Apoptotic cell death in mouse models of $G_{M2}$ gangliosidosis and observations on human Tay–Sachs and Sandhoff diseases

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INTRODUCTION

Tay–Sachs and Sandhoff diseases are autosomal recessive neurodegenerative diseases resulting from the inability to catabolize $G_{M2}$ ganglioside by $\beta$-hexosaminidase A (Hex A) due to mutations of the $\alpha$ subunit (Tay–Sachs disease) or $\beta$ subunit (Sandhoff disease) of Hex A. Hex B ($\beta\beta$ homodimer) is also defective in Sandhoff disease. We previously developed mouse models of both diseases and showed that $Hexa^{-/-}$ (Tay–Sachs) mice remain asymptomatic to at least 1 year of age while $Hexb^{-/-}$ (Sandhoff) mice succumb to a profound neurodegenerative disease by 4–6 months of age. Here we find that neuron death in $Hexb^{-/-}$ mice is associated with apoptosis occurring throughout the CNS, while $Hexa^{-/-}$ mice were minimally involved at the same age. Studies of autopsy samples of brain and spinal cord from human Tay–Sachs and Sandhoff diseases revealed apoptosis in both instances, in keeping with the severe expression of both diseases. We suggest that neuron death is caused by unscheduled apoptosis, implicating accumulated $G_{M2}$ ganglioside or a derivative in triggering of the apoptotic cascade.

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Tay–Sachs and Sandhoff diseases are autosomal recessive neurodegenerative diseases resulting from the inability to catabolize $G_{M2}$ ganglioside and related glycolipids by $\beta$-hexosaminidase (Hex) (1). There are two major forms of Hex: Hex A, a heterodimer composed of one $\alpha$ and one $\beta$ subunit, and Hex B, composed of two $\beta$ subunits. Hydrolysis of $G_{M2}$ ganglioside, in a complex with $G_{M2}$ activator protein, is catalysed by Hex A but not by Hex B. Tay–Sachs disease, prevalent in Ashkenazi Jews, is caused by mutations in the $HEXA$ gene encoding the $\alpha$ subunit of Hex A. It results in deficiency of Hex A activity but does not affect Hex B. The much rarer Sandhoff disease is caused by mutations in the $HEXB$ gene encoding the $\beta$ subunit, with resulting deficiency of both Hex A and Hex B activities. In their most severe, infantile acute forms both diseases have in common a progressive neurological deterioration from infancy, culminating in death by 2–5 years of age. Subacute (juvenile) and chronic (adult) forms may also occur with progressively later onset and milder clinical course (1). At the cellular level, distended neurons engorged with swollen lysosomes (membranous cytoplasmic bodies, MCB) are found throughout the nervous system. Visceral organ pathology is also a feature of Sandhoff disease.

While the $G_{M2}$ gangliosidoses have become well understood at the biochemical level, more problematical is the cause of neuron death. Speculation has included disruption of subcellular structures due to lysosomal dysfunction with toxicity of accumulated glycolipids, including lysogangliosides (2), and disruption of neurotransmitter function (3). This enigma and the desire to develop treatments for neurodegenerative diseases have led us and others to develop mouse models of Tay–Sachs and Sandhoff diseases by homologous recombination (4–8).

Unexpectedly, $Hexa^{-/-}$ mice, which were expected to have the features of Tay–Sachs disease, failed to express an abnormal phenotype. These mice show behaviour and motor coordination indistinguishable from wild-type to >1 year of age. Nevertheless, $Hexa^{-/-}$ mice do show limited accumulation of $G_{M2}$ ganglioside and the presence of MCB in the brain (4,7,8). In contrast, $Hexb^{-/-}$ mice, developed as models of Sandhoff disease, exhibit a severe neurodegenerative disease with profound depletion of spinal cord axons (6,8). With onset at ~3 months of age, these mice have tremor, spasticity, hind limb rigidity, ataxic gait and muscle wasting and weakness, leading to paralysis and death by 4–6 weeks after onset. The brain and spinal cord are replete with swollen neurons due to lysosomes accumulating dramatic levels of $G_{M2}$ ganglioside and its asialo derivative, glycolipid $G_{A2}$. Visceral organs are also involved, with subcellular vacuolation of liver parenchyma and

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proximal kidney tubules in Hexb–/– but not Hexa–/– mice. The Hexa–/– mice avoid disease due to their greater capacity than humans to catabolize GM2 ganglioside via glycolipid GA2, using sialidase and Hex B activities rather than through GM3 ganglioside, which is blocked due to the Hex A defect (6,8). In Hexb–/– mice the additional absence of Hex B activity also blocks this secondary route. The GA2 bypass was confirmed as a major catabolic route in wild-type as well as mutant mouse embryonic fibroblasts by metabolic labelling of ganglioside precursor (6).

