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ORIGINAL ARTICLE

Epigallocatechin-3-gallate suppresses transforming growth factor-beta signaling by interacting with the transforming growth factor-beta type Ⅱ receptor

Masaki Tabuchi, Sumio Hayakawa, Eiko Honda, Kana Ooshima, Tatsuki Itoh, Koji Yoshida, Ah-Mee Park, Hideaki Higashino, Mamoru Isemura, Hiroshi Munakata

Masaki Tabuchi, Koji Yoshida, Ah-Mee Park, Hiroshi Munakata, Department of Biochemistry, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Sumio Hayakawa, Division of Signaling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan
Eiko Honda, Life Science Research Institute, Kinki University, Osaka-Sayama 589-8511, Japan
Kana Ooshima, Hideaki Higashino, Department of Pharmacology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Tatsuki Itoh, Department of Pathology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Mamoru Isemura, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
Author contributions: Tabuchi M and Hayakawa S contributed equally to this work; Tabuchi M and Hayakawa S designed studies; Tabuchi M, Hayakawa S, Honda E, Ooshima K, Itoh T, Yoshida K, Park AM, Higashino H and Isemura M performed the research; Tabuchi M, Hayakawa S and Munakata H analyzed the data; Munakata H wrote the paper.
Correspondence to: Hiroshi Munakata, Professor, Department of Biochemistry, Kinki University Faculty of Medicine, 377-2 Ohnohigashi, Osaka-Sayama 589-8511, Japan. munakata@med.kindai.ac.jp
Telephone: +81-72-3660221 Fax: +81-72-3660245
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Abstract

AIM: To investigate the (-)-epigallocatechin-3-gallate (EGCG) binding to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ).

METHODS: The expression of α-smooth muscle actin (α-SMA) was used as a marker for fibrotic change in human lung fibroblast MRC-5 cells. The α-SMA expression level was determined by western blotting and immunohistological analysis. We examined whether the anti-fibrotic effects of EGCG on MRC-5 cells was dependent on antioxidant mechanism by using edaravone and *N*-acetylcysteine (NAC). The suppression effects of EGCG on Smad2/3 activation were studied by confocal fluorescence microscopy. The binding of EGCG to recombinant TGFRⅡ protein was analyzed by immunoprecipitation and affinity chromatography.

RESULTS: When MRC-5 cells were treated with TGF-β, EGCG decreased the expression of α-SMA in a dose dependent manner, whereas catechin did not influence the α-SMA expression in the cells. Except for EGCG, antioxidant compounds (*e.g.,* edaravone and NAC) had no effects on the TGF-β-induced α-SMA expression. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment; however, EGCG treatment attenuated the nuclear transportation of Smad2/3 in the presence or absence of TGF-β. After a TGFRⅡ expression vector was introduced into COS-7 cells, cell lysates were untreated or treated with EGCG or catechin. The immunoprecipitation experiments using the lysates showed that EGCG dose-dependently bound to TGFRⅡ and that catechin did not at all. Affinity chromatography study indicated that EGCG would bind to TGFRⅡ.

CONCLUSION: Our results demonstrate that EGCG interacts with TGFRⅡ and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in human lung fibroblast MRC-5 cells.

Key words: Epigallocatechin-3-gallate; Transforming

growth factor-β; Myofibroblast; α-smooth muscle actin; Fibrosis

Core tip: (-)-Epigallocatechin-3-gallate (EGCG) binds to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ) and inhibits TGF-β action by interfering with the interaction between TGF-β and TGFRⅡ. Because TGF-β is considered to be the strongest inducer of tissue fibrosis, the obtained data from this investigation suggest that EGCG may be a new therapeutic agent for organ fibrosis.

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INTRODUCTION

(-)-epigallocatechin-3-gallate (EGCG), the most biologically active constituent in green tea, has been recognized as a component that provides the beverage with potential benefits for human health[1]. The reported health-promoting properties of green tea include anti-cancer[1-3], anti-obesity[4], anti-diabetic[5,6], anti-atherosclerotic[7], anti-viral[8-10], anti-bacterial[11-13] and neuroprotective[14-16] effects. The anti-fibrotic effects of green tea and its constituents, especially EGCG, on liver fibrosis[17-19], pancreatic fibrosis[20] and pulmonary fibrosis[21] have been also reported.

