Sphingosine-1-phosphate stimulated connective tissue growth factor expression in human buccal fibroblasts: Inhibition by epigallocatechin-3-gallate

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BACKGROUND/PURPOSE: Connective tissue growth factor (CCN2) has been associated with the pathogenesis of various fibrotic diseases, including oral submucous fibrosis (OSF). The chemical constituents of areca nut along with the mechanical trauma cause OSF. The coarse fibers of areca nut injure the mucosa and hence sphingosine-1-phosphate (S1P) is released at the wounded sites. Recent studies have shown that S1P is involved in wound healing and the development of fibrosis. The aims of this study were to investigate the effects of S1P on CCN2 expression in human buccal fibroblasts (HBFs) and identify the potential targets for drug intervention or chemoprevention of OSF.

METHODS: Western blot analyses were used to study the effects of S1P on CCN2 expression and its signaling pathways in HBFs and whether epigallocatechin-3-gallate (EGCG), the main and most significant polyphenol in green tea, could inhibit this pathway.

RESULTS: S1P significantly enhanced CCN2 synthesis in HBFs. This effect can be inhibited by c-Jun NH2-terminal kinase (JNK) inhibitor and extracellular signal-regulated kinase inhibitor but not by P38 mitogen-activated protein kinase inhibitor. Interestingly, EGCG completely blocked S1P-induced CCN2 expression via suppressing S1P-induced JNK phosphorylation.

KEYWORDS
- c-Jun NH2-terminal kinase
- connective tissue growth factor
- epigallocatechin-3-gallate
- oral submucous fibrosis
- sphingosine-1-phosphate
Introduction

It is generally accepted that areca nut (AN) chewing is closely related to oral submucous fibrosis (OSF). Epidemiological studies and current investigations suggest that the chemical constituents and the persistent mucosal trauma seem to be key to the etiology. This fibrotic disease is characterized by epithelial atrophy, juxta-epithelial inflammatory reaction, and progressive fibrosis in the lamina propria and the submucosal tissues.1–3 These changes gradually lead to mucosal stiffness, trismus, limited tongue movement, and eventually cause problems with eating, maintaining oral hygiene, and even speech. 

The major alkaloid of areca nut, known as arecoline, has been found to have several biological effects such as stimulating collagen synthesis,4 upregulating tissue inhibitor metalloproteinase-15 and connective tissue growth factor (CCN2),6 and promoting myofibroblast differentiation7 in human fibroblasts. When chewing AN, the coarse fibers cause trauma to the oral mucosa and the chemical constituents consecutively diffuse into the subepithelial connective tissue.1 Furthermore, this traumatization causes thrombin production, which has been found to correlate with the pathogenesis of OSF.9 However, the molecular pathologic mechanism(s) of OSF still remain to be further clarified.

CCN2 is a 36–38 kDa secreted matricellular protein that belongs to the CCN family and has been found overexpressed in many fibrotic diseases.10 Normally, CCN2 expression is restricted to the glomerular mesangial and hepatic stellate cells.11 When it is induced by growth factors, CCN2 is secreted into the extracellular environment and interacts with extracellular matrix components. It is an important mediator. It regulates many biological processes including cell proliferation, adhesion, and migration, and plays a major role in wound healing.12,11 In addition, it has also been considered to be a regulator of inflammation and creates a favorable environment for fibrogenic stimuli to interact.12,13 Moreover, its overexpression in human fibrotic diseases is an interesting issue.6,11 By blocking CCN2 action with neutralizing antibodies or with antisense oligonucleotides abrogates both transforming growth factor-β (TGFβ)- and CCN2-mediated extracellular matrix production in vitro and in vivo.14 Collectively, CCN2 may therefore represent a new target for antifibrotic therapy.

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, is an important pleiotropic lysosphospholipid mediator that induces diverse biological activities via transmitting signals through specific G protein-coupled receptors. For example, wound healing, cell proliferation, cell migration, cell differentiation, and the development of fibrosis.15,16 It presents in human plasma at concentrations ranging from 100 ng/mL to 1000 ng/mL.17 To date, five members (EDG-1/51P1, EDG-5/51P2, EDG-3/51P3, EDG-6/51P4, and EDG-8/51P5) have been identified. According to previous studies, S1P1 and S1P3 are involved in the development of human liver fibrosis.18 Furthermore, Ikeda et al.19 reported that the incidence of carbon tetrachloride- and dimethylnitrosamine-induced liver fibrosis is significantly reduced in S1P2 knockout mice as compared with the wild-type. Reports have shown that S1P induces CCN2 expression in human endothelial20 and rat glomerular mesangial cells.21 However, the effects of S1P on CCN2 expression in human buccal fibroblasts (HBFs) remain obscure.

