Glucocerebroside Ameliorates the Metabolic Syndrome in OB/OB Mice

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ABSTRACT

Glucocerebroside (GC) is a naturally occurring glycolipid that may alter natural killer T (NKT) cell function. To determine the effect of GC on the metabolic derangements and immune profile in leptin-deficient mice, Ob/Ob mice were treated by daily injections of GC for 8 weeks and followed for various metabolic and immunological parameters. Marked amelioration of the metabolic alterations characteristic of leptin-deficient mice was observed in GC-treated animals compared with controls. A significant decrease in liver size and hepatic fat content were observed in GC-treated mice. Near-normalization of glucose tolerance and decreased serum triglyceride levels were observed. Fluorescence-activated cell sorting analysis of peripheral and intrahepatic lymphocytes revealed a 1.6-fold increase of the peripheral/intrahepatic NKT lymphocyte ratio. A 33% decrease of serum interferon-γ level and a 2.6-fold increase of serum interleukin 10 level were noted in GC-treated mice. Immune modulation by GC may have a role in the treatment of nonalcoholic steatohepatitis and other immune-mediated disorders.

Obesity is strongly associated with nonalcoholic fatty liver disease, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Hepatic steatosis (“first hit”) results from accumulation of lipids, predominantly triglycerides, within hepatocytes due to variable combinations of excess lipid uptake and synthesis and altered lipid secretion. The transition from simple steatosis to NASH is thought to involve a “second hit,” usually attributed to oxidative stress (Day and James, 1998).

The pathogenesis of NASH may also involve a number of immune mechanisms. A number of immunological derangements have been noted in leptin-deficient ob/ob mice, a murine model for NASH. These include impaired cell-mediated immunity (Lord et al., 1998; Howard et al., 1999), a reduction in the number of intrahepatic natural killer T (NKT) lymphocytes, impaired function of hepatic Kupffer cells (Lee et al., 1999), reduced serum levels of IL10 and IL15, and increased serum levels of IL12 (Loffreda et al., 1998; Tilg and Diehl, 2000; Li et al., 2002). The reduced number of intrahepatic NKT lymphocytes in ob/ob mice may result from chronic oxidative stress that promotes increased apoptosis. Altered secretion of IL15 by Kupffer cells, which is important for NKT cell differentiation, and decreased expression of leukocyte factor antigen 1, necessary for hepatic accumulation of CD4+ NKT lymphocytes, were also suggested to be responsible for this defect (Kennedy et al., 2000; Takeda et al., 2000). Leptin replenishment results in increased numbers of hepatic NKT lymphocytes and partial reversal of the associated immune derangements in these animals (Takeda et al., 2000).

NKT lymphocytes are a subset of regulatory lymphocytes that coexpress cell surface receptors characteristic of both T lymphocytes (e.g., CD3, α/β T-cell receptor) and natural killer cells (e.g., NK1.1) (Godfrey et al., 2000). These cells, which are abundant in the liver (Crispe and Mehal, 1996), recognize glycolipids in the context of the major histocompatibility complex class I-related molecule CD1d. α-Galactosylceramide (KRN-7000), a synthetic glycolipid, was found to be a potent activator of both mouse and human NKT cells (Kawano et al., 1997).

Glucocerebroside (β-glucosylceramide), a metabolic intermediate in the metabolic pathways of complex glycosphingolipids (Radin and Inokuchi, 1988), is a naturally occurring glycolipid that may alter NKT cell function.

