RESEARCH ARTICLE

Preventive Effects of *Spirogyra neglecta* and a Polysaccharide Extract against Dextran Sodium Sulfate Induced Colitis in Mice

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Abstract

Ulcerative colitis (UC) results from colonic epithelial barrier defects and impaired mucosal immune responses. In this study, we aimed to investigate the modifying effects of a *Spirogyra neglecta* extract (SNE), a polysaccharide extract (PE) and a chloroform fraction (CF) on dextran sodium sulfate (DSS)-induced colitis in mice and to determine the mechanisms. To induce colitis, ICR mice received 3% DSS in their drinking water for 7 days. Seven days preceding the DSS treatment, oral administration of SNE, PE and CF at doses of 50, 25 and 0.25 mg/kg body weight (low dose), 200, 100 and 1 mg/kg body weight (high dose) and vehicle was started and continued for 14 days. Histologic findings showed that DSS-induced damage of colonic epithelial structure and inflammation was attenuated in mice pre-treated with SNE, PE and CF. Furthermore, SNE and PE significantly protected colonic epithelial cells from DSS-induced cell cycle arrest, while SNE, PE and CF significantly diminished apoptosis. Proteome analysis demonstrated that SNE and PE might ameliorate DSS-induced colitis by inducing antioxidant enzymes, restoring impaired mitochondria function, and regulating inflammatory cytokines, proliferation and apoptosis. These results suggest that SNE and PE could prevent DSS-induced colitis in ICR mice by protection against and/or aiding recovery from damage to the colonic epithelium, reducing ROS and maintaining normal mitochondrial function and apoptosis.

Keywords: Colitis - dextran sodium sulfate - green alga - polysaccharide - proteome analysis - *Spirogyra neglecta*

Asian Pac J Cancer Prev, 17 (4), 2235-2245

Introduction

One of the inflammatory bowel diseases (IBD), ulcerative colitis (UC), is a chronic disorder of the intestinal tract which is known to be linked to progression of colorectal carcinogenesis (Itzkowitz and Yio, 2004). The pathogenesis of UC remains unclear, but many studies have reported that an imbalance between pro-inflammatory and anti-inflammatory cytokines plays a vital role (Ardizzone and Bianchi Porro, 2005). The dextran sodium sulfate (DSS) colitis mouse model is widely used to study the etiology of UC because many pathological symptoms observed with this model are similar to those of UC in man, such as body weight loss, diarrhea, bloody feces and colonic shortening (Baumgart and Sandborn, 2007). Histologically, DSS-induced colitis is characterized by erosion, ulceration, submucosal infiltration of inflammatory cells and crypt loss (Wirtz et al., 2007). It is commonly believed that DSS causes direct toxicity to gut epithelial cells and abrogates the integrity of the mucosal barrier, allowing lumen bacteria into the

lamina propria (Kitajima et al., 2000) and activating macrophages secreting pro-inflammatory cytokines such as TNF- α (Kaser et al., 2010).

Various kinds of drugs, include antibiotics, immunosuppressive agents and anti-inflammatory compounds, are used to cure UC, although they may have adverse side effects such as osteoporosis, neurotoxicity and gastrointestinal intolerance (Hendrickson et al., 2002). To overcome this problem, many scientists have attempted to use various natural products which could act as alternative approaches to therapy (Cho et al., 2011; Lee et al., 2013).

An extract of *Spirogyra neglecta* (SNE), a freshwater green alga found in the northern provinces of Thailand has been reported to exert various biological effects such as antioxidant (Thumvijit et al., 2013), antimutagenicity (Thumvijit et al., 2013), antihyperglycemia and antihyperlipidemia activities in rats with type 2 diabetes mellitus (Ontawong et al., 2013), as well as cancer chemopreventive activity with reference to development of diethylnitrosamine induced preneoplastic lesions in the

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rat liver (Thumvijit et al., 2014). Furthermore, not only different alga but also derivatives have been reported as bioactive agents (Wijesekara et al., 2011; Ngo and Kim, 2013). Importantly, the sulfated polysaccharides found in algae are known to exert antioxidant, anticoagulant, antiviral, anti-inflammatory and in vitro antitumor effects (Chattopadhyay et al., 2010; Shao et al., 2014). Furthermore, it has been reported that some herbal oligo- and polysaccharides could ameliorate chemically induced colitis (Yousef et al., 2012; Zhao et al., 2014).

As it has been found that *Spirogyra neglecta* mainly contains sulfated polysaccharides and chlorophyll (Thumvijit et al., 2014), we hypothesized that it could have preventive effects on DSS-induced colitis. To investigate modifying effects of SNE, polysaccharide (PE) and non-polysaccharide (CF) fractions on DSS-induced colitis in ICR mice and to elucidate mechanisms, we performed the present histopathological analysis, evaluating alteration in cellular proliferation and apoptosis, as well as identifying differentially expressed proteins and affected upstream regulators in the colon mucosa of mice after short-term administration of DSS and test agents.

Materials and Methods

Chemicals

3, 3'-diaminobenzidine tetrahydrochloride was obtained from Dojindo and dextran sodium sulfate (DSS) from ICN Biomedicals (Aurora, OH). An ApopTag® Peroxidase In Situ Apoptosis Detection Kit was purchased from Millipore and an iTRAQ Reagent 4 Plex Kit from AB Sciex. The rabbit monoclonal Ki-67 [SP6] antibody was the product of Abcam. VECTASTAIN Elite ABC was purchased from Vector Laboratories Ltd.

