Sialidase-mediated depletion of $G_{M2}$ ganglioside in Tay–Sachs neuroglia cells

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Received January 22, 1999; Revised and Accepted February 28, 1999

Tay–Sachs disease is a severe, inherited disease of the nervous system caused by accumulation of the brain lipid $G_{M2}$ ganglioside. Mouse models of Tay–Sachs disease have revealed a metabolic bypass of the genetic defect based on the more potent activity of the enzyme sialidase towards $G_{M2}$. To determine whether increasing the level of sialidase would produce a similar effect in human Tay–Sachs cells, we introduced a human sialidase cDNA into neuroglia cells derived from a Tay–Sachs fetus and demonstrated a dramatic reduction in the accumulated $G_{M2}$. This outcome confirmed the reversibility of $G_{M2}$ accumulation and opens the way to pharmacological induction or activation of sialidase for the treatment of human Tay–Sachs disease.

INTRODUCTION

Tay–Sachs and Sandhoff diseases are autosomal recessive genetic disorders of $G_{M2}$ ganglioside catabolism due to deficient activity of lysosomal $\beta$-hexosaminidase A (Hex A, structure $\alpha$) (1). Tay–Sachs disease is caused by mutation of the $\alpha$ subunit, resulting in deficient Hex A activity, while Sandhoff disease is caused by mutation of the $\beta$ subunit, with consequent deficiency of Hex A and Hex B (structure $\beta\beta$) activities. Both diseases cause $G_{M2}$ gangliosidosis leading to a devastating neurodegenerative disease which is fatal by 2–4 years of age.

We (2) and others (3,4) have established mouse models of Tay–Sachs and Sandhoff disease by targeted disruption of the Hexa ($\alpha$ subunit) and Hexb ($\beta$ subunit) genes, respectively. In contrast to the similarity of the human diseases, Hexb$^{-/-}$ mice suffer a profound, fatal neurodegenerative disease with onset at 3–4 months of age and death 4–6 weeks later, while Hexa$^{-/-}$ mice escape disease to >1 year of age. The difference between the human and mouse diseases stems from a more efficient conversion in mice of $G_{M2}$ ganglioside to its asialo derivative, glycolipid $G_{A2}$, which can be catabolized further by the Hex B isozyyme present in Hexa$^{-/-}$ but not Hexb$^{-/-}$ mice (Fig. 1) (2,3). This enzymatic sequence constitutes a metabolic bypass of the Hex A defect that permits disease avoidance in Hexa$^{-/-}$ mice. The enzyme responsible for initiating the bypass is lysosomal sialidase. It occurs in a multienzyme complex with $\beta$-galactosidase and cathepsin A (5–8). It is inactive outside the complex and, indeed, it is rapidly degraded in cells deficient in cathepsin A in the genetic disease galactosialidosis (9). The activity of lysosomal sialidase is independently deficient in the inborn error sialidosis (10).

In this study, we show that Tay–Sachs neuroglia cells, boosted in the level of functional sialidase by transfection, are able to catabolize accumulated $G_{M2}$, suggesting that pharmacological stimulation of endogenous sialidase might have therapeutic benefit in Tay–Sachs disease.

RESULTS

In order to evaluate the potential for the sialidase bypass to function in human cells, we made use of a neuroglia cell line, derived from fetal cerebellum from the terminated pregnancy of an Ashkenazi Jewish couple (11), as an in vitro model of the disease. The cells have been shown to have deficient Hex A activity and to accumulate $G_{M2}$ (12), confirming that they have a functional ganglioside biosynthetic pathway. We tested for the common Ashkenazi Jewish mutations using established DNA tests and determined that the fetus was a compound heterozygote for functionally null mutations in the $\alpha$ subunit ($\alpha$HexA gene mutations, 1278insTATC and IVS12+1G→C; data not shown), indicating that the cells were devoid of Hex A activity. We confirmed immunocytochemically that we could detect the accumulated $G_{M2}$ in the affected Tay–Sachs cells, but not in normal neuronal cells (Fig. 2A and B). We also confirmed the location of the $G_{M2}$ in lysosomes by co-localization with the lysosomal membrane protein LAMP2 (13; Fig. 2C1–C3) and cathepsin A (Fig. 2D1–D3).

