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Efficacy of ginsenoside treatment to alleviate anticancer-drug induced mucoenteritis in mice

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Abstract. 5-fluorouracil (5-FU), which is widely used as an anticancer drug, causes enteritis due to disruption of mucosal tissue, resulting in diarrhoea and body weight loss. Ginsenoside Rd (GRd) and ginsenoside Re (GRe) are two of the saponins contained in carrots. Their anti-inflammatory effects on the digestive system have been reported, but their effects on small intestinal mucositis have not been studied much. This study investigated the effect of GRd and GRe on 5-FU-induced mucoenteritis. GRd and GRe did not affect weight loss or exacerbation of diarrhoea due to 5-FU administration in mice. Histopathological observations showed that 5-FU reduced the shortening of intestinal villi and the number of crypts and cells compared to the vehicle group. However, co-administration of GRd and GRe, significantly suppressed the decrease in the number of small intestinal crypts and cells in the crypt by 5-FU treatment. It indicates that 5-FU-induced enteritis is attenuated by the co-administration of GRd and GRe. The expression of NF-κB was increased by 5-FU administration and this increase was clearly suppressed in the group that was co-administered GRd. Although the anti-inflammatory effects of GRd and GRe have been reported based on various mechanisms in many models, the mechanism of their enteritis-suppressing action in anticancer drug-induced enteritis has not been clarified. It was reported that the anti-inflammatory effect of GRd was due to the suppression of NF-κB expression; we concluded that NF-κB may be one of the molecules involved in the suppression of 5-FU-incuced enteritis.

Keywords: 5-fluorouracil, anticancer drug, Ginsenoside, mucositis, enteritis, NF-κB

Introduction

5-Fluorouracil (5-FU) is the most widely used antimetabolite anticancer drug in cancer chemotherapy. Its main mechanism of action is to exert antitumor effects by inhibiting thymidylate synthase (TS) and disrupting the intracellular deoxynucleotide pool required for DNA replication. In addition, it is said to act on RNA uptake, inhibition of RNA synthesis thereafter and fragmentation by uptake into DNA (Vodenkova et al. 2020). 5-FU is widely indicated for gastric cancer, colorectal cancer, breast cancer and pancreatic cancer. In fact, according to the National Database of Health Insurance Claims and Specific Health Checkup of Japan (NDB) in FY2018, the number of prescriptions for 5-FU treatment was 1,273,300, including the forerunner and generic products, making it the most prescribed drug among antimetabolites, and its sales reached 672,860,362 JPY.

However, 5-FU acts not only on cancer cells but also on normal cells with high growth rates, such as intestinal mucosal epithelial cells, hair root cells and blood cells. As such, patients treated with 5-FU have side effects such as stomatitis, leukopenia, thrombocytopenia and intestinal mucositis, with intestinal mucositis occurring in 50–80% of patients (Benson et al. 2004). Intestinal mucositis is problematic because it causes vomiting, abdominal distension and diarrhoea, which interferes with treatment. Indeed, dose reduction and discontinuation of treatment is often required, as well as increases in medical expenses due to extended hospital stays (Dranitsaris et al. 2005).

Anticancer drugs can cause early- or late-onset diarrhoea. Early-onset diarrhoea occurs within hours of drug administration through stimulation of parasympathetic nerves with topoisomerase inhibitors such as irinotecan and activating intestinal peristalsis. Late-onset diarrhoea occurs about one week after drug administration due to intestinal mucosal damage caused by 5-FU as well as other chemotherapeutic agents such as doxorubicin, cisplatin, etc. In addition, diarrhoea caused by immune checkpoint inhibitors such as nivolumab and pembrolizumab, which have been attracting attention recently, occurs about 2 weeks after drug administration, but the mechanism of onset remains unclear.

Although the molecular mechanism of 5-FU-induced intestinal mucositis has not been clarified yet, it has been reported that apoptosis is involved, in addition to inhibition of cell proliferation, resulting in shortening of small intestinal villi and destruction of intestinal crypts (Benson et al. 2004; Duncan, Grant 2003; Hamouda et al. 2017; Inomata et al. 2002). Villous epithelial cells are made up of crypt cells and move upwards in a spiral. Since the villi are worn by the passage of food, destruction of intestinal crypts causes the villi to shorten and the function of the villi deteriorates, causing diarrhoea and bleeding.

It was reported that traditional Kampo medicines such as Hangeshashinto were used to prevent side effects of anticancer drug treatment and alleviate diarrhoea in patients treated with irinotecan (Mori et al. 2003). The reduction of 5-FU-induced stomatitis (Ozawa et al. 2020) and preventive effect on cisplatin-induced small intestinal mucosal damage (Mori et al. 2003) were studied too. Many Kampo medicines such as Hangeshashinto, Daikenchuto and Rikkunshito contain ginseng, which is a crude drug, and ginsenoside is a saponin glycoside extracted from ginseng. Ginsenosides have a sugar moiety structure bound to the structure of oleanane and dammarane and are classified into protopanaxadiol (PPD) and protopanaxatriol (PPT) according to the position of the carbon bond of the sugar chain. Among ginsenosides, Ra1, 2, Rb1, 2, 3, Rc, Rd, Rf2, Rg3, Rh2 are classified as PPD and Re, Rf, Rg1, 2, Rh1 are classified as PPT. It was reported that ginsenoside Rd (GRd) had a protective effect in DSS-induced colitis models and TNBS-induced colitis models and balances the intestinal flora in autoimmune disease model mice (Jin et al. 2020; Liu et al. 2018; Yang et al. 2012). In addition, it was reported that ginsenoside Re (GRe) had an anti-neuroinflammation effect in addition to a protective effect against colitis (Lee et al. 2012; Zhang et al. 2016). Natural compounds in Kampo medicines attract attention as therapeutic agents for diseases with high efficacy and low toxicity (Khan et al. 2019; Yin et al. 2019).