Preparation of extracts of *Spirogyra neglecta* and its derivatives

Dried *S. neglecta* was milled and then an extract was prepared with distilled water at 100°C with mechanical stirring for 2 hours. After the residue was removed by filtration the supernatant was collected, dried by lyophilizer and named the *S. neglecta* extract (SNE). Next, a polysaccharide extract, namely PE, was prepared according to Paradossi, et al (Paradossi et al., 1999) with some modification. Briefly, the SNE was dissolved in distilled water and the aqueous extract was decolorized with the solvent mixture of chloroform and methanol at the ratio 3:1. The chloroform part was collected and dried by evaporation. This extract was called the chloroform fraction (CF). The aqueous part was precipitated with absolute ethanol (1:4 v/v) overnight. The crude polysaccharide extract (PE) was collected by centrifugation and dried with a lyophilizer.

Animals

Male Crl: CD1 (ICR) mice were purchased from Charles River Japan (Tokyo, Japan) and housed five mice per cage. They received commercial MF diet (Oriental Yeast Co., Tokyo, Japan) and fresh tap water ad libitum. The experimental protocol was approved by the Animal Care and Use Committee of Osaka City

University Medical School. All experimental procedures were conducted under the Guidelines set by the National Institute of Health and Public Health Service Policy on the Humane Use and Care of Laboratory Animals. Mice were maintained in a room controlled at 23±1°C with relative humidity of 44±5% and 12 h light/dark cycle (8:00-20:00). All mice at 4 weeks of age were acclimatized for 1 week before starting the experiment.

Induction of colitis by DSS

Colitis was induced by the protocol previously reported by Kwon, et al with modification (Kwon et al., 2005) featuring drinking water containing 3% DSS (w/v) given ad libitum. The mice were randomly divided into 13 groups. In groups 3 through 10, they received 3% DSS in their drinking water for 7 days starting one week after the commencement. In control groups (groups 1 and 2), animals were treated with Milli-Q water (vehicle control group for DSS control, SNE and PE groups) or 1% DMSO (vehicle control for CF group) by intragastric gavage (i.g.) for 2 weeks, and received tap water instead of DSS. In DSS control groups (groups 3 and 4), mice were administered Milli-Q water or 1% DMSO i.g. for 7 days and co-treated with 3% DSS for another 7 days. Mice in groups 5 through 10 orally received SNE (50 and 200 mg/kg body weight (b.w.) and PE (25 and 100 mg/kg b.w.) in Milli-Q water or CF (0.25 and 1 mg/kg b.w.) in 1% DMSO according to the same protocol as for the DSS control groups. Animals in groups 11 through 13 were orally administered SNE (50 mg/kg b.w.), PE (25 mg/kg b.w.) in Milli-Q water and CF (0.25 mg/kg b.w.) in 1% DMSO for 14 days. The body weight of each mouse was recorded. The diet and water intakes were measured twice a week. At day 14, mice were sacrificed under isoflurane anesthesia and colons were immediately removed and processed for histological assessment and immunohistochemical examination or frozen for molecular analysis. The weights of internal organs including liver, kidneys and spleen were measured.

Histopathological examination

Portions of colon were fixed in 10% neutral formalin solution and embedded in paraffin for sectioning at 3 µm and staining with hematoxylin and eosin. Histologically, the damage to colon mucosa was assessed as described previously (Cooper et al., 1993). In brief, the pathophysiology of the colon was characterized by the presence of edema, infiltration of inflammatory cells into submucosa and crypt loss. Scores were given for grades as follows; a) edema (0: none; 1: mild; 2: moderate and 3: severe); b) inflammatory cell infiltration into submucosa (0: none; 1: mild; 2: moderate and 3: severe); c) crypt loss (0: none; 1: 1/3 loss; 2: 2/3 loss; 3: entire loss). The histological score for the pathophysiology of the colon was calculated as the sum of these three parameters, giving a maximum score of 9.

Immunohistochemical assessment of Ki-67 and apoptosis

Changes in cell proliferation and cell cycle arrest were analyzed by Ki-67 immunohistochemistry. From paraffin embedded samples, 3 µm sections were cut, deparaffinized and rehydrated. After antigen retrieval, the endogenous

peroxidase was blocked by incubation in 3% hydrogen peroxide solution. Then, samples were treated with normal serum for 15 min. Incubation of tissue sections with anti-Ki-67 antibodies (SP6, Abcam, USA, dilution 1:500) was at 4°C overnight. After primary antibody incubation and washing, the secondary antibody was added onto the tissue sections and incubated for 30 min at room temperature. The VECTASTAIN Elite ABC solution was incubated with the specimens for 30 min at room temperature. Antigen visualization was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB). At least 3000 mucosa cell nuclei in each colon part were counted and labeling indices were calculated as the percentages of cells positive for Ki-67.

Apoptosis was investigated using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining kit (Riezzo et al., 2014). Tissue sections were pre-treated with proteinase K and incubated with the working strength TdT enzyme, followed by working strength stop/wash buffer and then anti-digoxigenin peroxidase conjugate. Antigens were visualized with DAB peroxidation. Labeling indices were calculated from 12 fields per section of each colon (proximal middle and distal) part and expressed as percentages of positive cells for TUNEL in all examined colonic mucosa cells.

