CMLS, Cell. Mol. Life Sci. 59 (2002) 694–707
1420-682X/02/040694-14 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Human Genome and Diseases: Review

Gaucher disease: perspectives on a prototype lysosomal disease

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Received 25 October 2001; received after revision 3 December 2001; accepted 3 December 2001

Abstract. Gaucher disease is an autosomal recessive trait and the most common lysosomal storage disease. The pathogenesis evolves from the diminished activity of the lysosomal hydrolase, acid β-glucosidase and the resultant accumulation of glucosylceramide within lysosomes. The pathogenic mechanisms are poorly understood. During the past 2 decades, progress has been made in understanding the biochemical basis and molecular biology of the disease, but more fundamental knowledge is required to relate these advances to the cell and whole body phenotypes. Despite this lack of understanding, enzyme replacement therapy has proved a successful and effective management for Gaucher disease. However, basic details of this therapeutic efficacy require elucidation. Here, we review the current state of the molecular pathogenesis and provide our perspective of some major issues for continued advances in this prototype lysosomal storage disease.

Key words. Glucosidase; glucocerebrosidase; glucocerebroside; glycosphingolipid; macrophage; chitotriosidase; cytokines; enzyme therapy; gene therapy.

Introduction

In 1882, Dr Phillipe Gaucher, a French dermatologist, first described a neurologically normal 32-year-old female with massive hepatosplenomegaly [1]. He thought her disease was an epitheloma of the spleen because of the presence of peculiar cells in that organ (fig. 1A). In 1901, Brill recognized the systematic and familial nature of the disease and coined the term 'Gaucher's disease'. In 1907 the biochemical nature of Gaucher disease was recognized and, later, Aghion [2] characterized the storage material as glucosylceramide (GC). Since that time, much of the phenotypic diversity of Gaucher disease has been delineated, particularly the recognition of neuronopathic variants and variation within the defined types [3–6].

The disease encompasses a heterogeneous group of disorders with highly variable phenotypes caused by the defective lysosomal hydrolysis of GCs and related glucosphingolipids. Brady and co-workers, and Patrick identified a glucocerebrosidase, a GC glucohydrolase, as the enzymatic defect in Gaucher disease [7,8]. Later, this enzyme was shown to be a β-glucosidase [9]. The complementary DNA (cDNA) and gene were characterized, and about 200 mutations at this locus on human chromosome 1q 21–23 have been found in Gaucher disease patients [3]. Although an uncommon metabolic disorder, Gaucher disease is the most common lysosomal storage disease, with an estimated birth frequency of 1/50,000 in the Caucasian population [10]. The disease is panethnic and has its highest prevalence in the Ashkenazi Jewish population. The past 2 decades have witnessed much progress in understanding of biochemical and molecular basis of the disease, and their association with clinical presentation. Major advances include the availability of safe and effective enzyme therapy as a prototype for other such intracellular protein deficiency diseases. This re-

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view will provide an overview of some of the outstanding issues in understanding Gaucher disease as a prototype for other monogenic diseases due to enzyme deficiencies. Detailed reviews of the clinical and biochemical aspects of this disease are available [3,11–16].

Clinical and pathologic features

Clinical presentation

Development of perspectives on the cellular, biochemical and molecular aspects of Gaucher disease requires some insight into the clinical and pathologic phenotypes. Gaucher disease is classically divided into three variants based on the absence or presence and progressivity of neuronopathic disease [3, 6]. Table 1 presents a current summary of Gaucher disease classification. However, extensive variability exists within each phenotype, and the spectrum of involvement is great within each type. All variants have differing degrees of enlargement of the liver and spleen, anemia, thrombocytopenia and skeletal disease. These can range from very severe to mild within each type, although the rate of progression generally is greater in younger patients. Also, the degrees of visceral organ involvement are not concordant in patients. For example, massive involvement of the liver and spleen is not necessarily accompanied by severe bony disease. The reverse also is true. In addition, this classification is not age dependent, but depends on the primary involvement of the CNS by Gaucher disease at any age.

Gaucher disease type 1 patients are free of primary CNS involvement. The variability of the phenotype of visceral manifestations ranges from severe fatal disease in the first 2 decades to essentially asymptomatic nonagenarians. Gaucher disease types 2 and 3 have primary CNS neuronopathic involvement. Types 2 and 3 represent a continuum of disease phenotypes that differ, primarily in their rates of CNS and visceral disease progression. This continuum encompasses phenotypes leading to death in utero, or in the first few days of life, to rapidly progressive CNS and visceral diseases that are fatal in the first years, to more slowly progressive (yet severe) CNS (with mild to severe visceral disease) deterioration over a period of 2–3 years to decades [17–21]. For the neuronopathic variants, the brainstem and cranial nerve nuclei are variably involved, but eye movement abnormalities generally are the first findings. Brain stem (bulbar) findings predominate in the type 2 variants, but oculomotor apraxia may be the only finding in the type 3 variants for many years. Clinically, the distinctions between types 2 (acute neuronopathic) and 3 (subacute neuronopathic) are useful and have therapeutic import. Biologically, the distinctions may relate more to rate of substrate accumulation than qualitative mechanistic differences.

Table 1. Gaucher disease: General Grouping of Clinical Variants.