In this study, we developed a mouse enteritis model and investigated the effects of GRd and GRe on 5-FU induced enteritis to evaluate their usefulness in the prevention and treatment of anticancer drug-induced enteritis, particularly small intestinal mucositis.

Materials and methods

Animals

Eight-week-old male C57BL/6N mice (20 to 25 g) (Japan SLC, Inc.) were used. In all experiments, 4 to 6 animals were used per group. All animals were housed in cages with free access to food and water and kept at 22 ± 1 °C with a 12-h:12-h light/dark cycle. The experimental protocol was carried out in strict accordance with guidelines for experiments involving animals.

Drugs

5-FU (Sigma) was dissolved in physiological saline, while GRd and GRe obtained from natural product library of Kyoto Pharmaceutical University were suspended in 0.5% carboxymethyl cellulose CMC (Nacalai Tesque, Japan) before use. 5-FU, GRd and GRe were prepared immediately before use, in readiness to intraperitoneally or orally administer a volume of 0.1 mL/10 g body weight.

Preparation of 5-FU-induced enteritis

Enteritis was induced by intraperitoneally administering 5-FU (50 mg/kg) once daily for 7 or 8 days (day 0–6 or 7) to the mice. GRd (60 and 100 mg/kg) and GRe (100 mg/kg) were orally administered 30 min before 5-FU administration. The body weight of the animals was measured, and extent of diarrhoea was daily observed, throughout the period of 5-FU administration. The degree of diarrhoea was assigned a 5-point score, specifically one of 0: Normal stool, 1: Soft stool, 2: Severely soft stool, 3: Diarrhoea and 4: Severe diarrhoea.

Hematoxylin & Eosin stain

Twenty-four hours after the last 5-FU dose, the mice were euthanised, their small intestines removed and washed with physiological saline. The small intestine was incised along the opposite side of the mesentery and fixed overnight in 10% formalin solution. The small intestinal tissue was dehydrated with a tissue dehydration solution (Fuji Film Wako Pure Chemical Industries, Ltd., Japan) and the alcohol in the tissue was replaced with xylene (Fuji Film Wako Pure Chemical Industries, Ltd.) as an intermediate solvent. After replacing the intermediate solvent in the tissue with paraffin (Fuji Film Wako Pure Chemical Industries, Ltd.), a paraffin block was prepared and sliced to a thickness of 4 μm using a microtome (Leica, Germany). H&E staining was performed, and the small intestinal tissue was observed under an optical microscope (40 X to 400 X Olympus, Japan) to measure the length of villi, the number of intestinal crypts and the number of cells in the intestinal crypts.

Measurement of MPO activity

Twenty-four hours after the last 5-FU dose, the mice were euthanised, their small intestines removed and washed with ice-cooled physiological saline, before cryopreserving approx. 5 mm of small intestinal tissue sections in a freezer (–30 °C). Then, the small intestinal tissue sections were homogenised in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Fuji Film Wako Pure Chemical Industries, Ltd.) and subjected to 3 rounds of freeze-thawing before centrifuging (at 20 °C and 3,000 rpm). The resulting supernatant was used as samples. Sample volumes of 5 μL were placed in a 96-well microplate. Thereafter, 95 μL of 10 mM phosphate buffer and 50 μL of 20 mM o-dianisidine hydrochloride: 0.44 M hydrogen peroxide were added. The changes in absorption at a wavelength of 450 nm were measured using a microplate reader (CORONA Electric Co., Ltd.). The protein level in the sample was determined using the Pierce BCA Protein Assay Kit (Thermo). The MPO activity was calculated using the following equation: MPO activity (nmol H_2O_2 /min/mg protein) = (OD/min) / (OD/min H_2O_2) × mg protein.

Quantitative analysis of TNF-α mRNA expression

Twenty-four hours after the last 5-FU dose, the mice were euthanised, their small intestines removed and washed with ice-cooled physiological saline. Approximately 5 mm of small intestinal tissue section was cryopreserved in a deep freezer (–80°C). Then, small intestinal tissue sections were homogenised in Sepasol–RNA I (Nacalai Tesque) and centrifuged at 20°C and 12,000 rpm for 15 minutes to extract total RNA. The extracted total RNA was mixed with PrimeScript™ RT Master Mix (Perfect Real Time) (Takara) and complementary DNA (cDNA) was synthesised using a thermal cycler (Takara Thermal Cycler Dice Touch). The cDNA was mixed with Power Up™ SYBR Green Master Mix (Thermo) and primers and the mRNA expression levels were measured by real-time PCR (Step OnePlus Real-Time PCR System; Applied Biosystems). The primers used in this experiment are shown. [TNF-α; forward: 5′-ACTCCAGGCGGTGCCTATGT-3′; reverse: 5′-GTGAGGGTCTGGGCCATAGAA-3′, GAPDH; forward: 5′-TGTGTCCGTCGTGGATCTGA-3′ reverse: 5′-TTGCTGTTGAAGTCGCAGGAG-3′]. The mRNA expression level in each group was standardised by the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each group and further expressed as a ratio to the mRNA expression level in the vehicle group.

Immunohistochemical staining

Twenty-four hours after the last 5-FU dose, the mice were euthanised, their small intestines removed and washed with ice-cooled physiological

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saline. A 2 cm section of small intestinal tissue was fixed overnight in 4% paraformaldehyde/phosphate buffer (Fujifilm Wako Pure Chemical Industries, Ltd.). Moisture in the tissue was replaced with 10, 20 and 30% sucrose phosphate buffers. The tissue was then embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Japan) and cryostat (Leica) was used to prepare frozen slices with 15 μm thickness. The localisation of NF-κB p65 in the small intestinal tissue was studied using mouse monoclonal NF-κB p65 antibody (Santa Cruz Biotechnology, Inc., CA, US) as the primary antibody. After blocking with a mouse IgG blocking reagent (goat serum), the primary antibody reacted at 20 °C for 30 min and then the biotin-labeled anti-mouse IgG antibody was used as the secondary antibody. A Mouse on Mouse (M.O.M.) Immunodetection Kit (Vector) was used as the blocking reagent and secondary antibody. Nuclear staining was performed using the Mounting Medium with DAPI (Vector). Localisation of NF-κB was observed under a confocal laser scanning microscope (FV10i; Olympus).