LC-MS/MS analysis

Pooled samples of mice colonic mucosa were prepared in 9M Urea/2% CHAPS and T-PER lysis buffers with protease inhibitor. The samples were homogenized and then centrifuged at 10,000 rpm for 10 min at 4°C. For 9M Urea/2% CHAPS tissue lysates acetone precipitation was performed and the protein concentration in each sample was determined using BCA Protein Assay Kit (Pierce, IL, USA). Each pooled sample contained 40 µg protein. Protein Reduction, Alkylation and iTRAQ labeling were performed using an iTRAQ Reagent 4 Plex Kit according to standard procedures (Gluckmann et al., 2007). Pooled samples were labeled as described below: iTRAQ isobaric reagents 114, normal colon mucosa of mice in the vehicle control group; 115, colon mucosa of mice in the DSS control group; 116, colon mucosa of mice in the DSS and SNE-treated group; 117, colon mucosa of mice in the DSS and PE-treated group. The iTRAQ labeled samples were combined and applied to ICAT cation exchange column and eluted as six fractions with 10, 50, 70, 100, 200 and 350 mM KCl followed by evaporation in a vacuum centrifuge. The peptides in each fraction were resuspended in 2% acetonitrile and desalted with a Sep-Pak C18 Plus light cartridge. After desalting, the peptides in each fraction were evaporated and dissolved in 0.1% (v/v) formic acid. Proteome analysis was performed with a DiNa-AI nano system coupled to a QSTAR Elite Hybrid mass spectrometer through a Nano Spray ion source. MS/MS data were searched against the Swiss Protein database (MOUSE) using ProteinPilot™ software (version 2.0, AB Sciex, Concord, ON, Canada) with trypsin set as the digestion enzyme and methyl methanethiosulfonate as the cysteine modification. The search results were further processed by ProteinPilot™ software using the Paragon

Algorithm for redundant hits removal and comparative quantitation, resulting in a minimal set of justifiable identified proteins. Each sample was measured 3 times and all reported data were used at the 95% confidence cut-off limit created by the ProteinPilot™ software. The same program was used to remove the bias for proteins expressed at very low levels. Protein ratios with a p-value less than 0.05 were considered reliable. Previously standard deviations of the protein ratio, which stem from technical variation, were reported to be less than 0.3 in 90% of iTRAQ experimental runs (Song et al., 2008). In this experiment, expression changes greater than 1.2-fold or less than 0.83-fold in normalized expression levels were considered to be outside the range of technical variability.

Ingenuity Pathway Analysis (IPA)

To assign biological significance to differentially labeled proteins, to identify function and networks of interacting proteins, functional groups and pathways, and to predict activated up-stream regulators by DSS, DSS and SNE or DSS and PE treatment groups, the Ingenuity program (Ingenuity Systems, Mountain View, CA) was utilized. Transcriptional regulation was measured by the z-score. The basis for z-score predictions are relationships in the molecular pathways (networks) which represent experimentally observed protein expression or transcription events. A z-score of above 2 or lower than -2 was considered significant.

Statistical analysis

The results are presented as mean value ± standard deviation (SD). Data were analyzed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan), and the significance of differences for each parameter (excluding general conditions) was analyzed and evaluated at the level of a) $p < 0.05$ vs vehicle control group or b) $p < 0.05$ vs DSS control group. Statistical comparisons of numerical data between the DSS control group and DSS and SNE or DSS and PE treatment groups were conducted using the Bartlett's test. If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (two-sided), and if not, with the Steel's test (two-sided). Statistical comparisons between Milli-Q and tap water control and DSS control groups for numerical data were assessed using the F test. If homogeneous, the data were analyzed with the Student's t-test (two-sided), and if not, with the Welch test.

Results

Symptomatic change

The trends for body weight decrease, which is one of the symptomatic parameters of colitis, were observed in all groups administered DSS at days 5, 6 and 7 after starting application (data not shown). No significant differences of final body weights were found in groups administered DSS and pre-treated with SNE, PE or CF, as compared to DSS controls (Table 1). Furthermore, no significant body weight changes were found in mice of vehicle control, SNE, PE and CF alone groups.

DSS treatment caused an increase in relative liver

weight when compared to control group, but without significance. Interestingly, a significant reduction and a tendency for decrease of relative liver weights were observed in PE and SNE-treated groups, respectively, at both low and high doses as compared to the DSS control group (Table 1). No significant changes in relative liver, kidney and spleen weights were detected in mice administered vehicles, SNE, PE and CF alone.

Histopathological changes in the colonic mucosa

The histological scores of pathological changes in colonic mucosa of mice are summarized in Table 2. A normal colonic architecture was observed in mice of vehicle control groups (Figure 1A and B). The colon of DSS-treated mice presented submucosal edema, infiltration of inflammatory cells to submucosa, loss of crypts, epithelial erosion and ulceration (Figure 1C and D).

Interestingly, treatment with SNE, PE and to a lesser extent CF prior to and in combination with DSS ameliorated those pathological changes in the colon (Figure 1E and F, 1G and H and 1I and J). Crypt structures were preserved and the numbers of infiltrated inflammatory cells were decreased, resulting in the low histological scores for colons of SNE, PE and CF-treated mice (Table 2).

Induction of cell cycle arrest and apoptosis by DSS and its prevention by SNE and PE

The percentage of Ki-67 positive cells tended to decrease in DSS-treated mice colons, indicating induction of cell cycle arrest due to significant damage to colon epithelial cells. However, administration of SNE and PE caused recovery of Ki-67 positive cell number up to the level observed in the vehicle control group (Table 2). No significant differences in Ki-67 positive cell indices were

Table 1. Final Body and Relative Liver, Kidneys and Spleen Weights of Mice Treated with DSS or Vehicles and Administered SNE, PE and CF Extracts at Low and High Doses

