Reducing glycosphingolipid biosynthesis in airway cells partially ameliorates disease manifestations in a mouse model of asthma

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Abstract

Lipid rafts reportedly play an important role in modulating the activation of mast cells and granulocytes, the primary effector cells of airway hyperresponsiveness and asthma. Activation is mediated through resident signaling molecules whose activity, in part, may be modulated by the composition of glycosphingolipids (GSLs) in membrane rafts. In this study, we evaluated the impact of inhibiting GSL biosynthesis in mast cells and in the ovalbumin (OVA)-induced mouse model of asthma using either a small molecule inhibitor or anti-sense oligonucleotides (ASOs) directed against specific enzymes in the GSL pathway. Lowering GSL levels in mast cells through inhibition of glucosylceramide synthase (GCS) reduced phosphorylation of Syk tyrosine kinase and phospholipase C gamma 2 (PLC-γ2) as well as cytoplasmic Ca²⁺ levels. Modulating these intracellular signaling events also resulted in a significant decrease in mast cell degranulation. Primary mast cells isolated from a GM3 synthase (GM3S) knockout mouse exhibited suppressed activation-induced degranulation activity further supporting a role of GSLs in this process. In previously OVA-sensitized mice, intra-nasal administration of ASOs to GCS, GM3S or lactosylceramide synthase (LCS) significantly suppressed metacholine-induced airway hyperresponsiveness and pulmonary inflammation to a subsequent local challenge with OVA. However, administration of the ASOs into mice that had been sensitized and locally challenged with the allergen did not abate the consequent pulmonary inflammatory sequelae. These results suggest that GSLs contribute to the initiation phase of the pathogenesis of airway hyperreactivity and asthma and lowering GSL levels may offer a novel strategy to modulate these manifestations.

Keywords: asthma, glycosphingolipids, lipid rafts, mast cells

Introduction

Mast cells and granulocytes represent the key effector cells in allergy and asthma (1). Mast cells and granulocytes bind allergen-specific IgE molecules through the IgE Fc receptors FcεRI and FcεRII. When exposed to allergen, mast cells and granulocytes respond by secreting a variety of key mediators that affect the development of symptoms associated with allergy and asthma. These mediators include histamine, serotonin, various cytokines and enzymes (2). Allergen-mediated cross-linking of IgE-FcεRI/FcεRII complexes on the surface of mast cells and granulocytes results in phosphorylation of key tyrosine residues on the intracellular signaling entities Syk kinase and phospholipase C gamma (PLC-γ) leading to their activation. This, in turn, initiates the cascade of events that leads subsequently to elevation of intracellular Ca²⁺ levels and culminating in the degranulation of cells and the secretion of allergy and asthma mediators.

Cross-linking of IgE-FcεRI/FcεRII complexes by allergen can purportedly induce the segregation of these complexes into lipid rafts thereby facilitating their interaction with various intracellular signaling molecules such as palmitoylated Syk kinase located in the inner leaflet of the plasma membrane (3). However, whether the segregation of IgE-FcεRI/FcεRII complexes into these detergent-resistant membrane fractions or lipid rafts is an important component of mast cell activation...
remains controversial (4–12). Other results showing that altering the lipid composition of membrane rafts can affect the extent of mast cell activation support the view that lipid rafts are essential components of productive mast cell activation. For example, lowering cholesterol levels in mast cells inhibits degranulation, whereas incorporation of additional cholesterol into lipid rafts induces degranulation (4, 11). Also, relocation of Syk kinase into lipid rafts through altering the glycosphin-golipid (GSL) composition of mast cell membranes induces their activation (9, 10). Moreover, using a monoclonal antibody that cross-links several GSL species thereby artificially induces formation of lipid rafts also results in mast cell activation (10). Finally, disruption of lipid rafts by treating with short-chain ceramides strongly inhibits signal transduction from IgE-FceRI/FcεRII complexes and the subsequent degranulation of mast cells (7, 8). Although these studies support the contention that lowering GSL levels may attenuate activation of airway cells, there are reports suggesting that the contrary may apply (6). For example, another study showed that lowering cholesterol levels in mast cell membranes increases mast cell degranulation (12), in contrast to that reported by Baumrucker et al. (4). Together, these results suggest that although the lipid composition of the cell membranes in mast cells is an important determinant of mast cell activation, their specific role in mast cell biology remains unclear.