Statistical analysis

The data are shown as a sample mean ± standard error (SEM) for 4 to 6 animals per group. Statistical significance was determined with GraphPad Prism software version 8 (San Diego, CA) using the Student's t-test and results were considered to be significant when $p < 0.05$.

Results

Effect of GRd on 5-FU-induced enteritis

Body weight and diarrhoea

The body weight decreased over time by continuous administration of 5-FU and a significant decrease was observed from day 4 as compared with the vehicle group. The body weight at day 8 of continuous 5-FU administration was 82.9 ± 0.9% of the body weight before 5-FU administration. The animals co-administered GRd (60 and 100 mg/kg) also showed progressive weight loss from day 4 of continuous 5-FU administration. The body weight of the GRd (60 mg/kg) group on day 8 of continuous 5-FU administration was 84.0 ± 0.6% of the weight before 5-FU administration, while that in the GRd (100 mg/kg) group was 84.6 ± 1.6% of the weight before 5-FU administration (Fig. 1A).

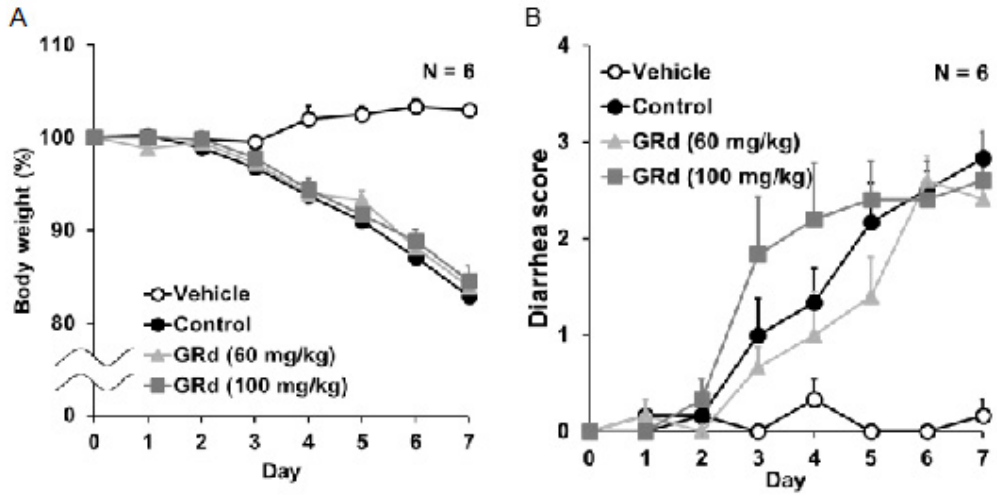

Fig. 1. Effect of co-administered GRd on body weight loss, and diarrhoea due to 5-fluorouracil (5-FU). Animals received an intraperitoneal injection of 50 mg/kg 5-FU once daily for 8 days (days 0–7), with 60 mg/kg GRd (n = 6) and 100 mg/kg GRd (n = 6) co-administered once daily. Control animals were administered 5-FU with 0.5 % CMC (vehicle for GRd). Body-weight was measured daily (A), while diarrhoea was scored daily using a 5-point scale (0–4) (B). Data are expressed as a mean ± S.E.M.

Continuous 5-FU administration also showed progressive exacerbation of diarrhoea, and a significant at $p < 0.05$ exacerbation of diarrhoea was noted in the 5-FU-treated groups from day 4 of continuous administration relative to the vehicle group. The degree of diarrhoea score on day 8 of continuous 5-FU administration was 2.8 ± 0.3. The degree of diarrhoea was noted on day 4 of continuous 5-FU administration even in the GRd groups. On day 8 of continuous 5-FU administration, the diarrhoea score was 2.4 ± 0.5 in the GRd (60 mg/kg) group and 2.6 ± 0.5 in the GRd (100 mg/kg) group (Fig. 1B).

Histological assessment

The length of the small intestinal villi in the vehicle group was 203.2 ± 13.6 μm. The length of the small intestinal villi on day 8 of continuous 5-FU administration was 115.0 ± 10.4 μm, which was significantly shorter than that in the vehicle group. When GRd was co-administered, the length of the small intestinal villi on day 8 of continuous 5-FU administration was 134.0 ± 8 μm in the GRd (100 mg/kg) group, which was significantly shorter than that in the vehicle group (Fig. 2A, B).

The number of intestinal crypts in the vehicle group was 18.2 ± 1.4 crypts/mm. The number of intestinal crypts on day 8 of continuous 5-FU administration was 7.7 ± 0.6 crypts/mm, which was significantly at $p < 0.05$ less than in the vehicle group. When GRd was co-administered, the number of intestinal crypts on day 8 of continuous 5-FU administration was 8.7 ± 0.5 crypts/mm in the GRd (60 mg/kg) group and 10.7 ± 1.0 crypts/mm in the GRd (100 mg/kg) group, both significantly lower than in the vehicle group. Compared to the animals

