Hyaluronic Acid Induces COX-2 Expression via CD44 in Orbital Fibroblasts From Patients With Thyroid-Associated Ophthalmopathy

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Purpose. The aim of this study was to determine the effect of hyaluronic acid (HA) on cyclooxygenase (COX)-2 expression in orbital fibroblasts from patients with thyroid-associated ophthalmopathy (TAO).

Methods. Primary cultured orbital fibroblasts were obtained from patients with TAO and non-TAO subjects. Dermal and conjunctival fibroblasts were cultured from the eyelid skin of subjects undergoing cosmetic lid surgery or cataract surgery, respectively. The cells were treated with HA and the transcriptional and translational levels of COX-2 were measured. The expression of CD44 on each type of cells was determined, and the involvement of CD44 in the HA-induced COX-2 increase in orbital fibroblasts from patients with TAO was evaluated by using CD44 knockdown cells and by pretreatment with neutralizing antibody. The relevance of the mitogen-activated protein kinase (MAPK) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated signaling pathway was assessed by immunoblotting for the phosphorylated form of each MAPK or IκB and by using specific inhibitors to these pathways.

Results. Hyaluronic acid increased COX-2 expression in orbital fibroblasts from patients with TAO, which was not observed in the cells from non-TAO subjects and conjunctival or dermal fibroblasts. Orbital fibroblasts from patients with TAO expressed significantly higher level of CD44 than non-TAO cells, and the increased *COX-2* expression by HA in these cells was attenuated by knockdown or neutralizing of CD44. Hyaluronic acid induced MAPK and IκB phosphorylation; and cotreatment with specific MAPK or NF-κB inhibitors halted HA-induced transcription of *COX-2*, suggesting the involvement of these signaling pathways.

Conclusions. Hyaluronic acid induced COX-2 expression in orbital fibroblasts from patients with TAO via CD44 through the MAPK and NF-κB-mediated signaling pathways. These results suggest that HA may have a proinflammatory role in the pathogenesis of TAO by inducing COX-2.

Keywords: CD44, COX-2, hyaluronic acid, orbital fibroblast, thyroid-associated ophthalmopathy

Clinically overt thyroid-associated ophthalmopathy (TAO) accompanies in 25% to 50% of patients with Graves disease, and is one of the most common causes of proptosis or eyelid retraction.[1] Most signs and symptoms of TAO are explained by the volumetric expansion of orbital tissues such as fatty connective tissue and extraocular muscles in the confined bony orbital space. This expansion of orbital tissues is mainly composed of fat expansion and tissue edema. Although the pathogenesis is not fully understood, the edematous tissue changes are known to come from extracellular accumulation of hyaluronic acid (HA), which is a histological hallmark of TAO.[2,3] In vitro studies have shown that orbital fibroblasts produce a significant amount of HA in response to several cytokines and growth factors such as interleukin (IL)-1β and transforming growth factor-β,[4–6] suggesting that the source of HA accumulated in orbital tissue is orbital fibroblasts.

Hyaluronic acid is a polymer composed of repeating disaccharide units composed of D-glucuronic acid and D-N-acetylglucosamine. Hyaluronic acid attracts water into the corresponding tissue resulting in edema due to its extremely hydrophilic characteristics, polyanionic charge, and high osmotic pressure.[7,8] In addition to its pathologic role in the hydrophilic property, the possible roles of HA as a stimulant for inflammation in certain conditions have been proposed. Hyaluronic acid upregulates expression of costimulatory molecules such as CD40, CD80, and CD86 in dendritic cells.[9] Hyaluronic acid also plays proinflammatory roles in several in vivo circumstances such as pulmonary fibrosis, diabetes, and intervertebral disc degeneration.[10–12]

Similar to other stimulatory molecules, several HA binding proteins have been identified. These include receptors such as CD44, receptor for hyaluronan-mediated motility expressed

protein, and lymphatic vessel endothelial hyaluronan receptor-1.[13] Among these, CD44 is expressed in a large number of mammalian cell types and is considered a major HA receptor on most cell types.[14] The role of CD44 in promoting inflammation, together with its ligand HA, has been suggested by a number of studies.[15] In addition to an ability to recruit inflammatory cells,[16,17] Hyaluronic acid–CD44 interactions induce several inflammatory mediators such as IP-10, CXCL12, CXCL13, and CXCR5.[18,19] It is clear that CD44 transduces intracellular signaling events such as mitogen-activated protein kinase (MAPK) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in macrophage/microglia and epithelial cells, leading to alterations in gene expression in response to ligand binding.[20–23]

In contrast to the weak CD44 immunoreactivity in normal orbital tissues, orbital tissues and orbital fibroblasts obtained from patients with TAO display strong CD44 immunoreactivity.[24] The hyaluronic acid–CD44 interaction induces the formation of prostacyclin (PGI2) and cyclooxygenase (COX)-2,[25] in human vascular endothelial cells (HUVECs), which are well-known cytokines that modulate the inflammatory process of TAO.[26] However, the significance of CD44 expression in orbital fibroblasts from patients with TAO has not been elucidated, and previous studies to evaluate the role of HA in the pathogenesis of TAO have been limited to its hydrophilic characteristics, which causes tissue edema.

In this study, we show that HA increases COX-2 expression in orbital fibroblasts from patients with TAO, which is not observed in the cells from non-TAO subjects and conjunctival or dermal fibroblasts. Moreover, the increased *COX-2* expression by HA was attenuated by CD44 knockdown or pretreatment with neutralizing antibody in orbital fibroblasts from patients with TAO, indicating that the HA-induced COX-2 increase occurred through CD44. Hyaluronic acid induced phosphorylation of MAPKs and IκB; and cotreatment with specific MAPK or NF-κB inhibitors halted HA-induced transcription of *COX-2* suggesting the involvement of these signaling pathways. These data propose that HA is an important pro-inflammatory mediator in the pathogenesis of TAO.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Caisson (North Logan, UT, USA). Hyaluronic acid and specific MAPK inhibitors—SB 203580 (p38 MAPK inhibitor), PD 98059 (MAPK kinase [MEK] 1 inhibitor), and SP 600125 (c-Jun N-terminal kinase [JNK] inhibitor)—were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ammonium pyrrolidinedithiocarbamate (PDTC), an NF-κB inhibitor, was obtained from Sigma-Aldrich Corp. Rabbit anti-human COX-2 antibody, rabbit anti-human CD44 antibody, goat anti-rabbit immunoglobulin G (IgG), and DyLight 488 goat anti-rabbit IgG were purchased from Abcam (Cambridge, UK). For neutralization, mouse anti-human CD44 neutralizing antibody (clone BU75; Ancell, Bayport, MN, USA) and isotype-matched mouse IgG2a (Abcam) was used. Mouse anti-human ß-actin, horseradish peroxidase (HRP)–conjugated anti-rabbit and anti-mouse antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell Culture

