

JNK and p38 Inhibitors Prevent Transforming Growth Factor- β 1-Induced Myofibroblasts Transdifferentiation in Graves' Orbital Fibroblasts

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Research Article

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Abstract

Transforming growth factor- β 1 (TGF- β 1)-induced myofibroblasts transdifferentiation from orbital fibroblasts is known to dominate tissue remodeling and fibrosis in Graves' ophthalmopathy (GO). However, the signaling pathway through which TGF- β 1 activates Graves' orbital fibroblasts is unclear. This study investigated the role of mitogen-activated protein kinase (MAPK) pathways in TGF- β 1-induced myofibroblasts transdifferentiation of Graves' orbital fibroblasts. MAPK pathways were assessed by measuring the phosphorylation levels of p38, c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) using Western blot analysis. The expression levels of connective tissue growth factor (CTGF), α -smooth muscle actin (α -SMA), fibronectin, and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-3) representing fibrogenesis processes were analysed. Specific pharmacologic kinase inhibitors were used to confirm the involvement of MAPK pathways. After treating Graves' orbital fibroblasts with TGF- β 1, the phosphorylation levels of p38 and JNK but not ERK were increased. Meanwhile, CTGF, α -SMA and fibronectin were overexpressed. After pre-incubation with p38, JNK and ERK inhibitors respectively, the TGF- β 1-induced expression of CTGF, α -SMA, fibronectin, TIMP-1 and TIMP3 was abolished by p38 and JNK inhibitors but not ERK inhibitors. This study confirmed TGF- β 1-induced myofibroblasts transdifferentiation in Graves' orbital fibroblasts via p38 and JNK signaling. Thus, MAPK pathways may be potential targets for the management of GO.

Introduction

Graves' ophthalmopathy (GO) is an autoimmune disorder characterised by initial inflammation and later tissue expansion, remodeling and/or fibrosis, causing cosmetically disfiguring and vision-threatening morbidities [1, 2]. Orbital fibroblasts are known to be the most important effector cells [3]. The definite pathogenesis, however, is incompletely understood, especially in late-stage GO when the disease progresses into orbital soft tissue fibrosis, leading to proptosis, exposure keratopathy, diplopia, and compressive optic neuropathy that are usually refractory to most medical treatments and require surgical rehabilitation [4–6].

Transforming growth factor- β (TGF- β) is believed to induce tissue remodeling and fibrosis via myofibroblasts transdifferentiation in Graves' orbital fibroblasts [6, 7]. In addition, the protein and messenger ribonucleic acid (mRNA) expression levels of TGF- β 1 are higher in Graves' orbital tissues and orbital fibroblasts [8–10]. TGF- β 1 can promote various fibrotic diseases by stimulating either canonical (Smad-based) or non-canonical (non-Smad-based) signaling pathways, through which myofibroblasts are activated and lead to an overproduction of extracellular matrix (ECM) proteins [11, 12].

The deposition of ECM is due to an imbalance between the activity of matrix degrading proteinases and their inhibitors, which apparently plays an important role in fibrosis [13]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases collectively capable of degrading essentially all ECM components, while tissue inhibitors of metalloproteinases (TIMPs) are specific endogenous inhibitors of MMPs. Several studies have reported that TGF- β 1-mediated ECM remodeling via the

alteration of MMPs and TIMPs contributes to fibrotic disorders including in systemic sclerosis, idiopathic pulmonary fibrosis and myelofibrosis [14–16]. However, the molecular mechanisms responsible for TGF- β 1-induced myofibroblasts transdifferentiation in GO have not been investigated. Our prior study demonstrated that connective tissue growth factor (CTGF) was an essential downstream mediator of TGF- β 1 to promote this reaction, identified by upregulation of alpha-smooth muscle actin (α -SMA) and fibronectin [6]. In this study, we further investigated the role of mitogen-activated protein kinase (MAPK) pathways including p38, c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) signaling cascades in TGF- β 1-induced myofibroblasts transdifferentiation from Graves' orbital fibroblasts.