Recently, we found that epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, inhibits TGFβ- and thrombin-induced CCN2 synthesis completely in HBFs.5,22 The aim of the present study is to investigate the effect of S1P on CCN2 expression in HBFs and whether or not EGCG can affect this signaling pathway. Our findings may provide further useful information for the treatment of OSF caused by CCN2 overexpression.

Materials and methods

Reagents

S1P, EGCG, and curcumin were from Sigma-Aldrich (St Louis, MO, USA). Lovastatin was from Standard Chem. & Pharm. Co. (Tainan, Taiwan). Specific extracellular signal-regulated kinase (ERK) inhibitor PD98059, c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 were obtained from Calbiochem (San Diego, CA, USA). Goat anti-CCN2 polyclonal antibody, rabbit anti-total JNK polyclonal antibody, mouse anti-β-actin monoclonal antibody, and donkey anti-goat, goat anti-mouse, and goat anti-rabbit horseradish peroxidase-link secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibodies against phospho-JNK antibodies were from Cell Signaling (Beverly, MA, USA). All biological tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

After receiving approval from the National Taiwan University Hospital Research Ethics Committee, four primary HBF cultures were established by an explant technique as previously described.6,9 Cells at 4–10 passages were plated on 60 mm Petri dishes at a density of 2 × 105 cells and subjected to various treatments. Prior to treatment, the cells were incubated in serum-free media for 18 hours. In

Conclusion: S1P released by repetitive mechanical trauma during AN chewing may contribute to the pathogenesis of OSF through upregulating CCN2 expression in HBFs. EGCG could be an adjuvant to the current offered therapy options or the prevention of OSF through suppression of JNK activation.

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selected experiments, three groups of HBFs were pre-
treated with 10 μM ERK inhibitor PD98059, 10 μM JNK
inhibitor SP600125, or 10 μM of p38 MAP kinase inhibitor
SB203580 for 1 hour prior to exposure to 1 μM S1P for 2
hours. To study the effects of EGCG, curcumin, and lova-
statin, HBFs were pretreated with 10 μM curcumin, 10 μM
EGCG, or 20 μM lovastatin for 1 hour prior to S1P treatment.
Data presented herein are the means of four experiments.

Western blot analysis

Western blot analysis was performed as previously
described.6,9 In brief, cells were harvested, lysed in lysis buffer, separated by sodium dodecyl sulfa-
te–polyacrylamide gel electrophoresis, and transferred to
PVDF membranes. The membrane was then incubated with
antibodies against CCN2 (1:1000 dilution), β-actin (1:1000),
phosphor-JNK (1:750) or total JNK (1:1000). Primary anti-
bodies were detected with donkey anti-goat or goat anti-
mouse horseradish peroxidase-linked secondary antibodies
(1:10000). For quantitative analysis, we added the Western
Lighting Chemiluminescence Reagent (PerkinElmer, Wal-
tham, MA, USA) on the membrane and analysis was carried
out with a Fuji LAS-4000 lumino image analyzer (Fuji Photo
Film Co., Tokyo, Japan).

Statistical analysis

Group data are expressed as mean ± standard error.
The unpaired Student t test was used to compare the group
differences. A p value < 0.05 was considered statistically
significant.

Results

S1P has been detected in human plasma at concentrations
up to 1000 ng/mL. HBFs were therefore first treated with
1 μM S1P for various periods of time. As shown in Fig. 1,
treatment of HBFs with 1 μM S1P led to about three-fold
increase of CCN2 protein expression which reached its
peak level after 2 hours treatment and decreased to control
level thereafter. HBFs were then pretreated with 10 μM ERK
inhibitor PD98059, 10 μM JNK inhibitor SP600125, or 10 μM
p38 MAP kinase inhibitor SB203580 for 1 hour prior to
exposure to 1 μM S1P for 2 hours to investigate the MAPK
signaling pathway in the S1P-induced CCN2 protein
expression. Results revealed that after pretreatment with
SP600125 and PD98059, the S1P-induced CCN2 protein was
reduced by approximately 79.7% and 62.1%, respectively
(Fig. 2). However, pretreatment with SB203580 had no
significant effect on the S1P-induced CCN2 protein
expression. We next examined whether EGCG, curcumin, or
lovastatin inhibited S1P-induced CCN2 protein expression in
HBFs. As shown in Fig. 3, only EGCG significantly inhibited
S1P-induced CCN2 protein expression and the inhibition is
dose-dependent. At concentration of 10 μM, EGCG almost
completely inhibited S1P-induced CCN2 protein expression.
EGCG significantly decreased the S1P-induced JNK phos-
phorylation (Fig. 4).

Discussion

Elevated levels of CCN2 were found in almost all human
fibrotic disorders and are suggested to be involved in the
development and progression of fibrotic diseases.11 CCN2
was recently found to be overexpressed in OSF.9 Constitu-
tive CCN2 overexpression may play an important role in the
pathogenesis of OSF. In this study, we found CCN2 expres-
sion stimulated by S1P in HBFs. Repetitive microtrauma
during AN chewing causes S1P release and may contribute
to the pathogenesis of OSF by inducing CCN2 expression.