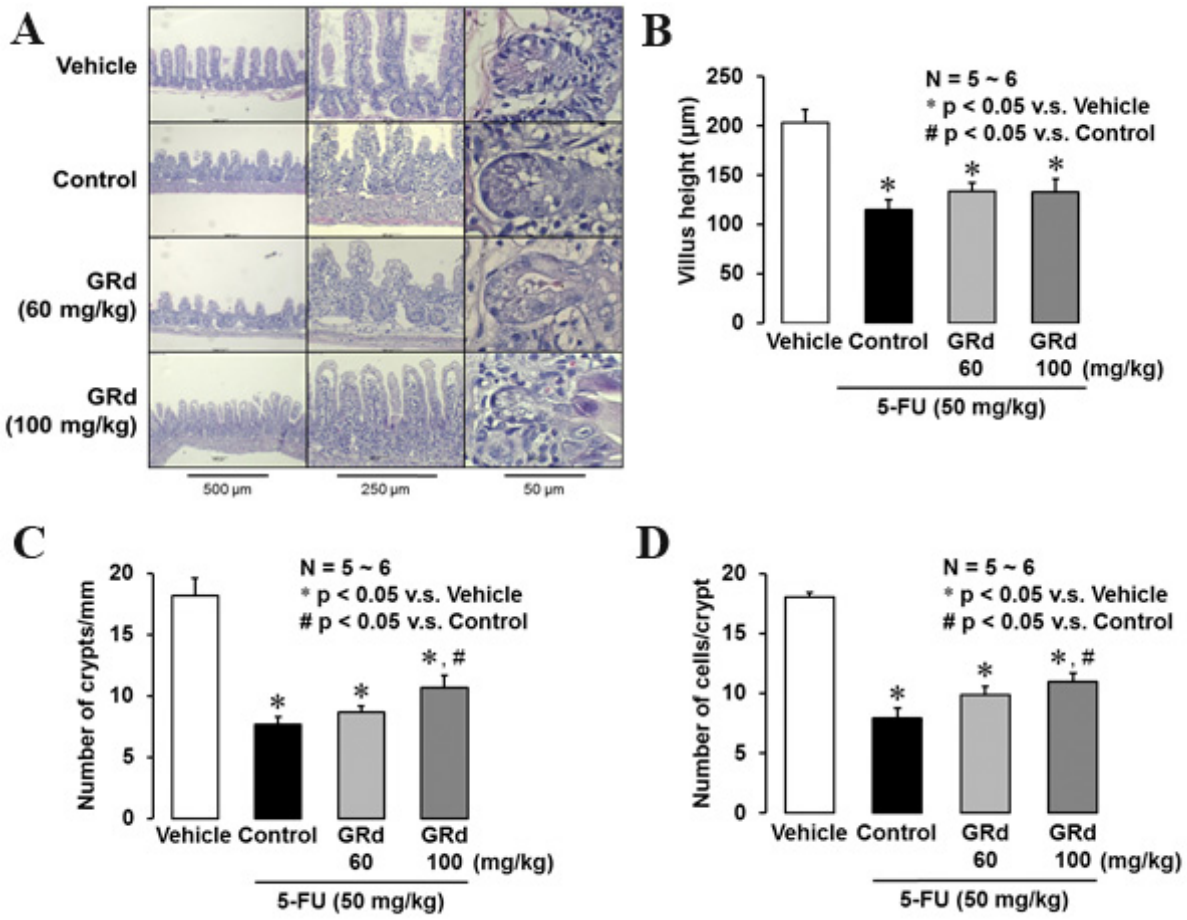

Fig. 2. Effect of co-administered GRd on intestinal mucositis due to 5-fluorouracil (5-FU). Animals received an intraperitoneal injection of 50 mg/kg 5-FU once daily for 8 days (days 0–7), with 60 mg/kg GRd (n = 6) and 100 mg/kg GRd (n = 6) co-administered once daily. Jejunum tissues collected on day 7 were stained with hematoxylin/eosin and imaged at ×40, ×100, and ×400 (A). The height from the top of the villus to the villus-crypt junction (B), the number of crypts per millimeter (C) and cells per crypt (D) were measured by light microscopy. *$p < 0.05$ versus untreated animals from vehicle (not treated with 5-FU, n = 6); #$p < 0.05$ versus animals treated with 5-FU only (n = 6).

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administered only 5-FU, those co-administered with GRd (100 mg/kg) showed significant suppression of the reduction in the number of intestinal crypts by 5-FU (Fig. 2C).

The number of cells in the intestinal crypts was 18.0 ± 0.4 cells/crypt in the vehicle group. In addition, the number of cells in the intestinal crypts on day 8 of continuous 5-FU administration was 7.9 ± 0.8 cells/crypt, which was significantly lower than in the vehicle group. When GRd was co-administered, the number of cells in the intestinal crypts on day 8 of continuous 5-FU administration was 9.9 ± 0.7 cells/crypt in the GRd (60 mg/kg) group and 11.0 ± 0.7 cells/crypt in the GRd (100 mg/kg) group, both significantly lower than in the vehicle group. Compared to the animals administered only 5-FU, those co-administered with GRd (100 mg/kg) showed significant suppression of the reduction in the number of intestinal crypt cells by 5-FU (Fig. 2D).

Changes in MPO activity

The MPO activity in the vehicle group was 21.7 ± 3.5 (nmol H_2O_2/min/mg protein). The MPO activity 24 h after the last 5-FU dose increased to 45.8 ± 3.0 (nmol H_2O_2/min/mg protein) and significantly increased relative to the vehicle group. When GRd was co-administered, the MPO activity on day 8 of continuous 5-FU administration was 33.1 ± 5.3 (nmol H_2O_2/min/mg protein) in the GRd (60 mg/kg) group and 31.9 ± 2.9 (nmol H_2O_2/min/mg protein) in the GRd (100 mg/kg) group. The animals co-administered GRd (100 mg/kg) had a significant increase in the MPO activity compared to the animals administered only 5-FU (Fig. 3).

TNF-α mRNA expression

The TNF-α mRNA expression 24 h after the last 5-FU dose was increased to 1.4 ± 0.2-fold that of the vehicle group; however, it was not significant statistically. When GRd (100 mg/kg) was administered, the TNF-α mRNA expression was 0.7 ± 0.3-fold that of the vehicle group, which meant there was a significant suppression of the increase in the TNF-α mRNA expression compared to the animals that were administered only 5-FU (Fig. 4).

