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Full Paper

Echinacoside Stimulates Cell Proliferation and Prevents Cell Apoptosis in Intestinal Epithelial MODE-K Cells by Up-Regulation of Transforming Growth Factor-β1 Expression

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Abstract. *Cistanche deserticola* Ma (*C. deserticola*) has been widely used as a laxative herbal in herbal medicine for the treatment of irritable bowel syndrome or constipation, and echinacoside (ECH) is one of the major bioactive ingredients in this herbal. Our aim was to investigate the effect of ECH on intestinal epithelial cell growth and death. MODE-K, an intestinal epithelial cell line, was used as an in vitro model of the intestine. Cell proliferation was measured by methylthiazol tetrazolium (MTT) assay. Cell apoptosis was determined with Annexin-V staining. Here we showed that in cultured MODE-K cells, ECH significantly stimulated cell proliferation and enhanced cell survival by reducing cell apoptosis in the presence of H_2O_2 or the mixture of pro-inflammatory cytokines, while transforming growth factor (TGF)-β1 expression was up-regulated in a dose-dependent manner. Knockdown of TGF-β1 expression disrupted both the proliferative and cytoprotective activities of ECH, which was further confirmed by neutralization of TGF-β1 activity using anti–TGF-β1 antibody. These data suggest that ECH as one of bioactive ingredients in herbal *C. deserticola* and others may improve mucosal tissue repair by stimulating intestinal epithelial cell proliferation and preventing cell death via up-regulation of TGF-β.

Keywords: *Cistanche deserticola*, intestinal epithelium, cell proliferation, cell apoptosis, transforming growth factor (TGF)-β

Introduction

Cistanche deserticola Ma (*C. deserticola*) is commonly known as *Rou Cong Rong* in Chinese herbal medicine, and it has become a new crop in China due to its medical use (1). Its clinical benefit has been reported in the treatment of many health problems including chronic constipation (2, 3) and its beneficial effects are associated with its laxative activity in the intestine; it enhances moistening the stool, promoting bowel movement in patients (4, 5), as well as in animal models (6, 7). Our recent study demonstrates that oral administration of a water-extract of *C. deserticola* significantly reduces the number of inflammatory hyperplasia in the gut of colorectal cancer–prone mice (8).

Echinacoside (ECH) is a major component of phenylethanoid glycosides (PhG) (> 50% of total PhG) isolated from *C. deserticola* (9) and has been used as a biomarker for quality control of the *C. deserticola*–containing laxative drug *Ronggui Tongbian* Capsule (10) or for the production quality control of cultivated *C. deserticola* (11). ECH has been demonstrated to be cytoprotective both in an animal model of Parkinson's disease (12, 13) and a model of lung injury (14) and in cultured pheochromocytoma cells (PC12) (15, 16); and it has been shown to enhance cellular division of human fibroblastic cells (MRC-5) (17). In this study, the effect of ECH as a major bioactive ingredient of *C. deserticola* on cell proliferation and survival in cultured intestinal epithelial cells was examined in order to understand the pharmacological action of *C. deserticola*–containing herbal medicine in the treatment of intestinal disorders.

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Materials and Methods

Cell culture

MODE-K is an intestinal epithelial cell line derived from C3H/HeJ mice (18) that was kindly provided by Dr. Theodore Steiner (Vancouver Coastal Health Research Institute, Vancouver, Canada). MODE-K cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C under a 5% CO_2 in air atmosphere.

ECH preparation

ECH was further purified from commercial *C. tubulosa* extract (containing ≥ 70% ECH, Tianlife TLCT-E70; Sinphar Tian-Li Pharmaceutical Co., Ltd., Hangzhou, China) by repeated ethanol extraction, followed by air-drying in a chemical fume hood. The remaining substance was further dried by lyophilization. ECH in the end product was confirmed by high-performance liquid chromatography (Fig. 1).

Knock-down of transforming growth factor (TGF)-β1 expression

TGF-β1 expression in MODE-K cells was knock-down by stable expression of shRNA specifically against TGF-β1 transcript (19). In brief, oligonucleotide sequences for generation of shRNA targeting mouse TGF-β1 mRNA (5′-AAC CAA GGA GAC GGA ATA CAG-3′) and non-specific control (scrambled) (5′-AAT CGC ATA GCG TAT GCC GTT-3′) were previously described (19, 20), and they were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA). Each sequence was ligated into pHEX-siRNA vector (21). The vectors were transfected into MODE-K cells using Lipofectamine2000 (Invitrogen-Gibco, Carlsbad, CA, USA) following the manufacturer's protocol. Both anti–TGF-β1 shRNA-transfected (MODE-K^{tgfb}) and scrambled shRNA-transfected (MODE-K^{scr}) cells were grown in the presence of Zeocin (up to 500 μg/mL) (Invitrogen-Gibco) and selected by cell sorting using a flow cytometry for green fluorescence protein (GFP). More than 95% of transfected cells showed strong green fluorescence in a flow cytometry or microscopy.

Methylthiazol tetrazolium (MTT) assay

The MTT assay was used to measure cell proliferation or viability in cultures. Cells were seeded at 2500 per well in 96-well microplates, and each condition was tested at least in quadruplicate. In brief, 10 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Canada, Oakville, ON, Canada) were added to each well and incubated at 37°C for 4 h. The formazan crystals in viable cells were then dissolved in 100 μL/well of dimethyl sulfoxide (DMSO, Sigma-Aldrich Canada). The absorbance of the color in each well was quantified as absorbance units (AU) at 560 nm wavelength using an ELx808 Ultra Microplate Reader (BioTek, Winooski, VT, USA). The percentage of cell proliferation change in drug-treated cultures against nondrug-treated control was calculated as follows: % = (drug-treated − Control) / Control × 100%. The growth rate was calculated as follows: Growth rate (AU/day) = (AU^{end} − AU^{begin}) / time (day), where time represented the period of examination, AU^{end} represented the absorbance units at the end of the examination, and AU^{begin} represented the absorbance units at the beginning of the examination.