Results

After treating the orbital fibroblasts from GO patients with 5 ng/ml TGF- β 1, the phosphorylation levels of p38 and JNK but not ERK were significantly increased at 3, 6 and 9 hours in a time-dependent manner compared with those of the control without TGF- β 1 treatment (Fig. 1). Meanwhile, CTGF, α -SMA and fibronectin were overexpressed in TGF- β 1-treating orbital fibroblasts. After incubating the orbital fibroblasts from GO patients with 20 μ M p38 and JNK inhibitors (SB202190 and SP600125) for 1 hour, followed by 5 ng/ml TGF- β 1 treatment for another 9 hours, the phosphorylation levels of p38 and JNK were significantly reduced respectively (Fig. 2). After incubating the orbital fibroblasts from GO patients with one of the MAPK inhibitors for 1 hour, followed by 5 ng/ml TGF- β 1 treatment for another 24 hours, the expression levels of CTGF, α -SMA and fibronectin were significantly reduced in orbital fibroblasts pre-incubated with 20 μ M p38 and JNK inhibitors (SB202190 and SP600125) respectively but not in those pre-incubated with 20 μ M ERK inhibitors (PD98059) (Fig. 3). On the other hand, when treating orbital fibroblasts with 20 μ M p38 and JNK inhibitors (SB202190 and SP600125, respectively) for 1 hour, followed by 5 ng/ml TGF- β 1 treatment for another 24 hours, the expression levels of TGF- β 1-induced TIMP-1 and TIMP-3 were significantly reduced (Figs. 4A and 4B). Further, we analysed the gelatinase activities of MMP-2 and MMP-9 from cultured medium. The MMP-2/MMP-9 activities were significantly inhibited by 5 ng/ml TGF- β 1 treatment for 24 hours, which could be recovered by pre-treatment with 20 μ M p38 and JNK inhibitors (SB202190 and SP600125) respectively (Fig. 4C).

Discussion

The pathological changes of GO involve primarily orbital extraocular muscles and adipose tissues that expand within the unyielding confines of the bony orbit, consequently leading to proptosis, increased orbital pressure and limited ocular motility [19]. Evidence has shown that TGF- β 1 induces Graves' orbital fibroblasts to differentiate into myofibroblasts, which dominates the pathogenic processes [6, 7]. This study confirmed that both p38 and JNK pathways could transduce signals from TGF- β 1 to promote myofibroblasts transdifferentiation in GO. The MAPK family belongs to the non-canonical (non-Smad-based) pathway of TGF- β 1 signaling. Normal activation of MAPK pathways is physiologically essential for the stress response and cell survival, while excessive activation of these pathways accelerates

inflammation and leads to pathologies [20]. The TGF- β 1-dependent MAPK pathway has been implicated in stimulating myofibroblasts transdifferentiation from fibroblasts and subsequent ECM production in several fibrotic pathologies. Cui *et al.* [21] illustrated that p38 and ERK mediated TGF- β 1-induced overexpression of the ECM in keloid fibroblasts. Yu *et al.* [22] proposed that TGF- β 1 activated hepatic stellate cells via p38 and ERK pathways in liver fibrosis. Tian *et al.* [23] reported the correlation of TGF- β 1-dependent p38 and ERK pathways with nickel oxide nanoparticle-induced pulmonary fibrosis. Additionally, the contribution of p38 and JNK pathways for TGF- β to activate renal fibrosis has been addressed [24, 25]. Together with our current findings, the p38 and JNK inhibitors may provide a potential target in the treatment of GO [21–25].

Dysregulation of ECM composition and/or structure is associated with various pathological fibrotic diseases [13, 26]. It is especially important that TGF- β contributes to ECM homeostasis by influencing the production and turnover of ECM components via regulation of matrix degrading proteolytic enzymes (e.g., MMPs) and proteinase inhibitors (e.g., TIMP) [14, 27, 28]. TIMPs maintain the balance between the deposition and degradation of ECM in physiological as well as pathological processes [14]. In current study, we found that the expression of TIMP-1 and TIMP-3 was upregulated by TGF- β 1 in the primary cultured orbital fibroblasts from GO patients, and could be manipulated by p38 and JNK signaling transduction pathway. Further, the gelatinase activities of MMP-2/MMP-9 from cultured medium were reduced by TGF- β 1 in GO orbital fibroblasts, which could be recovered by pre-treatment of those cells with p38 and JNK inhibitors respectively. Based on the above findings, TGF- β 1-mediated overexpression of TIMP-1 and TIMP-3 as well as reduction in the activities of MMP-2/MMP-9 in GO orbital fibroblasts may contribute to abnormal tissue remodeling in GO. In addition, p38 and JNK inhibitors could ameliorate TGF- β 1-induced imbalance between ECM deposition and degradation in GO orbital fibroblasts.

