The CLN3 Gene is a Novel Molecular Target for Cancer Drug Discovery

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INTRODUCTION

Mutations in the CLN3 gene are responsible for the JNCL (1). JNCL is a recessively inherited neurodegenerative disorder of childhood (1–3). The clinical hallmarks are progressive loss of vision, seizures, and mental deterioration. These symptoms are attributable to massive cortical neuronal death and gradual loss of photoreceptor cells (2, 3). Apoptosis has been shown to be the mechanism of neurodegeneration in the brain of patients with the juvenile form of Batten disease (4). Moreover, up-regulation of Bcl-2 and elevation of endogenous ceramide levels in the brain from affected individuals provides mechanistic evidence for apoptotic death of neurons in this disorder (5).

We have demonstrated previously that stable CLN3 overexpression protects NT2 neuronal precursor cells from serum starvation-induced growth inhibition and also rescues these cells from death caused by treatment with vincristine, etoposide, and staurosporine (6). We have also shown that CLN3 up-regulation decreases the level of the lipid second messenger, ceramide, in these cells and also attenuates vincristine-induced activation of ceramide (6). However, overexpression of CLN3 fails to protect NT2 cells from exogenous ceramide-induced killing. These facts place CLN3 upstream of ceramide in apoptosis signaling and suggest that CLN3 plays an important role in mechanisms of cell death and survival. The fact that CLN3 is highly conserved across species from human to yeast and also in Caenorhabditis elegans and Drosophila underscores its importance for cell function. CLN3 has also been found to be developmentally regulated in differentiating hNT neurons and in neonatal rat brain. Peaks of expression are noted just after hNT cells exit the cell cycle, and on day Po in neonatal rat brain, which corresponds to the period of maximum neuronal growth (7). This suggests that CLN3 is an oncelfal, antiapoptotic gene. These facts led us to investigate whether CLN3 could be differentially expressed in some cancers.

Defects in proapoptotic events can contribute to cancer formation by allowing cells to survive and to proliferate beyond their normal life span (8–12). Regulation of apoptosis is involved in the development of tumors and plays an essential role in their treatment. A variety of chemotherapeutic agents and radiation kill tumor cells by inducing apoptosis (10, 13, 14). It has been established that many tumor types are resistant to chemotherapy-induced apoptosis. This can occur either because of inactivation of tumor suppressor genes, such as p53 or retinoblastoma, or because of overexpression of antiapoptotic oncogenes, such as Bcl-2 (B-cell lymphoma), Bcr-Abl (myelogenous leukemia), and survivin in a number of cancers and lymphomas (15–18). Identification of novel antiapoptotic genes expressed in cancer cells provides a basis for a better understanding of the biology of these tumors and may lead to discovery of new targets for anticancer drug development (9, 19–21). Direct inactivation of antiapoptotic gene expression may promote cancer cell death. Suppression of antiapoptotic genes can be achieved by antisense strategies, which in some instances may improve the efficacy of conventional chemotherapy (9). Antisense-based therapies have already been developed to block Bcl-2 overexpression in non-Hodgkin’s lymphoma (22).

In this study we establish that CLN3 mRNA and protein are overexpressed in a number of cancer cell lines including breast, colon, malignant melanoma, prostate, ovarian, neuroblastoma, and glioblastoma multiforme but not lung or pancreatic cancer cell lines. We also show that CLN3 is overexpressed in 8 of 10 solid human colon cancer cases. Additionally, we demonstrate that blocking CLN3 protein expression in these cancer cell lines, using adenosine-mediated antisense CLN3 methodology, inhibits cancer cell growth and viability. Treatment of cancer cells with Ad-AS CLN3 virus also affects the de novo ceramide synthetic pathway and results in ceramide elevation and cancer cell death by apoptosis.