The relationship between ganglioside accumulation and neuron death remains to be defined. In this study we explore the possibility that neuron death in Hexb–/– mice is due to inappropriate apoptosis. Apoptosis is a regulated physiological process that leads to elimination of unwanted cells during development or the renewal of cell populations in mature tissues (9,10). It has also been proposed that inappropriate or accelerated rates of apoptosis may underlie neuronal loss in neurodegenerative diseases (11). At the cellular level, apoptosis is a multistage process involving chromatin condensation, cell compaction and membrane blebbing (12). This is a rapid process that terminates in the removal of apoptotic cells by phagocytosis by adjacent native cells.

We obtained evidence of apoptosis throughout the brain and spinal cord of Hexb–/– mice. Hexa–/– mice, in contrast, were minimally affected, in keeping with the absence of disease. Comparing the observations with autopsy samples from patients who died of Tay–Sachs or Sandhoff diseases, we detected apoptosis throughout the CNS in both diseases. We speculate that inappropriate apoptosis in some lysosomal storage diseases is caused by accumulation of gangliosides or related glycolipids to a critical threshold that triggers pathways of programmed cell death.

RESULTS

Groups of four to seven mice of each genotype were examined for apoptosis in tissues at the age matching the onset of symptomatic phenotype (tremor, weakness, ataxic gait) of the Hexb–/– mice, which corresponded to ~3.5–4 months of age. Genotypes were determined in control, asymptomatic Hexa–/– mice and symptomatic Hexb–/– mice by diagnostic PCR conducted on tail DNA that detected the presence of a neomycin-resistance gene interrupting exons of the two genes.

DNA from cerebral cortex, brain stem, cerebellum and spinal cord were subjected to agarose gel electrophoresis. Ethidium bromide staining of the gel revealed that the DNA from all of these tissues from Hexb–/– mice migrated as discrete bands at intervals of 180–200 bp compared with the 1 kb DNA marker (Fig. 1, lanes S). In contrast, the DNA from the brain and spinal cord of Hexa–/– mice failed to show DNA laddering (Fig. 1, T), except in one mouse where it was only faintly visible in cerebral cortex (not shown). A similar number of sibling control mice failed to show any DNA laddering in any part of the brain or spinal cord (Fig. 1, +).

Using the in situ DNA end-labeling technique to detect free 3′-OH ends of newly cleaved DNA, apoptotic cells were identified as brown staining over highly condensed, distored cell structures with extremely shrunken and dark nuclei which were often fragmented into several small, dense droplets (illustrated in Fig. 2b).

Experimental animals

These studies were done at the time of symptomatic onset in the Hexb–/– mice. Sibling control mice (Hexb+/+ or Hexb+/–) were essentially devoid of apoptotic cells in brain stem, cerebellum and spinal cord but had a significant number of positive cells in the cortex (Fig. 3). These cells predominated among the interstitial cells of the corpus callosum and external capsule. Hexa–/– mice were comparable with controls for all four areas of the brain and spinal cord. Only one of six mice examined for apoptotic cells in the cortex was significantly increased over control, although this did not extend to the other regions examined. In Hexb–/– mice apoptotic cells were identified in cerebral cortex, brain stem (Fig. 2b), cerebellum and spinal cord (Fig. 2d). They were sparsely distributed but in greater numbers than controls in all but cerebral cortex (Fig. 3). The cell types of origin were not discernible because of their distorted morphology. Intact neurons in affected mice were grossly distended and filled with enlarged lysosomes. This was as true for Hexa–/– mice (e.g. Fig. 2a) as it was for Hexb–/– mice, but at a less extensive level. The lysosomal swelling was shown to be due to GM2 ganglioside accumulation, detected with an anti-GM2 human–mouse chimeric monoclonal antibody (Fig. 2c). Lysosomal swelling and GM2 ganglioside storage were never observed in control mice.