Clinical features	Type 1	Type 2	Type 3
Onset	early childhood/ adulthood	infancy	early childhood/ adolescence
Hepatosplenomegaly	+→+++	+++	+→+++
Hypersplenism	+→+++	+	+→+++
Bone Crises/ Fractures	+→+++	–	++
Neurodegenerative Course	–	+++	+→+++
Survival	< 6–80 + years	< 2 year	1st – 4th decade
Ethnic Predilection	panethnic Ashkenazi Jewish	panethnic	panethnic Northern Sweden

The gaucher cell

The presence of Gaucher cells in various tissues is the hallmark of this disease (fig. 1A). The Gaucher cell results from the accumulation of excessive glucocerebrosides in lysosomal compartments of monocyte/macrophage derived cells. Histologically, Gaucher cells are enlarged macrophages (up to 100 μm) with cytoplasmic linear inclusions. Ultrastructurally, these inclusions are tubule-like structures that contain GC (fig. 1B), i.e., the stored GC and phospholipids (~90%) and about 0.3% protein [22]. Ultrastructural studies of monocytes from Gaucher patients also show smaller membrane-bound tubule-like structures similar to those in Gaucher cells. These results indicate that monocytic cells are precursors of tissue-bound Gaucher cells [23]. The relationship of the accumulation of these cells to the overt clinical findings is not clear. For example, in some Gaucher disease patients, the liver and spleen may exceed 25% of body weight (normal < 3% of BW), but GC does not account for this excess mass. Normal tissue components with fibrosis and parenchymal expansion also are found, and may be a reaction to the Gaucher cells or the by-products of tissue injury. Thus, growth of normal tissue and reaction to the Gaucher cells requires explanation. The evolution of differential gene expression and pathologic response in these involved tissue and from Gaucher cells remains a fertile area for research.

The relationships of Gaucher cells to cortical bone loss (osteopenia and osteoporosis) and bone marrow disease are even more obscure. Necrosis, fibrosis and Gaucher cell infiltration account for some of this pathology, but hyperemia and ischemia also may be critical. A recent study showed increased cathepsin K expression in Gaucher cells [24]. This protein has high-level expression in osteoclasts, a cell to which its expression is restricted. Cathepsin K has roles in bone resorption, modeling and turnover [25,26]. This protease and other cytokines overexpressed by Gaucher

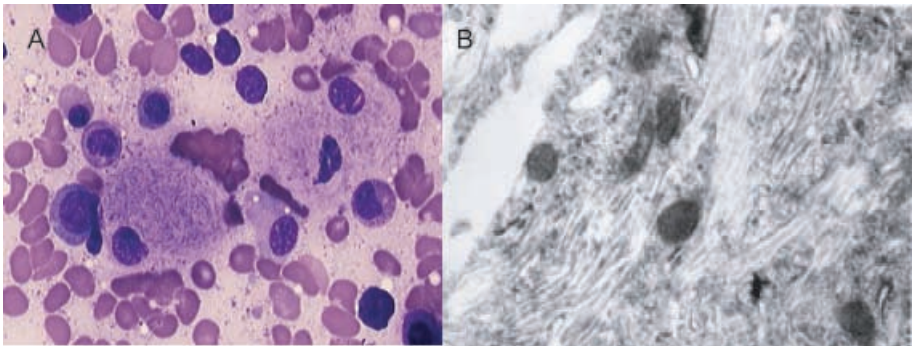

Figure 1. Light (*A*) and electron (*B*) micrographs (EMS) of Gaucher cells from bone marrow. Numerous cytoplasmic striations are present in (*A*), which under EM appear more tubular. These tubules are twisted bilayers of GC, phospholipid and small amounts of protein that are stacked. Most of these structures are unit membrane bound. A typical Gaucher cell is packed with these structures, whereas in macrophage/Gaucher cell precursors these are sparse.

cells (macrophage) may prove important to the pathogenesis of architectural bone disease in Gaucher patients.

Unlike visceral tissues, the CNS disease in the type 2 and 3 variants does not derive from accumulations of Gaucher cells nor large amounts of GC storage. Histologically, the major consistent finding has been the progressive loss of neuronal cells [27–31]. Gaucher-like glial derived cells are present within brain substance, but their numbers are not great and this finding is inconsistent. In comparison with GC, the accumulation of the GC-deacylated analogue, glucosylsphingosine, is greatly increased and has been proposed to be a toxic agent leading to neuronal toxicity [32–34]. These findings indicate that the fundamental pathophysiology in the CNS and visceral tissues differs significantly.

Cytokines

The accumulated GC and glucosylsphingosine have been suggested to activate macrophages and induce inflammatory responses by releasing cytokines. Immunohistochemical studies of bone marrow biopsies and spleens of patients with Gaucher disease and chronic myeloid leukemia showed similar origins of Gaucher cells and pseudo-Gaucher cells, respectively. In particular, human leukocyte antigen (HLA)-DR antigens are expressed at higher levels in Gaucher cells compared with pseudo-Gaucher cells [35]. This level of expression suggested that cytokines might be increased in the Gaucher cell or in surrounding cells that interact with these macrophages [36]. Indeed, interleukin (IL)-6 and IL-10 are elevated in sera from patients with Gaucher disease [37]. Increased expression of IL-1β messenger RNA (mRNA) and a trend toward elevated tumor necrosis factor α (TNF-α) mRNA also was found in Gaucher disease patients [38]. A variety of other cytokines were variably elevated in Gaucher patient sera, including IL-1β, IL-1Ra, IL-6, soluble IL-2 receptor (sIL-2R) and transforming growth factor β (TGF-β) [39].

In summary, elevated cytokines have been variably increased in Gaucher cells, and plasma/serum from Gaucher patients. Furthermore, there is a trend to increasing levels of these cytokines in serum associated with disease severity. How these cytokines are triggered to be produced, what regulates their production and how they impact the pathogenesis of Gaucher disease remain unclear. In addition, the local concentrations and effects of elevated cytokines may have greater impact on the disease manifestations than is reflected by serum levels.

Molecular genetics

Gaucher disease is transmitted as an autosomal recessive trait. The 7.5-kb (11 exons) gene is located on chromosome 1q21 (see fig. 2) and encodes acid β-glucosidase (GCase, glucocerebrosidase, *N*-acyl-sphingosyl-β-D-glucose: glucohydrolase, EC 3.21.45). Importantly, many mutations causal to Gaucher disease appear to arise from gene conversion events with the pseudogene: this remains to be formally proven [40]. The GCase pseudogene is highly homologous to normal gene (96% identity), ~5 kb in length, and is 16 kb downstream from the functional gene [41]. The GCase pseudogene is transcribed, but no functional protein can result because of numerous mutations that generate stop codons [40–42]. The major differences between the functional and pseudogenes are the presence of several small deletions in introns 2, 4, 6 and 7, numerous exonic missense mutations, and a 55-bp deletion in exon 9 in the pseudogene. Several other genes surround the GCase gene [43–48] (fig. 2). The genomic structure in this region is dense and contains pseudogenes, indicating an evolutionarily recent duplication (fig. 2). Any functional relationships between these genes and the pathophysiology of Gaucher disease are, at present, speculative.