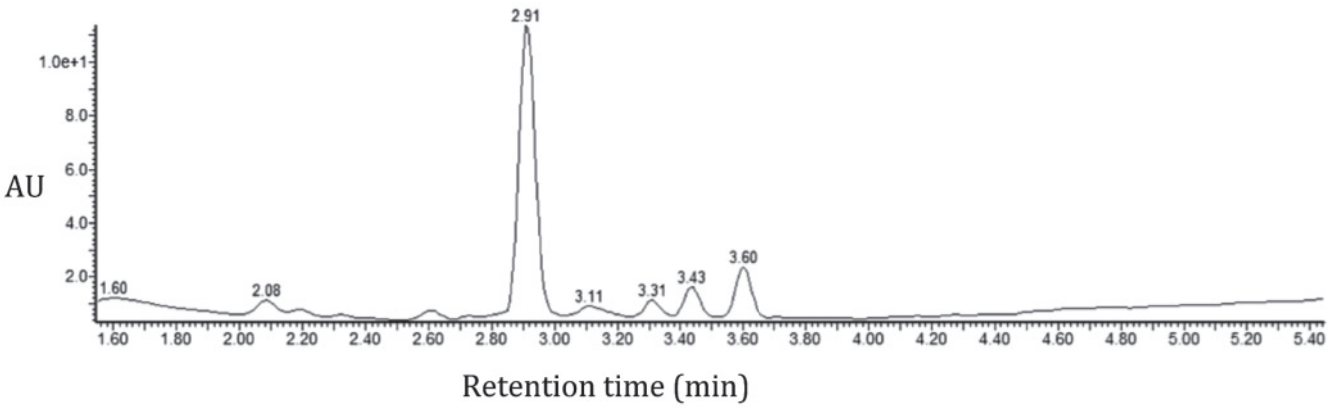

Fig. 1. HPLC fingerprint of echinacoside preparation. The batch-to-batch variations of purified echinacoside were monitored by HPLC analysis with a C-18 column and methanol/water (70:30, v/v) as eluent solution. AU: absorbance unit at UV absorption. The peak at 2.91 is echinacoside (major). Based on the molecular weight in the HPLC spectrum, other minor chemicals could be acteoside (MW: 624.594) at 3.43 min and cistanoside A (MW: 800.755) at 3.60 min.

Fluorescence-activated cell sorting (FACS) analysis of apoptosis

FACS analysis with Annexin-V–phycoerythrin (Annexin-V-PE) and 7-amino-actinomycin D (7-AAD) staining was used for the determination of cell apoptosis (Annexin-V positive: early apoptosis; 7-AAD: late apoptosis/necrotic) following the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). Thus, in the FACS graph, viable cells were in the lower left quadrant, late apoptotic cells in the upper left quadrant (7-AAD positive only), apoptotic cells in the upper right quadrant (Both Annexin-V and 7-AAD positive), and early apoptotic cells in the lower right quadrant (Annexin-V positive only). In brief, after treatment, cells were released by a brief incubation with trypsin-EDTA solution (Sigma-Aldrich Canada) and then incubated with Annexin-V-PE in 1 × binding buffer for 15 min. After they were washed with PBS, the cells were stained with 7-AAD. The intensity of fluorescence of apoptotic cells was measured using a flow cytometry and analyzed as compared to background controls using CELLQUEST (BD Biosciences) or FlowJo (Tree Star, Inc., Ashland, OR, USA) software.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels of TGF-β1 were semiquantitatively determined as compared to those of glyseraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control using RT-PCR kits (Invitrogen, Carlsbad, CA, USA) as described previously (19). Both TGF-β1 and GAPDH transcripts in the same cDNA preparation were amplified by PCR with 35 cycles of 94°C for 1 min, 57°C for 1 min and 15 s, and 72°C for 1 min and 30 s after the initial denaturation step at 94°C for 1 min. A final extension step at 72°C for 10 min was added. The specific primer pairs for TGF-β1 and GAPDH were derived from a previous report (19) (mTGF-β1: sense, 5′-TAC TAT GCT AAA GAG GTC ACC CGC, and anti-sense, 5′-CTG TAT TCC GTC TCC TTG GTT CAG; mGAPDH: sense, 5′-ATC ACT GCC ACC CAG AAG ACT G, and anti-sense, 5′-CCC TGT TGC TGT AGC CGT ATT C). The resultant PCR product (25 μL) was subjected to electrophoresis on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer containing 0.5 μg/mL of ethidium bromide and visualized under UV light. The expression levels of TGF-β1 were semiquantitatively compared to those of GAPDH.

Western blot

Cellular levels of TGF-β1 were examined by western blotting as described previously (19). TGF-β1 protein bands in the blot were identified with monoclonal anti–TGF-β1 (A75-3) (BD Biosciences). The same blot was re-probed using anti–β-actin IgG (Sigma-Aldrich Canada) for confirmation of loaded protein in each sample.

Enzyme-linked immunosorbent assay (ELISA)

The levels of secreted TGF-β1 in culture supernatants were determined by ELISA. MODE-K cells (1.8×10^5 cells/well in 0.5 mL of DMEM medium) in 24-well plates in triplicate were grown overnight, followed by incubation in the absence or presence of ECH. The culture supernatants were collected after 24 h of treatment, and the amount of TGF-β1 release was determined using the TGFβ1 $E_{max}^{®}$ ImmunoAssay System following the manufacturer's protocol (Promega, Madison, WI, USA).

