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Lipid composition of membrane rafts, isolated with and without detergent, from the spleen of a mouse model of Gaucher disease

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ABSTRACT

Biological membranes are composed of functionally relevant liquid-ordered and liquid-disordered domains that coexist. Within the liquid-ordered domains are low-density microdomains known as rafts with a unique lipid composition that is crucial for their structure and function. Lipid raft composition is altered in sphingolipid storage disorders, and here we determined the lipid composition using a detergent and detergent-free method in spleen tissue, the primary site of pathology, in a mouse model of the sphingolipid storage disorder, Gaucher disease. The accumulating lipid, glucosylceramide, was 30- and 50-fold elevated in the rafts with the detergent and detergent-free method, respectively. Secondary accumulation of di- and trihexosylceramide resided primarily in the rafts with both methods. The phospholipids distributed differently with more than half residing in the rafts with the detergent-free method and less than 10% with the detergent method, with the exception of the fully saturated species that were primarily in the rafts. Individual isoforms of sphingomyelin correlated with detergent-free extraction and more than half resided in the raft fractions. However, this correlation was not seen with the detergent extraction method as sphingomyelin species were spread across both the raft and non-raft domains. Therefore caution must be exercised when interpreting phospholipid distribution in raft domains as it differs considerably depending on the method of isolation. Importantly, both methods revealed the same lipid alterations in the raft domains in the spleen of the Gaucher disease mouse model highlighting that either method is appropriate to determine membrane lipid changes in the diseased state.

1. Introduction

Gaucher disease (GD) results from more than 300 known mutations in the gene encoding the lysosomal hydrolase, acid β-glucosidase. This enzyme is a key catabolic hydrolase in sphingolipid metabolism, responsible for cleaving the sphingolipid, glucosylceramide (GC), into its subunits glucose and ceramide. The loss of acid β-glucosidase activity results in GC accumulation in affected cells primarily of the mononuclear phagocyte system (macrophages), especially those in the spleen, liver, lung and bone marrow. The resultant pathology is complex, multi-factorial and progressive, and is associated with hepatosplenomegaly, anaemia, bone disease and central nervous system impairment in the rarer variants [1].

It has been suggested that the primary defect in GC catabolism affects secondary biochemical pathways that are the actual cause of cell and tissue damage [2]. For example, bone disease in a mouse model of GD has been attributed to a defect in osteoblast activity caused by inhibition of protein kinase C by both GC and its lyso derivative [3]. This is perhaps not surprising given that sphingolipid metabolism is a complex network of interdependent events with equally complex regulation, the loss of a lysosomal enzyme activity within sphingolipid metabolism is likely to have significant consequences for the cell.

Previously we have shown that the block in GC catabolism in the lysosome does indeed result in secondary increases in other sphingolipids, and that these increases extend beyond the lysosome [4,5]. This suggests that intracellular trafficking of sphingolipids through the cell to the lysosome is restricted and retrograde transport of GC occurs concurrently with build up in the lysosome [4]. One hypothesis that may explain the sequelae of extra-lysosomal sphingolipid accumulation is the "jamming of the endolysosomal system" with a consequent lack of feedback regulation to

Abbreviations: BMP, bis(monoacylglycero)phosphate; Cer, ceramide; DHC, dihexosylceramide; GD, Gaucher disease; GC, glucosylceramide; LC/ESI-MS/MS, liquid chromatography/electrospray-ionization tandem mass spectrometry; MBS, MES buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; THC, trihexosylceramide.

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the Golgi network for synthesis. This is believed to be mediated by organised membrane microdomains, termed rafts, which are formed by the preferential association of sphingolipids with cholesterol [6]. We have evidence to suggest that the lipid composition of membrane rafts is altered in a macrophage model of GD possibly explaining the altered lipid and protein trafficking seen in this disorder [7,8].

To date, the work addressing the role of raft lipid composition in GD has been restricted to cell models; cell culture artefacts and/or unknown actions of chemical inhibitors on cells cannot be ruled out. Here we have eliminated potential limitations of cell culture by taking a physiological angle and characterised the lipid composition of rafts from an acid β-glucosidase conditional knock-out mouse model in which the resulting phenotype mimics the high levels of visceral GC storage seen in human GD patients [9]. Rafts were isolated from the spleen, the primary site of pathology, to enable an assessment of lipid raft composition within the diseased organ as a whole, rather than isolated cell systems. As far as the variable nature of raft isolation procedures is concerned we used both a detergent and a detergent-free method, and compared the lipid composition of the rafts isolated by both protocols in the diseased and non-diseased state.

2. Materials and methods

2.1. Tissue preparation

All breeding and experimental procedures were performed according to the protocols approved by the institutional Animal Ethics Committee in accordance with the Guidelines of the National Health and Medical Research Council of Australia. The acid β-glucosidase conditional knockout mouse model of GD has been previously described [9]. The spleens from three control (unaffected littermates) and three GD six month old mice were harvested, snap frozen in 98% hexane and stored at −80 °C. Keeping the spleen frozen, approximately 50 mg was cut from the end. After thawing on ice the samples were cut up into smaller pieces (approximately 2 mm) prior to membrane microdomain isolation.