Our current findings agreed with previous reports that the subfamilies of the MAPK pathway had respective physiological actions. Briefly, the JNK and p38 pathways were stimulated by stress-related effectors or cytokines to cause inflammatory responses, autophagy or apoptosis, while the ERK pathway was stimulated by mitogens or growth factors, resulting in cell cycle progression, cell proliferation and differentiation [29]. Besides, this study was in accordance with our previous research that CTGF could be a downstream mediator for TGF- β 1-induced myofibroblasts transdifferentiation and ECM production in Graves' orbital fibroblasts [6]. This study further showed that TGF- β 1-induced upregulation of CTGF in GO orbital fibroblasts could be abolished by p38 and JNK inhibitors.

In conclusion, our study provided an evidence that p38 and JNK but not ERK contributed to the signal transduction of TGF- β 1 to activate myofibroblasts transdifferentiation and ECM production in GO orbital fibroblasts. Therefore, blocking the p38 or JNK pathway may be a potential therapeutic target for prevention or treatment of abnormal tissue remodeling and fibrosis in GO.

Methods

Tissues acquisition and cell culture.

The surgical specimens of 4 subjects with GO (G01-G04) during decompression surgery were recruited. The disease was in the inactive stage in all the subjects who had achieved stable euthyroidism for at least 6 months prior to the surgery by using methimazole. All the subjects were precluded from radiotherapy and systemic corticosteroids for at least 1 month before the surgery. The primary cultures of orbital fibroblasts were collected from the surgical orbital tissues in an aseptic technique. Briefly, the orbital tissues were minced and digested with a sterile phosphate-buffered saline (PBS) containing collagenase (130 U/ml) and dispase (1 mg/ml) in an incubator filled with an atmosphere of 5% CO₂ at 37 °C [6, 17]. After 24 hours, the digested orbital tissues were collected, and the resuspended in the cultured medium containing 10% fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin sulfate (100 µg/ml). Cultured orbital fibroblasts were recruited between the 3rd and 5th passages whereby the cell cultures at the same passage number were selected for the same set of experiments. All study protocols were approved by the Institutional Review Board of Taipei Veterans General Hospital (TVGH) and were designed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the subjects.

Chemicals and antibodies.

The recombinant protein for human TGF-β1 (#P01137), the mouse monoclonal antibodies against human TIMP-1 (#MAB970) and TIMP-3 (#MAB973) were acquired from R&D Systems, Inc (MN, USA), respectively. The rabbit polyclonal antibodies against the CTGF (#ab6992), fibronectin (#ab2413) and α-SMA (#ab5694) were acquired from Abcam Inc. (Cambridge, UK). The mouse monoclonal antibodies against the p38 (#ab31828), p38 phosphorylation (p-p38, #ab4822), JNK (#ab208035), JNK phosphorylation (p-p38, #ab76572), ERK (#ab184699) and ERK phosphorylation (p-p38, #ab201015) were all acquired from Abcam Inc. (Cambridge, UK). The secondary antibodies against rabbit (#A5795) and mouse (#A9044) as well as GAPDH (#G5262) and β-actin (#A5441) were acquired from Sigma-Aldrich (St. Louis, MO, USA) [6].

Western blot analysis.

The protein was extracted from cells lysate with lysis buffer containing a protease inhibitor cocktail (Biomax, Taipei, Taiwan) as our previous study [17]. An aliquot of 60–100 µg proteins was separated on 10% SDS-PAGE and blotted onto the PVDF membrane (Amersham-Pharmacia Biotech Inc., Buckinghamshire, UK). After blocking with 5% skim milk, the PVDF membrane was incubated with the primary antibody, followed by a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody. The protein expression signals were detected by an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech Inc., Buckinghamshire, UK) according to the manufacturer's instruction, and were quantified by ImageScanner III with LabScan 6.0 software (GE Healthcare BioSciences Corp., Piscataway, NJ, USA).