Activation of myofibroblasts is the one of the critical events during fibrosis development. Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is pivotal in the regulation of myofibroblast activation, differentiation, migration, and extracellular matrix production; it also plays an important role in the initiation and progression of fibrosis[22]. However, the mechanisms by which EGCG influences TGF-β action on myofibroblast activation remain incompletely defined.

Tachibana *et al*[23] identified a catechin receptor for EGCG, and showed that this receptor partially mediates the function of EGCG. It is also known that EGCG shows its biological action by interacting with receptors other than the catechin receptor[24,25]. In the present study, we investigated the possibility that EGCG might bind to the TGF-β type Ⅱ receptor (TGFRⅡ).

MATERIALS AND METHODS

Cell culture

The MRC-5 and COS-7 cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan), and were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, United States) at 37 ℃ under 5% carbon dioxide and 95% air.

Chemicals

Catechin and EGCG were obtained from Funakoshi Co. (Tokyo, Japan) and dissolved in PBS. *N*-acetylcysteine (NAC) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and dissolved in dimethyl sulfoxide. Edaravone was the product of Mitsubishi Tanabe Pharma (Osaka, Japan). TGF-β was obtained from R&D Systems (Minneapolis, MN, United States).

Antibodies

The following antibodies were used in this study: monoclonal anti-FLAG antibody produced in mouse (anti-Flag) (Sigma); monoclonal anti-α-smooth muscle actin antibody (anti-α-SMA) produced in mouse (Sigma); monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (anti-GAPDH) produced in mouse (Sigma); rabbit anti-Smad2/3 antibody (anti-Smad2/3) (Cell Signaling Technology, Danvers, MA, United States); and goat anti-human TGFRⅡ antibody (anti-TGFRⅡ), which recognizes extracellular domain of the receptor (R and D).

Western blotting

After washing with ice-cold phosphate buffer saline (PBS), cells were treated with 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, United States), suspended in growth medium and collected by centrifugation at 700 *g* for 5 min. The pellets were washed with PBS, resuspended in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate), which contained a cocktail of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) on ice, and centrifuged at 18000 *g* at 4 ℃ for 10 min.

Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, United States). Cell lysates were suspended in SDS electrophoresis sample buffer and boiled for 5 min. Samples (2.5 μg of protein per lane) were separated on 10% polyacrylamide gels and then transferred to an Immobilon P membrane (Millipore, Billerica, MA, United States). Antibody binding was detected by ECL Plus (GE Healthcare, Buckinghamshire, United Kingdom).

Immunohistological studies

Cells were seeded on BD Falcon 8-well CultureSlide. Cells were cultured under indicated conditions. Medium was removed, and cells were washed with PBS, fixed by 3% paraformaldehyde in PBS for 10 min. After washing with PBS, cells were permeabilized by 0.1% Triton X-100 in PBS. Fixed cells were sequentially treated with anti-α-smooth muscle actin (SMA) antibody (1/100, 37 ℃, 1 h), and fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin G (37 ℃, 30 min). Actin stress fibers were visualized by rhodamine-labeled phalloidin (1/50,

37 ℃, 10 min). For staining the nuclei, cells were treated with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 20 min. Cells were examined with a fluorescence microscope (Nikon ECLIPSE E-800, Nikon Corporation, Tokyo, Japan) equipped with a fluoresecence digital microscope camera controller (VB-7000; Keyence Co., Osaka, Japan).

Plasmid construction

Plasmid was constructed according to standard recombinant DNA techniques. The fragment encoding the human TGFR Ⅱ cDNA (Met1-Lys567, GenBank accession no. M85079) was amplified from a human fetal liver cDNA library (OriGene Technologies, Rockville, MD, United States) by polymerase chain reaction (PCR) with KOD Plus DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) using the primers 5'-TTTGAATTCGCCATGGGTCGGGGGCTGCTC-3' (forward) and 5'-TTTGGATCCTTTGGTAGTGTTTAGGGAGCC-3' (reverse). The forward and reverse primers were designed to introduce an *Eco*R Ⅰ and a *Bam*H Ⅰ restriction site (underlined), respectively, for subcloning purposes. The PCR product was cloned into the pFlag-CMV-5a vector (Sigma). The construct was verified by DNA sequencing.