GSLs and sphinogolipids are reportedly important regulators of lipid raft function and other cellular processes (13, 14). The pathogenesis of several metabolic diseases has been shown to be associated with altered GSL compositions (13). For example, in mouse models of type 2 diabetes, lowering GSL levels by inhibiting glucosylceramide synthase (GCS), the rate-limiting enzyme responsible for the synthesis of many GSL species, leads to amelioration of disease symptoms (15, 16). An altered GSL composition may also be a significant contributor to sustained autoreactive T- and B-cell activation in systemic lupus erythematosus (17, 18). Inducing lipid raft formation on immune cells by administering cholera toxin B subunit increases the severity of systemic lupus erythematosus-like symptoms in a mouse model of the disease (19). Based on these observations, we examined whether lowering GSL levels in the effector cells of airway hyperreactivity through inhibition of GSL synthesis can attenuate mast cell and granulocyte activation and thereby abate the development of airway hyperreactivity and asthma. To lower GSL levels in mast cells, we deployed a small molecule inhibitor of GCS (Genz-123346) and studied its impact on mast cell activation. This molecule has previously been shown to be effective at addressing insulin insensitivity in a mouse model of type 2 diabetes and at effecting substrate reduction therapy of Gaucher disease (15, 16, 20). We also examined mast cell function from mice that are deficient in GM3 synthase (GM3S) as another approach to probe the impact of reduced GSL levels (21). Finally, the effect of reducing the synthesis of GSLs using anti-sense oligonucleotides (ASOs) against GCS, GM3S or lactosylceramide synthase (LCS) in the ovalbumin (OVA)-induced mouse model of asthma was examined. We showed that inhibition of GSL synthesis reduces mast cell activation in vitro and prevented development of airway hyperreactivity in vivo. These studies offer novel biochemical insights on mast cell activation and degranulation in allergic diseases such as asthma.

**Methods**

**Animals, cells and reagents**

Balb/Cj and C57BL/6J mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). GM3S knockout mice (GM3S−/−) (21), heterozygous (GM3S+/-) and wild-type (GM3S+/+) littermates were bred at Charles River Laboratories (Waltham, MA, USA). All mice were 6–8 weeks old at the start of the studies. All animal-handling procedures had been approved by the Institutional Care and Use Committee at Genzyme Corporation.

The cell lines RBL-2H3 and MC/9 were obtained from the American Type Culture Collection (Manassas, VA, USA). IL-3 was purchased from Peprotech, Inc. (Princeton, NJ, USA). Fluorescently labeled FcεRI and CD117-specific antibodies were obtained from BD Biosciences (San Jose, CA, USA). Fluorescently labeled recombinant chola toxin B subunit and Fura-2-acetoxymethyl ester dye were from Invitrogen (Carlsbad, CA, USA). Phosphotyrosine, Syk, phospho-Syk, PLC-γ and phospho-PLC-γ-specific antibodies for western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA). Genz-123346 was synthesized as described previously (16). ASOs specific for GCS, GM3S and LCS (see Table 1) were developed and synthesized by Isis Pharmaceuticals (Carlsbad, CA, USA). ASOs were designed by Isis Pharmaceuticals using proprietary algorithms and synthesized using the chimeric ASO design of 2-O-methoxyethylribosylphosphorothioate backbone (22, 23). All ASOs were evaluated in wild-type C57BL/6 mice for specific reduction of their target molecule by Isis (data not shown). An outline of the GSL biosynthetic pathway and the junctures where these inhibitors were designed to act are shown in Fig. 1(A). Dexamethasone was purchased from Sigma–Aldrich (St Louis, MO, USA).