Human orbital fibroblasts were cultured from orbital fatty connective tissue obtained as surgical waste during decompression surgery for patients with TAO ($n = 5$) or cosmetic surgery for non-TAO healthy subjects ($n = 3$) as described previously.[27] In addition, dermal and conjunctival fibroblasts were cultured from the eyelid skin of subjects undergoing cosmetic lid surgery or cataract surgery, respectively ($n = 3$). These activities were undertaken after informed consent was obtained from the donors according to procedures approved by the Institutional Review Board of Ajou University Hospital, and following the tenets of the Declaration of Helsinki. Written informed consent was obtained from all donors. All patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid condition before the decompression surgery. The cell cultures were maintained in a humidified 5% CO_2 incubator at 37°C covered with DMEM containing 10% FBS and antibiotics. Once a fibroblast monolayer was obtained, the cultures were serially passaged after gentle treatment using trypsin/EDTA. Medium was changed every 3 days, and cells at passages 3 through 8 were used.

RT-PCR Analysis

Cells (5×10^5 cells) were seeded in six-well cell culture plates and treated with HA (0.5 or 1 mg/mL) for 4, 8, or 12 hours. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was generated from RNA (1 μg) using a reverse transcription kit (QuantiTect; Qiagen, Hilden, Germany). We performed PCR using 1 μL cDNA, 0.25 mM dNTP, 1 U of *Pfu* DNA polymerase, and 10 ρmol of primer pairs with a thermal cycler. The specific primer sequences were as follows. COX-2: forward, 5′-GTT CCA CCC GCA GTA CAG-3′ and reverse, 5′-GGA GCG GGA AGA ACT TGC-3′; CD44: forward, 5′-CTC GGG TGT GCT ATG GAT GG-3′ and reverse, 5′-ACT AGT ACA CCC CAA CCT CAG T-3′; β-actin: forward, 5′-CCA AGG CCA ACC GCG AGA AGA TGA C-3′ and reverse, 5′-AGG GTA CAT GGT GGT GCC GCC AGA C-3′. The polymerase chain reaction products were electrophoresed on 1.5% agarose gels and visualized by staining (SafePinky; GenDEPOT, Barker, TX, USA). Band densities were quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The measured intensities were corrected to the ß-actin level in each lane and subjected to statistical analyses.

Quantitative Real-Time PCR (qRT-PCR) Analysis

As described above, cDNA was prepared and qRT-PCR was performed on a real-time PCR machine (ABI PRISM 7500; Applied Biosystems, Foster City, CA, USA) using a commercial reagent (SYBR Premix Ex Taq II; Takara, Shiga, Japan) according to the manufacturer's protocol. The standard PCR conditions were 30 seconds at 95°C, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 34 seconds. The specific primer sequences were as follows: COX-2: forward, 5′- AGA ACT GGT ACA TCA GCA AG-3′ and reverse, 5′-GAG TTT ACA GGA AGC AGA CA-3′; CD44: forward, 5′-TTT GCA TTG CAG TCA ACA GTC-3′ and reverse, 5′-GTT ACA CCC CAA TCT TCA TGT CCA C-3′; β-actin: forward, 5′-TCA CCC ACA CTG TGC CCA T-3′ and reverse, 5′-TCC TTA ATG TCA CGC ACG ATT T-3′. All polymerase chain reactions were carried out in triplicate for each sample. The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method, after being normalized to the Ct value of the ß-actin housekeeping gene.

Western Blot Analysis

Cells were scraped into lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail

(GenDEPOT), and centrifuged at 14,171 *g* for 20 minutes at 4°C. Proteins were separated by SDS-PAGE on 10% (wt/vol) gels and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH, USA). Each membrane was blocked by a 1-hour incubation at room temperature with Tris-buffered saline (TBS; pH 7.4) containing 5% (wt/vol) bovine serum albumin (BSA), and then incubated at 4°C overnight with primary rabbit anti-human COX-2, total or phosphorylated SAPK/JNK, p38, extracellular signal-regulated kinases (ERK), IkBα, or mouse anti-human ß-actin (Cell Signaling Technology) antibody. After three washes with TBST (TBS containing 0.05% [vol/vol] Tween-20), membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 hour. The immunoreactive proteins were detected by incubation with enhanced chemiluminescent blotting detection agent (Pierce Biotechnology, Rockford, IL, USA) using a commercial imaging system (LAS Imaging System; FUJIFILM, Tokyo, Japan). Band densities were quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and subjected to statistical analyses.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Analysis with FACS was performed to determine CD44 expression on each type of cells. After detaching and washing with ice-cold PBS, one million cells were stained with anti-human CD44 (clone IM7; Abcam) or control rat IgG2b (Abcam) for 1 hour at 4°C, washed, and incubated with AlexaFluor 488-conjugated goat anti-rat IgG (H+L; Molecular Probes, Eugene, OR, USA) for 30 minutes at 4°C in the dark. After washing off the unbound antibody, we analyzed 10,000 cells per sample using a fluorescence-activated cell sorter (FACSAria III; Becton Dickinson, San Jose, CA, USA). The data were analyzed using commercial software (FACSDiva ver. 6.1.3; Becton Dickinson).

Immunofluorescence Assay

Cells cultured on poly-D-lysine-coated slides were fixed in 4% paraformaldehyde, blocked with 1% normal goat serum/1% BSA/0.1% Tween-20 in PBS for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies: anti-CD44 Ab (10 μg/mL) or isotype control (10 ug/mL). After five washes in PBS, the cells were incubated with secondary antibodies for 1 hour at room temperature in the dark. Nuclei were stained for 15 minutes with Hoechst 33342 (Molecular Probes) and examined with a fluorescence/live cell imaging microscope (Axiovert 200M; Carl Zeiss Meditec, Jena, Germany).

Small Interfering RNA (siRNA) Transfection

Cells were plated and transiently transfected using a transfection reagent (Lipofectamine RNAiMAX; Invitrogen) with either 25 nM of CD44 siRNA or negative control siRNA (Samchully Pharm Co. Ltd., Seoul, Korea), according to the manufacturer's instructions. Cells were allowed to recover for 24 hours before treatment with 1 mg/mL HA for 4 or 8 hours, and then analyzed by RT-PCR.

