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Aqueous Extract of *Schizandra chinensis* Suppresses Dextran Sulfate Sodium-induced Generation of IL-8 and ROS in the Colonic Epithelial Cell Line HT-29

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Abstract – Intestinal epithelial cells (IEC) play an important role in the mucosal immune system. IEC-derived mediators of inflammatory cascades play a principal role in the development of colon inflammation. The aim of this study was to investigate the inhibitory effect of aqueous extracts of *Schizandra chinensis* fruits (SC-Ex) on the production of inflammatory mediators by the human colonic epithelial cells. HT-29 cells were stimulated with dextran sulfate sodium in the presence or absence of SC-Ex to examine the cytoprotection and production of IL-8 and reactive oxygen species (ROS). It was shown that dextran sulfate sodium (DSS) caused the reduction of cell viability and production of IL-8 and ROS in DSS-treated HT-29 cells. We observed that the treatment of SC-Ex protected significantly cell proliferation from DSS-induced damage in dose-dependent manner. SC-Ex (10 and 100 μg/ml) also suppressed DSS-induced production of IL-8 mRNA and protein. Moreover, DSS-induced ROS production was inhibited markedly by the treatment of 100 μg/ml SC-Ex. These results suggest that SC-Ex has the protective effects on DSS-induced cell damage and the release of inflammatory mediators in the intestinal epithelial cells.
Keywords – *Schizandra chinensis*, Intestinal epithelial cells, Reactive oxygen species, IL-8, Dextran sulfate sodium

Introduction

The fruits of *Schizandra chinensis* (SC), a member of the Magnoliaceae family, have been used as tonic and astringent in traditional Oriental medicine. In the Chinese medicine, a formula known as 'Shengmai San' comprising SC has been commonly used for treating coronary heart disease (Chen *et al.*, 1993; Li *et al.*, 1995; Ko *et al.*, 1995). Since SC had the capacity to increase resistance to a wide range of physical, chemical, and emotional stresses, it has been classically prescribed for the treatment of chronic cough and dyspnea, diarrhea, night sweats, wasting disorders and irritability (Sinclair, 1998). The current studies have shown that SC has the anti-hepatotoxic and anti-neurotoxic effects through the properties of antioxidation and lipid peroxidation inhibition (Hikino *et al.*, 1984; Mak *et al.*, 1996; Ip *et al.*, 1995). The crude extracts of SC have the anti-ulcer and anti-secretory activity (Daniel *et al.*, 1998; Long *et al.*, 1985). Moreover, scientific evidences for use of SC-Ex in various diseases are being investigated because of the solid clinical observations in Oriental medicine.

Intestinal epithelial cells (IEC) play an important role in the mucosal immune system. IEC is the first physical barrier of defense against the aggressive gut milieu responding to many inflammatory stimuli. During the progress of gut inflammation, IEC receive the activating signals from basically two sources; the inflammatory cytokines released by the immune cells of the gut, and bacteria or bacterial products (Sartor *et al.*, 1999; Hyams, 2000). These signals activate IEC to produce chemokine IL-8 and recruit the inflammatory cells into the inflamed tissue. An enhanced synthesis of IL-8 has been shown in the mucosa from patients with inflammatory bowel disease (IBD) (Fiocchi, 1998; Okayasu *et al.*, 1990). Reactive oxygen species (ROS) also can initiate inflammatory cascades and cause subsequent tissue damage. ROS results in the upregulation of a number of different genes involved in the inflammatory response through activation of NF-κB which is a ubiquitous transcription factor regulating several genes involved in

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the inflammation responses (Kaplan *et al.*, 2007; Albermann *et al.*, 1992). In addition, ROS is a potent unopposed oxidative stress which appears to be a major pathogenic mechanism in IBD (Grisham, 1994; Gross *et al.*, 1994).