Treatment	No. of mice	Final body weight (g)	Relative organ weight (%)		
			Liver	Kidneys	Spleen
Tap water + Milli-Q	5	32.9±1.4	3.56±0.24	1.41±0.11	0.31±0.05
Tap water + 1% DMSO	5	32.6±1.7	3.70±0.26	1.25±0.11	0.30±0.03
3% DSS + Milli-Q	5	31.9±1.3	4.58±0.48	1.33±0.13	0.73±0.37
3% DSS + 1% DMSO	5	31.4±1.4	3.92±0.43	1.34±0.11	0.43±0.11
3% DSS + SNE 50 mg/kg b.w.	5	30.1±2.8	4.14±0.30	1.28±0.16	0.76±0.20
3% DSS + SNE 200 mg/kg b.w.	4	31.6±1.2	4.06±0.54	1.37±0.12	0.64±0.33
3% DSS + PE 25 mg/kg b.w.	5	30.5±1.5	3.90±0.28 ^b	1.50±0.16	0.63±0.25
3% DSS + PE 100 mg/kg b.w.	5	31.8±0.6	3.89±0.23 ^b	1.43±0.15	0.59±0.11
3% DSS + CF 0.25 mg/kg b.w. in 1% DMSO	5	32.3±1.4	3.85±0.29	1.37±0.15	0.65±0.23
3% DSS + CF 1 mg/kg b.w. in 1% DMSO	5	31.1±1.4	3.77±0.48	1.36±0.07	0.42±0.17
Tap water + SNE 50 mg/kg b.w.	4	32.4±0.9	3.55±0.17	1.39±0.09	0.28±0.05
Tap water + PE 25 mg/kg b.w.	5	31.0±2.1	3.72±0.44	1.39±0.05	0.33±0.20
Tap water + CF 0.25 mg/kg b.w. in 1% DMSO	4	32.4±0.8	3.54±0.20	1.31±0.06	0.26±0.04

Data are mean ± SD. ^bp<0.05 v.s. the DSS+ Milli-Q control group.

Table 2. Histological Scores, and Ki-67 and TUNEL Indices in the Colons of Mice Treated with DSS or Vehicles and Administered SNE, PE and CF Extracts at low and High Doses

Treatment	Histological score	Ki-67 positive cell index (%)	TUNEL positive cell index (%)
Tap water + Milli-Q	0.0±0.0	33.4±3.7	0.5±0.1
Tap water + 1% DMSO	0.0±0.0	28.9±2.9	0.4±0.1
3% DSS + Milli-Q	3.9±2.1 ^a	19.3±4.3	2.2±0.7 ^a
3% DSS + 1% DMSO	4.2±1.2 ^a	23.3±6.1	2.0±0.3 ^a
3% DSS + SNE 50 mg/kg b.w.	3.5±0.7	41.2±5.9 ^b	1.4±0.2 ^b
3% DSS + SNE 200 mg/kg b.w.	3.0±1.7	33.2±4.8 ^b	1.1±0.5 ^b
3% DSS + PE 25 mg/kg b.w.	2.8±0.9	35.4±8.1 ^b	0.9±0.3 ^b
3% DSS + PE 100 mg/kg b.w.	3.4±0.8	36.5±9.0 ^b	1.3±0.5 ^b
3% DSS + CF 0.25 mg/kg b.w. in 1% DMSO	3.4±1.0	29.5±4.8	1.2±0.3 ^b
3% DSS + CF 1 mg/kg b.w. in 1% DMSO	3.4±1.0	23.9±6.0	1.3±0.3 ^b
Tap water + SNE 50 mg/kg b.w.	0.0±0.0	26.8±0.7 ^a	0.6±0.0
Tap water + PE 25 mg/kg b.w.	0.0±0.0	28.7±2.1 ^a	0.7±0.1
Tap water + CF 0.25 mg/kg b.w. in 1% DMSO	0.0±0.0	24.9±5.3	0.7±0.2

Data are mean ± SD; ^ap<0.05 v.s. Tap water + Milli-Q or Tap water + 1% DMSO control group; ^bp<0.05 versus the DSS+ Milli-Q or DSS+1% DMSO control group.

found in CF and DSS-administered mice as compared to the concomitant DSS control group.

The number of apoptotic cells was low in the vehicle control groups but significantly increased in DSS-administered animals. Similarly to observed alterations of Ki-67, SNE, PE and CF application prior to DSS application led to reduction of apoptosis positive cell indices becoming close to the level observed in the vehicle control group (Table 2).

Alteration of protein expression in mouse colons and IPA analysis

In this study, we investigated alterations of protein expression in the colon mucosa which might explain the mechanisms of DSS-induced colitis in mice and further

explain the preventive effects of SNE and PE. The results of QSTAR Elite LC-MS/MS and IPA are summarized in Table 3. A total of 49 differentially expressed proteins were identified in 3 sample pairs for comparison: a) DSS control and vehicle control; b) SNE→DSS and vehicle control; and c) PE→DSS-treated and vehicle control colon mucosa. It was observed that differentially expressed proteins in mice treated with DSS were involved in xenobiotic metabolism, oxidation-reduction process, glutathione and lipid metabolism, mitochondrial function, calcium metabolism, transcription, protein synthesis, protein peroxisome proliferation, cytoskeleton organization, cell proliferation and apoptosis processes.

According to the IPA upstream regulator analysis, after 7 days on DSS, in colons of mice proteins regulated by c-Myc, NMYC, TNF- α and transcriptional factor X-box-binding protein 1 (XBP1) associated with cell proliferation and angiogenesis were up-regulated but those promoting apoptosis, such as a tumor suppressor gene cellular tumor antigen p53 (p53), were inhibited, which could be the adaptive response to the severe cellular damage, cell cycle arrest and cell death induced by DSS (Table 4).