Transfection

COS-7 cells, grown to 50%-70% confluence, were transfected using Lipofectamine plus (Invitrogen) according to the manufacturer's instructions. The transfectants were grown in DMEM containing 10% FBS. After 3 d, the medium was removed and expression of TGFR Ⅱ in the cells was examined by western blotting.

Immunoprecipitation

Cell lysates were treated with Protein G Sepharose (GE Healthcare) for 30 min at 4 ℃ to remove proteins nonspecifically bound to Protein G Sepharose. Anti- TGFR Ⅱ antibody was then added to the above lysate, and incubated for 2 h at 4 ℃. Next, Protein G Sepharose was added and incubated for 1 h at 4 ℃. Protein G Sepharose was recovered by centrifugation and washed three times with PBS. The immunoprecipitated proteins were removed from the Protein G Sepharose by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min and then separated by electrophoresis.

Affinity chromatography

EGCG was coupled to CNBr-activated Sepharose 4B (GE Healthcare) at a concentration of 5 mg/mL of wet gel. Cell lysate was applied to a column of EGCG-Sepharose 4B and washed with PBS. Bound proteins were eluted with 4 mol/L urea, 1 mol/L NaCl in PBS, and fractions of 0.25 mL were collected. An aliquot of each fraction was spotted onto polyvinylidene difluoride (PVDF) membrane and stained with Coomassie Brilliant Blue. A portion of each fraction was also examined by western blot analysis after SDS-PAGE using anti-TGFR Ⅱ antibody.

RESULTS

Effects of EGCG on the expression of α-smooth muscle actin

The MRC-5 cell line, which is derived from human fetal lung fibroblasts, expresses α-SMA and is considered to be a myofibroblast cell line[26,27]. Therefore, this cell line was used in this study.

MRC-5 cells were grown to 85% confluence, and then serum-starved (0.5% FBS) for 48 h. After serum starvation, cells were treated with TGF-β. We and others usually use 1-2 ng/mL of TGF-β in culture media[27-31]. A representative and frequently used marker of myofibroblast activation is α-SMA[32,33]. Western blot analysis and immunohistological examination showed that expression of α-SMA was increased by TGF-β (Figure 1). Whereas a catechin control showed no effects on α-SMA expression, EGCG dose-dependently abolished the increase in expression of α-SMA induced by TGF-β (Figure 1B). The EGCG concentration used in this study was reasonable[34]. The expression of GAPDH also seemed to be decreased by a high dose of EGCG. The band densities of α-SMA and GAPDH were compared (Figure 1B), and the result clearly showed the effects of EGCG on α-SMA.

Because EGCG is an antioxidant compound, we examined whether edaravone and NAC, two well-known antioxidant compounds, have similar effects. Neither treatment with edaravone (Figure 2A) nor treatment with NAC (Figure 2B) affected the increase in expression of α-SMA induced by TGF-β.

EGCG suppresses SMAD activation

The effects of TGF-β are largely mediated by Smad proteins. TGF-β causes phosphorylation of Smad2/3, and then phosphorylated Smads enter into the nucleus. After treatment with TGF-β, MRC-5 cells were examined by confocal fluorescence microscopy. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment, whereas EGCG treatment clearly decreased the nuclear transportation of Smad2/3 (Figure 3).

EGCG binds to TGFR Ⅱ

Next, we examined the possibility that EGCG interferes with binding of TGF-β to the TGFR Ⅱ. To this end, cells expressing large amounts of the receptor are preferable. Because COS-7 cells showed high transformation efficiency and marked expression of exogenous cDNA, these cells were used for transformation experiments. A TGFR Ⅱ expression vector was introduced into COS-7 cells. Cell lysates were untreated or treated with EGCG or catechin, and then subjected to immunoprecipitation with anti-TGFR Ⅱ antibody. In untreated lysate and lysate treated with catechin, TGFR Ⅱ was precipitated by the antibody. When lysate was treated with EGCG, however, anti-TGFR did not precipitate TGFR Ⅱ (Figure 4).