Statistical Analysis

Data are expressed as mean ± standard deviation. Student's *t*-test was performed to evaluate intergroup differences. Values of $P < 0.05$ were considered significant. All statistical analyses were performed using commercial (SPSS, version. 15.0; SPSS, Inc., Chicago, IL, USA).

Results

HA Induces COX-2 Production in Orbital Fibroblasts From Patients With TAO

As HA has been proposed as a proinflammatory stimulant by inducing COX-2 expression in HUVECs,[25] we initially examined the effects of HA on the COX-2 expression level in orbital fibroblasts obtained from patients with TAO and from non-TAO subjects. The cells were treated with incremental doses of HA (0.5 to 1.0 mg/mL) for 4, 8, or 12 hours and COX-2 mRNA levels were measured. As shown in Figure 1A, COX-2 mRNA expression in orbital fibroblasts from patients with TAO showed an enhanced response to HA treatment in a time- and dose-dependent manner, whereas no definite increases were shown in orbital fibroblasts from non-TAO subjects. These PCR results were verified by qRT-PCR after treatment with 1.0 mg/mL HA (Fig. 1B). The level of COX-2 transcription increased strikingly by HA treatment in a time-dependent manner, reaching to 8-fold of the control in orbital fibroblasts from TAO patients, but no significant increase was noted in the ones from non-TAO subjects. This increase of COX-2 mRNA in orbital fibroblasts from TAO patients was associated with an increase in protein expression level, as revealed by immunoblotting (Fig. 1C). As shown in Figures 1D and 1E, no significant increase of COX-2 expression was noted in conjunctival or dermal fibroblasts when they were treated by HA with the same protocol as the one used for orbital fibroblasts.

CD44 Expression and Effect of HA on its Expression Level in Orbital Fibroblasts From Patients With TAO

Based on the concept that CD44 is a major HA receptor,[14] and on a previous report showing the strong CD44 expression in orbital tissues from patients with TAO,[24] we examined whether orbital fibroblasts from patients with TAO would express CD44 in our system. As shown in Figure 2A, FACS analysis showed that most cells (99.1%) expressed CD44. Immunofluorescence analysis verified this result by showing that CD44 was constitutively expressed in orbital fibroblasts from patients with TAO (Fig. 2B). The expression level of CD44 in orbital fibroblasts from patients with TAO was significantly higher than that in non-TAO orbital fibroblasts (Fig. 2C). Conjunctival and dermal fibroblasts expressed CD44, as it is known that CD44 is expressed in a large number of mammalian cell types including orbital fibroblasts (Fig. 2D). A previous study showed that IL-1α, tumor necrosis factor (TNF)-α, insulin like growth factor (IGF-1) and TAO-IgGs can significantly stimulate CD44 expression in orbital fibroblasts.[24] Therefore, to evaluate the effect of HA on the CD44 expression level in orbital fibroblasts from patients with TAO, the cells were treated with 1.0 mg/mL HA for 4, 8, or 12 hours and RT-PCR was performed. As shown in Figure 2E, no definite change was found in CD44 expression level following treatment with HA. In addition, the quantification of CD44 mRNA transcription level by qRT-PCR analysis showed no significant changes in CD44 expression level (Fig. 2F), which was in accordance with the RT-PCR findings.

Involvement of CD44 in HA-Induced COX-2 Expression

To investigate whether CD44 is involved in HA-induced COX-2 expression in orbital fibroblasts from patients with TAO, cells were treated with 1.0 mg/mL HA for 4 or 8 hours following transient transfection with CD44 siRNA, and RT-PCR was performed. Orbital fibroblasts transfected with CD44 siRNA displayed reduced levels of endogenous CD44 expression (Fig.

