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Potential Therapeutic Role of Bone Morphogenic Protein 7 (BMP7) in the Pathogenesis of Graves' Orbitopathy

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Purpose. We investigated a role of bone morphogenic protein 7 (BMP7), a member of the TGF-β superfamily on pathogenic mechanism of Graves' orbitopathy (GO). The therapeutic effects of BMP7 on inflammation and fibrosis were evaluated in cultured Graves' orbital fibroblasts.

Methods. Expression of BMP7 was compared in cultured orbital tissue explants from GO (n = 12) and normal control (n = 12) subjects using real-time PCR. Orbital fibroblasts were cultured from orbital connective tissues obtained from GO (n = 3) and normal control patients (n = 3). Cells were pretreated with recombinant human BMP7 (rhBMP7) before stimulation with TGF-β, IL-1β, and TNF-α. Fibrosis-related proteins and inflammatory cytokines were analyzed by Western blotting. The activation of signaling molecules in inflammation and fibrosis was also analyzed.

Results. The expressions of *BMP7* mRNA were lower in GO orbital tissues than control. Fibrosis-related proteins, fibronectin, collagen 1α, and α-SMA induced by TGF-β were suppressed by treating rhBMP7, and rhBMP7 upregulated TGF-β induced SMAD1/5/8 protein expression, whereas downregulated SMAD2/3. Increased pro-inflammatory molecules, IL-6, IL-8, and intercellular adhesion molecule-1 (ICAM-1) by IL-1β or TNF-α were blocked by rhBMP7 treatment, and the expression of phosphorylated NFκB and Akt was suppressed by rhBMP7 treatment.

Conclusions. *BMP7* transcript levels were downregulated in Graves' orbital tissues. Exogenous BMP7 treatment showed inhibitory effects on the production of profibrotic proteins and proinflammatory cytokines in orbital fibroblasts. Our results provide a molecular basis of BMP7 as a new potential therapeutic agent through the opposing mechanism of profibrotic TGF-β/SMAD signaling and proinflammatory cytokine production.

Keywords: graves' orbitopathy, inflammation, fibrosis, bone morphogenic protein 7, orbital fibroblast

Graves' orbitopathy (GO) is an autoimmune disease that targets the orbit, which occurs in patients with hyperthyroidism caused by Graves' disease. In progression of GO, mononuclear cells such as B cells, T cells, and orbital fibroblasts play a significant role in pathogenic inflammation and fibrosis.[1] Interactions of T cells and autoantibodies produced by B cells stimulate orbital fibroblasts to make cytokines and chemokines and act as a bridgehead to exaggerated inflammation, proliferation, and adipocyte differentiation.[1,2] Recent studies in GO pathogenesis have shown that proinflammatory cytokines produced by activated T cells induce collagen fibrils and glycosaminoglycan production with hyaluronan predominating, leading to fibrosis and edematous changes.[3,4] Chronic inflammation eventually leads to collagen accumulation and excessive extracellular matrix production, which can cause mechanical and functional damage of orbital tissues. Fibrotic tissue formation of extraocular muscles in GO can contribute to diplopia or vision loss associated with motility restriction. To reduce irreversible tissue destruction, systemic glucocorticoid therapy has been a main treatment in moderate-to-severe and active GO[5]; however, there is a limitation of efficacy because of adverse effects with chronic therapy.[6] Despite the emergence of antifibrotic drugs that have shown encouraging results in experimental models, the translational outcomes have not been as successful. Investigation of novel treatments suppressing the development of GO with a deeper understanding of the underlying pathogenesis is necessary.

Bone morphogenic protein (BMP), first identified as a factor involved in bone formation in 1990,[7] physiologically acts as a major survival factor in the development of kidney, bone, and eye.[7,8] This is supported by studies showing that BMP7 mutants predominantly displayed abnormalities confined to the developing kidney and eye.[9] It is the member of the TGF-β superfamily, counteracting the profibrotic activity of TGF-β in various disease models and inhibits epithelial-to-mesenchymal transition (EMT), which provokes the fibrogenic response by the formation of myofibroblasts. Several studies have shown that induction of BMP7 by recombinant protein or adenovirus-mediated overexpression could reverse TGF-β induced EMT or peritoneal fibrosis in vitro or in vivo models.[10,11] Administration of exogenous BMP7 or overexpression of BMP7 protects organs such as the kidney,[12] liver,[13] lung,[14] and heart[15] from fibrosis. In recent years, the use of BMP7 has been extended to several other inflammatory diseases, including cardiovascular diseases and neurological disorders. Several studies suggest that BMP7 has anti-inflammatory activity by enhancing M2 macrophage differentiation and anti-inflammatory cytokine production.[16,17] In addition to enhancing monocyte polarization into the M2 macrophage, BMP7 significantly suppressed levels of proinflammatory cytokines, IL-6, TNF-α and MCP-1 in THP-1 cells.[16] BMP7 can also protect neural cells from oxidative stress[18] and exert an important role in brown fat development and differentiation.[19]

We are unaware of any prior studies investigating a therapeutic role of BMP7 in GO. This study was undertaken to evaluate the effect of exogenous recombinant human BMP7 on the fibrotic and inflammatory pathogenesis of GO in primary cultured orbital fibroblasts.