MATERIALS AND METHODS

Cell Culture. Cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37°C and 5% CO₂ except for...
SW1116 cells, which are cultured under CO₂-free conditions by sealing the flasks with parafilm. Human cancer cell lines were maintained in medium as described below. Normal fibroblasts and A 375 (melanoma) cells were cultured in DMEM with 10% FBS; SW 626 (ovarian adenocarcinoma) cells were cultured in DMEM with 1 mm sodium pyruvate, 10% FBS; PC3 (prostate adenocarcinoma) cells were propagated in Ham’s F12K medium with 7% FBS; A549 (lung carcinoma) cells were cultured in Ham’s F12K medium with 2 mm glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 10% FBS; SW1116 (colon adenocarcinoma) cells were cultured in Leibovitz’s L-15 medium with 10% FBS; SW-480 (colon adenocarcinoma) cells were cultured in Leibovitz’s L-15 medium with L-glutamine, 10% FBS; SK-OV-3 (ovary adenocarcinoma) cells were cultured in MEM with Earle’s BSS, 0.1 mm nonessential amino acids, and 10% FBS (heat inactivated for PA-1 cells); T98G and U-373 MG (glioblastomas) cells and IMR-32 and SK-N-MC (neuroblastoma) cells were cultured in MEM with L-glutamine and Earle’s BSS adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mm nonessential amino acids, 1 mm sodium pyruvate, 10% FBS; LNCaP (prostate carcinoma) and BT 549 (breast carcinoma) were cultured in RPMI 1640 with 10% FBS; CAPAN-1 and As-PC-1 (pancreas adenocarcinomas) cells were cultured in RPMI 1640 with 15% and 20% FBS, respectively; and NCI-H520 (lung carcinoma) and BT 474 (breast carcinoma) cells were cultured in RPMI 1640 with 2 mm glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mm HEPES, 1 mm sodium pyruvate, and 10% FBS. Penicillin-streptomycin (1%) were added in all of the mediums. Mouse B16 (melanoma) cells and 4T1 (breast carcinoma) cells were cultured in DMEM high glucose medium with 10% heat inactivated FBS, 1% penicillin-streptomycin-fungene; mouse embryo and contact sensitive C3H10T1/2 cells were used as a control and were cultured in BME medium with Earle’s BSS, 10% heat inactivated FBS, and 1% penicillin-streptomycin-fungene.

Human Colon Cancer and Normal Colon Tissues. Samples of human colon cancer and normal colon tissue from the same patient were obtained using an Institutional Review Board-approved procedure for collecting left-over, “deidentified,” or “unlinked” samples from surgical pathology. In brief, at the time of frozen section analysis of the patient tumor, samples of representative normal or neoplastic tissue. In all of the cases, the colon cancer specimens consisted mostly of neoplastic glands invading into the muscularis externa. The normal colon specimens contained mucosa, submucosa, and muscularis externa.

Construction of Ad-AS-CLN3. Ad-AS-CLN3 virus was constructed using Ad-Easy method (23). CLN3 cDNA was cloned in the antisense direction into the multiple cloning site of pShuttle-CMV vector. The Ad-AS-CLN3 vector was constructed by homologous recombination using cotransformation of linearized AS-CLN3 pShuttle-CMV plasmid and pAdEasy (adenoviral backbone) into Escherichia coli BJ5183-competent cells. The Ad-AS-CLN3 vector was isolated, propagated, and purified exactly as described previously (24). Infection units were determined by limiting dilution assay in 293 cells.

Limiting Dilution Assay. Serial dilutions of viral stock from 1 × 10³ to 1 × 10⁵ were made. B6 cells were plated to 80% confluency in 24-well plates. Serial dilutions of virus were then added in each row of the 24-well plate and allowed to infect for 1 h. Additional medium was added, and the plates were incubated in a 37°C incubator at 5% CO₂ for 2 weeks. Limiting dilution was determined by the wells with the lowest viral titer that showed cytopathic effect.

Transduction of Cancer Cells with Control Vector or Ad-AS-CLN3 Virus. Transduction of cancer cells was carried out in 60-mm dishes for Western blot and in 12-well plates for growth curves and proliferation assays. At 70% confluence, the number of cells was determined. Cells were infected with the MOI (number of viral particles used per cell) of virus indicated in the figures by incubation with virus in a minimum amount of medium for 1 h and addition of medium to a final volume after that.

