Therapeutic effect of nintedanib, a triple angiokinase inhibitor, in orbital fibroblasts from patients with Graves' orbitopathy

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ABSTRACT

Therapeutic effect of nintedanib, a triple angiokinase inhibitor, in orbital fibroblasts from patients with Graves' orbitopathy

Graves' orbitopathy (GO) is an autoimmune disorder of the orbit, characterized by inflammation, adipogenesis, and fibrosis. Nintedanib is a triple angiokinase inhibitor approved for various fibrotic lung diseases and its potential therapeutic effect in various inflammatory diseases are under discover. Here, we investigated the potential therapeutic effect of nintedanib on adipogenesis and fibrosis in orbital fibroblasts of GO. Orbital connective tissue from GO and normal controls were obtained, and orbital fibroblasts were cultured. Cells were pretreated with nintedanib prior to stimulation with either IL-1β, TGF-β, IGF-1, or IL-11. The expression of fibrosis-related protein and intracellular signaling protein production were assessed via Western blotting. Hyaluronan and procollagen concentration were quantified using ELISA. To evaluate adipogenesis, Oil Red O staining was performed, and the levels of adipogenic transcriptional factors were determined by Western blot analysis. TGF-β induced fibronectin, collagen 1 and 3 protein expression were abrogated by nintedanib treatment. Nintedaninb decreased the phosphorylation of STAT3, SMAD 2/3, AKT, JNK, and ERK. Exposure to nintedanib also hindered adipocyte differentiation and the expression of adipogenic transcription factors including PPAR-γ, C/EBP α/β, SREBP-1, aP2, adiponectin, and leptin. Similarly, nintedanib reduced procollagen and hyaluronan secretion. Nintedanib demonstrated suppressive effect on profibrotic protein production, adipogenesis, and extracellular matrix production in *in vitro* model of GO. These findings suggest the potential therapeutic efficacy of nintedanib in GO management.

Key words : Adipogenesis, Fibrosis, Graves' orbitopathy, Nintedanib, Receptor tyrosine kinase

I. INTRODUCTION

Graves' orbitopathy (GO) is an autoimmune disorder that targets the orbit, leading to orbital connective tissue inflammation and tissue remodeling [1]. The clinical presentation of GO includes pain, eyelid swelling, proptosis, and even compressive optic neuropathy. Central to the disease process is an aberrant immune reaction on orbital fibroblasts, stimulated by autoantibodies targeting the thyroid-stimulating hormone receptor (TSH-R) and insulin-like growth factor-1 receptor (IGF-1-R) [2,3]. Activated orbital fibroblasts trigger extensive autoreactive lymphocytes infiltration and secretion of inflammatory cytokines and mediators, exacerbating the inflammatory milieu. The infiltration of T cells, B cells, and macrophages results in extensive extracellular matrix tissue accumulation, adipogenesis, and eventually fibrosis [4-6]. The clinical course of GO is variable, but usually initiates with inflammatory and adipogenic phase, followed by a chronic fibrotic phase [7]. Conventionally, high-dose systemic glucocorticoid therapy is the mainstay treatment for moderate-to-severe GO. However, its effectiveness in managing fibrosis remains limited [2,8]. Recently, new therapeutic strategies targeting immune responses at molecular level have emerged [9]. In 2020, teprotumumab, a human IGF-1-R blocking monoclonal antibody was approved for GO treatment, demonstrating efficacy in reducing proptosis [10]. Despite these advancements, the underlying fibrogenic mechanisms are not fully understood, and conventional therapies are still limited in controlling fibrosis.

Nintedanib, a small-molecule tyrosine kinase inhibitor with antifibrotic properties, was the first drug to be approved for interstitial lung diseases [11]. Nintedanib targets receptor tyrosine kinase including vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGF-R), and platelet-derived growth factor receptor (PDGF-R), as well as non-receptor tyrosine kinase such as Src family and Flt-3 [12,13]. Nintedanib prevents the downstream consequences of growth factor receptors and fibroblast activation, resulting in inhibition of angiogenesis and fibrosis [14]. Nintedanib inhibited transforming growth factor (TGF)-β induced transformation to myofibroblast in human lung fibroblasts derived from patients with idiopathic pulmonary fibrosis [15]. Additionally, nintedanib has been demonstrated to slow the progression of pulmonary fibrosis associated with scleroderma and other rheumatoid diseases and to reduce immune cell infiltration in a mouse dermatitis model [16-18].

Several studies have investigated the role of PDGF, FGF in GO pathogenesis. Serum PDGF and basic-FGF (b-FGF) levels were increased in active GO compared to inactive GO or normal controls, and their levels correlated with clinical activity scores (CAS) [19]. It has been also reported that FGF and PDGF synergistically induced hyaluronan and interleukin (IL)-6 production in GO orbital fibroblasts, and nintedanib abrogated FGF- and PDGF- induced adipogenesis [20,21].

Despite the potential anti-fibrotic and anti-inflammatory effects of nintedanib in numerous inflammatory disorders, the precise biological impact on GO pathogenesis remains unclear. Thus, this study aimed to investigate the therapeutic effects of nintedanib on the fibrotic phenotype, hyaluronan production and adipogenesis in an *in vitro* model of GO.

II. MATERIALS AND METHODS

2.1. Reagents and Cell Culture

Table 1 lists the reagents used in this study. Orbital adipose and connective tissue explants were obtained from 10 patients with GO during orbital decompression surgery and from 11 normal healthy controls during upper or lower blepharoplasty (Table 2). All GO patients were in stable euthyroid status, stable inactive GO (CAS less than 3) and were free from radiation therapy or steroid treatment for at least 3 months preoperatively. Normal healthy controls were free from thyroid diseases and autoimmune diseases. The study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (IRB approve No. 4-2023-0762). Written informed consent was obtained from all patients and this study adhered to the tenets of Declaration of Helsinki.

Table 1. The source of reagents in this study

Reagents	Source
Fetal bovine serum (FBS)	Gibco, Waltham, MA, USA
Nintedanib Oil Red O staining Antibody for α-SMA	Sigma Aldrich, Inc., St. Louis, MO, USA
Dulbecco's modified Eagle's medium (DMEM) Phosphate buffer saline (PBS) Penicillin and streptomycin	Welgene, Gyeongsangbuk-do, Gyeongsang-si, South Korea
Annexin, FITC assay Antibody for fibronectin	BD, Franklin Lakes, NJ, USA
Recombinant human interleukin IL-1β, TGF-β, IL-11, IGF-1, ELISA kit for hyaluronan and procollagen	R&D systems, Minneapolis, MN, USA
Antibodies for phosphorylated (p)- and total (t)- Akt, p-p38 mitogen-activated protein kinase (MAPK), t-p38, p-c-Jun N-terminal kinase (JNK), t-JNK, p-extracellular signal-regulated kinase (ERK), t-ERK, p-nuclear factor κ-light-	Cell signaling technology, danvers, MA, USA

chain-enhancer of activated B-cells (NF-κB), t-NF-κB, p-STAT3, t-STAT3, p-SMAD 1/2/3, t-SMAD 1/2/3/4, C/EBPα, IL-6, IL-8, MCP-1, ICAM-1, COX-2	
Antibodies for PPARγ, C/EBPβ, β-actin, collagen 3, adiponectin, leptin, aP2	Santa Cruz biotechnology, CA, USA
Antibody for collagen 1	Abcam, Cambridge, UK
Antibody for p-SMAD4	Invitrogen, Carlsbad, CA, USA

