CD40 Enhances Sphingolipids in Orbital Fibroblasts: Potential Role of Sphingosine-1-Phosphate in Inflammatory T-Cell Migration in Graves’ Orbitopathy

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Purpose. Graves’ orbitopathy (GO) is an autoimmune orbital disorder associated with Graves’ disease caused by thyrotropin receptor autoantibodies. Orbital fibroblasts (OFs) and CD40 play a key role in disease pathogenesis. The bioactive lipid sphingosine-1-phosphate (S1P) has been implicated in promoting adipogenesis, fibrosis, and inflammation in OFs. We investigated the role of CD40 signaling in inducing S1P activity in orbital inflammation.

Methods. OFs and T cells were derived from GO patients and healthy control (Ctl) persons. S1P abundance in orbital tissues was evaluated by immunofluorescence. OFs were stimulated with CD40 ligand and S1P levels were determined by ELISA. Further, activities of acid sphingomyelinase (ASM), acid ceramidase, and sphingosine kinase were measured by ultraperformance liquid chromatography. Sphingosine and ceramide contents were analyzed by mass spectrometry. Finally, the role for S1P in T-cell attraction was investigated by T-cell migration assays.

Results. GO orbital tissue showed elevated amounts of S1P as compared to control samples. Stimulation of CD40 induced S1P expression in GO-derived OFs, while Ctl-OFs remained unaffected. A significant increase of ASM and sphingosine kinase activities, as well as lipid formation, was observed in GO-derived OFs. Migration assay of T cells in the presence of SpHKi inhibitor revealed that S1P released by GO-OFs attracted T cells for migration.

Conclusions. The results demonstrated that CD40 ligand stimulates GO fibroblast to produce S1P, which is a driving force for T-cell migration. The results support the use of S1P receptor signaling modulators in GO management.

Keywords: Grave’s orbitopathy, sphingosine-1-phosphate, sphingolipids, inflammation

Graves’ orbitopathy (GO) is an autoimmune orbital disorder associated with Graves’ disease where pathogenic autoantibodies to the thyrotropin hormone receptor (TSHR) lead to stimulation of the thyroid gland and overproduction of thyroid hormones. Orbital fibroblasts (OFs) in the orbital tissue express TSHR and are activated by autoantibodies or T cells. The binding and activation of OFs by T cells via CD40 ligation results in increased proliferation, release of cytokines such as IL-6, IL-8, or prostaglandins, and adipogenic differentiation.

Sphingolipids like ceramide, sphingosine, and sphinosine-1-phosphate (S1P) play a crucial role in a multitude of diseases like cancer, bacterial infection, and autoimmune diseases. While ceramide and sphingosine are described as mediators of apoptosis and cell cycle arrest, S1P plays an important role in inflammation, proliferation, and survival. The controversial roles of these sphingolipids require a strict regulation of sphingolipid metabolism and the involved enzymes. Acid sphingomyelinase (ASM), hydrolyzing sphingomyelin to ceramide, is activated in signaling pathways of death receptors like tumor necrosis factor receptor 1 (TNFR1), CD95, DR4 and DR5 inducing apoptosis. Ceramide can be converted to sphingosine by acid ceramidase (AC), which has antibacterial function in lung disease via the production of sphingosine. On the other hand, overproduction of AC protects tumor cells from cell death. The phosphorylation of sphingosine by sphingosine kinase 1 or 2 (SphK1/2) results in the formation of S1P, a sphingolipid that induces intracellular signaling pathways but can also be released and initiates signaling cascades from outside the cell. S1P is well known as a chemoattractant for T cells and is central for T-cell egress from lymphatic organs. Recently, it has been shown that S1P is involved in GO by promoting inflammation, adipogenesis, and fibrosis. In this study we investigated the role of S1P produced by orbital fibroblasts in T-cell trafficking in GO. Our studies demonstrated that CD40 ligation stimulates GO fibroblast to produce S1P, which in turn triggers T-cell migration into orbital tissues.
METHODS

Cell Culture and T-Cell Isolation

Orbital adipose/connective tissue biopsy specimens and blood samples of GO patients with severe GO (clinical activity score >4) and of healthy control persons undergoing cosmetic surgery for other reasons were collected according to the guidelines of the Declaration of Helsinki and were approved by the local ethics committee. Orbital fibroblasts were obtained from orbital fat tissues of 12 GO patients and 10 healthy control persons by Ficoll density gradient centrifugation. T cells were isolated by using a human CD4 T-cell isolation kit and AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured in RPMI medium containing 10% FBS and 5% each HEPES (pH 7.4), penicillin/streptomycin, sodium pyruvate, and glutamine (cell lines GO-TC1-3 and Ctl-TC1, 2).

