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ORIGINAL ARTICLE

EGCG blocks TGFβ1-induced CCN2 by suppressing JNK and p38 in buccal fibroblasts

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Abstract
Objectives Transforming growth factor β (TGFβ) has been suggested as the main trigger for the increased collagen production and decreased matrix degradation pathways in oral submucous fibrosis (OSF). Connective tissue growth factor (CTGF/CCN2) and cyclooxygenase-2 (COX-2) were found to overexpress in OSF. The aim of this study was to investigate the molecular mechanism underlying the TGFβ-induced CCN2 expressions in human buccal mucosal fibroblasts (BMFs) to identify the potential targets for drug intervention or chemoprevention of OSF.
Materials and methods TGFβ-induced CCN2 expression and its signaling pathways were assessed by Western blot analyses in BMFs.
Results TGFβ1 stimulated CCN2 synthesis in BMFs. Pretreatment with c-Jun NH_2-terminal kinase (JNK) inhibitor SP600125, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, and activin receptor-like kinase 5 (ALK5) inhibitor SB431542 significantly reduced TGFβ1-induced CCN2 synthesis. Epigallocatechin-3-gallate (EGCG) completely blocked TGFβ1-induced CCN2 synthesis by inhibiting the phosphorylation of JNK and p38 MAPK. Prostaglandin E_2 (PGE_2) inhibited the TGFβ1-induced CCN2 synthesis in human fetal lung fibroblasts IMR90 but not in BMFs.
Conclusions The TGFβ1-induced CCN2 synthesis in BMFs could be mediated by the ALK5, JNK, and p38 MAPK pathways. EGCG blocks TGFβ1-induced CCN2 by suppressing JNK and p38 in BMFs.
Clinical relevance The exceptional signal transduction pathways of TGFβ1-induced CCN2 production in BMFs contribute to the resistance of PGE_2 downregulation of CCN2 expression; therefore, the CTGF/CCN2 levels are maintained in the OSF tissues in the presence of COX-2. EGCG may serve as a useful agent in controlling OSF.

Keywords CTGF/CCN2 · Connective tissue growth factor · EGCG · Oral submucous fibrosis · TGFβ1 · Transforming growth factor β

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Introduction

Oral submucous fibrosis (OSF) is a precancerous chronic oral mucosal disease with a malignant transformation rate of 7.6% over a 17-year period [1, 2]. It is characterized by irreversible generalized fibrosis and epithelial atrophy affecting the oral cavity, pharynx, and upper digestive tract [1, 2]. OSF leads to restriction of mouth opening and tongue movement, intolerance to spicy food, xerostomia, limitation of food consumption, difficulty in maintaining oral health, and impaired ability to speak [1, 2]. Over five million of victims in the world suffer from this disease [3]. OSF is generally considered as a collagen metabolic disorder with excessive deposition of extracellular matrix (ECM) components and collagens in the connective tissue [1]. The regular use of areca nut (AN, *Areca catechu*) preparations is the main etiological factor [1, 2]. Exposure of oral buccal mucosal fibroblasts (BMFs) to the chemical constituents of AN results in fibroblast proliferation, increased collagen synthesis, stabilization of collagen structures, and inhibition of collagen phagocytosis [4]. However, the molecular

mechanisms underlying the pathogenesis and progression of OSF are not entirely clear.

Mediators such as growth factors, hormones, cytokines, and lymphokines influence the synthesis of ECM and collagens. Among which, transforming growth factor β (TGFβ) has been suggested as the main trigger for both the increased collagen production and decreased matrix degradation pathways in OSF [4]. Submucosal tissues from early stages of OSF specimen demonstrated elevated expression of TGFβ; however, no difference was found between normal specimen and those obtained from late stages of OSF [5]. Recently, Moutasim et al. showed that arecoline, the major alkaloid of AN, upregulated αvβ6 integrin expression in oral keratinocytes and modulated TGFβ1 activation [3]. TGFβ1 induced transdifferentiation of oral fibroblasts into myofibroblasts and resulted in upregulation of genes associated with tissue fibrosis [3]. Taken together, these findings suggest that the TGFβ isoforms may play an important role in the earlier stages of OSF development and that the pathogenesis of OSF may be initially epithelial-driven. Anti-TGFβ drugs such as anti-TGFβ antibodies or peptide mimetics have been proposed for the treatment of OSF [1]. However, TGFβ is also involved in tumor suppression [6] and immunosuppression [7]. Broadly suppressing TGFβ action could cause deleterious effects when treating chronic disorders such as fibrosis.