Methods

Reagents and Chemicals

The source for antibodies used in this study was as follows. Dulbecco's modified Eagle medium (DMEM):F12 and penicillin-streptomycin were obtained from Welgene, Inc. (Daegu, Gyeongsangbuk-do, Korea). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). Recombinant human BMP7, recombinant TNF-α, IL-1β, and TGF-β were obtained from R&D Systems (Minneapolis, MN, USA). Antibodies for phosphorylated (p)-SMAD1/5/8, total (t)-SMAD1/5/8, p-SMAD2, t-SMAD2, p-SMAD3, t-SMAD3, p-extracellular signal-related kinase (ERK), t-ERK, p-p38, t-p38, TNF receptor–associated factor 6 (TRAF6), intercellular adhesion molecule 1 (ICAM-1), p-nuclear factor (NF)-κB, and t-NFκB, p-Akt, t-Akt, p-Jun N-terminal kinase (Jnk), and t-Jnk were obtained from Cell Signaling Technology (Danvers, MA, USA). Fibronectin antibody was obtained from BD Bioscience (Franklin Lakes, NJ, USA), α-SMA antibody were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA), and IL-6 antibody was obtained from Novus Biologicals (Centennial, CO, USA). Collagen 1α and IL-8 antibodies were obtained from Abcam (Cambridge, MA, USA). BMP7 and β-actin antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The antibodies used in the study are listed in detail in Supplementary Table S1.

Tissue and cell Preparation

We obtained orbital adipose/connective tissue samples from 12 GO patients undergoing decompression surgery for severe proptosis (six males, six females; age 31–72 years). All patients were euthyroid at the time of surgery, and clinical activity scores were recorded. Euthyroid status was defined as normal serum level of thyroid-stimulating hormone and free thyroxine (T4). All GO patients were in a stable euthyroid state and free from steroid and radiotherapy treatment for at least three months before surgery. Normal adipose/connective tissues for control group were obtained from 12 patients undergoing upper or lower lid blepharoplasty who had no history of GO or autoimmune thyroid disease (five males, seven females; age 35–78 years). There was no significant age difference between GO (48.6 ± 14.0) and non-GO (59.8 ± 12.6) groups ($P > 0.05$), and all patients involved in the study were Asian (Supplementary Table S2). All processes of obtaining tissue samples were performed by one surgeon in one institution. The institutional review board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) approved the study (IRB No.: 4-2020-1431), and we followed the tenets of the Declaration of Helsinki. All patients agreed with written informed consent.

Orbital fibroblasts were cultured as described in our previous study.[20] Minced tissues were distributed in DMEM:F12 (1:1) containing 20% FBS, penicillin-streptomycin. After a monolayer of fibroblasts was grown, cells were treated gently with trypsin/ethylenediaminetetraacetic acid and remained in DMEM cell culture media with 10% FBS and antibiotics. Strains were stored in liquid nitrogen, and cells were grown in a humidified 5% CO_2 incubator at 37°C. Cells between the second and fifth passages were used for all experiments.

Cell Viability Assay

The effect of rhBMP on cell viability was evaluated by methyl tetrazolium bromide (MTT) assay, according to the manufacturer's (Sigma-Aldrich Corp.) protocol. Orbital fibroblasts from GO and non-GO subjects were seeded into 24-well culture plates (1×10^5 cells/well) and treated with different dosages of rhBMP7 (10, 50, 100, 200, and 400 ng/mL) for 24 hours and for different times (24, 48, and 72 hours/10 ng/mL). After treatment, cells were washed and treated with MTT (5 mg/mL) solution for four hours at 37°C. The medium was then removed, and cells were solubilized in ice-cold isopropanol. Absorbency was measured at 560 nm with background subtraction at 630 nm using a microplate reader (EL 340 Bio Kinetics Reader; Bio-Tek Instruments, Winooski, VT, USA).

Real-Time PCR

Orbital tissues were homogenized with a tissue homogenizer (Precellys 24; Bertin Instruments, Montigeny-le-Bretonneux, France) using a Precellys lysing kit (Bertin Instruments) with Trizol (Invitrogen). The RNA concentration was determined using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). 1 µg of mRNA was reverse-transcribed into cDNA (SensiFAST cDNA Synthesis Kit; Meridian Life Science, Inc., Memphis, TN, USA) and amplified with SYBR green PCR master mix (Takara Bio, Inc., Shiga, Japan) with The LightCycler 480 System (Roche, Basel, Swiss). The primers sequences for target genes were as follows: *BMP7*; 5′-GCTTCTCCTACCCCTACAAGG-3′ (forward), 5′- TCGGTGAGGAAATGGCTATC-3′(reverse), GAPDH; 5'- TGCTGTAGCCAA ATTCGTTG-3' (forward), and 5'- CACCCACTCCTCCACCTT T-3' (reverse). All PCRs were

performed in triplicate. In normalization, GAPDH expression was used. The results as relative changes of fold in the threshold cycle (Ct) value were obtained based on control group, using $2-\Delta\Delta Ct$ method.