**Isolation and differentiation of mast cells from bone marrow**

Mast cells were differentiated from freshly isolated mouse bone marrow cells using IL-3 as described previously (10). Briefly, mononuclear cell suspensions from bone marrow were cultured for 4 weeks in the presence of 30 ng ml−1 IL-3 with periodic replacement of the culture medium. Mast cells were then treated with the GCS inhibitor Genz-123346 for 48 h and induced to degranulate by incubating overnight with 1 μg ml−1 dinitrophenol (DNP)-specific IgE (Sigma–Aldrich) and treating with 20 ng ml−1 BSA-coupled DNP (DNP-BSA; Sigma–Aldrich) (10). Degranulation activity was determined by measuring the levels of β-hexosaminidase secreted into the medium using a colorimetric enzyme activity assay as described (10).

**Assays**

Intracellular Ca2+ release in mast cells in response to antigen-mediated activation was measured using the Ca2+-sensitive fluorescent dye Fura-2 (Invitrogen). Briefly,
anti-DNP IgE-loaded cells were incubated with 1 μM Fura-2 for 30 min at 37°C, washed and then activated with 20 ng ml⁻¹ DNP-BSA. Changes in Ca²⁺-dependent fluorescence were followed using a spectrofluorimeter (Shimadzu Corporation, Columbia, MD, USA). In each experiment, each condition had at least two replicates, from which the data were then averaged.

Phosphorylation of Syk and PLC-γ was assayed by western blotting of cell lysates obtained by lysing cells in Nonidet P-40 lysis buffer (Boston Bioproducts, Worcester, MA, USA) supplemented with protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Normalization of the amount of phosphorylated Syk or PLC-γ2 was performed as follows. First, a western blot using anti-phosphotyrosine (Fig. 2) or anti-phospho-PLC-γ2 (Fig. 3) antibodies was performed. A separate western blot of the same samples was probed with anti-total Syk or PLC-γ2 antibodies. The same amount of total protein was loaded from each sample. Normalization was performed by expressing the phosphorylated fraction as a ratio to the total amount of Syk or PLC-γ2 in each sample based on intensities detected on the two blots.

The MTT assay used to measure cell viability was purchased from Promega (Madison, WI, USA). For flow cytometry, 10⁶ cells were stained with fluorescently labeled antibodies or cholera toxin in PBS containing 1% BSA. Data were collected on a Becton Dickinson FACS Calibur (San Jose, CA, USA) and analyzed using the FlowJo version 7.2 program (Treestar, Eugene, OR, USA).

### Table 1. Sequences of ASOs used in the study against enzymes in the GSL biosynthesis pathway

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<th>Symbol</th>
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<tr>
<td>Control ASO</td>
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<td>N/A</td>
<td>141923</td>
<td>5'-CCTCCCTGAAGTTCCC-3'</td>
</tr>
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Fig. 1. (A) The GSL biosynthetic pathway. The enzymes targeted in the studies and the interventional agents utilized are indicated in the figure. (B) Study design of the ‘prophylactic’ model used in the animal studies. (C) Study design of the ‘therapeutic’ model used in the animal studies.
Fig. 2. (A) Effect of Genz-123346-mediated inhibition of GSL synthesis on mast cell degranulation. RBL mast cells were treated with the Genz-123346 at the concentrations indicated for 48 h, loaded with DNP-specific IgE overnight in the presence of Genz-123346 and activated with antigen. The percentage of total enzyme content was measured. (B) Graph showing percentage of total enzyme content over time after antigen exposure. (C) Bar graph showing area ratio normalized to cell number for different GSL species (CRM, GL-1, GL-2, GL-3, GM1, GM3) under untreated and Genz-123346 1 μM conditions. (D) Graph showing Ca$^{2+}$ concentration over time (30 sec) with untreated and Genz-123346 1 μM conditions. (E) Western blot analysis showing anti-phosphotyrosine and anti-Syk antibodies for untreated and Genz-123346 1 μM conditions. (F) Graph showing fold increase in phosphorylation over untreated conditions. (G) Western blot analysis showing anti-phosphotyrosine and anti-PLC-γ2 antibodies for untreated and Genz-123346 1 μM conditions. (H) Graph showing fold increase in phosphorylation over untreated conditions.
Measurement of mRNA and lipid levels