Connective tissue growth factor (CTGF/CCN2) is a matricellular protein belonging to the CCN protein family. It is significantly correlated with fibrotic diseases [8]. Studies have shown that CCN2 is required for most of the increased ECM production and other profibrotic activities generally observed in response to TGFβ [9]. While TGFβ plays an important role in the initial induction of fibrotic tissue formation, CCN2 promotes a sustained fibrotic response in which the presence of TGFβ is no longer required [10–12]. Recently, we have found the overexpression of CCN2 protein in OSF tissues [13]. Constitutive overexpression of CCN2 may play an important role in the pathogenesis of OSF [13].

TGFβ has been reported to stimulate CCN2 expression in lung [14], dermal [15], and gingival fibroblasts [14]. Blockade of CCN2 by antisense mRNA or by anti-CCN2 antibodies inhibits both CCN2-mediated and TGFβ-mediated ECM production in vitro and in vivo [16]. Thus, CCN2 is regarded as a better treatment target than TGFβ once fibrosis has occurred [10]. Inhibition of the CCN2 might block the profibrotic effects of TGFβ without affecting the anti-proliferative and immunosuppressive effects of TGFβ. Detailed understanding of the molecular mechanism underlying the TGFβ-induced CCN2 expressions in human BMFs may shed some light in identifying the targets for drug intervention or chemoprevention of OSF. We therefore examined the possible signal transduction pathways involved in TGFβ-induced CCN2 expression in BMFs. We also investigated whether epigallocatechin-3-gallate (EGCG), the major polyphenol found in green tea, affected the TGFβ-stimulated induction of CCN2 in BMFs. The underlying mechanism of the inhibition of CCN2 by EGCG was further examined.

Materials and methods

Materials

Human TGFβ1 was obtained from R&D Systems (Minneapolis, MN, USA). Extracellular signal-regulated kinase (ERK) inhibitor PD98059, c-Jun NH_2-terminal kinase (JNK) inhibitor SP600125, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, activin receptor-like kinase 5 (ALK5) inhibitor SB431542, and prostaglandin E_2 (PGE_2) were from Calbiochem (San Diego, CA, USA). EGCG was from Sigma-Aldrich (St. Louis, MO, USA). All tissue culture biologicals were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

The protocol of this study has been reviewed and approved by the Institutional Ethical Committee. Informed consents were obtained before surgical removal of impacted mandibular third molars. Five primary human BMF cultures were established by an explant technique as previously described [17]. Cells between four and ten passages were plated on 60-mm Petri dishes at a density of 2×10^5 cells and subjected to various treatments. Human fetal lung fibroblasts IMR90 were from ATCC. The cells were serum starved for 18 h before treatment.

Western blot analysis

Western blot analysis was performed as previously described [13]. In brief, cells were lysed in lysis buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transfer to PVDF membranes. Antibodies against phospho-JNK, total JNK, phospho-p38, total p38, phospho-Smad3, total Smad3 (Cell Signaling Technology, Danvers, MA, USA), CCN2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added at concentrations recommended by the manufacturers. Proteins were detected with horseradish peroxidase-link secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the Western Lighting Chemiluminescence Reagent (PerkinElmer, Waltham, MA, USA). The membranes were then analyzed with a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Statistical analysis

Group data are expressed as mean ± SEM. Student's *t* test was used to compare differences between the groups studied. $P<0.05$ was considered statistically significant. Data given herein are the means of four experiments.