Interestingly, IPA indicated that pretreatment of mice with SNE and PE protected against activation of up-stream regulators TNF- α , nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1), heat shock transcription factor 2 (HSF2), XBP1 and pancreatic and duodenal homeobox 1 (PDX1), and suppressed activity of several genes and cell kinases involved in cell proliferation. Those included SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), cAMP responsive element binding protein 1 (CREB1), erb-b2 receptor tyrosine kinase 4 (ERBB4), prohibitin 2 (PHB2), insulin-like growth factor 1 receptor (IGF1R), presenilin 2 (PSEN2), Raf kinase 1 (RAF1), mitogen-activated protein kinases (MAPK) p38 MAPK, ERK1/2 and MAP2K1, peroxisome proliferator-activated receptors alpha (PPARA), gamma, coactivator 1 alpha (PPARGC1A) and beta (PPARGC1B) (Tables 3 and 4). Furthermore, DSS suppressed the expression of keratins 8 (KRT8) and 18 (KRT18) in the colons of mice. We also found that SNE and PE ameliorated down-regulation of proteins involved in cytoskeleton organization including tropomyosin 1, alpha (Tpm 1) and tropomyosin 2, beta (Tpm 2) and inhibited expression of cytokeratin 19 (CK19) (Table 3).

Importantly, it was observed that DSS treatment induced activation of nuclear receptor subfamily 1, group I, member 2 (PXR) which could result in generation of reactive oxygen species (ROS) in the colon mucosa (Table 4). Again, SNE and PE blocked this activation, in coordination with preventive effects on cell proliferation. Furthermore, PE suppressed up-regulation of proteins such as cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) involved in generation of oxidative stress (Table 3). In addition, in proteome analysis, down-regulation of cytochrome c oxidase subunit Va (COX5A) and glutathione reductase (GSR) enzymes participating in maintenance of normal mitochondrial function and antioxidant response was observed in DSS-induced colitis as compared to vehicle controls (Table 3). However, PE

Figure 1. Histological Changes in Colonic Mucosa in Mice with DSS-induced Colitis and Modifying Effects of SNE, PE and CF. (A–J) Representative microphotographs of HE stained mice colons from Milli-Q control (A), DMSO control (B), DSS control (C), DSS-DMSO control (D), DSS-SNE (50 mg/kg b.w.) (E), DSS-SNE (200 mg/kg b.w.) (F), DSS-PE (25 mg/kg b.w.) (G), DSS-PE (100 mg/kg b.w.) (H), DSS-CF (0.25 mg/kg b.w.) (I), and DSS-CF (1 mg/kg b.w.) (J) groups

Table 3. Differentially Expressed Proteins in the Colon Mucosa of Mice Treated with DSS Alone or in Combination with SNE and PE, Identified by QSTAR Elite LC-MS/MS and IPA

Protein Name (Symbol)	GI Number	DSS vs vehicle		DSS SNE vs vehicle		DSS PE vs vehicle		Location	Type	Function	Up-stream regulator
		FC	p-value	FC	P-value	FC	p-value				
carboxylesterase 2A (Ces2a)	19527178	NC		NC		-1.43	0.022	C	E	M	NR1/2, NR1/3
carboxylesterase 2C (Ces2c)	21704206	-1.17	0.0008	-1.27	0	-1.62	0	C	E	M	NR1/2, NR1/3
aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)	85861182	-1.32	0	NC		-1.73	0	C	E	M	N1/2, N1/3
adenosylhomocysteinase (AHCY)	262263372	-1.09	0.047	-1.19	0.0021	-1.22	0.034	C	E	M	N1/2
cytochrome P450, fam. 2, subfam. C, polypep. 9 (CYP2C9)	268607516	1.2	0.031	1.09	0.0001	-1.2	0.022	C	E	XM, OR	N1/2, N1/3, PPARGC1A
cytochrome P450, fam. 2, subfam. C, polypep. 18 (CYP2C18)	13386282	-1.1	0.0005	NC		-2.12	0	C	E	XM, OR	
glutathione S-transferase, alpha 4 (Gsta4)	160298217	1.15	0.048	NC		-1.24	0.0013	O	E	GM	N1/2, PPARA
glutathione S-transferase mu 1 (GSTM1)	6680121	-1.36	0.0009	-1.67	0.0003	-1.63	0	C	E	GM	N1/2, N1/3, TP53
glutathione S-transferase mu 5 (GSTM5)	6754084	-1.95	0	-2.1	0	-2.4	0	C	E	GM	N1/2, SRF, TP53
sulfotransferase family 1A, phenol-pref., member 1 (Sult1a1)	19526822	NC		-1.36	0.0007	-2.24	0.002	C	E	XM	N1/3
3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2)	61098088	1.14	0.0013	1.22	0.0012	NC		C	E	SM	N1/3
calnexin (CANX)	6671664	1.37	0	1.42	0.0007	1.38	0.0004	C	O	CaM	c-MYC
cytochrome c oxidase subunit Va (COX5A)	112181182	-1.54	0	-1.32	0	-1.34	0.0002	C	E	MitF	
glutathione reductase (GSR)	160298213	-1.85	0	-1.62	0	NC		C	E	MitF	
prohibitin 2 (PHB2)	126723336	NC		-1.18	0.022	-1.45	0.02	C	TR	TRA	c-MYC
nucleophosmin (NPM1)	6679108	1.31	0.001	2.09	0	1.95	0	N	TR	TRA	c-MYC, MYCN, SRF
nucleolin (NCL)	84875537	1.22	0	1.37	0	1.5	0	N	O	CP	c-MYC, MYCN, ERBB4
ribosomal protein L10a (RPL10A)	255003735	2.44	0	2.84	0	2.94	0	N	SCR	PS	c-MYC, MYCN
ribosomal protein L13 (RPL13)	33186863	2.19	0.0022	2.58	0.0043	2.45	0.0076	N	SCR	PS	c-MYC, MYCN
ribosomal protein L14 (RPL14)	13385472	1.95	0	2.79	0	2.91	0	C	SCR	PS	c-MYC, MYCN