Table 2. Patients' demographics in this study

Age (years)	Sex	CAS	Duration (years)	Proptosis R/L (mm)	Type of surgery
GO patients					
57	F	0	1	18/16	Orbital wall decompression
45	M	1	1	23/23	Orbital wall decompression
22	F	1	5	21/22	Orbital wall decompression
27	F	2	2	20/23	Orbital wall decompression
23	M	0	3	21/20	Orbital wall decompression
25	F	0	3	20.5/21	Orbital wall decompression
36	M	0	3	25/26	Orbital wall decompression
48	F	0	13	20/20	Orbital wall decompression
32	F	1	4	21/22	Orbital wall decompression
41	F	1	3	22/22	Orbital wall decompression
Normal control patients					
75	F	n/a	n/a	n/a	Upper lid blepharoplasty
74	M	n/a	n/a	n/a	Lower lid blepharoplasty
68	M	n/a	n/a	n/a	Upper lid blepharoplasty
37	F	n/a	n/a	n/a	Upper lid blepharoplatsy
61	M	n/a	n/a	n/a	Upper lid blepharoplasty
57	F	n/a	n/a	n/a	Upper lid blepharoplasty

54	M	n/a	n/a	n/a	Lower lid blepharoplasty
61	F	n/a	n/a	n/a	Upper lid blepharoplasty
60	F	n/a	n/a	n/a	Upper lid blepharoplasty
59	M	n/a	n/a	n/a	Lower lid blepharoplasty
58	M	n/a	n/a	n/a	Upper lid blepharoplasty

CAS, clinical activity score; R, right eye; L, left eye; F, female; M, male; GO, Graves' orbitopathy; n/a, not applicable.

Orbital fibroblasts were isolated from orbital tissue explants and cultured as described previously [22]. After being minced, the tissue explant was placed in 1:1 Dulbecco's modified Eagle's medium (DMEM):F12 medium containing 20% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) to promote fibroblast proliferation. After confirming cell growth, the monolayers were serially passaged using trypsin/EDTA. The cell strains were stored in liquid nitrogen and cells between the third and sixth passages were used for experiments.

2.2. Cell Viability Assay

Cell viability was assessed with the Annexin/FITC assay, following the manufacture's protocol. Orbital fibroblasts were seeded into 24-well culture plates (1 x 10^5 cells/well) and exposed to serial concentrations of nintedanib (0, 0.1, 1, 2, 3, 5 μM). Subsequently, the supernatant and cells were harvested and washed with PBS. The samples were incubated with Annexin V labeled with FITC (5μL) and propidium iodine solution (5μL) for 15 minutes at room temperature in the dark. The stained cells were immediately analyzed using a flow cytometer (BD FACSymphony A5; BD Biosciences Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.3. Western Blotting Assay

Confluent orbital fibroblasts were pretreated with nintedanib (1 μM, 3 hours), followed by stimulation with TGF-β (5 ng/mL, 1, 3, or 24 hours) or IL-1β (10 ng/mL, 24 hours), to assess the fibrosis-related protein expression, signaling molecule, and inflammatory cytokine secretion by TGF-β or IL-1β and its inhibition by nintedanib. Orbital fibroblasts were incubated in an adipogenic medium for 14 days with or without simultaneous nintedanib (1 μM) treatment to

evaluate the adipogenic transcription factors. Western blot analysis was performed on orbital fibroblasts subjected to different study conditions. In detail, orbital fibroblasts were washed with DMEM and then lysed using a cell lysis buffer containing a protease inhibitor cocktail. Equal amounts of protein (50 μg) were separated by 8~15% SDS-PAGE, transferred to nitrocelluous membranes, and incubated with primary antibodies overnight at 4 ºC. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescent substrate, using an image reader (LAS-4000 mini;Fuji Photo Film, Tokyo, Japan). The relative intensity of the protein in each immunoreactive band was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland) and normalized to β-actin (positive control) levels in the same sample. The protein activation level was assessed by calculating the ratio of phosphorylated protein level to total protein level.

2.4. ELISA

Hyaluronan and procollagen secretion levels from GO orbital fibroblasts cultures in 1% FBS under nintedanib pretreatment (1μM, 1hour), followed by IGF-1 (200ng/mL, 96hours), recombinant IL-11 (rIL-11) (15ng/mL, 96hours), or TGF-β (5ng/mL, 96hours) were analyzed using commercially available ELISA kit, according to the manufacturer's protocol. After measuring the absorbance at 450 nm and calculating the binding percentage of each sample, a standard binding curve was generated to determine the concentration.

2.5. Adipogenesis

Orbital fibroblasts from GO and normal controls were incubated for 14 days to induce adipogenic differentiation. The cells were seeded into a 6-well plate. To evaluate the effect of nintedanib on adipogenesis, cells were exposed to nintedanib (1μM) and were cultured simultaneously in adipogenic solution (serum-free DMED supplemented with insulin, carbaprostaglandin, dexamethasone, T3, biotin, pantothenic acid, transferrin, and rosiglitazone, which is peroxisome proliferator activator γ (PPAR-γ) agonist) for the entire differentiation period of 14 days to evaluate the effect of nintedanib on adipogenesis. The adipogenic medium was replaced every 2 to 3 days.

2.6. Oil Red O Staining

To assess the adipocyte differentiation, orbital fibroblasts were stained with Oil Red O. Differentiated cells were washed with 1% PBS and fixated with 10% formalin for 1 hour at 4°C. Then the cells were stained with Oil Red O solution for 2 hours at room temperature and then washed with distilled water. Stained cells were visualized under light microscope (Olympus BX60; Olympus Corp., Melville, NY, USA). The optical density of each sample was measured at 490nm using a spectrophotometer.

2.7. Statistical Analysis

All experiments were performed in duplicate for at least three strains from three different individuals. The Mann-Whitney U-test and Kruskal-Wallis test were used for nonparametric data and the Kolmogorov-Smirnov test was performed for data without normal distribution. SPSS Statistics for Mac 26 (IBM, Armonk, NY, USA) was used for statistical analysis and p-value less than 0.05 was assumed to be statistically significant.

III. RESULTS

3.1. Effect of Nintedanib on Viability of Orbital Fibroblasts

The effect of nintedanib on the cellular viability of orbital fibroblasts from patients with GO and normal subjects was assessed using an Annexin V/FITC kit. The cells were treated with serial concentration of nintedanib (0 - 5 μM) for 24 hours. The result indicated that over 98% of both GO and normal orbital fibroblasts were viable at nintedanib concentrations up to 1 μM (Figure 1). Based on this result, 1 μM of nintedanib was applied for the subsequent assays.