Results

Human BMFs were first treated with various concentrations of TGFβ1. As shown in Fig. 1a, TGFβ1 dose-dependently increased CCN2 synthesis. The levels of CCN2 protein increased about 11-fold after 6 h of exposure to 2 ng/mL of TGFβ1. We then treated BMFs with 2 ng/mL TGFβ1 for various incubation periods to study the kinetics of TGFβ1-induced CCN2 synthesis. Figure 1b showed that TGFβ1 time-dependently increased CCN2 synthesis.

It has been shown that cyclooxygenase-2 (COX-2), the key enzyme in PGE_2 production, was significantly higher in OSF specimens [18]. While PGE_2 has been reported to inhibit TGFβ1-induced CCN2 expression in human lung (IMR-90) and kidney fibroblasts but not in gingival fibroblasts [14], we therefore investigated the effects of PGE_2 on TGFβ1-induced CCN2 expression in BMFs and IMR90 lung fibroblasts. Results revealed that pretreatment with 1 μM PGE_2 completely blocked the TGFβ1-induced CCN2 protein expression in IMR90 lung fibroblasts, whereas the TGFβ1-induced CCN2 protein expression in BMFs was not inhibited by PGE_2 pretreatment (Fig. 2a). These imply a tissue-specific mechanism to maintain CCN2 expression in OSF tissues.

TGFβ initiates its cellular action by binding to cell membrane serine/threonine kinase receptors [19]. ALK5, the TGF-β type I receptor, is the principal receptor that mediates most cellular responses to TGFβ [20]. TGFβ signaling is also controlled by MAPK signaling cascades [9, 19]. To identify the specific signal transduction pathways involved in TGFβ1-induced CTGF/CCN2 expression in BMFs, cells were pretreated with 10 μM of ERK inhibitor PD98059, 10 μM of JNK inhibitor SP600125, 10 μM of p38 MAP kinase inhibitor SB203580, or 20 μM of ALK5 inhibitor SB431542 for 1 h before exposure to 2 ng/mL TGFβ1 for 6 h. Western blots from different experiments demonstrated that SP600125, SB203580, and SB431542 reduced about 47, 41.7, and 100% of the TGFβ1-induced levels of CCN2 protein, respectively (Fig. 2b, c). Combined pretreatment with SP600125 and SB203580 showed additive effect (82 % inhibition) (Fig. 2d). However, PD98059 had no effect on the TGFβ1-induced CCN2 protein expression.

We recently found that EGCG completely inhibited thrombin-induced CTGF/CCN2 synthesis in BMFs [17]. We therefore investigated whether EGCG could affect TGFβ1-induced CTGF/CCN2 expression in human BMFs. As shown in Fig. 3, EGCG dose-dependently inhibited TGFβ1-induced CCN2 expression. At the concentration of 10 μg/mL, EGCG completely blocked the expression of TGFβ1-induced CTGF/CCN2. Since the activation of ALK5 directly phosphorylated Smad3 [9], and that our results revealed the JNK and p38 involvement in the TGFβ1-induced CTGF/CCN2 expression in

