Activation of p38 Mitogen-Activated Protein Kinase in Gaucher’s Disease

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Abstract

Gaucher’s disease is caused by defects in acid β-glucosidase 1 (GBA1) and has been also proposed as an inflammatory disease. GBA1 cleaves glucosylceramide to form ceramide, an established bioactive lipid, and defects in GBA1 lead to aberrant accumulation in glucosylceramide and insufficient formation of ceramide. We investigated if the pro-inflammatory kinase p38 is activated in Gaucher’s disease, since ceramide has been proposed to suppress p38 activation. Three Gaucher’s disease mouse models were employed, and p38 was found to be activated in lung and liver tissues of all Gaucher’s disease mice. Most interestingly, neuronopathic Gaucher’s disease type mice, but not non-neuronopathic ones, displayed significant activation of p38 and up-regulation of p38-inducible proinflammatory cytokines in brain tissues. In addition, all type of Gaucher’s disease mice also showed increases in serum IL-6. As cellular signalling is believed to represent an in vivo inflammatory phenotype in Gaucher’s disease, activation of p38 and possibly its-associated formation of proinflammatory cytokines were assessed in fibroblasts established from neuronopathic Gaucher’s disease mice. In mouse Gaucher’s disease cells, p38 activation and IL-6 formation by TNF-α treatment were enhanced as compared to those of wild type. Furthermore, human fibroblasts from Gaucher’s disease patients also displayed increases in p38 activation and IL-6 formation as comparison to healthy counterpart. These results raise the potential that proinflammatory responses such as p38 activation and IL-6 formation are augmented in Gaucher’s disease.

Introduction

Gaucher’s disease is the most common lysosomal storage disorder caused by mutations in a gene encoding a lysosomal enzyme, acid β-glucosidase 1 (GBA1) [1–5]. The enzyme degrades
glucosylceramide to form ceramide, and the defects in the catalytic activity in Gaucher’s disease result in intracellular accumulation of undegraded substrates (glucosylceramide and glucosylsphingosine). The unifying primary clinical manifestations in Gaucher’s disease include hepatosplenomegaly, hematologic abnormalities, anemia, thrombocytopenia, and bony lesions [6]. The disease has classically been divided into three major subtypes, namely types I, II, and III, which are distinguished by the presence of or absence of neuronopathic manifestations [7].

The classical hallmark of the disease is the presence of the Gaucher cell, a macrophage containing much of the stored glucosylceramide found in tissues, which is believed to cause the clinical manifestations of the disease. However, the pathogenesis of Gaucher’s disease appears to extend beyond simple accumulation of glucosylceramide in Gaucher cells and seems to involve systemic inflammatory responses [8, 9]. Importantly, Gaucher’s disease patients have been reported to display elevated serum concentrations of hematopoietic growth factors and proinflammatory cytokines, including monocyte/macrophage colony-stimulating factor, TNF-α, interleukin (IL)-1β, IL-8, and IL-6 [10–12]. In addition, Mizukami et al. also demonstrated that Gaucher’s disease is linked to systemic inflammation in a mouse model [13]. Interestingly, brain inflammation is likely to contribute to neuronal cells death in neuronopathic Gaucher’s disease mice [8]. In fact, neurodegenerative diseases are associated with brain inflammation, and preclinical studies demonstrated that sphingolipidoses such as Sandhoff disease [14] and Niemann-Pick disease [15] benefited from anti-inflammatory therapy. Collectively, inflammation is suggested to play a role in the pathogenesis of Gaucher’s disease.

Our previous studies suggested that GBA1 participates in a pathway that leads to the formation of ceramide which in turn serves as an inhibitory lipid in activation of the proinflammatory kinase p38 [16, 17], raising the potential of ceramide as an anti-inflammatory lipid. Thus, insufficient formation of ceramide caused by GBA1 defects was assumed to promote p38 activation and its-driven inflammatory responses in Gaucher’s disease.

In preclinical and clinical studies, p38 is a therapeutic target in inflammatory disease such as rheumatoid arthritis, Crohn’s disease, and neurodegenerative diseases [18–20]. In the present studies, we assessed if p38 is activated in Gaucher’s disease in vivo and in vitro. Those studies provide insight for potential anti-inflammatory therapy targeting p38 for Gaucher’s disease.