Total mRNA was prepared from cells using the RNeasy Mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. GM3S mRNA levels were quantified by real-time PCR assays using primer and probe sets supplied by Applied Biosystems (Foster City, CA, USA). Lipid levels were quantified using liquid chromatography-coupled mass spectrometric analysis of cell lysates. Briefly, cell pellets were homogenized in a methanol extraction buffer and briefly centrifuged to pellet cell debris. Supernatants were collected and added to vials containing internal standards and analyzed on an HPLC-coupled mass spectrometry system consisting of an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1200 HPLC and an API-4000 mass spectrometer (Applied Biosystems). Samples were stored at 9°C during analysis in the autosampler. The HPLC was run in isocratic mode using a normal phase silica column. Mass spectrometry was performed in the multiple reactions monitoring mode.

Mouse model of airway hyperresponsiveness

We used two different treatment regimens in a mouse model of airway hyperresponsiveness: a prevention (prophylactic) or treatment (active therapeutic) model. In the prophylactic model, mice were pre-sensitized by intra-peritoneal injections of OVA/Alum or PBS/Alum on days 1 and 14 (‘sensitization’) and then challenged with OVA in PBS intra-nasally between days 41 and 44 (‘challenge’). Mice were treated between days 28 and 44 by intra-nasal instillation of 200 μg kg⁻¹ ASO delivered in 100 μl PBS intra-nasally (‘treatment’). In the therapeutic model, mice were pre-sensitized with OVA/Alum or PBS/Alum on days 1 and 14 (‘sensitization’) and then challenged with OVA in PBS intra-nasally between days 27 and 30 followed by treatment with 500 μg kg⁻¹ ASO between days 55 and 73 (‘treatment’) using intra-nasal instillation and rechallenge with OVA between days 69 and 73 (‘challenge’). The study outlines of both the prophylactic and the therapeutic models are shown in Fig. 1(B and C). All intra-nasal instillations were performed under anesthesia using 3% isoflurane in oxygen. Plethysmography was performed as per the manufacturer’s instructions (Buxco Research Systems, Wilmington, NC, USA) 1 day prior to euthanasia. Briefly, mice were exposed to increasing concentrations of metacholine (Sigma–Aldrich) aerosolized in PBS and enhanced pause was measured. Bronchoalveolar lavage fluid (BALF) was collected using 25-gauge blunt needles. Cells from BALF were stained using DiffQuik reagents and the different cell types (eosinophil and neutrophil granulocytes, monocytes/macrophages and lymphocytes) counted using the DiffQuik staining kit (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Cytokine levels in BALF were measured using cytokine-specific ELISA kits (eBioscience, San Diego, CA, USA and R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Statistics

Groups were compared using the Mann–Whitney nonparametric test. A value of P < 0.05 was considered statistically significant.

Results and discussion

Reducing GSL biosynthesis through inhibition of GCS attenuates mast cell activation and degranulation

To investigate the potential role of GSLs in the pathogenesis of asthma, we examined the relative abilities of a small molecule inhibitor of GCS, Genz-123346, at ameliorating the cascade of signaling and cellular events that contribute to the presentation of this disease. Genz-123346 inhibits the first rate-limiting step in the biosynthesis of GSLs (15, 16) and thereby blocks the synthesis of several GSL species in the GSL pathway.