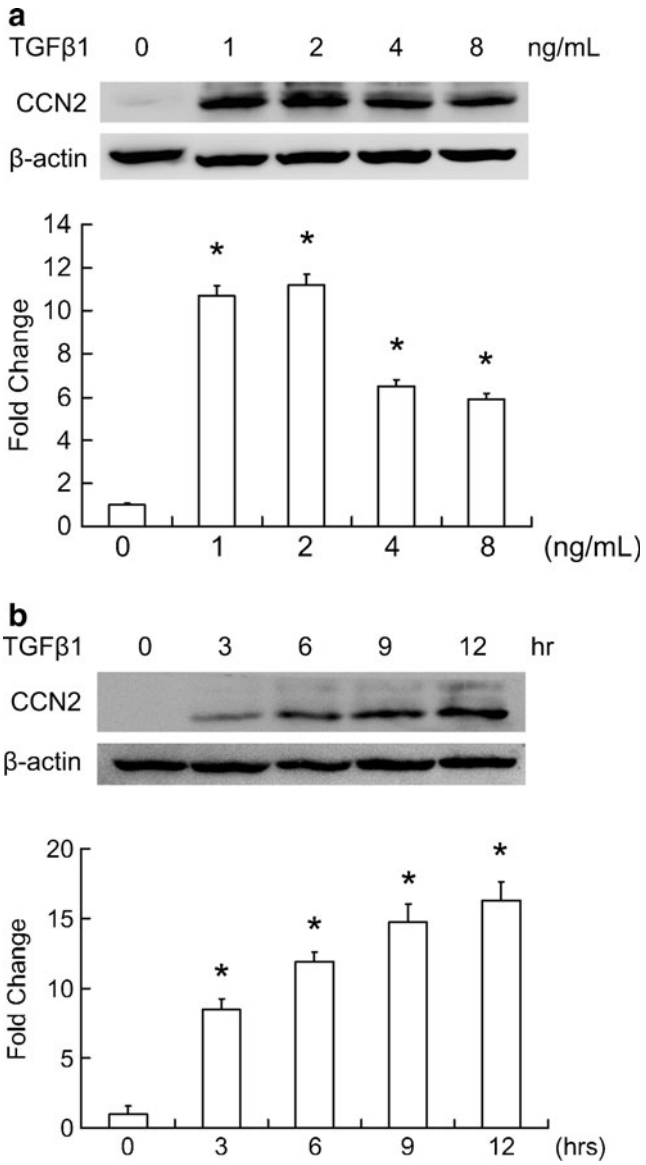
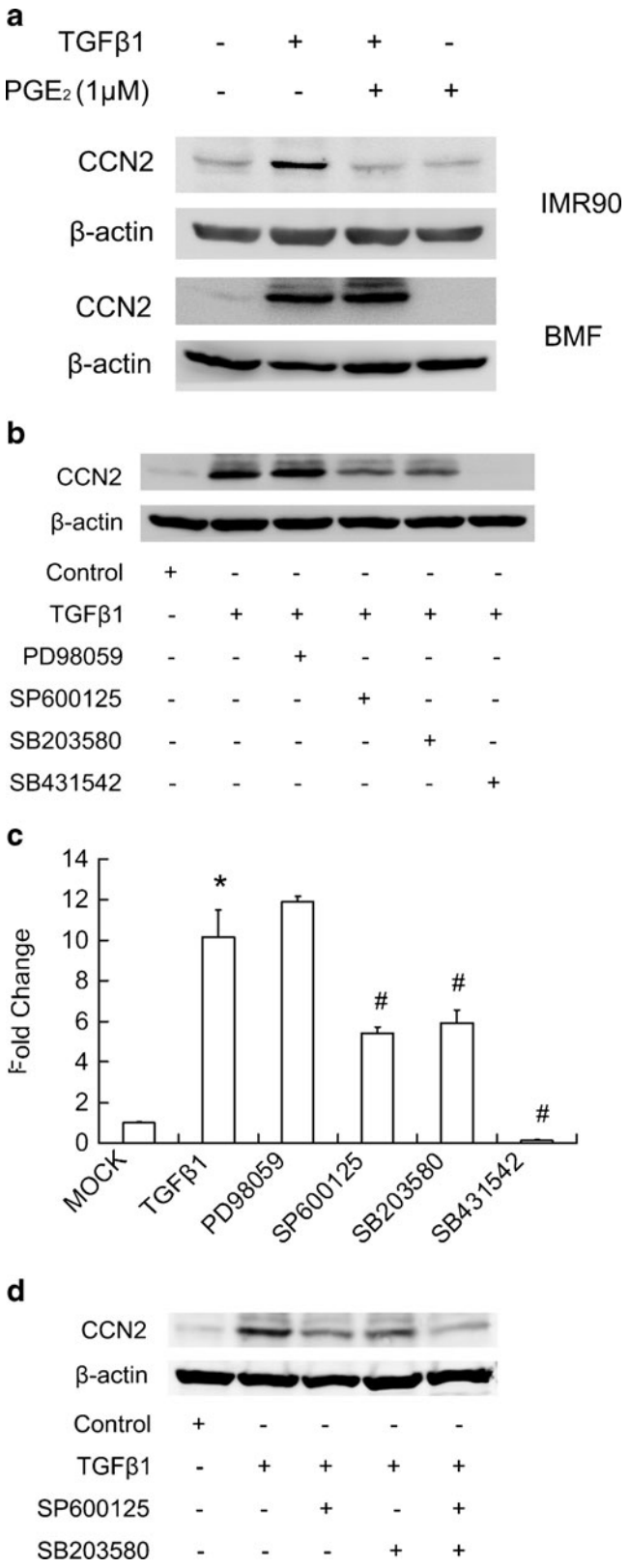


