Inhibitory Effect of Idelalisib, a Selective Phosphatidylinositol 3-Kinase δ Inhibitor, on Adipogenesis in an In Vitro Model of Graves’ Orbitopathy

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PURPOSE. Emerging evidence indicates that the phosphatidylinositol 3-kinase (PI3K)-AKT pathway is involved in the pathogenesis of Graves’ orbitopathy (GO). In this study, the therapeutic effects of idelalisib, a selective PI3Kδ inhibitor, on adipogenesis were evaluated in GO orbital fibroblasts in vitro.

METHODS. Orbital fibroblasts were cultured from orbital connective tissues obtained from individuals with GO and healthy control subjects. Cells were pretreated with idelalisib for 1 hour before stimulation with IL-1β. Inflammatory cytokine expression was measured by Western blotting and ELISAs. The adipogenesis-related downstream mediators of the PI3K/AKT cascade, that is, forkhead box protein O1 (FOXO1) and mammalian target of rapamycin (mTOR), also were measured by Western blotting. After adipogenic differentiation and idelalisib treatment, cells were stained with Oil Red O and the levels of peroxisome proliferator activator γ (PPARγ) and CCAAT-enhancer-binding proteins (C/EBP) α/β were determined by Western blot analyses.

RESULTS. AKT phosphorylation decreased in a dose-dependent manner upon treatment with idelalisib in GO and non-GO orbital fibroblasts. Treatment with idelalisib inhibited the IL-1β-induced expression of IL-6 and IL-8. Idelalisib attenuated the phosphorylation of mTOR and FOXO1, downstream regulators of the PI3K pathway. Oil Red-O staining results revealed a decrease in lipid droplets and suppressed expression of PPARγ and C/EBPα/β upon treatment with idelalisib during adipose differentiation.

CONCLUSIONS. Idelalisib inhibited proinflammatory cytokine production and adipogenesis in GO orbital fibroblasts in vitro. These results support the potential use of PI3K inhibitors in GO management.

Keywords: adipogenesis, Graves’ orbitopathy, idelalisib, orbital fibroblast, phosphatidylinositol 3-kinase

Graves’ orbitopathy (GO) is an autoimmune disorder of the orbit involving the infiltration of T cells, B cells, plasma cells, and macrophages.1,2 Inflammation causes extensive remodeling of connective tissues in the orbit leading to enlargement of extraocular muscles and orbital adipose tissue.3 Current evidence indicates that orbital fibroblasts are the key effector cells in GO, and the inflammatory response in orbital fibroblasts is triggered by stimulation of thyrotropin receptor (TSHR) and insulin-like growth factor-1 receptor (IGF-1R).3–5

The adenylyl cyclase-cAMP cascade was traditionally thought to act as a primary intracellular signaling pathway in orbital fibroblasts in GO, but recent studies have highlighted the role of the phosphatidylinositol 3-kinase (PI3K)-AKT signaling cascade.3,6 PI3K is a lipid kinase that generates phosphatidylinositol-3,4,5-trisphosphate (PI(3, 4, 5)P3), a second messenger essential for the translocation of AKT to the plasma membrane, where it is phosphorylated and activated.7 It acts as an intracellular signal transduction pathway, mediating cell survival/apoptosis, protein synthesis, cell growth, and cell proliferation.8,9 Emerging evidence indicates that the PI3K-AKT pathway is involved in GO, in addition to several autoimmune diseases.3,6,10

PI3K has important roles in T cell development, survival, proliferation, and differentiation.11 As PI3K has complementary roles in many aspects of immune function, it is a potential target for anti-inflammatory treatments.12,13 Moreover, PI3Kδ, which is required for effective humoral and cell-mediated immune responses, recently has been investigated as a potential therapeutic target in autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus.10,13 Given the fundamental role of inflammation in the pathogenesis of GO, we investigated the therapeutic effect of idelalisib, a recently developed potent and selective inhibitor of the kinase activity of PI3Kδ, in an in vitro model of GO. Idelalisib is globally approved as an oral treatment for B cell hematologic malignancies.14,15
**Materials and Methods**

**Reagents and Chemicals**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). Idelalisib, a selective inhibitor of phosphatidylinositol 3-kinase δ, was purchased from Selleck Chemicals (Houston, TX, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay solution was purchased from Promega Corporation (Madison, WI, USA). The ELISA kit for IL-6 and IL-8 was purchased from R&D Systems. Oil Red O was purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA).