RT-PCR for CLN3. Total RNA was isolated from cancer cells using the RNeasy mini kit (Qiagen Inc., Valencia, CA). Then mRNA was isolated from total RNA by the Oligotex mRNA spin-column Mini kit (Qiagen Inc.). First-strand cDNA was then synthesized from mRNA by reverse transcription using Omniscript Reverse Transcription kit (Qiagen Inc.). PCR reaction was set up with first-strand cDNA, 2.5 units of Ampli Taq Gold (PE Applied Biosystems, Foster City, CA), and 5 μM of α–32PdCTP in each reaction. The reaction was performed in PCR buffer (Applied Biosystem) containing 2.5 mM MgCl₂, 50 μM of dCTP, dATP, dGTP, and dTTP. The primers used for amplification of human CLN3 were 5’ primer, 5’-GGTGCACTGTATTCAAGGG-3’ (958–976), and 3’ primer, 5’-CTTGGCCAGAAAGCGAAAC-3’ (1229–1246). Cyclophilin was used as an internal control, and primers used for amplification of cyclophilin were the 5’ primer, 5’-AATGCGTGGCAA-CACATGCTGTC-3’ (357–334), and 3’ primer, 5’-AAAAACCACTGCTGTC-3’ (384–401). The primers used for amplification of mouse CLN3 were 5’ primer, 5’-AGGTGGAGCAGGTCTCAAGGTC-3’ (961–982), and 3’ primer, 5’-GGAAAGATTACGACTAAGGCGC-3’ (1300–1321). The primers used for amplification of mouse cyclophiline were 5’ primer, 5’-ACAGCAAG TTCGCTACGTTCACCAC-3’ (285–307), and 3’ primer, 5’-TGCTCTTTCCTCCTG GCCATC-3’ (347–368). The reaction conditions used for human cDNA were 10 min at 95°C, and then 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, for 20 cycles. The reaction conditions used for mouse cDNA were 10 min at 95°C, and then 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, for 20 cycles. PCR amplified products were separated on 8% nondenaturing polyacrylamide gel. Bands of interest were quantitated using a Phospholmager ( Molecular Dynamics Inc., Sunnyvale, CA). The results are expressed as the ratio of CLN3 signal to that of the internal control, cyclophilin.

Immunocytochemistry. Cells were grown on glass coverslips until confluent, fixed in 4% paraformaldehyde and 4% sucrose in PBS at 4°C, and permeabilized in 0.2% Triton X-100 in PBS at room temperature. After blocking in 3% BSA in PBS, cells were incubated with human CLN3 antisem. The CLN3 antibody used in this study is a polyclonal antibody raised against the peptide sequence AAHDLHSVDRSGNHVDP corresponding to amino acids 58–77 of the CLN3 protein (Research Genetics, Huntsville, AL). An additional glass coverslip of each cell type was exposed to rabbit IgG at the same concentration under the same conditions Cells were washed and then incubated with 1:500 dilution of biotin-conjugated goat antirabbit IgG (Zymed, San Francisco, CA) for 1 h. This was followed with exposure to streptavidin-conjugated horseradish peroxidase (Zymed) 1:300 for 30 min. Cells were stained with 3,3’-diaminobenzidine in 0.2 ml Tris-HCl, counterstained with hematoxylin blue, dehydrated in ethanol, washed in xylene, and mounted on glass slides with Permount.

Western Blot. Western Blot for CLN3 protein detection was performed as described previously (6). Cells were harvested on day 3 after infection and lysed in buffer containing 250 mM NaCl, 0.1% NP40, 50 mM HEPES (pH 7.0), 5 mM EDTA, 1 mM DTT, and 10% protease inhibitor mixture (Sigma Chemical Co.). After 40 min of incubation at 4°C, lysates were centrifuged at 12,000 × g, and the supernatants were quantitated for total proteins using the Bio-Rad protein assay. Equal amounts of protein (200 μg) were separated by SDS-PAGE using 4.5% and 12% acrylamide for stacking and resolving gels, respectively. Proteins were transferred to nitrocellulose membrane and probed with a polyclonal CLN3 antibody raised against the peptide sequence AAHDLHSVDRSGNHVDP corresponding to amino acids 58–77 of the CLN3 protein (Research Genetics). CLN3 primary antibody complexes were detected using goat antirabbit IgG conjugated with horseradish peroxidase and visualized by SuperWest Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

