JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2022, 73, 4, 539-546
www.jpp.krakow.pl | DOI: 10.26402/jpp.2022.4.07

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ALLEVIATIVE EFFECTS OF GLUTAMATE AGAINST CHEMOTHERAPEUTIC AGENT-INDUCED INTESTINAL MUCOSITIS

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5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents; however, it often causes intestinal mucositis with severe diarrhea. An efficient treatment strategy to reduce this side effect is lacking. Glutamate (Glu), a nonessential amino acid, is the most important energy source in the small intestine and has been shown to maintain intestinal morphology, barrier function, and antioxidative capacity. However, the effects of Glu on intestinal mucositis induced by chemotherapeutic agents have not been explored. This study aimed to demonstrate the alleviative effects of Glu on 5-FU-induced intestinal mucositis. Mucositis was induced in C57B/6N mice by intraperitoneal injection of 5-FU (50 mg/kg) for 6 days and assessed by histological and physiological analyses. Glu (500 or 1000 mg/kg) was orally administered as a pretreatment twice daily for 7 days before the initial treatment of 5-FU. Cellular proliferation and apoptosis were assessed using Ki-67 immunostaining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, respectively. Furthermore, fluorescein isothiocyanate-dextran infiltration was assessed to measure intestinal permeability. *In vitro* experiments using rat intestinal epithelial cells (IEC-6 cells) were performed to clarify the effect of Glu on 5-FU-induced barrier dysfunction. Glu alleviated 5-FU-induced intestinal mucositis by reducing villi shortening, enhancing cell proliferation, and suppressing apoptosis. It also alleviated the 5-FU-induced increased intestinal permeability. *In vitro* studies revealed significantly increased trans-epithelial electrical resistance (TEER) in Glu-pretreated IEC-6 cells compared to that in 5-FU-treated and control cells. In conclusion, the findings of this study provide evidence for the potential of Glu to protect against 5-FU-induced intestinal mucositis in patients with cancer.

Key words: *intestinal mucositis, chemotherapeutic agents, 5-fluorouracil, glutamate, cell proliferation, apoptosis, intestinal permeability, trans-epithelial electrical resistance*

INTRODUCTION

Chemotherapeutic agents used to treat cancers often cause diarrhea, nausea, vomiting, mucositis, hepatotoxicity, and weight loss. These dose-limiting effects have a marked negative impact on patients, resulting in chemotherapy schedule failure (1, 2). Furthermore, these conditions are associated with impaired quality of life and increased healthcare costs due to the associated additive care (3). Therefore, it is crucial to manage the chemotherapeutics-induced side effects to improve the quality of life of patients with cancer. Although several studies have assessed the prevention of chemotherapeutic agent-induced mucositis, fundamental treatment approaches have not yet been fully developed (4).

5-Fluorouracil (5-FU), a fluoropyrimidine widely used chemotherapeutic agent in the treatment of various cancers, has been reported to cause intestinal mucositis (1, 3). Accumulating evidence shows that the sequential events in the development of 5-FU-induced mucositis involve apoptosis of the crypts, a suppressed proliferation of intestinal cells, dysbiosis, and expression of inflammatory cytokines (5-7); however, the exact underlying mechanisms have been poorly investigated.

Patients with cancer often experience eating disorders with chemotherapy, which results in weight loss and a decreased median survival (8). Recently, several studies have demonstrated the protective effects of glutamine (Gln) against 5-FU-induced mucositis (9-12). In addition, other amino acids, such as arginine (12-14), glycine (15, 16), and cysteine (17), also exhibit protective roles in several intestinal disorders, including chemotherapy-induced intestinal mucositis. Glutamate (Glu), a nonessential amino acid, is formed from Gln *via* glutaminase in the enterocytes (18, 19). It is the most abundant amino acid in dietary proteins in daily meals (20). Moreover, Glu is the most important energy source in the small intestine, plays several important roles in the intestine and is the precursor of several molecules produced within the intestinal mucosa, including amino acids (20). Dietary Glu in