Sulfasalazine and mesalazine are commonly used for the treatment of IBD. These drugs inhibit the activation of NF-κB in inflamed mucosa of patients with IBD and inhibit TNF-α-stimulated IL-8 expression by reduction of NF-κB activation in IEC (Simmonds *et al.*, 1993). A variety of herbal preparations have been considered to be useful in the treatment of inflammatory disorders. Herbs with antioxidant and anti-inflammatory properties have displayed benefits in treating inflammatory disorders. Since inflammation in IBD is also mediated by ROS, these same herbal therapies can be expected to have favorable effects in treating this disorder. In one study, curcumin displayed anti-inflammatory properties in an animal model of colitis (Sugimoto *et al.*, 2002).

Our recent study has shown that aqueous extracts of *S. chinensis* (SC-Ex) attenuated the dextran sulfate sodium (DSS)-induced experimental colitis in mice which is a useful animal model exhibiting similar phenotype to human IBD (Kang *et al.*, 2007). It is possible that antioxidative activity of SC-Ex contributes beneficial effects in pathogenesis of DSS-induced colitis. The aim of this study was to investigate an inhibitory effect of SC-Ex on the activation of colonic epithelial cells.

Materials and methods

Cell culture and reagents – Human colonic cell lines HT-29 were used in this experiment. Cells were grown in Dulbecco's Modified Eagle medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 2 mM glutamine, and 100 μg/ml penicillin/streptomycin at 37 ºC in a humidified 5% CO_2 atmosphere. For each experiment, HT29 cells were seeded onto each well at an appropriate cell density and incubated for 48 h in the complete medium. DSS (mol wt; 36,000 - 50,000) was purchased from ICN Biomedicals (Aurora, OH), All chemical reagents were from Sigma Co. (St. Louis, MO).

Plant extracts – *Schizandra chinensis* (Turcz.) Baill. fruit (SC) was obtained from a local market and ground using a commercial food mixer. Dried SC (100 g) was extracted consecutively under reflux with water (1 liter) for 1 h and then filtered through 0.45 μm filter. The resulting aqueous extract was concentrated using a rotary vacuum evaporator, and finally freeze-dried (29.2 g). For the experiment, the SC-extract (SC-Ex) was dissolved with distilled water at a concentration of 100 mg/ml. To determine the content of schizandin and gomisin A in SC-Ex, the dried extract was resuspended with 90% methanol and then analyzed by high performance liquid chromatograpy (Gilson Co.) using a proper condition [column: Sunfire^{TM} C18 (5.0 μm), 4.6 mm × 250 mm; flow rate: 1,0 ml/min; elution solvent: acetonitrile-methanol-water (11 : 11 : 8); detector: UV 254 nm]. Data were obtained from triplicate experiments.

Cell viability – In order to measure cell viability by DSS or SC-Ex, cells (1×10^4/well) were seeded onto a 96-well plate and then incubated with complete medium including 10% FBS. The cells were stimulated with DSS or SC-Ex at various concentrations in medium including 1% FBS for 24, 48, or 72 h. The percentages of viable cells were assayed by counting with a hemocytometer.

RNA preparation and RT-PCR – Total RNA was extracted from HT-29 cells stimulated with DSS (10 μg/ml) for 8 h in the presence or absence of SC-Ex (0 to 100 μg/ml) using a Trizol reagent (Invitrogen, Carlsbad, CA). A reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows. Complementary DNA (cDNA) was synthesized from 2 μg total RNA in a reaction mixture (20 μl) containing 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM of dNTPs, 10 mM DTT, 0.5 μg oligo $(dT)_{15}$, and 200 U of Maloney murine leukemia virus-reverse transcriptase (iNtRON Biotech, Gyunggido) at 42 ºC for 50 min. The reaction was terminated by heating at 72 ºC for 15 min. The reaction mixture (25 μl) for the subsequent PCR contained 10 mM Tris (pH 9.0), 50 mM KCl, 1.25 mM $MgCl_2$, 1 μl synthesized cDNA solution, 2 U of Taq polymerase, and 25 pM of each primer. PCR amplification was performed for 30 cycles of denaturation at 94 ºC for 45 sec, annealing at 60 ºC for 45 sec, and extension at 72 ºC for 2 min. Each PCR product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide to visualize bands on UV lamp. A cDNA for GAPDH was amplified as an internal control. Primers used in this study were as follows; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5'-CCA TGT TCG TCA TGG GTG TGA ACC A-3' and reverse primer 5'-GCC AGT AGA GGC AGG GAT GAT GTT C-3': 251 bp), IL-8 (forward primer 5'-ATG ACT TCC AAG CTG GCC GTG-3' and reverse primer 5'-TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC-3': 302 bp).