Table 3 (continued). Differentially Expressed Proteins in the Colon Mucosa of Mice Treated with DSS Alone or in Combination with SNE and PE, Identified by QSTAR Elite LC-MS/MS and IPA

ribosomal protein L24 (RPL24)	18250296	1.84	0.0007	2.42	0.0001	2.58	0.0001	C	SCR	PS, AP	c-MYC, MYCN
ribosomal protein L28 (RPL28)	6677779	2.41	0.0028	3.09	0.0024	3.2	0.0012	C	SCR	PS	c-MYC, MYCN
ribosomal protein L32 (Rpl32)	25742730	2.2	0.0018	2.2	0.0012	2.55	0.0016	C	SCR	PS	c-MYC, MYCN
ribosomal protein L4 (RPL4)	30794450	2.13	0	2.29	0	2.24	0	C	E	PS	c-MYC, MYCN
ribosomal protein L6 (RPL6)	84662736	2.46	0	2.67	0	2.56	0	N	SCR	PS	c-MYC, MYCN
ribosomal protein L8 (RPL8)	6755358	2	0.003	2.11	0.0039	2.07	0.0076	O	SCR	PS, PM	c-MYC, MYCN
ribosomal protein S24 (RPS24)	46519158	2.21	0.0005	2.35	0.0004	2.41	0.0004	C	SCR	PS	c-MYC, MYCN
ribosomal protein S3A1 (Rps3a1)	254553321	2.07	0	2.3	0	2.6	0	C	SCR	PS	c-MYC, MYCN
ribosomal protein S6, pseudogene 4 (Rps6-ps4)	94367038	3.09	0.0006	3.82	0.0044	4.23	0.0027	O	SCR	PS	c-MYC, MYCN
S100 calcium binding protein A10 (S100A10)	6677833	1.45	0.013	1.78	0.0009	1.37	0.0013	C	O	CaM	c-MYC, MYCN
eukaryotic translation elongation factor 1 gamma (EEF1G)	110625979	1.47	0	1.6	0	1.57	0	C	TR	TRA	MYCN
peroxiredoxin 6 (PRDX6)	6671549	-1.42	0	-2.11	0	-1.93	0	C	E	AP	TP53
4-aminobutyrate aminotransferase (ABAT)	37202121	-1.64	0.0011	-1.86	0.0001	-2.24	0.0003	C	E	AP	TP53
calreticulin (CALR)	6680836	1.16	0	1.41	0.0003	1.51	0.0003	C	TR	TRA	TNF, XBP1
actinin, alpha 1 (ACTN1)	61097906	-1.13	0.011	-1.28	0.0006	-1.41	0	C	TR	TRA	TP53, c-MYC
actin, alpha 2, smooth muscle, aorta (ACTA2)	6671507	-1.48	0.0001	-1.56	0.0004	-2.67	0	C	CS	CO	TP53, SP1, ERBB4
transgelin (TAGLN)	6755714	-1.98	0	-2.13	0	-2.59	0	C	CS	CO	c-MYC, MYCN
transgelin 2 (TAGLN2)	30519911	-1.19	0.0098	-1.19	0.0009	-1.44	0	C	CS	CO	c-MYC, MYCN, TP53
tropomyosin 1, alpha (Tpm1)	31560030	-2.84	0	-2.18	0	-2.23	0	PM	CS	CO	c-MYC, SRF
tropomyosin 2, beta (Tpm2)	482677666	-3.02	0	-2.65	0	-2.15	0	C	CS	CO	c-MYC, RAF, SRF
vimentin (VIM)	31982755	-1.22	0	-1.61	0	-1.23	0.0002	C	CS	CO	c-MYC, MYCN, TP53
keratin 8, type II (KRT8)	114145561	-1.73	0	-1.23	0	-1.64	0	C	CS	CO, AP	TP53, PPARA
keratin 18, type I (KRT18)	254540068	-2.06	0.0001	-1.93	0	-2.23	0	C	CS	CO	TP53, SP1, SMARCA4
keratin 19, type I (KRT19)	6680606	NC		-1.32	0	-1.84	0	C	CS	CO	OSM, IGFR1, SP1
myosin, light chain 9, regulatory (MYL9)	198278553	-2.59	0.0003	-2.54	0.0001	-2.67	0.0017	C	CS	CO	c-MYC
myosin, heavy chain 11, smooth muscle (MYH11)	241982718	-1.42	0	-1.52	0	-2.17	0	C	CS	CO	SRF

Table 3 (continued). Differentially Expressed Proteins in the Colon Mucosa of Mice Treated with DSS Alone or in Combination with SNE and PE, Identified by QSTAR Elite LC-MS/MS and IPA

anterior gradient 2 (AGR2)	6753010	-1.52	0	-1.61	0	-1.91	0	EM	O	PP	SMARCA4, ERBB2
acetyl-CoA acyltransferase 2 (ACAA2)	29126205	-1.21	0.0012	-1.15	0.022	-1.53	0	C	E	PP, LM	PPARA, PPARGC1A
acyl-CoA dehydrogenase, C-4 to C-12 str. chain (ACADM)	6680618	NC		-1.14	0.026	-1.69	0.01	C	E	PP, LM	PPARA, PPARGC1A

1.C: cytoplasm; ES: extracellular space; EPR: endoplasmic reticulum; G: Golgi apparatus; Mi: mitochondria; N: nucleus; P: peroxisome; PM: plasma membrane; 2. E: enzyme; IC, ion channel; K: kinase; Pe: peptidase; Ph: phosphatase; SCR: structural constituent of ribosome; T: transporter; TR: transcriptional regulator; O: other; 3. AP: apoptotic process; CaM: calcium metabolism; CM: cellular migration; CO: cytoskeleton organization; CP: cell proliferation; GM: glutathione metabolism; LM: lipid metabolism; M: metabolism; PP: protein peroxisome proliferation; PS: protein synthesis; OR: oxidation-reduction process; SM: sulfur metabolism; TRA: transcription; XM: xenobiotic metabolism; Ratio: ratio to vehicle control group; NC: no change