[3H]Thymidine Incorporation Cell Proliferation Assay. Cells were plated in 12-well plates at a density of 7 × 10⁴ cells/well and were incubated with 0.5 μCi/ml [3H]thymidine (DuPont) in medium for 2 h before harvesting. For viral transduction experiments, cells were infected with 5, 10, 15, 20, and 40 MOI of control or Ad-AS-CLN3 virus as described above. On day 2 after infection cells were incubated with 0.5 μCi/ml [3H]thymidine (DuPont) in medium for 2 h. The cells were washed twice with ice-cold PBS and the DNA precipitated with 5% trichloracetic acid. The DNA precipitate was dissolved in 0.4 ml of 0.25 M NaOH, and incorporated [3H]thymidine was determined by liquid scintillation counting. Each time point was determined in triplicate.
PBS, and lipids were extracted using the Bligh and Dyer method. Fumonisin, an inhibitor of de novo ceramide biosynthesis (Biomol, Plymouth, PA) was added at a concentration of 25 μM to two dishes of DU 145 cells 12 h before transduction with virus. Ceramide levels in the cells were measured using diacylglycerol kinase assay, as described previously (6). The results are expressed as pmols of ceramide per nmol of total phospholipids and represent an average from three experiments.

Measurement of Sphingomyelin Content. Lipids were extracted from 10 × 10^5 cells using the method of Bligh and Dyer. Lipid extracts in 1 ml of chloroform each were subjected to hydrolysis by adding 0.1 ml of 0.2 N NaOH in methanol. Aliquots were taken for quantitation of total phospholipids before hydrolysis. After neutralization with an equal amount of 2 N HCl, lipids were reextracted by adding 1 ml of chloroform and 1 ml of water and vortexing. The lower organic phase was then evaporated under the nitrogen gas, resuspended in 75 μl of chloroform-methanol (2:1), and 50 μl out of it was spotted onto a TLC plate (Whatman Inc., Clifton, NJ). The plate was developed in CHCl3-CH3OH-acetic acid-H2O (50:30:8:5). Lipids were visualized using iodine vapor, and bands corresponding to sphingomyelin standard were scraped. The amount of phosphor was determined using phosphomolybdate phosphate assay. The content of sphingomyelin was expressed in nmol per nmol of total phospholipids, and percentage change in sphingomyelin in patient cells in comparison to control fibroblasts was calculated.

PI Staining. DU145 prostate cancer cells were grown on glass coverslips and transduced with 40 MOI of Ad-AS CLN3 virus or control virus. After 72 h, cells were stained with PI in PBS at a concentration of 5 μg/ml for 5 min at 4°C. After that, cells were washed with PBS and mounted in PBS/glycerol (1:1). PI-positive apoptotic cells were observed under fluorescence (excitation wavelength: 525 nm, emission wavelength: 600 nm) at ×100 magnification.

JC-1 Staining. JC-1 is a cationic lipophilic dye and exhibits potential-dependent accumulation in mitochondria (25, 26). Cell were grown on glass coverslips and transduced with 40 MOI of Ad-AS CLN3 virus or control virus. After 72 h, cells were stained with 1 μg/ml JC-1 in medium for 15 min at 37°C. After that, cells were washed twice with PBS and mounted on glass slides with PBS/glycerol (1:1). At the onset of apoptosis, a drop in potential was indicated by a fluorescence emission shift from green to red (525 to 590 nm) attributable to formation of J-aggregates. These appear as bright dots in Fig. 9. J-aggregates in Du145 prostate cancer cells are visualized using ×400 magnification. Percentage of apoptotic cells is calculated from two fields (60–150 cells/field), and the data are presented as the average of both counts. The statistical significance of the difference in the number of apoptotic cells after transduction with control or Ad-AS CLN3 virus is determined by the two-tailed Student t test.

RESULTS

CLN3 Is Overexpressed in Cancer Cell Lines Both at the RNA and Protein Levels. We had shown previously that CLN3 is involved in antiapoptotic pathways. Because defects in apoptosis play an important role in carcinogenesis, we compared CLN3 expression at the RNA level in neuroblastoma, glioblastoma, prostate, ovarian, colon, breast, melanoma, pancreas, and lung cancer cell lines to CLN3 expression in normal fibroblasts. Mouse CLN3 expression at the RNA level is also measured in cells derived from naturally occurring mouse melanoma and mouse breast carcinoma (Figs. 1 and 2). The results demonstrate that expression of CLN3 is increased 3.5-fold in glioblastoma, 1–4-fold in neuroblastoma, 2–4-fold in prostate cancer cell lines, 1.5–2-fold in ovarian cancer cell lines, and 2–4-fold in breast carcinomas in comparison to normal fibroblasts. CLN3 mRNA expression in C 32 (melanoma)
demonstrate in 7 tumors that CLN3 expression is 50–330% higher than in the corresponding normal colon tissue. In an eighth case CLN3 expression was increased by 22% (Fig. 4).