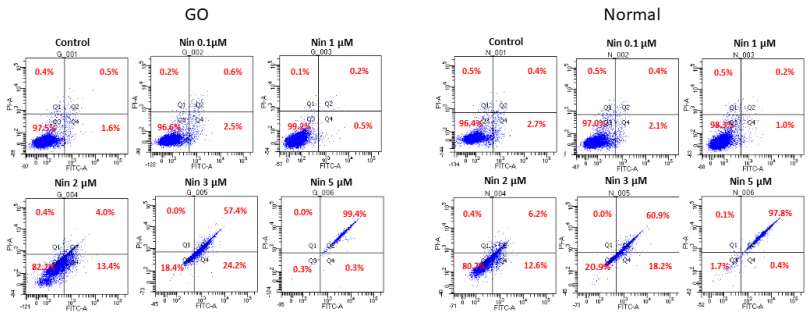

Figure 1. Effect of nintedanib on viability of orbital fibroblasts. Cell viability was determined via Annexin V/Fluorescein Isothiocyanate (FITC) assay. Various density of nintedanib (0, 0.1, 1, 2, 3, 5 μM) was treated in both GO and normal control orbital fibroblasts for 24 hours. Annexin V/Flow-Cytometric Apoptotic Assay was performed in duplicate cells from three different donors. Treatment of nintedanib concentration of 1μM or less showed viability more than 98%.

3.2. Effect of Nintedanib on Fibrotic Protein and Procollagen Production

TGF-β is crucial in fibrosis and tissue remodeling response in GO. Hence, orbital fibroblasts were stimulated with TGF-β to induce fibrotic response. Orbital fibroblasts from patients with GO and normal controls were pretreated with nintedanib (1μM, 3hours) before TGF-β (5ng/mL, 24hours) stimulation. Western blot results showed that stimulation with TGF-β led to increase fibronectin,

collagen 1 and 3 expression, which was significantly attenuated by nintendanib treatment in both GO and normal orbital fibroblasts (Figure 2A, 2B).

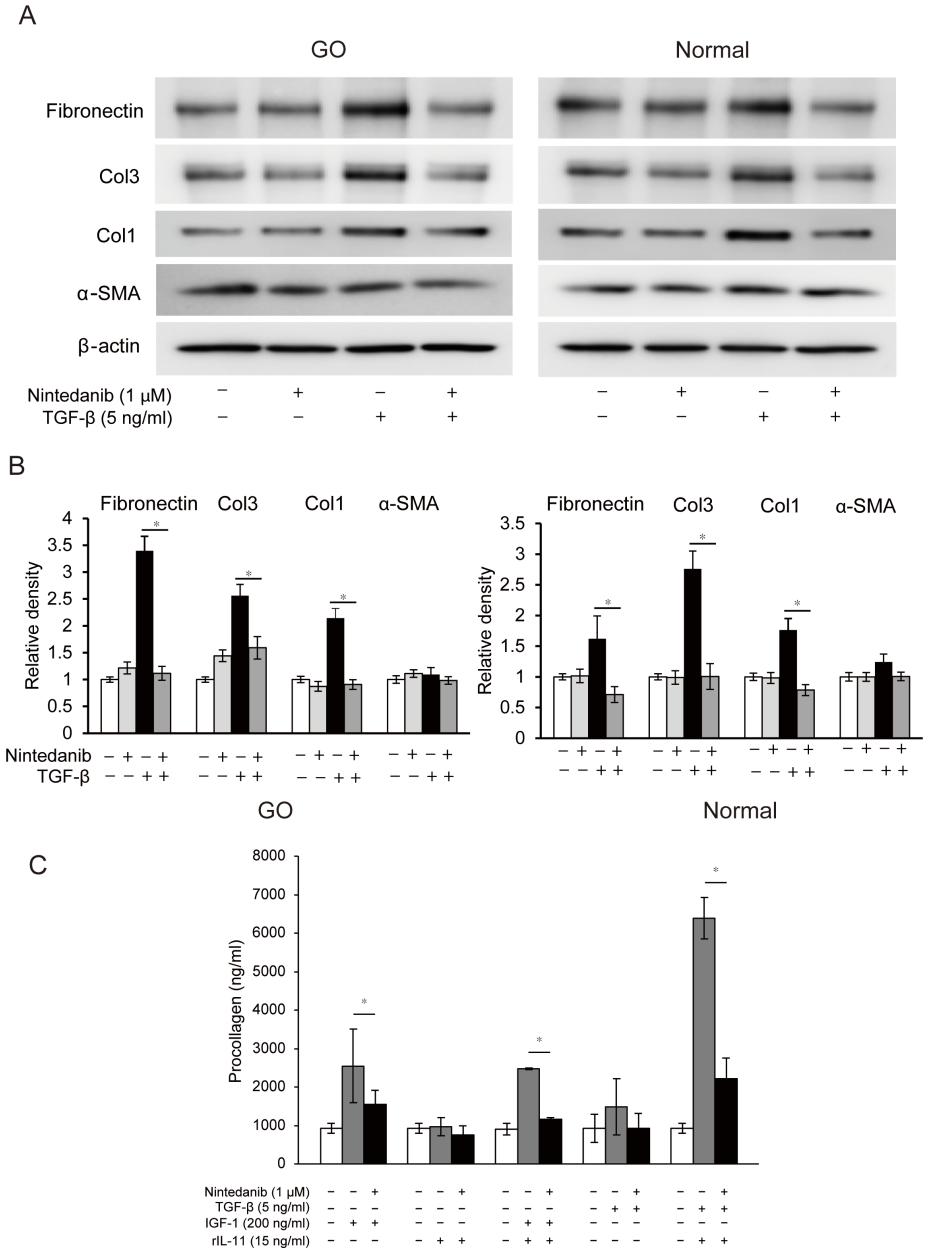

Figure 2. Effect of nintedanib on TGF-β induced fibrosis-related protein and procollagen secretion. (A, B) Orbital fibroblasts from both GO (n=3) and normal controls (n=3) were pretreated with nintedanib (1μM, 3hours) before being stimulated with TGF-β (5ng/mL, 24 hours). (A) Western blot analysis was conducted to assess the fibrosis-related protein expression, including fibronectin, collagen 3 (Col3), collagen 1 (Col1), and α-smooth muscle actin (α-SMA). (B) Quantification was performed using densitometry. The results are presented as the relative mean expression ratio ± standard deviation, normalized to the level of β-actin in each sample. Nintedanib treatment abrogated TGF-β induced fibronectin, Col3, and Col1 production. (C) Orbital fibroblasts from GO (n=3) were pretreated with nintedanib (1μM, 1hour), followed by stimulation with IGF-1 (200ng/mL, 96hours) or TGF-β (5ng/mL, 96hours) either alone or in combination with rIL-11 (15ng/mL, 96hours). ELISA analysis demonstrated that nintedanib reduced procollagen secretion induced by IGF-1 alone, as well as IGF-1 or TGF-β combined with rIL-11. (*$p<0.05$)

rIL-11, an emerging member of IL-6 family of cytokines, has gained attention for its role in promoting profibrotic gene translation in fibroblasts [23,24]. Hence, orbital fibroblasts from patients with GO were stimulated with IGF-1 (200ng/mL, 96hours) or TGF-β (5ng/mL, 96hours) either individually or in combination with rIL-11 (15ng/mL, 96hours) to induce procollagen production, with or without nintedanib (1μM, 1hour) pretreatment. Nintedanib treatment also suppressed procollagen secretion induced by IGF-1 alone, as well as by IGF-1 or TGF-β in combination with rIL-11, in GO orbital fibroblasts (Figure 2C).