Table 4. Up-stream Regulators Affected in DSS Control, SNE and PE Pre-Treated Mice Detected by IPA

Up-stream regulators	3% DSS vs vehicle	3% DSS + SNE vs vehicle	3% DSS + PE vs vehicle	Function
NR1/2 (PXR)	2	-0.05	-0.66	XMS, OR
NR1/3 (CAR)	-0.24	-0.54	-2.04	XMS, OR
PPARA	-1.76	-1.48	-2.33	PP
PPARGC1A	-3.03	-3.25	-4.17	PP
PPARGC1B	-1.46	-2.59	-1.63	PP
MYC (c-Myc)	1.7	2.28	1.78	P
MYCN	4.87	4.54	4.82	P
ERBB4	2.4	1.71	1.18	P
TNF- α	2	1.49	-0.14	P, A
NR3C1	2	NC	NC	P
HSF2	2.24	0.76	NC	P
XBP1	2.59	-0.79	3.34	P
PDX1	2.14	1.09	0.71	P
SMARCA4	-1.79	-2.18	-2.54	CCP
CREB1	NC	-2.71	0.4	P
IGF1R	-1.72	-3.5	-1.99	P
P38 MAPK	NC	-2.22	NC	P
ERK1/2	NC	-2.17	NC	P
MAP2K1	NC	-2.2	NC	P
RAF1	NC	-2	-2	P
PRKG1	NC	-2	NC	P
PSEN2	NC	-2	NC	P
TP53	-3.02	-3.25	-2.88	A
SP1	-2.03	-2.13	-1.2	A, TRA
SRF	-2.34	-2.78	-2.68	ACO

NC: no change; A: apoptosis; ACO: actin cytoskeleton organization; CCP: cell cycle progression; D: differentiation; OR: oxidation-reduction process; P: proliferation; PP: peroxisome proliferation; TRA: transcription; XMS: xenobiotic metabolism signaling; z-score: <-2.0 regulator significantly inhibited; >2.0 regulator significantly activated

greatly and to the less extent SNE, ameliorated these effects, with expected lower levels of ROS generation in mouse colons.

Discussion

The present study demonstrated a range of changes

in clinical symptoms, colonic mucosa histology and pathobiology, cell proliferation, apoptosis and protein expression dependent on the SNE, PE and CF exposure in mice with DSS-induced colitis. The trends for decrease of body weights in DSS-treated mice were observed after 5-7 days of its application. Previously, body weight loss was reported as one of the clinical symptoms of DSS-induced colitis (Baumgart and Sandborn, 2007). On the other hand, DSS-induced significant increase of mouse liver weight, with SNE, PE and CF ameliorating this effect. It is likely that the liver is the main target organ for the extracts because it is the major site of their xenobiotic metabolism (Sturgill and Lambert, 1997).

At the microscopic level, DSS-induced colitis exhibits histological features characterized by edema, ulcer, erosion, inflammatory cell infiltration and crypt loss (Cooper et al., 1993), which were all detected in our study. From our results, SNE, PE, and to a lesser extent CF, caused reduction in the damaged histological appearance of colon mucosa architecture induced by DSS treatment. After injury, wound healing of colonic mucosa consists of several steps including regeneration of crypts through epithelial proliferation and compensation of epithelial monolayer through epithelial cell migration (Tessner et al., 1998). In addition, apoptosis has been reported to be involved in colonic epithelial cell injury (Iwamoto et al., 1996). In this study, DSS administration resulted in the cell cycle arrest and elevated cell death in the colon epithelium. Our results are consistent with those of the previous study report that DSS induces apoptosis and leads to cell cycle arrest in colonic epithelial cells (Araki et al., 2010). These events might further result in destruction of the epithelium barrier, incorporation of intestinal bacteria into the mucosal layer and induction of inflammation (Hans et al., 2000). Importantly, administration of SNE and PE significantly protected colonic epithelium against cell cycle arrest and apoptosis, indicating that they may have suppressive effect on DSS induced colitis by preventing damage to the colonic mucosa.

Next, we focused on protein expression changes which could be related to the preventive effects of SNE and PE on DSS-induced colitis in mice. The pathogenesis of UC is multifactorial and many aspects remain unclear. Several studies reported that mitochondrial dysfunction may play an important role in the pathogenesis of UC

(Hsieh et al., 2006; Sifroni et al., 2010). Dysfunction of mitochondria usually leads to reduction in energy supply, increased ROS and induction of apoptotic cell death (Hsieh et al., 2006). The novel finding of the present study was that DSS treatment induced activation of PXR and up-regulated its downstream protein CYP2C9, thus promoting undesirable ROS generation if enzyme induction were not to be reversed (Chen et al., 2004). From IPA analysis, in the colon mucosa of mice with DSS-induced colitis, numerous biological functions, especially those related to the quantity and generation of ROS were altered. We further found that application of SNE and PE ameliorated this effect of DSS. Moreover, proteome analysis indicated that DSS application led to down-regulation of COX5A, which is a subunit of cytochrome c oxidase (complex IV). Previously, Sifroni et al. reported activities of mitochondrial respiratory chain enzymes such as complex II-IV to be decreased in UC patients (Sifroni et al., 2010). Moreover, there are several studies providing evidence of ROS production in UC patients (Nishikawa et al., 2005; Rana et al., 2014) and in chemical-induced colitis using animal models (Damiani et al., 2007). When over-production of ROS occurs in cells, generation of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and increase of levels of glutathione (GSH) are necessary for removal of ROS (Medhi et al., 2008). In DSS-induced colitis, we found down-regulation of GSR, which is related to maintenance of GSH levels. Therefore, it was suggested that down-regulation of COX5A and GSR might impair mitochondria function and lead to increased ROS production during DSS-induced colitis in mice. This is supported by the fact that expression of GSR, and to the lesser extent COX5A, recovered in mice supplemented with SNE and PE, which could be related to improvement of mitochondria function and antioxidant effects.