Determination of IL-8 protein by ELISA – HT29 cells (3×10^5 cells/ml) were seeded into 24-well tissue culture plates. To examine IL-8 expression, cells were

stimulated with various concentration of DSS for 16 h. Level of IL-8 in supernatants was measured using commercial instruction (Pharmingen, SanDiego, CA). Briefly, microplates (Falcon, Becton Dickinson Labware) were coated with anti-human IL-8 mAb diluted in coating buffer (0.1 M carbonate, pH 9.5) overnight at 4°C. Coated plates were clearly washed with PBS containing 0.05% Tween-20. The non-specific binding of antibody was blocked with assay diluents (PBS with 10% FBS, pH 7.0) for at least 1 h. Samples or IL-8 standards diluted in assay diluents were applied to wells and incubated for 2 h. After washing, the working detector (biotinylated anti-human IL-8 mAb and avidin-HRP reagent) was applied to wells for 1 h. Substrate solution (tetramethylbenzidine and hydrogen peroxidase) was added to wells and incubated for 30 min at room temperature in the dark. Stop solution (50 µl of 2N-H_2SO_4) finally was added and absorbance read at 450 nm within 30 min of stopping reaction. All samples were assayed in triplicate.

Assay of intracellular ROS generation – For assay of intracellular ROS production, cells (1×10^5) were seeded directly onto one-chamber slides (Falcon Co.). After incubation for 48 h, the culture medium was replaced to 500 µl of serum free medium and then treated with 10 µM of 2,7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 20 min. The cells washed with PBS were pretreated with SC-Ex (10 or 100 µg/ml) for 20 min and than exposed to DSS (10 µg/ml) for 30 min. PBS was used as a negative control. Generation of intracellular ROS was measured by fluorescence microscope (Olympus, Tokyo, Japan) (original magnification 1:200) and fluorescence microplate reader (Molecular Devices). Values were represented as relative fluorescence intensity.

Statistical analysis – Each experiment was performed at least 3 times and the data are shown as mean ± standard deviation (SD). Statistically significant differences between samples in each assay were determined using the Student's *t*-test. Values with $p < 0.05$ were considered significant.

Results

Effects of SC-Ex on the viability of HT-29 cells – We first examined the cytotoxic influence of SC-Ex in human colonic cell line HT-29. Cells were incubated with medium including 1% FBS at various concentrations (0, 1, 10, 100, and 500 µg/ml) of SC-Ex for 48 h. The cell viability was not affected by up to 100 µg/ml SC-Ex, whereas 500 µg/ml of SC-Ex inhibited slightly the growth of HT-29 cells without cell death (Fig. 1). To therefore examine the inhibitory effect, SC-Ex was applied up to 100 µg/ml concentration. In this study, we used DSS as a stimuli to investigate the activation of HT-29 cells. To examine the cytotoxic activity of DSS, the cells were stimulated with a various concentration of DSS (0, 0.1, 1, 10, or 25 µg/ml) for 72 hours. We observed that cell viability was inhibited at the concentration above 10 µg/

