Procyanidin C1 from Cinnamomi Cortex inhibits TGF-β-induced epithelial-to-mesenchymal transition in the A549 lung cancer cell line

RYOEI KIN[1], SHINICHIRO KATO[1], NAOKI KANETO[1], HIROAKI SAKURAI[1,2], YOSHIHIRO HAYAKAWA[1], FENG LI[2], KEN TANAKA[3], IKUO SAIKI[1] and SATORU YOKOYAMA[1]

[1]Division of Pathogenic Biochemistry, Institute of Natural Medicine, [2]Department of Cancer Cell Biology, Graduate School of Medicine and Pharmaceutical Science, [3]Research Promotion Office, [4]Division of Pharmacognosy, Institute of Natural Medicine, University of Toyama, Toyama 930-0194, Japan

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Abstract. Cancer metastasis is one of the most critical events in cancer patients, and the median overall survival of stage IIIb or IV patients with metastatic lung cancer in the TNM classification is only 8 or 5 months, respectively. We previously demonstrated that *Juzentaihoto*, a Japanese traditional medicine, can inhibit cancer metastasis through the activation of macrophages and T cells in mouse cancer metastatic models; however, the mechanism(s) through which *Juzentaihoto* directly affects tumor cells during the metastasis process and which herbal components from *Juzentaihoto* inhibit the metastatic potential have not been elucidated. In this study, we focused on the epithelial-to-mesenchymal transition (EMT), which plays an important role in the formation of cancer metastasis. We newly determined that only the Cinnamomi Cortex (CC) extract, one of 10 herbal components of *Juzentaihoto*, inhibits TGF-β-induced EMT. Moreover, the contents of catechin trimer in CC extracts were significantly correlated with the efficacy of inhibiting TGF-β-induced EMT. Finally, the structure of the catechin trimer from CC extract was chemically identified as procyanidin C1 and the compound showed inhibitory activity against TGF-β-induced EMT. This illustrates that procyanidin C1 is the main active compound in the CC extract responsible for EMT inhibition and that procyanidin C1 could be useful as a lead compound to develop inhibitors of cancer metastasis and other diseases related to EMT.

Correspondence to: Dr Satoru Yokoyama, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
E-mail: yokoyama@inm.u-toyama.ac.jp

Abbreviations: EMT, epithelial-to-mesenchymal transition; CC, Cinnamomi Cortex

Key words: Cinnamomi Cortex, epithelial-to-mesenchymal transition, lung cancer, procyanidin C1

Introduction

The essential characteristics of cancer are the ability to invade surrounding tissues and metastasize to distal tissues, known as cancer metastasis, which is the major cause of mortality in cancer patients. In non-small cell lung cancer (NSCLC) patients, the median overall survival of patients with metastatic lung cancer (stage IIIb or IV in the TNM classification) is limited (only 8 or 5 months, respectively) (1); therefore, suppression of cancer metastasis results in the improved survival of lung cancer patients.

Cancer metastasis consists of several steps: intravasation, attachment to a vessel, extravasation, angiogenesis and growth in distal tissues (2-5). Among them, epithelial-to-mesenchymal transition (EMT) is involved in an early step of metastasis (6,7). EMT is a phenomenon in which cobblestone-like epithelial cells change into spindle-like mesenchymal cells with down-regulation of epithelial markers such as E-cadherin and also upregulation of mesenchymal markers such as N-cadherin (8). In addition to metastasis, this physiological phenomenon is important for resistance to apoptosis, maintenance of cancer stem cells and production of extracellular matrix. Thus, EMT would be an attractive therapeutic target in metastatic cancers.

Juzentaihoto, which is a Japanese Kampo medicine, has been widely used for the decline of physical strength, general debility, cold hands and feet, fatigue, night sweats, circulatory problems and anemia (9). Interestingly, our previous studies showed that *Juzentaihoto* indirectly inhibited cancer metastasis through the activation of macrophages and T cells in mouse models (10-12). Although *Juzentaihoto* could suppress cancer metastasis, it is not well known how *Juzentaihoto* can directly affect tumor cells during the metastasis process and which herbal ingredients of *Juzentaihoto* are involved in the regulation of EMT.