Statistical analyses

One-way or two-way analysis of variance (ANOVA) was used as appropriate for comparisons between groups. Data were collected from 3 to 4 individual experiments in each study for statistical analysis. A P-value of ≤ 0.05 was considered significant.

Results

Stimulation of cell proliferation in MODE-K cell cultures

Cell proliferation is essential for tissue repair. The effect of ECH on cell proliferation was examined in cultured MODE-K cells. As shown in Fig. 2A, addition of ECH stimulated cell proliferation, measured by the increase in cell viability using MTT assay, in a dose-dependent manner. Compared to the cell number in nondrug-treated cultures, the cell number in ECH-treated cultured was increased 25.6% ± 14.82% in cultures with 6.26 μg/mL of ECH, 33.6% ± 14.0% with 25 μg/mL of ECH, 64.4% ± 9.0% with 50 μg/mL of ECH, and 99.4% ± 11.7% with 100 μg/mL of ECH after 48-h incubation ($P < 0.0001$). To further confirm this observation, the effect of ECH on MODE-K cell growth curve in a 4-day period of time was examined. As shown in Fig. 2B, addition of optimized 10 μg/mL of ECH significantly increased the viable cell number following the time of incubation as compared to those in nondrug-treated cultures (a basal control), indicated by the increase in AU for cultures with ECH from 0.30 ± 0.02 at the beginning of ECH treatment (0 time point) to 1.49 ± 0.08 after 2 days of incubation and 2.20 ± 0.11 after 4 days of incubation, whereas for the nondrug-treated cultures (medium only) the increase was from 0.24 ± 0.01 at the beginning to 1.22 ± 0.12 after 2 days and 1.68 ± 0.11 after 4 days (medium vs. ECH, $P < 0.0001$). The growth rates in these two cultures, calculated based on the growth curve

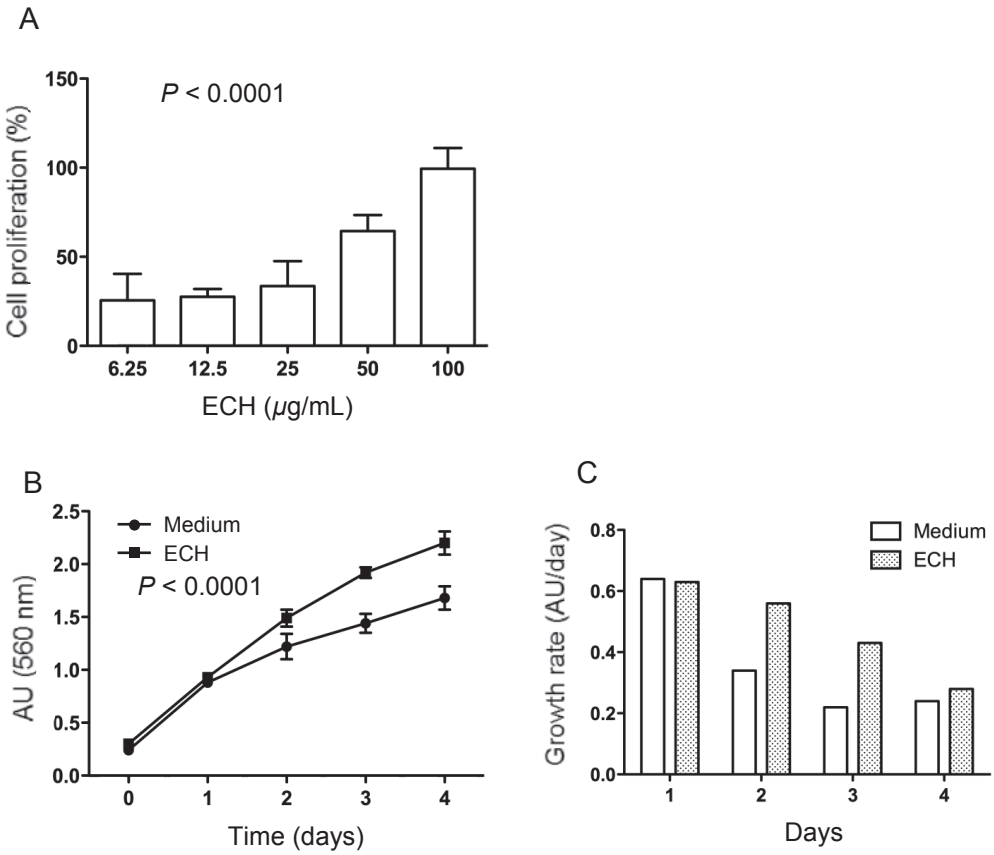

Fig. 2. Stimulation of cell growth in cultured intestinal epithelial cells by ECH. MODE-K cells (2,500 cells/well) were seeded in 96-well plates overnight, followed by incubation in the presence or the absence of ECH. The number of viable cells in cultures was measured by MTT assay. A) Cultures were treated with various concentrations of ECH for 48 h. The increase (%) in cell number in ECH-treated cultures was calculated based on the nondrug-treated cultures as a basal reference. $P < 0.0001$ (one-way ANOVA). B) Cultures were treated with 10 μg/mL of ECH for 4 days. Growth curves of ECH-treated vs. nondrug-treated cultures (medium only) were determined by the absorbance units (AU) at 560 nm in the MTT assay. $P < 0.0001$ (medium vs. ECH, two-way ANOVA). Data are presented as the mean ± standard derivation (S.D.) of independent experiments. C) Growth rate (AU change/day) was calculated based on the growth curve, the mean number on each day.

