

Celecoxib and Pioglitazone as Potential Therapeutics for Regulating TGF- β -Induced Hyaluronan in Dysthyroid Myopathy

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PURPOSE. To investigate the role of extraocular muscles (EOM) myoblasts in Graves ophthalmopathy (GO) pathology and the effect of a cyclooxygenase (COX)-2 inhibitor and a peroxisome proliferator-activated receptor (PPAR)- γ agonist in its treatment.

METHODS. Myoblasts were isolated and cultured from EOM of 10 patients with GO and 4 without (non-GO). The cultured myoblasts were treated with IFN- γ , insulin-like growth factor (IGF)-1, IL-1 β , and TNF- α , and the effect on PPAR- γ , COX-2, TGF- β , and thyroid stimulating hormone receptor (TSH-R) expressions were assessed using real-time (RT)-PCR, ELISA, and Western blot. The effect of a COX-2 inhibitor and a PPAR- γ agonist on the expression of TGF- β , hyaluronan synthases (HAS)-1, -2, and -3, and hyaluronan (HA) were further evaluated.

RESULTS. Real-time PCR showed significant upregulation in PPAR- γ , COX-2, TGF- β , and TSH-R mRNA expression in GO myoblasts when treated with TNF- α but not in the non-GO. While IFN- γ and IGF-1 had no significant effect, IL-1 β did upregulate COX-2 expression. These results were further confirmed by ELISA and Western blotting. Tumor necrosis factor α -induced TGF- β in turn significantly increased HA expression and HAS3 level, but not HAS1 and HAS2. The cyclooxygenase 2 inhibitor and PPAR- γ agonist substantially diminished this TNF- α -induced TGF- β , HA, and HAS3 expression.

CONCLUSIONS. These results demonstrate the role of EOM myoblasts in the pathogenesis of GO. The cyclooxygenase 2 inhibitor and PPAR- γ agonist in this study are potential treatments for GO due to their ability to suppress TNF- α -induced TGF- β , HAS, and HA upregulation.

Keywords: cytokines, extraocular muscles, Graves' ophthalmopathy, hyaluronan, myoblast, transforming growth factor-beta

Graves ophthalmopathy (GO) is an autoimmune disease closely associated with Graves disease (GD), which results in proptosis, periorbital swelling, ocular motility restriction, diplopia, eyelid retraction, and optic neuropathy.¹ Progress has been made in the understanding of the pathogenesis of GO; however, there are still no effective means of preventing or treating GO. Histochemical examination of the extraocular muscles (EOM) and adipose tissues in GO has revealed bone marrow-derived cell infiltration and inflammatory cytokine activation.^{2,3} The profile of these cells include lymphocytes, mast cells, and macrophages,⁴⁻⁶ which produce inflammatory cytokines including IFN- γ , IL-1 β , TGF- β , TNF- α , leukoregulin, CD154 (CD40 ligand), IL-4, -6, and -10.^{5,7} This inflammation has been suggested to be a critical factor in the pathogenesis of GO.

Current GO models postulate that autoantibodies such as thyroid-stimulating hormone receptor (TSH-R)^{8,9} or insulin-like growth factor-1 receptor (IGF-1R)¹⁰ trigger the inflammatory cascade and activate orbital fibroblasts. The activated orbital fibroblasts then secrete cytokines such as IL-1 β to upregulate the production of prostaglandin (PG)-E2 via

increased prostaglandin endoperoxide H synthase-2 (also known as cyclooxygenase [COX]-2).¹¹⁻¹⁵ Prostaglandin E2 mediates cellular immunity by playing vital roles in GO.^{16,17} Current evidence suggests orbital fibroblasts are target cells of autoimmunity in GO¹⁸⁻²⁰ and they are able to differentiate into myofibroblasts and adipocytes when treated with TGF- β ^{21,22} and peroxisome proliferator-activated receptor (PPAR)- γ agonists,^{11,22-24} respectively. However, it remains unclear whether myoblasts have comparable autoimmune properties as fibroblasts in GO.

Based on clinical manifestation and imaging, GO patients have an enlargement of both EOM and adipose tissue in orbit.^{25,26} A hallmark feature of EOM enlargement in GO is the accumulation of granular material primarily consisting of collagen fibrils and glycosaminoglycans, among which hydrophilic hyaluronic acid (HA) predominates.²⁷ Hyaluronic acid production is increased by orbital fibroblasts when exposed to cytokines such as TGF- β ^{14,28,29} and this may be accomplished with multiple underlying signaling pathways.³⁰⁻³² In addition to an increase in HA production, TGF- β also stimulates the



synthesis and deposition of other various extracellular matrix proteins, promotes cell-matrix interactions, and induces adhesiveness. These collective effects lead to edema and fibrosis, which make TGF- β a particularly intriguing molecule to study in GO pathogenesis.^{21,22} Previously, COX-2 inhibition and agonist PPAR- γ have been found to inhibit the proinflammatory cascade caused by TGF- β in GO patient's orbital fibroblasts^{32,33}; however, there is limited evidence present in the literature regarding the involvement of EOM. This study attempts to provide new insights into the extraocular muscles' involvement in GO. In addition, we will evaluate whether EOM involvement can be inhibited by celecoxib (COX-2 inhibitor) and pioglitazone (PPAR- γ agonist), as they do on orbital fibroblasts in vivo.