Here we firstly report that Cinnamomi Cortex (CC), one of the herbal medicines from *Juzentaihoto*, inhibits TGF-β-induced EMT phenotypes. After fractionation of CC extract, the content of catechin trimer was well-associated with the inhibitory activities of CC extract. Finally, procyanidin C1

from CC extract is newly identified as the responsible molecule for the EMT inhibition.

Materials and methods

Herbal medicine extraction. Astagali Radix (AsR), Glycyrrhizae Radix (GlR), Cinnamomi Cortex (CC), Rehmanniae Radix (RR), Paeoniae Radix (PR), Cnidii Rhizoma (CR), Angelicae Radix (AnR), Ginseng Radix (GiR), Hoelen (Ho), and Atractylodis Lanceae Radix (ALR) were purchased from Uchida-Wakanyaku Co., Ltd. (Tokyo, Japan). In this study, Cinnamomi Cortex was added to an appropriate volume of distilled water (w/v, 1:10) and extracted at 100˚C for 1 h. The extracted solution was filtered and then freeze dried to obtain dried powder. A voucher sample of this extract (INM 10000007, University of Toyama) was preserved in the Research Promotion Office, Institute of Natural Medicine, University of Toyama, Toyama, Japan. Six other Cinnamomi Cortex samples were collected from different regions of Vietnam and China, coded as CC-1 (Vietnam), CC-2 (Guang Xi, China), CC-3 (Guang Xi, China), CC-4 (Guang Dong, China), CC-5 (Guang Xi, China), CC-6 (Vietnam). Water extracts of the above-mentioned six CC samples were prepared and provided by the National Institute of Biomedical Innovation, Osaka, Japan, and a part of each extract was deposited at our institute (voucher specimen no. INM 10000001-10000006).

Cell cultures. Human lung adenocarcinoma A549 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37˚C. The cells were treated with recombinant human TGF-β (5 ng/ml) (Peprotech, London, UK) for various times as indicated, after pretreatment with TGF-β receptor I kinase inhibitor (10 μM) (Merck, Whitehouse, NJ, USA), each herbal component from *Juzentaihoto*, or Cinnamomi Cortex extracts (CC, or CC-1 to CC-6) for 30 min.

Protein preparation and western blotting. Whole cell lysates were collected in lysis buffer supplemented with some protease and phosphatase inhibitors as described previously (13). Equal amounts of protein were resolved by electrophoresis on acrylamide gels and transferred to PVDF membranes. Antibodies against phospho-specific Smad-2 (Ser 465/467), E-cadherin, and N-cadherin were purchased from Cell Signaling Technology (Beverly, MA, USA) and an antibody against PCNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell morphology and cell migration. Cell morphology was determined by BZ-8000 (Keyence, Osaka, Japan) after staining with hematoxylin and eosin. For the migration assay, Transwell cell culture chambers were used as described previously (14). Briefly, the filters were precoated with 1.25 μg fibronectin on the lower surfaces. The cell suspension ($3x10^4$ cells/100 μl) in serum-free medium was added to the upper compartment and incubated for 6 h. The migrated cells were stained with hematoxylin and eosin, and counted under the microscope in three predetermined fields at a magnification of x400.