S1P Immunostaining

One-micrometer sections from orbital fat tissue of control persons and GO patients were analyzed for S1P expression. The sections were rehydrated, antigens were retrieved, and tissue sections were blocked followed by overnight incubation at 4°C with anti-SIP antibody (from S1P-ELISA Kit; Echelon Biosciences, Inc., Salt Lake City, UT, USA). Secondary Alexa-Fluor-488–labeled anti-mouse IgG (ThermoFisher Scientific, Waltham, MA, USA) was applied to each section. Sections were rehydrated, antigens were retrieved, and tissue sections were blocked followed by overnight incubation at 4°C with anti-SIP antibody (from S1P-ELISA Kit; Echelon Biosciences, Inc., Salt Lake City, UT, USA). Secondary Alexa-Fluor-488–labeled anti-mouse IgG (ThermoFisher Scientific, Waltham, MA, USA) was applied to each section.

CD40 Stimulation

1.5 × 10⁷ cells (GO-OFs or Ctl-OFs) were seeded in six-well plates and cultured overnight by using standard conditions previously described. Before stimulation, medium was removed and cells were washed and incubated with PBS for 15 minutes on ice. The stimulation was started by removal of the PBS and the addition of 400 μL of either prewarmed PBS or 1 mL starvation medium (0.5% FBS; for longer time points) containing 100 ng/mL recombinant human soluble CD40L (formally called CD154, extracellular domain; Enzo Life Sciences, Farmingdale, NY, USA). Cells were incubated at 37°C for the indicated time points and the reaction was stopped by removing the liquid and shock freezing the cells in liquid nitrogen.

SIP ELISA

Cell lines were stimulated as described above and lysed by using 25 mM Tris/HCl (pH 7.4), 0.1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 10 mM sodium fluoride and sonicated for 5 minutes. Protein determination was performed by using Bradford reagent. SIP ELISA Kit (Echelon Biosciences) was used according to manufacturer’s instructions, and absorbance at 450 nm was read with a microplate reader. SIP contents were determined by using a standard curve.

Enzyme Activity Assays

For ASM activity assay, cell lysates were diluted 1:5 and incubated (1:1, vol/vol) with substrate buffer (0.05 mM Bodipy-C12-sphingomyelin [Life Technologies, Carlsbad, CA, USA], 0.2% Igepal, 0.2 M sodium acetate buffer [pH 5.0], 0.2 mM ZnCl2) at 37°C for 30 minutes. For AC activity assay, cell lysates were incubated (1:1, vol/vol) with substrate buffer (0.2 mM NBD-C12-ceramide [Cayman-Chemical, Ann Arbor, MI, USA], 0.2% Igepal, 0.2 M citrate/phosphate buffer [pH 4.5], 0.3 M NaCl, 10% FBS) at 37°C for 2 to 4 hours depending on protein concentration. For SphK assay, cell lysates were incubated (1:1, vol/vol) with substrate buffer (30 μM NBD-sphingosine [Avanti-Polar-Lipids, Alabaster, AL, USA], 50 mM HEPES, 250 mM NaCl, 2 mM ATP, 0.2% Triton X-100, 30 mM MgCl₂, 4 mg/mL BSA [pH 7.5]). All reactions were stopped by adding ethanol (10×) and samples were centrifuged (13,000g for 10 minutes). Supernatants were analyzed with an Acquity UHPLC system (Waters, Milford, MA, USA). Quantification of the product peak was performed by using Waters Empower Software according to standard curves.

T-Cell Migration Assay

1 × 10⁵ OFs were seeded in 24 wells and starved for 24 hours (OF medium with 0.5% FBS) followed by stimulation with 100 ng/mL CD40L in starved medium for 3 hours/24 hours with or without 1 hour prestimulation with 0.5 μmol sphingosine kinase inhibitor (SKH-1; Abcam, Berlin, Germany) at 37°C. Isolated T cells were starved (T-cell medium with 0.5% FBS) for at least 2 hours at 37°C. In this assay 0.75 × 10⁵ T cells were applied to each insert (PET membrane, 8-μm pore size [T-cell diameter: 7 μm]; Corning GmbH, Karlsruhe, Germany), which were placed into the wells containing the orbital fibroblasts of GO patients or control persons. After 24 hours of incubation at 37°C inserts were removed and the supernatants of every well were centrifuged at 300g for 10 minutes. T cells were resuspended in cell dissociation buffer and stained by addition of calcein (1:400; Thermo Fisher Scientific) followed by fluorescence reading at 485 nm excitation and 520 nm emission. The amount of T cells was determined by using a standard curve.