MATERIALS AND METHODS

Collection of clinical data and EOM specimens from GO patients and non-GO group were in conformity with the institutional review board of the National Taiwan University Hospital. The study followed the tenets of the Declaration of Helsinki and written informed consent was obtained from each patient.

Subjects Recruitment and Specimens Collection

Extraocular muscle tissue was obtained from GO patients ($n = 10$) during rectus myectomy. When the restricted muscle was identified and exposed, scissors (Westcott; Fairfield, CT, USA) were used to cut the middle part of muscle belly. In non-GO control group ($n = 4$), the EOM tissue was obtained from patients with different clinical entities including esotropia ($n = 2$), posttraumatic oculomotor palsy ($n = 1$), and alternating esotropia ($n = 1$) who underwent strabismus resection surgery. None of the non-GO patients had inflammatory orbital diseases. In addition, all patients from GO group were biochemically euthyroid at the time of surgery. Clinical data including sex, age, duration from onset of GD and GO to time of surgery, clinical activity score (CAS), steroid therapy, thyroidectomy, thyrotropin binding inhibitory immunoglobulin (TBII), and history of smoking were collected.

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from HyClone (Logan, UT, USA). Recombinant human IFN- γ , IL-1 β , IGF-1, TNF- α were obtained from ProSpec Biotech (East Brunswick, NJ, USA). Tumor growth factor β 1 (active form) and HA ELISA kit were obtained from R&D Systems (Minneapolis, MN, USA). Pioglitazone and celecoxib were purchased from Biovision, Inc. (Milpitas, CA, USA). We obtained anti-COX-2 rabbit monoclonal Ab (cat no. 2836-1) from Epitomics (Burlingame, CA, USA). We purchased anti-PPAR- γ polyclonal Ab (101711) from Cayman Chemical (Ann Arbor, MI, USA) and anti-HAS1 Ab (3E10) from Novus Biological (Littleton, CO, USA). We purchased anti-HAS2 Ab (sc 365262) and antimyosin heavy chain (MyHC) Ab (sc-53088) from Santa Cruz Biotechnologies (Heidelberg, Germany). We obtained anti-HAS3 polyclonal Ab (cat no. 156091AP) from Proteintech (Chicago, IL, USA) and anti-desmin antibody (ab15200) was from Abcam (Cambridge, MA, USA). Guanidinium thiocyanate phenol chloroform medium, phalloidin, AlexaFluor 488 and 594 conjugated secondary antibodies were purchased from Invitrogen (TRIZOL, Carlsbad, CA, USA) and random hexamers were purchased from Amersham Pharmacia Biotech (Uppsala,

Sweden). We obtained deoxyribonucleoside triphosphates from Boehringer-Mannheim (Mannheim, Germany) and the reverse transcriptase was purchased from Invitrogen. Taq polymerase and thermal cycler were purchased from Perkin Elmer (Norwalk, CA, USA).

Cell Culture

Cells were isolated and digested with a mixture of 0.24% trypsin, 0.1% collagenase in Ca²⁺, Mg²⁺ free PBS followed by purification with fibroblast-specific micromagnetic beads (Miltenyi Biotec, Auburn, CA, USA) that conjugated to monoclonal mouse antifibroblast antibodies (isotype: mouse IgG2a) to prevent fibroblast contamination. These cells were kept in liquid nitrogen for later use. Upon thawing, the cells were transferred to a sterile tube containing 10 mL Media 199 supplemented with 20% FBS, penicillin (100 U/mL), gentamicin (20 μ g/mL), L-glutamine (2 mM; designated as media 199/FBS), and centrifugation was performed at 282g for 5 minutes. The supernatants were aspirated and the cell pellets were resuspended in 0.5 mL Media 199/FBS, propagated, and cultured in same medium in a humidified 5% CO₂ incubated at 37°C. Cells were grown to 70% confluent (in ~3-5 days) with the medium changed every 2 to 3 days. For cell passage, cells were trypsinized with 0.25% trypsin/2.2 mM EDTA solution at 37°C for 5 minutes. Trypsin was neutralized by adding 7 mL Media 199/FBS per T-75 flask, and all cells were split at 1:4 under the same culture condition. Myoblasts cells were propagated for 2 to 3 passages before use. All cultures were stained for myogenic differentiation (myoD), desmin (1:500), MyHC (1:250) as well as phalloidin, and counterstained with 4,6 diamidino-2-phenylindole (DAPI) to confirm myoblast identity. AlexaFluor 488 or 596 conjugated secondary antibodies (1:1000) were applied afterward. Antibodies were diluted with PBS containing 2% BAS and 0.01% saponin. After staining, cells were imaged with confocal microscopy (LSM700; Carl Zeiss Microscopy GmbH, Jena, Germany).

Quantitative Real-Time (RT)-PCR to Measure mRNA Expression After Cytokine Treatment

The concentrations of cytokines were selected in preliminary experiments to obtain the highest responses. The effects of cytokines on selected genes expressions are mediated through a time-dependent upregulation of mRNA levels. Upon reaching confluence, myoblasts were treated with IFN- γ (5000 U/mL), IGF-1 (320 ng/mL), IL-1 β (20 ng/mL), and TNF- α (40 ng/mL) for 24 hours in both GO ($n = 10$) and non-GO ($n = 4$). Total RNA was extracted using the guanidinium thiocyanate phenol chloroform solution TRIZOL method developed by Chomynski and Sacchi (Invitrogen, Carlsbad, CA, USA). The real-time PCR was performed with a sequence detection system (ABI PRISM 7700; Applied Biosystems, Foster City, CA, USA) based on the manufacturer's protocol for quantifying the expression of COX-2, PPAR- γ , TGF- β , and mRNA level of TSH-R. The real-time PCR amplification reaction contained cDNA converted from 1.875 ng of total RNA from the cultured myoblasts. For each reaction, a total volume of 25 μ L containing 80 to 160 nM of respective target gene's forward, reverse primers and 2X SYBR Green PCR master mixes (Applied Biosystems) were used. The product of RT-PCR was examined on 1% agarose gels with 10 mg/mL ethidium bromide, visualized under ultraviolet light. The relative mRNA level or each gene was corrected with the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level as reference. Primers for RT-PCR are shown in Table 1.