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ABBREVIATIONS: NASH, nonalcoholic steatohepatitis; NKT, natural killer T; GC, glucocerebroside; IL, interleukin; PBS, phosphate-buffered saline; MRI, magnetic resonance imaging; BSA, bovine serum albumin; IFN-γ, interferon-γ; SI, signal intensity; TNF-α, tumor necrosis factor; MFI, MRI fatty liver index.
glycolipid. The effect of glucocerebroside on NKT cells was examined in recent studies. CD1d-bound glucocerebroside does not activate NKT cells directly and may inhibit activation of NKT cells by α-galactosylceramide (Stanic et al., 2003). In an in vitro study, glucocerebroside was shown to inhibit NKT lymphocyte proliferation in the presence of dendritic cells but not in their absence. In an in vivo study, glucocerebroside prevented development of NKT-mediated concanavalin A-induced hepatitis in a murine model (Margalit et al., 2005). Clinically, inherited deficiency of glucocerebrosidase, a lysosomal hydrolase, results in Gaucher’s disease, characterized by accumulation of glucocerebroside (Elstein et al., 2002). Patients with Gaucher’s disease were recently shown to have an altered number of peripheral blood NKT lymphocytes (Margalit et al., 2003).

The aim of the present study was to determine the immune modulatory effect of GC on the metabolic and immunological derangements in leptin-deficient ob/ob mice. Administration of GC induced a marked reduction in hepatic fat content and improved glucose tolerance. This effect was associated with alteration of the intrahepatic lymphocyte subset profile.

Materials and Methods

Animals. Eight-week-old male ob/ob mice were purchased from Jackson laboratories (Bar Harbor, ME). Animals were housed in laminar flow hoods in sterilized cages and given irradiated food and sterile acidified water. Animal experiments were carried out in accordance with the guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals and with the committee’s approval.

Preparation of Glycolipids. Glucocerebroside was purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in ethanol. Emulsification in PBS was then performed.

Experimental Groups. Four groups of mice were studied. Ob/Ob mice (Groups A and B, n = 12 per group) and lean C57BL mice (Groups C and D, n = 12/group) were treated by daily injections of GC (1.5 μg intraperitonially; Groups A and C) or PBS with similar ethanol concentration (100 μl; Groups B and D) for 8 weeks.

Follow-Up Parameters. Mice were followed by glucose tolerance tests; determination of serum alanine aminotransferase, aspartate aminotransferase, and triglyceride levels; and assessment of liver size and hepatic fat content by magnetic resonance imaging (MRI) and histological examination.

Glucose Tolerance Test. Mice underwent a glucose tolerance test on day 60. Glucose was administered orally (1 g per kg). Serum glucose measurements were performed on tail-vein blood every 15 min for 1 h. Glucose levels were measured by a standard glucometer.

MRI Hepatic Fat Content Measurement. Mice underwent MRI on day 60. Liver size was assessed, and hepatic fat content was measured by a double-echo chemical shift gradient-echo sequence technique that provides in-phase and out-of-phase images in a single acquisition for assessment and quantification of liver fat. T1-weighted out-of-phase MR imaging is sensitive for detection of relatively small proportions of tissue fat (Mitchell et al., 1991; Namimoto et al., 2001). Magnetic resonance images were acquired by a 1.5-T system (Signa LX; General Electric, Milwaukee, WI). Double-echo MRI was performed with a repetition time of 125 ms, double-echo times of 4 and 6.5 ms, and a flip angle of 80°. Imaging parameters included section thickness of 3 mm, a 13-cm field of view, and a 256 × 160 matrix. Axial and coronal images were obtained. Signal intensity (SI) changes between in-phase and out-of-phase images were computed. The SI index was calculated as follows: SI index = (SI1p − SI1o)/(SI1p + SI1o).

Triglyceride Measurement. On day 60, serum triglyceride levels were tested using standard techniques.

Liver Steatohepatitis Score. A liver segment from each mouse was fixed in 10% formaldehyde and embedded in paraffin for histological analysis. Five sections (5 μm) were stained with hematoxylin/eosin and reviewed by two pathologists in a blinded fashion. Histological examination and the steatohepatitis grading (NASH score) were performed using the steatohepatitis scoring system (Brunt, 2001).

Measurement of Serum Cytokines. Serum IFN-γ, IL10, and IL4 levels were measured by a “sandwich” enzyme-linked immunosorbent assay method using Genzyme Diagnostic kits (Genzyme Diagnostics, MA), according to manufacturer’s instructions.