3.3. Effect of Nintedanib on TGF-β Signaling Pathways

To investigate the effect of nintedanib on the TGF-β downstream signaling pathway, the expression of intracellular signaling molecules and SMAD proteins were determined using Western blot. In GO orbital fibroblasts, nintedanib treatment attenuated TGF-β (5ng/mL, 3hours) induced phosphorylation of STAT3, AKT, ERK, and JNK, whereas only STAT3 and AKT phosphorylation was suppressed in normal orbital fibroblasts (Figure 3). Similarly, nintedanib exposure suppressed TGF-β induced SMAD 1, 2, and 3 phosphorylation in GO orbital fibroblasts, whereas SMAD 2 and SMAD 3 phosphorylation was suppressed in normal orbital fibroblasts (Figure 4).

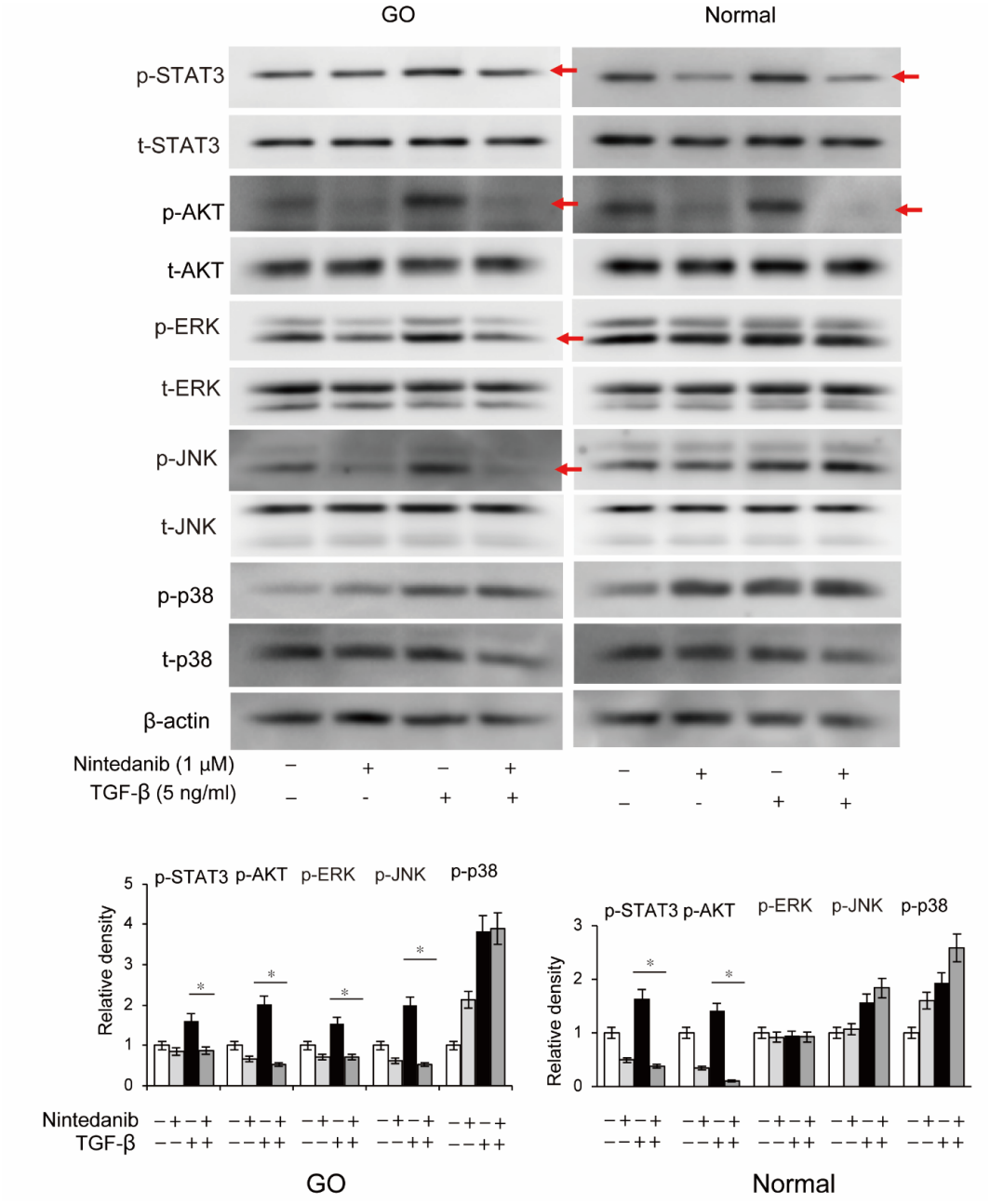

Figure 3. Effect of nintedanib on transcription factor activation.

Orbital fibroblasts were exposed to nintedanib (1μM, 3hours) and stimulated with TGF-β (5ng/mL, 3hours). Western blot assays were performed to evaluate the expression levels and phosphorylation levels of multiple transcriptional factors. Cell lysates were analyzed by Western blotting to evaluate total (t-) and phosphorylated (p-) protein expression. Treatment with nintedanib significantly blunted TGF-β induced phosphorylation of AKT, ERK, JNK, and STAT3 in GO orbital fibroblasts and AKT and STAT3 in normal orbital fibroblasts. Each column represents the mean expression ratio ± standard deviation. (*p<0.05)