Western Blot Assay

Using orbital fibroblasts from GO patients and control non-GO subjects, Western blot assay was performed to investigate the therapeutic effects of rhBMP7. Noncytotoxic concentration, 100 ng/mL rhBMP7 was used to pretreat orbital fibroblasts for one hour before the challenge with stimulants (5 ng/mL TGF-β, 10 ng/mL IL-1β, or 10 ng/mL TNF-α, 24 hours) for analysis of profibrotic and proinflammatory proteins. Each group of orbital fibroblasts in the study plan was washed with Dulbecco's phosphate-buffered saline solution (Welgene, Inc.) and then lysed with RIPA lysis buffer (Welgene, Inc.) that contained protease inhibitor cocktail (Thermo Fisher Scientific). The cell lysates were resolved in 10% SDS-PAGE and then transferred to nitrocellulose membranes (Millipore Corp., Billerica, MA, USA). The membranes were then treated with primary antibodies overnight at 4°C. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescent substrate (Thermo Fisher Scientific) using an image reader (LAS-4000 mini; Fuji Photo Film, Tokyo, Japan). The intensities of bands, which reflect protein quantities, were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized to that of the β-actin in the same sample. The experiments were performed three times in cells from each unique subject and repeated in triplicate per each sample. The relative amount of each immunoreactive band was quantified by densitometry analysis and normalized to the β-actin in the same sample.

Statistical Analysis

For statistical analyses, IBM SPSS statistic for Windows v. 20.0 (IBM Corp., Armonk, NY, USA) was used. All results are notated as mean values ± standard deviation. The Mann-Whitney U-test and Kruskal-Wallis test were used for nonparametric data, and the Kolmogorov-Smirnov test was used for data that was not normally distributed. $P < 0.05$ was considered statistically significant.

Results

Effect of rhBMP7 on Cell Viability

We used the MTT assay to evaluate the effect of rhBMP7 on cellular viability in both GO and non-GO orbital fibroblasts. Cell viability remained above 95% in both GO and non-GO orbital fibroblasts treated with 10 to 400 ng/mL of rhBMP7 for 24 hours and with 100 ng/mL of rhBMP7 for 24 to 72 hours (Supplementary Fig. S1).

GO Tissues Show Increased Expression of *BMP7*

To identify its potential role in GO, RNA was extracted and *BMP7* expressions were quantified from all orbital tissue explants of GO and non-GO patients. *BMP7* transcript levels were compared between GO and non-GO patients by RT-PCR (Fig. 1). The results from RT-PCR showed that *BMP7* mRNA levels were significantly lower in GO orbital tissue explants than in the normal control orbital tissues.

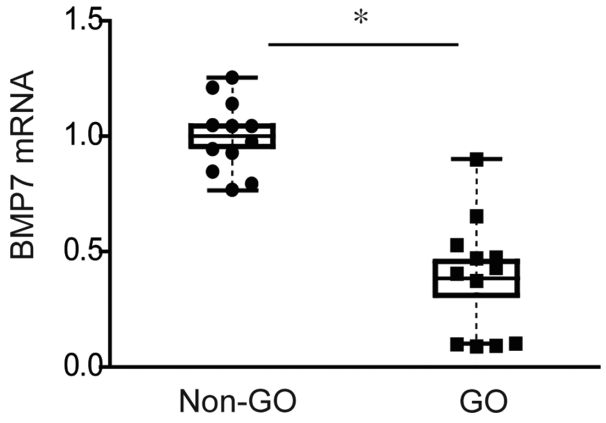

Figure 1. Expression of *BMP7* mRNA in GO tissues in comparison to non-GO normal control tissues. RNA was extracted form GO (n = 12) and non-GO (n = 12) orbital tissues. Using real-time PCR, *BMP7* mRNA transcript levels were compared between GO and normal control tissues. A single dot represents an average value obtained from the experiments on a single donor (*$P < 0.05$ vs. non-GO tissues). The samples were assayed in triplicate. The results are expressed as the median and interquartile ranges by box plot.

BMP7 Suppresses Production of TGF-β-Induced Profibrotic Proteins

TGF-β is a central mediator that drives fibrosis in the chronic stage of GO as in other progressive fibrotic diseases which results in a pathologic excess of extracellular matrix deposition, leading to disrupted tissue architecture.[21] In this study, TGF-β (5 ng/mL, 24 hours) induced expression of fibronectin, collagen Iα, and α-SMA in Western blot analyses. When orbital fibroblasts were pretreated with rhBMP7 (100 ng/mL, one hour), enhanced expression of fibronectin, collagen Iα and α-SMA after TGF-β treatment was significantly blunted in both GO and non-GO orbital fibroblasts (Fig. 2).