TABLE 1. Primers for RT-PCR

COX-2 160 nM	
Forward	5'-GCTCAAACATGATGTTTGCATTC
Reverse	5'-GCTGGCCCTCGCTTATGA
PPAR- γ 160 nM	
Forward	5'-CGGGCCCTGGCAAAAC-3'
Reverse	5'-AAGATCGCCCTCGCCTTT-3'
TGF- β 160 nM	
Forward	5'-CCTGCAAGACTATCGACATGGA
Reverse	5'-AGGATCTGGCCGCGGA
TSH-R 160 nM	
Forward	5'-GAGTTATCGGTGTATACGCTGACG-3'
Reverse	5'-GCGCATGGCGAAGGTG-3'
GAPDH 80 nM	
Forward	5'-CCCCTCTCCACCTTTGAC-3'
Reverse	5'-CATACCAGGAAATGAGCTTGA-3'

ELISA Analysis for TGF- β and HA With Cytokines and Drug Treatment

The myoblast cell cultures (GO [$n = 10$] and non-GO [$n = 4$]) were grown to confluence in six-well plates in Media 199/FBS. Upon reaching confluence, myoblasts were treated with or without IFN- γ (5000 U/mL), IGF-1 (320 ng/mL), IL-1 β (20 ng/mL), and TNF- α (40 ng/mL). For the drug treatment, myoblasts were treated with TNF- α (40 ng/mL) alone or TNF- α plus either celecoxib (100 μ M) or pioglitazone (28 μ M). The group that received no treatment was used as a negative control. After stimulation for 24 hours, the two groups of myoblasts and supernatants were harvested to measure TGF- β 1 (active form) with ELISA according to manufacturer's protocol (R&D Systems). Similarly, myoblasts were treated with TGF- β (2 ng/mL) alone or TGF- β plus either celecoxib (100 μ M) or pioglitazone (28 μ M) for 24 hours. Level of HA was measured with the aforementioned ELISA protocol.

Detection of Protein Production in Myoblast Cells After Treatment of Cytokines and Drugs by Western Blot

Myoblast cells isolated from EOM of normal subjects and patients (non-GO [$n = 4$] and GO [$n = 7$]) were grown to confluence in six-well plates in Media 199/FBS. Upon confluence, myoblasts were treated individually with or without IFN- γ (5000 U/mL), IGF-1 (320 ng/mL), IL-1 β (20 ng/mL), and TNF- α (40 ng/mL) for 24 hours. For the drug treatment, myoblasts were treated with TGF- β (2 ng/mL) alone or TGF- β (2 ng/mL) plus either celecoxib (100 μ M) or pioglitazone (28 μ M) for 24 hours. Then, the cell culture supernatants were collected and concentrated using centrifugal filter system (Centricon-10; Amicon, Beverly, MA, USA). Cells were extracted with 0.5% SDS in the presence of protease inhibitor complex containing antipain-hydrochloric acid, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, pefabloc SC, ethylenediaminetetraacetic acid, and aprotinin (Boehringer Mannheim Biochemicals; Penzberg, Upper Bavaria, Germany). The cell supernatant and cell lysate samples were mixed with $\times 2$ SDS gel-loading buffer (100 mM Tris-Cl [pH 6.8], 4% [wt/vol] SDS, 0.2% [wt/vol] bromophenol blue, 20% [vol/vol] glycerol, 200 mM dithiothreitol) and heated at 95°C for 4 minutes. An equivalent amount of total proteins at 20 μ g/lane was loaded onto gels (4% acrylamide stacking gel and 12% acrylamide resolving gel). Gel electrophoresis was

TABLE 2. Demographic Data of GO and Non-GO Patients

	GO, $n = 10$	Non-GO, $n = 4$	P Value
Age, y	49.6 \pm 11.8	38.3 \pm 23.1	0.2
Sex, F:M	5:5	2:2	1
Duration of GD, m	21.2 \pm 7.9		
Duration of GO, m	9.3 \pm 2.3		
CAS at muscle surgery	3.2 \pm 0.8		
Pulse therapy	2		
Oral steroid therapy	2		
Thyroidectomy	7		
TBII	5		
Smoking	2	0	0.3