MMP-2 and MMP-9 enzyme activity assay.

The MMP-2 and MMP-9 enzyme activities in the culture medium were measured from InnoZyme™ Gelatinase Activity Assay kit (Merck KGaA, Darmstadt, Germany) by a fluorogenic method according to the product instruction [18]. Briefly, 100 µl cultured medium were diluted in activation buffer, and incubated for 3 hours with thiopeptide substrate specific for type IV collagenases (MMP-2 and MMP-9). The released fluorescence of the cleaved substrate of MMP-2 and MMP-9 (ex: 320 nm; em: 405 nm) was monitored. Triplicate tests were performed for each reaction in a 96-well, and the relative fluorescence unit ratios to control set were plotted.

Statistical analysis.

The SPSS software and Microsoft Excel 2019 statistical package were used for statistical analysis. The results from the one-way ANOVA followed by an L.S.D. test and the Student's t test were obtained from three independent experiments, respectively. Data were presented as means ± S.D. and p value less than 0.05 was considered statistically significant.

Declarations

Data Availability

All data supporting the findings of this study are available from the corresponding author on reasonable request.

Acknowledgments

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Author Contributions

C.C.T. conceived and designed the experiments. S.B.W. performed the experiments. C.C.T., H.C.K. and S.B.W. analyzed the data. C.C.T., S.B.W. and T.Y.H. wrote the manuscript. C.C.T. supervised the study. All authors commented on and approved the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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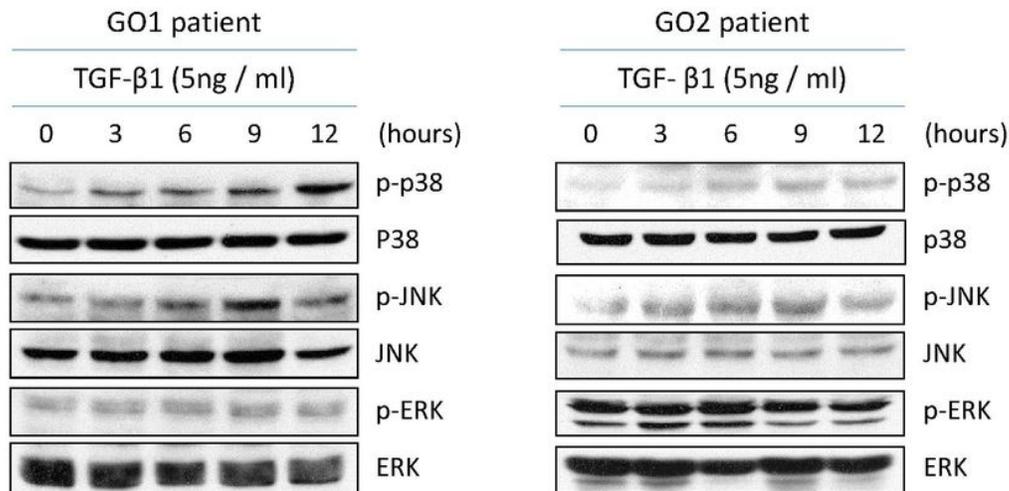
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Figures

(a)



(b)