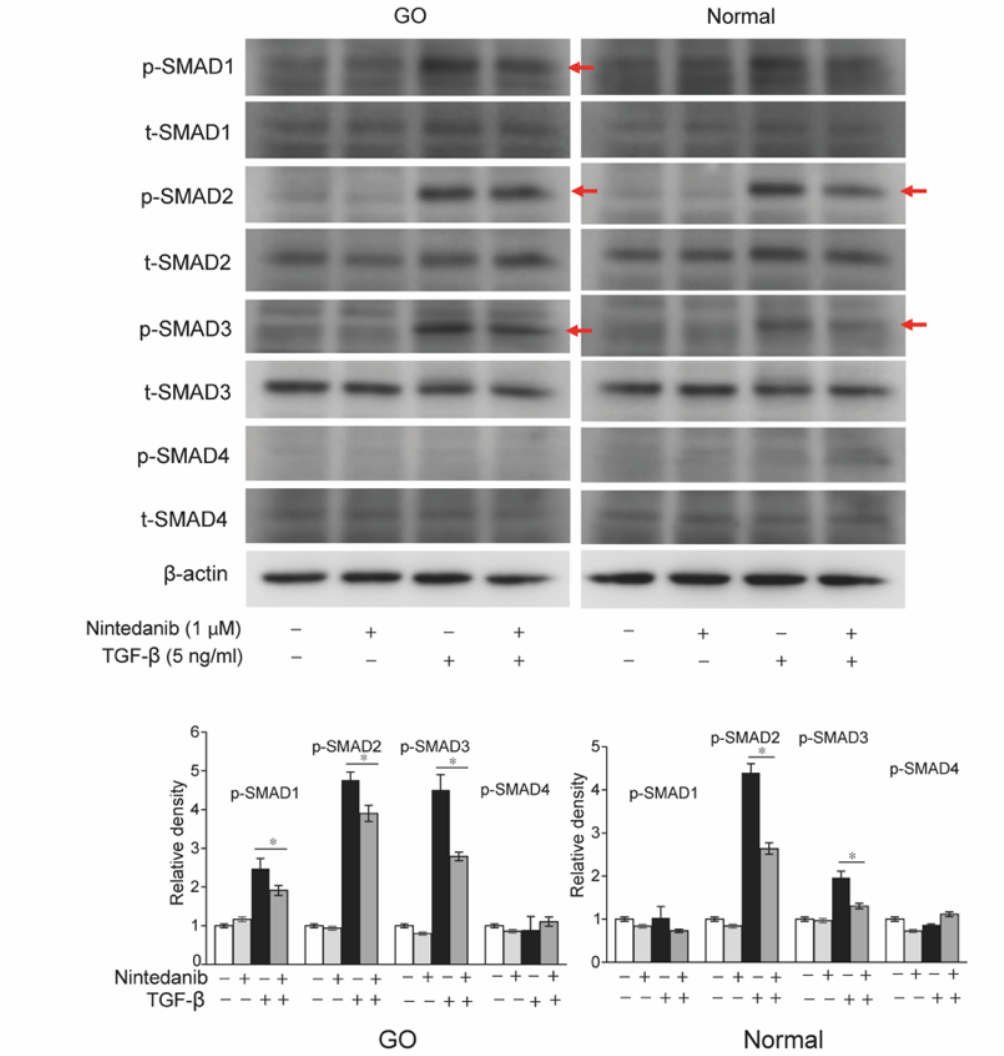

Figure 4. Effect of nintedanib on SMAD activation. Orbital fibroblasts were exposed to nintedanib (1μM, 3hours) and then stimulated with TGF-β (5ng/mL, 1hour). Western blot assay revealed that nintedanib treatment significantly suppressed the phosphorylation of SMAD 1, SMAD 2, and SMAD 3 on GO orbital fibroblasts and SMAD 2 and SMAD 3 on normal orbital fibroblasts. The results are expressed as the relative density of p-SMAD/total-SMAD and were presented as mean ± standard deviation. (* $p<0.05$)

3.4. Effect of Nintedanib on Adipogenesis

Orbital fibroblasts from patients with GO and normal controls were cultured in adipogenic medium for 14 days to induce adipogenesis, with or without simultaneous nintedanib exposure to investigate the effect of nintedanib on adipogenesis in GO. Nintedanib treatment significantly reduced the number of adipocytes and decreased lipid accumulation in GO orbital fibroblasts (Figure 5). Nintedanib substantially inhibited expression of adipogenic transcription factors including PPAR-γ, C/EBP α/β, aP2, adiponectin, and leptin during adipogenesis in Western blot analysis (Figure 6).

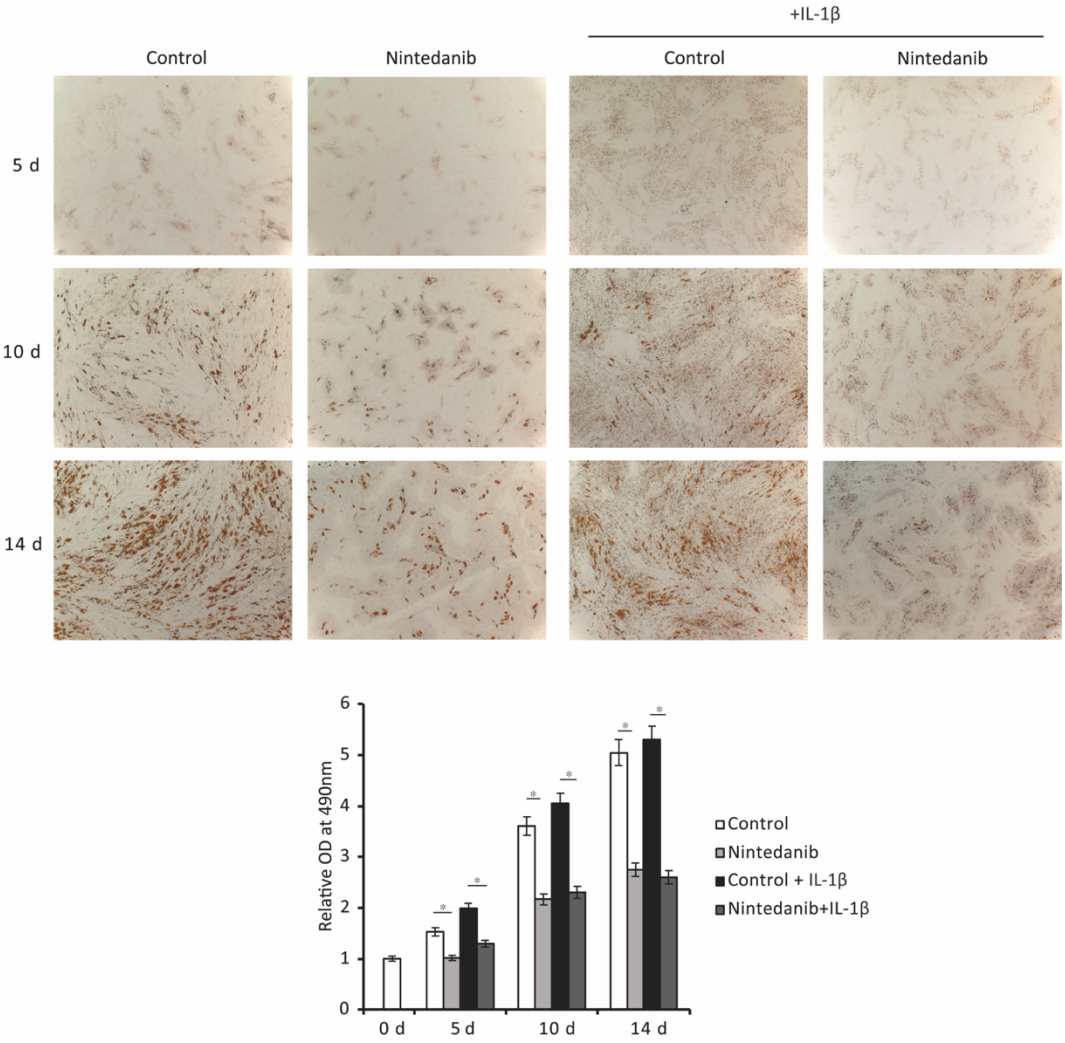

Figure 5. Effect of nintedanib on IL-β induced adipogenesis. Orbital fibroblasts from GO were incubated in adipogenic medium for 14 days with or without nintedanib treatment (1μM). Orbital fibroblasts were stimulated with IL-1β (1ng/mL). Oil red O staining revealed nintedanib exposure significantly attenuated IL-1β induced adipogenesis in GO orbital fibroblasts. Quantification by measuring optical density of Oil-red O stained lysates at 490nm also expressed same pattern. The data in the column indicate the relative optical density of each group ± standard deviation, normalized to the level of control group at day 0. (*$p<0.05$)