Two major and two minor haplotypes have been delineated at the GCase locus (GBA) [49]. The N370S and c84

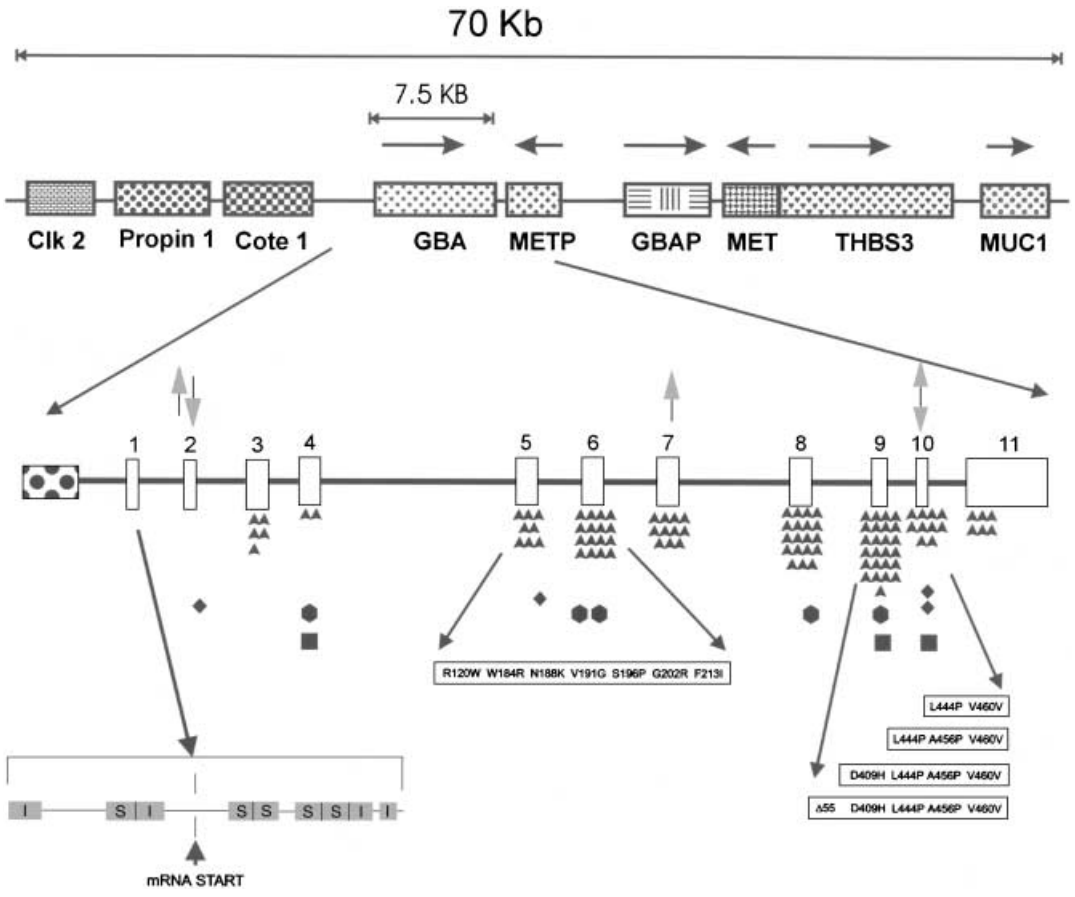

Figure 2. Schematic of the GBA locus and its surrounding genes on chromosome 1. Clk2, a gene resembling a serine/threonine kinase; propin 1, a gene of unknown function; cote 1, a gene of unknown function; METP, pseudogene of metaxin; MET, metaxin, a mitochondrial membrane protein; GBAP, pseudogene of GBA. THBS3, thrombospondin; MUCI, polymorphic epithelial mucin gene. The location of many GBA mutations is indicated throughout its 11 exons as follows: exonic missense mutations (▲), splice junction mutation (◆), large deletions (■), point deletions (↑), point insertions (↓), complex mutations (rectangles with text indicating multiple point mutations) and termination mutations (●). The rectangular block containing several mutations represents known 'complex alleles' that may have arisen from recombination with GBAP. Not shown is a complete gene deletion and a recombinant allele that includes pseudogene sequences spanning exons 2–11. In the lower left is an expansion of exon 1 showing putative stimulatory (S) and inhibitory (I) sequences for transcription of GCase.

ins G alleles are in linkage disequilibrium with the haplotypes. Using these data, the dates of separation and potential reoccurrence of the common Jewish mutation were assigned to 48 generations (N370S) and 55.5 generations (c84 ins G) [50]. Extragenic sequences of the glucocerebrosidase gene region also are polymorphic [51, 52]. In addition, linkage disequilibrium of these haplotypes and an extragenic polymorphism was used to assess the dates of separation, and potential reoccurrence of the common Jewish mutations has assigned to be 40–1000 generations (1000–25,000 years) for N370S mutation and 50–4800 generations (1300–120,000 years) for the c84 ins G mutation [53]. This linkage indicates the recent origin of the N370S mutation. Their functional significance is unknown.

There are two upstream potential ATG start codons in the GCase full-length open (2.2–2.5-kb cDNA) reading frame. Both in-frame ATGs can function to produce active enzyme in cultured fibroblasts [54–56]. The preferential use of either ATG in various tissues is not known, nor is the potential physiologic relevance of their use. Upstream TATA and CAAT boxes have been identified by S1 analysis, but their exact function has not been defined [41, 57]. Also, positive and negative regulatory sequences were detected in the first exon and intron in tissue culture systems [58, 59] (fig. 2). Their in vivo physiologic importance is not known. By in situ hybridization with antisense GCase mRNA, differential expression was shown particularly within the brain of mice [60]. During nearly all of embryonic and fetal development GCase mRNA is at a low level in most visceral tissues. In skin epidermis, the expression becomes high shortly before birth and is maintained throughout adult life. In comparison, a generalized low-level expression of GCase mRNA in the brain of early embryos transforms shortly before birth into specific high-level expression in neurons of the

cerebral cortex, hippocampus, basal ganglia, dentate nucleus and in the Purkinje cell layer of the cerebellar cortex. This pattern of expression is maintained throughout adult life. Studies are needed to confirm the relevance to humans, but these results indicate the need to examine the role of gene expression control in the expression of the phenotypes.