performed with a standard buffer system at a constant voltage of 90 V for 2 hours. Gels were presoaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) before electrophoretic transfer (at 90 mA for 3 hours) of proteins onto nitrocelluloses. Nitrocellulose membranes were incubated in blocking buffer containing 1X Tris-buffered saline (TBS; 10 mM Tris, pH 7.5, 150 mM sodium chloride), 0.05% Tween 20, and 5% nonfat dry milk for 3 hours at 37°C. After that, primary antibodies (COX-2, PPAR- γ , HAS1, HAS2, and HAS3 diluted at 1.0 μ g/mL in 1X TBS and 1% nonfat dry milk) were incubated with the membranes overnight at 4°C. After being washed, nitrocellulose membranes were incubated with respective alkaline phosphatase-conjugated immunoglobulin (1 μ g/mL) for 2 hours at room temperature. After washes with TBS/0.05% Tween 20, proteins were visualized using nitroblue tetrazolium and bromochlorodimethyl phosphate chromogenic substrates in developing buffer (100 mM Tris, pH 9.5, 100 mM sodium chloride, 500 mM magnesium chloride). Protein sizes were determined by comparison with a protein molecular weight marker (Amersham Life Science, Braunschweig, Germany). For quantitation, immunoblots of COX-2, TSH-R, HAS1, HAS2, and HAS3 were measured by scanning densitometry using a dual-wave length densitometer (Shimadzu TCL Scanner; Shimadzu Scientific, Columbia, MD, USA) connected to a data recorder (Shimadzu DR2; Shimadzu Scientific). Band intensities were quantitated and were normalized to the corresponding levels of Actin using commercial software (Phoretix 1D Advanced, version 4.01; Phoretix International, Newcastle upon Tyne, UK).

Statistical Analysis

Values were given as mean \pm SD. Data were analyzed using the ANOVA test. Results were considered significant if $P < 0.05$.

RESULTS

Isolation, Cultivation, and Identification of Myoblasts From Non-GO and GO Patients

We collected the EOM specimens from 10 GO and 4 non-GO patients for culture and further investigation. The demographic data are shown in Table 2. There is no significant difference in age, sex, and smoking between the two groups. The duration of GO was 9.3 \pm 2.3 months and they presented with 3.2 \pm 0.8 CAS during muscle surgery. Cultivated cells were stained for myogenic markers MyoD (Fig. 1A) and desmin (Fig. 1B), or differentiation marker MyHC (Fig. 1C). The detection of MyoD in every nucleus and desmin in the cytoplasm clearly demonstrated that these cells are myogenic lineage (Figs. 1A, 1B). In addition, the myoblast-like morphology shown by phalloidin staining (Fig.

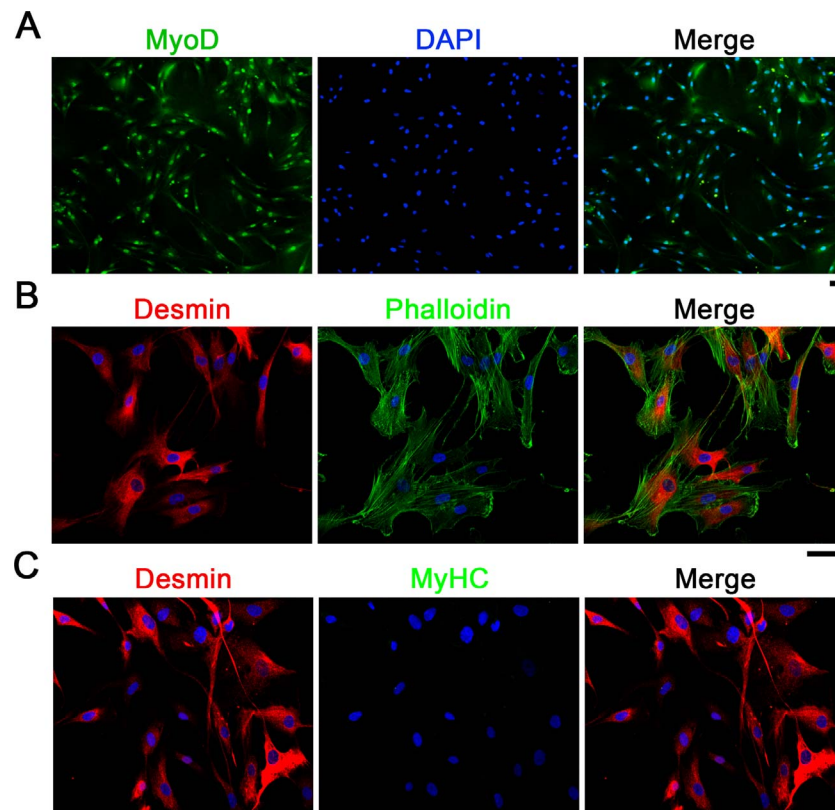


FIGURE 1. Myoblasts identity. Representative EOM tissue obtained from a GO patient during rectus myectomy surgery. Isolated myoblasts were stained with MyoD (A), desmin and phalloidin (B), or desmin and MyHC (C) to demonstrate the cells were undifferentiated, single nuclear myoblasts. Nucleus was counterstained with DAPI. Scale bars: 50 μ m.

1B) and the lack of MyHC signals (Fig. 1C) further manifests that these cells are undifferentiated and single nuclear myoblasts (Figs. 1B, 1C).