To confirm the binding of EGCG to TGFR Ⅱ, we next performed affinity chromatography. Namely, cell ly-

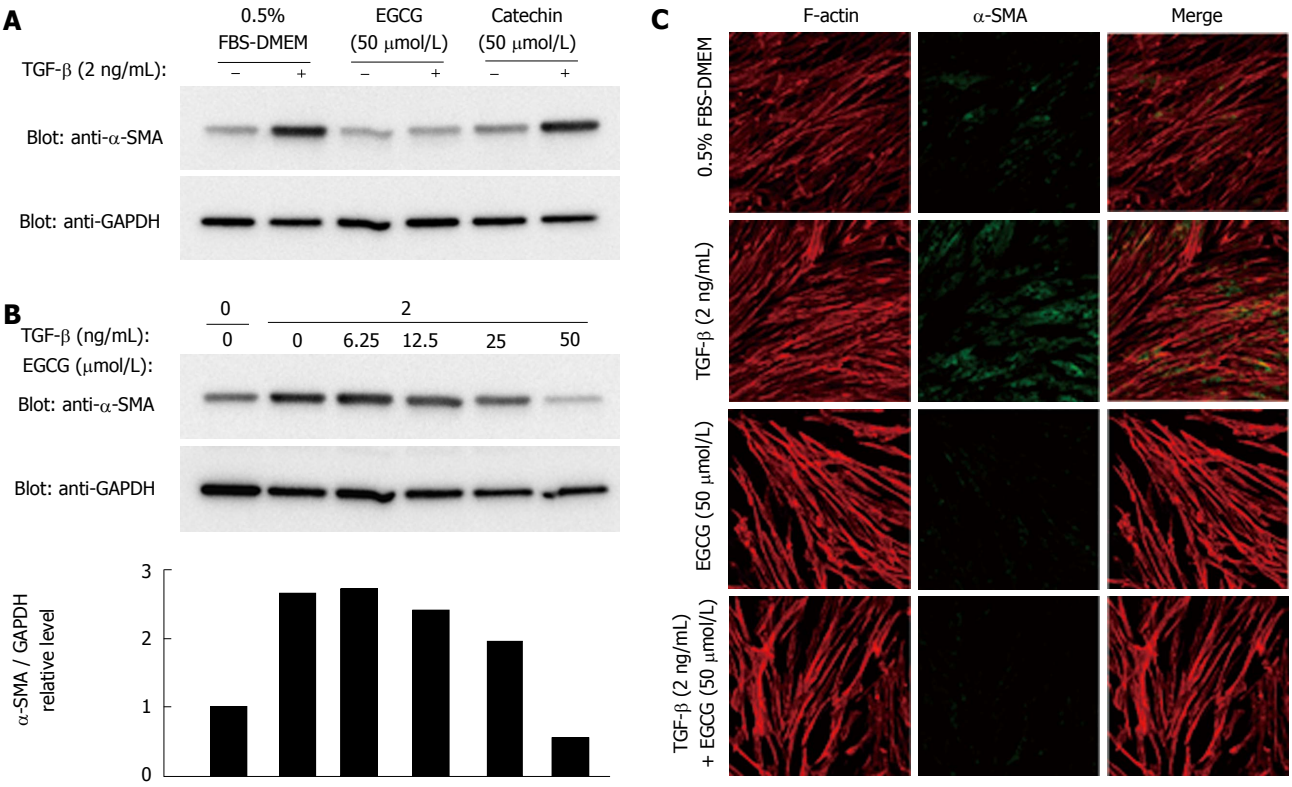

Figure 1 Effects of (-)-epigallocatechin-3-gallate on expression of α-smooth muscle actin. A: Lysates of MRC-5 cells were obtained from cells treated with 0.5% FBS in DMEM alone, (-)-epigallocatechin-3-gallate (EGCG) (50 μmol/L), or catechin (50 μmol/L) for 24 h. After SDS-PAGE, proteins were blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA) antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control; B: MRC-5 cells were treated with the indicated amounts of EGCG. α-SMA was detected in the same manner as in (A). The expression levels of α-SMA were normalized to those of GAPDH; C: Expression of α-SMA in cells treated with EGCG (50 μmol/L) in the absence (-) or presence (+) of transforming growth factor-β (TGF-β) (2 ng/mL) were examined by confocal microscopy. Green: α-SMA (fluorescein isothiocyanate conjugated goat anti-mouse IgG); Red: Actin stress fiber (rhodamine-labeled phalloidin); blue: Nuclei (DAPI). FBS: fetal bovine serum.

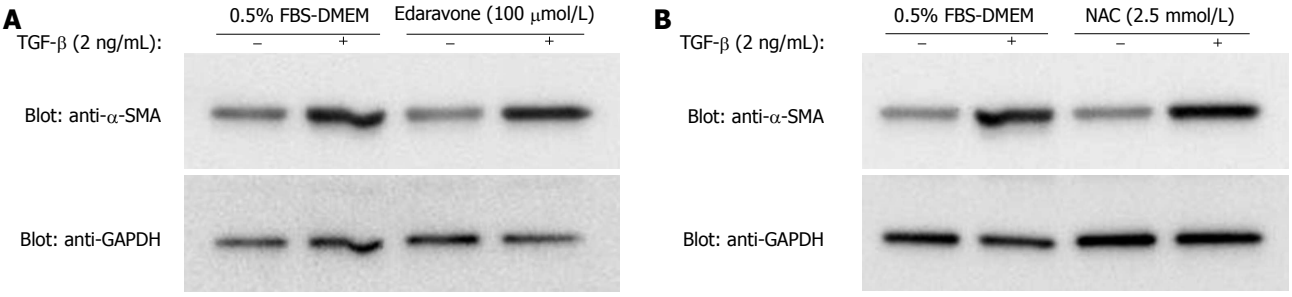