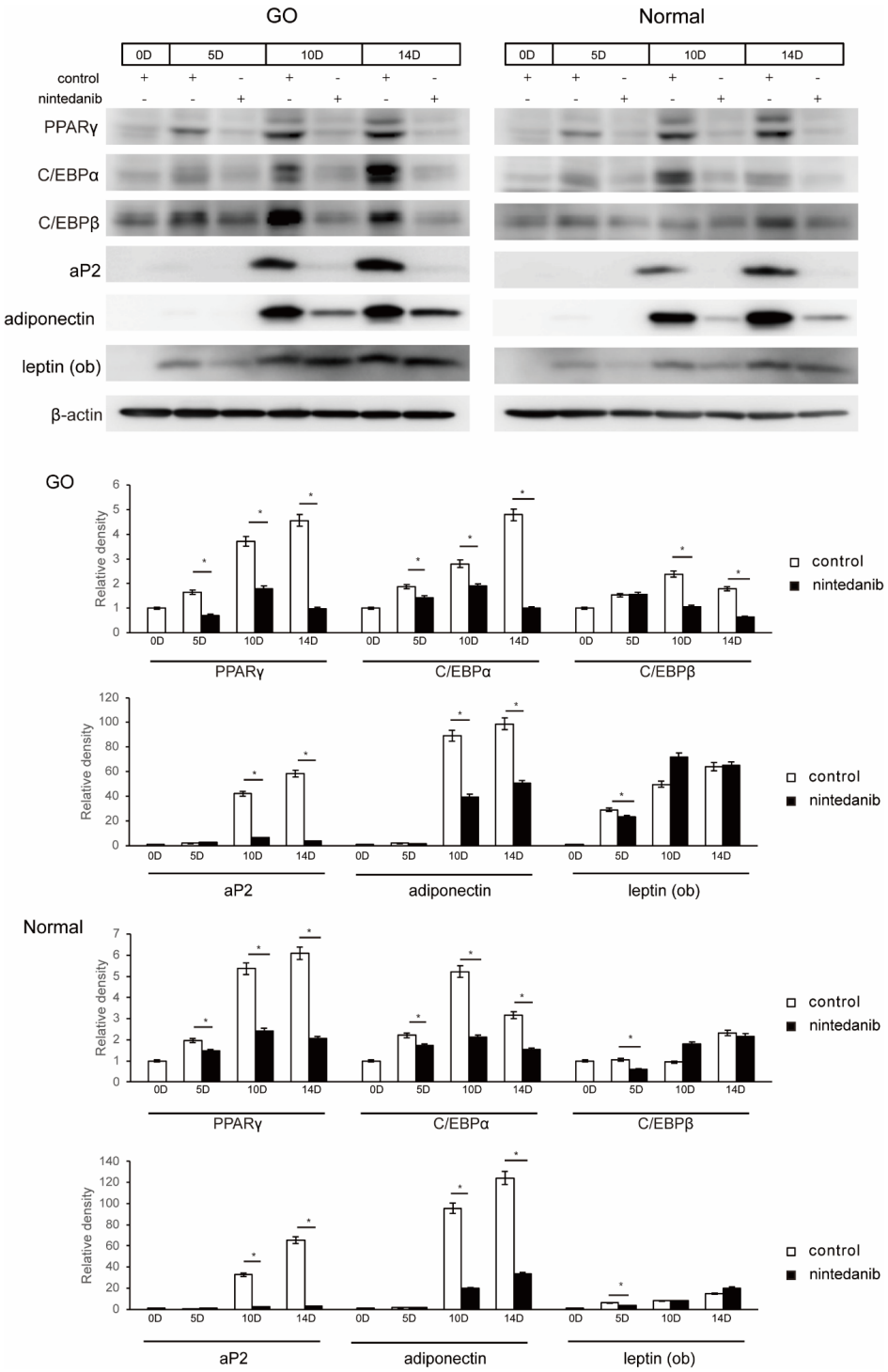

Figure 6. Effect of nintedanib on IL-β induced adipogenic transcription factor expression. Western blot analysis was conducted to determine the adipogenic transcription factor in both GO and normal orbital fibroblasts with or without nintedanib exposure. Throughout the 14-day period of adipogenesis, the cell lysates of orbital fibroblasts were collected and underwent Western blot analysis. Nintedanib treatment hindered expression of PPAR-γ, C/EBP α/β, aP2, adiponectin, and leptin expression in GO orbital fibroblasts. The results are expressed as the mean relative ratio of each adipogenic factor and were presented as mean ± standard deviation. (*$p<0.05$)

3.5. Effect of Nintedanib on Proinflammatory Cytokine Expression

Orbital fibroblasts from both GO and normal controls were treated with nintedanib and then stimulated with IL-1β. Western blot analyses revealed that nintedanib treatment suppressed IL-1β induced COX-2 expression in GO orbital fibroblast and IL-1β induced IL-6, IL-8, and COX-2 expression in normal orbital fibroblast (Figure 7).

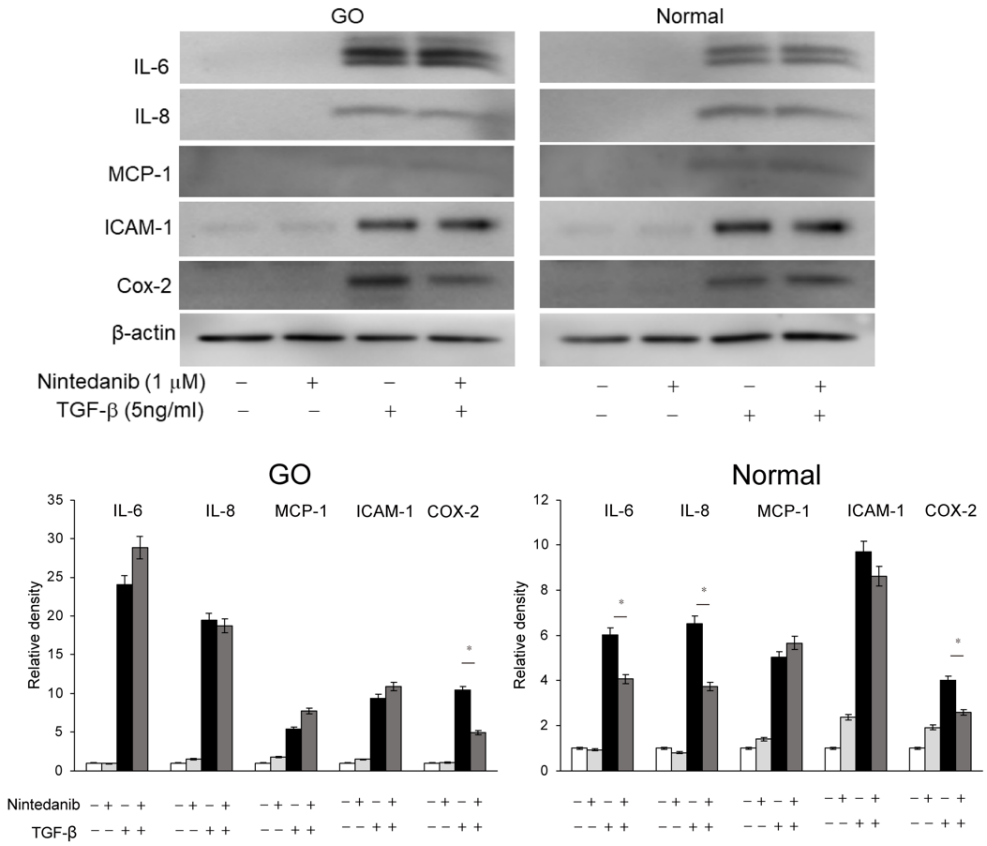

Figure 7. Effect of nintedanib on TGF-β induced inflammatory cytokine secretion. Orbital fibroblasts from both GO and normal controls were stimulated with TGF-β (5ng/mL, 24 hours) after exposure to nintedanib (1μM, 3hours). Nintedaninb treatment significantly abrogated TGF-β induced COX-2 expression in GO orbital fibroblasts and IL-6, IL-8 and COX-2 expression in normal orbital fibroblasts. (*$p<0.05$)

3.6. Effect of Nintedanib on Hyaluronan Production

To assess the effect of nintedanib on extracellular matrix synthesis, GO orbital fibroblasts were stimulated with either IGF-1 (200ng/mL, 96hours) alone or together with rIL-11 (15ng/mL, 96hours),

with or without pretreatment with nintedanib (1μM, 1hour). IGF-1 treatment significantly induced hyaluronan production and co-treatment with rIL-11 synergistically enhanced this effect nintedanib treatment significantly attenuated IGF-1 alone or in combination with rIL-11 induced hyaluronan secretion in GO orbital fibroblasts (Figure 8).