Fig. 1 TGFβ1 stimulated connective tissue growth factor (CTGF/CCN2) expression in human buccal mucosal fibroblasts (BMFs). **a** Cells were treated with various concentrations (0 to 8 ng/mL) of TGFβ1 for 6 h. CCN2 protein levels were measured by Western blot analysis. **b** Kinetics of CCN2 induction by 2 ng/mL TGFβ1 in BMFs. Results of **a** and **b** were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to untreated controls. *Bars* represent means ± SEM ($n=4$, $^{*}P<0.05$ compare to control)



◀ **Fig. 2** Effects of PGE_2 and various inhibitors on the TGFβ1-induced CCN2 expression in BMFs. **a** IMR90 lung fibroblasts and BMFs were treated with 1 μM PGE_2 for 1 h before exposure to 2 ng/mL of TGFβ1 for 6 h. CCN2 protein levels were measured by Western blot analysis. **b** BMFs were pretreated with 10 μM of ERK inhibitor PD98059, 10 μM of JNK inhibitor SP600125, 10 μM of p38 MAP kinase inhibitor SB203580, or 20 μM of ALK5 inhibitor SB431542 for 1 h before exposure to 2 ng/mL of TGFβ1 for 6 h. CCN2 levels were measured by Western blot analysis. **c** Results of **b** were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to untreated controls. **d** BMFs were pretreated with 10 μM of SP600125, 10 μM SB203580, or 10 μM of SP600125 plus 10 μM SB203580 for 1 h then treated with 2 ng/mL TGFβ1 for 6 h. CCN2 levels were measured by Western blot analysis. Data are presented as means ± SEM (n=4, $^{*}P$<0.05 compared to control, $^{\#}P$<0.05 compared to 2 ng/mL TGFβ1)

BMFs, we next examined the effect of EGCG on the TGFβ1-stimulated phosphorylation of Smad3, JNK, and p38. Results revealed that EGCG significantly attenuated the TGFβ1-induced phosphorylation of JNK and p38 but not Smad3 (Fig. 4).

Discussion

TGFβ is the main trigger for both the increased collagen production and decreased matrix degradation pathways in OSF. It has been shown that CCN2 creates an environment favorable for fibrosis and is responsible for mediating the profibrotic effects both direct and indirect of TGFβ [10, 21]. Recently, we have found the overexpression of CTGF/CCN2 in OSF [13]. Constitutive CTGF/CCN2 overexpression may play an important role in the pathogenesis of OSF [13]. CTGF/CCN2 could serve as a novel target for the treatment of OSF [13]. This study showed that TGFβ1 strongly upregulated CCN2 expression in cultured primary human BMFs. The induction of CCN2 by TGFβ1 could contribute to the pathogenesis of OSF.