Fractionation of CC and identification of procyanidin C1 from CC. CC extract, 2.0 g, was subjected to reversed-phase silica gel (Cosmosil $75C_{18}$-OPN; Nacalai Tesque Inc., Kyoto, Japan) using medium pressure liquid chromatography (MPLC; Buchi, Flawil, Switzerland) with a H_2O-CH_3CN gradient system (98:2→96:4→92:8→90:10→80:20→60:40→40:60→10:90) to obtain eight fractions (fr. 1, 435 mg; fr. 2, 282 mg; fr. 3, 123 mg; fr. 4, 212 mg; fr. 5, 105 mg; fr. 6, 305 mg; fr. 7, 205 mg; fr. 8, 105 mg). The bioactive fraction 4 (fr. 4, 200 mg) was further subjected to preparative HPLC (Discovery C18 column; 10x250 mm i.d., 5 μm particle size; Supelco, PA, USA) with H_2O-CH_3CN (92:8) containing 0.01% trifluoroacetic acid (TFA) at a flow rate of 2 ml/min to yield a procyanidin trimer (**1**, 5.5 mg, t_R 23.3 min). The molecular formula of compound **1** was determined by HR-TOF-MS to be $C_{45}H_{38}O_{18}$ [*m/z* 865.2003 $(M - H)^+$]. Its chemical structure was further identified to be epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (procyanidin C1, Fig. 3C) by comparing the [^{1}H] nuclear magnetic resonance (NMR), [^{13}C] NMR, and circular dichroism (CD) spectral data with those in the literature (15).

Liquid chromatography-mass spectrometry (LC-MS) analysis. For chemical profiling of 7 CC extracts, liquid chromatography-mass spectrometry (LC-MS) analysis was performed with a Shimadzu LC-IT-TOF mass spectrometer (Kyoto, Japan) equipped with an ESI interface (Shimadzu). The ESI parameters were as follows: source voltage +4.5 kV, capillary temperature 200˚C and nebulizer gas 1.5 l/min. The mass spectrometer was operated in positive ion mode scanning from *m/z* 200 to 2,000. A Waters Atlantis T3 column (2.1 mm i.d. x 150 mm, 3 m; Milford, MA, USA) was used and the column temperature was maintained at 40˚C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) CH_3CN under the following gradient conditions: 0-30 min linear gradient from 10 to 100% B, 30-40 min isocratic at 100% B. The flow rate was 0.15 ml/min. Mass spectrometry data obtained from the extract were deposited in the MassBank Database and stored with pharmacological information on the extract in the Wakan-Yaku Database System, Institute of Natural Medicine, University of Toyama.

Results

Cinnamomi Cortex (CC) extract suppresses TGF-β-induced EMT. Our previous studies showed that *Juzentaihoto*, a Japanese Kampo medicine, prevents metastasis in mouse models (10-12). Because *Juzentaihoto* contains ten ('Ju' means 'ten' in Japanese) kinds of herbal ingredients, we thus investigated whether each component can suppress TGF-β-induced EMT. We firstly screened the expression of an epithelial marker, E-cadherin, in a human non-small-cell lung cancer cell line, A549 cells, treated with TGF-β, TGF-β receptor kinase inhibitor (TGFRi), or herbal medicines (Fig. 1A). Known as EMT, the reduction of E-cadherin expression in A549 cells was detected after TGF-β treatment in A549 cells. Among the ten herbal components, only Cinnamomi Cortex (CC) extract strikingly suppressed TGF-β-induced EMT, as indicated by the restoration of E-cadherin. We could not detect any inhibition of the cell viabilities by CC extract (data not shown).