BMP7 Increases Activation of Antifibrotic Signaling Molecule

The canonical and noncanonical signal pathways of TGF-β impact the fibrotic process at the cellular and molecular levels, inducing profibrotic gene expression with the consequent increase in proteins such as α-SMA, fibronectin, collagen, and other extracellular matrix proteins.[21] The canonical TGF-β signaling pathway uses SMAD2, SMAD3, or both to transfer signals,[22] and noncanonical TGF-β signaling pathways involve ERK/MAPK kinase.[23] To investigate the effect of BMP7 on further downstream signal pathways, expression levels of total and phosphorylated forms of SMAD1/5/8, SMAD2/3, ERK and p38 were analyzed by Western blot. Treatment of orbital fibroblasts with 100 ng/mL of rhBMP7 significantly increased phosphorylation of SMAD1/5/8 in both non-GO and GO fibroblasts, even more in cells stimulated with TGF-β ($P < 0.05$). The increase of SMAD1/5/8 by BMP7 was more predominant in GO cells ($P < 0.05$). Phosphorylation of SMAD2 and SMAD3 was increased when TGF-β was treated and was suppressed when pretreated with rhBMP7. ERK and p38 signal proteins were increased by

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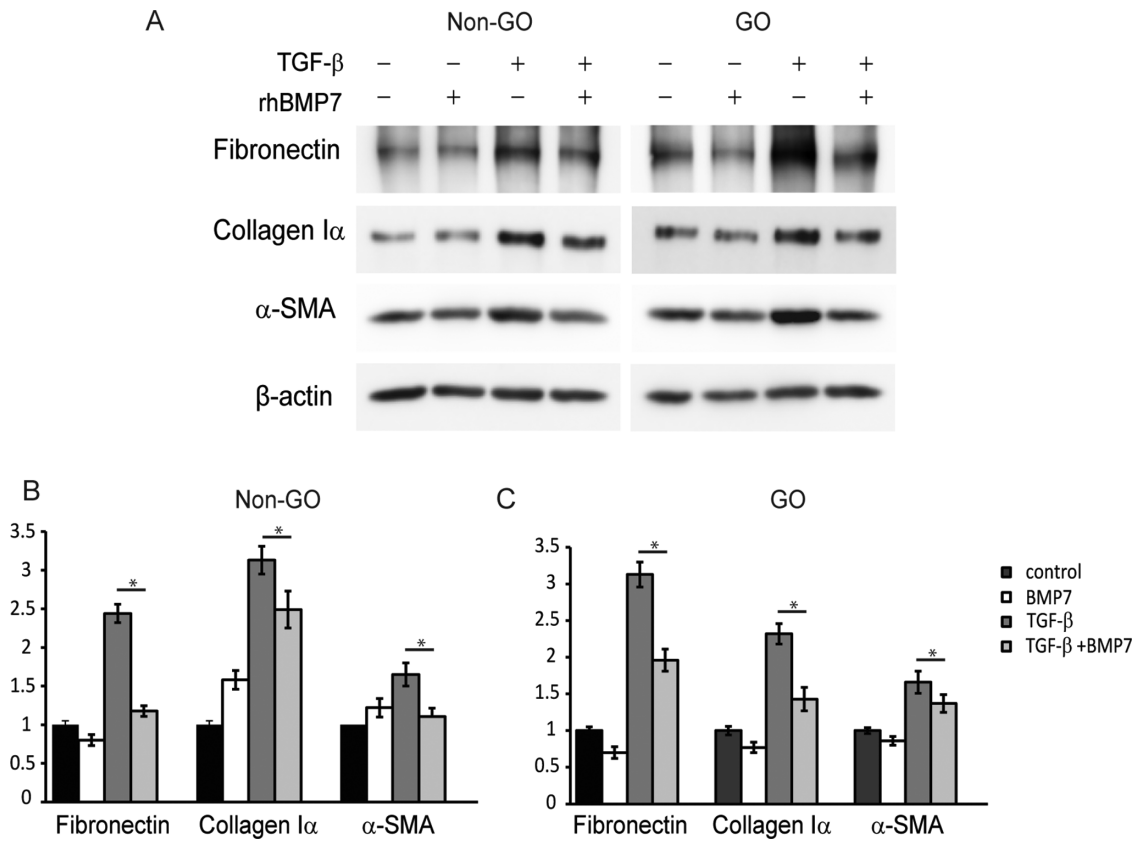

Figure 2. Effect of BMP7 on TGF-β-induced profibrotic protein production. Orbital fibroblasts from GO patients (n = 3) and control non-GO subjects (n = 3) were treated with 5 ng/mL TGF-β for 24 hours. The cells were also pretreated with 100 ng/mL rhBMP7 for one hour before the challenge with the stimulant. (**A**) Western blot analyses were conducted to compare protein expressions of the profibrotic proteins, fibronectin, collagen Iα and α-SMA. Experiments were performed in three GO and three non-GO cells from different individuals. The representative gel images are shown. (**B, C**) Quantification was performed using densitometry. The results are presented as the mean density ratio $\pm$ SD, normalized to the level of β-actin in the same sample (*$P < 0.05$).

TGF-β but, however, were not affected by rhBMP7 stimulation (Fig. 3).

BMP7 Suppresses Production of IL-1β or TNF-α-Induced Proinflammatory Mediators

As shown in prior study, IL-1β and TNF-α stimulate proinflammatory cytokine and chemokine production involved in pathogenesis of GO.[24] To evaluate the effect of BMP7 on the inflammatory pathogenesis, GO and non-GO orbital fibroblasts were pretreated with rhBMP7 for one hour before IL-1β or TNF-α (10 ng/mL) stimulation for 24 hours. The production of proinflammatory cytokines, IL-6, IL-8, ICAM-1 protein expressions were increased after stimulation with IL-1β or TNF-α in Western blot analyses, which were significantly attenuated by rhBMP7 pretreatment in both GO and normal control orbital fibroblasts (Fig. 4).