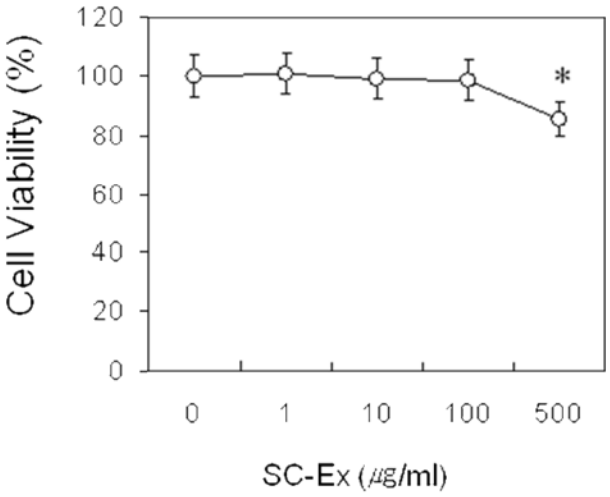

Fig. 1. Effect of SC-Ex on the viability of HT-29 cells. Cells were cultured with various concentration of SC-Ex (1 - 500 µg/ml) for 48 h. Cell viability was evaluated by counting after trypan blue staining. Values were represented as percent of control (mean ± SD) obtained from three independent experiments (*$p < 0.05$ vs. negative control).

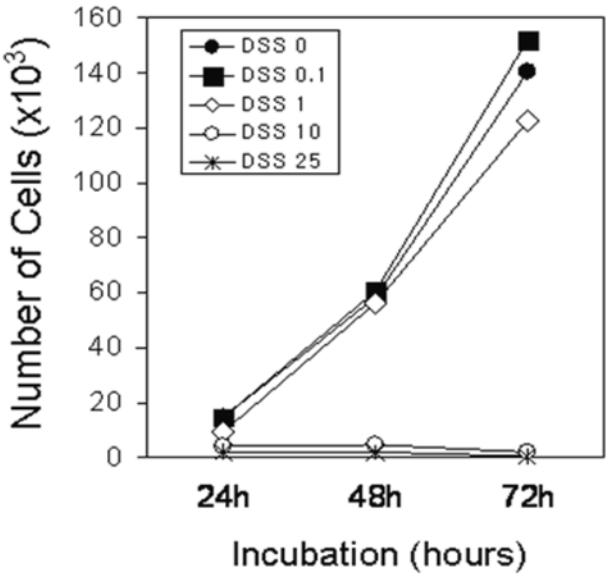

Fig. 2. Suppression of DSS on the viability in HT-29 cells. Cells were cultured with various concentration of DSS (0 - 25 µg/ml) at 37 °C for 24, 48, or 72 h under a humidified atmosphere of 5% CO_2. Cell viability was evaluated by counting after trypan blue staining. Values were obtained from three independent experiments.

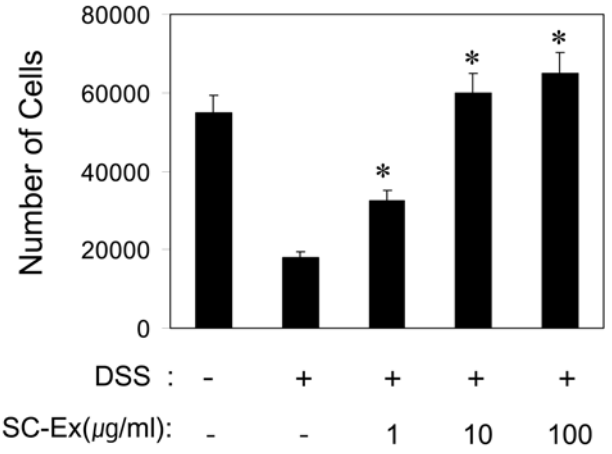

Fig. 3. The cytoprotective effect of SC-Ex in HT-29 cells stimulated with DSS. Cells were pretreated with SC-Ex for 20 min and stimulated with DSS (10 μg/ml) at 37 °C for 48 h. Cell viability was evaluated by counting after trypan blue staining. Values were obtained from three independent experiments and represented as the mean ± SD (*$p < 0.05$ vs. DSS alone).