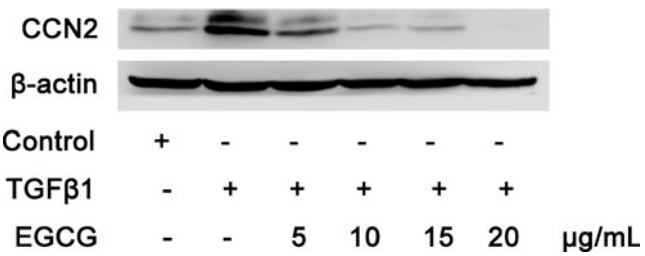


Fig. 3 EGCG inhibited TGFβ1-induced CTGF/CCN2 expression in buccal mucosal fibroblasts. Cells were pretreated with increasing concentrations of EGCG (0–20 μg/mL) for 1 h then treated with 2 ng/mL TGFβ1 for 6 h. CTGF/CCN2 levels were measured by Western blot analysis. A representative result of four independent experiments is shown

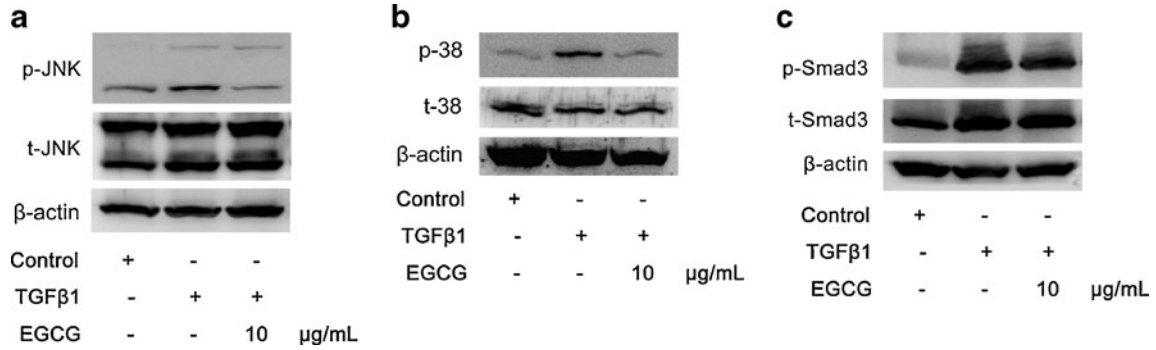


Fig. 4 EGCG significantly inhibited the TGFβ1-induced phosphorylation of JNK and p38 but not Smad3. A representative result of four independent experiments is shown

Although anti-TGFβ therapy has been proposed as a potential remedy for fibrosis, treating early-stage diffuse cutaneous systemic sclerosis with neutralizing anti-TGFβ antibody resulted in increased morbidity and mortality in a multicenter randomized clinical trial [22]. As TGFβ is involved in both normal and pathological processes in human physiology [19], generally blocking its actions is expected to have deleterious effects. Thus, identifying the signaling associated with TGFβ-induced CCN2 expression in BMFs is important in developing more appropriate antifibrotic strategies for the drug intervention or chemoprevention of OSF. In fibroblasts, TGFβ initiates its cellular action by binding to a serine/threonine kinase receptor (TGFβ receptor II) which then recruits and phosphorylates another serine/threonine kinase (TGFβ receptor I/ALK5), leading to the subsequent activation of transcriptional co-regulators, Smad2 and Smad3 [20, 23]. Smad3 mediates most profibrotic activities of TGFβ [24]. TGFβ-induced CCN2 expression is dependent on Smad3 but not Smad2 in dermal fibroblasts [10]. However, very few reports directly examined the effect of ALK5 inhibition on TGFβ-induced CCN2 expression. Recently, Thompson et al. reported that ALK5 inhibition blocked TGFβ1-induced CCN2 expression in gingival fibroblasts [25]. Our present study is the first to show that SB431542 completely suppresses TGFβ1-induced CCN2 expression in BMFs. This result suggests that ALK5 mediates the induction of CCN2 by TGFβ1 in the pathogenesis of OSF. Our result is consistent with the notions that Smad3 regulates TGFβ1-induced CCN2 expression in fibroblasts and that activation of TGFβ signaling through the ALK5 receptor may play a key role in fibroblast activation in the oral cavity [25].