Mutations

Nearly 200 different mutations at the GBA locus have been identified in patients with Gaucher disease. These mutations include missense, termination, deletion and insertions [61–66]. Most of these are rare and/or private mutations but several have significant frequencies. The occurrence of pseudogene-like mutations in the functional gene from affected patients is important for diagnostic and potentially mechanistic studies. Several point mutations including L444P – the most common mutation worldwide – arise from apparent recurrent gene conversion or other rearrangement between the functional and pseudogenes. Similarly, Gaucher disease alleles containing 2, 3 or more point mutations and/or deletions and insertions, identical to those in the pseudogene, have been found around the world in apparently distantly related populations [65, 67, 68]. These observations provide credence to the 'gene conversion' origin of many of the common alleles. In comparison, the most frequent Gaucher disease allele in the Ashkenazi Jewish population, N370S, does not appear in the pseudogene, and is likely due to a founder effect [50]. The spontaneous mutational event that established this allele appears to have occurred or entered the European population nearly simultaneously with the migration of Middle Eastern Jews to that continent. The perpetuation of the N370S alleles in the Ashkenazi population likely resulted from sociopolitical forces, but heterozygote selective advantage cannot be excluded. Table 2 summarizes the frequencies of the more common alleles in Jewish and non-Jewish populations. The biochemical effects of several point mutations are discussed later.

Table 2. Allele distribution in Gaucher disease Type 1 Patients.

Allele	Jewish (%) (n = 1160)*	Non-Jewish (%) (n = 419)
N370S	71.82	43.6
c84 ins G	11.20	0.2
L444P	2.84	25.6
IVS 2^{+1} g→a	1.72	0.7
Rec*	1.42	3.5
Alleles detected	89	74
Other Alleles	11	26

* n = total alleles. Rec, recombinant alleles (RecNci and RecTL) that include the L444P missense mutation and at least one additional pseudogene mutation.

Genotype and phenotype correlations and threshold effects

Beyond the obvious clinical and personal impact, elucidation of genotype and phenotype relationships has significant biological implications. In particular, the delineation of specific organ involvement or variation in overall phenotype could reflect specific functions or levels of GCase needed for normal metabolism and/or the participation of other loci in the expressivity of the trait(s). To date, the genotype and phenotype relationships in Gaucher disease have been restricted by population numbers to the major mutation alleles, N370S, L444P and D409H. Review of all reported cases (~400) and the personal experience of one of us (G.A.G.) shows that the presence of the N370S allele, in the homoallelic state or as the heteroallele with another mutant GCase allele, absolutely correlates with type 1, nonneuronopathic Gaucher disease. The recent soft correlations with 'Parkinsonian-like' neurologic disease in such patients [69–71] requires careful attention since a subpopulation of nonneuronopathic Gaucher disease patients may be predisposed to such neuronopathic manifestations. By comparison, L444P/L444P and D409H/D409H are highly associated with development of neuronopathic disease at some time in life. In addition, a striking difference in tissue involvement is present in L444P/L444P vs. D409H/D409H patients [4, 72, 73]. The neuronopathic patients with L444P/L444P have varying degrees of visceral (hepatic, splenic, bony and lung) disease, but do not have the cardiac valvular calcifications, cataracts and hydrocephalus, and milder other visceral involvement characteristic of D409H/D409H. The clinical neuronopathic involvement in L444P/L444P and D409H/D409H patients is quite similar. Also, both mutations produce catalytically defective, unstable proteins with similar properties [74]. Thus, the bases for the discrepancies of visceral phenotypes of patients with these two genotypes are not explained by the known biochemical properties of the mutant enzymes and remain an intriguing area for investigation. Also, well-characterized L444P/L444P patients are known to us, and reported by others who have apparent nonneuronopathic disease into the 3rd decade [18, 75]. Even if such patients do develop neuronopathic deterioration later in life, other major influences must impact the rate of progression.

The Swedish population of Gaucher disease patients is instructive in this regard. This variant of Gaucher disease derived from a single founder couple in the 17th century in the Norbottnian region of Northern Sweden [18, 75, 76]. A single GCase genotype, L444P/L444P, is present. However, the variation in phenotype ranges from severe infantile neuronopathic and visceral disease to milder variants with neuronopathic disease onset in the 4th to 6th decades. Since the environment is relatively similar, this major variation in phenotype must have a significant genetic basis, i.e. modifier genes.

The variation among Gaucher disease patients with N370S alleles also is great. Shown in figure 3 is a distribution of ages of onset and diagnosis of symptomatic Ashkenazi Jewish patients with N370S/N370S or N370S/'other mutant allele' genotypes [77]. Allowing for the vagaries of 'age at onset' and some genotyping errors, the results correspond closely to those from a larger, more diverse, population [78]. Importantly, these data represent patients from several European and American geographical regions. Similar relationships are present when hepatic or splenic volumes are used as parameters, with smaller organs being present in N370S/N370S patients [14, 77, 79]. Clearly, the N370S/N370S is associated with a less severe phenotype [3, 78]. Indeed, based on heterozygote frequencies, a paucity (~50%) of N370S/N370S genotypes are represented among the 'significantly' symptomatic patients with N370S alleles [3, 11]. The N370S/'other GCase mutant allele' phenotypes are more severe, and have ages of onset from 1 to 2 decades earlier than those with N370S/N370S [77, 78]. Since these data have been collected from widely different geographic and cultural milieus, the environmental impact on phenotype will be minimized, albeit not eliminated. Thus, the variation within each genotypic group probably represents genetic influences, i.e. modifier genes. However, the great variation between the N370S/N370S genotype and the other genotypes represents a major impact of the primary, GCase, genetic mutation.