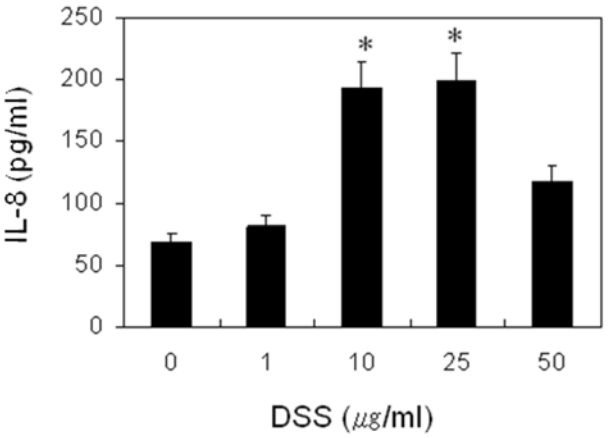

Fig. 4. IL-8 secretion in DSS-stimulated HT-29 cells. Cells were stimulated with DSS (1 - 50 μg/ml) for 16 h. IL-8 levels in the supernatants were analyzed by ELISA. Values were represented as the mean ± SD of three separate experiments (*$p < 0.05$ vs. negative control).

ml of DSS (Fig. 2). Thereby, 10 μg/ml of DSS was used for all experiments to examine the inhibitory effect of SC-Ex in HT-29 cells. Cells were pretreated with three different doses of SC-Ex (1, 10 and 100 μg/ml) for 20 min before stimulation with DSS. SC-Ex attenuated significantly DSS-induced inhibition of cell proliferation in a dose-dependent manner (Fig. 3). In particular, the 10 μg/ml SC-Ex resulted in extremely reduction of cell viability caused by DSS in HT-29 cells.

Effect of SC-Ex on DSS-Induced IL-8 production – We observed that DSS induced an increase in IL-8 secretion by HT29 cells and level of IL-8 secretion

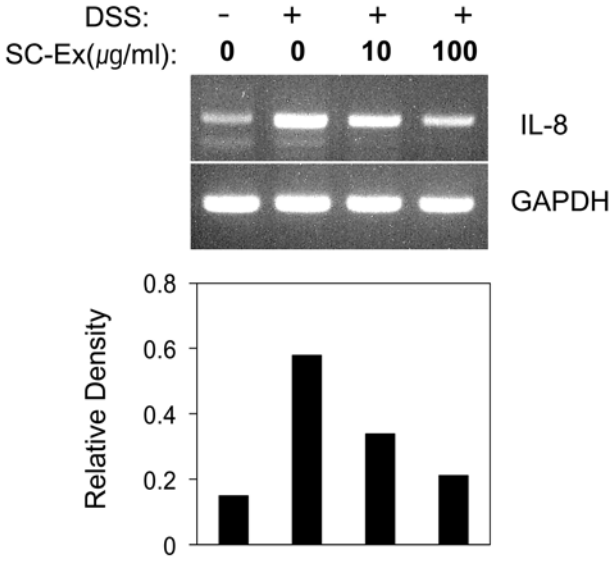

Fig. 5. Inhibitory effect of SC-Ex on DSS-induced expression of IL-8 mRNA inHT-29 cells. Cells were pretreated with SC-Ex (10 and 100 μg/ml) for 20 min and stimulated with DSS (10 μg/ml) for 8 h. Transcripts of IL-8 were amplified by PCR and GAPDH was used as an internal control. Values were represented as the relative density of IL-8 band against GAPDH.

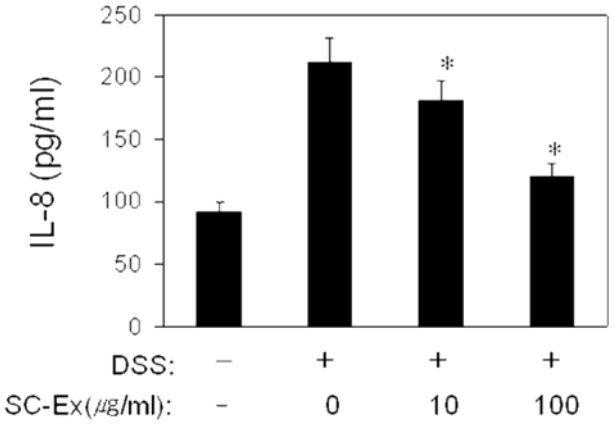

Fig. 6. Inhibitory effect of SC-Ex on DSS-induced IL-8 secretion from HT-29 cells. Cells were pretreated with SC-Ex for 20 min and than stimulated with DSS for 16 h. IL-8 levels in the supernatants were analyzed by ELISA. Values were represented as the mean ± SD of three separate experiments (*$p < 0.05$ vs. DSS alone).