NF-κB localisation

There was a clear increase in the expression of NF-κB in the small intestine 24 h after the last 5-FU dose compared to the vehicle group. When GRd (100 mg/kg) was co-administered, there was a clear suppression of the increase in the NF-κB expression relative to the group of animals administered only 5-FU. The NF-κB expression in the 5-FU monotherapy group particularly remarkably increased around the intestinal crypts,

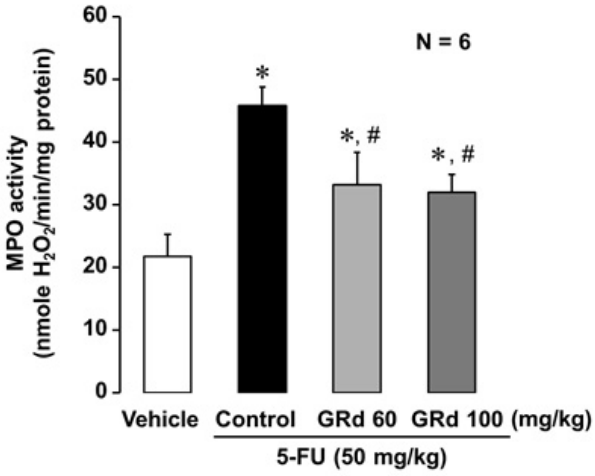

Fig. 3. Effect of co-administered GRd on 5-fluorouracil (5-FU)-induced increase in intestinal myeloperoxidase (MPO) activity. The animals received 50 mg/kg 5-FU by intraperitoneal injection once daily for 8 days (days 0–7), with 100 mg/kg GRd (n = 6) co-administered once daily. The jejunum was obtained on day 7. The MPO activity was measured with o-dianisidine. Data are expressed as a mean ± S.E.M. *p < 0.05 versus vehicle (not treated with 5-FU, n = 6); #p < 0.05 versus the animals treated with 5-FU only (n = 6).

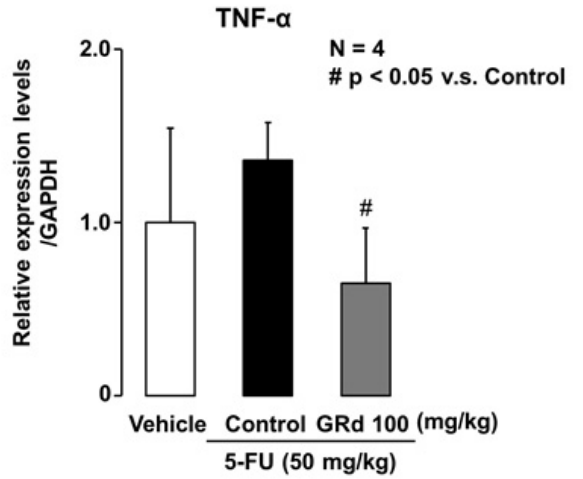

Fig. 4. Effect of co-administered GRd on 5-fluorouracil (5-FU)-induced increase in intestinal TNF-α mRNA expression. The animals received 50 mg/kg 5-FU by intraperitoneal injection once daily for 8 days (days 0–7), with 100 mg/kg GRd (n = 4) co-administered once daily. The jejunum was obtained on day 7. The TNF-α expression was quantified by real-time RT-PCR. The expression was normalised to GAPDH and to the mean value in the vehicle mice not treated with 5-FU. Data are expressed as a mean ± S.E.M. #p < 0.05 versus animals treated with 5-FU only (n = 4).

and co-administration of GRd (100 mg/kg) was suppressing this increase (Fig. 5).

Effect of GRe on 5-FU-induced enteritis

Body weight and diarrhoea

The body weight decreased over time by continuous administration of 5-FU, and a significant decrease was observed from day 2 as compared to the vehicle group. The body weight at day 7 of continuous 5-FU administration was 84.9 ± 1.4% of the body weight before 5-FU administration. When GRe (100 mg/kg) was co-administered, there was a gradual decrease in the body weight from day 2 of 5-FU administration, and the body weight on day 7 of 5-FU administration was 86.2 ± 0.2% of the body weight before the start of 5-FU administration (Fig. 6A).

Exacerbation of diarrhoea was noted from day 2 of 5-FU administration, with the degree of the diarrhoea score being 2.7 ± 0.3. Even in the animals that were administered GRe at 100 mg/kg, diarrhoea was observed from day 2 of 5-FU administration, with a score of 2.6 ± 0.2 (Fig. 6B). Diarrhoea was observed on day 3 in the GRe group; therefore, GRe administration delayed the diarrhoea.

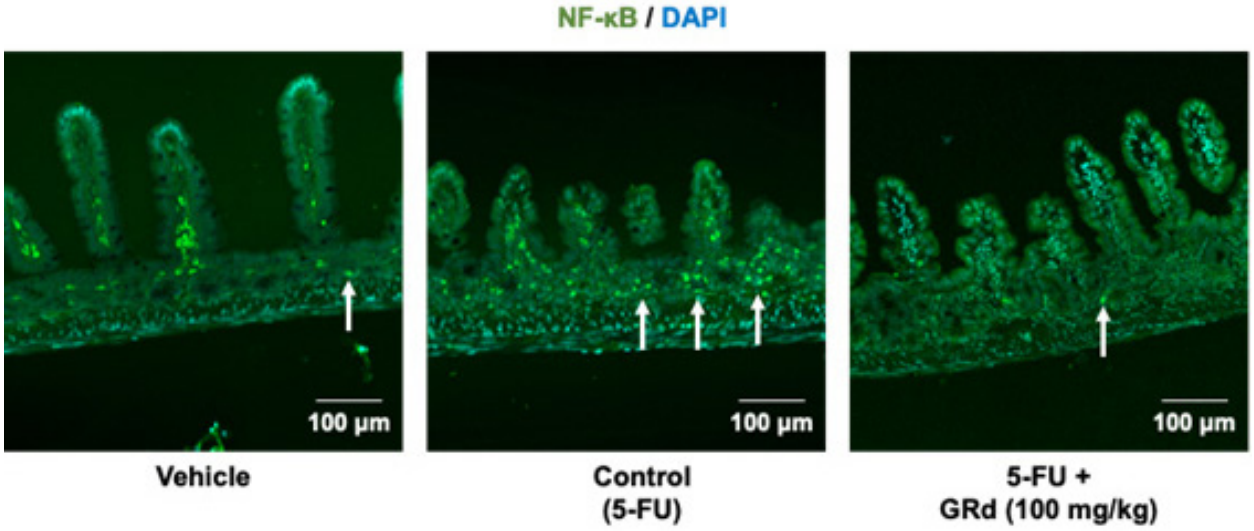

Fig. 5. Immunostainig of NF-κB in the small intestinal mucosa with or without 5-FU and GRd. NF-κB was determined immunohistochemically using anti-NF-κB antibody 24 h after 5-FU injection. Immunohistochemical images of stained intestinal tissues were observed. Arrows show NF-kB positive cells.