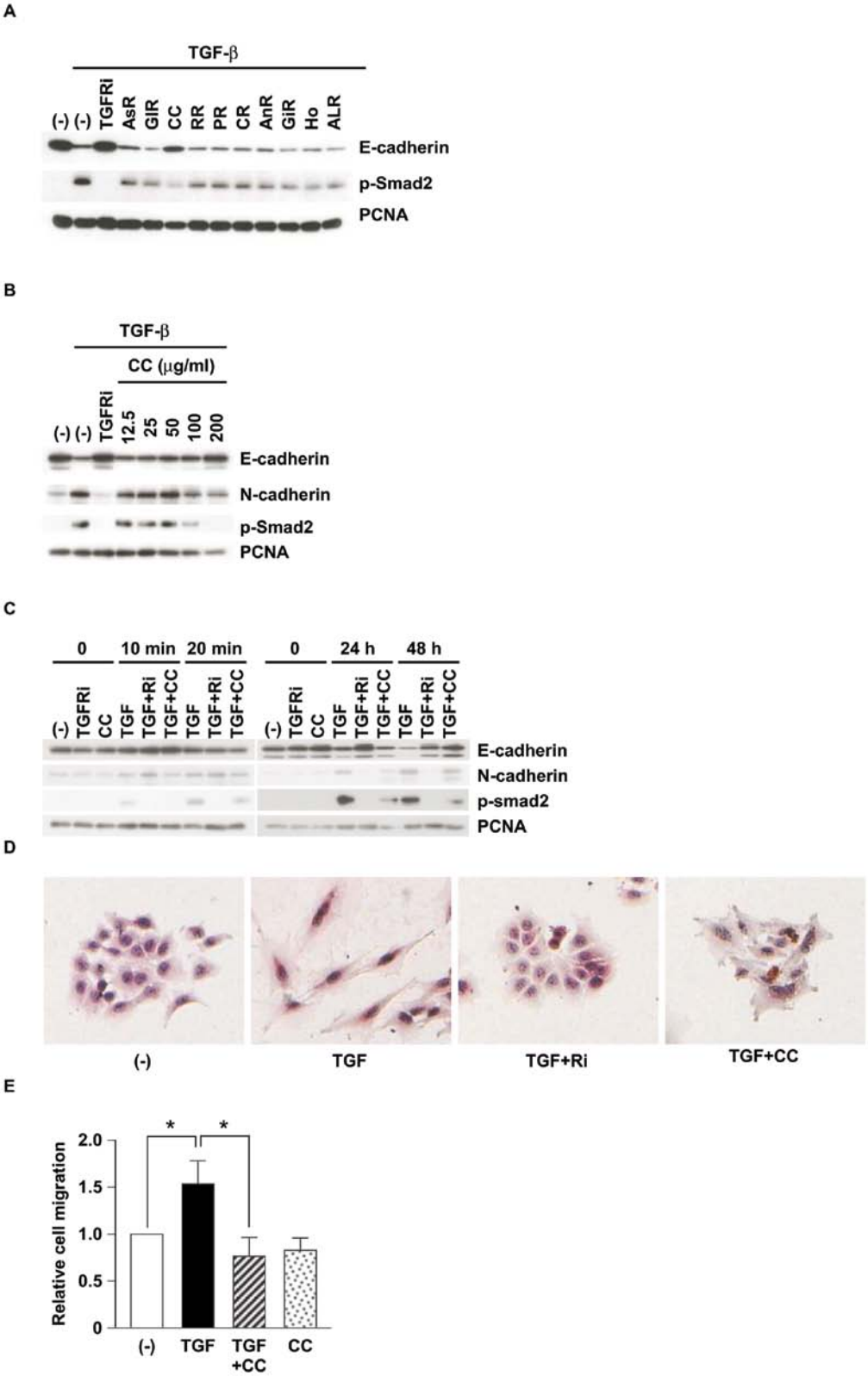

Figure 1. Cinnamomi Cortex (CC) suppresses TGF-β-induced EMT. (A) Effects of ten herbal components from *Juzentaihoto*. AsR, Astagali Radix; GlR, Glycyrrhizae Radix; CC, Cinnamomi Cortex; RR, Rehmanniae Radix; PR, Paeoniae Radix; CR, Cnidii Rhizoma; AnR, Angelicae Radix, GiR, Ginseng Radix; Ho, Hoelen; ALR, Atractylodis Lanceae Radix. A549 cells were pretreated with various herbal ingredients (100 μg/ml) or TGF-β receptor I kinase inhibitor (TGFRi; 10 μM) for 30 min and stimulated with TGF-β (5 ng/ml) for 48 h. Expression of each protein was detected by western blotting. (B) Dose-dependent inhibition of TGF-β-induced EMT with CC treatment. A549 cells were pretreated with various concentrations of CC (12.5, 25, 50, 100 and 200 μg/ml) or TGF-β receptor I kinase inhibitor (TGFRi; 10 μM) for 30 min and stimulated with TGF-β (5 ng/ml) for 48 h. Expression of each protein was detected by western blotting. (C) Time-dependent inhibition of TGF-β-induced EMT with CC treatment. A549 cells were pretreated with CC (100 μg/ml) for 30 min and stimulated with TGF-β (5 ng/ml) for the indicated times. Expression of each protein was detected by western blotting. (D) Cell morphologies with CC treatment. A549 cells were stained by H&E after treatment as in (C) for 48 h. (E) Migration with CC treatment. A549 cells treated as in (D) were seeded in Transwell chambers for 6 h. No. of migrated cells per field (x400) was counted. Relative cell migration with TGF-β (TGF, filled bar), TGF-β with CC extract (TGF+CC, shadow bar), or CC extract (CC, dotted bar) was normalized by untreated cell migration (open bar). Data are the mean ± SD of four independent experiments. *p<0.01, compared among each pair, by one-way ANOVA and Bonferroni's multiple comparision test.