Lipid Analysis

Ceramide and sphingosine were extracted and quantified as recently described. Briefly, lipid extraction of cells was performed by using C17-ceramide and C17-SPH as internal standards. Sample analysis was carried out by rapid-resolution liquid chromatography–MS/MS using a Q-TOF 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive ES mode. The precursor ions of sphingomyelin (m/z 500.289), C17-SPH (m/z 286.274), and ceramides (C16-ceramide [m/z 520.508], C17-ceramide [m/z 534.524], C18-ceramide [m/z 548.540], C18:1-ceramide [m/z 546.524], C20-ceramide [m/z 576.571], C22-ceramide [m/z 604.602], C24-ceramide [m/z 632.634], C24:1-ceramide [m/z 630.618]) were cleaved into the fragment ions of m/z 282.280, m/z 268.264, and m/z 264.270, respectively. Quantification was performed with Mass Hunter Software (Agilent Technologies).

Statistics

Statistical analyses were performed with GraphPad Prism 7 (GraphPad, San Diego, CA, USA) and carried out with unpaired t-test, 1-way-ANOVA, or 2-way-ANOVA with Bonferroni multi-comparison post hoc test. Data are presented with ± standard
RESULTS

S1P Is Increased in GO Orbital Fat Tissue and Derived OFs

Semiquantitative analysis of S1P abundance in orbital fat tissues from three GO patients and three Ctl persons was performed by immunofluorescent staining (Figs. 1A, 1B). Adipose tissue of GO patients showed higher amounts of S1P than that of healthy persons (Figs. 1A, 1B; additionally, orbital fat tissue morphology is shown in Supplementary Fig. S1A). Furthermore, localization of S1P was limited to cell nuclei in Ctl tissue, whereas S1P was also localized in the cell membrane area of adipose cells in GO tissue. OFs present in the orbital adipose tissue play an important role in the development of GO. Therefore, OFs were derived from Ctl or GO patients and the S1P expression was investigated. Basal levels of S1P were not different between Ctl- and GO-OFs; however, GO-OFs showed a significant increase in S1P expression in response to CD40 stimulation, while Ctl-OFs remained unaffected (Fig. 1C). Ctl- and GO-OFs displayed similar expression of CD40 (Supplementary Figs. S1B, S1C).

Stimulation of CD40 Upregulates Sphingolipid Pathway in GO-OFs

To investigate why GO-OFs displayed higher S1P levels, the cells were stimulated with CD40L, and sphingolipid levels and activity of enzymes were analyzed by mass spectrometry and ultra performance liquid chromatography. Ceramide and sphingosine levels were enhanced in GO-OFs compared to Ctl-OFs after 60 minutes of stimulation with CD40L (Figs. 2A, 2B). The activity of ASM was significantly increased in GO-derived OFs after 30 minutes of stimulation, whereas control OFs remained unaffected (Fig. 3A). However, the activity of AC was not altered upon CD40L stimulation in both types of fibroblasts (Fig. 3B). An increase of SphK activity in OFs derived from GO patients was detected after 120 minutes of stimulation (Fig. 3C). The results indicate that CD40 stimulation activates main enzymes of the sphingolipid pathway. The observed elevated SphK activities are most likely responsible for enhanced S1P expression in GO-OFs after CD40 ligation.

Increase in S1P Leads to Enhanced Attraction of T Cells Toward GO-OFs

Since S1P is known to act as a chemoattractant for T cells, migration assays were performed to investigate the interaction between GO-OFs and T cells. T cells isolated from healthy persons migrated toward S1P in a concentration-dependent manner (Supplementary Fig. S2A). Furthermore, T cells migrated toward S1P in a concentration-dependent manner (Supplementary Fig. S2A). Furthermore, T cells isolated from either GO patients (Fig. 4A) or healthy individuals (Fig. 4B) showed significantly increased migration toward CD40L-stimulated OFs derived from GO patients, whereas T-cell movement toward stimulated Ctl-OFs remained unaffected (Figs. 4A, 4B). To prove whether the increase in T-cell migration is due to enhanced production of S1P by GO-OFs, a migration assay was
performed with an SphK inhibitor (SKI-I) to block S1P production in OFs. By using the SphK inhibitor the elevation of T-cell migration toward GO-OFs was abolished (Fig. 4C). The results suggest S1P as a chemoattractant for T cells involved in GO.