reached a peak by 10 to 25 μg/ml (Fig. 4). To examine the inhibitory effect of SC-Ex on the expression of IL-8 mRNA and protein in DSS-stimulated HT-29 cells, we pretreated with two different doses of SC-Ex (10 and 100 μg/ml) for 20 min before DSS stimulation. SC-Ex inhibited remarkably the expression of IL-8 mRNA (Fig. 5). DSS-induced secretion of IL-8 protein also was significantly reduced by treated with SC-Ex (Fig. 6). High

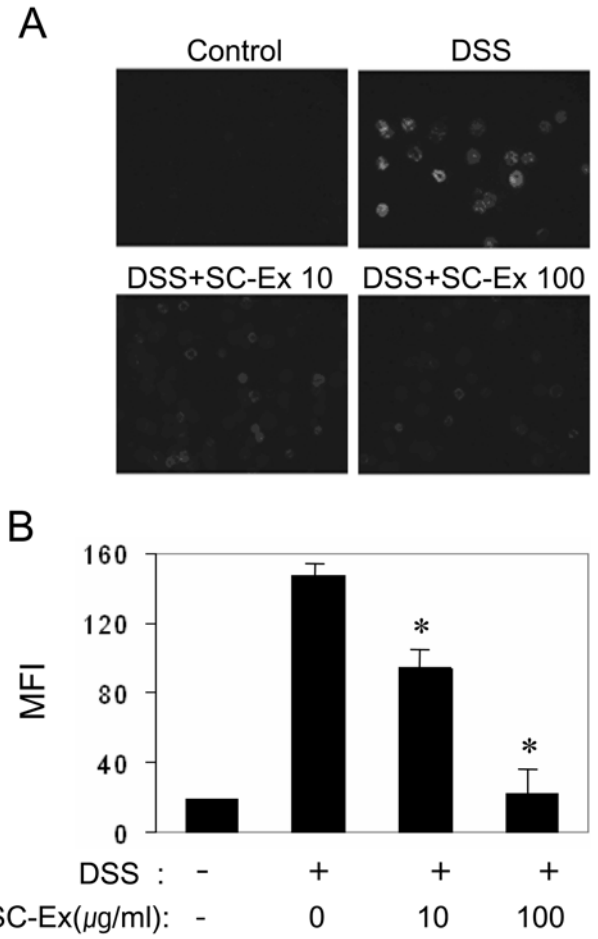

Fig. 7. Inhibitory effect of SC-Ex on DSS-induced ROS production by HT-29 cells. Cells were pretreated with SC-Ex for 20 min and than stimulated with DSS for 30 min. (A) ROS production was observed by fluorescence microscopy as described in the materials and methods. (B) Values were represented as the fluorescence density of ROS using fluorescence microplate reader (*$p < 0.05$ vs. DSS alone).

doses of SC-Ex (100 μg/ml) resulted in approximately 76% reduction of IL-8 secretion induced by DSS stimulation.

Effect of SC-Ex on DSS-Induced ROS production – To examine the inhibitory effect of SC-Ex on the DSS-induced ROS production by HT-29 cells, cells were pretreated with two different concentration of SC-Ex (10 and 100 μg/ml) for 20 min and then stimulated with DSS for 30 min. We observed that ROS production was markedly induced by DSS stimulation using fluorescence microscopy. In this condition, SC-Ex pretreatment reduced DSS-induced remarkably ROS producing cells and significantly MFI (mean fluorescence intensity) (Fig. 7). In particular, high concentration of SC-Ex (100 μg/ml) reduced approximately 95% of DSS-induced ROS production.

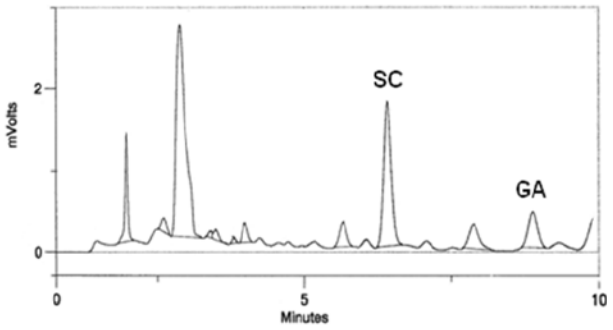

Fig. 8. Chromatogram of SC-Ex by HPLC. The contents of schizandin (SC) and gomisin A (GA) were analyzed as described in the materials and methods.