(Fig. 2B), indicated that ECH significantly enhanced the growth rates in cultures at both day 2 (0.56 in ECH-treated cultures vs. 0.35 in nondrug-treated controls) and day 3 (0.43 in ECH-treated cultures vs. 0.22 in nondrug-treated controls) (Fig. 2C). Taken together, these data indicate that ECH stimulates cell proliferation in cultured intestinal epithelial cells.

Protection of MODE-K cells from apoptosis

Protecting cells from apoptosis is a mechanism of preventing further tissue damage. The cytoprotective activity of ECH was examined in cultured MODE-K cells stimulated with hydrogen peroxide (H_2O_2) or pro-inflammatory cytokine mixture (TNF-α and IFN-γ). H_2O_2 is a reactive oxygen species generated by oxidative metabolism and represent a scenario of oxidative stress, while TNF-α and IFN-γ are prominent cytokines produced by activated immune cells that reflected the situation under the immune-mediated inflammation. As shown in Fig. 3, addition of ECH (50 μg/mL) significantly reduced apoptosis including its basal level in cultures treated with H_2O_2, evidenced by a decrease in apoptosis from the basal levels of 5.30% ± 1.24% in nondrug-treated cultures to 3.76% ± 1.01% in ECH-treated cultures, from 26.93% ± 1.86% in nondrug-treated cultures to 14.08% ± 1.30% in ECH-treated cultures in the presence of 0.3 μM of H_2O_2, and from 38.90% ± 1.76% in nondrug-treated cultures to 19.88% ± 1.02% in ECH-treated cultures in the presence of 0.6 μM of H_2O_2 ($P < 0.0001$, medium/nondrug-treated vs. ECH-treated). The similar cytoprotective effect of ECH was seen in the cultures stimulated with cytokine mixture (Fig. 4); apoptosis (51.5% ± 6.30%) in cultures without addition of ECH (medium/nondrug-treated controls) was significantly higher than that (38.50% ± 5.60%) in ECH-treated cultures ($P = 0.0204$, medium/nondrug-treated vs. ECH-treated), suggesting that ECH protected MODE-K cells from pro-inflammatory cytokine-induced apoptosis. Taken together, ECH has cytoprotective activity against cell apoptosis induced by H_2O_2 oxidation as well as the cytotoxicity of pro-inflammatory cytokines.

Up-regulation of TGF-β expression in cultured MODE-K cells

Evidence in the literature suggests that TGF-β1 plays an important role in tissue injury and repair (22, 23) and in the recovery of the gastrointestinal tract from colitis or inflammatory bowel disease (24). To examine if TGF-β1 plays a role in ECH-induced cell proliferation and cytoprotection in cultured intestinal epithelial cells as demon-

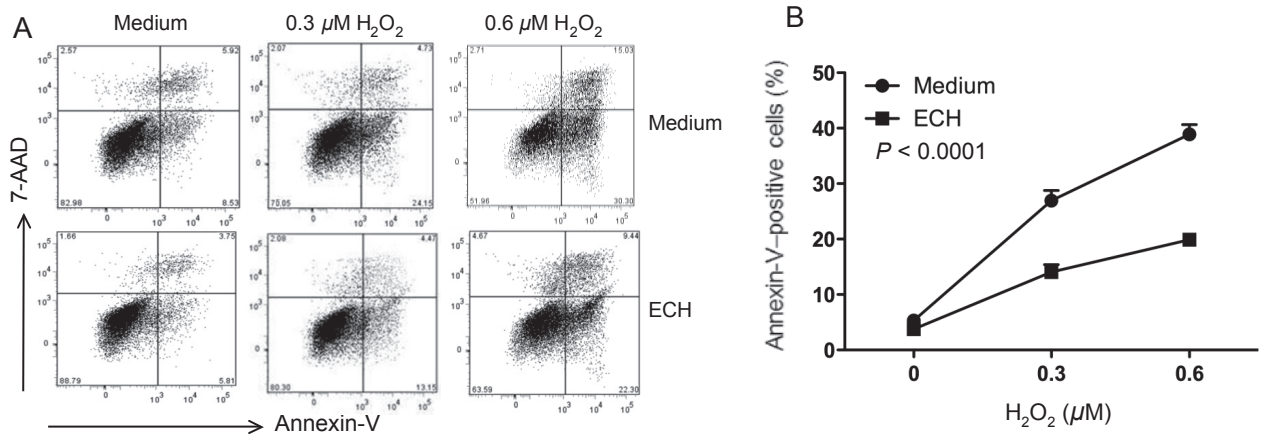

Fig. 3. ECH protects MODE-K cells from apoptosis induced by H_2O_2. MODE-K cells (0.18×10^6 cells/well) in 24-well plates were grown overnight, followed by treatment with various concentrations of H_2O_2 in the absence (medium) or the presence of ECH (50 μg/mL) for 24 h. Apoptosis was determined by FACS analysis with staining of Annexin-V-PE (early apoptosis) and 7-AAD (late apoptosis). Data are presented as a representative experiment (A) or mean ± S.D. of early apoptosis (single Annexin-V-PE–positive including those in the left upper quadrant) (B), $P < 0.0001$ (medium vs. ECH, two-way ANOVA, n = 4).