Figure 2 Effects of scavenging compounds on expression of α-smooth muscle actin. MRC-5 cells were treated with edaravone (100 μmol/L) (A) or *N*-acetylcysteine (NAC) (2.5 mmol/L) (B) for 1 h, and then stimulated with transforming growth factor-β (TGF-β) for 24 h. Cell lysates were electrophoresed, blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. FBS: Fetal bovine serum.

sates were applied to an EGCG-conjugated agarose column and proteins bound to the column were examined by western blotting. Figure 5 shows that TGFR Ⅱ bound to the column, indicating that EGCG binds to TGFR Ⅱ.

DISCUSSION

In this study, we have demonstrated that EGCG both inhibits the signal transduction of TGF-β by binding to TGFR Ⅱ and attenuates the expression of α-SMA in MRC-5 cells, which is a myofibroblast cell line, when it is stimulated by TGF-β. Myofibroblasts play crucial roles in the pathogenesis of tissue fibrosis[35]. Stimulation by TGF-β and other cytokines leads myofibroblasts to an activated state[36]. Activated myofibroblasts then secrete collagen and other components of the extracellular matrix, which can result in fibrosis[37].

TGF-β is the most potent cytokine causing fibrosis. Both Smad-dependent and Smad-independent TGF-β signaling pathways are known. Initiation of both pathways takes place via binding of TGF-β to its receptor. TGF-β binds to a type Ⅱ receptor, which then phosphorylates a TGF-β type I receptor. Subsequently, the type I receptor phosphorylates R-Smads (receptor-