BMP7 Decreases Activation of Proinflammatory Signal Molecules

Along with traditional adenylyl cyclase-cAMP cascade, the phosphoinositide 3-kinase (PI3K)–Akt pathway has been reported to be involved in GO pathology[25] and mitogen-activated protein kinase (MAPK) is well known for its involvement in inflammatory disease.[26] To demonstrate whether BMP7 is involved in PI3k-Akt pathway and MAPK pathway, GO and non-GO orbital fibroblasts were pretreated with an rhBMP7 for one hour before IL-1β or TNF-α stimulation (10 ng/mL, one hour). Phosphorylation of NFκB and Akt protein was increased under IL-1β or TNF-α stimulation in western blot analyses, and was significantly attenuated by treatment with rhBMP7, particularly in GO cells (Fig. 5).

Discussion

In the current study, we found that gene expression of *BMP7* was significantly downregulated in GO tissue explants compared to normal controls, suggesting a possible association of BMP7 in the pathogenesis of GO. Treatment of exogenous BMP7 suppressed TGF-β induced production of profibrotic proteins and activated SMAD1/5/8 phosphorylation and suppressed SMAD2/3 activated by TGF-β. In addition, exogenous BMP7 attenuated IL-1β or TNF-α induced proinflammatory cytokine production and also phosphorylation of NFkB and Akt signal protein expression.

Previous studies have shown the importance of BMP7 in organ homeostasis and as an opposing mechanism to the profibrogenic activity of TGF-β, and it has been extensively studied in multiple organs (Supplementary Table S3).[27] It is also reported to function in protecting the kidney in experimental models of chronic kidney disease and interstitial