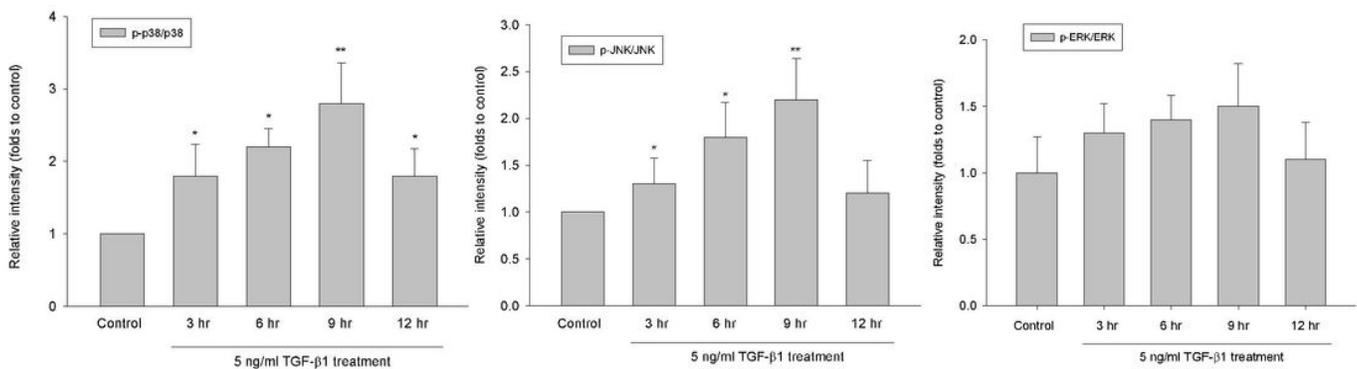


Figure 1

Upregulation of p38 and JNK phosphorylation by TGF-β1 in the primary cultures of orbital fibroblasts from patients with Graves' ophthalmopathy (GO). (a) After treating the orbital fibroblasts from GO patients with 5 ng/ml TGF-β1, the phosphorylation levels of p38, JNK and ERK were observed for 3, 6, 9 and 12 hours by Western blots. (b) The expression ratio of p-p38 to p38, p-JNK to JNK and p-ERK to ERK from the control without TGF-β1 treatment was defined as 1.0; then the other relative intensity (folds) was presented respectively. By three independent