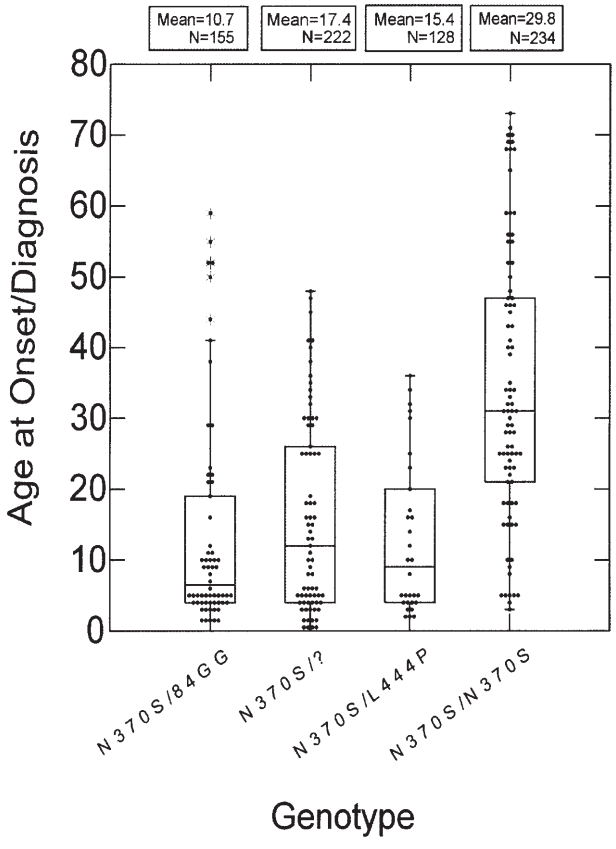

Figure 3. Age at onset and diagnosis for the patients with differing genotypes. The data were obtained from medical records and/or histories of symptomatic Ashkenazi Jewish patients. The boxes represent the median and first quartile boundaries. Each dot represents a single patient.

The N370S allele expresses a defective protein with substantial (~10–20% of normal) catalytic activity. In comparison, the c 84 ins G and IVS+2 alleles are null, and the L444P allele produces a highly defective protein with very little (but not zero) catalytic activity. Thus, most of the N370S/ 'other mutant alleles' or N370S/L444P patients have functionally half or less, respectively, the residual enzyme activity in vitro compared with N370S/N370S patients. Notably, the L444P/L444P patients have an average age at onset/diagnosis of 2.3 years [14].

The variable expressivity of the phenotypes would appear to be determined as follows: (i) For whole body phenotype, the residual activity of the mutant GCase sets the boundaries of potential variation in response to other influences. For example, an N370S/N370S patient could have a very wide range of potential response to other genetic polymorphisms or environmental stimuli and factors because of the amount of available residual enzyme. These stimuli and factors could influence substrate influx and production and/or mutant enzyme stability or activity. Thus, great variation in expression is expected. In comparison, the much lower residual activity in L444P/L444P patients would lead to much lower toleration of endogenous (genetic) or exogenous variations, with most being significantly deleterious. The least phenotypic variation, would be anticipated in phenotypes resulting from a null mutation that would allow for little, if any, response to varying input. (ii) For tissue-specific phenotypes, the effects of the mutant enzymes may vary with the tissue. For example, the compositions of glycosphingolipids present in cardiac valves, skin or brain differ and may interact with the mutant enzyme differently. GC in brain has shorter fatty acid acyl chains (~C_{16-18}) compared with that from visceral sources (~C_{18-21}) [80–82]. In comparison, skin has very long fatty acid acyl chains (~C_{27-30}) [83–87]. If in vitro differences in substrate specificity are reflected in vivo, some tissues could be 'protected' or not from disease manifestations even if the level of in vitro residual activities were identical with a particular substrate for the two mutant enzymes.

Recently, we have created mice with GCase point mutations, including N370S. Surprisingly, the N370S/N370S genotype in mice is lethal, with death due to disruption of the skin permeability barrier and the development of the severe ichthyosis [88]. This is identical to the GCase null mouse created nearly a decade ago [89]. In vitro initial characterization indicates that the mouse

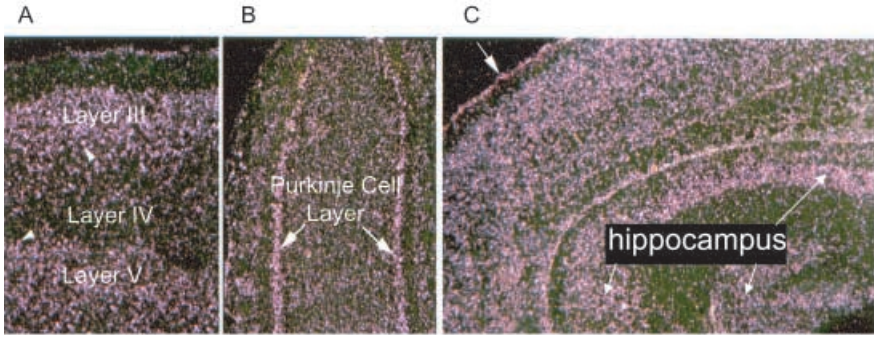

Figure 4. Expression of GCase mRNA in adult mouse CNS. Fluorescence in situ hybridization was performed with antisense GCase mouse RNA as probe. GCase mRNA signals are white to pink in the dark field. (*A*) Cerebral cortex neurons of layers III and V (arrowheads) have higher signal than those in layer IV (darker region in between III and V. (*B*) Cerebellar cortex with highly stained Purkinje cell layer. Both Purkinje (arrowheads) and granular cells have more intense signal than the surrounding tissues. (*C*) Intense staining of the hippocampal pyramidal cells (h). The meninges also have high signals (arrowheads).

N370S protein has very similar properties to the human N370S. However, more 'natural' assays with GCs containing very long chain fatty acid will need to be tested to evaluate the differences between the human and mouse wild-type and N370S enzymes to explain this phenotype.