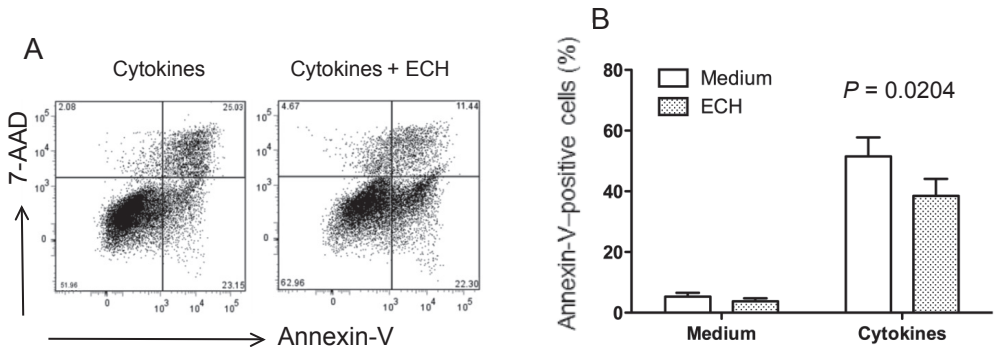

Fig. 4. ECH protects MODE-K cells from apoptosis induced by pro-inflammatory cytokines (TNF-α and IFN-γ). MODE-K cells (0.18×10^6 cells/well) in 24-well plates were grown overnight, followed by incubation with a mixture of TNF-α and IFN-γ (10 ng/mL each) in the absence or the presence of ECH (50 μg/mL) for 24 h. Apoptosis was determined by FACS analysis with staining of Annexin-V-PE and 7-AAD. Data are presented as a representative experiment (A) or mean ± S.D. of early apoptosis (single Annexin-V-PE–positive including those in the left upper quadrant) (B), $P = 0.0204$ (medium vs. ECH, two-way ANOVA, n = 4).

strated above, the effect of ECH on TGF-β1 expression in cultured MODE-K cells was examined. The transcript levels of TGF-β1 were examined by semiquantitative RT-PCR, while its protein levels were determined by western blotting (total cellular levels) or by ELISA (secreted protein levels). As shown in Fig. 5A, the dose-dependent up-regulation of TGF-β1 mRNA levels were seen in cultures treated with ECH at both 24 and 48 h after addition of ECH. The similar trend of increase in TGF-β1 protein levels inside the cells was demonstrated by western blotting analysis (Fig. 5B). After 24-h incubation in 0.5 mL of culture medium containing various concentrations of ECH, the secreted levels of TGF-β1 were also increased in a dose-independent manner; from 186.4 ± 18.39 pg/mL in nondrug-treated cultures (basal levels) to 186.4 ± 18.39 pg/mL in cultures with 25 μg/mL of ECH, 263.4 ± 14.14 pg/mL with 50 μg/mL of ECH, and 266.4 ± 15.56 pg/mL with 100 μg/mL of ECH ($P < 0.0001$). All these data clearly indicate that ECH increases TGF-β1 production in intestinal epithelial cells in cultures.

Loss of growth stimulation and cytoprotection of ECH by genetic knockdown of TGF-β1 expression

To clarify if TGF-β1 contributed to growth stimulation and apoptosis reduction in ECH-treated cells, TGF-β1 expression in MODE-K cells was knockdown by stably expressing anti–TGF-β1 shRNA, by which a new cell

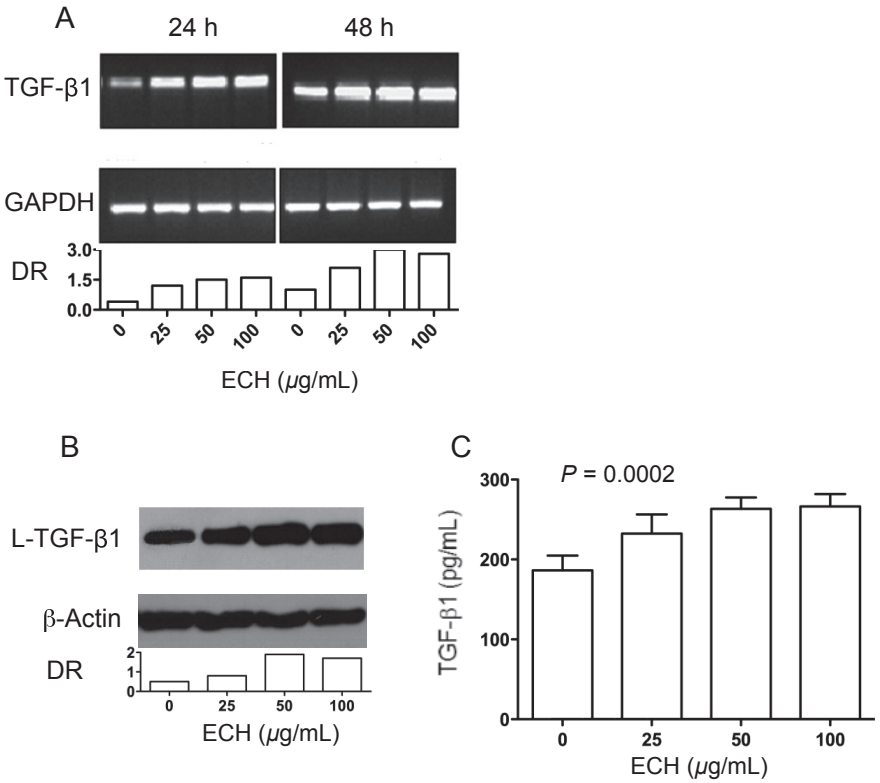

Fig. 5. ECH up-regulates TGF-β1 expression in MODE-K cells. MODE-K cells (0.75×10^6 cells/well) in 6-well plates were grown overnight, followed by incubation with various concentrations of ECH. A) Total RNA was extracted from MODE-K cells after 24- and 48-h ECH treatment. The mRNA levels of TGF-β1 and internal control GAPDH were determined by semiquantitative RT-PCR. DR: the density ratio of TGF-β1 to GAPDH. Data are a representative of three separate experiments, in which results were consistent. B) Cellular protein extract from MODE-K cells was prepared after 48-h ECH treatment. The protein levels of L(Latent)-TGF-β1 and internal control β-actin were determined by western blot analysis. DR: the density ratio of L-TGF-β1 to β-actin. Data are representatives of three separate experiments. C) MODE-K cells (0.18×10^6 cells/well) in 24-well plates were grown overnight, followed by incubation with various concentrations of ECH in 0.5 mL of culture medium for 24 h. The levels of secreted TGF-β1 in culture supernatants were determined by ELISA analysis. Data are presented as the mean ± S.D. of independent experiments, $P = 0.0002$ (one-way ANOVA, n = 3).