As mast cell activation and degranulation is a primary mediator of asthma and allergy, the effects of inhibiting GSL synthesis on allergen-mediated signal transduction and degranulation in mast cells were first evaluated. The immortalized RBL-2H3 mast cell line or primary mast cells differentiated from normal mouse bone marrow were treated with Genz-123346 for 48 h and then loaded with DNP-specific IgE in the presence of the drug. Following loading with IgE over a period of 16 h, antigen–IgE-complex-mediated activation of cellular events was measured. Figure 2 shows that treatment of RBL-2H3 cells (Fig. 2A) or primary mast cells (Fig. 2B) with Genz-123346 significantly inhibited degranulation and soluble enzyme secretion in a dose-dependent manner. This effect was somewhat more pronounced on the RBL-2H3 cell line than on the primary mast cells (Fig. 2A and B). The reason for this discrepancy is unclear but may be due to differences in the metabolism and physiology of the two cell types. For example, RBL-2H3 and bone marrow-derived mast cells harbored different composition of GSLs as detected by mass spectrometry and different GSL species were affected differently in RBL-2H3 and bone marrow-derived mast cells by Genz-123346 treatment (data not shown). The observed inhibitory effect (even at the highest drug concentration) was not due to either induction of cell death as the viability of the cells treated with the drug was...
not different from those of untreated controls or loss of FcεRI levels on the cell surface as the treatment did not alter cell surface expression levels of FcεRI as assessed by flow cytometry (data not shown). Inhibition of GCS did not lead to an increase in ceramide levels (Fig. 2C), consistent with previous reports (15, 16). However, drug-mediated reduction of several GSL species levels was noted by mass spectrometry (Fig. 2C). We surmise from these in vitro studies that reducing GSL biosynthesis through inhibition of GCS can lead to significant suppression of mast cell function.

To investigate the basis for this suppression of mast cell activity, the intracellular signaling events following treatment with Genz-123346 were examined. Cross-linking of FcεRI in the cell membrane of mast cells reportedly induces the phosphorylation of the immunoreceptor tyrosine-based activation motif motifs in the intracellular chains of the receptor complex (5). This, in turn, leads to activation of the Syk tyrosine kinase and PLC-γ2 through intermolecular phosphorylation, elevation of cytoplasmic Ca²⁺ concentration and consequent initiation of the cascade of events that trigger mast cell degranulation. Examination of DNP-BSA-activated primary mast cells showed that treatment with 1 μM Genz-123346 resulted in a reduction in the magnitude of Ca²⁺ flux in the cells (Fig. 2D). Treatment with 1 μM Genz-123346 also significantly decreased tyrosine phosphorylation of both Syk kinase (Fig. 2E and F) and PLC-γ2 (Fig. 2G and H). This suggests that inhibition of GSL synthesis following treatment with 1 μM Genz-123346 had reduced the activities of these intracellular signaling pathways. Hence, the basis by which Genz-123346 attenuated the activated mast cell response was likely due in part through modulation of the intracellular signaling events mediated by Syk kinase and PLC-γ2. We elected not to study the effect of transfecting mast cells with ASOs designed to inhibit the targeted enzymes in the GSL pathway as they were sensitive to the transfection reagents and methodologies attempted (data not shown).

**Mast cells lacking GM3S exhibited reduced degranulation upon activation**

Inhibition of GCS activity by Genz-123346 led to a reduction not only of cellular glucosylceramide levels but also the broad spectrum of GSLs that resided downstream of this metabolic pathway. To probe further the role and identity of the GSLs that may be involved in modulating mast cell activation, additional studies were performed using primary mast cells isolated from a GM3S knockout mouse (21).
These mice exhibit a decreased incidence of insulin-resistant diabetes but are otherwise viable and healthy. Heterozygous mice carrying one copy of GM3S display a 50% lower level of GM3 (21). We chose to study GM3 because this particular GSL may play an important role in lipid raft activation and cellular adhesion (24–26). It is also a key precursor for the synthesis of a variety of GSLs that reside downstream of this biosynthetic step (21).