Biochemistry and enzymology

Control of GCase expression

Although GCase is considered a housekeeping gene, its expression is controlled at the transcriptional, translational and posttranslational levels. The in vivo promoters that convey specificity to GCase expression have not been defined. However, developmental and tissue-specific GCase mRNA expression has been documented, particularly within regions of the brain [60]. In mice through about two-thirds of pregnancy, GCase mRNA expression is relatively low and ubiquitous [60]. Near term higher levels of expression are noted in neurons of the cerebrum, cerebellum, brain stem and spinal cord. This expression reaches a maximum following birth and into adulthood. Striking expression is observed in Purkinje cells and dentate nuclei of the cerebellum (fig. 4) and in the hippocampus. The differential signals result from increased concentrations of cells with relatively high expression surrounded by areas of much lower cell densities, rather than only high levels of mRNA per cell. General visceral expression is much lower and ubiquitous, except for higher levels in the epidermis of the skin. Thus, significant control of the timing of GCase mRNA expression is evident, and expression in neurons of the CNS and epidermis is higher than that in other cellular types. The temporal control promoters remain to be defined in vivo.

During overexpression studies of GCase mRNA in several mammalian cell types, a discrepancy (> 100-fold) was detected between mRNA expression and GCase protein production. This was due to the constitutive presence of a translational control protein (TCP), M_r ~80,000–90,000 that bound to double stranded RNAs [90–92]. The protein is identical to a variant of NF90, a protein originally implicated in the control of adenovirus gene expression following infection of cells. This protein binds specifically to a 180-nucleotide region of GCase mRNA and prevents its interaction with polysomes, and therefore translation cannot be initiated. In addition, TCP80/NF90 can be phosphorylated by the classical protein kinase C (PKC) pathway, and deficient phosphorylation of TCP80/NF90 facilitates GCase mRNA translation inhibition. The exact role of this protein in the control of GCase steady-state levels is unknown, but such inhibition of translation clearly impacts the overexpression of this protein for therapeutic purposes. Elimination of the translation inhibition by TCP80/NF90 may allow for substantial increases in protein expression and enhanced secretion.

Posttranslational modifications do not normally play a great role in the control of GCase expression and maintenance of steady-state levels. However, occupancy of the first glycosylation site is essential to GCase activity, but not proteolytic stability [93]. In comparison, occupancy of the other four glycosylation sites are not critical to activity [93]. This is likely due to the maintenance of hydrophilicity in this region, and vectorial cotranslational glycosylation may be important to disulfide bond formation between residues C4, C16, C18 and C23. Thus, mutations in this region may have global effects on protein folding if either glycosylation or disulfide structure is affected.

Biochemistry and cell biology

GCase is a membrane-associated lysosomal β-glucosidase composed of 497 or 496 amino acids in the human or mouse, respectively [56, 94, 95]. The enzyme is a

glycoprotein that contains four of five occupied N-glycosylation sequences [96]. The occupied sites are in the N-terminal 60% of the sequence, while the unoccupied site is in the COOH-terminal 10% of the protein. The seven cysteines are at residues C4, C16, C18, C23, C128, C248 and C342. The first four cysteines participate in disulfide formation, while that at residue C128 is free and those at C248 and C342 may be free, although this is not resolved [E. Ponce and G. A. Grabowski, unpublished observation]. Occupancy of the first N-glycosylation site at N19, A20, T21 is essential for the development of a catalytically active conformer, probably by directing disulfide formation. Mutagenesis studies showed that substitution of C4, C16, C18 or C23 with serine leads to catalytically inactive proteins as a result of disulfide disruption. Similarly mutated enzymes at residues C128 or C248 retain complete and partial activity, respectively. Substitution at C342 with glycine leads to a catalytically defective protein due to its proximity to the catalytic nucleophile at residue 340 [97].

GCase is synthesized, its leader peptide cleaved upon trans-ER (endoplasmic reticulum) membrane passage, and is cotranslationally glycosylated in the ER with subsequent sequential oligosaccharide modifications with movement through the cis, mid and trans Golgi [98–100]. The enzyme is not phosphorylated, nor does it contain mannose-6-phosphate residues for lysosomal sorting [101, 102]. Little GCase is normally secreted from cells, but significant amounts are secreted in overexpressed states [90, 103, 104]. This finding implies a saturable sorting system for delivery of GCase to the lysosomal compartments [101]. Normal human GCase does not contain any of the recognized COOH-terminal hydrophobic signals used for targeting of lysosomal membrane proteins [105]. However, newly synthesized GCase sorts to the lysosome in an unglycosylated state following treatment of cells with tunicamycin [101]. This treatment also does not appear to alter the degree of membrane binding of GCase as assessed by saponin solubilization. Membrane attachment is important for the survival of GCase in cells. In overexpressed systems, in which significant amounts of enzyme remain free in the lysosomal lumen, the half-life of the free enzyme (non-secreted) protein is much reduced compared with that of the membrane bound form [101]. Clearly, the association of the enzyme and membrane is essential for maintaining sufficient steady-state amounts of enzyme activity for normalization of GC flux through the lysosomal compartments. The implications of these observations for enzyme and gene therapies are discussed below.

