Molecular pathophysiology in Tay–Sachs and Sandhoff diseases as revealed by gene expression profiling

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Tay–Sachs and Sandhoff diseases are lysosomal storage disorders characterized by the absence of β-hexosaminidase activity and the accumulation of GM2 ganglioside in neurons. In each disorder, a virtually identical course of neurodegeneration begins in infancy and leads to demise generally by 4–6 years of age. Through serial analysis of gene expression (SAGE), we determined gene expression profiles in cerebral cortex from a Tay–Sachs patient, a Sandhoff disease patient and a pediatric control. Examination of genes that showed altered expression in both patients revealed molecular details of the pathophysiology of the disorders relating to neuronal dysfunction and loss. A large fraction of the elevated genes in the patients could be attributed to activated macrophages/microglia and astrocytes, and included class II histocompatibility antigens, the pro-inflammatory cytokine osteopontin, complement components, proteinases and inhibitors, galectins, osteonectin/SPARC, and prostaglandin D2 synthase. The results are consistent with a model of neurodegeneration that includes inflammation as a factor leading to the precipitous loss of neurons in individuals with these disorders.

INTRODUCTION

Lysosomal storage diseases are a group of inherited disorders that result from defective acid hydrolase function. Tay–Sachs and Sandhoff diseases are examples of such disorders, and represent members of a subcategory called the GM2 gangliosidoses that are so named because GM2 ganglioside accumulates in cells owing to its impaired degradation (reviewed in 1). The absence of β-hexosaminidase A in Tay–Sachs disease and of β-hexosaminidase A and B in Sandhoff disease are the primary enzyme deficiencies. Since only β-hexosaminidase A is able to degrade GM2 ganglioside, the substrate accumulates similarly in each disease. Affected individuals with either disease exhibit a virtually identical clinical course of neurodegeneration leading to death in early childhood. Apoptosis of neurons is demonstrable in patient samples and in mouse models (2,3). While it is clear that a primary insult to neurons is the accumulation of ganglioside substrates, the exact molecular mechanisms that translate the primary insult into neuronal cell death remain to be determined.

One alluring approach to gain insight into pathophysiology is to generate gene expression profiles of the central nervous system (CNS) in patients affected with these disorders (4). Such profiles would reveal how gene expression in the diseased state differed from that of the normal. Subsequent scrutiny of those genes exhibiting altered expression could be a wellspring for hypotheses regarding the pathways leading to the observed neurodegeneration. Advances, including serial analysis of gene expression (SAGE) (5) and microarray analysis (6), have made the undertaking of such studies feasible and are being used in the study of neurodegenerative diseases (4). In an earlier study on a mouse model of Sandhoff disease, we applied microarray analysis to monitor changes in gene expression. An extensive upregulation of genes related to an inflammatory process dominated by activated microglia was found in the disease model (3). Moreover, activation of microglia was found to precede massive neuronal death, suggesting that an inflammatory process may participate in neurodegeneration.

In the present study, we have utilized the SAGE methodology in which 10 base tags obtained from the 3’ end of each gene

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transcript are concatenated and sequenced to develop gene expression profiles for the GM2 gangliosidoses. This method, in principle, can provide both a qualitative and a quantitative profile of all the genes expressed in the tissues. Genes of known function, unknown function (expressed sequence tags, ESTs) and sequences yet to be identified as protein coding (novel genes) are included in SAGE analysis. The data thus generated can serve as a reference of the expressed genes in these diseased states. The gene expression profiles that we have obtained provide molecular details of the pathophysiology and demonstrate an intense inflammatory process that may be directly involved in the neurodegenerative process.

RESULTS

Generation of SAGE data

SAGE libraries were constructed from mRNAs isolated from cerebral cortex tissue samples derived from a normal child, a child with Tay–Sachs disease and a child with Sandhoff disease. A total of 107,976 tags were generated; 34,137 from normal, 38,940 from Tay–Sachs and 34,899 from Sandhoff tissues (Table 1). Sequence analysis showed that between 39% and 47% of the tags in each of the libraries were unique and corresponded to known genes, ESTs or potentially novel transcripts. Disregard for sequence tags that matched to more than one gene in each SAGE library allowed us to discern that at most 9,231 genes were represented in the pediatric control, 11,679 in the Tay–Sachs and 11,111 in the Sandhoff. While the majority of these corresponded to a sequence in GenBank, approximately 30% in either the Tay–Sachs or Sandhoff libraries did not match any GenBank entry and constitute a cadre of potentially novel transcripts awaiting characterization.