**Cell Culture and Differentiation Protocols**

Orbital adipose/connective tissue explants were obtained as surgical waste during decompression surgery in five patients with GO (2 females, 3 males; age, 44–57 years). Normal control tissues were harvested during upper lid blepharoplasty from five individuals with no history or clinical evidence of thyroid disease or GO (3 females, 2 males; age, 47–62 years). All five patients with GO achieved stable euthyroidism at surgery, and their clinical activity scores at surgery were less than 4. Additionally, no patient with GO received steroid treatment or radiotherapy for at least 3 months preoperatively. The institutional review board of the Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) approved the study (4-2017-0839), and written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. This study followed the tenets of the Declaration of Helsinki.

Primary cultures of orbital fibroblasts were established as described in our previous study. Briefly, minced tissue was placed directly in 1:1 DMEM: F12 medium with 20% FBS and antibiotics. When growth of fibroblasts was observed, monolayers were passaged serially with trypsin/EDTA solution, and cultures were maintained in DMEM with 10% FBS and antibiotics. Cells between the second and fifth passages were used for analyses.

To evaluate the antiadipogenic effects of idelalisib, orbital fibroblasts were differentiated into adipocytes under a previously reported protocol. Briefly, cells were cultured in serum-free DMEM supplemented with T3, insulin (Boehringer-Mannheim, Mannheim, Germany), carboprostacyclin (cPGI2; Calbiochem, La Jolla, CA, USA), and dexamethasone. A peroxisome proliferator activator γ (PPARγ) agonist, rosiglitazone (10 μM; Cayman, Ann Arbor, MI, USA), also was added from day 1 for further stimulation of adipogenesis.

**Cell Viability Assay**

To evaluate the effect of idelalisib on orbital fibroblast viability, orbital fibroblasts obtained from patients with GO were seeded on 24-well culture plates (1 × 10^4 cells/well) and treated with various concentrations of idelalisib (1, 10, and 100 nM, and 1 and 10 μM) for 48 and 72 hours with or without 10 ng/mL IL-1β. Thereafter, MTS solution was added and the plate was incubated again for 4 hours under the same conditions. dye absorbance was measured at 490 nm using an ELISA plate reader. Cell viability is expressed as a percentage relative to untreated control cells.

**Western Blotting**

Western blot analyses were performed as described previously. To assess activation of the PI3K pathway by IL-1β and its inhibition by idelalisib, confluent orbital fibroblasts were pre-exposed to serial concentrations of idelalisib for 24 hours, followed by stimulation with IL-1β for 1 hour. The phosphorylation levels of AKT, forkhead box protein O1 (FOXO1), and mammalian target of rapamycin (mTOR) were assessed by comparing the total and phosphorylated protein levels. The relative amount of protein in each immunoreactive band was quantified by densitometry and normalized to the concentration of β-actin in the same sample.

**RESULTS**

Nuclear proteins for the FOXO1 Western blot analysis were isolated from GO orbital fibroblasts using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer’s protocol. The relative amount of nuclear protein in each immunoreactive band was normalized to the concentration of Lamin A/C in the same sample.

Anti-PI3Kδ, anti-phosphorylated-AKT, anti-AKT, anti-ICAM-1, anti-phosphorylated-NFκB, anti-NFκB, anti-phosphorylated-FOXO1, anti-FOXO1, anti-phosphorylated-mTOR, and anti-mTOR antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IL-6, anti-IL-8, anti-PPARγ, anti-CCAAT-enhancer-binding protein (C/EBP) α, anti-C/EBPβ, anti-Lamin A/C, and anti-β-actin antibodies were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Enzyme-Linked Immunosorbent Assay**

Supernatants from culture media of confluent orbital fibroblasts from individuals with and without GO were collected, and IL-6 and IL-8 levels were determined using a commercially available ELISA kit as described previously. Samples were diluted 1:10 before analysis, and the average value of five assays was used for statistical analyses.

**Oil Red O Staining**

Orbital fibroblasts differentiated into adipocytes were stained with Oil Red O as described previously. After 10 days of adipocyte differentiation, cells were stained with an Oil Red O working solution. Stained cells were visualized and photographed at ×40, ×100, and ×200 magnifications using an Olympus BX60 light microscope (Olympus, Melville, NY, USA). To measure lipid accumulation quantitatively, cell-bound Oil Red O was solubilized with 100% isopropanol, and the optical density of the solution was measured using a spectrophotometer at 490 nm. Experiments for quantitative assessment of adipogenic differentiation were performed in duplicate using cells from different donors, and the results were normalized to the absorbance of untreated differentiated control cells.