Our approach was to transfact the Tay–Sachs neuroglia cells with an expressible sialidase cDNA and to assay the cells for clearance of $G_{M2}$ ganglioside. We showed previously that an SV40-transformed derivative of Tay–Sachs neuroglia cells transfected with a cDNA clone encoding the $\alpha$ subunit of Hex A (pCMV-Hex $\alpha$) produced active Hex A as assayed with a synthetic substrate (14). We repeated this experiment as a positive control, except that we used the untransformed cell line as host, in order to confirm whether the increased Hex A activity would result in depletion of the accumulated $G_{M2}$. While
untransfected cells remained positive for G\textsubscript{M\textsubscript{2}}, those expressing a high level of Hex A (44 cells counted) were cleared of the lipid (Fig. 3A–C). This illustrated the capacity of the accumulated G\textsubscript{M\textsubscript{2}} to be successfully degraded in cells with a restored catabolic sequence.

In order to determine whether increasing the activity of lysosomal sialidase would have a similar impact on G\textsubscript{M\textsubscript{2}} degradation, we performed similar experiments, this time making use of a cDNA encoding the lysosomal sialidase instead of the Hex \(\alpha\) subunit. In order to detect the transfected sialidase alone and not the endogenous enzyme, a pCMV vector was prepared with a polyhistidine-tagged human sialidase cDNA (pCMV-HisSial) which could be detected with an anti-polyhistidine antibody (Fig. 4A and B). We first confirmed that the polyhistidine-tagged sialidase retained the ability to form a complex with \(\beta\)-galactosidase and cathepsin A. This was accomplished by co-transfecting the Tay–Sachs neuroglial cells with pCMV-HisSial and pCMV-CA (cathepsin A), the latter to promote formation of the multi-enzyme complex (15,16). The cells were radiolabeled with \(^{35}\text{S}\)methionine and the resulting radioactive proteins were immunoprecipitated using the anti-polyhistidine antibody and resolved using SDS–PAGE. Radioactive polypeptides corresponding in size to those of \(\beta\)-galactosidase (78 kDa) and cathepsin A (20 kDa), as well as sialidase (doublet of 44 and 46 kDa), were detected, indicating that the polyhistidine tag does not interfere with assembly of the complex (Fig. 4C).

We could now test the effect of increased sialidase on G\textsubscript{M\textsubscript{2}} levels. Tay–Sachs neuroglial cells were co-transfected with pCMV-HisSial and pCMV-CA. Two days after transfection, the cells were fixed and immunolabeled with anti-G\textsubscript{M\textsubscript{2}} and anti-polyhistidine antibodies. Figure 3D–F illustrates that neuroglia cells expressing high levels of sialidase show virtually complete depletion of their accumulated G\textsubscript{M\textsubscript{2}}. All cells strongly positive for the transfected sialidase (270 cells counted) gave complete or near complete reduction of G\textsubscript{M\textsubscript{2}} ganglioside to background levels.

In an independent test of the efficacy of human sialidase to act on G\textsubscript{M\textsubscript{2}}, cultured Tay–Sachs and Sandhoff fibroblasts were preloaded with a ganglioside mixture (1 mg/ml), co-transfected with pCMV-HisSial and pCMV-CA and immunostained for G\textsubscript{M\textsubscript{2}} and sialidase as before. The fibroblast cultures were from skin biopsies and do not normally synthesize gangliosides, so that cells positively staining for G\textsubscript{M\textsubscript{2}} confirmed successful loading of the lipid in the lysosomes. Two days after transfection, both Tay–Sachs (Fig. 5A–C) and Sandhoff fibroblasts (Fig. 5D–F), shown to be overexpressing sialidase by immunostaining (35 and 48 cells counted, respectively), had dramatically reduced levels of G\textsubscript{M\textsubscript{2}} when compared with surrounding untransfected cells.