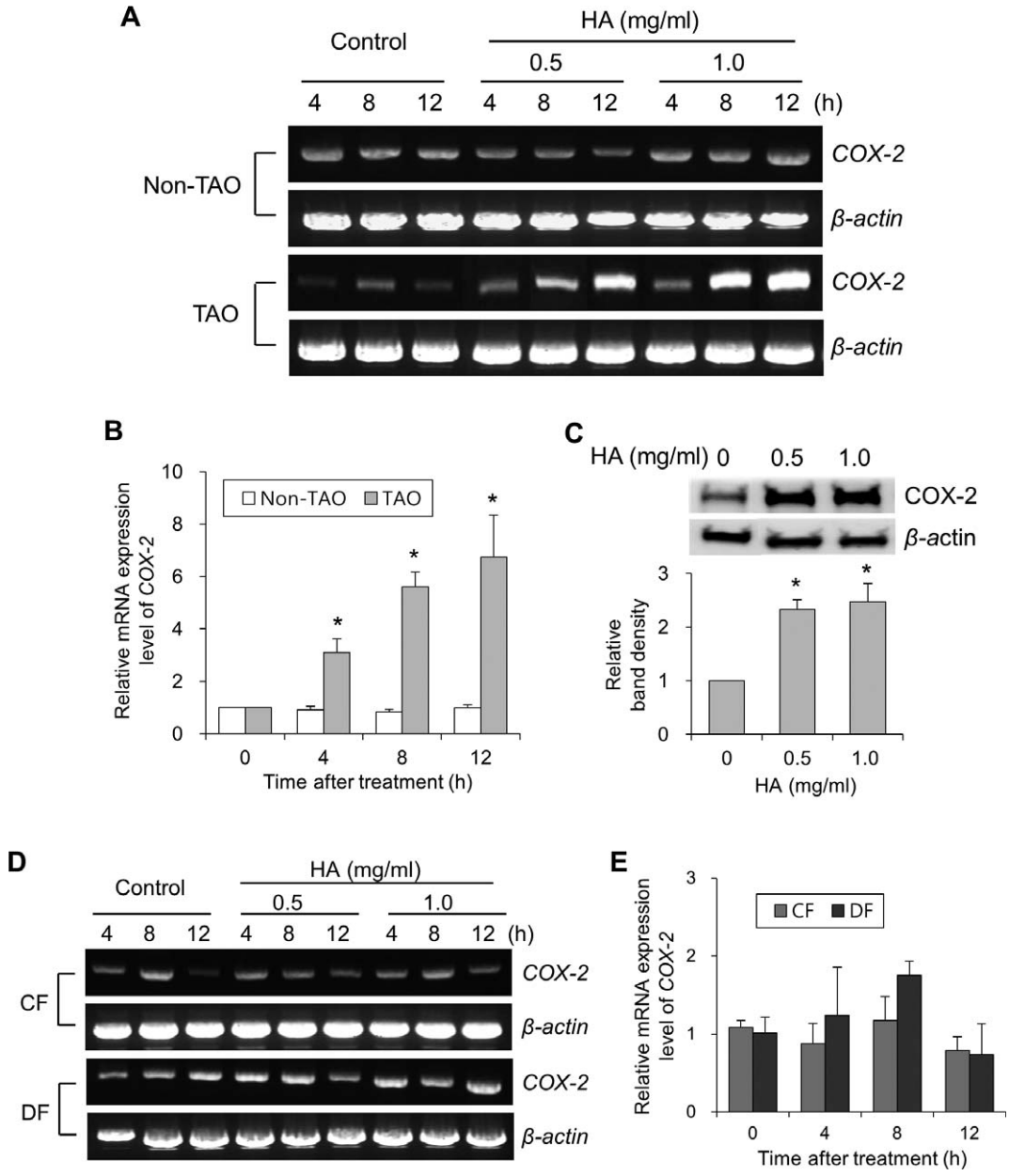

Figure 1. Hyaluronic acid (HA) increases COX-2 production in orbital fibroblasts from patients with TAO. Orbital fibroblasts were cultivated from patients with TAO ($n = 5$) and non-TAO subjects ($n = 3$). Conjunctival fibroblasts (CF) and dermal fibroblasts (DF) were obtained from non-TAO patients ($n = 3$). (**A**) Orbital fibroblasts were treated with HA (0.5 or 1.0 mg/mL) for the indicated times (4, 8, or 12 hours) and the RT-PCR analysis for COX-2 was performed. Representative data from independent experiments for cells from each TAO patients or non-TAO subjects are shown. (**B**) Following treatment of orbital fibroblasts with 1.0 mg/mL HA for the indicated times, transcriptional level of COX-2 in cells were measured by quantitative real-time PCR. *$P < 0.05$ versus untreated control, representative of three experiments. (**C**) Western blot analysis for COX-2 protein levels in orbital fibroblasts from patients with TAO. Cells were treated with 0.5 or 1.0 mg/mL of HA for 24 hours, and the levels of COX-2 and β-actin protein expression were verified. Representative blots from independent experiments for cells from each TAO patients are shown. Quantification of COX-2 protein (fold increase) was calculated by dividing the densitometric value of each lane by the corresponding β-actin value. *$P < 0.05$ versus untreated control. (**D**, **E**) Reverse transcription PCR (**D**) and quantitative real-time PCR analysis (**E**) for COX-2 expression in CF and DF following the same experimental protocol as described in (**A**) and (**B**), respectively. No significant change in COX-2 expression level was noted.

3A). Hyaluronic acid induced an increase in COX-2 expression in cells transfected with control siRNA (lanes 1, 3, and 5 in Fig. 3B), whereas no significant increase of COX-2 expression by HA was noted in CD44 knockdown cells (lanes 2, 4, and 6 in Figs. 3B, 3C). Both steady-state and HA-induced COX-2 expression levels were significantly reduced by CD44 knockdown in orbital fibroblasts from patients with TAO (Fig. 3D). To verify the relevance of CD44 in HA-induced COX-2 increase in orbital fibroblasts, cells were incubated with CD44-neutralizing antibody (5 μg/mL) for 1 hour prior to HA treatment (1.0 mg/mL for 4 or 8 hours). The same concentration of isotype-matched IgG was used for negative control. Pretreatment of CD44-neutralizing antibody significantly attenuated HA-induced COX-2 expression (Figs. 3E, 3F). Taken together, these data indicate that CD44 is involved in HA-induced COX-2 increase in TAO orbital fibroblasts.