**Statistical Analysis**

All experiments were performed using cells from at least three different samples, which were assayed in duplicate each time. Differences in parameter estimates between the experimental and control groups were assessed by the Student’s t-test or Wilcoxon rank-sum test using R version 3.1.2 (R Foundation, Vienna, Austria). P < 0.05 was considered significant.
and GO orbital fibroblasts (Fig. 1). Treatment with 10 ng/mL IL-1β for 72 hours induced proliferation of normal and GO orbital fibroblasts and idelalisib did not decrease viability to <95% in either cells (Supplementary Fig. S1).

**Effect of Idelalisib on the PI3K Pathway**

The major effector protein of the PI3K pathway is phosphorylated AKT (p-AKT), which is a potent protein kinase. To investigate the effect of idelalisib on the PI3K pathway, a Western blot analysis of PI3Kδ, AKT, and p-AKT was performed using GO and non-GO orbital fibroblasts and the ratio of p-AKT and total AKT was evaluated. PI3Kδ was expressed in orbital fibroblasts from individuals with and without GO and was more highly expressed in cells from individuals with GO than in cells from controls (Fig. 2). As shown in Figure 3, AKT phosphorylation was dose-dependently inhibited by idelalisib in GO and non-GO cells.

**Idelalisib Reduces IL-1β–Induced Inflammatory Cytokines in GO Orbital Fibroblasts**

We investigated the effects of various doses of idelalisib on IL-6, IL-8, and ICAM-1 expression in response to IL-1β (10 ng/mL, 16 hours) challenge in orbital fibroblasts from patients with GO and normal control orbital fibroblasts. Western blotting of protein extracts from orbital fibroblast cultures showed that IL-1β induced IL-6, IL-8, and ICAM-1 production compared to baseline levels in GO and non-GO cells. In the idelalisib-pretreated samples, IL-1β–induced increases in IL-6, IL-8, and ICAM-1 were inhibited in a dose-dependent manner in GO cells, but not in non-GO cells (Fig. 4). ELISA of the supernatant of cultured cells showed that pretreatment with 50 nM idelalisib for 24 hours resulted in significant inhibition of the IL-1β–induced secretion of IL-6 and IL-8 in primary cultured orbital fibroblasts from patients with GO, while increased secretion was maintained in cells from individuals without GO (Fig. 5). IL-1β–induced phosphorylation of NFκB decreased in a dose-dependent manner in response to treatment with idelalisib in orbital fibroblasts from GO individuals, but was unaffected in cells from individuals without GO (Supplementary Fig. S2).
Phosphorylation Levels of FOXO1 and mTOR Were Reduced by Idelalisib in GO Orbital Fibroblasts

We examined the effect of idelalisib on adipogenesis-related downstream effectors, that is, FOXO1 and mTOR, in the AKT pathway, in primary cultured orbital fibroblasts from individuals with GO. In particular, we investigated the effects of increasing doses of idelalisib on phosphorylated FOXO1 (p-FOXO1), total FOXO1, phosphorylated mTOR (p-mTOR), and total mTOR in response to IL-1β (10 ng/mL, 1 hour) in orbital fibroblasts from GO orbital fibroblasts. Idelalisib had a dose-dependent inhibitory effect on the IL-1β-induced phosphorylation of FOXO1 and mTOR in orbital fibroblasts from individuals with GO (Fig. 6A). FOXO1 is translocated from the nucleus to cytoplasm when it is phosphorylated and its activity is correlated with its extranuclear translocation; we also evaluated the nuclear and cytosolic fractions of this protein after treatment with IL-1β and idelalisib. Activation of GO orbital fibroblasts by IL-1β decreased the nuclear fraction of FOXO1 and increased the cytoplasmic fraction, and idelalisib dose-dependently reversed these effects (Fig. 6B).

Idelalisib Reduces IL-1β-Induced Adipogenesis in GO Orbital Fibroblasts

To determine the effect of idelalisib on inflammation-induced adipogenesis of orbital fibroblasts, GO cells were treated with idelalisib and 10 ng/mL IL-1β during adipocyte differentiation. After 10 days of adipocyte differentiation, addition of IL-1β to the adipogenic medium significantly increased adipogenesis compared to that observed under control conditions (Fig. 7A). When idelalisib (50–1000 nM) was added to the adipogenic medium, the number of adipocytes was reduced and accumulation of lipid droplets induced by IL-1β was suppressed. The optical density of Oil Red O-stained cell lysates showed that idelalisib-treated cells had decreased absorbance at 490 nm (Fig. 7A). Based on Western blot analyses, idelalisib had dose-dependent inhibitory effects on the expression of the adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ (Fig. 7B). Each experiment was performed using three GO cells from different patient samples and samples were assayed in duplicate.