2.2. Isolation of membrane microdomains using a detergent method

Membrane microdomains were extracted from the spleen using the method of Lisanti et al. [10]. Briefly, spleen pieces were placed into a glass Dounce homogeniser with 2 ml MES-buffered saline (MBS) (25 mM MES pH 6.5, 0.15 M NaCl), containing 1% (v/v) Triton X-100 and 1 mM PMSF and homogenised 20 times before being incubated on ice for 30 min. After incubation, homogenates were centrifuged at 425 g for 5 min at 4 °C, after which a 50 μl aliquot of the supernatant was taken for protein determination using the method of Lowry et al. [11]. The remainder of the supernatant was put into the bottom of a 12 ml Beckman (Palo Alto, CA) centrifuge tube and the sucrose concentration was adjusted to 40% (w/v) by the addition of 2 ml 80% (w/v) sucrose in MBS buffer, containing 1% (v/v) Triton X-100 and 1 mM PMSF. The sample was overlayed with 5 ml 30% (w/v) sucrose in MBS buffer, containing 1 mM PMSF and then 3 ml 5% (w/v) sucrose in MBS buffer, containing 1 mM PMSF. Samples were centrifuged at 270,500*g* for 18 h at 4 °C in a SW40 rotor and 1 ml fractions were collected from the top of the gradient.

2.3. Isolation of membrane microdomains using a detergent-free method

Membrane microdomains were isolated from the spleen using a detergent-free method based on the method of Persaud-Sawin et al. [12]. All steps were done on ice or at 4°C. Briefly, spleen pieces were placed into a glass Dounce homogeniser with 1 ml lysis buffer (Tris buffered saline (TBS) pH 8.0, 1% proteinase inhibitor cocktail, 1 mM PMSF, 1 mM $Na_4P_2O_7$, 1 mM Na_2MoO_4, 10 mM NaF, 1 mM NaV, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and homogenised 10–20 times. The homogenate was then sheared 20 times through a 20 gauge needle and centrifuged at 1625*g* for 4 min at 4°C. The supernatant was retained and the pellet was resuspended in 0.5 ml lysis buffer, sheared again and centrifuged at 1625*g* for 10 min at 4°C. The two supernatants were pooled and a 50 μl aliquot was taken for protein determination using the method of Lowry et al. [11]. A portion of the combined supernatant (750 μl) was put into the bottom of a 12 ml Beckman centrifuge tube and the sucrose concentration adjusted to 42.5% (w/v) by the addition of 750 μl 85% (w/v) sucrose in TBS. This was overlayed with 8.5 ml 35% (w/v) sucrose in TBS and then 2 ml 5% (w/v) sucrose in TBS. Samples were centrifuged at 270,500*g* for 18 h at 4°C in a SW40 rotor and twelve 1 ml fractions were collected from the top of the gradient.

2.4. Western blot analysis of membrane microdomains

An aliquot of each membrane microdomain fraction (13 μl) was run on 10% SDS–PAGE gels according to the method of Laemmli [13]. The gels were transferred to PVDF membrane at 30 V for 70 min. The membrane was incubated in block solution (TBS + 0.1% Tween 20 (TBST), 5% (w/v) skim milk pH 7.0) for 1 h at room temperature, washed for 5 min in TBST and then incubated overnight at 4°C in the presence of polyclonal rabbit flotillin-1 (1:1000 in block solution). The membrane was washed 3 × 5 min in TBST and then incubated for 1 h at room temperature in the presence of HRP-conjugated sheep-anti-rabbit immunoglobin (1:5000 in block solution). The membrane was washed 4 × 5 min and developed using the WestFemto ECL blotting system (Thermo Fisher) with detection using the LAS4000 Luminescent Image Analyser (Fujifilm Life Science, Stamford, CT USA).

2.5. Lipid extraction from membrane microdomains

Lipids were extracted from 750 μl of each fraction, using the method of Bligh and Dyer [14] with the inclusion of 400 pmol of the following internal standards: GC 18:1/16:0 (d_3), Cer 18:1/17:0, DHC 18:1/16:0 (d_3), THC 18:1/17:0, PC 14:0/14:0, PE 17:0/17:0, PG 14:0/14:0, PI 16:0/16:0, PS 17:0/17:0 and BMP 14:0/14:0 from Avanti Polar Lipids (Alabaster, AL, USA) and Matreya LLC (Pleasant Gap, PA, USA). The mixture was shaken for 10 min and incubated for 50 min at room temperature. Samples were partitioned by the addition of 950 μl $CHCl_3$ and 950 μl H_2O, shaken for 10 min and centrifuged at 2300*g* for 5 min. The lower hydrophobic phase was transferred to a clean tube and washed with 0.5 ml Bligh–Dyer synthetic upper phase (prepared by mixing 15 ml H_2O with 56 ml $CHCl_3$:CH_3OH (1:2), shaking vigorously for 1 min then adding 19 ml $CHCl_3$ followed by 19 ml H_2O and shaking for another min. The mixture was allowed to stand at room temperature overnight and the top aqueous layer retained for use as the synthetic upper phase), shaken for 10 min and centrifuged at 2300*g* for 5 min. The upper phase was discarded and the lower hydrophobic phase was dried under a gentle stream of N_2 at 40°C.

2.6. Liquid chromatography/electrospray ionisation-tandem mass spectrometry (LC/ESI-MS/MS) quantification of lipids

Prior to analysis the dried extracts were resuspended in 200 μl of CH_3OH containing 5 mM NH_4COOH and individual species of sphingolipids, phospholipids and cholesterol were quantified by LC/ESI-MS/MS in the multiple reaction monitoring mode on a PE Sciex API 4000 triple quadrupole mass spectrometer as previously