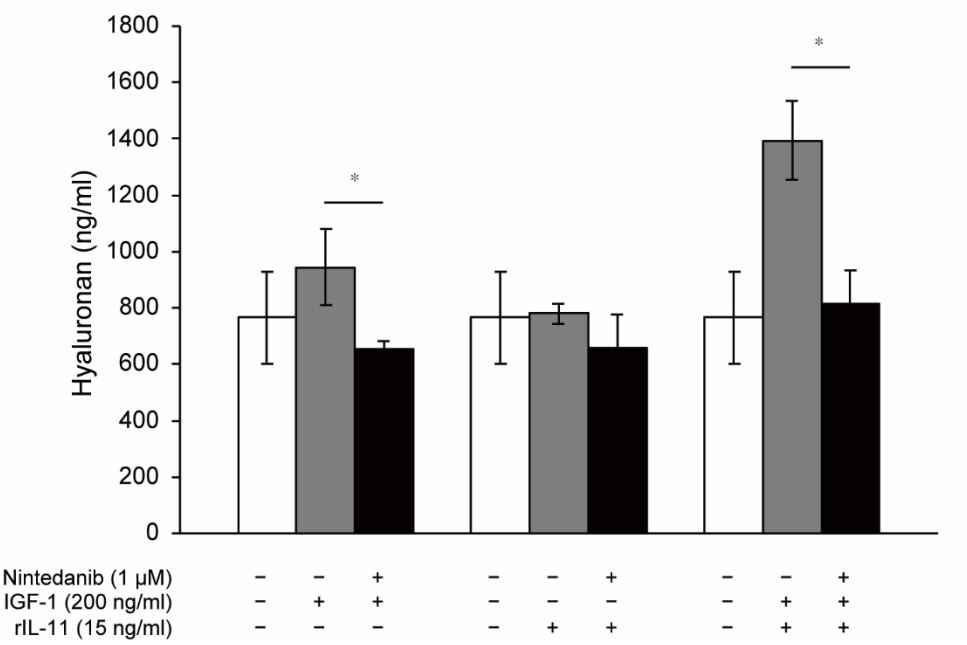

Figure 8. Effect of nintedanib on hyaluronan secretion. ELISA was performed to assess hyaluronan secretion level in GO orbital fibroblasts with or without nintedanib (1μM, 1hour) pretreatment followed by IGF-1 (200ng/mL, 96hours) or rIL-11 (15ng/mL, 96hours). Nintedanib treatment significantly inhibited hyaluronan secretion induced by IGF-1 alone or in combination with rIL-11. (*$p<0.05$)

IV. DISCUSSION

Nintedanib, a multiple tyrosine kinase involved in fibrotic mechanism, has demonstrated increased therapeutic efficacy in various fibrotic diseases [25]. Although some studies have identified the adipogenic effects of tyrosine kinases such as FGF and PDGF in GO, the mechanism and efficacy of nintedanib in GO pathogenesis has not yet been fully elucidated [20,21]. In this study, we demonstrated the ability of nintedanib to suppress GO pathogenesis, particularly in fibrosis and adipogenesis. Notably, nintedanib inhibited adipogenic differentiation and hyaluronan accumulation, which is consistent with previous research [20,21]. In addition, we discovered that nintedanib significantly curtailed TGF-β induced fibrosis-related protein expression, procollagen secretion, and adipogenic transcription factors in orbital fibroblasts. Our *in vitro* experiments suggest that nintedanib could be a promising therapeutic agent, as it significantly alleviated the chronic pathological mechanisms of GO.

Nintedanib was initially approved in idiopathic pulmonary fibrosis and chronic fibrotic interstitial lung diseases [12,16]. Numerous studies have demonstrated that nintedanib alleviates tissue fibrosis in multiple organs other than the lung. Nintedanib inhibited TGF-β induced expression of collagen 1 and α-SMA in a CCl_4-induced liver fibrogenesis mouse model and TGF-β induced fibronectin and tumor necrosis factor (TNF)-α production in primary human stellate cells [26]. In a chronic kidney disease mouse model, nintedanib reduced TGF-β induced α-SMA, collagen 1, and fibronectin expression [27]. In addition, nintedanib reduced the basal mRNA expression of collagen 1, 2, and fibronectin as well as PDGF- or TGF-β- induced collagen 1, 2, fibronectin, and α-SMA in a systemic sclerosis model [28]. Nintedanib has been shown to prevent TGF-β induced epithelial-mesenchymal transitions in retinal pigment epithelial cells [29]. As fibroblasts are activated, collagen types 1 and 3, which are major components of the extracellular matrix are overproduced, resulting in tissue scarring and fibrosis. Fibronectin, another crucial extracellular matrix protein, facilitates fibroblast recruitment and activation, thereby promoting fibrotic responses. Consistent with other cell types, nintedanib significantly curtailed TGF-β induced collagen 1, 3, and fibronectin production in orbital fibroblasts. Furthermore, our study revealed that nintedanib abrogated procollagen secretion stimulated by IGF-1 alone, as well as by the combination of IGF-1 or TGF-β with rIL-11. Procollagen, a precursor of collagen, is one of the key component

in extracellular matrix, plays a pivotal role in maintaining structural integrity. This indicates the substantial antifibrotic capability of nintedanib in the GO *in vitro* model.

TGF-β is pivotal signaling molecule in tissue fibrosis. The canonical pathway is the main mechanism of TGF-β downstream signal, which is SMAD-dependent pathway [30]. Receptor regulated SMADs, specifically SMAD 2/3 activation has been reported in various fibrotic diseases [31,32]. In human alveolar epithelial cells, nintedanib inhibited epithelial-mesenchymal transition by inhibiting TGF-β-SMAD pathway [33]. Besides the canonical pathway, TGF-β can also stimulate non-canonical pathways such as MAPK pathway, JAK/STAT3 pathway, and PI3K/Akt pathway [34]. In a pulmonary fibrosis model, it has been reported that nintedanib ameliorated fibrosis by modulating PI3K/Akt/mTOR pathway as well as ERK pathway [35,36]. The inhibitory effect of nintedanib on canonical and non-canonical pathways has been reported in various ocular diseases. Lin X et al. [37] reported that nintedanib reduced TGF-β induced phosphorylation of SMAD 2/3, p38 MAPK, and ERK 1/2 in human Tenon's fibroblasts. In a fibrotic cataract model, nintedanib relieved lens fibrosis by suppressing SMAD 2/3, Akt, ERK, JNK, and p38 [38]. We previously demonstrated that PI3K/Akt pathway and STAT 3 pathway may induce adipogenesis in GO pathogenesis [39,40]. Consistent with previous studies, we found that the SMAD 2/3 pathway, SMAD 1 pathway, STAT3 pathway, along with the MAPK pathway (ERK, JNK) and Akt pathway was suppressed by nintedanib treatment in GO orbital fibroblasts. In contrast, only SMAD 2/3, Akt, and STAT3 pathways were suppressed by nintedanib treatment in normal orbital fibroblasts. This suggests that nintedanib may exert anti-fibrotic and anti-adipogenic activity in GO orbital fibroblasts by blocking both canonical and noncanonical TGF-β signaling pathways.