**DISCUSSION**

We conclude that elevation of the level of lysosomal sialidase in Tay–Sachs neuroglia cells, as well as preloaded mutant fibroblasts, is sufficient to facilitate degradation of the
accumulated ganglioside and mimic the catabolic bypass that allows the murine model of Tay–Sachs disease to survive (Fig. 1). While our assay was limited to detecting clearance of GM2, we anticipate that continued catabolism through lactosylceramide likely occurs because of the much reduced level of glycolipid GA2 observed in Tay–Sachs compared with Sandhoff brain in the human disease (17). When the sialidase bypass was first documented in Hexa–/– mice, it was proposed that it was made possible by a higher affinity of the mouse enzyme for GM2 or that mice have a higher level of the enzyme in ganglioside-producing tissues (2,3). While our studies implicate quantity over quality of enzyme, they do not rule out a modest difference in substrate affinity that would favor the bypass pathway in mouse tissues. Resolution of the mechanism will likely require in vitro analysis of the two enzymes.

Our experiments took advantage of the potentiating effect of cathepsin A, transfected in combination with sialidase, to maximize lysosomal expression of the latter enzyme. Cathepsin A is required to facilitate incorporation of sialidase into the lysosomal β-galactosidase–cathepsin A–sialidase complex, either through ferrying the enzyme to the lysosome (15) or by protecting it from degradation prior to its entry into the lysosome (9). It remains to be determined if stimulating the endogenous synthesis of sialidase would require a concurrent increase in cathepsin A to result in a functional increase in sialidase activity.

As established for the Hexa–/– mouse, it is the presence of functional Hex B that makes it possible for lysosomal sialidase to function in the metabolic sparing of the Hex A defect (2,3,18). This is why the bypass can protect the Hexa–/– but not the Hexb–/– mouse, since the latter lacks both Hex A and Hex B. Thus, only in Tay–Sachs disease would it be possible to shunt G_{M2} to G_{A2} and lactosylceramide to permit completion of the catabolic sequence to ceramide.

Inherited neurodegenerative diseases pose an enormous challenge to treatment because of limited access to the brain. In Tay–Sachs and Sandhoff diseases, approaches to therapy have been directed toward the development of viral vectors encoding the deficient enzyme subunit (19–21), the injection of neural progenitor cells expressing functional enzymes (22), the use of bone marrow transplantation to provide enzyme through the

Figure 3. The effect of overexpressing Hex A or sialidase on GM2 ganglioside accumulation in Tay–Sachs neuroglia cells. The cells were transfected with pCMV-Hex α (A–C) or pCMV-HisSial/pCMV-CA (D–F), incubated for 72 h, fixed and double immunolabeled for GM2 ganglioside (A and D; green) and Hex A (B; red) or polyhistidine-tagged sialidase (E; red). Note that GM2 is not detected in cells expressing Hex A (C) or sialidase (F; minimal yellow), confirming the lysosomal degradation of the accumulated GM2. Bars: C, 11.5 μm; F, 8 μm.