the form of monosodium glutamate (MSG) dissociates in water and acts similarly to free Glu. Over 90% of the dietary Glu is metabolized in the first pass through the intestinal mucosa (21). Interestingly, plasma Glu levels are not strongly affected by dietary Glu; rather, they are tightly maintained at low concentrations (22, 23). Glu maintains growth and health as a major oxidative fuel (24), an important precursor of bioactive molecules (25-27), and a regulator of gene expression and cell signaling in the intestine (28). Although the beneficial effects of Glu on intestinal morphology, barrier, and antioxidative capacity has been reported (29, 30), its effects on intestinal mucositis have seldomly been investigated. Therefore, we hypothesized that evaluating the beneficial effects of Glu on inflammation-induced epithelial damage can be promising not only for maintaining the health of the intestine but also for the nutritional state of the entire body.

To test this hypothesis, in this study, we aimed to evaluate the effects of Glu on 5-FU-induced intestinal mucositis in mice.

MATERIALS AND METHODS

Chemicals

5-FU was obtained from Sigma-Aldrich (St. Louis, MO, USA), and sodium hydrogen L(+)-glutamate monohydrate was obtained from Wako Pure Chemicals (Osaka, Japan). 5-FU was dissolved in saline (Otsuka, Tokyo, Japan) to inject the animals. To treat cells, 5-FU dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemicals, Osaka, Japan) and L-glutamate (Wako Pure Chemicals, Osaka, Japan) was used.

Animals

Male C57BL/6N mice aged 7–8 weeks were purchased from Japan SLC, Inc., (Shizuoka, Japan). The mice were housed under standard laboratory conditions with a 12 h light/dark cycle and free access to water and food.

All experimental procedures were approved by the Experimental Animal Ethics Committee of Ritsumeikan University (No. 2018-039).

Mice model for chemotherapy-induced mucositis

Mice were randomly divided into three groups:
1) Control group (saline, intraperitoneally (i.p.) injection; and water, gavaged orally (p.o.));
2) 5-FU group (50 mg/kg 5-FU, i.p., and water, p.o.); and
3) 5-FU + Glu group (50 mg/kg 5-FU, i.p., and 500 or 1000 mg/kg Glu, p.o.).

All mice in the treatment groups were administered 5-FU (50 mg/kg, i.p.) once daily, while control mice were administered saline. Glu (500 or 1000 mg/kg) was administered twice daily for 13 days, starting 7 days before the initial treatment with 5-FU. Disease severity was assessed daily among the mice that were administered 5-FU treatments by measuring the body weight and scoring the consistency of stool (on a scale of 0–4) as previously described (5): 0, normal; 1, soft; 2, very soft; 3, diarrhea; and 4, severe diarrhea.

Histological staining

Twenty-four hours after the final administration of 5-FU, the mice were sacrificed by cervical dislocation. The ileum tissues were excised and immersed overnight in 10% formalin. Tissue samples were excised, embedded in paraffin, sectioned at 4 μm with a microtome (Leica Microsystems, Nussloch, Germany), and stained with hematoxylin and eosin (H&E). The length from the top of the villus to the villus-crypt junction was measured using a microscope (CX43; Olympus, Tokyo, Japan) at a magnification of 10×0.25 (numerical aperture; NA).

Immunohistochemistry

Ileum tissue was fixed with 4% paraformaldehyde, embedded with OCT compound (Sakura Finetek, Tokyo, Japan), and sectioned (14 μm) at –15°C using a cryostat micro-tome (Leica Microsystems, Nussloch, Germany). Proliferative cells were detected with donkey anti-Ki-67 antibody (Abcam, Cambridge, USA; 1:400). Sections were mounted using ProLong Glass Antifade Mountant with NucBlue (Thermo Scientific, Waltham, MA, USA). Images were acquired using a fluorescence microscope (BZ-X710 Keyence, Osaka, Japan) at a magnification of 10×0.3 (NA). Apoptotic cells were detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and an *in situ* Apoptosis Detection Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Sections were mounted using a Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Newark, CA, USA). Images were acquired using a confocal laser scanning microscope (LSM900; Airyscan-Carl Zeiss, Oberkochen, Germany) at a magnification of 10×0.8 (NA).