TNF- α Induces COX-2, PPAR- γ , TGF- β , and TSH-R mRNA Expression in GO Human Cultured Myoblasts

The 24-hour time point was chosen based on previous time-course studies³⁴⁻³⁸ and also confirmed from our supplemental experiments that indicated maximal upregulation of gene expression at 24 hours and tapered expression thereafter (Supplementary Fig. S1). At 24 hours, there was no significant upregulation of COX-2, PPAR- γ , TGF- β , and TSH-R mRNA expression among IFN- γ (fold increase: 0.9 ± 0.8 , 1.4 ± 1.1 , 1.1 ± 0.7 , and 1.3 ± 1.1 , respectively; Fig. 2A) and IGF-1 (fold increase: 1.4 ± 2.3 , 1.1 ± 0.7 , 1.1 ± 0.6 , 1.3 ± 1.2 , respectively; Fig. 2B) treatment compared with the nontreated GO myoblasts. Interleukin 1 β significantly increased COX-2 expression (fold increase: 5.2 ± 1.9 ; $P = 0.001$) but not PPAR- γ (fold increase: 1.7 ± 1.6), TGF- β (fold increase: 1.3 ± 0.3), and TSH-R (fold increase: 1.3 ± 1.2 ; Fig. 2C) compared with the nontreated GO myoblasts. Addition of TNF- α significantly upregulated ($P < 0.001$) the expression of COX-2 (fold increase: 5.3 ± 4.2), PPAR- γ (fold increase: 2.7 ± 1.2), TGF- β (fold increase: 1.8 ± 0.4), and TSH-R (fold increase: 2 ± 2) compared with the nontreated GO myoblasts (Fig. 2D). In contrast, all cytokines were unable to significantly stimulate the selected genes mRNA expression in the non-GO myoblasts (Figs. 2A-D).

TNF- α Induces TGF- β , COX-2 and TSH-R Protein Synthesis

The amount of TGF- β protein measured by ELISA was significantly increased by TNF- α (0.2 ± 0.04 ng/mL, $P < 0.01$) in GO myoblasts compared with the nontreated GO myoblasts (0.09 ± 0.03 ng/mL; Fig. 3). However, the increase of TGF- β protein by IFN- γ (0.1 ± 0.05 ng/mL), IGF-1 (0.1 ± 0.07 ng/mL), and IL-1 β (0.1 ± 0.04 ng/mL) was not significant (all $P > 0.05$). The protein levels of TGF- β measured by ELISA were consistent with the relative amounts of mRNA expression of TGF- β by PCR. In contrast, the TGF- β protein expression in non-GO myoblasts under the treatment of these cytokines did not change (Fig. 3).

Western blot analysis was performed to identify and compare the protein expression of COX-2 and TSH-R in myoblasts treated by different cytokines. The band intensities of COX-2 and TSH-R were normalized to the respective actin levels following quantitation. The intensity of the protein bands of COX-2 and TSH-R was consistent with their mRNA expression. The intensity of COX-2 band was more pronounced when treated with IGF-1 (13-fold), IL-1 β (fold increase: 29.9 ± 23.6), and TNF- α (fold increase: 11 ± 10) in GO myoblasts ($P < 0.001$; Fig. 4A) than that of their corresponding nontreated GO myoblasts. The amount of TSH-R expressed by stimulation with TNF- α (fold increase: 1.9 ± 0.8 , $P = 0.04$) was higher than that of the other cytokines (Fig. 4B) or than that of corresponding nontreated GO myoblasts. In contrast, the COX-2 and TSH-R protein expression did not increase under the treatment of any

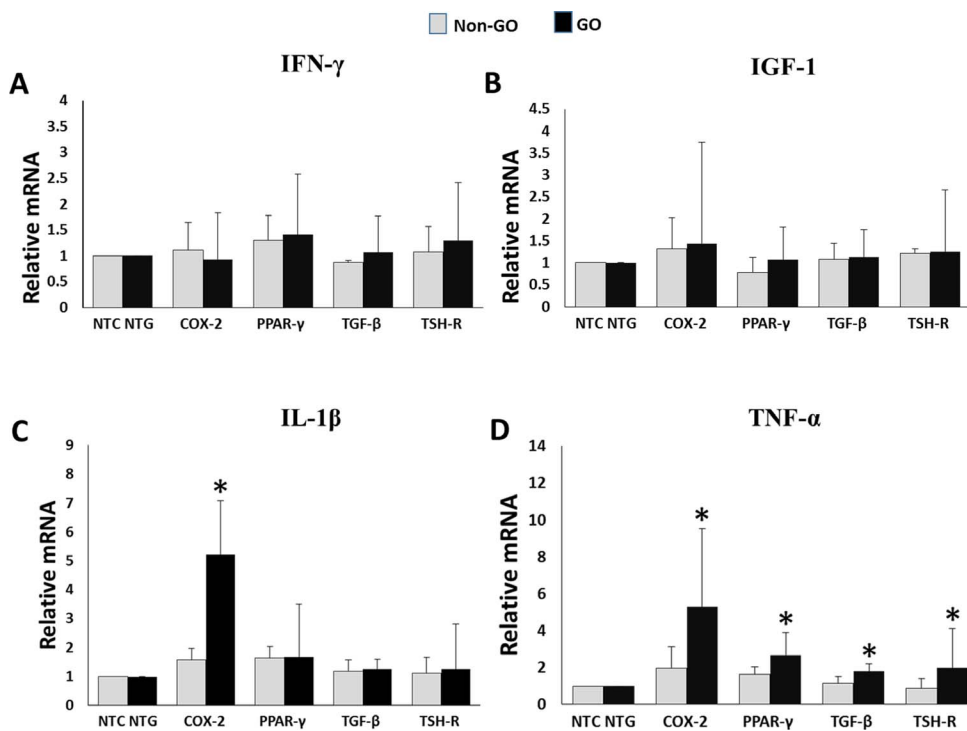


FIGURE 2. Effect of different cytokines treatment on the mRNA expressions of *COX-2*, *TGF-β*, *PPAR-γ*, *TSH-R* in cultured myoblasts. The cultures of EOM from GO ($n = 10$) and non-GO ($n = 4$) patients were incubated with or without IFN- γ (A), IGF-1 (B), IL-1 β (C) or TNF- α (D) for 24 hours. After treatments, the gene expressions were determined by RT-PCR and normalized to the control gene GAPDH. Results are expressed as relative expression units which were calculated by their cycle differences from nontreated non-GO (NTC) or nontreated GO (NTG). * $P < 0.05$, statistically significant difference compared with NTG.

cytokines in non-GO myoblasts (Fig. 4). This data closely corresponded with its respective mRNA expression.