DISCUSSION

We used IL-1β-treated primary cultured orbital fibroblasts to evaluate the pathogenesis of GO. AKT was activated by IL-1β, and IL-1β-induced inflammatory cytokine production and adipogenesis, which are the major pathologic mechanisms associated with the development of GO, were inhibited by idelalisib, a specific PI3Kδ inhibitor.

PI3K signaling has a complex role in regulation of the immune system and has been identified as a therapeutic target for autoimmune diseases. In a model of rheumatoid arthritis, inflammatory cytokines induced PI3Kδ mRNA in cultured synoviocytes, and the selective inhibition of PI3Kδ diminished joint erosion in an animal model. PI3Kδ also has potential roles in the pathogenesis of systemic lupus erythematosus and multiple sclerosis. Likewise, studies have identified a role of the PI3K pathway in the pathogenesis of GO. The PI3K pathway is activated by either TSHR or IGF-1R stimulation in GO orbital fibroblasts, and its activation is correlated with hyaluronan production and adipogenesis. We demonstrated that PI3Kδ also is expressed in GO orbital fibroblasts, and idelalisib, a specific PI3Kδ inhibitor, inhibits disease pathogenesis in an in vitro model of GO.

PI3K-AKT is involved in adipocyte differentiation in 3T3-L1 preadipocytes; the activation of PI3K-AKT is required during IGF-1-mediated adipogenesis, and treatment with PI3K inhibitors leads to complete blockade of adipocyte differentiation. Likewise, in GO orbital fibroblasts, AKT phosphorylation is increased by TSH and IGF-1 stimulation, which are major pathogenic stimuli in GO, and adipocyte differentiation and adipogenic markers (adiponectin, c/EBPα, and PPARγ) are reduced by treatment with the PI3K inhibitor LY294002. However, care should be taken when interpreting the results of studies using LY294002 to demonstrate the role of PI3K. Several reports have shown that LY294002 is not highly selective for PI3Ks, and it also acts as nonspecific protein kinase inhibitor. It inhibits not only mTOR,
a DNA-dependent protein kinase, but also other protein kinases, such as casein kinase 2 and Pim-1. Zhang et al. compared the inhibitory effects of LY294002 and rapamycin (mTOR inhibitor) on adipocyte differentiation in GO orbital fibroblasts. Cotreatment with rapamycin and LY294002 during adipocyte differentiation did not have an additive effect compared to treatment with rapamycin alone; the investigators concluded that mTOR is a critical driver of adipogenesis, not PI3K. Moreover, despite extensive use of LY294002 for in vitro PI3K studies, it is too toxic for in vivo analyses, resulting in severe respiratory depression and lethargy.

Proinflammatory cytokines, such as IL-6 and IL-8, are strongly associated with development of GO. The orbital fibroblasts are the major sources of inflammatory cytokines and have an important role in the development of GO. IL-6, a potent proinflammatory cytokine that activates B-cells to produce functional auto-antibodies, and IL-8, a chemotactic cytokine that recruits T-lymphocytes to the orbital tissue, have promoter regions that possess NFκB binding sites. Activation of NFκB, a ubiquitous transcription factor involved in inflammatory responses, is involved in the CD40-activated induction of proinflammatory cytokines in orbital fibroblasts.
FIGURE 5. Effect of idelalisib on IL-1β-induced secretion of IL-6 and IL-8 in GO and non-GO orbital fibroblasts. Confluent orbital fibroblasts derived from individuals with (black columns) and without (white columns) GO were untreated or pretreated with various concentrations of idelalisib for 24 hours before treatment with 10 ng/mL IL-1β for 16 hours. The supernatant of culture medium was collected and evaluated by ELISA for IL-6 and IL-8. The results are expressed as the means ± SD of three individual samples and the graphs are representative of three independent experiments. (*P < 0.05 versus IL-1β-treated cells without pretreatment).

FIGURE 6. Effect of idelalisib on the IL-1β–induced phosphorylation of FOXO1 and mTOR in GO orbital fibroblasts. Confluent orbital fibroblasts derived from individuals with GO were untreated or pretreated with various concentrations of idelalisib for 24 hours before treatment with 10 ng/mL IL-1β for 1 hour. (A) Total and p-FOXO1 levels as well as total and p-mTOR levels were determined in total protein extracts by Western blotting. (B) Expression levels of FOXO1 were evaluated by Western blotting using nuclear and cytosolic fractions. Results are presented as the mean ratio of phosphorylation and total levels of FOXO1 or mTOR ± SD of three experiments. (*P < 0.05 and **P < 0.01 versus IL-1β-treated cells without pretreatment).
We showed that idelalisib decreases the IL-1β-induced expression of IL-6 and IL-8 in GO orbital fibroblasts, but not in non-GO cells. Lack of an inhibitory effect of idelalisib on proinflammatory cytokine expression in non-GO cells probably was because idelalisib does not affect the IL-1β-induced activation of NFκB.