Comparison of gene expression profiles in normal and GM2 gangliosidosis cerebral cortex tissues

Results summarized in Table 2 show that of the 11,679 genes and novel tags expressed in the Tay–Sachs SAGE library, 631 (5.4%) showed differential expression at a statistically significant level (P < 0.05). A bout an equivalent number were elevated in expression as were depressed, and 10% of these showed a 10-fold or greater differential expression when compared with the pediatric normal. Of the 16 transcripts in the Tay–Sachs library displaying the highest fold elevation of expression (between 30 and 100), 9 belong to the novel tag category. Similar results were obtained with the Sandhoff library: 4.9% of the expressed genes and novel tags showed significant differential expression, with an approximately equal number elevated as depressed. To increase the probability of focusing on genes related to the pathophysiology of the GM2 gangliosidoses rather than individual variation, we confined our scrutiny to those genes whose expression was altered in both the Tay–Sachs and Sandhoff disease patients relative to the normal individual. Of the gene tags displaying differential expression, 111 elevated and 232 depressed were in common to both patients. This represents more than 50% of genes displaying differential expression in each of the disease libraries. These gene expression profiles can be found in the Supplementary Material. Those genes with elevated expression in the patients that could be functionally classified are listed in Table 3. Assignment of genes to a particular functional category is indicated by its color code. A category containing the largest fraction of the overexpressed genes included those associated with inflammation and injury responses. Many of these genes were associated with activated macrophages/microglia and astrocytes, the cell types that mediate inflammation and injury responses in the CNS. Genes that have been reported to be expressed by activated macrophages include cartilage gp-39 (7), galectin 3 (8), glycoprotein nmb (9,10), HLA-DR (7) and the complement component 1q β chain (11,12) (Table 3). A tag corresponding to CD68 (13), a classic macrophage marker, was also elevated in both disease libraries (Tay–Sachs, 13; Sandhoff, 4; normal, 0). The expression of two genes characteristic of activated astrocytes – glial fibrillary acidic protein (GFAP) and vimentin (14) – were both very highly elevated. Other genes elevated in both disease libraries and generally associated with astrocytes included αB-crystallin (15), apolipoprotein E (15,16), calcyclin (17) and the gap junction protein, connexin 43 (14). Elevated genes related to inflammatory responses and reported to be expressed by activated glia were prostaglandin D2 synthase (18), clusterin (12), cathepsin B (7) and the proinflammatory cytokine, osteopontin (19). Osteonectin (SPARC), a gene found to be upregulated in astrocytes after neural damage was also elevated in both patients (20,21).

In the category related to stress/apoptosis, several heat-shock proteins and the ubiquitin-conjugating enzyme E2H were elevated. Also of potential interest was the elevation of death-associated protein 6 (DAKX), encoding a protein that associates with the Fas receptor to mediate activation of Jun N-terminal kinase (JNK) and programmed cell death induced by Fas (22).

Other functional categories included genes involved in protein synthesis, cytoskeleton, signaling/gene expression and metabolism/housekeeping. A neuron-specific gene significantly elevated in both patients was growth-associated protein 43

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Known genes</th>
<th>ESTs</th>
<th>Novel tags</th>
<th>Elevated</th>
<th>Depressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tay–Sachs</td>
<td>516</td>
<td>39</td>
<td>84</td>
<td>314</td>
<td>317</td>
</tr>
<tr>
<td>Sandhoff</td>
<td>437</td>
<td>42</td>
<td>50</td>
<td>252</td>
<td>273</td>
</tr>
</tbody>
</table>

*P < 0.05.

*Does not include 8 genes in Tay–Sachs disease and 4 genes in Sandhoff disease with multiple tags that display both elevation and depression relative to normal.