Human tissue samples

In order to determine if apoptosis is also associated with human GM2 gangliosidoses, sections from autopsy samples of brain and spinal cord from cases of Tay–Sachs and Sandhoff diseases were examined for apoptosis using the in situ labelling technique. Very extensive staining was observed in all sections examined from both cases, including cerebral cortex, brain stem, cerebellum and spinal cord (Fig. 4b–d, spinal cord). Based on visual observation, the positively stained cells from the Sandhoff brain appeared to include all classes of cells, including neurons, oligodendrocytes, astrocytes, microglia and/or small granulocytes, Purkinje cells and also vascular pericytes. Positive staining was also observed among epithelial-appearing cells of the ependyma. In Tay–Sachs disease cell staining was also extensive, though not as much as in the Sandhoff sections. In the spinal cord anterior horn cells entering the earliest stages of apoptosis (brown stain over intact nucleus) were occasionally observed (Fig. 4d). These cells were highly distended and filled with enlarged lysosomes. Pericytes
Figure 2. Apoptosis, G\textsubscript{M2} ganglioside accumulation and lysosomal swelling in mouse tissue sections. (a) Toluidine blue staining of a semithin section of neurons from cerebral cortex of a Hex\textsuperscript{a–/–} mouse showing swollen lysosomes surrounding the nucleus, 630×. (b) Formation of apoptotic bodies in the brain stem of a Hex\textsuperscript{b–/–} mouse detected by in situ end-labelling of DNA, 630×. (c) Anterior horn cells of spinal cord from a Hex\textsuperscript{b–/–} mouse showing accumulated G\textsubscript{M2} ganglioside detected with anti-G\textsubscript{M2} monoclonal antibody, 630×. (d) Apoptotic cells (arrows) from the grey matter of the spinal cord of a Hex\textsuperscript{b–/–} mouse detected by in situ end-labelling of DNA, 200×.

were largely unaffected in the Tay–Sachs sections (compare Figs 4b and c). The control brains and spinal cords were negative (Fig. 4a). Also, in one control case, who died at 13 months, a few shrunk, dark brown nuclei were observed in the granular layer of the cerebellum which were likely reflective of this stage of development (not shown).

As observed for the mouse models, the apoptotic cells in the human disease cases were found among morphologically involved neurons swollen with engorged lysosomes. This was readily revealed by haematoxylin and eosin staining of anterior horn cells of the spinal cord. Both Tay–Sachs and Sandhoff cells showed highly distended, ballooned neurons (Fig. 5b and c). No such cells were observed in control sections (Fig. 5a).

**DISCUSSION**

The implication of apoptosis as a factor in disease has been a relatively recent phenomenon, despite the potentially crucial pathogenic role of this fundamental cellular process. This has been due at least in part to the difficulty of recognizing apoptotic cells using routine histological techniques. With the introduction of DNA laddering and DNA end-labelling techniques it has become apparent that apoptosis is a significant participant in the neurodegenerative disease process (11). Using these techniques we have observed apoptosis of degenerating nervous tissue in Hex\textsuperscript{b–/–} mice, as well as in initial autopsy samples of brains from patients with Tay–Sachs and Sandhoff diseases. Significantly, the
Ceramide is also produced by sequential hydrolysis, from the reducing end, of the oligosaccharide portion of gangliosides, a pathway of significance in the brain. Since mature nervous tissue is not subject to significant apoptosis, it is not likely that ganglioside catabolism normally generates signals mediating apoptosis. Nonetheless, numerous neurodegenerative diseases are characterized by gradual loss of specific sets of neurons which is thought to occur through inappropriate apoptosis. Examples include Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, retinitis pigmentosa, spinal muscular atrophy and various forms of cerebellar degeneration (reviewed in 11). In these diseases gradual cell death results in progressive deterio-ration of central nervous system function. Despite evidence for increased apoptotic cell death, the underlying defect in cell death control has not been defined in most of these disorders.

It is in this context that the Hexa\(^+/-\) and Hexb\(^+/-\) mice provide clues to one mechanism of neuron death. We previously showed that the dramatic phenotypic difference between these two mouse models could be explained by the extent of ganglioside (or glycolipid) accumulation (8). At phenotypic onset in several Hexd\(^+/-\) mice the combined glycolipid (GM\(_2\) + GA\(_2\)) levels ranged from 440 to 1452 mmol/g wet wt. In contrast, the range obtained for Hexa\(^+/-\) mice, which did not exhibit behavioural changes, was 194–234 mmol/g wet wt. These data suggested a threshold level of GM\(_2\) or GA\(_2\) accumulation associated with symptomatic disease. While more detailed correlative studies are required to determine the causal relationships among disease phenotype, ganglioside levels and apoptosis, it is tempting to speculate that the initiating event is the accumulation of GM\(_2\) ganglioside or the side product glycolipid GA\(_2\) or a related metabolite which induces the apoptotic cascade. It would be informative to examine apoptosis in Hexb\(^+/-\) mice at a younger age to determine the onset of apoptosis in relation to ganglioside level. This will have to await a more sensitive marker of apoptosis than the in situ method for DNA ends used here, given the low frequency of positive cells observed in the symptomatic mice.