Figure 1 Sphingosine-1-phosphate (S1P) stimulates CCN2
expression in human buccal fibroblasts (HBFs). Cells were
treated with 1 μM S1P for various periods of time. CCN2 protein
levels were measured by western blot analysis.

Figure 2 Effects of MAPK inhibitors on the S1P-induced CCN2
expression in HBFs. (A) Cells were pretreated with 10 μM of p38
MAP kinase inhibitor SB203580, 10 μM of JNK inhibitor SP600125
or 10 μM of ERK inhibitor PD98059 for 1 hour, then treated with
1 μM S1P for 2 hours. CCN2 levels were measured by western
blot analysis. (B) Results of (A) were quantified using computer-
zied image analysis, normalized by the level of β-actin, and
expressed as fold change relative to untreated controls. Data
are expressed as mean ± standard error of the mean (n = 4,
*p < 0.05 compared with control, **p < 0.05 compared to 1 μM
S1P).

Figure 3 Effects of EGCG, curcumin, or lovastatin on the
S1P-induced CCN2 protein expression in HBFs. Cells were
pretreated with 10 μM EGCG, 10 μM curcumin, or 20 μM
lovastatin for 1 hour prior to S1P treatment. CCN2 levels were
measured by western blot analysis. Data are expressed as mean ±
standard error of the mean (n = 4, *p < 0.05 compared with control).

Figure 4 EGCG significantly decreased the S1P-induced JNK
phosphorylation.
EGCG inhibits S1P-induced CCN2 in buccal cells

![Table A](image)

**Figure 3** Epigallocatechin-3-gallate (EGCG) inhibits S1P-induced CCN2 in HBMFs. Cells were pretreated with 20 μM lovastatin, 10 μM EGCG, or 10 μM curcumin for 1 hour, then (A) treated with 1 μM S1P for 2 hours or (B) pretreated with increasing concentrations of EGCG (0–15 μM) for 1 hour prior to exposure to 1 μM S1P for 2 hours (B). CCN2 levels were measured by western blot analysis.

![Table B](image)

**Figure 4** Epigallocatechin-3-gallate (EGCG) significantly inhibited S1P-induced JNK phosphorylation.

small GTPases of the Rho family in human endothelial and rat glomerular mesangial cells. Most of these small GTPases undergo post-translational isoprenylation, which is essential for their proper subcellular localization and biological function. Statins have been shown to abrogate the biological functions of the small GTPases through their inhibition of protein isoprenylation. Pretreatment HBFs with lovastatin did not significantly affect the expression of CCN2 protein in S1P treated HGFs indicating that small GTPases of the Rho family are not involved in the S1P-induced CCN2 expression.

In addition to the canonical Smad-mediated pathway, TGFβ-induced CCN2 expression in fibroblasts is also modulated by MAPK signaling. In gingival fibroblasts, Black et al found that TGFβ induced CCN2 expression through JNK, but not Ras/MEK/ERK in gingival fibroblasts. Stratton et al reported that Ras/MEK/ERK stimulates TGFβ-induced CCN2 expression in dermal fibroblasts whereas JNK suppresses the reaction. Recently, we found that TGFβ1 induced CCN2 expression through JNK and p38 MAPK but not ERK in HBFs. Our present study shows that inhibitors of JNK and ERK but not MAPK suppress S1P-induced CCN2 expression in HBFs. This result suggests that S1P-induced CCN2 expression is mediated via JNK and ERK but not p38 MAPK in HBFs and is consistent with the opinions that CCN2 expression is regulated in a cell type and context specific manner.

To date, no known treatment for OSF is effective in reversing the disease. Our previous studies revealed that curcumin can completely inhibit arecoline-induced CCN2 synthesis in HBFs. EGCG inhibits TGFβ- and thrombin-induced CCN2 synthesis completely by suppressing JNK in HBFs. In addition, EGCG can also inhibit arecoline-induced CCN2 synthesis (our unpublished observations). We therefore pretreated primary HBFs with curcumin or EGCG to test their inhibitory effects on S1P-induced CCN2 expression in HBFs. Our results revealed that EGCG, but not curcumin, completely blocks the S1P-induced CCN2 expression and JNK phosphorylation in HGFs. These results strengthen the usage of EGCG for the prevention and treatment of OSF.

In conclusion, this study demonstrates, for the first time to our knowledge, that S1P stimulates CCN2 protein production in HBFs. Constitutive CCN2 overexpression may enhance the profibrotic activity in OSF. The S1P-induced CCN2 expression could be completely blocked by EGCG. EGCG could be an adjuvant to the current offered therapy options or the prevention of OSF through suppression of JNK activation.

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