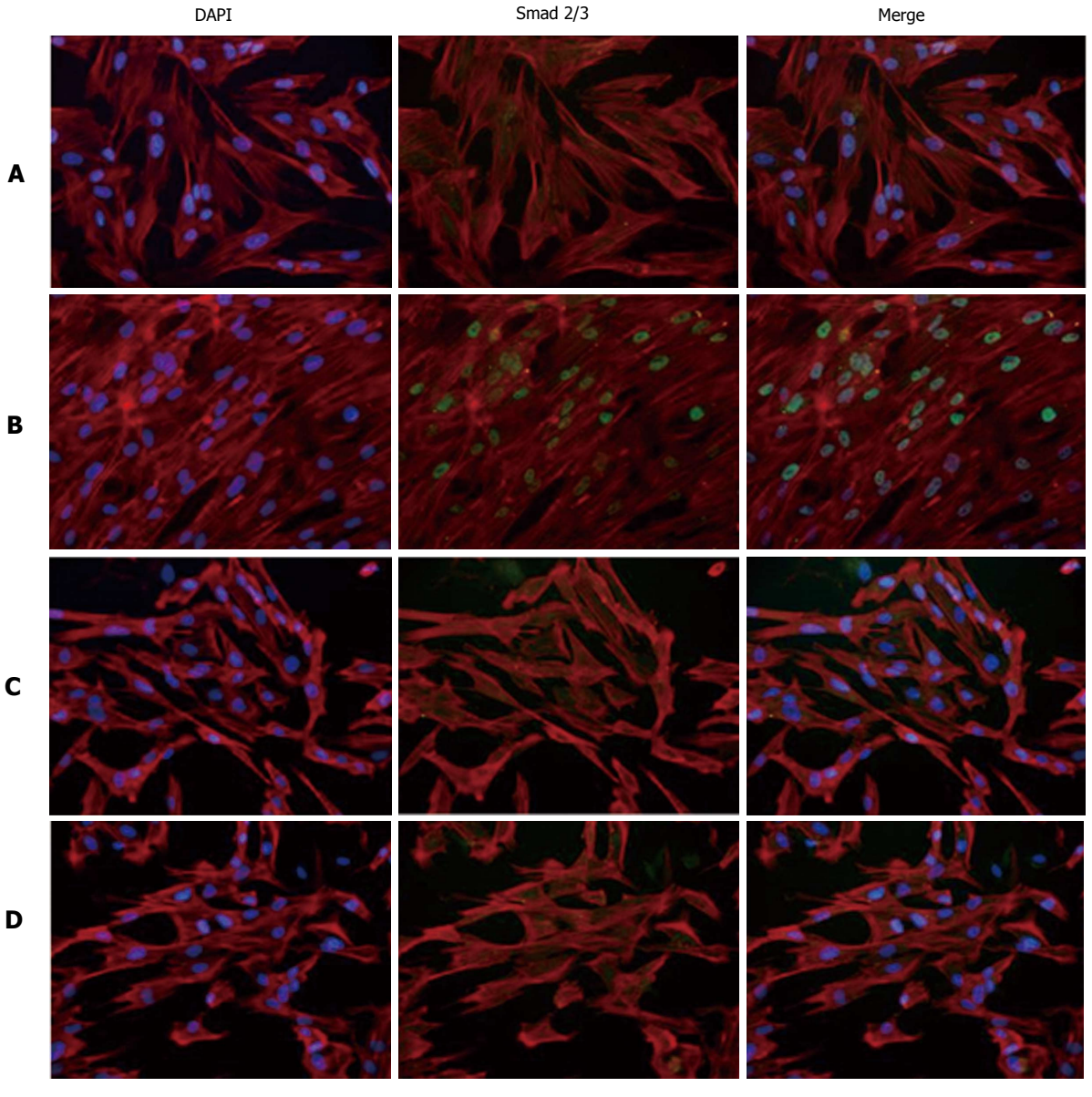

Figure 3 Effects of (-)-epigallocatechin-3-gallate on activation and localization of Smad2/3. MRC-5 cells were treated with transforming growth factor-β (TGF-β) and/or (-)-epigallocatechin-3-gallate (EGCG). Cells were examined by confocal microscopy. Subcellular localization of Smad2/3 (green) and actin stress fibers (red) are shown. Nuclei were stained by DAPI (blue). A: Control; B: Treated with TGF-β; C: Treated with EGCG; D: Treated with TGF-β and EGCG. DAPI: 4',6-diamidino-2-phenylindole.