Recent studies have shown that TGFβ-induced CCN2 expression in fibroblasts was also modulated by MAPK signaling. Stratton et al. found that Ras/MEK/ERK potentiated but that JNK suppressed TGFβ-induced CTGF expression in dermal fibroblasts [15]. In contrast, Black et al. reported that TGF-β induces CCN2 expression through JNK but not Ras/MEK/ERK in gingival fibroblasts [14]. We found that TGFβ1 induced CCN2 expression in BMFs through JNK and p38 MAPK but not ERK signaling. Our results are different from those in dermal and gingival fibroblasts and are consistent with the findings that different types of fibroblasts may differ in their crosstalk between TGFβ and MAPK signaling [26].

PGE_2 is a proinflammatory agent. PGE_2 inhibits the TGFβ1-induced CCN2 expression in lung and kidney fibroblasts [14]. COX-2 is the key enzyme in PGE_2 production. COX-2 expression is significantly upregulated and is associated with the inflammatory responses in OSF tissues [18] while the expression of CCN2 is also elevated in OSF specimens [13]. We thus inspected the effects of PGE_2 on the TGFβ1-induced CCN2 expression in both BMFs and IMR90 lung fibroblasts. Comparative results revealed that PGE_2 pretreatment completely blocked the TGFβ1-induced CCN2 protein expression in IMR90 lung fibroblasts but not in BMFs. These imply that the exceptional signal transduction pathways of TGFβ1-induced CCN2 production in BMFs contribute to the resistance of PGE_2 downregulation of CCN2 expression in these cells. Therefore the CCN2 levels are maintained in the OSF tissues in the presence of COX-2. Our results are comparable to the results from Black et al. [14] that gingival fibroblasts are resistant while lung fibroblasts are highly sensitive to the PGE_2-mediated inhibition of TGFβ1-induced CCN2 expression.

Green tea is one of the most widely consumed beverages in the world and is reported to have significant health benefits for humans. The health-promoting effects of green tea are mainly attributed to EGCG, the most prevalent polyphenol contained in green tea. Animal studies showed that EGCG suppressed bleomycin-induced pulmonary fibrosis [27], injury-induced liver fibrosis [28], and myocardial fibrosis [29]. EGCG has been shown to modulate multiple signal transduction pathways such as the MAPKs. Recently, Dooley et al. found that EGCG suppressed TGFβ-induced ROS production, ERK activation, and CCN2 in dermal fibroblasts [30]. At present, there is no effective therapy to reverse or directly inhibit the process of OSF. We previously

showed EGCG completely blocked the thrombin-induced CCN2 in BMFs [17]. Our present study demonstrated that EGCG completely blocked the TGFβ1-induced CCN2 expression through inhibition of JNK and p38 activation in BMFs. To our knowledge, this is the first time to demonstrate that EGCG can reduce TGFβ1-stimulated CCN2 production through the suppression of the JNK and p38 MAPK activation. Interestingly, EGCG could also inhibit arecoline-induced CCN2 synthesis (data not shown). EGCG, which has a long history of safe beverage consumption, may be a useful agent for the prevention and treatment of OSF.

Conclusion

In conclusion, this study demonstrates that TGFβ1 stimulated CCN2 production in BMFs. The TGFβ1-induced CCN2 synthesis in BMFs could be mediated by the ALK5, JNK, and p38 MAPK pathways. EGCG effectively blocks the TGFβ1-induced CCN2 by suppressing JNK and p38 in BMFs. Thus EGCG may serve as a useful agent in controlling OSF. The exceptional signal transduction pathways of TGFβ1-induced CCN2 production in BMFs contribute to the resistance of PGE_2 downregulation of CCN2 expression; therefore, the CTGF/CCN2 levels are maintained in the OSF tissues in the presence of COX-2.

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Conflict of interest The authors declare that they have no conflict of interest.

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