Western blots experiments, together data were averaged from the same patient strain. Then the means of the different patient (GO1-GO4, N=4) strains were averaged. The representative histogram was constructed based on the mean values of protein expression levels in the primary cultures of orbital fibroblasts from the four GO patients. Data were presented as means ± S.D. of the results from three independent experiments (*p < 0.05 vs. control without TGF-β1 treatment; **p < 0.01 vs. control without TGF-β1 treatment).

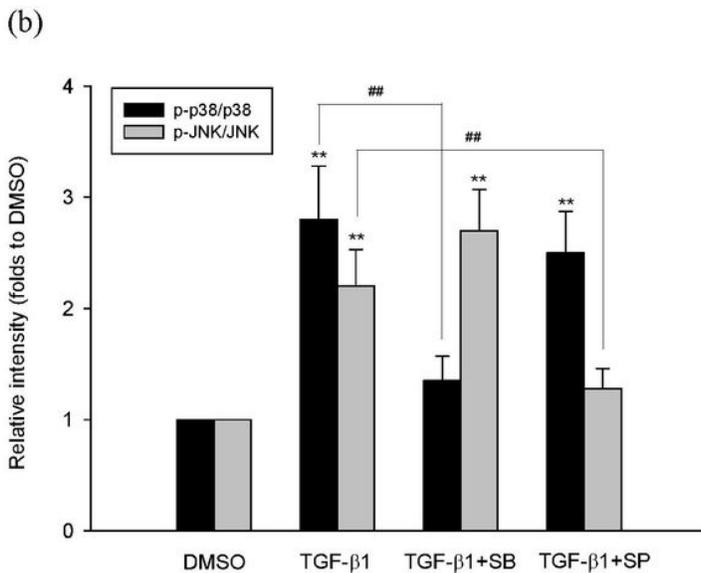
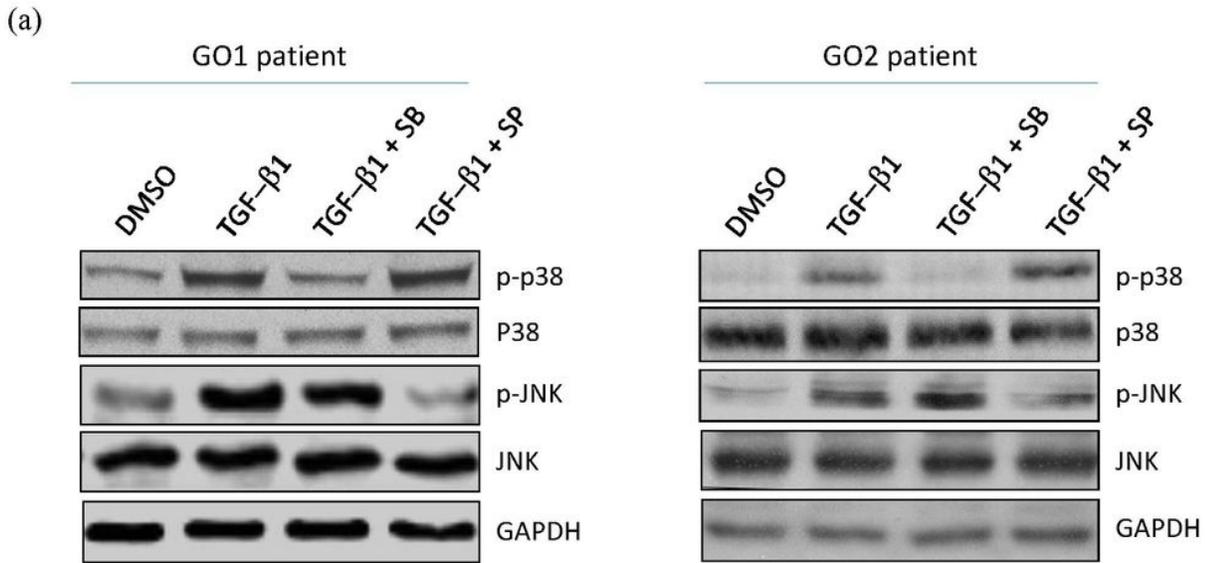