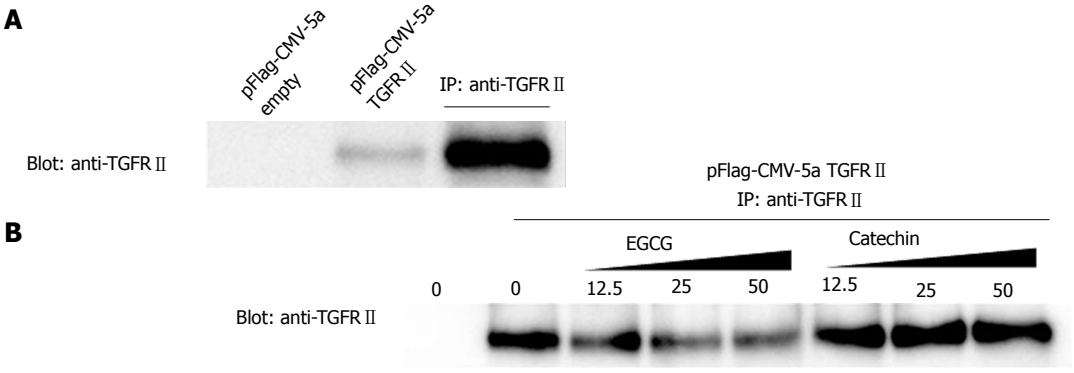

Figure 4 (-)-Epigallocatechin-3-gallate interferes with binding between transforming growth factor-β and its type Ⅱ receptor. A: Positive control of the immunoprecipitation experiment. Cell lysates from transfected COS-7 cells were treated with anti-transforming growth factor-β type Ⅱ receptor (TGFR Ⅱ). TGFR Ⅱ was recovered in the immunoprecipitation product of the lysate; B: Effects of (-)-epigallocatechin-3-gallate (EGCG) and catechin on the antigen-antibody interaction. After cells were treated with EGCG or catechin, anti-TGFR Ⅱ bound to Protein G was added to each lysate. Western blotting was performed using anti-TGFR Ⅱ.

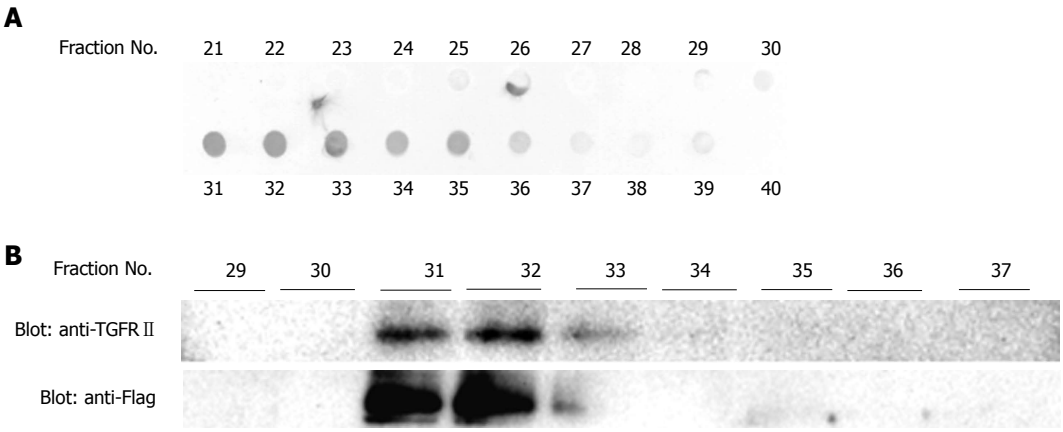

Figure 5 Transforming growth factor-β type Ⅱ receptor binds to (-)-epigallocatechin-3-gallate. A: Proteins in the fractions were detected by staining with Coomassie Brilliant Blue. An aliquot of each fraction was blotted on polyvinylidene difluoride membrane and stained; B: The protein-containing fractions revealed in (A) were electrophoresed and Western blotting was performed using anti-Flag antibody. TGFR Ⅱ: Transforming growth factor-β type Ⅱ receptor.

regulated Smads), and phosphorylated R-Smads bind to Co-Smad (common-mediator Smad). R-Smad/Co-Smad complexes translocate into the nucleus, where they act as transcription factors[38]. In this manner, regulation of TGF-β target gene expression is carried out. Expression of many proteins in MRC-5 cells changes after stimulation by TGF-β[39]. A frequently used marker of the activation of myofibroblast is α-SMA; therefore, this protein was also used as a marker in this study. Expression changes after TGF-β stimulation in cells other than MRC-5 has been observed, for example, in IMR-90 human lung fibroblasts[33] and WI38-VA13 cells[40]. Besides α-SMA, upregulation of collagen I [41], fibronectin[27]and CTGF[42] has been reported when human lung fibroblasts are treated with TGF-β.