Isolation of Splenic and Hepatic Lymphocytes for Determination of T Cell Subpopulations. Mice were sacrificed on day 60 of the experiment. Splenic lymphocytes and NKT cells were isolated, and red blood cells were removed as described previously (Trop and Ilan, 2002). Intrahepatic lymphocytes were isolated as follows. After cutting the inferior vena cava above the diaphragm, the liver was flushed with ice-cold PBS until it become pale followed by removal of connective tissue and gall bladder. Livers and spleens were crushed through a stainless mesh (size 60; Sigma Chemical Co., St. Louis MO). The cell suspension was placed in a 50-ml tube for 3 min and washed twice in ice-cold PBS (1250 rpm for 10 min). Cells were resuspended in PBS, cell suspension was placed through a nylon mesh presoaked in PBS, and unbound cells were collected. For liver and spleen lymphocyte isolation, 20 ml of histopagae 1077 (Sigma Diagnostics, St. Louis, MO) was placed under the cells. The tube was centrifuged at 1640 rpm for 15 min in room temperature. Cells at the interface were collected, diluted in a 50-ml tube, and washed twice with ice-cold PBS (1250 rpm for 10 min). Approximately 1 × 108 cells/mouse liver were recovered. The viability by trypan blue staining was above 95%.

Flow-Cytometry Analysis for Determination of CD4, CD8, and NKT Lymphocyte Populations. After lymphocyte isolation, triplicates of 2 to 5 × 106 cells/500 μl of PBS were placed in Falcon 2052 tubes, incubated with 4 ml of 1% BSA for 10 min, and centrifuged at 1400 rpm for 5 min. Cells were resuspended in 10 μl of fetal calf serum with 1:20 fluorescein isothiocyanate-anti-mouse CD3 antibody, 1:20 phycoerythrin-anti-mouse CD4 antibody, 1:20 anti-mouse CD8 antibody, or 1:20 fluorescein isothiocyanate-anti-mouse NK1.1 antibody (NKR-P1C; BD PharMingen, San Diego, CA), and mixed every 10 min for 30 min. Cells were washed twice in 1% BSA and kept at 4°C until reading. For the control group, only 5 μl of 1% BSA was added. Analytical cell sorting was performed on 1 × 106 cells from each group with a fluorescence-activated cell sorter (PAC-STAR plus; Becton Dickinson, Oxnard, CA). Only live cells were counted, and background fluorescence from nonantibody-treated lymphocytes was subtracted. Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. Data were analyzed by the Consort 30 two-color contour plot (Becton Dickinson) or CellQuest programs.

Statistical Analysis. The Student’s t test was used for data analysis; p < 0.05 was considered statistically significant.

Results

Effect of Glucocerebroside on Liver Size and Hepatic Fat Content. Livers of ob/ob mice that were treated by glucocerebroside (Group A) were significantly smaller than livers of untreated control ob/ob mice (Group B, 20.63 versus 24.12 cm2, respectively; p < 0.05). Glucocerebroside did not affect liver size in lean C57BL mice (Groups C and D, 10.7 and 11.75 cm2, respectively). There was a 10.9% decrease in the hepatic SI index in the glucocerebroside-treated ob/ob group compared with control ob/ob mice (SI index 0.620 versus 0.688 in Groups A and B, respectively, p < 0.05; Figs. 1B
and 2), indicating a significant reduction in the hepatic fat content. Because both the liver area and the SI index are correlated with the degree of hepatic fat, their product, MFI, was calculated to increase the accuracy in detecting a difference between the groups. There was an 18.7% reduction of the MFI in treated (Group A) ob/ob mice compared with untreated ob/ob controls (MFI 13.1 versus 15.55 in Groups A and B, respectively, \( p < 0.05 \)). Glucocerebroside treatment also resulted in marked reduction of the hepatic fat content in lean C57BL mice. The SI index was 0.1 in glucocerebroside-treated Group C mice compared with 0.18 in untreated Group D controls, a reduction of 80% in the intrahepatic fat content. The MFI index decreased from 1.92 to 1.17 in Groups D and C, respectively (Fig. 1C).