Figure 2

Inhibition of TGF- β 1-induced p38 and JNK phosphorylation by specific inhibitors in the primary cultures of orbital fibroblasts from patients with Graves' ophthalmopathy (GO). (a) Orbital fibroblasts from GO patients were pre-incubated with 20 μ M p38 and JNK inhibitors (SB202190 and SP600125) for 1 hour respectively, followed by 5 ng/ml TGF- β 1 treatment for another 9 hours. The phosphorylation levels of p38 and JNK were analysed by Western blots. (b) The expression ratio of p-p38 to p38 and p-JNK to JNK from the DMSO control without TGF- β 1 treatment was defined as 1.0; then the other relative intensity (folds) was presented respectively. By three independent Western blots experiments, together data were averaged from the same patient strain. Then the means of the different patient (GO1-GO4, N=4) strains were averaged. The representative histogram was constructed based on the mean values of protein expression levels in the primary cultures of orbital fibroblasts from the four GO patients. Data were

presented as means \pm S.D. of the results from three independent experiments (** $p < 0.01$ vs. control without TGF- $\beta 1$ treatment; ## $p < 0.01$ vs. TGF- $\beta 1$ treatment).

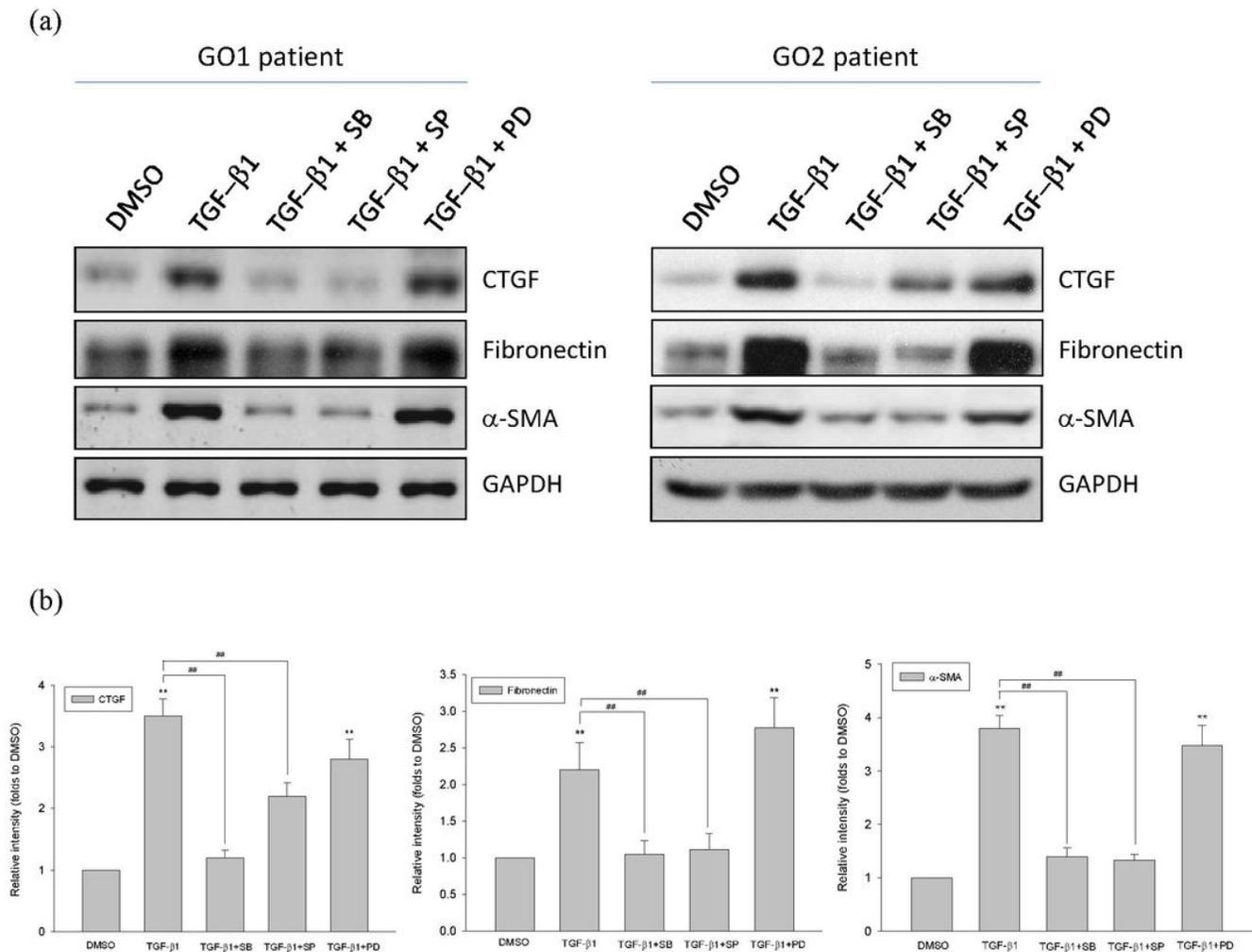


Figure 3

Abolishment of TGF- $\beta 1$ -induced fibrosis by p38 and JNK inhibitors but not ERK inhibitors in the primary cultures of orbital fibroblasts from patients with Graves' ophthalmopathy (GO). (a) Orbital fibroblasts from GO patients were pre-incubated with 20 μ M p38, JNK and ERK inhibitors (SB202190, SP600125 and PD98059) for 1 hour respectively, followed by 5 ng/ml TGF- $\beta 1$ treatment for another 24 hours. Then the expression levels of CTGF, fibronectin and α -SMA were analysed by Western blots. (b) The relative intensity of CTGF, fibronectin and α -SMA expression normalised to each GAPDH control and DMSO control without TGF- $\beta 1$ treatment was defined as 1.0; then the other relative intensity (folds) was presented respectively. By three independent Western blots experiments, together data from the same patient strain were averaged. Then the means of different patient (GO1-GO4, N=4) strains were averaged.

The representative histogram was constructed based on the mean values of protein expression levels in the primary cultures of orbital fibroblasts from the four GO patients. Data were presented as means \pm S.D. of the results from three independent experiments (** $p < 0.01$ vs. control without TGF- β 1 treatment; ## $p < 0.01$ vs. TGF- β 1 treatment).