Ad-AS-CLN3 Virus Blocks CLN3 Protein Expression and Decreases Viability of Cancer Cells. To demonstrate the impact of CLN3 overexpression on cancer cells we engineered an adenoviral vector with an antisense CLN3 cDNA construct capable of blocking CLN3 overexpression and studied its effect on cancer cell growth and apoptosis. Infection of prostate DU 145, neuroblastoma SK-N-MC, and breast BT-20 cancer cells with 40 MOI Ad-AS-CLN3 virus and colon SW1116 cells with 5 MOI appreciably block expression of the CLN3 protein in comparison to cells infected with the same amount of control virus (Fig. 5).

We then analyzed the effect of different MOIs of Ad-AS-CLN3 virus on cancer cell growth. Incorporation of \(^{3}H\)thymidine was measured in DU 145, SK-N-MC, BT-20, and SW1116 cells infected with 5, 10, 15, 20, and 40 MOI of Ad-As CLN3 virus or control virus. Incorporation of \(^{3}H\)thymidine into cancer cells was inversely related to an increase in MOI or the number of Ad-AS-CLN3 viral particles infecting each cell (Fig. 6, a, c, e, and g). Ad-AS-CLN3 at a dose of

![Image](93x341 to 247x552)

Fig. 2. CLN3 expression in mouse melanoma, breast carcinoma cell lines, and C3H10T1/2 control mouse fibroblasts. mRNA was isolated from different cell lines, cDNA synthesized, and PCR was performed in the presence of CLN3 5' and 3' primers as described in “Materials and Methods.” Cyclophilin was used as an internal control. The results are expressed as the ratio of CLN3 signal to that of cyclophilin. Average of four separate experiments; bars, ± SD; *P < 0.05 versus the control; au, arbitrary units.

![Image](104x627 to 237x741)

Fig. 3. CLN3 protein expression by immunocytochemistry. Confluent cells were fixed, permeabilized, and incubated with human CLN3 antiserum for 12 h. After that cells were exposed to goat antirabbit biotin-conjugated antibody for 1.5 h and to horseradish peroxidase conjugated streptavidin for 30 min. Next, cells were stained with diaminobenzidine, dehydrated, and mounted in Permount. Brown staining correlates with CLN3 protein expression. Note increased amount of staining in Du145 prostate, SK-N-MC neuroblastoma, BT-549 breast, PA-1 ovarian, and T98g glioblastoma cancer cells compared with normal human fibroblasts.

![Image](314x128 to 554x283)

Fig. 4. CLN3 mRNA expression in human colon tumors compared with normal colon tissue. mRNA is isolated from 10 solid colon cancers, and expression of CLN3 is compared with normal colon tissue obtained from the same patient using RT-PCR. Cyclophilin is used as an internal control. Results are expressed as percentage change of CLN3 expression in colon cancer samples compared with corresponding normal colon controls. Each experiment was repeated twice.

![Image](352x359 to 516x504)

Fig. 5. Western Blot. Cancer cells were transduced with 10 or 40 MOI of Ad-AS-CLN3 virus or control virus. Lane 1, control cells; Lane 2, control virus 10 MOI; Lane 3, Ad-AS-CLN3 virus 10 MOI; Lane 4, control virus 40 MOI; Lane 5, Ad-AS-CLN3 virus 40 MOI; Lane 6, Ad-AS-CLN3 virus 2 MOI; Lane 7, Ad-AS-CLN3 virus 5 MOI. Protein was isolated 3 days after infection and quantitated. Equal amounts of protein were separated by SDS-PAGE then transferred to nitrocellulose membrane and probed with CLN3 antibody. To confirm equal protein loading, the membrane was stained with Ponceau red dye. a, prostate carcinoma DU145 cells; b, colon adenocarcinoma SW1116 cells; c, neuroblastoma SK-N-MC cells; d, breast carcinoma BT-20 cells.