line MODE-K^{tgfb} was generated. TGF-β1 protein expression was largely reduced in MODE-K^{tgfb} cells as compared with that in MODE-K^{scr} cells that were stably expressing scrambled shRNA (Fig. 6A). As shown in Fig. 6B, the proliferative stimulation of ECH was significantly impaired in MODE-K^{tgfb} cells; similar to the proliferative response to ECH in parental MODE-K cells (Fig. 2), the viable cell number was increased in MODE-K^{scr} cells following ECH stimulation, 28.3% ± 12.4% increase in cultures with 25 μg/mL of ECH, 54.2% ± 6.8% increase with 50 μg/mL of ECH, and 122.7% ± 20.8% increase in cultures with 100 μg/mL of ECH. In contrast, the cell number was decreased in the presence of ECH in MODE-K^{tgfb} cell cultures, 12.0% ± 5.0% decrease in cultures with 25 μg/mL of ECH, 18.3% ± 4.1% decrease with 50 μg/mL of ECH, and 30.4% ± 3.3% decreased with 100 μg/mL of ECH. Statistical analysis with two-way ANOVA indicated the significant difference of proliferative response to ECH between these two groups ($P < 0.0001$). These data suggest that loss of TGF-β1 expression in intestinal epithelial cells impairs growth stimulation of ECH.

To further examine if the up-regulated TGF-β1 expression in ECH-treated cells resulted in apoptosis reduction, the anti-apoptotic activity of ECH was examined in MODE-K^{tgfb} cells *versus* MODE-K^{scr} cells. As shown in Fig. 6C, as compared to MODE-K^{scr} cell cultures, the levels of apoptosis were higher in MODE-K^{tgfb} cell cultures in the absence of cytokines (11.2% ± 0.42% in MODE-K^{tgfb} vs. 9.6% ± 0.99% in MODE-K^{scr}) and in the presence of cytokines (20.95% ± 0.07% in MODE-K^{tgfb} vs. 15.4% ± 1.56% in MODE-K^{scr}) ($P = 0.0013$), suggesting that TGF-β1 is an anti-apoptotic factor in MODE-K cells. Addition of ECH (50 μg/mL) to MODE-K^{scr} cell cultures significantly reduced the basal levels of apoptosis (9.6% ± 0.99% in nondrug treated vs. 8.3% ± 0.43% in ECH-treated) and 30.2% of apoptosis reduction was found in cytokines-stimulated cultures (15.4% ± 1.56% in nondrug treated vs. 10.75% ± 1.05% in ECH-treated). However, in MODE-K^{tgfb} cell cultures, ECH did not reduce the basal levels of apoptosis (11.2% ± 0.42% in nondrug treated vs. 12.0% ± 0.43% in ECH-treated), and only 16.71% of apoptosis reduction, compared to 30.2% in MODE-K^{scr} cell cultures, was seen in cytokines-stimulated cultures (20.95% ± 0.07% in nondrug treated vs. 17.45% ± 0.35% in ECH-treated). These data indicate that knockdown of TGF-β1 expression in intestinal epithelial cells worsens apoptosis and decreases the cytoprotective activity of ECH.

Loss of growth stimulation and cytoprotection of ECH by neutralization of TGF-β1 activity using antibody

To further confirm that TGF-β1 might be required for the growth stimulation and cytoprotection of ECH in