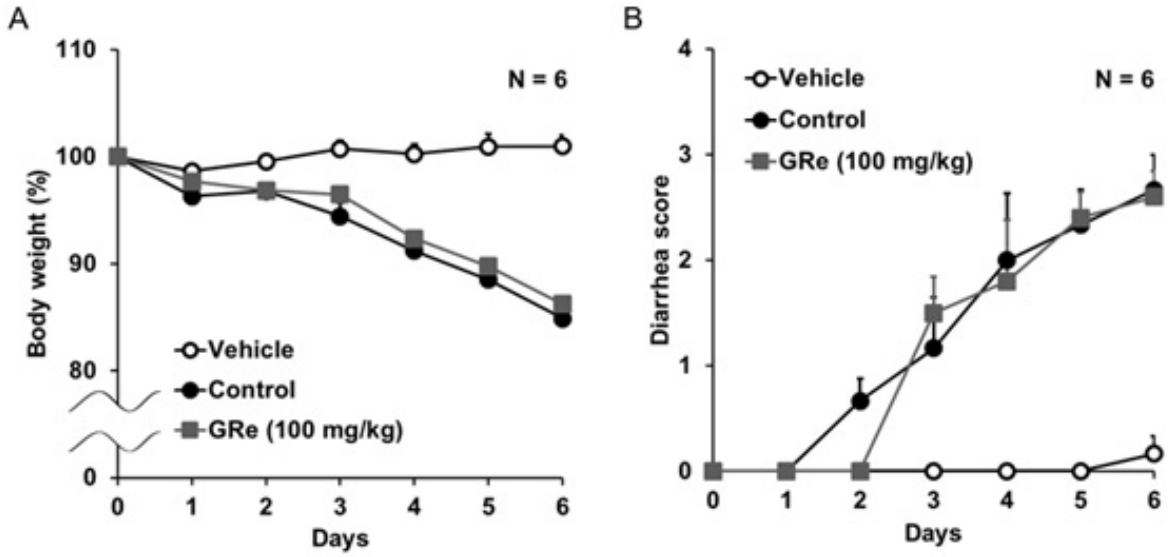

Fig. 6. Effect of co-administered GRe on body weight loss, and diarrhoea due to 5-fluorouracil (5-FU). The animals received an intraperitoneal injection of 50 mg/kg 5-FU once daily for 7 days (days 0–6), with 100 mg/kg GRe (n = 6) co-administered once daily. The control animals were administered 5-FU with 0.5 % CMC (vehicle for GRe). The body-weight was measured daily (A), while diarrhoea was scored daily using a 5-point scale (0–4) (B). Data are expressed as a mean ± S.E.M.

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Histological assessment

The length of the small intestinal villi in the vehicle group was 180.6 ± 14.1 μm. The length of the small intestinal villi on day 7 of 5-FU administration was 102.2 ± 9.1 μm, which was significantly shorter than that in the vehicle group. When GRe was co-administered, the length of the small intestinal villi on day 7 of 5-FU administration was 168.7 ± 10.8 μm in the GRe (100 mg/kg) group, and the shortening of villi was significantly suppressed compared to the 5-FU monotherapy group (Fig. 7A, B).

The number of intestinal crypts in the vehicle group was 14.7 ± 0.8 crypts/mm. The number of intestinal crypts on day 7 of 5-FU administration was 9.5 ± 0.6 crypts/mm, which was significantly lower than in the vehicle group. When GRe (100 mg/kg) was co-administered, the number of intestinal crypts on day 7 of 5-FU administration was 11.7 ± 0.8 crypts/mm, which was significantly lower than in the vehicle group (Fig. 7C).

The number of cells in the intestinal crypts in the vehicle group was 17.9 ± 1.0 cells/crypt. Furthermore, the number of cells in the intestinal crypts on day 7 of 5-FU administration was 9.7 ± 0.5 cells/crypt, which was significantly lower than in the vehicle group. When GRe (100 mg/kg) was co-administered, the number of cells in the intestinal crypts on day 7 of 5-FU administration was 10.7 ± 0.8 cells/crypt, which was significantly lower than in the vehicle group (Fig. 7D).

Changes in MPO activity

The MPO activity in the vehicle group was 28.6 ± 1.3 (nmol H_2O_2/min/mg protein). Furthermore, the MPO activity 24 h after the last 5-FU dose was 85.8 ± 9.9 (nmol H_2O_2/min/mg protein). When GRe (100 mg/kg) was co-administered, the MPO activity 24 h after the last 5-FU dose was 58.9 ± 6.0 (nmol H_2O_2/min/mg protein) and significantly (at $p < 0.05$) lower than in the vehicle group as well as showing