**Effect of Glucocerebroside on Serum Triglyceride Levels.** The serum triglyceride level was significantly lower in glucocerebroside-treated ob/ob mice compared with control ob/ob mice (1.57 versus 2.29 mM in Groups A and B, respectively). Serum triglyceride levels also decreased in lean C57BL mice (1.31 versus 1.56 mM in glucocerebroside-treated versus untreated animals, respectively).

**Effect of Glucocerebroside on Glucose Tolerance.** The glucose tolerance test was markedly abnormal in control ob/ob mice (339 mg/dl at 1 h in Group B; Fig. 4). In contrast, glucocerebroside-treated ob/ob mice had an almost normal glucose tolerance curve throughout the 3-h test (glucose level 153 mg/dl at 1 h; \( p < 0.05 \)). No significant effect on fasting plasma glucose levels was noted.

**Effect of Glucocerebroside on Serum Liver Enzymes.** The serum aspartate aminotransferase and alanine aminotransferase levels did not differ significantly between the groups.

**Effect of Glucocerebroside on Body Weight.** Treatment had no significant effect on body weights, and no statistically significant differences were noted between treated mice and controls.

**Effect of Glucocerebroside on Intrahepatic and Intrasplenic Lymphocyte Subsets.** Fluorescence-activated cell sorting analysis of peripheral and intrahepatic lymphocytes revealed a 1.6-fold increase of the peripheral/intrahepatic...
patic NKT lymphocyte ratio in glucocerebroside-treated ob/ob mice compared with controls [peripheral/intrahepatic NKT lymphocyte ratio (3.46 versus 2.13 in Groups A and B, respectively; \( p < 0.05 \); Figs. 4 and 5)]; this ratio increased 3.73-fold in glucocerebroside-treated lean C57BL mice compared with control lean C57BL mice (peripheral/intrahepatic NKT lymphocyte ratio 2.13 versus 0.57 in Groups C and D, respectively; \( p < 0.05 \); Fig. 5). The intrahepatic CD4⁺/CD8⁺ lymphocyte ratio was significantly increased in the GC-treated group compared with nontreated controls (4.09 versus 2.18 in Groups A and B, respectively; \( p < 0.05 \); Fig. 5). This change was specific to the liver and was not accompanied by a similar change in the peripheral CD4/CD8 ratio (1.53 versus 1.33 in Groups A and B, respectively). Thus, the peripheral to intrahepatic CD4/CD8 ratio increased 1.64-fold in GC-treated mice (0.61 versus 0.37 in Groups A and B, respectively; \( p < 0.05 \)), suggesting increased intrahepatic CD8 lymphocyte trapping.

**Effect of Glucocerebroside on Serum Cytokine Levels.** A 33% decrease of the serum IFN-γ level (6.5 versus 9.75 pg/ml in Groups A and B, respectively) and a 2.6-fold increase of the serum IL10 level (140 versus 53 pg/ml in Groups A and B, respectively; \( p < 0.05 \); Fig. 6) were noted in glucocerebroside-treated ob/ob mice compared with control ob/ob mice. The serum IL4 level did not differ among these groups. Glucocerebroside did not affect serum cytokine levels in lean C57BL mice.

**Discussion**

Administration of glucocerebroside alleviated hepatic steatosis and the metabolic syndrome in leptin-deficient ob/ob mice. In treated mice, lower serum triglyceride levels, almost normal glucose tolerance curves, and significantly reduced liver size and liver fat content were observed. Glucocerebroside-treated ob/ob mice had an altered immunological profile, including an increased peripheral/intrahepatic NKT lymphocyte ratio and increased CD8⁺ T lymphocyte trapping.

The concomitant improvement in steatosis and glucose tolerance that was observed in this study following administration of glucocerebroside reinforces the concept of a close interaction between steatosis and the metabolic syndrome in NASH (Koteish and Diehl, 2001). Glucocerebroside had an impressive effect on glucose tolerance that was comparable with that observed for currently available drugs, without causing hypoglycemia. The effect of glucocerebroside on the hepatic fat content and serum triglyceride levels in ob/ob and lean C57BL mice may suggest that the metabolic syndrome is a result of several pathogenic mechanisms that can be manipulated separately.