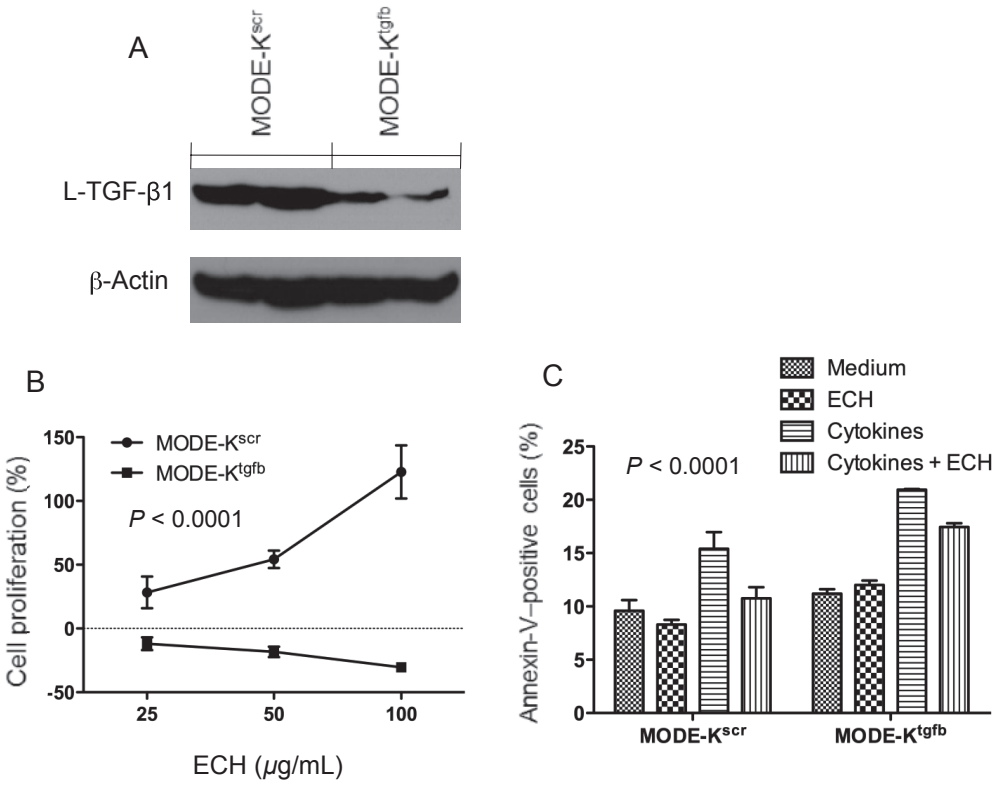

Fig. 6. Suppression of both proliferative and anti-apoptotic activities of ECH in MODE-K cells by stable knockdown of TGF-β1 expression. A) TGF-β1 expression in MODE-K cells was largely reduced by expression of anti–TGF-β1 shRNA. MODE-K^{tgfb} cells were generated by the stable expression of pHEX-siRNA vector containing a shRNA sequence against TGF-β1 mRNA in MODE-K cells, while MODE-K^{scr} cells were MODE-K cells with stable expression of pHEX-siRNA containing a scrambled shRNA sequence. TGF-β1 protein levels in MODE-K^{scr} cells vs. MODE-K^{tgfb} cells. Data are presented as a typical image of three separate examinations. B) Knockdown of TGF-β1 expression disrupts ECH-induced cell proliferation in MODE-K cells. Twenty-five hundred cells (MODE-K^{scr} vs. TGF-β1–deficient MODE-K^{tgfb} cells) per well were seeded in 96-well plates overnight, followed by incubation in the absence or the presence of ECH for 24 h. Cell viability in cultures was measured by MTT assay, and the increase (fold) in cell growth in ECH-treated cultures was calculated based on the untreated (medium) cultures as a basal reference. Data are presented as the mean ± S.D. of independent experiments, $P < 0.0001$ (two-way ANOVA). C) Knockdown of TGF-β1 expression reduces ECH-mediated cell survival in MODE-K cells. MODE-K^{scr} vs. TGF-β1–deficient MODE-K^{tgfb} cells (0.18×10^6 cells/well) in 24-well plates were grown overnight, followed by incubation with a mixture of TNF-α and IFN-γ (10 ng/mL each) in the absence or the presence of ECH (50 μg/mL) for 24 h. Apoptosis was determined by FACS analysis with staining of Annexin-V-PE and 7-AAD. Data are presented as the mean ± S.D. of early apoptosis (Annexin-V-PE–positive including those in the left upper quadrant), $P < 0.0001$ (MODE-K^{scr} vs. MODE-K^{tgfb}, two-way ANOVA, n = 4).

intestinal epithelial cells as described above (Fig. 6), the effect of neutralizing anti–TGF-β1 antibody (Clone 9016; R&D Systems Inc. Minneapolis, MN, USA) on ECH-mediated cell proliferation and survival was examined in MODE-K cell cultures. As shown in Fig. 7A, addition of ECH (25 μg/mL) stimulated 44.3% ± 8.2% increase in cell number in non-antibody-treated controls, but in the presence of anti–TGF-β1 antibody, 11.3% ± 9.10% loss in the cell number was seen in the same ECH-treated cultures, suggesting that neutralizing TGF-β1 activity diminished the growth stimulation of ECH in intestinal epithelial cells. The further experiments demonstrated that the anti–TGF-β1 antibody also neutralized the cytoprotective activity of ECH in MODE-K cell cultures; addition of ECH decreased the apoptosis from 4.37% ± 1.19% in nondrug-treated cultures to 1.72% ± 0.01% in ECH-treated cultures after 24 h of incubation and from 11.13% ± 1.12% in nondrug-treated cultures to 9.13% ± 0.02% in ECH-treated cultures after 48 h of incubation (nondrug/medium vs. ECH, two-way ANOVA, $P = 0.0001$). However, in the presence of anti–TGF-β1 antibody the apoptosis in ECH-treated cultures (2.88% ± 0.01% after 24-h incubation; 12.58% ± 0.02% after 48-h incubation) was not significantly different from those in control cultures (nondrug-treated) (two-way ANOVA, $P = 0.9427$), implying that anti–

