER-induced apoptosis. CLN3P significantly increased the survival rate of the SH-SY5Y cells in this system. This study provides additional evidence that the function of CLN3P is related to preventing neuronal apoptosis.

Keywords: Batten disease; Neuronal ceroid lipofuscinosis; Inherited neurodegenerative disease; Neuronal apoptosis; Cytokines; Pancreatic-derived factor

Introduction

The neuronal ceroid-lipofuscinoses (NCLs) are the most common group of autosomal recessive inherited neurodegenerative disorders of childhood, with an overall incidence of up to 1 in 12,500 [1]. There are nine known distinct forms of NCL, which have been designated, CLN1–9, for which six genes have been currently identified. The clinical courses of the NCLs overlap. They are progressive disorders, characterized by loss of vision, seizures, loss of motor function, and behavioral alterations leading to a vegetative state and early death [2]. The juvenile form of NCL (JNCL; CLN3; Batten or Spielmeyer–Vogt–Sjogren disease; OMIM #204200) occurs at a frequency of 0.7–7 per 100,000 live births per year [3,4]. JNCL results from mutations in *CLN3* on chromosome 16p12.1 [5]. A 1.02 kb deletion comprising exons 7 and 8 results in the removal of nucleotides 461–677 of the CLN3 coding region and accounts for the majority of alleles from Batten disease patients. An additional 39 mutations and five polymorphisms have now been identified in *CLN3* [[6], http://www.ucl.ac.uk/ncl/CLN3.html (accessed on November 21, 2005)]. *CLN3* encodes a 438 amino acid protein (CLN3P, Battenin) with an estimated molecular weight of 48 kDa. CLN3P is a predicted transmembrane protein of unknown function that localizes to membrane lipid rafts and may play a role in neuronal proteo-lipid

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trafficking and synaptic transmission [7]. Localization studies using Northern analysis demonstrated the ubiquitous presence of CLN3 mRNA with the highest expression in pancreas [5]. In neuronal cell types, co-localization of CLN3P to synaptic vesicles as well as to late endosomes, golgi, and lysosomes has been observed [8,9]. In another report, CLN3P was localized to synaptosomes but not to synaptic vesicles, suggesting that CLN3P may be involved in neurotransmission [10].

We recently reported the presence of a novel 33 kDa CLN3-like protein (CLN3LP) in normal human brain, which is over-expressed in brain from human Batten disease patients and in the *Cln3*$^{-/-}$ mice brain [11]. Oswald et al. [12] have suggested the presence of one species of *CLN3* alternative splice product that may have pathophysiologic significance in neurons.

Given that there is massive neuronal death in Batten disease, it is logical to hypothesize that CLN3P functions in an anti-cell death pathway and that genetic loss of CLN3P disrupts this pathway leading to uncontrolled neuronal death. Epitope-specific deletion experiments within motifs of CLN3P by Persaud-Sawin et al. [13] have suggested the presence of several important domains that modulate the growth of the cells and are involved in the regulation of apoptosis and Puranam et al. [14,15] have demonstrated that neuronal death is apoptotic in CLN3 and that CLN3P suppresses apoptosis by impacting generation of ceramide. To further investigate the role of CLN3P in apoptosis, we used PANDER (PANcreatic-DERived factor, FAM3B), a novel 235 amino acid pro-apoptotic cytokine, that was recently cloned from pancreatic islet cells. PANDER is expressed in the islets of Langerhans, small intestine, testis, prostate, and some neurons of the central nervous system [16]. The previous studies have revealed that recombinant PANDER or over-expression of PANDER in the pancreatic beta cells induces cell apoptosis in a time- and dose-dependent manner [17,18]. Recently, we have demonstrated that PANDER is co-secreted with insulin in the same granule through the same mechanism from pancreatic beta cells, showing that PANDER may play a role in glucose homeostasis in combination with or without insulin. This novel discovery has expanded our understanding of PANDER and implied that pancreas might not be the only target tissue of PANDER. We speculate that PANDER, secreted in response to glucose or other fuels or signaling pathways, has some metabolic effects on other tissues [19]. Our present study indicates that PANDER also induces apoptosis of SH-SY5Y neuroblastoma cells, but the cell death process can be blocked by increased expression of CLN3P.