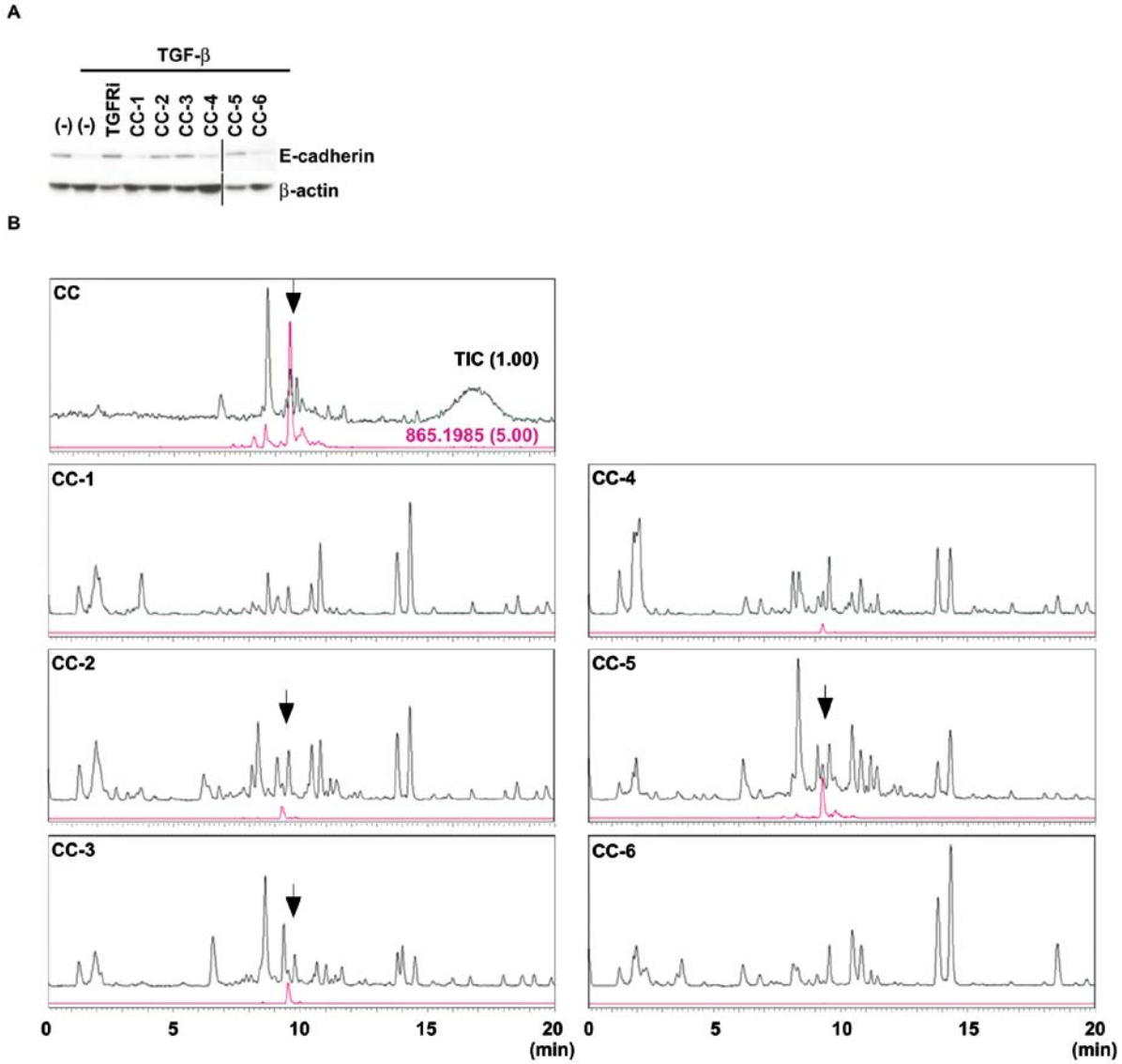

Figure 2. CCs show variability in the activity of TGF-β-induced EMT. (A) Effects of various CCs. A549 cells were pretreated with various CCs (100 μg/ml), but other conditions were similar to Fig. 1C. Expression of each protein was detected by western blotting. (B) Inhibition activities of CCs correlated with the content of catechin trimer. Total ion chromatogram (TIC) profiles of various CC extracts are shown. Arrows show the catechin trimer fractions.

In order to further determine the inhibition of TGF-β-induced EMT by CC extract, E-cadherin, N-cadherin, and phosphorylated Smad-2 were examined. Similarly to Fig. 1A, restoration of E-cadherin expression by CC extract was detected in a concentration/time-dependent manner (Fig. 1B and C). Interestingly, phosphorylation of Smad-2 by TGF-β, a downstream molecule of TGF-β signaling, was suppressed by CC extract after the early phase (10 and 20 min) as well as the late phase (24 and 48 h). We also detected the inhibition of TGF-β-induced EMT by CC extract at mRNA levels of snail, *E-cadherin* and *fibronectin* (data not shown). In addition to expression levels of EMT markers, we observed morphological changes with CC extract (Fig. 1D). A549 cells treated with TGF-β showed spindle-like shapes as compared with untreated cells. On the other hand, TGF-β-stimulated cells treated together with CC extract showed cobblestone-like shapes similar to the untreated cells or the cells with TGFRi and TGF-β. Moreover, consistent with cell morphology, TGF-β-induced cell migration was also suppressed with CC extract (Fig. 1E). These results support that CC extract suppresses TGF-β-induced EMT in A549 cells and consequently inhibits cell migration.

Identification of an active fraction inhibiting TGF-β-induced EMT. To gain insight into the variations of CC extracts in EMT-inhibitory activities, we examined six additional CC (CC-1 to -6) extracts, which differ according to the harvest location (Fig. 2A). CC-2, CC-3 and CC-5 extracts showed restoration of E-cadherin expression stronger than CC-1, CC-4, and CC-6 extracts; therefore, an active component(s) for the inhibition of EMT might be included in CC-2, CC-3 and CC-5 extracts more than in CC-1, CC-4 and CC-6.