Materials and Methods

Antibodies and reagents

Phospho/active p38 antibody (V1211) was purchased from Promega (Madison, WI). Horse-radish-peroxidase-conjugated antibodies for mouse (sc2005) and rabbit (sc2004) IgG were from Santa Cruz Biotechnology. A mouse monoclonal antibody specific for β-actin (A5441) was from Sigma. Recombinant human tumor necrosis factor-α (TNF-α, 300-01A) was from Peprotech. Halt Phosphatase Inhibitor Cocktail was from Pierce. Phorbol 12-myristate 13-acetate (PMA, 524400) was from Calbiochem. SuperSignal West Dura Extended Duration Substrate was from Thermo Scientific.

Human fibroblasts from healthy (wild type GBA1: catalog number AG14580) and Gaucher’s disease patients (case-1, catalog number GM10915; case-2, catalog number GM00877) were purchased from the Coriell Institute For Medical Research. The case-1 patient harboring homozygous L444P mutation (expired at age 7.5) was classified into type 1 according to the profile of the Coriell Institute For Medical Research and has clinical manifestations including severe hepatosplenomegaly, bilateral sensorineural hearing loss, but no other CNS findings. The case-2 patient also harboring homozygous L444P mutation (expired at age 1) was classified into type 2 with clinical manifestations including hepatosplenomegaly, strabismus, and trismus.
Ethics statement
The use of murine models and experimental protocols was reviewed and approved by the institutional animal care and use committee at Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA) prior to the start of this study and. The ID number is 2D12113.

Gaucher’s disease mice
Three types of Gaucher’s disease mouse models including V394L/V394L (V394L) [21], D409H/D409H (D409H) [21], and V394L/PS-NA [22] were maintained in a microisolator pathogen-free environment. Mice (10 weeks, female) were euthanized with CO2 suffocation followed by cervical dislocation and then tissues and serum were collected.

Cell culture
Human fibroblasts established from patients with Gaucher’s disease and healthy volunteer were grown in α-MEM medium supplemented with 10–15% fetal bovine serum (FBS). Mouse fibroblasts established from wild type or Gaucher’s disease model mouse were grown in DMEM medium supplemented with 10% FBS. Cells were maintained at <80% confluence under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37°C).

IL-6 ELISA assay
For measuring mouse serum IL-6, blood samples were allowed to clot for 2 h at room temperature. The clotted materials were removed by centrifugation at 1,000 × g for 10 min, and then the supernatant serum was collected. IL-6 production in cell culture supernatants was normalized to proteins extracted from adherent cells. Levels of IL-6 in culture supernatants or mouse serum were measured using commercially available IL-6 ELISA (Quantikine; R&D Systems).

Immunoblotting
Tissues from mice (10 weeks of age, female) were homogenized in M-PER (Mammalian Protein Extraction Reagent), and then proteins were extracted as described [22]. For human or mouse fibroblast studies, cultured cells were washed three times with phosphate-buffered saline (PBS) supplemented with Halt Phosphatase Inhibitor Cocktail and lysed using Laemmli buffer. Protein concentrations were determined using the BCA method [23]. The protein samples (20 μg) were subjected to SDS-PAGE (4–20% gradient gels). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with PBS/0.1% Tween 20 (PBS-T) containing 5% nonfat dried milk, washed with PBS-T, and incubated with antibodies for β-actin or phospho-p38 in PBS-T containing 5% nonfat dried milk. The blots were washed with PBS-T and then incubated with a secondary antibody conjugated with horseradish peroxidase in PBS-T containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence reagent, and quantification of the chemiluminescent signals was performed with a digital imaging system (VersaDoc, Bio-Rad).