The pattern of tissue pathology and/or GM\(_2\) ganglioside accumulation correlates well with the pattern of inappropriate apoptosis we observed in the mouse models. In Hexb\(^+/-\) mice all brain compartments contained swollen neurons with perinuclear accumulation of MCB. That the neuronal swelling was due primarily to accumulation of GM\(_2\) ganglioside was demonstrated immunohistochemically with a human–mouse chimeric antibody to detect GM\(_2\) ganglioside. In Hexa\(^+/-\) mice, which were generally devoid of apoptotic cells or not increased over controls (cortex), the brain and spinal cord showed reduced histopathology and GM\(_2\) ganglioside accumulation. Significantly, the one mouse with an increase in the number of apoptotic cells, mainly in the white matter of the corpus callosum and external capsule, was asymptomatic at the time of sacrifice, compatible with the view that these are remnants of subplate neurons (13).

Ceramide is also generated biosynthetically from sphinganine and fatty acid by ceramide synthase. It can be activated by the anticancer drug daunorubicin in P388 and U937 cells (20). The importance of acid sphingomyelinase in the signalling pathway has been demonstrated genetically in lymphoblasts established from patients with Niemann–Pick disease with inherited defects of the enzyme and, recently, in knock-out mouse models of the human disease (21). Both disorders are defective in radiation-induced apoptosis (21).

Figure 3. Distribution of positively stained ‘apoptotic’ cells in cerebral cortex, brain stem, cerebellum (cereb.) and spinal cord. Whole sections were counted from three contiguous sections in control (shown as +, including three homozygous wild-type and three heterozygous mice), Hexa\(^+/-\) (T) and Hexb\(^+/-\) (S) mice. Results are given as no. positive cells/section ± SEM (*P \(\leq 0.05\)). The + and T values for cerebellum and spinal cord are 0.

association of increased apoptosis with symptomatic disease in the Hexb\(^+/-\) mice contrasted markedly with the absence of an increase in apoptosis and absence of overt disease in the Hexa\(^+/-\) mice. This was readily apparent for the brain stem, cerebellum and spinal cord, since these tissues were virtually devoid of positive cells in the control or Hexa\(^+/-\) mice. In the cortex a significant frequency of positive cells was identified in controls that concentrated around the interstitial cells of the white matter. These latter cells are thought to be remnants of early subplate neurons that are continuing to die off in the adult brain (13).

Because of the high variation in apoptotic cell counts in the cortex, a much more extensive series of analyses would be needed to determine if the cortex is compromised at the time of overt disease (21). Both disorders are defective in radiation-induced apoptosis (21).

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Our observation of apoptosis in human brain samples general-izes this mechanism of cell death to human Tay–Sachs and Sandhoff diseases. It is significant that the human Tay–Sachs samples were positive for increased apoptosis while those of the Hexa\(^+/-\) mouse counterpart were not. This difference is linked to the asymptomatic, fatal disease of human Tay–Sachs patients. In contrast, the Hexa\(^+/-\) mice showed essentially no change in apoptosis and no disease. A major difference between the human and mouse diseases was the extent of apoptotic cell staining, which was much more dramatic in the human disorders. We
Figure 4. In situ DNA end-labelling of spinal cord sections showing the region of anterior horn cells from autopsy samples of human Tay–Sachs and Sandhoff disease cases. (a) Control, 200×; (b) Tay–Sachs disease with arrows indicating examples of apoptotic cells, 200×; (c) Sandhoff disease showing extensive pattern of apoptotic cells with arrows showing apoptotic cells at different stages and open arrowhead showing stained vascular pericyte, 200×; (d) Sandhoff disease with arrow indicating anterior horn cell with positively stained nucleus, 400×.

Our studies suggest that gangliosides or glycolipids should be examined as potential inducers of apoptosis. Although the studies summarized here point to the GM2/GA2 structures as candidate inducers, it is not apparent whether only these molecules, gangliosides with different lengths or complexity of the oligosaccharide chain or perhaps lysogangliosides should be considered as candidates for triggering apoptosis when abnormally accumulated. Since metabolic blocks are known at all of the steps in GM1 ganglioside catabolism, we suggest that brain tissue should be examined for apoptosis in all of these disorders. By taking advantage of the very metabolic blocks causing these diseases, it may be possible to define the initiating structures or conversely to show the breadth of oligosaccharide structures that can contribute to the apoptotic cascade.