Once delivered to the lysosome, GCase becomes bound to the inner membrane surface of this organelle. The enzyme is not an integral membrane protein with transmembrane domains, but is tightly membrane associated. No evidence is available to show that GCase will associate with the plasma membrane of cells, other than through specific oligosaccharide receptors, i.e. the mannose receptor used for enzyme therapy. Binding to the inner lysosomal membrane is thought to be mediated by resident phospholipids, although binding to a protein receptor has not been formally excluded. Using purified GCase, artificial liposomal membranes, pure or mixed phospholipids are essential activators of the enzyme's hydrolytic activity, i.e., in the absence of such lipids GCase is inactive. Negatively charged head groups on these lipids, e.g., phosphatidylserine, phosphatidic acid, phosphatidylinositols, lysobisphosphatidic acid [106, 107], are required for the activation effects. GCase also requires such negatively charged phospholipids (NCPs) to have an unsaturated fatty acid acyl chain covalently attached or in proximity, i.e. free oleic acid or present on another contiguous non-NCP phospholipid, for activation effects in liposomal systems [106, 107]. Phosphatidylcholine has no activation properties [108, 109]. Spectral studies using intrinsic GCase tryptophan fluorescence show a λ blue shift and an alteration in secondary structure of GCase by circular dichroism upon binding of the enzyme to such NCP membranes [107]. This binding is fast, submicrosecond time frame, and dissociation from the membrane cannot be detected within 12 h [X. Qi and G. A. Grabowski, unpublished observation]. Binding is tight, has specific structural requirements (albeit stereospecificity has not been shown) and is associated with reconformation of GCase into an active form. These and the above metabolic labeling analyses show that membrane attachment is key to enzyme stability (proteolytic) and full catalytic activity within cells. The actual NCP(s) that plays these roles in vivo is (are) unknown. Although a potent activator, phosphatidylserine is not present in significant amounts in the inner lysosomal membrane. Indeed, the asymmetric distribution of this lipid to the inner leaflet of the plasma membrane is maintained topologically through the endocytic pathway, and a 'flip' would be required for phosphatidylserine to be a major physiologic activator. Lysobisphosphatidic acid and phosphatidylinositol phosphates are prime candidates since they are resident in the interior of lysosomal compartments [110, 111]. Lysobisphosphatidic acid is resistant to phospholipases [110, 112] and could provide a relatively 'stable' group of binding sites for GCase.

Additionally, GCase membrane attachment in the lysosome is important in understanding how the enzyme gains access to insoluble lipid substrates (GC) as they are presented to the late endosomal/lysosomal compartments. Such understanding might provide insight into the aberrant hydrolysis of GC and other substrates by mutant enzymes in Gaucher disease and the development of newer therapeutic approaches. In the phagocytic pathway, glycosphingolipid substrates cross the plasma membrane,

enter the endosomal system, pass through the multivesicular body (MVB) and enter the lysosomal compartment. Topologically, access of GCase to GC would require lysosomal luminal orientation of GC, potentially by first being oriented on the outer surface of the small vesicles of the MVB. Alternatively, GC could be incorporated into the lysosomal membrane through a fusion/hybrid compartment of late endosomes and lysosomes. This possibility seems less likely since it requires preferential or exclusive sorting of GC to the lysosomal membrane and away from the endosomes. Such a degree of specificity might occur through lipid rafts [113–116], but the mechanism is obscure. The delivery of GC-containing MVB vesicles to the lysosomes as a surface for GCase action does not require such a high degree of specific sorting, since only the formation of a hybrid compartment is needed. The MVB small vesicles, containing many lipids, could be incorporated into the lysosomal compartment. Since GC is not present in aqueous solution and must be presented in a membrane form, either of these mechanisms requires that membrane bound GCase 'confront' GC in a membrane. Available data indicate that GCase requires membrane attachment, interaction with NCP and an activator protein (saposin C) for hydrolytic activity [106, 107, 117]. GCase membrane attachment appears tight and a 'scooting mode' of hydrolysis is likely, i.e. GCase functions only by hydrolysis of GC in the interfacial mode with both enzyme and substrate on the membrane surface at least in vitro. This implies that GCase and GC move to confront each other in the lysosomal membrane or that GCase or GC must move from the MVB small vesicles to another membrane to confront each other. Based on current knowledge of GCase, this enzyme, once bound, does not dissociate from the membrane. Consequently, only newly synthesized GCase that might be freed from the lysosomal membrane, albeit this is not known, could interact with MVB, or collision and/or restructuring of the small vesicles would be needed for GCase to access the substrate. Removal of the substrate from the membrane for presentation to GCase is unlikely, since, contrary to saposin B's mechanism with more water-soluble glycosphingolipids, saposin C requires GCase at the NCP interface for activation effects [118]. This interaction is independent of the presence of GC or other substrates [106]. This is summarized in figure 5.

Such mechanistic insights are needed to understand the potential for mutant enzyme interactions and potential in situ modifications that could be therapeutic. For example, the N370S enzyme in vitro has greater activation effect by NCPs than the wild-type counterpart [108, 119, 120]. Since this mutant enzyme protein is present in normal

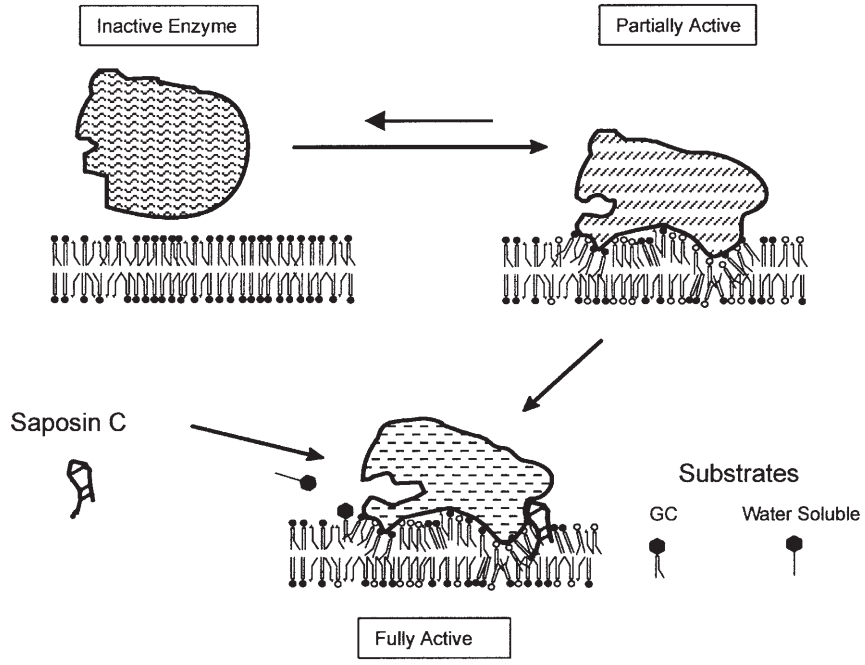

Figure 5. Schematic of GCase and its activation upon association with lysosomal membrane. In the soluble form, GCase is inactive. Upon attachment to negatively charged phospholipids (NCP, filled head groups), GCase undergoes a conformational change with realignment of residues in the active site. The enzyme then has catalytic activity. This attachment step is slowly reversible as indicated by the arrows. Saposin C is soluble and attaches to the GCase/NCP complex and induces a further conformational change. This leads to a fully active GCase that is capable of cleaving membrane-bound and water-soluble substrates. In the absence of saposin C, in vivo, GCase has low-level activity. In vitro and, potentially, in vivo, interaction of saposin C may require GCase bound to NCP: i.e. NCP, may make GCase conformation acceptable to saposin C.