Quantitative real-time PCR (Q-RT-PCR)
RNAs in mouse tissues were extracted using TOTALLY RNA kit (Ambion Inc., catalog number AM1910). Reverse transcription of total RNA (1 μg) was carried out using RT² first strand kit (QIAGEN, catalog number 330401). The resulting cDNAs were used in the quantitative real-time PCR to determine the mRNA levels. Real-time PCR was performed on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBRE Green Supermix (Bio-Rad). The mouse β-actin gene was used as an internal
reference control to normalize relative levels of gene expression, and expression of each transcript is presented as arbitrary units (AU). The following primers were used: β-actin (forward, TCCTCCCTGGAGAAGAGCTA; reverse, CCAGACAGCCTGTGTTGGC); IL-6 (forward, TCCAGTTGCTTCTGGGAG; reverse, GTGTAATTAGCCCTCCGACTTG), 4331182; p38α (forward, CAGTTCGAAGTAGCTTGCAA; reverse, TGGAAGACACTGCTGAGGTC); p38β (forward, CAGTCCTGAAGTTCTGGCAA; reverse, TGGAAGACACTGCTGAGGTC); p38γ (forward, GGCCCAGAAATATGACGACT; reverse, TCTCCTTTGGAACCTGGCT); p38δ (forward, TCGACATCTGGTCTGTTGGT; reverse, GGTCAGCTGGTCCAGGTAGT).

TNF-α (forward, AATGGCCTCCCTCTCATCAGTT; reverse, CCACTTGGTGGTTTGCTACGA).

Statistical analysis

Results are presented as means ± SEM. Statistical analyses were performed using GraphPad Prism and Instat. Comparison between two groups was carried out by unpaired Student’s t test.

Results

p38 is activated in Gaucher’s disease mice

To evaluate p38 activation in Gaucher’s disease in vivo, three types of Gaucher’s disease mouse models were employed, including V394L, D409H, and V394L/PS-NA. The former two types have been established as homozygous for GBA1 point mutant knock-in mice with minor glucosylceramide accumulation in viscera and without significant phenotypes such as neuro-degeneration and shorter life span [21]. The last has been generated by crossing V394L homozygous with the mouse with hypomorphic expression of the prosaposin transgenes (5–45% of wild type, PS-NA), showing glucosylceramide accumulation in multiple organs, several phenotypes including neuro-degeneration and shorter life span [22]. Phospho/active p38 in tissues was determined by immunoblotting. p38 in the brain tissues of neuronopathic mice (V394L/PS-NA) was activated as compared with those of wild type, whereas other Gaucher’s disease mice (V394L and D409H) did not show significant activation of p38 (Fig 1A and 1B). p38 activation in brain tissues is correlated with neuronopathic phenotype. As to other tissues including liver and lung, the levels of active p38 in all types of Gaucher’s disease mice were increased as compared with those of wild type (Fig 1B).

Proinflammatory cytokines in brain have pathological roles in neurodegenerative central nervous system. Campbell et al. have reported that cerebral overexpression of IL-6 induced neurologic disease [24]. mRNA levels of the major proinflammatory cytokine TNF-α were highly elevated in brain tissues of neuronopathic V394L/PS-NA mice relative to those of wild type mice (Fig 1C). IL-6 mRNA increases were not statistically significant (Fig 1C); however, its mRNA levels were prone to increase in brain tissues of neurodegenerative V394L/PS-NA. Therefore, neuropathic phenotype is believed to associate with the elevation of inflammatory cytokines.

Activation of p38 is involved in forming a myriad of inflammatory mediators, and p38 is a predominant kinase responsible for generating proinflammatory cytokines such as TNF-α and IL-6 [18]. In fact, previous studies showed that GBA1 knockdown potentiates p38-dependent formation of IL-6 in human cell lines [16], and IL-6 has been shown to increase in serum of patients with Gaucher’s disease [11, 12]. We determined serum IL-6 in Gaucher’s disease model mice using an ELISA system (Fig 1D). Serum IL-6 in the mice (V394L, D409L, and V394L/PS-NA) was 28.5 ± 7.8, 12.8 ± 2.7, and 23.1 ± 13.6 pg/ml, respectively. All values were significantly higher as compared with that of wild type (7.1 ± 1.0 pg/ml). Those results show that serum IL-6 is increased with GBA1 defects. This elevation is consistent with that of patients with Gaucher’s disease [11].
p38 is activated in cellular proinflammatory responses in mouse Gaucher’s disease fibroblasts

The in vivo studies above raised the possibility of a pathological role for p38 activation in Gaucher disease. p38 is known to serve as a proinflammatory kinase that is activated in response to proinflammatory cytokines or inflammation inducers such as PMA [25]. To determine if p38 is activated in proinflammatory responses of fibroblast cells derived from Gaucher’s disease mouse (V394L/PS-NA), those cells were stimulated with TNF-α, known as a potent inflammatory cytokine. Both basal and TNF-α-treated fibroblasts established from V394L/PS-NA mouse displayed higher p38 activation as compared to those of wild type, respectively (Fig 2A). Consistent with potentiated activation of p38, IL-6 formation was also facilitated in mouse Gaucher’s disease fibroblasts (Fig 2B).