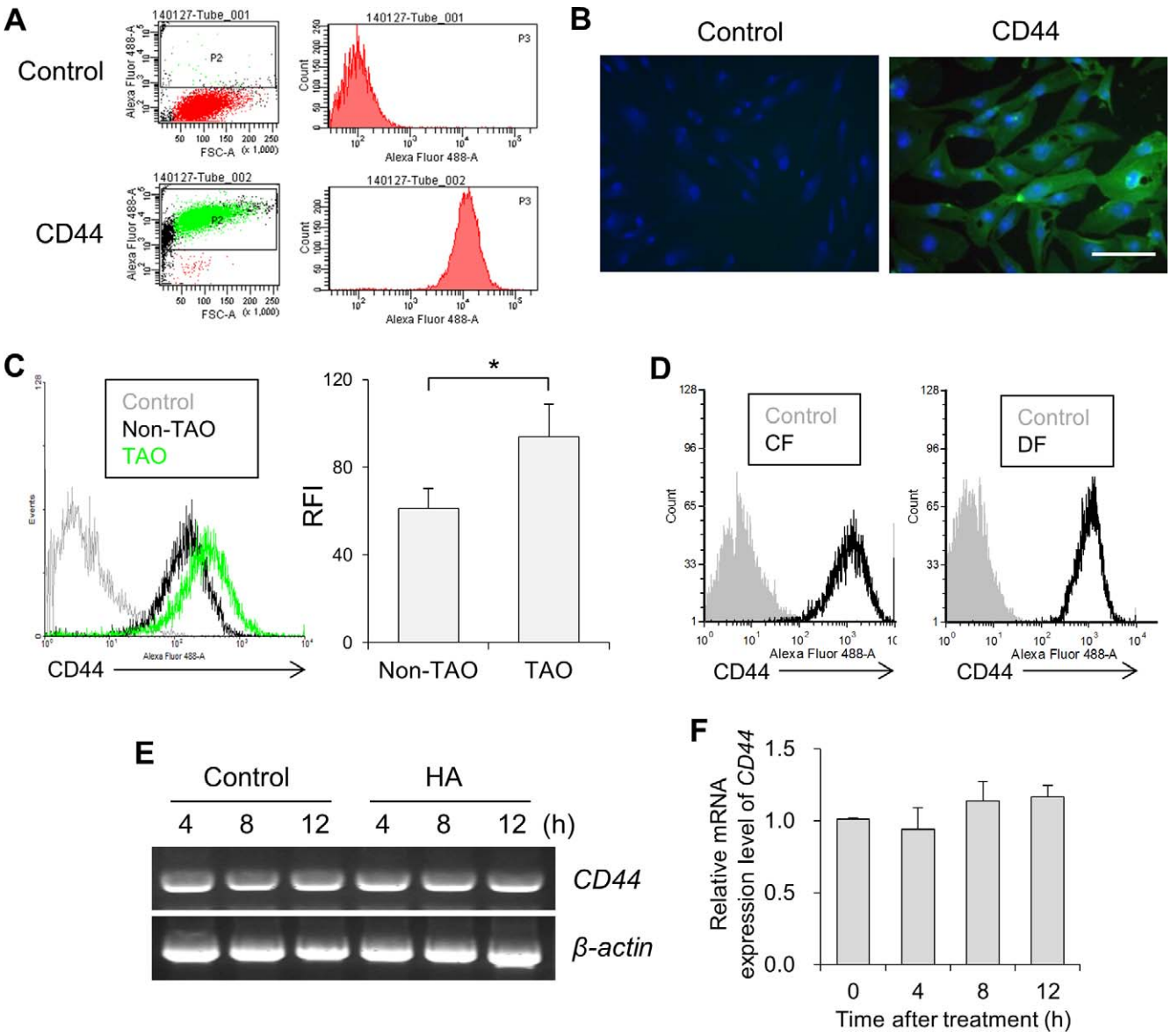

Figure 2. Expression of CD44 and the effect of HA on its expression in orbital fibroblasts from patients with TAO. (**A**) Orbital fibroblasts from patients with TAO ($n = 5$) were subjected to FACS analysis for CD44 expression. Representative data show that 99.1% of orbital fibroblasts were CD44 (+). (**B**) Immunofluorescence assay showing constitutive expression of CD44 (*green*) in orbital fibroblasts from patients with TAO. Representative merged images (CD44, *green*; nucleus, *blue*) of immunofluorescence staining examined by microscopy from independent experiments for cells from each TAO patients are shown. *Scale bars*: 100 μm. (**C**) Analysis with FACS for CD44 expression in orbital fibroblasts from TAO patients and non-TAO subjects (*left panel*). Relative fluorescence intensity of CD44 was calculated by dividing the value of fluorescence intensity by one of each isotype control (*right panel*). (**D**) Conjunctival and dermal fibroblasts obtained from non-TAO subjects ($n = 3$) were subjected to FACS analysis for CD44 expression. (**E**) Orbital fibroblasts were treated with 1.0 mg/mL HA for the indicated times (4, 8, or 12 hours) and the RT-PCR for CD44 was performed. Representative bands from independent experiments for cells from each TAO patients are shown. (**F**) Following treatment with HA using the same protocol as described (**D**), the levels of CD44 mRNA were measured by quantitative real-time PCR. No significant change in CD44 expression level was noted.

Participation of the MAPK Signaling Pathway in HA-Induced COX-2 Expression

Mitogen-activated protein kinases participate in HA-CD44–induced signaling in various cells.[20–22] To determine their involvement in orbital fibroblasts, cells were treated with 1 mg/mL HA for 5 minutes and immunoblotting was performed for total and phosphorylated form of MAPKs. As shown in Figure 4A, HA increased JNK, p-38, and ERK phosphorylation. In addition, to verify the contribution of MAPK signaling pathway to HA-induced COX-2 expression in orbital fibroblasts from patients with TAO, we utilized specific inhibitors of MAPKs, including SB203580, PD98059, or SP600125. The messenger RNA levels of COX-2 in cells cotreated with 1 mg/mL HA and incremental doses (0.1, 1, 10, 20 μM) of each specific inhibitor for 12 hours were identified by qRT-PCR. Inhibitors of MAPK, SB203580, PD98059, or SP600125, significantly attenuated HA-induced COX-2 expression in a dose-dependent manner (Fig. 4B). As shown in Figure 4C, cotreatment with the MAPK inhibitors for 4, 8, or 12 hours successfully abrogated HA-induced enhancement of COX-2 expression. Analysis with qRT-PCR showed that MAPK inhibitors significantly attenuated HA-induced COX-2 expres-