amounts in cells from humans with Gaucher disease, the potential exists to enhance the activity above a threshold for corrections of GC flux through cells. In addition, this enzyme attaches to NCP membranes and occupies binding sites in NCP liposomes. This suggests a potential for competition between different enzyme forms for binding sites within the lysosome and, depending upon the mechanism of enzyme interaction with lysosomal substrates, could interfere with therapeutic supplementation by enzyme.

Pathogenesis

Two competing hypotheses for the pathogenesis of Gaucher disease include (i) Gaucher cells as relatively inert long-lived space-occupying lesions or the constipated lysosome hypothesis, and (ii) the transduction box hypothesis in which there is a deficiency of an essential signal that normally would leave the lysosome following cleavage of GC and have subsequent effects on cellular functions. These two hypotheses are not mutually exclusive, and the components may exist for both. The first has been assumed as a principle for more than 2 decades and envisions a passive role for the lysosomal storage material. Except for the lysosphingolipid hypothesis of Hannun and Bell [121], the second hypothesis has received little attention. This second hypothesis is different than the lysosphingolipid hypothesis since it assumes an active normal role for sphingolipid products that leave the lysosome, rather than a toxic role for deacylated analogues of glycosphingolipids. Lacking an animal model, for direct studies, pathogenic analyses of Gaucher disease have focused primarily on static pathologic samples that may reflect end-stage organ or cellular disease, rather than an early pathophysiologic processes. The constipated lysosome hypothesis is based primarily on the apparent accumulation of engorged macrophages in various tissues that could result from either filling up of the macrophages with GC, or accumulation of GC with resultant proliferation of macrophages. This is a relatively late event in tissues. Evidence for either is lacking, and mitotic figures of macrophage precursors as they enter various organs have not been obvious. Thus, lacking, direct analyses by markers of cellular proliferation, the space-occupying lesion hypothesis cannot be formally excluded. Similarly, the active hypothesis requires additional in vivo data for support. However, for these authors, this is a more attractive working hypothesis. Developing evidence supports elaboration of substances by activated macrophages due to GCase defects including cytokines, proteases and other markers such as angiotensin-converting enzyme and chitotriosidase. Several of these are secreted or lost from macrophages when activated by exogenous agents during immunologic responses. Analysis of bony lesions in Gaucher disease with particular attention to the cortical bone indicates an active metabolic process whereby bone structure is lost [122]. Thus, a reasonable hypothesis would include activation of the macrophage system by some lipid component that would normally move from the lysosome to the cytoplasm with subsequent enhancement of a cascade of transcriptome and proteome effects. A volumetric effect of the engorged lysosomes might also lead to cellular activation, but there is no evidence for this. It should be noted that the lysosphingolipid hypothesis and/or the activation hypothesis might be relevant to the CNS disease in which neuronal cell death is the major pathologic consequence, and not macrophage proliferation and cellular accumulation.

The overall hypothesis includes the following: a tissue macrophage becomes bound, and GC presented to the lysosome through phagocytosis cannot be broken down and accumulates, but the major event is the lack of effector egress from the lysosome. This effector is unknown. However, ceramide seems an attractive candidate since it has been shown, at least when generated from sphingomyelin in the plasma membrane, to become phosphorylated and lead to a series of events including apoptosis. It could be envisioned that the loss of this effector would lead to increased production of cytokines by the loss of feedback signals to the nucleus of active agents, such as cytokines or other markers of macrophage activation, and local hyperplastic or hyperfunction effects on macrophage-function. This has been demonstrated in the lysosomal acid lipase deficient mouse in which macrophage-colony stimulating factor (mCSF) is upregulated due to a deficiency of a fatty acid being blocked from egress from the lysosome [123]. Thus, lipids could have repressor effects either directly as signals or mediated through other events, such as the plasma membrane composition, producing activation of the macrophage and proliferation of these cells. The continual presentation of GC to the macrophages and the inability to digest this lipid to its components can be viewed as a secondary event. The primary event would be the lack of effector egress and expansion of the macrophage space into which GC could accumulate. This dysregulation of such activators would lead to more generalized macrophage production and increased synthesis of macrophage activation markers as described above. Furthermore, the elaboration of such compounds would produce hyperplasia of the macrophage compartment, and a self-perpetuating vicious cycle would result due to the lack of a signal to turn off the continuous production of macrophages. Whether the presence of additional macrophages alone would have a continuing detrimental effect or whether these secondary cytokines or other pathologic agents would have an effect requires direct experimental support. The availability of viable mouse models with Gaucher disease provides the opportunity to address such hypotheses to then close the

loop between the molecular biology and the cell biology and pathogenesis of Gaucher disease [88]. Through such studies of transcriptome and proteome analysis, additional targets for improved or adjuvant therapies would become available potentially to improve current responses to therapy.

Enzyme and gene therapy

The outstanding accomplishment by Dr Roscoe Brady and co-workers [124–126] of effective and safe enzyme therapy for Gaucher disease has been reviewed extensively [12]. The readers are referred to these references for details. Succinctly put, after a decade of enzyme therapy, the population of Gaucher patients have had major improvements in health and reversal of many aspects of their pathology. The treatment of CNS and established bone disease remain major challenges for clinical researchers. The improvement of therapeutic efficiency and reduction of cost are major challenges for the next decade. Gaucher disease type 1 is also a prime candidate for hematopoietic stem cell gene therapy since the major primary pathology derives from the bone-marrow-originating monocyte and macrophage cells. Recent successes in gene therapy for immunodeficiency diseases [127] indicate that progress is being made in this therapeutic strategy.

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