Figure 4. Construction of an expression vector containing polyhistidine-tagged sialidase cDNA. (A) Nucleotide sequence of the polyhistidine insert within the human sialidase cDNA. (B) Construction of the pCMV-HisSial vector. The cDNA was digested at nt 152 with PshAI and at nt 171 with BsiEII. The synthetic adapter was prepared as two separate oligonucleotides (sense and antisense), annealed and inserted into dephosphorylated pCMV-sialidase (pCMV-hneu) to generate pCMV-HisSial. (C) Coomassie blue staining (I) and radiograph (II) of 12% SDS–PAGE of anti-polyhistidine antibody immunoprecipitated from Tay–Sachs neuroglia cells 48 h after transfection with pCMV-HisSial/pCMV-CA. The cells were pulsed with [35S]methionine for 30 min and chased for 30 min. Immunoprecipitation and electrophoresis were performed as described previously (30). Note that radioactive bands with molecular masses corresponding to β-galactosidase (pro-form, 78 kDa) and cathepsin A (20 kDa) co-precipitated with polyhistidine-tagged sialidase (44 and 46 kDa) indicating that the polyhistidine tag does not interfere with complex formation.
circulation (23) and the drug-mediated inhibition of ganglioside biosynthesis (24). Bone marrow transplantation extended the lifespan of Hexb–/– mice, but central nervous system pathology was unchanged by the treatment (23). Drug inhibition of ganglioside synthesis is a promising prospect for limiting the accumulation of GM2. Platt et al. (24) treated Hexa–/– mice with N-butyldeoxynojirimycin and estimated that they reduced the synthesis of gangliosides in brain by ~10%. This limited biosynthetic impairment was sufficient to reduce the presence of swollen lysosomes characteristic of the pathology of GM2 storage. A side-effect of the drug used was severe destruction of the spleen, an issue that would need to be addressed in a chronic treatment method. Also uncertain is the potential impact of interfering with ganglioside biosynthesis in humans. Nevertheless, these are early stage experiments that challenge the notion that Tay–Sachs disease is inaccessible to treatment. Our studies, along with those of Platt et al. (24), indicate that manipulations of brain metabolism are candidates for approaches to treatment. If safe, effective pharmacological intervention can be developed for stimulation of the sialidase bypass, perhaps in combination with marginal inhibition of ganglioside biosynthesis, then an effective treatment may be on the horizon for Tay–Sachs disease.

**MATERIALS AND METHODS**

**Cell lines**

Normal and Tay–Sachs neuroglia cells were generous gifts from L. Hoffman (Neuroscience Centre, Kingsbrook Jewish Medical Center, New York, NY), Tay–Sachs (WG107) and Sandhoff (WG150) fibroblasts were obtained from the Mutant Human Cell Repository (Montreal Children’s Hospital, Montreal, Canada) and their molecular defects have been characterized previously. Cell lines were maintained in modified Eagle’s medium supplemented with 15% fetal calf serum and antibiotics.

**Antibodies**

Mouse monoclonal antibody against human LAMP2 was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD). Texas Red-conjugated goat anti-rabbit IgG, Texas Red-conjugated goat anti-mouse IgG, BODIPY FL-conjugated avidin and biotinylated anti-human antibody were purchased from Molecular Probes (Eugene, OR). The mouse anti-polylhistidine peptide antibody was purchased from Qiagen (Germany). The mouse/human chimeric monoclonal anti-GM2 antibody KM966 was provided by N. Hanai (Tokyo, Japan) and has been shown to be highly specific for GM2 ganglioside (25,26). The rabbit anti-cathepsin A antibody was provided by A. Pshezhetsky (Montreal, Canada) (9). The anti-Hex A antibody was provided by D. Mahuran (Toronto, Canada) (27).

**Subcloning into expression vectors**

The human sialidase cDNA (1.9 kb) flanked by XmaIII restriction sites was subcloned into the NotI site of a pCMV vector (28). In order to insert a nucleotide sequence encoding a polyhistidine tag, a synthetic adapter made up of two oligonucleotides (5′-CATGCGCGGATCTCATCATCATCACCA-TCACCTCGTGACGCGCTG and 5′-GTCACCACCGGCTGCACGCCATG) was prepared. The oligonucleotides were allowed to anneal, creating a blunt 5′-end and a cohesive BseII-compatible 3′-end. The pCMV-Sial vector was digested at unique restriction sites using PshAI (position 152) and BseII (position 171), dephosphorylated and then ligated with the polyhistidine adapter. The ligation mixture was used to transform TOP10F cells. Positive clones of pCMV-HisSial were identified by digesting with Hhal and XhoII (Fig. 4A and B). The final pCMV-HisSial vector contained the amino acid sequence MRGSHHHHH immediately following the signal peptide cleavage site. The pCMV-Hex A and pCMV-cathepsin A vectors were prepared as described before (14,16). At the
end of this study, we learned from Dr H. Sakuraba (Tokyo) that our cDNA contained an amino acid difference, Leu90Pro (269T→C), compared with the published sequence (29). A polyhistidine-tagged cDNA prepared subsequently with the corrected sequence confirmed the results obtained with the original clone.