Materials and methods

SH-SY5Y cell culture

SH-SY5Y neuroblastoma cells were cultured in T175 flasks in high glucose DMEM (11 mmol/L glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine at 37 °C under conditions of 95% air and 5% CO_2. The medium was changed twice a week and on the day before the experiment. Cells were trypsinized weekly and were used exclusively between passages 10 and 20.

Development of stable SH-SY5Y cells expressing CLN3P

SH-SY5Y cells were plated in 6-well plates (density of 1×10^6 cells/well) on the day before the experiment. Cells were transfected with pCTAP vector containing Cln3 cDNA using GeneJammer reagent (Stratagene, USA), following the manufacturer's protocol. In brief, cells were incubated with a mixture of plasmid and transfection reagent in serum-free medium for 3 h. At the end of the incubation, cells were supplemented with additional medium (supplemented with 10% FBS) and incubated for 72 h. Cells were trypsinized and split at a ratio of 1:5 and allowed to attach overnight. The next day, cells were transferred to fresh medium containing the selection antibiotic (500 μg/mL of G418, with 10% FBS). Cells were grown in selection medium until all the control cells were dead and the surviving cells were pooled and grown in the maintenance medium with lower concentration of antibiotic (100 μg/mL of G418, 10% FBS). All further cultures of this stable cell line were performed using medium containing low dose of selection antibiotic.

Cell viability determination–C,N-diphenyl-N′4-5-dimethyl thiazol-2-yl tetrazolium bromide assay

The *C,N*-diphenyl-*N*′4–5-dimethyl thiazol-2-yl tetrazolium bromide (MTT) assay is an indirect measure of cell viability [20]. The assay is based on the ability of viable cells to reduce MTT to insoluble colored formazan crystals. SH-SY5Y cells were plated in 6-well plates (density of 1×10^6 cells/well) on the day before the experiment. The next day cells were washed once with serum-free medium (DMEM) and were infected with PANDER containing adenovirus using serum-free (DMEM) medium at a concentration of 5000 viral particles/cell and incubated for 3 h. Control cells were incubated with adenoviral particles without PANDER. The serum-free medium was replaced with regular medium (DMEM with 10% FBS) after 3-h incubation and allowed to incubate for 72 h before performing MTT assay. Cells were washed twice with Krebs–Hepes buffer (115 mmol/L NaCl, 24 mmol/L $NaHCO_3$, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 25 mmol/L Hepes, and 1% BSA, pH 7.4) with no glucose and incubated in 2 mL Krebs–Hepes buffer with 0.5 mg/mL MTT for 60 min at 37 °C. The supernatant was discarded, and cells in each well were lysed with 2 mL of 2-propanol and incubated for 1 h at room temperature. The optical density of the resultant colored 2-propanol was measured at 560 nm using CARY Bio-100 UV–Vis Spectrophotometer (Varian Technologies).

Western blot analysis

Western blots were performed on samples containing 10 μg of protein each. Four to 20% linear gradient precast gels (Bio-Rad Laboratories, Hercules, CA, USA) were used for electrophoresis. They were then transferred onto nitrocellulose membranes at a constant voltage (100 V for 1 h). The membranes were incubated in blocking buffer (5% membrane blocking agent in 1% TBS-T (Tris-buffered saline with Tween 20); Amersham Biosciences, Piscataway, NJ, USA) overnight at 4 °C. The next day, the membranes were incubated with the CLN3P, PANDER or cleaved caspase 3 primary antibody at a concentration of 5 μg/mL (TBS-T containing 1% blocking reagent) at 4 °C overnight. Immunoreactivity was visualized with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Amersham Pharmacia Biotech, Piscataway, NJ, USA), followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) was performed to determine the extent of over-expression of *Cln3*. Total RNA was isolated from SH-SY5Y cells and SH-SY5Y-pCTAP/*Cln3* stable cell