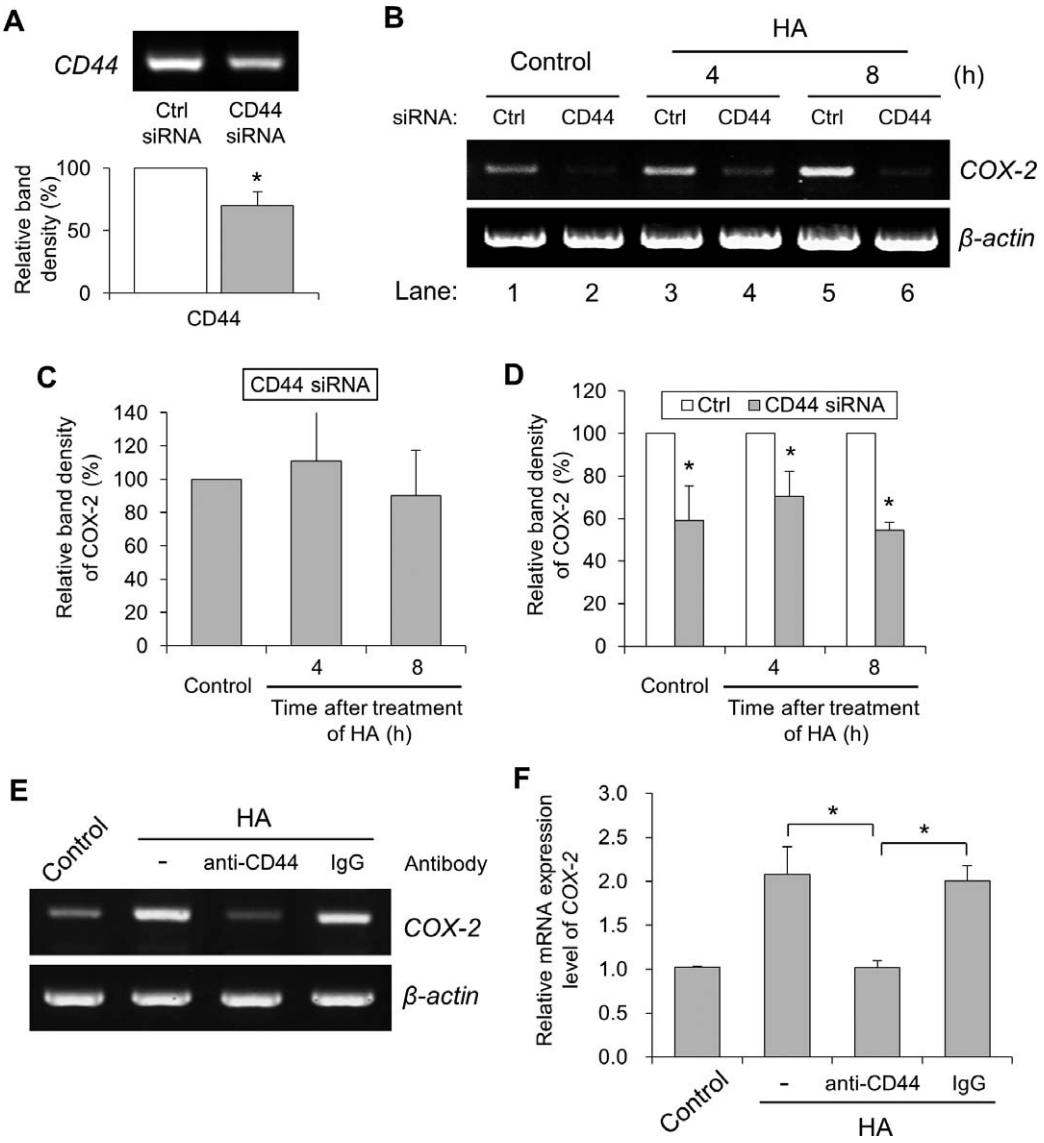

FIGURE 3. Involvement of CD44 in the HA-induced increase in COX-2 in orbital fibroblasts. (**A**) After knockdown of CD44 using siRNA for orbital fibroblasts from patients with TAO ($n = 5$), the expression level of CD44 was evaluated by RT-PCR. Representative bands from independent experiments for cells from each TAO patients are shown. The expression level of CD44 was significantly decreased in CD44 knockdown cells compared with the one of cells transfected with negative control siRNA. *$P < 0.05$. Ctrl, control. (**B**) The cells were treated with 1.0 mg/mL HA for 4 or 8 hours, and the RT-PCR analysis for COX-2 expression was performed. Representative bands from independent experiments of cells from each TAO patients are shown. (**C**, **D**) Densitometric quantification of COX-2 mRNA levels of cells transfected with control siRNA (*empty bars*) and cells transfected with CD44 siRNA (*filled bars*). The measured intensities of COX-2 mRNA were corrected to their corresponding β-actin values, and the relative percent decrease to each control was analyzed (*$P < 0.05$ versus each control). (**E**) Following pretreatment with CD44-neutralizing antibody or isotype IgG (5 μg/mL) for 1 hour, cells were treated with 1.0 mg/mL HA for 12 hour and then RT-PCR analysis for COX-2 expression was performed. (**F**) Following treatment using the same protocol as described in (**E**), the levels of CD44 mRNA were measured by quantitative real-time PCR. *$P < 0.05$ versus untreated control, representative of three experiments.

sion (Fig. 4D). These results suggest that MAPK signaling participates in HA-induced COX-2 expression in orbital fibroblasts from patients with TAO.

Involvement of the NF-κB Signaling Pathway in HA-Induced COX-2 Expression

Based on a previous report demonstrating the involvement of NF-κB in HA–CD44 interaction-induced signaling pathway,[23] we examined whether NF-κB is involved in HA-induced COX-2 expression in orbital fibroblasts from patients with TAO. Immunoblotting for cells treated with 1 mg/mL HA for 30 minutes showed an increase in the phosphorylated form of IκB, compared with that in the untreated control (Fig. 5A). Following cotreatment with 1 mg/mL HA and incremental doses (0.1, 1, 10, 20 μM) of PDTC, a specific NF-κB inhibitor, for 12 hours, the mRNA levels of COX-2 were analyzed by qRT-PCR (Fig. 5B). Cotreatment with PDTC significantly attenuated HA-induced enhancement of COX-2 expression in a dose-dependent manner. Cells cotreated with HA and PDTC for 4 to 12 hours also displayed reduced levels of COX-2 expression (Figs. 5C, 5D), suggesting that NF-κB signaling is involved in HA-mediated COX-2 induction in orbital fibroblasts from patients with TAO.