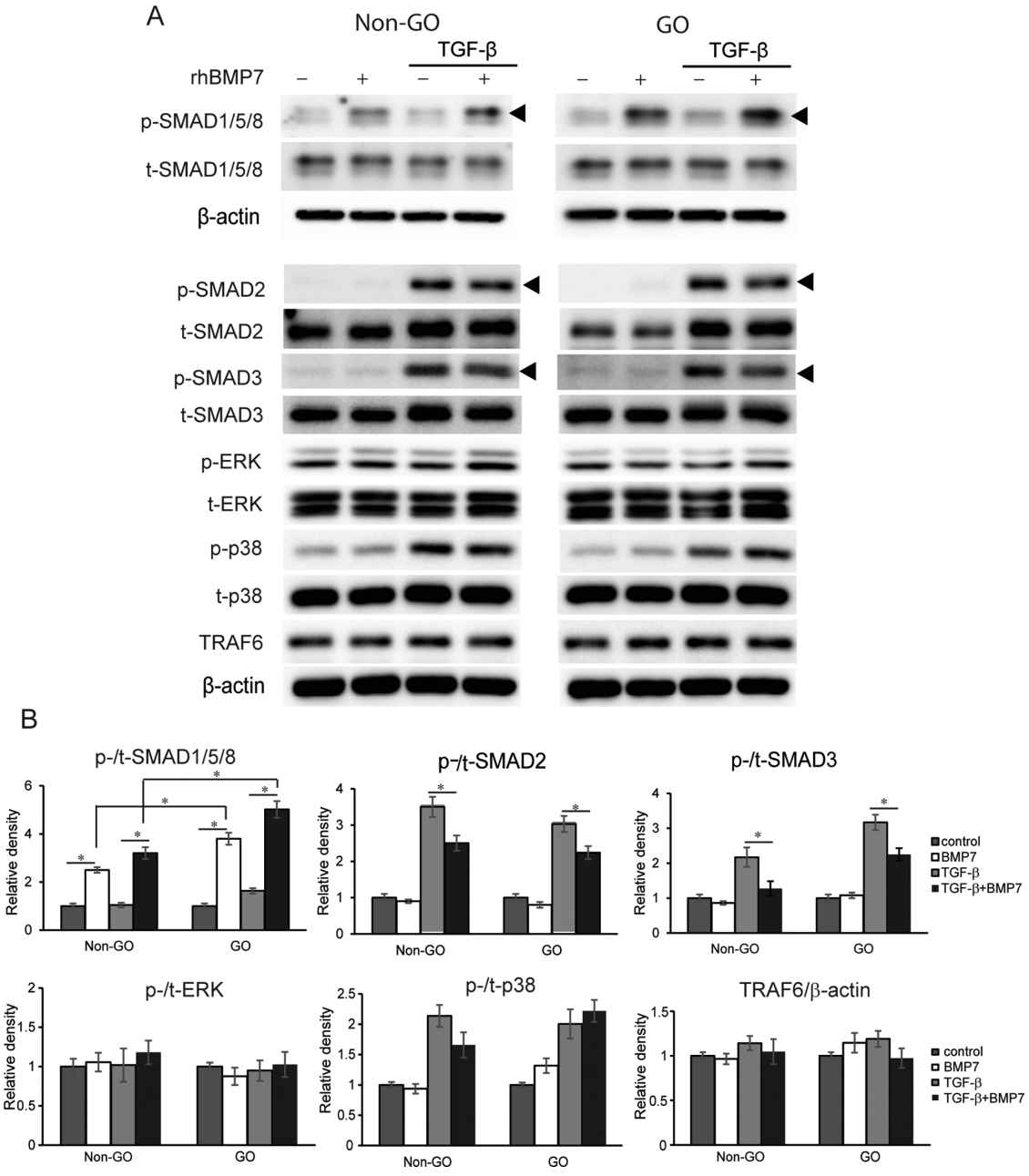

Figure 3. Effect of BMP7 on the activation of signal proteins in response to TGF-β treatment. Orbital fibroblasts from GO patients (n = 3) and normal control non-GO subjects (n = 3) were treated with 5 ng/mL TGF-β for one hour with or without pretreatment with 100 ng/mL rhBMP7 for one hour. (**A**) In Western blot analyses, treatment of rhBMP7 resulted in a significant increase in the levels of phosphorylated forms of SMAD1/5/8 more predominantly in GO cells, while suppressed phosphorylation of SMAD2/3 was activated by TGF-β. Experiments were performed in three GO and three non-GO cells from different individuals. The representative gel images are shown. (**B**) Quantification of signal protein markers was performed using densitometry. The relative band intensity of each protein was indicated by the ratio of phosphorylated protein and total protein and normalized to the level of β-actin in the same sample. The results are presented as the mean density ratio ± SD (*$P < 0.05$).

fibrosis by reversing EMT.[28] In an animal model of transverse aortic constriction, BMP7 exerted beneficial effects on left ventricular pathologic remodeling and repressed TGF-β induced transcriptional activation of Col1A1 promoter in NIH-3T3 fibroblasts.[29] Induction of BMP7 by Ad-BMP7 infection, as well as exogenous BMP7, inhibited α-SMA and collagen formation via upregulation of SMAD1/5/8 phosphorylation in hepatic stellate cells and improved liver fibrosis in mice.[13,30] Based on our data of downregulated BMP7 in Graves' orbital tissue explants, we hypothesize that BMP7 might play a role in normal homeostasis in healthy orbit. The repression of BMP7 could have resulted in progression to fibrosis on exposure to chronic inflammatory stimuli in GO. Exogenous BMP7 could inhibit TGF-β–mediated production of profibrotic proteins, confirming a protective role of BMP7 signaling in an in vitro model of GO.

Different TGF-β members exert distinct biological activity with high specificity. TGF-β and BMP7 counter-regulate each

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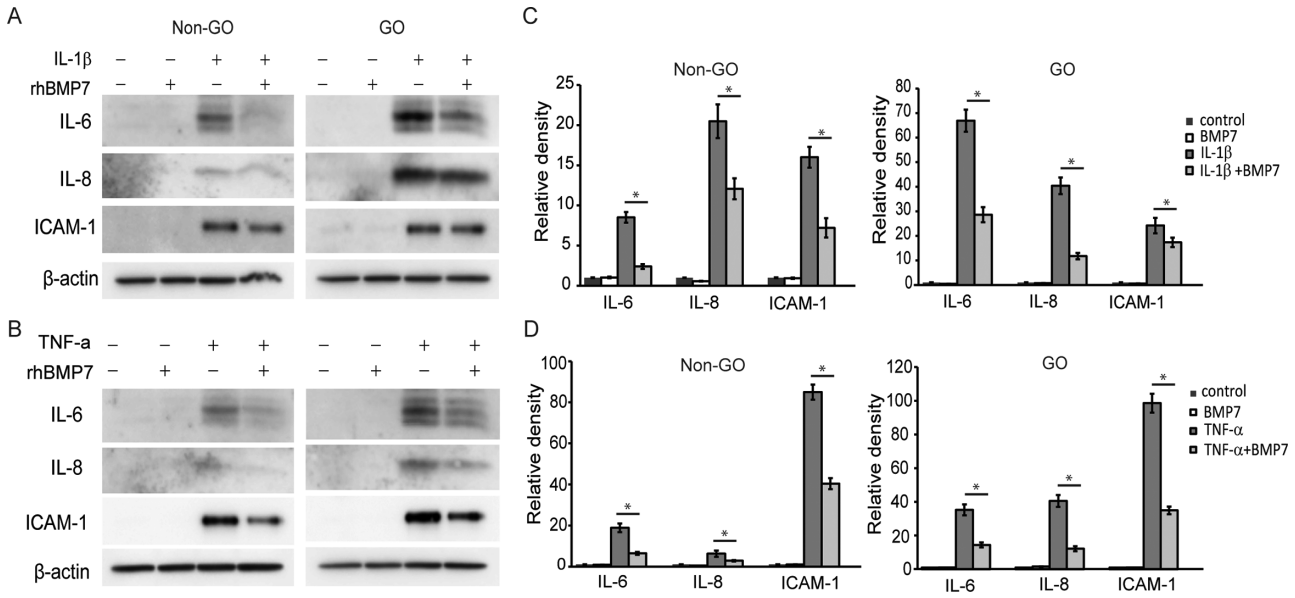