NKT lymphocytes, a subpopulation of regulatory lymphocytes, have a role in various infectious, inflammatory, and neoplastic processes (Vincent et al., 2003). These cells, which are abundant in the liver, are considered to be a link between...
innate and adaptive immune responses (Bendelac and Aron, 1997) and were shown to have a role in a number of immune-mediated disorders. In NOD mice, reduced numbers of NKT cells are associated with increased susceptibility to diabetes (Baxter et al., 1997); in experimental allergic encephalomyelitis, activation of NKT attenuates the disease (Miyamoto et al., 2001); in an animal model of systemic lupus erythematosus, a selective reduction in NKT cells precedes the development of autoimmune phenomena (Takeda and Dennert, 1993). NKT cells are known to induce hepatic injury in several models, including concanavalin A-induced (Eberl and McDonald, 1998; Takeda et al., 2000) and salmonella infection-induced (Ishigami et al., 1999) liver damage. Leptin-deficient ob/ob mice feature a dysfunctional immune response, manifested by depletion of hepatic NKT lymphocytes, and impaired function of hepatic Kupffer cells (Loffreda et al., 1998; Lee et al., 1999; Li et al., 2002). These alterations may explain the relatively increased sensitivity to LPS-induced hepatotoxicity and resistance to concanavalin A-induced hepatitis and experimental allergic encephalomyelitis observed in these animals (Matarese et al., 2001). Because NKT cells were suggested to have a central role in the pathogenesis of steatosis and steatohepatitis in leptin-deficient ob/ob mice, it can be expected that alteration of their distribution, number, or function may change the course of these disorders. The mechanism by which an effect on NKT lymphocytes results in metabolic consequences remains to be determined. Because TNF-α receptor knockout ob/ob mice feature improved insulin sensitivity and administration of antidiabetic drugs, such as metformin and pioglitazone that block TNF-α signaling, results in improved steatosis, it appears that TNF-α signaling may be involved in this process (Solomon et al., 1997; Uysal et al., 1997).

Glucocerebrosides were shown to have an immune modulatory effect on NKT cells. In an in vitro study, exposure of NKT cells to glucocerebrosides in the presence of dendritic cells inhibited NKT cell proliferation (Margalit et al., 2005). In the present study, the beneficial effect of GC was associated with a decreased intrahepatic NKT cell number. One possible explanation for this finding is redistribution of NKT cells, i.e. expulsion of these cells from the liver to the periphery, thus alleviating NKT-mediated liver damage. Other possible explanations for the decreased number of intrahepatic NKT cells in this study may include glucocerebrosides-mediated inhibition of NKT cell proliferation, glucocerebrosides-mediated apoptosis, and altered subpopulations of NKT lymphocytes. NKT lymphocytes include subpopulations that are phenotypically and functionally diverse. The CD3+DX5+ NKT cells identified in this study are one population of NKT cells made up mostly of “classical” Vα14+ NKT lymphocytes; because other surrogate markers for identification of NKT cells were not used, the decreased number of intrahepatic NKT lymphocytes may reflect a relative reduction in the proportion of this subpopulation of NKT cells, rather than a truly lower total NKT lymphocyte number.

Previous studies suggested that a relatively low number of intrahepatic NKT lymphocytes in ob/ob mice may be associated with the pathogenesis of steatosis in these animals. The results of the present study imply that the absolute number of these lymphocytes may be less important than previously suggested and that a qualitative defect of these cells may be of more significance. The association between a decreased number of NKT lymphocytes and the beneficial clinical effect observed in this study may reflect an inhibitory effect on a subset of “injurious” NKT cells. A similar phenomenon was observed in the concanavalin A-induced hepatitis model (Margalit et al., 2005).