Ganglioside loading of mutant fibroblasts

Confluent Tay–Sachs and Sandhoff fibroblast cultures were incubated for 72 h with OptiPrep medium (Gibco BRL, Burlington, Ontario, Canada) containing 1 mg/ml bovine ganglioside mixture (Sigma, Oakville, Ontario, Canada). Twenty-four hours before transfection, the cells were trypsinized and replated at 50–70% confluency on single chamber LabTek slides (Nunc, Burlington, Ontario, Canada).

Expression in mammalian cells

For transient expression, cells were transfected with either pCMV-Hex α vector or pCMV-HisSial/pCMV-CA. Transfection was done using lipofectamine [1 μg vector(s) and 6 μl lipofectamine solution in 1 ml OptiPrep solution for one chamber-LabTek slides] as described by the manufacturer (Gibco BRL). For controls, a pCMV vector containing no insert or cathepsin A cDNA was used. After transfection, cells were incubated for 24 h in modified Eagle’s medium containing 15% fetal calf serum with no antibiotics. On the second day, the medium was replaced with medium containing 15% fetal calf serum and antibiotics. Transfection efficiency was <1% as detected by immunostaining for the expressed product (Hex A or polyhistidine tag).

Immunocytochemical localization of lysosomal sialidase

Transfected cells expressing human sialidase and cathepsin A cDNAs, grown on chambered slides, were washed in phosphate-buffered saline (PBS) and fixed for 30 min in 3.8% paraformaldehyde in PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. After washing twice in cold PBS, cells were blocked with 10% goat serum in PBS for 1 h. For double labeling, primary antibodies (diluted 1:200 in PBS) were incubated with the cells at 4°C overnight. Cells were washed three times for 5 min each in PBS containing 0.1% Tween-20. Cells were then incubated with secondary antibodies (either biotinylated or Texas Red conjugated) for 1 h at room temperature and again washed in PBS containing 0.1% Tween-20. Cells were incubated with BODIPY FL-conjugated avidin for 30–45 min, then were washed again in PBS containing 0.1% Tween-20 three times for 5 min each. A final wash was performed in distilled water to remove residual salts. Cells were mounted on slides using Pro-Long antifade solution (Molecular Probes). Double antibody labeling experiments were analyzed on a Zeiss LSM 410 inverted confocal microscope (Carl Zeiss, Thornwood, NY) as described previously (16). The fluorescein signal was imaged by exciting the sample with the 488 nm line from an argon or an argon/krypton laser and the resulting fluorescence was collected on a photomultiplier after passage through the FT510, FT560 and LP590 filter sets. Likewise, the same field was excited with a helium/neon (543 nm line) laser and the Texas Red signal was imaged on a second photomultiplier after passage through the FT510, FT560 and LP590 filter sets. The green and red images were overlaid and pseudo-coloured using built-in LSM software. Images were obtained with 25×/0.8 or 63×/1.4 plan-Apochromat (Zeiss) oil objectives and printed on a Kodak XLS8300 color printer.

ACKNOWLEDGEMENTS

S.A.I. is a recipient of a Montreal Children’s Hospital Research Institute post-doctoral fellowship. C.M. is a recipient of an Eileen Peters McGill Major Fellowship. J.M.T. is a Medical Research Council of Canada Scientist and scholar of the Fonds de la Recherche en Santé du Québec. These studies were supported by the Medical Research Council of Canada.

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