Several studies have implicated the role of basic FGF, and PDGF in GO pathogenesis. Orbital FGF expression and serum FGF levels were positively correlated with the CAS [19]. Reportedly, serum FGF1 and FGF2 levels were increased in patients with GO. FGF1 increased GO fibroblasts proliferation and differentiation into adipocytes, while FGF1 inhibitor reversed these effects [41]. Virakul S. et al. [20] reported that basic FGF stimulated the production of orbital fibroblast with hyaluronan production, and co-stimulation with basic FGF and PDGF-BB synergistically enhanced IL-6 and hyaluronan production, whereas nintedanib blocked these effects. Furthermore, basic FGF induced adipogenesis in orbital fibroblasts in a concentration-dependent manner, while nintedanib had an inhibitory effect [21]. Consistent with previous reports, nintedanib significantly alleviated IL-1β induced adipogenesis in our study. In addition, nintedanib treatment

resulted in corresponding decreases in PPAR γ, C/EBP α/β, aP2, adiponectin, and leptin in GO and normal orbital fibroblasts, which indicates that nintedanib exerts its anti-adipogenic effect at the transcription level.

Studies on the role of nintedanib in inflammation have shown distinct biological effects in different cell types. Nintedanib significantly decreased the number of M1 macrophages and downregulated the phosphorylation levels of ERK, JNK, p38, PI3K, and Akt in a mouse osteoarthritis model [42]. In addition, nintedanib significantly suppressed MCP-1, TNF-α, and IL-6 expression, and macrophage infiltration in a mouse peritoneal fibrosis model and a chronic kidney disease model [27,43]. In our study, nintedanib only suppressed IL-1β induced COX-2 expression in GO orbital fibroblasts, indicating that its suppressive effect on proinflammatory cytokine production was relatively modest compared to its effects on fibrosis and adipogenesis. This suggests that nintedanib may alter adipogenesis and fibrosis rather than inflammatory milieu of orbital tissue in GO.

Excessive hyaluronan secretion and accumulation in the orbital connective tissue result in tissue remodeling in GO. It has been reported that bFGF and PDGF-BB co-stimulation enhances hyaluronan synthesis in GO orbital fibroblasts and nintedanib inhibited hyaluronan secretion [20]. Similarly, our findings confirmed that nintedanib effectively reduces hyaluronan secretion triggered by IGF-1 alone or in combination with rIL-11, which is known to promote fibrosis in GO [24]. While the precise mechanism behind extracellular matrix production is not fully understood, it seems likely that nintedanib works by blocking fibrotic and adipogenic pathways downstream of and IGF-1.

Among the various tyrosine kinases, our study focused on triple angiokinase inhibitor with anti-fibrotic activity, in an *in vitro* GO model. Several tyrosine kinases have been implicated in GO pathogenesis. We previously revealed that ibrutinib, Bruton's tyrosine kinase inhibitor, demonstrated anti-inflammatory effect by suppressing phosphorylation of Akt and NF-κB [44]. Furthermore, we demonstrated that selective IL-2 inducible tyrosine kinase inhibitor exerts anti-inflammatory effects [45]. Recently, an orally available IGF-1R inhibitor, linsitinib was proven to prevent the development and progression of GO in a murine model, especially in inflammation and adipogenesis [46].

Despite the anti-fibrotic and anti-adipogenic potential of nintedanib in GO, several limitations remain. Nintedanib is a multiple tyrosine kinase inhibitor; therefore, its precise mechanism and specific target in GO pathogenesis remain unclear and warrant further investigation.

In terms of anti-inflammatory activity, nintedanib did not show significant effects, suggesting that combined use with anti-inflammatory agents may enhance therapeutic outcomes in GO by addressing all the main pathogenic mechanisms. Additionally, the lack of an established *in vivo* model for GO limits the ability to evaluate nintedanib's therapeutic effects in a physiological setting.

To conclude, we demonstrated that nintedanib, an orally available triple angiokinase inhibitor, exert significant anti-fibrotic, anti-adipogenic effect and inhibitory effect on hyaluronan production in GO orbital fibroblasts. Nintedanib abrogated the activation of adipogenic transcription factor production and multiple transcription factors while suppressing fibrosis phenotype. Nintedanib treatment also attenuated hyaluronan production, demonstrating its potential as a therapeutic agent for GO. Further *in vivo* and clinical studies are necessary to validate the therapeutic potential of nintedanib in clinical settings.

V. CONCLUSION

Nintedanib, a triple angiokinase inhibitor, exhibited significant anti-fibrotic and anti-adipogenic effects in primary cultured orbital fibroblasts from GO. Nintedanib also inhibited hyaluronan and procollagen secretion induced by TGF-β, IGF-1, and rIL-11. These findings underscore the therapeutic potential of nintedanib in the treatment of GO.

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Abstract in Korean

삼중 angiokinase inhibitor인 nintedanib의 갑상선 안병증에서의 병태생리에 대한 치료적 효과

갑상선 안병증은 갑상선 항진증 환자에서 발생할 수 있는 자가면역질환의 일종으로, 염증, 지방생성, 섬유화를 특징으로 한다. Nintedanib은 다양한 섬유화성 폐질환의 치료로 승인된 삼중 angiokinase inhibitor로, 본 연구에서는 갑상선 안병증에서 안와 섬유모세포의 지방형성과 섬유화에 대한 nintedanib의 치료제로서의 잠재적 역할을 분석하였다. 갑상선 안병증 환자와 정상 대조군 환자의 안와 결합 조직에서 안와 섬유모세포를 배양하였다. 안와섬유모세포에 nintedanib을 처리한 후 IL-1β, TGF-β, IGF-1, 또는 rIL-11로 자극하여 염증성 cytokine 생성, 섬유화 관련 단백질 및 세포 내 신호 전달 단백질, 지방생성 전사 인자를 웨스턴 블랏 (Western blot)으로 분석하였다. 히알루론산과 프로콜라겐 농도를 ELISA로 정량화하였고, 지방세포로의 분화를 Oil Red O 염색을 통해 확인하였다. Nintedanib은 TGF-β로 유도된 fibronectin, collagen 1, 3 단백질 발현을 억제하였고, STAT3, SMAD 2/3, AKT, JNK, 및 ERK의 인산화를 감소시켰다. 또한, nintedanib은 지방세포 분화와 PPAR-ɣ, C/EBP α/β, SREBP-1, aP2, adiponectin, leptin을 포함한 지방형성 전사 인자의 발현을 저해하였다. 추가적으로, 히알루론산과 프로콜라겐의 분비도 유의미하게 감소하였다. 본 연구는 in vitro 에서 nintedanib이 갑상선 안병증 안와 섬유아세포에서 항섬유화 및 항지방형성 효과를 입증하였다. 즉, tyrosine kinase inhibitor가 갑상선 안병증 치료에 잠재적인 효과가 있음을 시사하는 바이다.

핵심되는 말 : 갑상선 안병증, 섬유화, 지방생성, Nintedanib, Receptor tyrosine kinase