Differentiated mast cells isolated from wild-type mice (GM3S+/−), GM3S knockout mice (GM3S−/−) and heterozygous (GM3S+/−) littermates were subjected to activation as described above. Measurement of their subsequent degranulation activity showed that degranulation activity of mast cell isolated from GM3S−/− mice was suppressed when compared with that of cells obtained from heterozygous GM3S+/− or wild-type mice (Fig. 3A). This suppression was similar to that noted earlier with mast cells from wild-type mice that had been treated with Genz-123346 (Fig. 2B). Moreover, progressively lower levels of GM3 in the heterozygous (GM3S+/−) and homozygous (GM3S−/−) mice displayed correspondingly smaller magnitudes in antigen–IgE complex-mediated increases in Ca2+ flux and protein phosphorylation (Fig. 3B–D). However, the extent of inhibition of degranulation and Ca2+ flux in the GM3S−/− mice was not as great as that observed in wild-type cells treated with Genz-123346. As only a subset of GSLs is absent in the GM3S−/− mice, this suggests that other GSLs in this metabolic pathway also have a role in modulating these functions (27). Hence, while GM3 and the different GSLs downstream of GM3 are important components of the observed activation-induced degranulation activity of mast cell isolated from GM3S−/− mice, it is also likely that other GSLs contribute to this effect. To test this hypothesis, GM3S−/− mast cells were treated with Genz-123346 to inhibit the synthesis of all GSLs. Treatment of GM3S−/− mast cells with Genz-123346 further decreased activation-induced degranulation (Fig. 3E). These results suggest that additional GSL species (in addition to those that reside downstream from the block of GM3S) are likely engaged in the regulation of mast cell degranulation.

The basis by which changes in GSL levels may alter mast cell function is of obvious interest. Previous in vitro studies have suggested a correlate between the aggregation status of lipid rafts and activation of mast cells (4, 7–11). However, the data supporting this premise remain controversial. For example, studies have shown that IgE–antigen complex-mediated cross-linking of FcεRI promoted the segregation of the FcεRI-IgE-antigen complexes into detergent-resistant membrane fractions (reviewed in ref. 28). Corroborating data supporting a role of the lipid rafts in mast cell function were the demonstration that disruption of cholesterol-rich microdomains in the membrane led to inhibition of mast cell degranulation (11). However, another study that sought to address the effect of cholesterol deprivation on mast cell degranulation (by treating with β-methyl-cyclohexatin) did not show an impact on degranulation (12). Several studies, including those by our laboratory, have revealed a possible link between the composition of GSLs in lipid rafts and intracellular signaling (6, 15, 16, 24, 26, 28). For example, we have reported that decreasing the GSL levels in mouse models of type 2 diabetes can restore insulin receptor signaling (16). Moreover, reducing the synthesis of GSLs in T cells similarly could abate the key intracellular signaling events that lead to their activation (Y. Zhu, unpublished data). Taken together, these studies indicate that altering the composition of GSLs on lipid rafts can significantly attenuate activation-induced degranulation of mast cells. A nonoptimal composition of GSLs in the rafts may have rendered the resident intracellular signaling molecules less responsive to physiological stimuli.

Inhibitors of GSL biosynthesis abated airway hyperresponsiveness in a mouse model of asthma