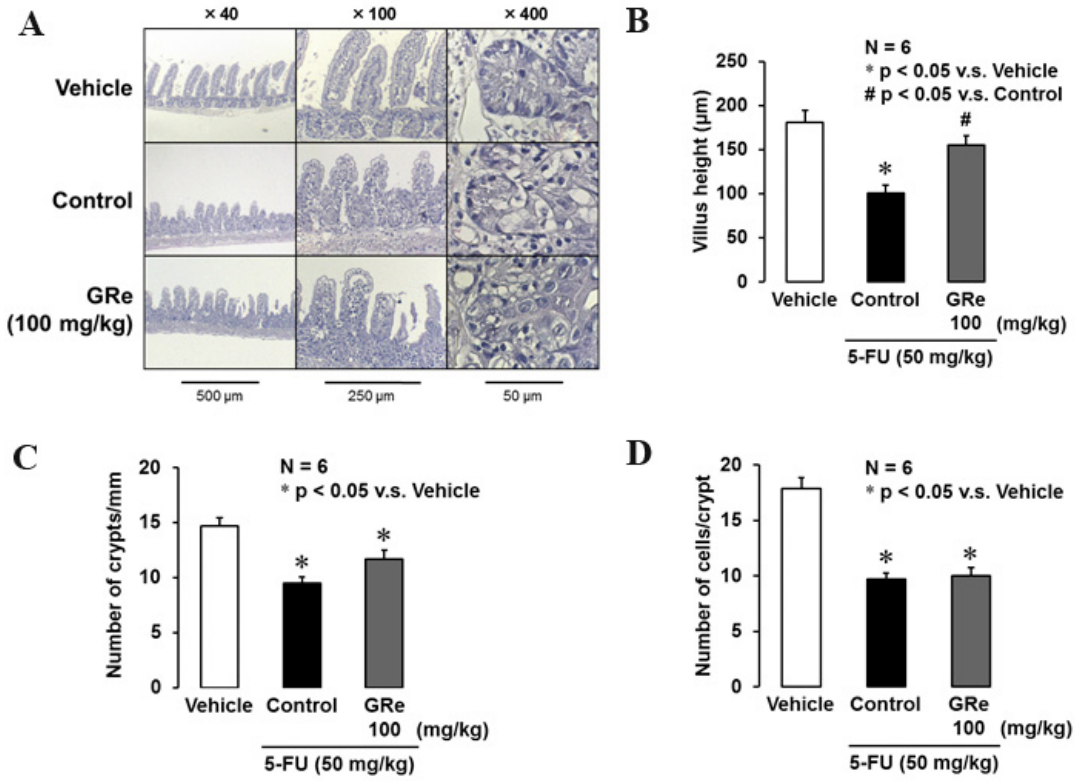

Fig. 7. Effect of co-administered GRe on intestinal mucositis due to 5-fluorouracil (5-FU). The animals received an intraperitoneal injection of 50 mg/kg 5-FU once daily for 7 days (days 0–6), with 100 mg/kg GRe (n = 6) co-administered once daily. Jejunum tissues collected on day 6 were stained with hematoxylin/eosin and imaged at ×40, ×100, and ×400 (A). The height from the top of the villus to the villus–crypt junction (B), the number of crypts per millimeter (C) and cells per crypt (D) were measured by light microscopy. *p < 0.05 versus the untreated animals from vehicle (not treated with 5- FU, n = 6); #p < 0.05 versus the animals treated with 5-FU only (n = 6).

a significant suppression in the increase in the MPO activity relative to the 5-FU monotherapy group (Fig. 8).

Discussion

Previous studies reported that administration of 5-FU caused weight loss and diarrhoea (Benson et al. 2004; Hamouda et al. 2017). The mechanism of anticancer drug-induced enteritis has not been elucidated yet, but in addition to the inhibitory effect of anticancer drugs on cell proliferation, it was reported that apoptosis is involved and causes enteritis by shortening the villi of the small intestine and destroying the intestinal crypts (Duncan, Grant 2003; Hamouda et al. 2017; Inomata et al. 2002).

In this study, continuous administration of 5-FU reduced the body weight of the mice over time. In clinical practice, side effects of 5-FU include anorexia due to nausea and vomiting (Shih et al. 2007). Anticancer drugs are known to stimulate the secretion of 5-HT and substance P proteins in the gastrointestinal tract which bind to 5-HT3 and NK1 receptors to generate nerve impulses; these signals are transmitted to the vomiting centre to cause a vomiting reaction (Xi et al. 2021). The weight loss of mice by 5-FU administration observed in this experiment was considered to be due to the decrease in food intake caused by anorexia.

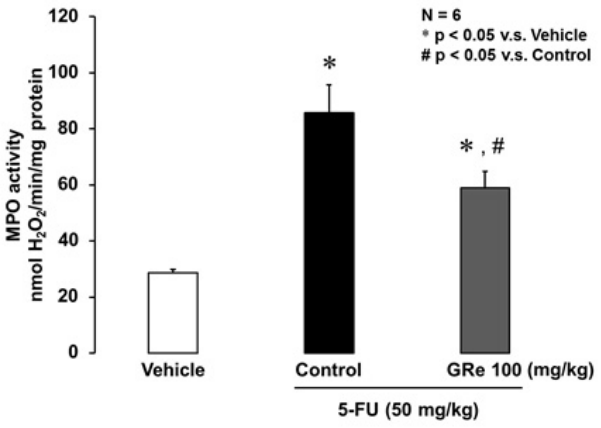

Fig. 8. Effect of co-administered GRe on 5-fluorouracil (5-FU)-induced increase in intestinal myeloperoxidase (MPO) activity. The animals received 50 mg/kg 5-FU by intraperitoneal injection once daily for 7 days (days 0–6), with 100 mg/kg GRe (n = 6) co-administered once daily. The jejunum was obtained on day 6. The MPO activity was measured with o-dianisidine. Data are expressed as a mean ± S.E.M. *p < 0.05 versus vehicle (not treated with 5-FU, n = 6); #p < 0.05 versus the animals treated with 5-FU only (n = 6).