To identify the active chemical compound(s) that is able to inhibit TGF-β-induced EMT, total ion chromatogram analysis was performed in CC and CC-1 to CC-6 extracts. Notably, the peaks of catechin-catechin-catechin trimer (catechin trimer)

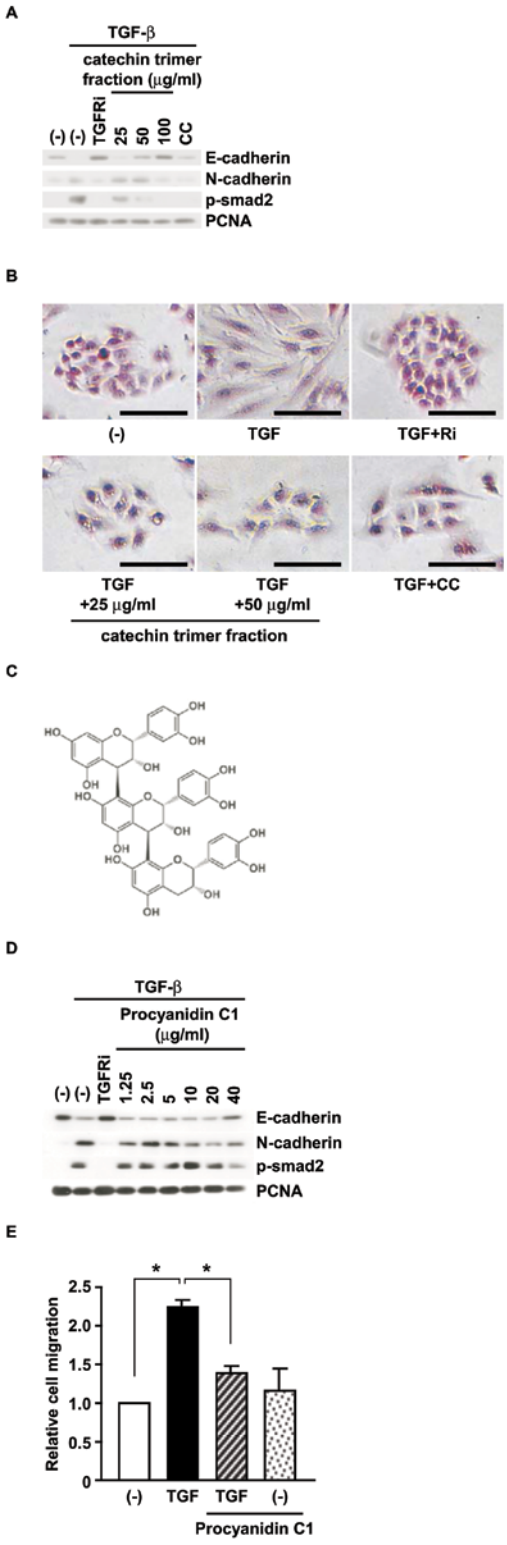

Figure 3. Procyanidin C1 from CC extract inhibits TGF-β-induced EMT. (A) Effects of catechin trimer fraction. A549 cells were pretreated with catechin trimer at the indicated concentration for 30 min, but other conditions are similar to Fig. 1C. Expression of each protein was detected by western blotting. (B) Cell morphologies with catechin trimer fraction. A549 cells were stained by H&E after treatment as in Fig. 1C for 48 h. (C) Structure of procyanidin C1. (D) Dose-dependent inhibition of TGF-β-induced EMT with procyanidin C1 from CC. Other conditions were similar to (A). Expression of each protein was detected by western blotting. (E) Migration with procyanidin C1 from CC. A549 cells were treated as in (C). Relative cell migration of the cells treated with TGF-β (TGF, filled bar), with both TGF-β and procyanidin C1 (shadow bar) or with procyanidin C1 (dotted bar) was normalized by untreated cells (open bar). Data are the mean ± SD of three independent experiments. *p<0.01, compared among each pair, by one-way ANOVA and Bonferroni's multiple comparision test.

significantly overlapped with EMT inhibitory activities in CC-2, CC-3 and CC-5 extracts, but were not detected in CC-1, CC-4, and CC-6 extracts (Fig. 2B), suggesting that the catechin trimer may have crucial roles in suppressing TGF-β-induced EMT.