The in vitro data suggesting an effect of inhibition of GSL synthesis on mast cell activation led us to consider testing this concept as a potential therapeutic intervention for asthma. The effects of GSL biosynthesis blockade were evaluated in an OVA-induced murine model of airway hyperresponsiveness. Balb/c mice were first sensitized by OVA/Alum immunization and subsequently subjected to additional challenges with OVA as described in Methods. As the exposure of Genz-123346 in the lung following oral delivery was relatively low (data not shown) and because intra-nasal instillation of the drug was limited by formulation considerations, we elected to block GSL biosynthesis in the lung using ASOs. Feasibility of therapy using intra-nasal delivery of ASOs has been validated in a similar mouse model of airway hyperreactivity by using ASOs that target the alpha chain of the IL-4 receptor complex or the expression of CD86 in the airways (22, 23). Three ASOs specific for GM3S, GCS and LCS were generated and validated for these studies. Intra-nasal administration of the ASO into the mice was facile and well tolerated even at high concentrations (500 μg kg−1). The effect of administering the different ASOs on several pulmonary parameters in the mouse model of airway hyperresponsiveness was evaluated. These included measurements of (i) enhanced pause using conscious unrestricted plethysmography, (ii) accumulation of inflammatory cells in the airways (22, 23). Three ASOs specific for GM3S, GCS and LCS were generated and validated for these studies. Intra-nasal administration of the ASO into the mice was facile and well tolerated even at high concentrations (500 μg kg−1). The effect of administering the different ASOs on several pulmonary parameters in the mouse model of airway hyperresponsiveness was evaluated. These included measurements of (i) enhanced pause using conscious unrestricted plethysmography, (ii) accumulation of inflammatory cells in the airways (22, 23). Three ASOs specific for GM3S, GCS and LCS were generated and validated for these studies. It was elected to use enhanced pause as a measurement of airway obstruction because it has been shown to be a good indicator of airway responsiveness in Balb/c mice in response to allergen challenge (31) and to correlate with airway resistance and intrapleural pressure in response to metacholine challenge (32). However, we appreciate that this approach to assessing airway resistance is controversial as it is thought to measure obstructions in both the nasal and the pulmonary compartments (29, 30).

As the above in vitro studies had suggested that GM3 is an important modulator of mast cell degranulation, an ASO specific for GM3S was first evaluated in the OVA-induced airway hyperresponsiveness mouse model. Initial studies were performed in a prophylactic model where the ASO was administered after immunization with OVA but prior to a subsequent challenge with antigen (see Methods). Dexamethasone administered at 2.5 mg kg−1 intra-peritoneally was used as positive control for treatment efficacy. Plethysmographic analysis of mice treated with the ASO targeting GM3S showed a significant reduction in enhanced pause as
Fig. 4. (A) Effect of administering a GM3S-specific ASO into a ‘prophylactic’ mouse model of airway hyperreactivity. Experimental airway hyperreactivity was induced as described in Methods and the mice were treated in the prophylactic mode. Graph depicts enhanced pause as a function of increased metacholine concentration following treatment with either a control or GM3S-specific ASO. (B) mRNA levels of GM3S were determined by real-time PCR of cells isolated from the BALF of mice immunized with OVA. (C) GSL levels in the control and GM3S ASO-treated animals were determined by mass spectrometry. (D) Absolute numbers of eosinophil granulocytes and (E) the percentage of eosinophil granulocytes in the BALF of the animals were also quantitated. (F) The concentration of CCL11 (eotaxin) in the BALF was measured using CCL11-specific ELISA.
compared with control ASO indicating that treatment had significantly decreased airway obstruction (Fig. 4A). Reduction of GM3S mRNA and GM3 lipid levels in GM3S ASO-treated mice were confirmed by real-time PCR and mass spectrometry, respectively (Fig. 4B and C). Administration of the ASO to GM3S resulted in an ~50% decrease in lung GM3S mRNA levels and 75% decrease in GM3 levels when compared with those administered a control ASO (Fig. 4B and C). The reduction in airway obstruction observed by plethysmography in the GM3S ASO-treated mice was correlated with a reduced number of airway cells and a lower percentage of eosinophil granulocytes in their BALF when compared with those administered the control ASO (Fig. 4D and E). We also detected lower levels of eotaxin (CCL11), a key chemokine responsible for the recruitment of eosinophil granulocytes in the GM3S ASO-treated animals (Fig. 4F and data not shown). Consistent with this observation is the finding of reduced numbers of eosinophils in the BALF (Fig. 4D and E). Levels of the inflammatory cytokines IL-5 and IL-13 (but not IL-4) were also reduced in the BALF of GM3S ASO-treated animals (data not shown). Although there was a trend toward lower mucus production and fibrosis in lungs of mice treated with the GM3S-specific ASO as compared with mice treated with the control ASO, these parameters did not reach statistical significance (data not shown). Neither were there any differences in the serum levels of OVA-specific IgG and IgE levels between control and GM3S-specific ASO-treated mice. These data demonstrate that treatment with the GM3S-specific ASO had modulated both the recruitment and degranulation activity of inflammatory cells in the lung. However, further studies will be required to determine the relative contribution of these two activities toward airway hyperreactivity. Hence, the observed decrease in airway obstruction following intra-nasal instillation of GM3S-specific ASO was likely due, at least in part, to inhibition of the local immune responses in the lung.