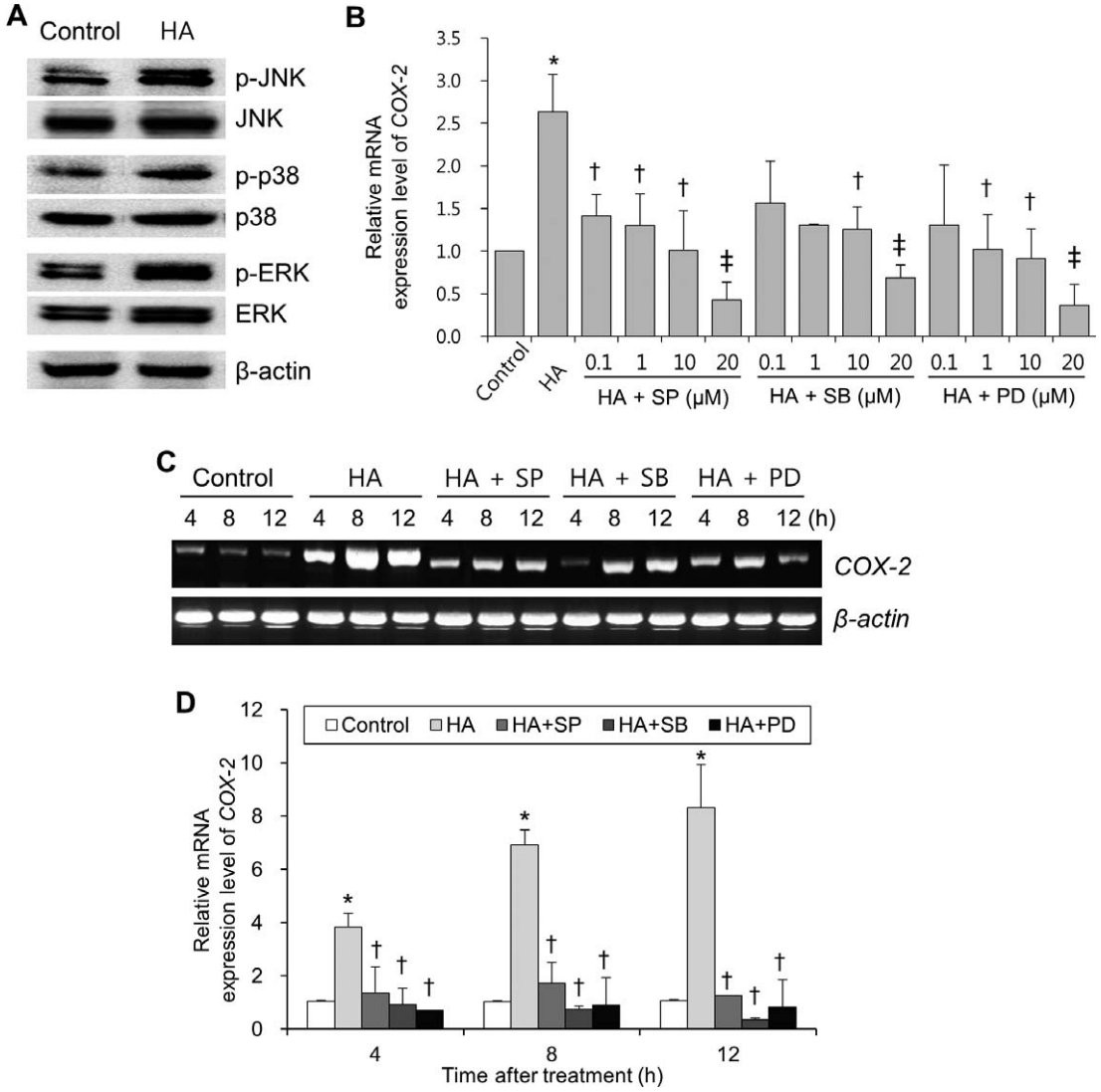

Figure 4. Participation of the MAPK signaling pathway in the HA-induced COX-2 increase in orbital fibroblasts. (**A**) Orbital fibroblasts from patients with TAO ($n = 5$) were treated with 1.0 mg/mL HA for 5 minutes, and immunoblotting was performed for total or phosphorylated forms of MAPKs. Representative bands from independent experiments of cells from each TAO patients are shown. (**B**) Quantitative real-time PCR analysis for the cells cotreated with 1.0 mg/mL HA and incremental doses of an MAPK inhibitor (SP600125 [SP]), a JNK inhibitor; SB203580 [SB], a p38 inhibitor; PD98059 [PD], an MEK inhibitor) for 12 hours. *$P < 0.05$ versus untreated control. †$P < 0.05$ versus HA-treated cells, representative of three experiments. ‡$P < 0.01$ versus HA-treated cells, representative of three experiments. (**C**) Cells were cotreated with each 20 μM MAPK inhibitor and 1.0 mg/mL HA for 4, 8, or 12 hours, and RT-PCR for COX-2 expression was performed. Representative bands from independent experiments of cells from each patient with TAO are shown. (**D**) Following the same experimental protocol as described in (**C**), COX-2 mRNA level was measured by qRT-PCR analysis. *$P < 0.05$ versus untreated control. †$P < 0.05$ versus HA-treated cells, representative of three experiments.

Discussion

In this study, our data show that HA increased COX-2 expression in orbital fibroblasts from patients with TAO. Cyclooxygenase is an enzyme that catalyzes the production of prostaglandin (PG)E_2, which modulates the inflammatory process.[28] The significance of COX-2 in the histopathology of TAO has been addressed, in which inflammation dominates, particularly in the active stage of the disease.[29] In vitro studies demonstrate proinflammatory cytokines such as leukoregulin and IL-1β induce upregulation of COX-2 in orbital fibroblasts.[30,31] In addition, COX-2 is expressed at higher levels in orbital fibroadipose tissues of patients with TAO and is positively correlated with increasing severity of TAO, suggesting a possible relationship between COX-2 expression and orbital inflammation in patients with TAO.[26,32]

Orbital fibroblasts originate from the neuroectoderm, unlike other fibroblasts,[33] and are believed to have unique site-specific characteristics compared with other types of fibroblasts.[34] Proinflammatory cytokines, such as leukoregulin and IL-1β, potentiate the induction of orbital fibroblast genes that play important roles in the orbital inflammatory responses, but do not stimulate the inflammatory response in dermal fibroblasts.[35,36] The magnitude of enhanced HA synthesis by stimulants, such as leukoregulin or immunoglobulins from patients with TAO, is greater in orbital than that in dermal fibroblasts.[37,38] In addition, orbital fibroblasts from patients with TAO produce significantly more IL-6, IL-8, and MCP-1 through CD40 ligation than do orbital fibroblasts of non-TAO donors.[39] Our data from this study show that HA induced the increase of COX-2 in orbital fibroblasts from patients with TAO, but failed to do so in dermal, conjunctival, or orbital fibroblasts