The expression of α-SMA is regulated by Smad[43]. TGF-β increases the nuclear translocation of Smad and expression of α-SMA. We examined the influence of EGCG treatment on Smad2/3 appearance in MRC-5 cells. Immunohistological experiments indicated that EGCG inhibits the nuclear transportation of Smad2/3.

Moreover, we found that EGCG had suppressive effects on the expression of α-SMA in MRC-5 cells, whereas catechin did not. These data suggest that the effects are dependent on the gallate or pyrogallol moiety of EGCG.

Next, we investigated the mechanism of the inhibitory effect on the Smad2/3 pathway. EGCG is a potent antioxidant and a lot of its health benefit effects are thought to be due to its antioxidative action[44-46]. EGCG attenuated the increase in α-SMA expression brought about by TGF-β, whereas edaravone and NAC did not. These results indicate that the inhibitory effect of EGCG on α-SMA expression is independent of its antioxidative action.

We thought that part of the EGCG's effects on α-SMA expression might arise through interference with receptor-ligand binding. Indeed, EGCG treated cell lysate containing TGFR Ⅱ showed no immunoprecipitation with anti-TGFR Ⅱ antibody. The interaction between EGCG and TGFR Ⅱ was also confirmed by the affinity chromatography experiment. A likely explanation for this observation is that EGCG binds to TGFR Ⅱ, thereby blocking the antibody from binding to TGFR Ⅱ. Similarly, if EGCG binds to the TGF-β receptor, TGF-β would not be able to bind to its receptor and downstream signaling pathways would be ineffective.

In conclusion, we have shown that EGCG interacts with TGFRII and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in MRC-5 cells, which are human lung fibroblasts. These results suggest that EGCG has anti-fibrotic effects that are crucial for the control of myofibroblast differentiation and extracellular matrix deposition, which are involved in fibrosis. The evidence that EGCG is effective in the suppression of fibrosis may lead not only to better understanding of the biological roles of EGCG but also to clinical applications of this flavonoid.

COMMENTS

Background

Fibrosis is an intractable disease. Effective treatments for it have not been developed yet. Catechin is a substance with a variety of physiological effects. However, the investigation on the antifibrotic effect of catechin has not been fully performed.

Research frontiers

Various physiological effects of catechin have been intensely studied. It has been reported that catechin has a variety of physiological activity (*e.g.*, regulation of blood pressure, blood cholesterol, blood sugar; antioxidant, anti-aging, anti-cancer effects).

Innovations and breakthroughs

Many studies have been performed about (-)-epigallocatechin-3-gallate (EGCG) relationship with transforming growth factor-β (TGF-β) and its antifibrotic properties. We demonstrated that EGCG inhibits the TGF-β activity through its binding to TGF-β type Ⅱ receptor (TGFR Ⅱ).

Applications

TGF-β is believed to be the strongest inducer of tissue fibrosis. EGCG inhibits TGF-β activity by interacting with TGFR Ⅱ. Therefore, EGCG may become an antifibrotic agent.

Terminology

Green tea contains four main catechin substances: Epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, all of which are

inclusively called catechin. Organ fibrosis is a clinical condition caused by an excessive deposition of extracellular matrix. The progression of fibrosis resulted in a loss of normal function.

Peer review

This paper reports a novel, interesting and important study. This is a basic work which shows that EGCG could bind to the TGFR Ⅱ abolishing myofibroblast activation. The original point in this work is the analysis that is done on the cytokine receptor. The authors soundly demonstrated the binding EGCG to TGFR Ⅱ by immunoprecipitation and affinity chromatography experiments.

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