MATERIALS AND METHODS

Animal models

The mutant Hexa<sup>+</sup> and Hexb<sup>+</sup> mice used in this study were derived by introduction of a neomycin-resistance gene within
Figure 5. Haematoxylin and eosin staining of spinal cord sections from human control (a), Tay–Sachs (b) and Sandhoff disease (c) cases, 200x. Note distended anterior horn neurons in (b) and (c) compared with (a).

exon 11 or 2 of the Hexa or Hexb genes respectively by homologous recombination (8). Heterozygotes were interbred to produce homozygous offspring (Hexa+/− and Hexb+/−). Wild-type littermates were used as controls. The mice were genotyped by diagnostic PCR typing of tail DNA to detect the presence of the inserted sequence and the products analysed on 1% agarose gels (8). The mice were killed at the age matching onset of the symptomatic phenotype of the Hexb+/− mice, which corresponded to 3.5–4 months of age (8).

For preparation of fresh frozen tissues mutant and control mice were killed using a CO2 chamber and were immediately dissected. Cerebral cortex, cerebellum, brain stem and spinal cord were collected and snap frozen between dry ice blocks and kept at –80°C prior to DNA extraction.

For in situ DNA end-labelling studies mice were anaesthetized with 4% sodium chloral hydrate solution and perfused through the heart with neutral buffered formalin. Following perfusion, cerebral cortex, cerebellum, brain stem and spinal cord were removed and left in the same fixative overnight at 4°C. The samples were dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin in a Tri-Matic automatic tissue processor. Paraffin sections (4–5 µm) were cut and collected on poly-L-lysine-coated slides (50 µg/ml in 10 mM Tris–HCl). The slides were air dried, heated in a 60°C oven overnight and stored at room temperature until used. For morphological examination of tissues semithin sections (0.5 µm) were prepared from the brains of perfused mice and stained with toluidine blue, as described previously (8). GM2 ganglioside was detected immunohistochemically using a monoclonal, human–mouse chimeric antibody, as described (8). It was shown to react with GM2 ganglioside and weakly with GD2 ganglioside but no other component of mixed bovine brain gangliosides or a defined set of gangliosides (22). It also does not react with neutral glycolipids from human spleen, which also contain significant amounts of globoside.

Human tissue samples

Human autopsy brain samples were obtained from Sainte-Justine Hospital, Montreal, from one patient with Tay–Sachs disease and one with Sandhoff disease. In both cases disease onset was at ~3 months of age. The diagnosis was made by enzymatic assay of blood leukocytes and serum. Both patients showed symptoms typical of these disorders, including weakness, startle reaction to sharp sound, absence of purposeful movement or response to stimulus, hypotonia and a neurodegenerative course with progression to spasticity. In each case death was due to complications of bronchopneumonia, at 19 months of age for the Sandhoff patient and 3 years of age for the Tay–Sachs patient. Control cases included one individual who died at 21 months due to heart failure secondary to endocardial fibroelastosis and another who died following surgery at 13 months of age for tetralogy of Fallot. The cases were kept at 4°C until autopsy, which was within 14 h of death. Paraffin blocks of tissue samples, including cerebral cortex, brain stem, cerebellum and spinal cord, prepared at autopsy were made available for this study. Sections (5 µm) from each block were cut and mounted on slides for in situ DNA end-labelling studies. Adjacent sections were stained with haematoxylin and eosin. In addition, four cases of infant deaths (2–3 months of age) due to bronchopneumonia were reviewed for whom brain sections were examined by in situ DNA end-labeling in an independent study (A.Côté, personal communication).

DNA extraction and agarose gel electrophoresis

Frozen tissue samples from control or affected mice were cut into small pieces and digested in lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 100 µg/ml proteinase K, 0.5%
at room temperature and rinsed through double distilled H\textsubscript{2}O and proteinase K, (Boehringer Mannheim; 10 
\textmu g/ml) for 15 min at 37°C. The tissue was then digested with 0.5% collagenase (Type IA, Sigma) in PBS for 30 min and rinsed again through PBS several times.

**In situ apoptosis detection (DNA end-labelling)**

Tissue sections were deparaffinized with xylene and immediately rehydrated through serial dilution in ethanol. After rehydration the slides were rinsed in phosphate-buffered saline (PBS). Nuclei of tissue sections were stripped of proteins by incubation with proteinase K, (Boehringer Mannheim; 10 
\textmu g/ml PBS) for 10 min at room temperature and rinsed through double distilled H\textsubscript{2}O and PBS. Endogenous peroxidase was inactivated by immersing the tissue sections in 2% H\textsubscript{2}O
d solution (0.05% 3,3′-diaminobenzidine, 0.03% H\textsubscript{2}O
d in PBS) for 10 min and stopped in double distilled H\textsubscript{2}O. Nuclear counterstaining was performed with haematoxylin solution. Positive controls were

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