The occurrence of diarrhoea was associated with abnormal intestinal and intestinal water secretion and reabsorption. Aquaporin (AQP) is a family of water channel membrane proteins with a molecular weight of approximately 30 kDa. There are 13 AQP subtypes (AQP 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) involved in water secretion and absorption pathways (Lv et al. 2022). Abnormal AQP expression causes impaired water absorption and secretion in the intestine, which affects intestinal membrane permeability and fluid transport, resulting in diarrhoea (Engevik et al. 2018). In fact, there are reports that the expression of AQP4 and AQP8 was reduced in the transverse and distal colons of mice in the 5-FU-induced diarrhoea model (Sakai et al. 2013). In this study, the co-administration of GRd and GRe did not affect the occurrence and exacerbation of diarrhoea due to continuous administration of 5-FU, but there is no report focusing on the relationship between ginsenosides and AQP in the small intestine. It is necessary to investigate the AQP expression level in the small intestine when 5-FU and ginsenosides are used in combination.

It was histopathologically confirmed that continuous administration of 5-FU shortened the villi of the small intestine and reduced the number of intestinal crypts and the number of cells in the intestinal crypts. Villous epithelial cells are made up of crypt cells and move upwards in a spiral. Since the villi are worn by the passage of food, destruction of intestinal crypts causes the villi to shorten, resulting in deteriorating functioning of the villi. The co-administration of GRd and GRe significantly improved the destruction of intestinal mucosal tissue structure, which is considered to be due to the anti-inflammatory action of GRd and GRe. In addition, GRd and GRe did not affect weight loss due to continuous administration of 5-FU, but the intestinal absorption capacity decreased due to the destruction of the intestinal mucosal tissue structure by 5-FU such that the protective effect of GRd and GRe may have decreased. Therefore, it is necessary to consider prophylactic administration of GRd and GRe before treatment with 5-FU.

There is a report which suggests that continuous administration of 5-FU to mice upset the balance of intestinal flora, reduced the abundance of gram-positive *phylum Bacillota* and increased the abundance of gram-negative *phyla Bacteroides* and *Verrucomicrobia* (Hamouda et al. 2017). The intestinal flora is involved in many functions such as maintenance of immune homeostasis and improvement of metabolic capacity and it is said that its imbalance will adversely affect health (Frank et al. 2007). *Akkermansia muciniphila*, which belongs

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to the *phylum Verrucomicrobia*, was reported to break down mucin (Derrien et al. 2011) and it is possible that the enteritis caused by the administration of 5-FU was associated with a decrease in the intestinal epithelial barrier due to a decrease in mucus volume and lipopolysaccharide (LPS) derived from Gram-negative bacteria. GRd was reported to balance the intestinal flora in an autoimmune disease model in mice (Jin et al. 2020). In this study, the MPO activity that increased after the continuous administration of 5-FU was significantly suppressed by co-administration of GRd and GRe. This suggests that ginsenosides may be involved in improving the balance of intestinal flora.

Continuous administration of 5-FU increased the TNF-α mRNA expression. According to the report by Chang et al. (2012), the expression of NF-κB was increased by administering 5-FU to mice and in this study, the expression of NF-κB was also confirmed by immunohistological examination. When NF-κB is activated by LPS or cytokines, it increases inflammatory cytokines such as TNF-α which further activate NF-κB. The deterioration of inflammation from this repetition is thought to destroy crypt cells and cause enteritis. The co-administration of GRd suppressed the increase in the TNF-α mRNA and NF-κB expression by 5-FU. It was reported that GRd exhibited an anti-inflammatory effect by blocking the NF-κB signalling pathway in a carrageenan footpad oedema model (Wang et al. 2012), while GRe suppressed the inflammatory cytokine expression and activation of NF-κB, their transcription factor, by binding competitively to the TLR4 receptor on macrophages (Lee et al. 2012). Taken together, the data suggest that the NF-κB pathway may be involved in the suppression (by GRd and GRe) of the inflammatory response caused by continuous administration of 5-FU seen in these experiments. By conducting a detailed study on the expression and localisation of NF-κB and its related molecules IκB, TLR4 and MyD88 involved in signal transduction in the pathophysiology of 5-FU-induced enteritis, the mechanism of enteritis and the action mechanisms of drugs that protect against it may be clarified.

The anti-inflammatory effects of dexamethasone, a synthetic steroid, are associated with reduced levels of NF-kB (p65) and decreased levels of TNF-α in gastrointestinal disorders (Yousefi-Manesh et al. 2020). In addition, dexamethasone regulates several signalling pathways, including NF-kB, TLR4, and IRAK-M signalling (Ribeiro et al. 2017). It is suggested that corticosterone could contribute to the suppression of the 5-FU-induced inflammatory response by GRd and GRe.

On the other hand, it is also possible that the protective action of GRd and GRe observed in this experiment is due to the attenuation of the anticancer effect of 5-FU or the inhibition of uptake. However, a report indicated that GRd suppressed tumour angiogenesis through increased coverage of pericytes around micro-vessels and decreased the VEGF expression in vivo and enhanced antitumor effects when used in combination with 5-FU (Zhong et al. 2020).

The results obtained in this study suggest that GRd and GRe show an anti-inflammatory effect against 5-FU-induced enteritis and that NF-κB is involved in their action.

Conflict of Interest

The authors declare that there is no conflict of interest, either existing or potential.

Ethics Approval

All procedures involving animals were approved by the Committees for Animal Research of Ritsumeikan University Institutional Animal Care and Use Committee.

Author Contributions

a. Daisuke Kato—conducted experiments, contributed to the interpretation of the results, drafted the original manuscript, reviewed the manuscript draft and revised it critically on intellectual content;

b. Shizuka Jonan—conducted experiments, contributed to the interpretation of the results, drafted the original manuscript, reviewed the manuscript draft and revised it critically on intellectual content;

c. Ryo Sugahara—conducted experiments, reviewed the manuscript draft and revised it critically on intellectual content;

d. Hikaru Otsuki—conducted experiments, reviewed the manuscript draft and revised it critically on intellectual content;

e. Seikou Nakamura—conceived the idea of the study, reviewed the manuscript draft and revised it critically on intellectual content;

f. Kikuko Amagase— conceived the idea of the study, supervised the conduct of this study, reviewed the manuscript draft and revised it critically on intellectual content.

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