Discussion

IEC has been known to be a physical barrier which is the first line of defense against the aggressive gut milieu responding to many inflammatory stimuli. Most of genes expressing in the activated IEC have the potential to initiate and perpetuate inflammation. IEC therefore play an important role in intestinal inflammation. (Dotan *et al.*, 2002; McCormack *et al.*, 2001). In the present study, we examined the inhibitory effect of SC-Ex on DSS-induced activation of HT-29 cells. SC-Ex inhibited not only production of IL-8 and ROS in DSS-stimulated cells, but also cell damage.

In general, DSS induces the expression of an oxidative DNA damage biomarker, 8-oxo-7,8-dihydro-2-deoxyguanosine, in rat colonic mucosa (Tardieu *et al.*, 1998; Korkina *et al.*, 2003). The oxidative damage by cellular macromolecules is a common characteristic of apoptosis induced by a wide variety of stimuli (Tseng *et al.*, 2007). Recent studies has reported several evidences that ROS induce MAPKs activation in many cell types (de Bernardo *et al.*, 2004; Schweyer *et al.*, 2004). Most of phagocytic leukocytes, such as neutrophils, eosinophils, monocytes, and macrophages, have been reported to be the source of ROS in the mucosal interstitium (Moslen *et al.*, 1994). It is well documented that NADPH oxidase is the major enzyme for ROS generation (Park *et al.*, 2003). DSS-induced ROS generation may be necessary for p38 MAPK and NF-κB activation, and these signaling cascades are able to elevate the induction of proinflammatory genes, including IL-1β, TNF-α, IL-6, and IL-8, in a transcriptional manner (van den Blink *et al.*, 2001, Simmonds *et al.*, 1993). Although we did not show the functional mechanism on IL-8 secretion and ROS generation in DSS-stimulated cells, our results implicate that SC-Ex maybe inhibit IL-8 expression via

blockage of DSS-induced ROS generation by IEC.

Schizandra has been used in the Orient as an astringent. Recent studies have shown that SC comprises enriched dibenzocyclooctadiene lignans (Nakajima *et al.*, 1983). Schizandrin and gomisin A are major lignan compounds in SC (Liu, 1989). In this study, we determined the contents of schizandrin and gomisin A in SC-Ex using HPLC (Fig. 8). Contents of schizandrin and gomisin A revealed 1.17 ± 0.18 and 0.37 ± 0.06 mg/g, respectively. Although we have little direct information on the chemical nature of the active components of SC-Ex, some studies showed that the lignan-enriched SC has a myocardial protective effect in vivo and ex vivo models in rats and an activating effect on the hepatic cell degeneration in carbon tetrachloride-induced experimental liver injury models (Li *et al.*, 1996; Ko *et al.*, 1995). Lignan compounds also are well known as one primary group of phytoestrogen compounds in the plants, and some of them have cardiovascular activities (Ghisalberti *et al.*, 1997). The pharmacological and molecular approaches have been shown that the expression of proinflammatory genes could be inhibited by blockade of NF-κB and MAPK pathway in IEC (Shindo *et al.*, 2006; Parhar *et al.*, 2003). Therefore, it suggest that lignan compounds, such as schizandrin and gomisin A, may be the candidates to suppress DSS-induced activation of HT-29 cells associated with NF-κB and MAPK pathway.

In conclusion, we demonstrated that SC-Ex has the inhibitory effect on cell damage, IL-8 secreation, and ROS production by DSS-stimulated HT-29 cells. This study may provide a clue to the role of SC-Ex, which has long been used to prevent the intestinal diseases. Further work is necessary for the isolation and identification of the active ingredient(s) of SC-Ex directly regulating the cellular responses of IEC.

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