Figure 4. Effect of BMP7 on the proinflammatory cytokine production. Orbital fibroblasts obtained from GO patient (n = 3) and normal control non-GO subjects (n = 3) were treated with IL-β (10 ng/mL, 24 hours) or TNF-α (10 ng/mL, 24 hours) with or without pretreatment with 100 ng/mL rhBMP7 for one hour. (**A, B**) Western blot analyses were performed to compare protein expressions of the proinflammatory cytokines, IL-6, IL-8, and ICAM-1. Experiments were performed in three GO and three non-GO cells from different individuals. The representative gel images are shown. (**C, D**) Quantification was performed using densitometry. The results are presented as the mean density ratio $\pm$ SD, normalized to the level of β-actin in the same sample (*$P < 0.05$).

other to preserve the balance of homeostasis of their biological activities. SMAD2/3 pathway activation is reported to produce profibrotic pathology in numerous organs, whereas SMAD1/5/8 activation plays an antifibrotic role.[31,32] The activation of BMP receptors by BMP7 results in phosphorylation of SMAD1/5/8 and their heterodimerization with SMAD4, followed by translocation into the nucleus, leading to suppression of SMAD2/3 by TGF-β1 stimulation.[13,33] In several chronic diseases, the counter-regulatory balance is significantly altered as TGF-β signaling is upregulated by activation of SMAD2/3, whereas BMP7 and its downstream SMAD1/5/8 are downregulated.[34,35] Similar to previous reports, our data show exogenous BMP7 induced phosphorylation of SMAD1/5/8 in either presence or absence of TGF-β stimulation and suppressed phosphorylation of SMAD2/3 in presence of TGF-β stimulation. These results suggest that the balance between profibrotic and antifibrotic signaling is the opposing action of BMP7 and TGF-β/SMAD signaling, which infers that BMP7 induces the phosphorylation of antifibrotic SMAD1/5/8 that opposes TGF-β mediated phosphorylation of SMAD2/3 and fibrogenic gene expression.[27] Although the canonical SMAD signaling pathway is considered a major downstream mechanism, TGF-β/BMP7 is also reported to stimulate noncanonical signaling pathway such as MAPK pathway (ERK, Jnk, p38).[36] BMP7 is also reported to orchestrate protective effect through PI3K/Akt alteration.[37,38] However in this study, BMP7 did not transduce signal through MAPK or Akt pathway in orbital fibrosis. We assume that BMP7 may exert antifibrotic activity in GO by transducing signal through activation of SMAD1/5/8 to alleviate fibrotic change in GO, not through the noncanonical signal pathway.

Our study demonstrates that exogenous BMP7 inhibited IL-1β or TNF-α induced production of proinflammatory molecules such as IL-6, IL-8, and ICAM-1 in orbital fibroblasts. Although not as thoroughly investigated as in fibrotic models, numerous studies regarding the anti-inflammatory effect of BMP7 were reported.[16,17,39,40] BMP7 prevented the loss of kidney function associated with ischemic injury by suppressing ICAM-1 expression and infiltration of neutrophil, monocyte, and macrophage.[39] BMP7 reduced expression of proinflammatory cytokines, like IL-6, IL-8, IL-1β, and MCP-1 in proximal tubule epithelial cells cocultured with TNF-α in kidney.[40] Treatment of BMP7 was associated with M2 polarization of macrophage and induced production of anti-inflammatory cytokines, IL-10 and IL-1Ra.[16,17] In addition, exogenous BMP7 treatment attenuated phosphorylation of NFκB and Akt protein in GO cells, suggesting an additional potential anti-inflammatory role of BMP7 in GO pathogenesis.

We found that BMP7 could modulate fibrogenesis driven by TGF-β in orbital fibroblasts as expected; however, further sophisticated design of BMP7 delivery is critically required for clinical application. Animal studies have revealed that current soluble rhBMPs exhibit a short half-life because of rapid clearance and enzyme degradation,[41] requiring an excess dose with initial burst release. Recently a pro-drug BMP7 with novel delivery mode of action fused with protein transduction domain has been developed.[42] Although BMP7 ligands directly interact with membrane BMP receptor II, the novel prodrug is transduced into the cells by endosomal transport and shows a long-range signaling activity in vivo. This pro-BMP7 drug successfully activated SMAD1/5/8, inhibited TGF-β mediated EMT and suppressed unilateral ureter obstruction-induced renal fibrosis in pigs.[43] Pro-BMP7 drug treatment also suppressed the progression of peritoneal dialysis fibrosis in the rat model.[44] Further in vivo confirmation of antifibrotic and anti-inflammatory roles of BMP7 is required with increased bioactivity of BMP7 to provide the evidence of possible BMP7 therapeutics in GO.