Table 1. Summary of SAGE analysis

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Total tags</th>
<th>Distinct tags</th>
<th>Genes a</th>
<th>Novel tags</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>34,137</td>
<td>13,290</td>
<td>5731</td>
<td>3500</td>
</tr>
<tr>
<td>Tay–Sachs</td>
<td>38,940</td>
<td>17,290</td>
<td>7312</td>
<td>4367</td>
</tr>
<tr>
<td>Sandhoff</td>
<td>34,899</td>
<td>16,419</td>
<td>7249</td>
<td>3862</td>
</tr>
</tbody>
</table>

*a*As defined by matches to GenBank entries.
(GAP43), a phosphoprotein expressed in the elongating terminals of neurites (23). However, GAP43 appeared to be unique as a gene with specific neuronal expression that was elevated in the patients’ libraries. A number of tags with specific neuron expression appeared to be significantly depressed in the patients’ gene profiles (Table 2 in Supplementary Material). These included neuronal-specific enolase (NSE, enolase 2) (24), β-synuclein (25), neuronal pentraxin receptor (26), metabotropic glutamate receptor 3 (27), neurogranin (28), complexin 1 (29) and hippocalcin (30). The myelin

Table 3. Classified genes elevated in both Tay–Sachs and Sandhoff diseases
Figure 1. Histologic analysis of neurons, microglia and astrocytes. Sections of cerebral cortex from control (A, D, G, J), the Tay-Sachs patient (B, E, H, K) and the Sandhoff patient (C, F, I, L) were immunostained with antibody to neuronal-specific enolase (NSE) to detect neurons, glial fibrillary acidic protein (GFAP) to detect astrocytes and CD68 to detect microglia/macrophages. Staining with antibody to phosphotyrosine was used to detect activated microglia/macrophages. Note the low density of neurons and the enlarged astrocytes and microglia/macrophages in the patients’ sections. Bar = 50 μm.

Figure 2. Confirmation of SAGE by immunostaining of cerebral cortex sections. Sections of cerebral cortex from control (A, D, G, J, M), the Tay-Sachs patient (B, E, H, K, N) and the Sandhoff patient (C, F, I, L, O) were immunostained with antibody to cathepsin B, αB-crystallin, ferritin, osteopontin and GAP43. Bars = 50 μm.
basic protein gene, expressed by oligodendrocytes, was also significantly depressed in the patients' SAGE libraries.

Confirmation of SAGE data
To confirm the results of our SAGE library data, we checked the expression of several genes through immunohistochemical staining of paraffin-embedded sections of cortex (Fig. 1). A antibody to NSE, a gene found to have decreased expression in the Tay–Sachs and Sandhoff SAGE libraries, demonstrated less intense staining in the patients' sections compared with the control (Fig. 1A–C). The patients' sections appeared to contain fewer neurons, and those that were present had an altered, swollen morphology. Immunostaining for glial fibrillary acidic protein (GFAP) showed astrocytes that were clearly enlarged in the patients' sections, characteristic of an activated state (31) (Fig. 1D–F). CD68 immunostaining indicated a significantly elevated number of microglia in the patients' sections (P < 0.001) (Fig. 1G–I). The patients' microglia were amoeboid with minimal processes, a morphology associated with their activation (32). Their activation was confirmed by intense staining with antibody to phosphotyrosine (Fig. 1J–L) (3).

The protein products of several other genes with altered expression were assessed by immunostaining. Cathepsin B expression was elevated in the patients' sections, and appeared to be expressed by the amoeboid microglia (Fig. 2A–C). Staining with αB-crystallin was more intense in the patients' sections than in the control, consistent with the SAGE results, and appeared to be localized to the enlarged astrocytes and neurons (Fig. 2D–F). ferritin immunostaining was also enhanced in patients' samples, as predicted from the elevated expression of ferritin heavy- and light-chain genes (Table 3), and appeared to be localized to amoeboid microglia as well as other cell types (Fig. 2G–I). Osteopontin was found to be highly overexpressed in the patients' sections (Fig. 2J–L). Double immunostaining revealed that the majority of osteopontin expression was confined to microglia (not shown). GAP43, a neuron-specific gene, was more intensely expressed in patients' sections and appeared in a punctuate pattern (Fig. 2M–O).