![Image](804)
15 MOI was sufficient and effective in the killing of cancer cells as seen from the dose-response curves in Fig. 6, a, c, e, and g.

The growth profile of DU145, BT-20, SW1116, and SK-N-MC cancer cells infected with control virus practically matches that of untreated cells (Fig. 6, b, d, f, and h). In contrast, infection of the cells with Ad-AS-CLN3 halts cancer cell growth. This effect was noticeable on day 2 after transduction with virus. At the end of the treatment the amount of live cells drastically dropped compared with control, which indirectly indicates an increase in the number of dying cells.

Increase in Ceramide Levels in CLN3 Deficient Fibroblasts Is Not Sphingomyelin-derived. We also measured ceramide levels in fibroblasts obtained from patients with juvenile Batten disease that are deficient in CLN3. There was a 78.5% increase in endogenous ceramide in CLN3-deficient fibroblasts compared with normal fibroblasts. At the same time there was no simultaneous drop in sphingomyelin content. In fact, it was 26.9% higher than in normal cells (Fig. 7b). This suggests that the increase in ceramide levels seen with diminished expression of CLN3 is not attributable to sphingomyelin breakdown or the sphingomyelin cycle but more likely suggests an impact of CLN3 on the de novo ceramide pathway.

Blocking of CLN3 Expression in Prostate Cancer Cells Results in Increased Ceramide Levels. We have shown previously that CLN3 negatively modulates ceramide generation in NT2 cells. Ceramide levels in DU145 prostate cancer cells were increased by 91.4% after transduction with Ad-AS CLN3 virus compared with cells transduced with control virus (Fig. 7a). Moreover, pretreatment of these cells with fumonisin, an inhibitor of ceramide synthase, abrogated ceramide elevation. Fumonisin equally blocks ceramide elevation in DU145 cells after transduction with Ad-AS CLN3 virus and control virus by 93.3% and 91.4%, respectively. Therefore, blocking of de
novo cereamide synthesis suppresses ceramide level increases induced by Ad-AS CLN3 virus, and this suggests that CLN3 impacts the de novo cereamide pathway (Fig. 7a).

Transfection of CLN3-deficient Lymphoblasts with CLN3 Containing Plasmid Restores Growth but Has No Effect on Thymidine Incorporation. CLN3-deficient lymphoblasts from patients manifest slowed growth compared with normal lymphoblasts (Fig. 8a). Reintroduction of CLN3 into JNCL patient cells, as opposed to the empty vector, restores growth almost to normal levels but has no effect on thymidine incorporation (Fig. 8b). This indicates that the CLN3 gene impacts apoptotic pathways and not cell proliferation.

Blocking of CLN3 Expression in Prostate Cancer Cells Increases Apoptosis. Transduction of DU145 prostate cancer cells with Ad-AS CLN3 results in increased apoptosis. There is a higher number of apoptotic cells in DU145 prostate cancer cells after transduction with Ad-AS CLN3 virus compared with transduction with empty plasmid (Fig. 9a). Note that CLN3 deficient lymphoblasts incorporate thymidine at a rate similar to lymphoblasts transfected with full CLN3 gene.
control virus shown by PI staining (Fig. 9, a and b). Also, the number of cells with J-aggregate formation is increased from 8.2 ± 3.7% to 34.5 ± 9% (P < 0.05) after transduction with Ad-AS CLN3 virus as shown by JC-1 staining. (Fig. 9, c and d).

DISCUSSION

Deregulation of antiapoptotic genes and/or mutated proapoptotic genes may contribute to carcinogenesis by allowing abnormal cells to survive and thereby become cancerous (21, 27, 28). Bcl-2 was one of the first antiapoptotic genes found to be up-regulated in cancer (29). Other genes have been discovered that also are overexpressed in cancer and that inhibit apoptosis. Examples include Bcr-Abl in chronic myeloid leukemia, BUG-1 in breast tumors, and Survivin in a number of cancers and lymphomas (10, 15, 16, 30–34).