**DISCUSSION**

We demonstrated that CD40 stimulation of GO patients’ OFs leads to increased activities of enzymes involved in the sphingolipid pathway. Subsequently, levels of ceramide, sphingosine, and S1P were elevated in GO-derived OFs compared to healthy control OFs. T cells showed a high migration potential toward CD40L-stimulated GO-OFs as a consequence of S1P production. Comparison of T cells from healthy and GO patients demonstrated that T-cell attraction depends on the origin of OFs and is independent from the source of T cells. The results support the notion that T-cell invasion into the orbital tissue of GO patients is triggered by S1P in response to CD40 ligation. Binding of T cells to OFs via CD40 could drive progression of the disease by increasing the amount of S1P, resulting in a vicious cycle (Fig. 5).

S1P is involved in several diseases like cancer, inflammatory bowel disease, chronic kidney diseases, and multiple sclerosis. Recently, it has been shown that S1P also plays a role in GO by promoting inflammation, adipogenesis, and fibrosis. In our study we found increased S1P expression in the orbital adipose/connective tissue of GO patients. Interestingly, S1P in orbital adipocytes of healthy persons was limited to the nucleus, whereas S1P in fat cells of GO patients was mainly found in the membrane area. Nuclear S1P is produced by SphK2 and is involved in gene regulation by inhibiting histone deacylases. Mechanisms for SphK2 activation are not yet fully understood. In contrast, it is well known that SphK1, which is mainly present in the cytoplasm, can be activated by...
proinflammatory cytokines like TNF-α and is known as a cell survival promotor. Further investigations are needed to support the hypothesis of whether SphK1 is activated in orbital adipose tissue of GO patients.

To elucidate the mechanisms of increased S1P levels in orbital tissue we examined the sphingolipid pathway in orbital fibroblasts, which—together with adipocytes—may be responsible for enhanced S1P levels in GO adipose tissue. OFs act as effector cells in the development of GO and can be activated by CD40, a key player in Grave’s disease and in autoimmunity in general. Furthermore, it is known that stimulation with CD40 leads to activation and translocation of SphK1 and increased S1P levels. We found a rapid increase of ASM activity combined with an increased ceramide amount. This is in line with findings of Grassmé and coworkers who demonstrated that upon CD40L stimulation ASM is activated, resulting in the formation of ceramide-enriched membrane platforms. The formation of ceramide-enriched membrane platforms results in the clustering of receptors, supporting the efficient signal transduction into the cells. The increased ceramide in patients’ OFs might therefore enhance so far unknown signals as compared to control cells. Ceramide platforms are necessary for efficient receptor internalization in a clathrin-dependent manner. In this context, Meyer zu...
Hörste and colleagues have demonstrated an increased clathrin phosphorylation in GO patients' cells. Clathrin, as a major component for endocytosis, is involved in the formation of vesicles for intracellular trafficking and although CD40 is so far not connected with clathrin-dependent internalization, the activation of CD40 and generation of ceramide could support the endocytosis of receptors or sphingolipids.

Further analysis revealed that sphingosine is also increased after 30 minutes of stimulation with CD40 ligand in GO-OFs. However, this effect is not due to enhanced activity of acid ceramidase, which plays a role in several diseases like prostate cancer, melanoma, or Alzheimer's disease. Other ceramidases such as the neutral or alkaline ceramidases are therefore most likely responsible for the increased sphingosine levels. They differ in cellular localization, function, and the preferred type of ceramide to degrade (for review see Ref. 48).

Recently, the importance of S1P has been demonstrated in fibrosis, adipogenesis, and in the inflammatory reaction in Graves' orbitopathy. It has also been demonstrated that mRNA for S1P receptors and SphK1/2 are upregulated after treatment with IL-1β, supporting our observations that proinflammatory events activate S1P formation. CD40 stimulation in B cells results in S1P formation necessary for optimal B-cell activation, differentiation, and IgG class switch, demonstrating the important role of S1P in the immune system. Our T-cell migration assay showed clearly that increased migration is triggered by GO patient OFs no matter if the T cells were derived from healthy controls or GO patients. It is already known that S1P functions as a chemoattractant for T cells. Studies with mouse T cells derived from spleen have demonstrated that full expression of S1P1 and S1P4 receptors is necessary for increased migration.

The stimulatory effects could be stopped by treating the T cells with fingenolind (FTY720) acting as an S1P receptor modulator. In this context, patients suffering from multiple sclerosis treated with the S1P analogue fingenolind (FTY720, Gilenya; Novartis, Nürnberg, Germany) show less T-cell invasion into the brain, resulting in milder progression of the disease. In other autoimmune diseases like autoimmunity uveoretinitis, systemic lupus erythematosus, rheumatic arthritis, or a pemphigus vulgaris mouse, application of anti-CD40L antibody improves the disease outcome. Since fingenolind is an already available drug, treatment of GO patients with active inflammation with fingenolind could be a therapeutically useful approach in combination with anti-CD40L antibodies to calm down inflammation and avoid progression via T-cell infiltration.

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