DISCUSSION
Both Tay–Sachs and Sandhoff diseases are characterized by a deficiency of β-hexosaminidases that result in the accumulation of GM2 ganglioside in lysosomes. As a consequence of a similar biochemical defect, the two diseases are virtually indistinguishable in their neurodegenerative courses. Although much is known about the enzymology and molecular genetics of these diseases (1), there is considerably less information regarding how the primary cellular insult - lipid storage - leads to massive neuronal cell death and what cellular and molecular mechanisms participate in this process. We hypothesized that gene expression profiles of cerebral tissue from patients with the GM2 gangliosidoses could provide clues that would prove valuable in elucidating the pathological mechanisms operating in these diseases. Toward that goal, we employed the SAGE methodology to obtain global gene expression profiles for cerebral cortex tissue from a Tay–Sachs and a Sandhoff disease patient. We confined our attention to those genes with significantly elevated or decreased expression in both the Tay–Sachs and Sandhoff disease patients to focus our attention on genes mostly likely to be relevant to the neurodegenerative process rather than individual variation.

Decreased expression of neuron-specific genes was characteristic of both patient libraries. Immunostaining with NSE, a gene specifically depressed in the patient libraries, confirmed a decreased density of neurons in the disease samples. These findings underscore the neuronal loss that occurs during the disease process. Apoptosis of neurons has been demonstrated in both Tay–Sachs and Sandhoff disease patients and in mouse models, and is thought to be the cause of the precipitous neuronal loss (2,3). Of potential relevance to the neuronal cell death in the disorders is the elevated expression of cathepsin B, a lysosomal protease that has been shown to be released by activated microglia and to directly cause neuronal apoptosis (33). In Alzheimer's disease, high levels of cathepsin B are in senile plaques (34). Prostaglandin D2 synthase, elevated in both patients' SAGE libraries, has also been demonstrated to directly induce apoptosis in neuronal cells (35).

The elevation of GAP43 expression in the patients may be indicative of dysfunction or damage to neurons during the pathogenesis of disorder. GAP43 is a phosphoprotein that promotes neurite formation and is expressed during axonal growth and regeneration (23,36). A characteristic feature in the GM2 gangliosidoses is the presence of ‘meganeurites’ - areas of swollen neuronal processes with abnormally high densities of neurites (37). An elevation of GAP43 expression provides a molecular explanation for this enhanced neuritogenesis, a process that has been suggested to cause neuronal dysfunction in the GM2 gangliosidoses (37). Abnormal neurite sprouting attributed to GAP43 expression has also been found in Alzheimer disease neurons (38). In addition, increased expression of GAP43 has been observed in neurons in amyotrophic lateral sclerosis patients (39).

Inflammation is believed to be a central factor causing neuronal cell death in a number of neurodegenerative disorders, including Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis and HIV dementia (40,41). We previously provided evidence that inflammation contributes to the neurodegeneration in the GM2 gangliosidoses using a mouse model of Sandhoff disease (3). Through cDNA microarray analysis, we found that a large fraction of overexpressed genes in the CNS from the disease-model mice were related to an inflammatory process. Our results with SAGE show a remarkably similar profile of increased expression of inflammatory genes. Elevated genes in common between the mouse and human gene profiles included class II histocompatibility antigens, the pro-inflammatory cytokine osteopontin, complement components, proteinases and inhibitors, galectin-3, and prostaglandin D2 synthase. Both activated macrophages/ microglia and activated astrocytes are known to mediate inflammatory responses in the CNS (40,42). In the SAGE study, the gene profiles indicated that both cell types were activated in the patients.

In addition to mediating inflammation, astrocytes along with microglia respond to neuronal damage by a process of reactive gliosis where the cells proliferate and migrate to sites of
damage to preserve tissue integrity (14,32). The elevated expression of genes concerned with protein synthesis and the cytoskeleton may be associated with the increased proliferative and migratory activity of the glia. The heightened expression gene encoding osteonectin (SPARC), an extracellular matrix protein involved in tissue remodeling, is likely related to an injury response in the patients (20,43).

The results obtained by SAGE allow new detail to be added to our previous understanding of the pathophysiology of the GM2 gangliosidoses. A proposed model is shown in Fig. 3. Neuronal storage of ganglioside, due to the \( \beta \)-hexosaminidase deficiency, is the primary insult in these diseases, and leads to neuronal dysfunction and damage. The molecular events involved in these early processes are still poorly understood. The overexpression of GAP43 and the formation of ectopic dendrites may be one of these events (37), but has not yet been determined experimentally. Both microglia and astrocytes become activated by sensing neuronal damage (14,32) or through their own accumulation of glycolipid, which may activate cells directly (44). The activated microglia and astrocytes express inflammatory proteins, including cytokines, proteases and complement proteins, and monocytes are recruited from the blood (45). The inflammatory milieu in the CNS causes an additional insult to the already compromised neurons, leading to a phase of rapid neuronal apoptosis. Demyelination, prominent in the disorders, occurs as a result of the loss of axons (46). The severe neuronal cell death also triggers reactive gliosis and may induce the expression of genes related to tissue remodeling such as osteonectin/SPARC.