To further examine the robustness of this therapeutic strategy, the ASOs were also analyzed in OVA-immunized mice after they had received a subsequent challenge with the antigen (see Methods). In this therapeutic modality, intra-nasal administration of the GM3S-specific ASO following a subsequent challenge with OVA was ineffective at ameliorating the airway obstruction or associated eosinophilia (Fig. 5A and B). This inability to address the disease manifestations was not due to a failure of the ASO to effect a reduction in GM3S mRNA levels (Fig. 5C). An ~50% decrease in GM3S mRNA levels was noted in the mice administered the GM3S-specific ASO when compared with mice given the
control ASO. Hence, although reducing GSL synthesis through inhibition of GM3S could impact disease pathogenesis, this approach was only limited to conditions where the inflammatory stimulus was modest. It was ineffective at subduing the increased inflammatory stimuli associated with repeated challenges with antigen.

To ascertain whether inhibition of other enzymes in the GSL biosynthetic pathway may similarly impact disease manifestations in this mouse model of asthma, ASOs to GCS and LCS were also evaluated. As these enzymes reside upstream of GM3S, ASO-mediated reduction of their levels should lead to decreases in additional GSLs, including GM3 as well as its downstream derivatives (Fig. 1A and ref. 33). Intra-nasal instillation of either of these two ASOs into mice using the prophylactic model was effective in dampening the inflammatory sequelae in the lungs (Fig. 6A and C). The magnitude of the effects was similar to that observed in mice treated with the ASO specific to GM3S. However, as with the ASO for GM3S, testing the ASOs for GCS and LCS in the more stringent therapeutic model of asthma was largely ineffective (Fig. 6B and D). In fact, contrary to our expectations, we detected a trend toward increased enhanced pause using LCS ASOs in the therapeutic model; however, this did not reach statistical significance (Fig. 6D). These data suggest that a number of different GSLs, besides GM3, can have a role in modulating the inflammatory response in a mouse model of asthma. Reducing the levels of these GSLs could partially abate but not prevent the pathogenesis of asthma in this murine model. In this regard, this strategy is less effective than those reported previously using ASO to CD86 and IL-4 receptor-α (22, 23). Importantly, comparing the effect of inhibiting GSL biosynthesis in the prophylactic and therapeutic models suggests that GSLs may play a more involved role during the induction phase of the disease. Our results also indicate that although GM3 is an important GSL, other GSLs are likely to be involved.

In summary, we have demonstrated that the composition of GSLs in airway cells could influence the allergic response to stimulation with OVA. Reducing the levels of these GSLs in the airways, using either a small molecule inhibitor of GCS or ASOs to GCS, GM3S or LCS abated the response to subsequent challenges with OVA in the prophylactic but not therapeutic model. The effects were likely directed toward either decreasing the recruitment of inflammatory cells or reducing their subsequent activation. As such, they contribute to our understanding of the biology of mast cells and granulocyte activation and of the local events associated with the pathogenesis of asthma.

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References