c/EBPs and PPARγ have been studied extensively as adipogenic transcription factors, and the FOXO transcription factor family also is thought to have a role in adipocyte differentiation. FOXO1, the most abundant FOXO isoform, is a negative regulator of adipogenesis; it inhibits adipogenesis by binding and disabling PPARγ. When FOXO1 is phosphorylated by AKT via the PI3K-AKT cascade, p-FOXO1 is transported from the nucleus to the cytosol, freeing PPARγ for activation, while cytosolic FOXO1 is ubiquitinated and degraded. In orbital fibroblasts from patients with GO, M22 (a stimulatory TSHR antibody) and IGF-1 increased cytosolic FOXO1 and decreased nuclear FOXO1, indicating that FOXO1 is a downstream mediator of the TSH and IGF1-initiated pathogenesis of GO. Moreover, FOXOs act as repressors of hyaluronan production and adipogenesis, as a FOXO enhancer (trifluoperazine hydrochloride) blocks adipogenesis in orbital fibroblasts. In this report, we showed that indirect inhibition of FOXO1 phosphorylation by idelalisib suppresses the action of PPARγ, thereby reducing adipogenic differentiation (Fig. 8).

mTOR is a downstream protein kinase of the PI3K-AKT cascade. It serves as a central signal integrator that receives signals from growth factors, nutrients, and cellular energy metabolism. In adipocytes, mTOR regulates protein synthesis, adipose tissue morphogenesis, and leptin synthesis/secretion. It is involved in the pathogenesis of GO; in particular, M22-induced hyaluronan production is downregulated by inhibition of mTOR in GO orbital fibroblasts. We showed that IL-1β induced phosphorylation of FOXO1 and mTOR, and idelalisib downregulated these phosphorylation levels, resulting in the inhibition of adipogenesis.

Orbital fibroblasts in the orbital tissues of patients with GO are constantly exposed to proinflammatory stimuli from infiltrated immune cells, predominantly T lymphocytes. In orbital fibroblasts (A) IL-1β (10 ng/mL) was added to adipogenic medium to stimulate adipogenesis during 10 days of adipocyte differentiation. Addition of idelalisib to the adipogenic medium attenuated adipocyte differentiation, as evidenced by microscopic examinations (×40) after Oil Red O staining. The optical density at 490 nm of stained cell lysates also quantitatively showed the inhibitory effect of idelalisib on the accumulation of lipid droplets. (B) Western blot analyses of PPARγ, C/EBPα and C/EBPβ expression after 10 days of adipogenic differentiation. The experiments were performed in duplicate with cells from three different donors. PPARγ, C/EBPα and C/EBPβ levels determined by densitometry were normalized to the level of β-actin in the same sample. The results are presented as the mean relative density (%) ± SD of three experiments (* P < 0.05 and ** P < 0.01 versus IL-1β-stimulated cells).
Anti-Adipogenic Effect of Idelalisib in GO

fore, primary cultures of orbital fibroblasts might not directly reflect the in vivo inflammatory condition. Proinflammatory conditions can be induced by stimulants to observe the effects of certain chemicals. IL-1β has been used widely as an effective proinflammatory stimulant in many in vitro studies of GO, and it is highly correlated with the phenotype of GO.40-42 IL-1β induces key pathogenic mechanisms, including adipogenesis, fibrosis, and the expression of proinflammatory cytokines and hyaluronan in primary culture of orbital fibroblasts from patients with GO.32,39,40,43 We showed that IL-1β induces the phosphorylation of AKT in GO orbital fibroblasts, and the subsequent production of inflammatory cytokines and adipogenesis are reversed by treatment with idelalisib.

In conclusion, we confirmed that idelalisib has antiadipogenic effects via the PI3K-AKT pathway and phosphorylation of FOXO1 and mTOR. In the pathogenesis of GO, PI3K is activated by thyrotropin, IGF-1, or cytokine receptors, of FOXO1 and mTOR. In the pathogenesis of GO, PI3K is idelalisib.

cytokines and adipogenesis are reversed by treatment with fibroblasts, and the subsequent production of inflammatory cytokines and hyaluronan in primary culture of orbital fibroblasts, and the subsequent production of inflammatory cytokines and adipogenesis are reversed by treatment with idelalisib.

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