Celecoxib and Pioglitazone Inhibit TNF- α -Induced TGF- β , HA and HAS3 Biosynthesis in Cultured GO Myoblasts

The above results demonstrated that TNF- α significantly upregulated TGF- β protein expression. We postulated TGF- β can induce HA production through HAS. To determine the effect of celecoxib (COX-2 inhibitor) and pioglitazone (PPAR- γ agonist) on the TGF- β and HA synthesis, cultured myoblasts were treated with 40 ng/mL TNF- α or 2 ng/mL TGF- β , in the presence of 100 μ M celecoxib or 28 μ M pioglitazone. As shown in Figure 5, TNF- α significantly increased the production of TGF- β protein (0.26 ± 0.09 ng/mL, $P = 0.004$; Fig. 5A) and TGF- β also significantly upregulated the production of HA

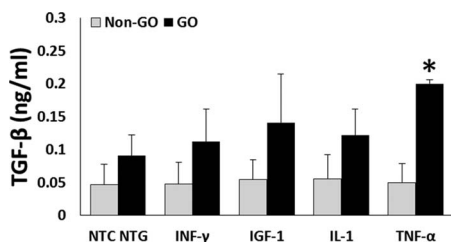


FIGURE 3. Effect of different cytokines treatment on the protein expression of TGF- β in cultured myoblasts. The protein expressions of TGF- β in patients with GO ($n = 10$) and non-GO ($n = 4$) culture supernatants incubated with or without different cytokines for 24 hours were determined by ELISA. * $P < 0.05$, statistically significant difference compared with NTG.

(936 ± 309 ng/mL, $P < 0.001$; Fig. 5B) in GO myoblasts compared with the amount produced in nontreated GO myoblasts (0.07 ± 0.06 and 191 ± 51 ng/mL, respectively). Celecoxib and pioglitazone treatment substantially diminished the TNF- α -induced TGF- β (0.08 ± 0.07 ng/mL and 0.08 ± 0.07 ng/mL, $P = 0.004$, respectively) (Fig. 5A) and HA synthesis (408 ± 228 ng/mL, $P = 0.04$ and 284 ± 93 ng/mL, $P < 0.001$, respectively; Fig. 5B). In contrast, TGF- β and HA production in non-GO myoblasts by TNF- α or TGF- β did not change compared with that in nontreated controls. Additionally, neither celecoxib nor pioglitazone inhibited TGF- β and HA synthesis in non-GO myoblasts.

Transforming growth factor β strongly induced HAS3 protein level (fold increase: 5.8 ± 0.3 ; $P < 0.001$; Fig. 5C), whereas HAS1 and HAS2 protein levels remained unchanged according to Western blot (data not shown). Both celecoxib and pioglitazone inhibited TGF- β -induced HAS3 protein expression by a fold increase of 3.2 ± 1.8 ($P = 0.008$) and 1.4 ± 0.3 ($P < 0.001$), respectively, but did not significantly affect HAS1 and HAS2 expressions ($P > 0.05$). In contrast, stimulation with TGF- β did not induce the HAS production relative to nontreated expression in non-GO myoblasts. Therefore, additional experiments using the drugs under study on non-GO myoblasts were not further performed.

DISCUSSION

Evidence supports lymphocyte activation and subsequent cytokine release play important roles in GO pathogenesis.² This study is the first in vitro experiment to demonstrate the effect of these cytokines on the gene expression pattern in GO cultured myoblasts. The results show increased cytokine-induced gene expression suggesting that the EOM is an

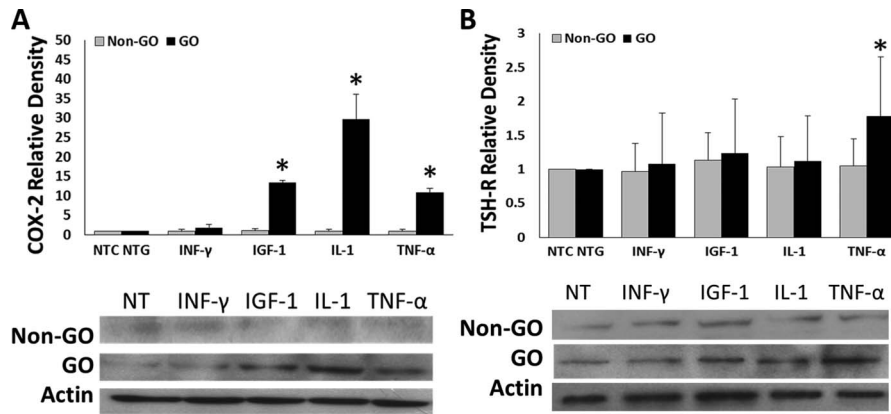


FIGURE 4. Protein expression of COX-2 and TSH-R in cultured myoblasts after treatment of different cytokines. Western blot analysis was performed to detect COX-2 (A) and TSH-R (B) protein level. Bar diagrams show the computed band intensities of COX-2 and TSH-R normalized compared with respective actin protein level in cultured GO ($n = 7$) and non-GO ($n = 4$) myoblasts. * $P < 0.05$, statistically significant difference compared with NTG.