Procyanidin C1 can suppress TGF-β-induced EMT. Next to assess whether catechin trimer fraction of CC extract has activity to inhibit EMT, we examined the protein expression levels with the catechin trimer fraction from CC (Fig. 3A). As predicted, the catechin trimer fraction inhibited E-cadherin reduction and N-cadherin induction. Similarly to protein levels, the change of cell morphology (Fig. 3B) also supported the inhibition of TGF-β-induced EMT.

We finally purified the catechin trimer from CC and identified its structure, which was an epicatechin-epicatechin-epicatechin trimer, procyanidin C1 (Fig. 3C). To check the inhibition of EMT by procyanidin C1, the protein expression and cell migration activities were examined (Fig. 3D and E). Strikingly, EMT markers represented the inhibition of EMT in western blotting, and TGF-β-induced cell migration was suppressed by procyanidin C1.

Discussion

In this study, we firstly identified Cinnamomi Cortex (CC) extract in 10 herbal components from *Juzentaihoto* as the only inhibitory herbal ingredient of TGF-β-induced epithelial-to-mesenchymal transition (EMT) in a human non-small cell lung cancer cell line, A549 cells. The inhibitory effects of CC extracts could be derived from the procyanidin C1 because of the correlation of the content of procyanidin C1 in CC extract with their inhibitory activities.

The inhibitory effect of CC extract on EMT phenotypes we showed here might be supported by the evidence gained from *Ninjinyoeito* and *Keishibukuryogan*, both of which are other Kampo medicines containing CC. *Ninjinyoeito* has also been reported to suppress cancer metastasis (16). On the other hand, *Keishibukuryogan* has been shown to suppress renal fibrosis, which is caused by TGF-β-induced EMT (17). Despite the inhibitory activity of CC extract, the possibility can not be excluded that other herbal medicines might play a role in concert with CC extract, because of the harmonization effects among various herbal medicines (11,12).

We identified procyanidin C1 as an active compound for the inhibition of EMT, but CC extract contains various chemical compounds, such as phenyl propanoids (cinnamic acid, cinnamaldehyde), terpenoids (cinnamonol, cassioside) and tannin (epicatechin) (18). There are many reports on the biological activities of cinnamic acid or cinnamaldehyde, for example, induction of ROS-mediated apoptosis (19). In addition, the procyanidin C1 we purified is known for its anti-inflammatory effects (20,21); however, it is the first time to show that procyanidin C1 has anti-metastatic or anti-EMT effects. Among the catechin sub-family, epigallocatechin-3 gallate in green tea has been reported to inhibit EMT in human melanoma cells (22,23). These reports raise the possibility that another catechin sub-family is involved in the inhibition of EMT besides procyanidin C1. The inhibition of cell migration by procyanidin C1 (Fig. 3E) and the clear linkage

of the amount of procyanidin C1 to the inhibition of TGF-β-induced EMT (Fig. 2B) suggest that procyanidin C1 is still the main compound in CC extracts to inhibit cell migration and EMT.

Although we need more efforts to identify the target molecule of procyanidin C1, the phosphorylation of Smad-2 (Fig. 3D) and its transcriptional activity (data not shown) were suppressed. Indeed, CC extract and procyanidin C1 did not affect basal cell migration in A549 cells (CC alone or procyanidin C1 alone in Figs. 1E and 3E), suggesting that the inhibition of EMT by CC extract and procyanidin C1 is dependent on inhibition of the TGF-β signal pathway and that mesenchymal-to-epithelial transition is not induced by CC extract or procyanidin C1, which might affect basal levels of cell migration.

Collectively, our data identified CC in *Juzentaihoto* as the only herbal component inhibiting TGF-β-induced EMT, which could be worthy of clinical study in a variety of settings associated with EMT, including cancer metastasis and/or tissue fibrosis. As procyanidin C1 showed inhibition of EMT, it is suggested that the utilization of procyanidin C1 as a lead compound would be attractive for the development of cancer metastasis inhibitors.

Acknowledgements

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