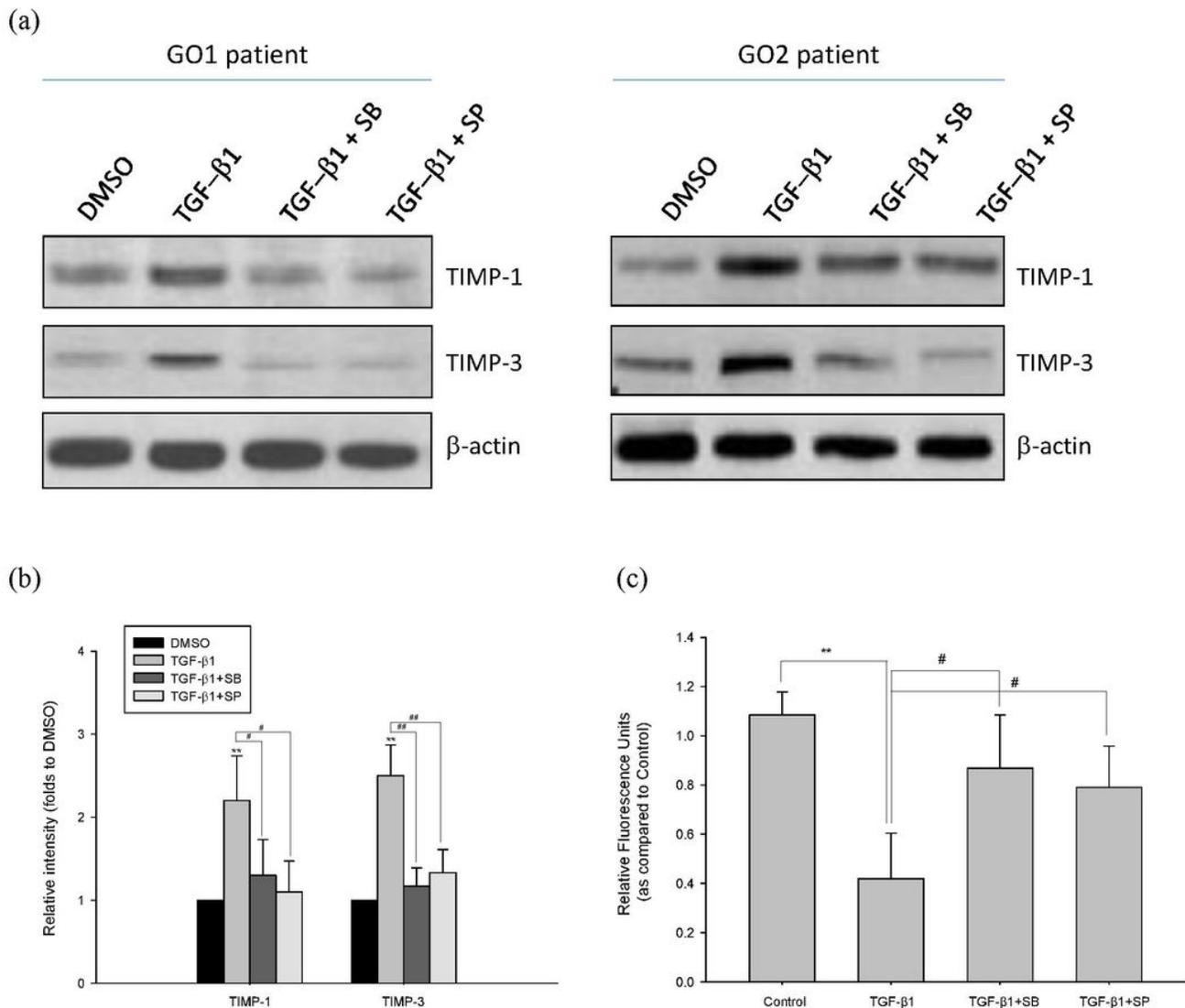


Figure 4

Abolishment of TGF- β 1-mediated matrix remodeling by p38 and JNK inhibitors in the primary cultures of orbital fibroblasts from patients with Graves' ophthalmopathy (GO). (a) Orbital fibroblasts from GO patients were pre-incubated with 20 μ M p38 and JNK and ERK inhibitors (SB202190 and SP600125) for 1 hour respectively, followed by 5 ng/ml TGF- β 1 treatment for another 24 hours. Then the expression levels of TIMP-1 and TIMP-3 were analysed by Western blots. (b) The relative intensity of TIMP-1 and TIMP-3 expression normalised to each GAPDH control and DMSO control without TGF- β 1 treatment was defined as 1.0; then the other relative intensity (folds) was presented respectively. By three independent Western

blots experiments, together data from the same patient strain were averaged. Then the means of different patient (GO1-GO4, N=4) strains were averaged. (c) The gelatinase activities of MMP-2 and MMP-9 enzymes from cultured medium were determined. The representative histogram was plotted based on the mean values of relative fluorescence units from the four GO patients cultured orbital fibroblasts. Data were presented as means \pm S.D. of the results from three independent experiments (**p< 0.01 vs. control without TGF- β 1 treatment; #p< 0.05 and ##p< 0.01 vs. TGF- β 1 treatment).

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