Abnormalities of mucosal structure or the epithelial barrier are associated with pathological UC. Keratin 8 (KRT8) and keratin 18 (KRT18) are the major cytoskeletal intermediate filaments (IFs) in the intestinal epithelia (Ding et al., 2010). KRT8 is the major type II keratin in the small and large intestine, along with type I keratins KRT18, 19, or 20 dependent on the cell and individual tissue. KRT8 has functional roles in maintenance of normal epithelial architecture (Coulombe and Omary, 2002). Here we observed down-regulation of both KRT8 and KRT18 in mouse colonic mucosa induced by DSS, perhaps as a reflexion of severe damage to the epithelium. Several studies have indicated that KRT8-null mice suffer chronic inflammation (Baribault et al., 1994; Habtezion et al., 2005). Thus, mutation of KRT8 in humans also leads to epithelial instability in the gut and may play a role in IBD (Owens et al., 2004). Furthermore, Treton et al. described changes in protein translation that altered colonic epithelial barrier function involving KRT8, from the results of genome-wide microarray analysis of polysome-bound messenger RNA from UC patients (Treton et al., 2011).

In addition, colon mucosa of mice with DSS-induced colitis may be characterized by lower levels of microfilament-associated proteins tropomyosin 1 and 2

(Tpm1 and Tpm2), which are present in all eukaryotic cells with organ-specific isoforms having distinct functions (Lin et al., 1997). However, this downregulation of Tpm1 and Tpm2 induced by DSS was also ameliorated in mice administered SNE and PE. It has further been reported that in trinitrobenzenesulphonic acid (TNBS)-induced rat colitis, the Tpm1 gene is down-regulated on day 7 of TNBS treatment (Martinez-Augustin et al., 2008). Therefore, down-regulation of cytoskeleton proteins such as KRTs and Tpm1s might impair the colonic epithelial structure, allowing bacteria to enter into the lamina propria and trigger mucosa inflammation, as observed in UC. Therefore, maintenance of colonic epithelial structure by SNE and PE clearly might play a role in protection against the strong inflammation which occurs with DSS-induced colitis.

Several reports exist of associations between chronic inflammation and colorectal cancer (Terzic et al., 2010; Rubin et al., 2012). Cytokines that encourage colitis-associated tumor development include TNF, IL-6, and IL-1 while many others were found to be unregulated in these tumor types (Rubin et al., 2012). TNF- α is involved in promotion and progression of experimental and human cancers (Balkwill, 2006). In our IPA upstream regulator analysis, TNF- α was found to be activated in DSS-treated mice. However, amelioration of its activity was observed with SNE and PE, which could be one of the mechanisms of their suppression of inflammation.

Moreover, IPA indicated that oncogenes c-MYC and NMYC were activated but p53 was suppressed in the colonic mucosa of DSS-treated mice. The c-MYC transcription factor is one of the most powerful and frequently deregulated oncoproteins in human cancers. Overexpression of c-MYC has been described in nearly 70% of colorectal cancers associated with dysplasia of colonocytes in ulcerative colitis (Chu et al., 2007). c-MYC modulates many biological activities including cell proliferation, cell growth and cell transformation. Deregulation observed in the present study could have promoted apoptosis and angiogenesis in line with previous results (Ponzielli et al., 2005). In addition, from our results, the p53 pathway, another important molecular checkpoint, is likely to be suppressed in response to the severe damage to colonic epithelium induced by DSS treatment. In the present model the mechanism of DSS-induced cell death and apoptosis appeared to be independent of p53 activation. In previous studies, an absence or low occurrence of p53 mutations were found during chemically-induced colon tumorigenesis in rodents (De Robertis et al., 2011).

Here we found that administration of SNE or PE prior to DSS impacted on numerous regulators of cell proliferation including NR3C1, HSF2, XBP1, PDX1 and suppressed activity of SMARCA4, CREB1, ERBB4, PHB2, IGF1R, PSEN2, RAF1 and MAP kinases such as p38 MAPK, ERK1/2 and MAP2K1, which could explain their effects on cell proliferation in colonic mucosa. Altogether, the results of proteome and IPA analyses imply that DSS-induced colitis in mice might be associated with colorectal carcinogenesis by regulating inflammatory cytokines, proliferation and apoptosis. Amelioration of

Figure 2. Proposed Mechanisms of DSS-induced Colitis in Mice (A) and the Modifying Effects of SNE and PE (B)

DSS effects on mouse colonic mucosa by pretreatment with SNE and PE could result in prevention of colorectal tumorigenesis.

In conclusion, our results indicated that SNE and PE exert protective effects against DSS-induced colitis in mice (as illustrated in Figure 2). These effects seem to be related to protection of colonic epithelium from DSS-induced cell cycle arrest and apoptosis, by maintaining normal mitochondrial function and elevating antioxidant levels, finally resulting in improvement of the colonic epithelial architecture.

Acknowledgements

This study was supported by a grant for a fellowship from the Japanese Association of University Women (JAUW). We are grateful to Rie Onodera, Yuko Hisabayashi and Keiko Sakata for their technical assistance and to Yukiko Iura for her help during preparation of this manuscript.

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