Previous work has shown that CLN3 is antiapoptotic (6). Ceramide levels are elevated in the brain of patients with the juvenile form of Batten disease compared with normal brain (5). NT2 cells stably or transiently overexpressing CLN3 protein have lower ceramide levels than control cells and are unable to increase ceramide levels in response to vincristine. CLN3 overexpression protects NT2 cells from apoptosis induced by the chemotherapeutic agents vincristine, staurosporine, etoposide, and serum starvation. Therefore, CLN3 negatively modulates endogenous ceramide levels. Alternatively, loss of functional CLN3 protein in juvenile Batten disease results in increased ceramide production and accelerated apoptosis. Up-regulation of CLN3 failed to prevent apoptosis caused by exogenous C2- or C6-ceramide, suggesting that CLN3 impacts ceramide generation upstream.

We demonstrate that CLN3 is overexpressed at the mRNA and protein levels in a number of human cancer cell lines including prostate, glioblastoma, neuroblastoma, ovarian, breast, colon, and malignant melanoma. Pancreas and lung cancer cell lines do not show increased CLN3. CLN3 was also found to be up-regulated in mouse melanoma and breast carcinoma cell lines. On the basis of these observations, it may become possible to develop semiquantitative assays of CLN3 expression for screening or to monitor effectiveness of therapy in some cancers. Overexpression of CLN3 in 80% of solid colon cancers tested corroborates data obtained from human cancer cell lines. Discovery of novel antiapoptotic genes involved in cancer may lead to a better understanding of cancer biology. Also, these novel genes may prove to be effective targets for cancer drug development. Potential strategies for induction of apoptosis in cancer cells include inactivation of anti-apoptotic genes by blocking their expression (9, 18). Reduction in Bcl-2 expression by antisense methods increases the susceptibility of cancer cells to induction of apoptosis by multiple chemotherapeutic agents (19, 22). Similarly, down-regulation of CLN3 expression with a resultant increase in ceramide generation may be explored as a potential cancer therapeutic strategy. We demonstrate that expression of the CLN3 protein drops significantly after transduction with 40 MOI of Ad-AS-CLN3 virus. Blocking of CLN3 expression in cancer cells by Ad-AS-CLN3 virus suppresses their growth and results in an increase in the number of apoptotic cells. CLN3-deficient lymphoblasts show no difference in thymidine incorporation when transfected with empty plasmid or plasmid carrying full-length CLN3 cDNA. This suggests that CLN3 impacts apoptotic pathways and not proliferative pathways.

Transduction of DU145 prostate cancer cells with Ad-AS CLN3 virus elevates ceramide levels in these cells compared with those transduced with control virus. The fact that less CLN3 expression results in increased ceramide was confirmed in CLN3-deficient fibroblasts derived from patients with juvenile Batten disease. These cells show a 78.5% increase in endogenous ceramide compared with normal fibroblasts. Different apoptotic stimuli induce ceramide generation via two different routes. One route involves activation of the sphingomyelin cycle, which results in cleavage of sphingomyelin and release of ceramide, the other is via activation of enzymes in the de novo ceramide synthetic pathway (35–43). Pretreatment of DU145 cells with fumonisin, an inhibitor of ceramide synthase, before transduction with the Ad-AS CLN3 virus completely blocks elevation in ceramide. This indicates that blocking of CLN3 protein expression induces an increase in ceramide levels not through sphingomyelin breakdown but via the de novo ceramide synthetic pathway. Additional evidence is the fact that increases in ceramide levels in CLN3-deficient brain and CLN3-deficient fibroblasts were not accompanied by a change in sphingomyelin levels.

Suppressing levels of CLN3 protein in cancer cells enhances ceramide production and results in death of cancer cells. There are a number of anticancer drugs of which the effects include boosting of ceramide levels in cancer cells (44).

In conclusion, CLN3 overexpression may be developed as yet another marker for some but not all cancers. Moreover, blocking CLN3 overexpression using an antisense adenoviral CLN3 construct inhibits cancer growth, increases ceramide levels, and promotes death of cancer cells. The implications are 2-fold. The use of antisense CLN3 methodology may be valid for treatment of some cancers. Additionally, the CLN3 gene provides a novel molecular target to screen for new drugs effective in combating cancer.

REFERENCES

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