Gaucher’s disease patients-derived fibroblasts show increased p38 activation and IL-6 formation

Genetic approaches and pharmacological approaches demonstrated that GBA1 defects facilitate p38 activation in vitro [16] and in vivo (above); however, whether p38 is highly activated in
human Gaucher’s disease is not known. Human fibroblasts established from Gaucher’s disease patients harboring homozygous L444P were employed to assess effects of Gaucher’s disease on p38 activation and IL-6 formation. Those cells were stimulated with PMA or TNF-α, and then p38 activation was determined by immunoblotting, p38 was activated in response to TNF-α treatment in fibroblasts from healthy, whereas fibroblasts from both two Gaucher’s patients displayed significant increases in p38 activation in response to TNF-α as compared to that of healthy (Fig 3A). Similar to mouse fibroblasts, human Gaucher’s fibroblasts also displayed increased formation of IL-6 in response to individual treatments with PMA and TNF-α (Fig 3B).

Discussion

The results from this study provide significant evidence to support the emergence of Gaucher’s disease as an inflammatory disease. Our studies ([16] and the current results) suggest that the p38 pro-inflammatory kinase is activated in Gaucher’s disease. Especially, p38 activation in brain tissues is associated with the neurodegeneration phenotype in Gaucher’s disease. Therefore, p38 activation is proposed to play a role in the inflammation-associated pathogenesis in Gaucher’s disease.

In brain tissues, p38 activation was specific to neuronopathic Gaucher’s disease V394L/PS-NA mice (Fig 1A and 1B). Several types of neuronopathic Gaucher’s disease mouse models have been established by genetic engineering. Neuronopathic Gaucher’s disease mouse model (GBA1 V394L/V394L, saposin C−/−) has been also generated, displaying shortened life span and brain proinflammation involving elevations in active p38, IL-6 and TNF-α mRNAs [26]. Importantly, in vivo treatment with a pharmacological chaperon, isofagomine, known to enhance GBA1 function in Gaucher’s disease fibroblasts, suppressed the increased p38 activation and TNF-α formation in brain tissues, and extended life span [26]. Therefore, GBA1 defects are suggested to facilitate p38 that serves as a proinflammatory kinase in brain tissues.

p38 MAPK is classified into four isoforms including p38α, p38β, p38γ, and p38δ [27, 28]. GBA1-regulated ceramide in cancer cell lines is suggested to down-regulate activation of p38δ.
by activating ceramide-activated protein phosphatases [16, 17]. Which p38 isoform is predominantly activated in Gaucher’s disease remains to be determined, although mRNA expression of all p38 isoforms was detected in mouse brain tissues (Fig 1C).

p38 has been implicated in cellular inflammatory responses [29], differentiation [30] and cell death [30], and is believed to serve as a key kinase that mediates the production of inflammatory mediators such as proinflammatory cytokines, prostaglandins and reactive oxygen species. Importantly, p38 serves as a crucial molecule that promotes the formation of IL-6 and TNF-α, in vivo and in vitro. Experimentally, overexpression of these cytokines in the central nervous system of transgenic mice triggers central nervous system inflammation and degeneration [24, 31–33]. Therefore, p38 activation and/or increased formation of these cytokines might play roles in the pathogenesis of Gaucher’s disease.

In summary, our studies demonstrate that p38 signaling is activated in Gaucher disease and raise the possibility that p38-driven inflammation is involved in the disease pathogenesis.

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Author Contributions

Conceived and designed the experiments: LMO NY GAG YAH. Performed the experiments: MW DP. Analyzed the data: YS. Contributed reagents/materials/analysis tools: TU. Wrote the paper: KK.
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