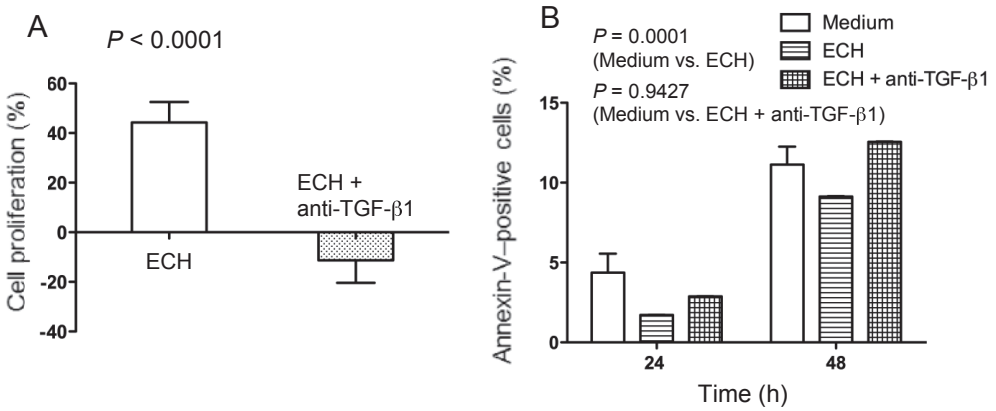

Fig. 7. Neutralization of TGF-β1 activity inhibits ECH-induced cell proliferation and survival in MODE-K cells. A) MODE-K cells (2,500 cells/well) were seeded in 96-well plates overnight, followed by treatment with 25 μg/mL of ECH in the absence or the presence of anti–TGF-β1 antibody (5 ng/mL, Clone 9016) for 48 h. Cell viability or proliferation in cultures was measured by MTT assay, and the increase (%) in ECH-treated cultures was calculated based on the nondrug-treated cultures as a basal reference. Data are presented as the mean ± S.D. of independent experiments, $P < 0.0001$ (ECH vs. ECH + anti–TGF-β1, n = 4). B) MODE-K cells (0.18×10^6 cells/well) in 24-well plates were grown overnight, followed by treatment with 25 μg/mL of ECH in the absence or the presence of anti–TGF-β1 antibody (5 ng/ml, Clone 9016) for 24 or 48 h. The apoptosis was determined by FACS analysis with staining of Annexin-V-PE and 7-AAD. Data are presented as the mean ± S.D. of early apoptosis (Annexin-V-PE–positive including those in the left upper quadrant), $P = 0.0001$ (medium vs. ECH), $P = 0.9427$ (medium vs. ECH + anti–TGF-β1), two-way ANOVA, n = 4.

TGF-β1 antibody interferes with ECH-mediated survival in intestinal epithelial cells.

Discussion

ECH is not only a major PhG in the extract of herbal *Cistanche spp*., but also found in many other medicinal plants such as *Echinacea spp*. (25, 26). This natural compound has been reported to have anti-oxidant activity in vitro and in vivo (13, 27, 28). However, the mechanism(s) of its action remains unknown. Our results from present study for the first time demonstrate in an in vitro model that ECH stimulates intestinal epithelial cell proliferation and survival, which is dependent on TGF-β1.

Addition of ECH reduces cell apoptosis in H_2O_2- or pro-inflammatory cytokine mixture (TNF-α and IFN-γ)-treated MODE-K cells (Figs. 3 and 4) and in other cell types as well (16, 29), and knockdown of TGF-β1 expression or blocking its activity results in abolishing its anti-apoptotic activity (Figs. 6 and 7), indicating that the cytoprotection of ECH depends on TGF-β1 production (Fig. 5). Our previous study has shown that TGF-β1 induces anti-apoptotic Bcl-2 expression and prevents TNF-α mediated apoptosis in renal tubular epithelial cells (19). Similar findings have been reported in other cell types; addition of TGF-β1 in cultured pulmonary artery endothelial cells up-regulates the baseline level of Bcl-2 and prevents apoptosis induced by serum deprivation and vascular endothelial growth factor (VEGF)-receptor blockade (30); and in cultured neural progenitor cells (NPCs), it enhances (by approximately 76%) cell survival against H_2O_2 treatment, which is correlated with up-regulation of Bcl-2 expression (31). Although the effect of ECH on Bcl-2 expression in MODE-K cells is not examined in this study, the correlation of Bcl-2 up-regulation with anti-apoptotic activity of ECH has been reported in TNF-α–treated SHSY5Y neuronal cells (29) or H_2O_2-treated PC12 cells (15). Taken together, these data suggest that the anti-apoptotic activity of ECH may be mediated by TGF-β1 via a Bcl-2 pathway.

It is possible that the cytoprotection of ECH could contribute to a slight increase in viable cell number by decreasing spontaneous cell death at the end of incubation, but addition of ECH (100 μg/mL) almost doubles the viable cells after 48-h treatment (Fig. 2A), suggesting that ECH indeed stimulates cell proliferation that is further confirmed by the data in Fig. 2, B and C. A similar effect of ECH has been reported in human fibroblastic cells, MRC-5 (17): in MRC-5 cell cultures, ECH could retard cellular senescence by inducing the cell cycle from the G1 phase to the S phase and G2 phase. Like the anti-apoptotic activity of ECH, the proliferative activity of ECH is dependent on TGF-β1 (Figs. 6 and 7) that has been well documented to be a multifunctional cytokine (32, 33). TGF-β can stimulate cell proliferation or in-

duces growth arrest depending on the cell type or extracellular microenvironment; it activates multiple signaling pathways including Smads and non-Smads (e.g., the mitogen-activated protein kinases) (34, 35), but which pathway mediates ECH/TGF-β–stimulated cell proliferation in MODE-K cells needs further investigation.

To date, little is known about the target protein(s) of ECH or the signal pathway(s) it regulates. One study shows that ECH treatment increases the cellular levels of cyclic guanosine monophosphate (cGMP) in endothelial cells (36), and addition of 8-bromo-cGMP modestly increases TGF-β1 expression in cardiac fibroblasts (37). However, whether ECH treatment elevates cGMP and by which ECH stimulates TGF-β1 expression in cultured intestinal epithelial cells need further investigation.

In conclusion, our study demonstrates that ECH induces TGF-β1 production that mediates cell proliferation and cell survival in cultured intestinal epithelial cells. TGF-β signaling in intestinal epithelium is required for mucosal injury repair or healing in mice with dextran sulfate sodium–induced colitis (38), suggesting that ECH, perhaps ECH-containing herbal *C. deserticola* or others, may have therapeutic potential for promoting mucosal injury repair in patients.

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