A number of genes elevated in GM2 gangliosidosis patients have been described as elevated in Alzheimer disease and other neurodegenerative disorders (19,47–51). The molecular similarities between the disorders of diverse etiology suggest common mechanisms for neurodegeneration, regardless of the nature of the primary insult. In many of these disorders, inflammation has been implicated as a central mechanism leading to neurodegeneration.

In addition to establishing molecular details of the pathophysiology of neurodegeneration in the GM2 gangliosidoses, the gene profiles can provide potential markers for monitoring the clinical progression of the disorders. The ability to assess the neurodegenerative course would be critical for the development of therapies for these disorders.

**MATERIALS AND METHODS**

**Tissue samples**

Samples of cerebral cortex from a 2-year-old male suffering from Sandhoff disease (#2081) (frozen < 24 hours postmortem) and from a normal 9-month-old male who died in an accident (#2844) (frozen 20 hours postmortem) were provided by the University of Miami Brain and Tissue Bank for Developmental Disorders. This represents a joint effort of the University of Miami and the University of Maryland Brain and Tissue Banks through NICHD Contract N01-HD-8-3284. The Tay-Sachs cerebral cortex sample was frozen 1.5 hours following the death of a 32-month-old male and was obtained from the Children’s National Medical Center, Washington, DC. A recent study has indicated that there was little degradation in human brain RNA up to 96 hours postmortem (52).

**RNA isolation**

Total cellular RNA from 5g of each cerebral cortex sample was isolated using Trizol reagent (Gibco BRL). Poly(A) RNA was then purified from total RNA using the Oligotex (Quiagen) spin column protocol.

**Construction and sequencing of SAGE libraries**

The SAGE method was performed essentially as previously described (5) and is accessible on the SAGE Home Page.
Data analysis and comparison of SAGE libraries

Tag sequences and their count in the libraries were organized and tallied via eSAGE software developed by Margulies and Innis (52). In order to eliminate the potential PCR bias in amplification of ditags during the SAGE protocol, we counted any ditag that was found more than one time in any given library only once for that library. Library comparisons were performed using eSAGE 2000 software. This software compared the relative abundance of each tag between a disease and the control library and produced a P-value for each comparison. Only tags that had P < 0.05 were considered statistically significant. Tags that matched to more than one gene were considered ambiguous and excluded from the analysis. Matching tags to specific genes in GenBank was achieved by comparing tags for each library with a SAGE tag to a Unigene map that had been downloaded from the NCBI SAGE website. The classification of function of individual genes was based in part on information obtained from the OMIM (www.ncbi.nlm.nih.gov/OMIM/) and LocusLink (www.ncbi.nlm.nih.gov/LocusLink/) databases.

Immunohistochemistry

Antibodies were from the following sources: ferritin, NSE, CD68 and GFAP from DAKO (Carpenteria, CA), phosphotyrosine from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), cathepsin B from Oncogene Research Products (Cambridge, MA), GAP43 from Sigma (St Louis, MO), Osteopontin from IBL (Fujoka-shi, Gunma, Japan) and α2-crystallin from Stressgen (Victoria, British Columbia). Antibodies to CD68 and GFAP were conjugated to peroxidase. For immunostaining, paraffin sections were deparaffinized and rehydrated; in some cases, antigen retrieval was accomplished by trypsin and microwave treatment. Sections were incubated with primary antibodies overnight at 4°C. When appropriate, the washed sections were incubated with labeled polymer peroxidase-conjugated mouse and rabbit secondary antibodies (DAKO Corp., Carpenteria, CA). The peroxidase reaction was visualized by diaminobenzidine and hydrogen peroxide.

SUPPLEMENTARY MATERIAL

For Supplementary Material, please refer to HMG Online.

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