Leptin-deficient ob/ob mice exhibit a unique type of immune profile (Trop and Ilan, 2002). In the present study, the beneficial effect of GC was associated with a significant decrease in the serum IFN-γ level and a significant increase in the serum IL10 level, compatible with further skewing of the cytokine profile in a Th2 direction. This finding suggests that, in ob/ob mice, the serum cytokine profile per se does not explain the characteristic metabolic derangements.

The immune modulatory effect of GC on NKT lymphocytes may be mediated by a direct metabolic effect or by displacement of an activating ligand from the CD1d molecule. Glucocerebrosides-induced apoptosis may be mediated by glucocerebrosides itself or by altered levels of other compounds in its metabolic pathway, such as ceramide, which has a well-characterized proapoptotic effect (Muranaka et al., 2004). Binding of glycolipids to CD1d is mediated by anchoring of their lipid tail to the hydrophobic pockets of the CD1d antigen-binding groove. Occupation of the CD1d molecule by the ceramide tail of GC, which does not activate NKT lymphocytes, may competitively inhibit binding and presentation of activating ligands in a similar manner to that recently demonstrated for CD1b, another glycolipid-presenting molecule (Brigl and Brenner, 2004). It was recently demonstrated that β-galactosylceramide binds to CD1d without activating NKT cells.

We have previously shown that a significant portion of radiolabeled glucocerebrosides is concentrated in the liver following oral or intraperitoneal administration (Margalit et al., 2005). The liver is a meeting place for subpopulations of lymphocytes and antigen-presenting cells. It has a capacity to preferentially trap activated CD8+ lymphocytes (Mehal et al., 1999), a process in which NKT cells were shown to participate (Trop and Ilan, 2002). Inflammatory conditions are characterized by an increase in the CD4/CD8 T cell ratio (Matsui, 1997; Guebre-Xabier et al., 2000). An opposite shift accompanies liver regeneration following partial hepatectomy (Minagawa, 2000) and alleviation of GVHD. The beneficial effect of GC in this study was associated with an increased peripheral to intrahepatic CD4/CD8 ratio, suggesting increased intrahepatic CD8+ lymphocyte trapping. This finding is in line with the correlation between CD8+ T lymphocyte trapping and an anti-inflammatory effect that was described previously in several models of immune-mediated conditions.

At present, there is no effective pharmacologic therapy for NASH. Attempts have been made to target the underlying metabolic process by administration of a number of pharmacologic agents, including vitamin E, metformin, pioglitazone, rosiglitazone, propucol, and betaine (Brunt, 2004), with variable effects on the hepatic fat content and serum amino transferase levels. Current treatment approaches are directed primarily at achieving control of the metabolic conditions associated with NASH. Very few studies have examined the therapeutic potential of immune modulation in the context of NASH and the metabolic syndrome. Although it cannot be ruled out that glucocerebrosides has a direct metabolic effect, the immune-modulatory influence observed in this
study suggests that its beneficial effect may have been immunemediated. Thus, glucocerebroside may exemplify the relatively new concept of immune-modulation of metabolic processes that has gained support from increasing experimental evidence that several factors, which influence metabolism, also have a role in the regulation of immune responses (Matarese and La Cava, 2004).

Despite the effect of glucocerebroside on hepatic fat content and the metabolic syndrome in ob/ob mice, serum amino- transferase levels did not differ significantly between the study groups. In this study, a therapeutic effect was sought after two months, a relatively short time period. It remains to be determined whether a further reduction in hepatic fat content and an effect on serum aminotransferases would result from a longer treatment regimen. The significance of the transition from a macrovesicular pattern of steatosis to a microvesicular pattern remains unclear. Interestingly, a similar finding was observed with other immune-modulatory interventions that had a beneficial effect in this model (Elia

In summary, the results of this study suggest that glucocerebroside has a remarkable beneficial effect on hepatic steatosis and the metabolic syndrome in ob/ob mice. This effect may be mediated by immune modulation of NKT lymphocytes, supporting a major role for the immune system in the pathogenesis of NASH. The long-term implications of administration of glucocerebroside in this model, as well as the effect of other dosing regimens of glucocerebroside for variable time durations, remain to be determined.

References