essential factor in the inflammation process of GO. Although a potential limitation of our study was the relatively small sample size, our results achieved clear statistical significance. We observed that cytokines upregulated selected gene expressions in a time-dependent manner. Our results are consistent with the finding that IFN- γ , IL-1 β , and TNF- α are detected more in eye muscle than orbital fat tissue⁵ and these results are also in agreement with studies³⁹⁻⁴² that proposed T-lymphocyte-mediated immune reactions against eye muscle membrane protein are the primary target of GO. Orbital EOM has been identified as a highly specific and sensitive diagnostic marker of GO as well.⁴³ A recent study also showed chemokines were released when stimulated by cytokines in cultured myoblasts, suggesting that EOM initiates and participates in a self-perpetuation of inflammation in GO.³⁴ Despite this knowledge, studies currently focus on orbital connective or adipose tissue as the initial target of GO.

In this study, we demonstrate that EOM is a vital target of the autoimmunity in GO and cytokines play important roles in this process. Messenger RNA and protein expression of PPAR- γ ,

COX-2, TGF- β , and TSH-R were significantly increased under TNF- α treatment in GO myoblasts but not in non-GO myoblasts. These results are consistent with others that have shown TNF- α plays a role in the pathogenesis of GO.^{12,34,44} The different results between GO and non-GO group may be due to the genetic and environmental factors; however, this requires further investigation.² Our finding that TNF- α and other cytokines play a crucial role in GO myoblasts is also evidenced in the current dogma of orbital fibroblasts. Current studies have shown patients with GO contain autoantibodies that stimulate orbital fibroblasts to synthesize chemoattractants which enhance recruitment of activated T cells and other mononuclear immune cells into the orbit.⁴⁵ The direct interaction of T cells with orbital fibroblasts results in fibroblast production of cytokines and inflammatory mediators. These cytokine stimulate orbital fibroblasts to produce high levels of PGE2 via increased COX-2. Prostaglandin E2 in turns influences B cell switching, T cell differentiation, and important cellular immunity.^{16,17} Through the activity of COX-2, proadipogenic

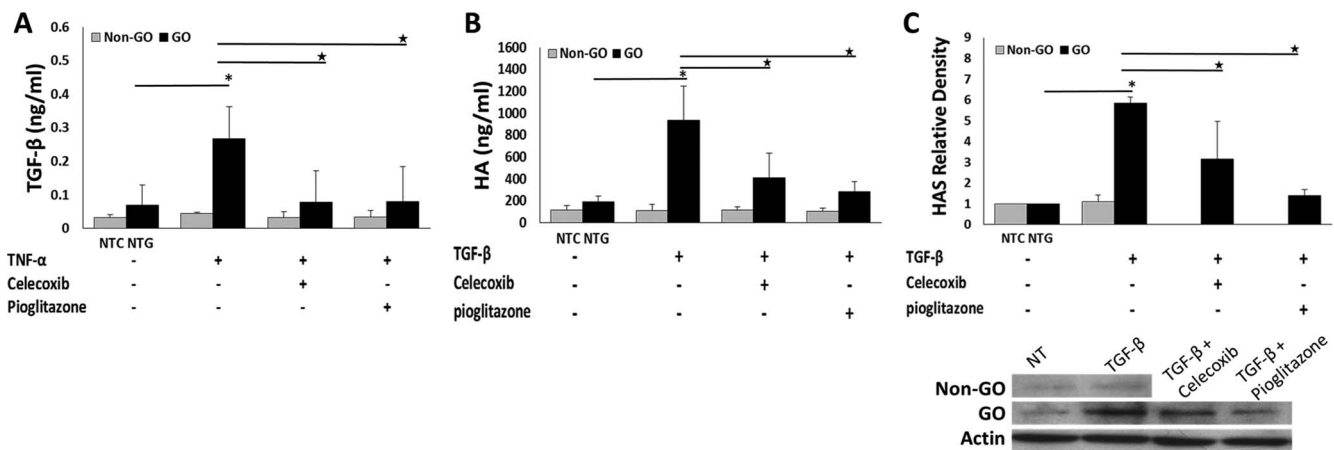


FIGURE 5. Effect of COX-2 inhibitor (celecoxib) and PPAR- γ agonist (pioglitazone) on TGF- β synthesis (A), HA synthesis (B), and HAS3 enzyme induction (C). We treated GO ($n = 10$) and non-GO ($n = 4$) myoblasts with TNF- α (40 ng/mL) alone or in the presence of either celecoxib (100 μ M) or pioglitazone (28 μ M) for 24 hours. The group that received no treatment was used as a negative control. (A) The protein expression of TGF- β in culture supernatants was assessed using ELISA. (B) Similarly, myoblasts were treated with TGF- β (2 ng/mL) alone or in the presence of either celecoxib (100 μ M) or pioglitazone (28 μ M) for 24 hours and HA level was assessed with ELISA. Expression of HAS3 was detected by Western blot analysis. (C) Bar diagrams show the computed band intensities of HAS3 normalized to the respective actin protein level in GO ($n = 7$) and non-GO ($n = 4$) myoblasts. * $P < 0.05$, statistically significant difference compared with NTG. $\star P < 0.05$ statistically significant difference compared with TNF- α -induced TGF- β , HA, and HAS expression.