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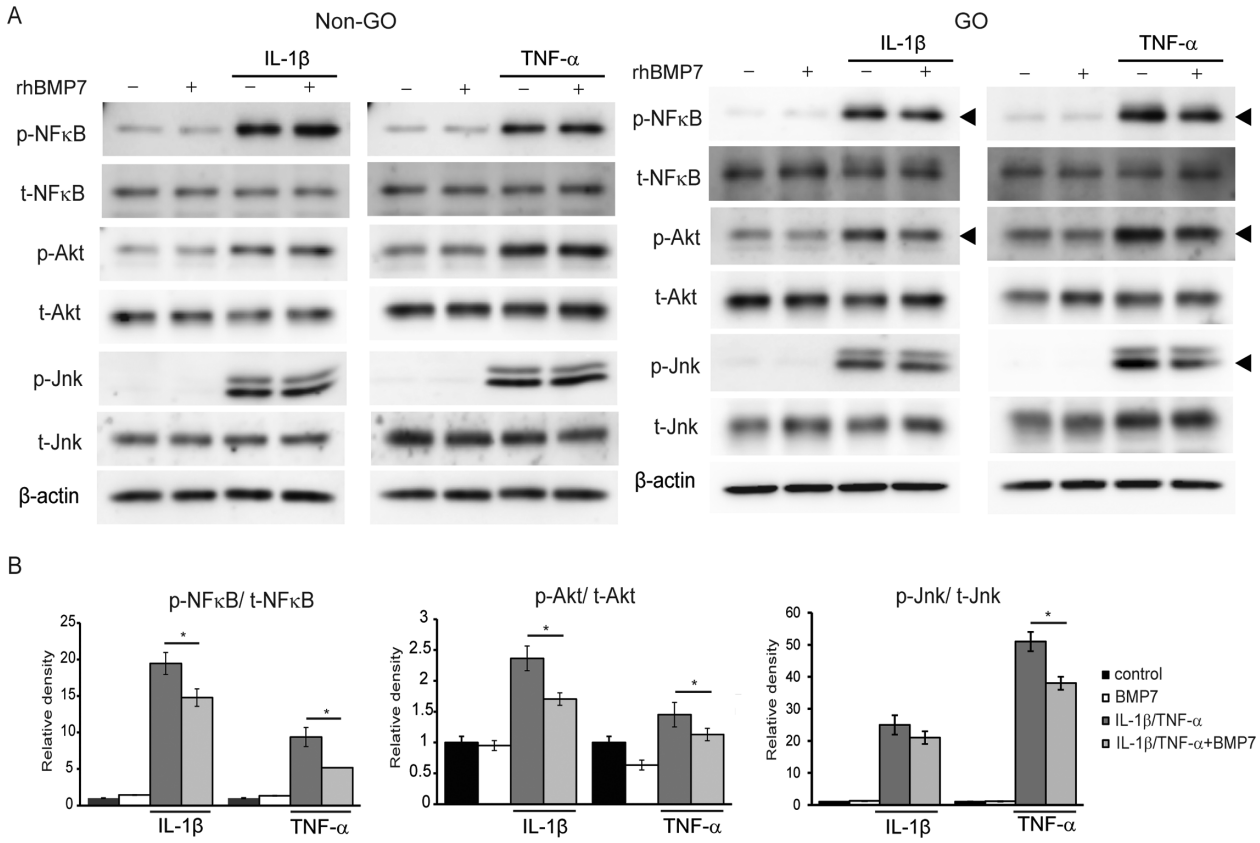

Figure 5. Effect of BMP7 on the activation of signal proteins in response to proinflammatory cytokines treatment. Orbital fibroblasts from GO patients (n = 3) and normal control non-GO subjects (n = 3) were treated with IL-β or TNF-α (10 ng/mL for one hour) with or without pretreatment with 100 ng rhBMP7 for one hour. (**A**) Western blot analyses were conducted to compare protein expressions. Treatment with rhBMP7 attenuated IL-1β or TNF-α-induced expression of p-NFκB, p-Akt and p-Jnk in GO cells. Experiments were performed in three GO and three non-GO cells from different individuals. The representative gel images are shown. (**B**) Quantification of signal pathway proteins was performed using densitometry analysis. The relative band intensity of each protein was indicated by the ratio of phosphorylated protein and total protein and normalized to the level of β-actin in the same sample. The results are presented as the mean density ratio ± SD (*$P < 0.05$).

Future studies of serum BMP7 level in GO patients correlated with clinical activity could confirm clinical relevance. In a recent study, direct local injection of 50 ng BMP7 to injured spinal cord for seven consecutive days showed promotion of neuronal regeneration and recovery of motor function in rats.[45] Series of experiments about pharmacological potential and local injection of BMP7 also suggest the therapeutic possibility of BMP7 via local injection.[42,46–52]

In conclusion, our studies are the first to shed light on the potential of therapeutic role of BMP7 in GO pathogenesis. Based on our results, the status of GO may be a function of disequilibrium of TGF-β/BMP7 levels, as BMP7 gene levels were significantly downregulated in Graves' orbital tissue explants than normal controls. BMP7 significantly inhibited TGF-β-mediated pro-fibrotic protein production, and attenuated IL-1β or TNF-α induced proinflammatory cytokine expression. Our in vitro data highlights the anti-inflammatory and antifibrotic mechanism of BMP7 in GO and may serve as a background for the validity and potential for future clinical studies examining BMP7 in GO.

Acknowledgments

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