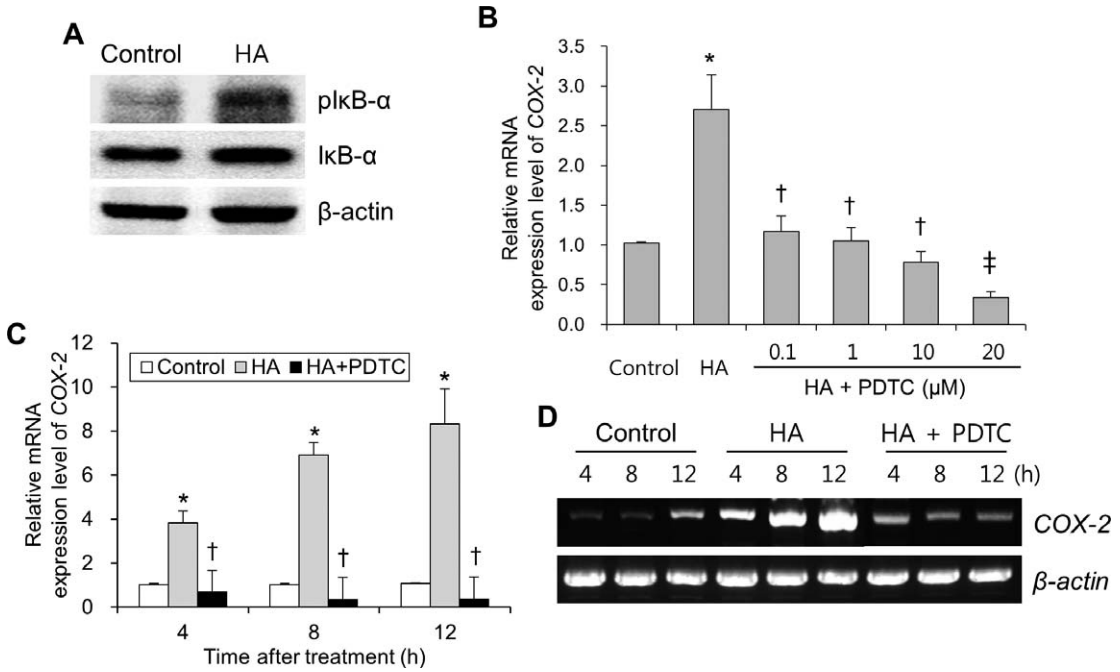

Figure 5. Involvement of the NF-κB signaling pathway in the HA-induced COX-2 increase in orbital fibroblasts. (**A**) Cells were treated with 1.0 mg/mL HA for 30 minutes, and immunoblotting was performed for total or phosphorylated forms of IκB. Representative bands from independent experiments of cells from each TAO patients ($n = 5$) are shown. (**B**) Quantitative real-time PCR analysis for the cells cotreated with 1.0 mg/mL HA and incremental doses of an inhibitor to NF-κB (PDTC) for 12 hours. $^*P < 0.05$ versus untreated control. †$P < 0.05$ versus HA-treated cells, representative of three experiments. ‡$P < 0.01$ versus HA-treated cells, representative of three experiments. (**C**) Cells were cotreated with 20 μM PDTC and 1.0 mg/mL HA for 4, 8, or 12 hours, and the qRT-PCR analysis was performed. $^*P < 0.05$ versus untreated control. †$P < 0.05$ versus HA-treated cells, representative of three experiments. (**D**) Following the same experimental protocol as described in (**C**), RT-PCR for COX-2 expression was performed. Representative bands from independent experiments of cells from each patient with TAO are shown.

from non-TAO subjects. Taken together with the clinical usage of an HA-based formula for patients with dry eye with few inflammation-related complications, these specific characteristics of orbital fibroblasts from patients with TAO may be relevant to our results.

Hyaluronic acid is synthesized by a class of integral membrane proteins called HA synthases, of which vertebrates have three types called HAS1, HAS2, and HAS3. These enzymes lengthen HA by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded via the ABC-transporter through the cell membrane into the extracellular space.[40] Its molecular weight often reaches the millions as high molecular weight (HMW)-HA.[41] The average molecular weight in human synovial fluid is 3000–4000 kDa, and HA purified from human umbilical cord is 3140 kDa.[42] One of the main differences between the HAS isoforms is the chain length of the HA molecules that they produce. Synthases HAS1 and HAS3 generate HA with broad size distributions (molecular weights of 200–2000 kDa), whereas HAS2 generate HA with a broad but extremely large size (average molecular weight >2000 kDa).[43] All of these HAS isoforms have been identified in orbital fibroblasts from patients with TAO, and their expression is induced by IL-1β.[44] The hyaluronic acid utilized in this study was from rooster combs. This particular HA was categorized as HMW-HA by a previous report showing its molecular weight to be 1400 kDa.[45] In a recent in vitro study, HA secreted from orbital fibroblasts of patients with TAO was primarily comprised of HMW polymers (>500 kDa).[46] We believe this recent finding supports the validity of our experimental design using HMW-HA, in terms of the pathological circumstance in patients with TAO.

Most hyaluronic acid exists in the extracellular matrix in a soluble form, and degradation is mediated by a family of enzymes called hyaluronidases. Degraded fragments of HA by hyaluronidases are usually low molecular weight (LMW) HA. Interestingly, HA is thought to possess distinct size-specific activities.[47] Low molecular weight HA can induce inflammatory responses in macrophages and dendritic cells during tissue injury and skin transplants, whereas the native HMW-HA cannot.[48,49] However, the role of HMW-HA is controversial regarding inflammation. In accordance with our results, HMW-HA increases COX-2 expression and production of PGI2 and vascular endothelial growth factor in HUVECs.[25] Taken together with a report that hyaluronidase activity is undetectable in orbital fibroblasts,[37] this discrepancy, depending on the molecular weight of HA, might be derived from different cellular characteristics.

Cell-adhesion molecule CD44, is a major HA binding protein that is ubiquitously expressed on a wide variety of cells[15]; it is highly expressed in orbital connective tissue of patients with TAO,[24] which is consistent with our findings that the expression level of CD44 in orbital fibroblasts from patients with TAO was significantly higher than that in non-TAO orbital fibroblasts (Fig. 2C). In previous reports, CD44 expression can be increased significantly following treatment with IL-1α, TNFα, IGF-1, or TAO-IgGs.[24] We examined the effects of HA on CD44 expression in orbital fibroblasts from patients with TAO; however, we did not find any changes in mRNA expression level of CD44 upon HA treatment, suggesting that HA is not a stimulator of CD44 expression. In dermal or conjunctival fibroblasts, the HA-induced COX-2 increase was not detected despite their possession of CD44, of which the mechanism has not been further elucidated here. In our system, HA-induced COX-2 expression was significantly reduced by knockdown or

neutralization of CD44 in orbital fibroblasts from patients with TAO (Fig. 3). These results strongly suggest that the HA-CD44 interaction plays a crucial role in the pathogenesis of TAO through upregulation of COX-2. In addition, HA increased the phosphorylation of MAPKs and IκB in orbital fibroblasts from patients with TAO, and HA-induced COX-2 expression was significantly abrogated by inhibiting MAPKs or NF-κB. These findings suggest their involvement in CD44-mediated signaling in orbital fibroblasts, which was supported by several reports revealing activation of p38,[20] MEK,[21] JNK,[22] and NF-κB[23] during CD44-mediated intracellular signaling in various cells.

Administration of anti-CD44 antibodies inhibits inflammation in murine models of inflammatory bowel disease,[50] collagen- and proteoglycan-induced arthritis cutaneous inflammation,[51] and experimental autoimmune encephalomyelitis.[52] Therefore, our findings suggest that CD44 could be a target for novel interventions for this disease.

In conclusion, our results provide evidence that HA has a possible role as an inflammatory stimulant by inducing the COX-2 production during TAO pathogenesis. Moreover, considering that the MAPK and NF-κB-mediated signaling pathways participated in HA-induced COX-2 increase through involvement of CD44, this also suggests the possibility that it could be one of a pathogenesis-based therapeutic target in patients with TAO.

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