prostaglandin PPAR- γ ligands are produced by the activated T cells.⁴⁶

There is clear enlargement of EOM in GO as evidenced by electron microscopy. Such material primarily consists of glycosaminoglycans, among which HA predominates.²⁷ The polyanionic charge and extremely high osmotic pressure of HA makes EOM extremely hydrophilic and capable of binding numerous water molecules. Consequently, the edematous muscle together with hyperplastic adipocytes lead to the classic expansion and enlargement in patients with GO. One of the major mediators implicated in HA synthesis is TGF- β , which is also suggested as a potential fibrotic mediator in GO.^{21,22} Studies have shown TGF- β can induce HAS expression and HA production in various tissue cells.⁴⁷⁻⁵¹ We postulated TNF- α stimulates TGF- β production, which induces HA production via HAS regulation in GO myoblasts. Results confirmed TGF- β protein elevation under TNF- α stimulation is compatible to the result of RT-PCR. We further demonstrated upregulated HAS3 and HA by TGF- β , which is consistent with Guo et al.³² and, interestingly, the HAS subtype and cell type culture are different. Although the stimulation of HA by TGF- β is recognized, there may be multiple signaling pathways by which TGF- β regulates HA synthesis in orbit. The major route by which TGF- β regulates cell activation is the Smad signaling cascade.⁵²⁻⁵⁴ Recent studies reported TGF- β -induced HA involves c-Abl-independent Smad signaling in orbital fibroblast.³¹ However, the phosphorylation and nuclear translocation of Smad2 and -3 was unaffected by treatment with PPAR- γ agonists that inhibited TGF- β -induced HA in orbital fibroblasts of GO patients.³² In addition, protein kinase C β II was demonstrated to be the enzyme to regulate HA synthesis by TGF- β in orbital fibroblasts.³⁰ Thus, transforming growth factor β may exert its biologic actions by activating multiple intracellular signal transduction pathways.

This is the first study to demonstrate a COX-2 inhibitor (celecoxib) substantially diminished and blocked HAS3-induced HA synthesis via TGF- β under TNF- α stimulation in GO myoblasts. The results are supported by a recent finding that cyclooxygenase inhibition demonstrated a significant decrease in TGF- β induced proliferation in orbital fibroblasts in the enlarged EOM of GO patients.³³ Furthermore, celecoxib has been reported to effectively treat GO patients with EOM enlargement who were refractory to corticosteroid treatment.⁵⁵

As reported, PPAR- γ agonists have an important role in modulating inflammation and inducing adipogenesis in orbital fibroblasts.^{22,24,56} A recent study also demonstrated PPAR- γ agonists might inhibit cytokine-induced chemokine expression in GO-cultured myoblasts.³⁴ However, the mechanisms whether PPAR- γ agonist inhibition pathways are PPAR- γ dependent or independent are still under investigation.^{57,58} Signaling pathways considered PPAR- γ dependent result through the repression of the activator protein 1 and nuclear factor kappa B, whereas PPAR- γ independent mechanisms may involve the agonists directly interfering with the TGF- β signaling. Our results demonstrated that PPAR- γ agonist (pioglitazone) treatment substantially diminished TGF- β and HA synthesis in GO myoblasts. The result is further supported by increasing evidence that showed PPAR- γ ligands negatively regulate TGF- β in various tissues.⁵⁷ Our results may suggest that pioglitazone functions through PPAR- γ independent molecular pathways in the cultured myoblasts. Therefore, the inhibition may occur downstream of the initial TGF- β signaling molecules or involve Smad/non-Smad-mediated TGF- β signaling. An encapsulating peritoneal sclerosis animal model demonstrated PPAR- γ agonist (pioglitazone) significantly inhibits matrix metalloproteinases and TGF- β accumulation in peritoneal dialysis patients.⁵⁹ Pioglitazone was also found to inhibit

TGF- β -induced fibroblast to myofibroblast differentiation in the cornea.⁶⁰ Although the interaction between the TGF- β and PPAR- γ pathway has recently been reported, their roles are still unclear. One limitation of this study is that the possible unknown PPAR- γ -mediated synergistic and antagonistic interactions between the pathways regulating HA production and adipogenesis had not been assessed. Despite this, our data has shown that COX-2 inhibitors and PPAR- γ agonists may be potential therapeutic modalities in patients with GO. These results are promising and suggest that the TGF- β pathway is a potential future target for the therapy of enlargement or fibrosis of the EOM in GO. However, further studies are needed to verify whether the anti-HA production effects of PPAR- γ activation can be exploited without the risk of retrobulbar fat proliferation in GO.

Although recent studies suggest that there are novel mechanisms involved in the development of GO,^{1,2,20,29,33,61} many remain to be explored. In this in vitro GO myoblasts study, we demonstrated that a COX-2 inhibitor (celecoxib) and PPAR- γ agonist (pioglitazone) substantially diminished TGF- β induced HAS3 and HA production by TNF- α stimulation. Although our results suggested that the targeting of TGF- β signaling could be effective, these results were achieved by using a single cell type (EOM myoblasts) and single mediator (TNF- α) culture system. However, the orbital pathophysiological process of GO comprises various different cell types and mediators. Furthermore, the doses of celecoxib and pioglitazone cannot be realistically determined by an in vitro culture system. Additional studies are necessary to verify the dose-dependent safety and efficacy of a COX-2 inhibitor and PPAR- γ agonist in the treatment of GO.

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