Analysis of intestinal permeability

Fluorescein isothiocyanate-dextran (FD-4, Sigma-Aldrich, St. Louis, MO, USA) at 15 mg/0.2 mL/mouse was dissolved in water and administered p.o. 4 h before tissue and blood sample collection. The concentration of FD-4 in the serum was calculated using a standard curve and a microplate reader (SH-9500Lab; Corona Electric, Ibaragi, Japan). The ileum tissues were snap-frozen with OCT compound and sectioned (14 μm) at –15°C using a cryostat microtome. Images were acquired using a microscope (CX43) at a magnification of 10×0.25 (NA).

Cell culture

Rat intestinal epithelial cells (IEC-6 cells, Riken, Yokohama, Japan) were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Kyoto, Japan) containing 5% fetal bovine serum (MP Biomedicals, Irvine, CA, USA), 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) and 0.1 IU/mL insulin (Wako Pure Chemicals, Osaka, Japan).

Trans-epithelial electrical resistance (TEER) measurement

IEC-6 cells were cultured on a transwell membrane (Corning, Kennebunk, ME, USA), and complete confluent cells that were incubated for more than 10 days were used as IEC-6 monolayers for experiments. The cells were treated with 1 mM Glu for 24 h before treating with 100 μM 5-FU; the control was treated with DMSO. TEER values were measured using a Millicel-ERS2 Volt ohmmeter (Millipore, Bedford, MA, USA).

Statistical analysis

Data are reported as mean ±standard error (SE) for 4–6 animals per group and 3 experiments per group. Data were analyzed with GraphPad Prism version 8 (La Jolla, CA, USA) using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical significance was set at $p<0.05$.

RESULTS

Effect of glutamate on 5-fluorouracil-induced body weight loss and diarrhea

The experimental schedule for the induction of intestinal mucositis by 5-FU and pretreatment with Glu (500 and 1000 mg/kg) to examine the effect of Glu on 5-FU-induced mucositis in mice is shown in *Fig. 1A*. Repeated administration of 5-FU caused significant weight loss beginning on day 4, with mean body weight decreasing to 87.3±2.0% on day 6 compared to 100% on day 0. Pretreatment with Glu (500 and 1000 mg/kg) did not affect the 5-FU-induced body weight loss, with mean body weight decreasing to 85.9±2.4 and 86.4±0.8% on day 6 compared to that on day 0 (100%), respectively (*Fig. 1B*). As shown in *Fig. 1C*, a significant difference in stool consistency was observed between the 5-FU (red) and 5-FU+Glu (1000 mg/kg) group (blue), with mean diarrhea scores of 3.0±0.3 and 2.0±0.3 on day 6, respectively. Although Glu 1000 mg/kg (blue) improved 5-FU-induced diarrhea (red) significantly only on day 6, the diarrhea score in 5-FU+Glu groups (green and blue) was higher than that in the control (black). Glu 500 mg/kg (green) showed slightly better effects at other time points. These findings suggest that pretreatment with Glu had no significant effect on 5-FU-induced body weight loss; however, it ameliorated 5-FU-induced diarrhea.

Effect of glutamate on histological changes caused by 5-fluorouracil

Compared to the crypt structure in the control group of mice, that in the 5-FU (50 mg/kg) group of mice was severely damaged (*Fig. 2A*). However, the structure of crypts in the intestine was improved in the 5-FU+Glu (1000 mg/kg) group compared to that in the 5-FU group, but comparable to that of the control group 24 h after the final administration of 5-FU. The villus length in the 5-FU group was significantly shortened (117.5±4.9 μm) compared to that of the control group (174.0±11.0 μm) (*Fig. 2B*). In contrast, Glu significantly improved the 5-FU-induced reduction in villus length (146.3±4.6 μm; $p<0.05$). These findings suggest that pretreatment with Glu protects against 5-FU-induced histological changes.

Changes in 5-fluorouracil-induced intestinal proliferation

Immunohistochemical analysis of the ileum tissues 24 h after the final administration of 5-FU identified Ki-67 positive cells, mainly in intestinal crypts. As shown in *Fig. 3A* and *3B*, 5-FU significantly decreased the proliferation of the intestinal cells (1.1±0.2 cells/crypt) compared to the control (9.3±0.8 cells/crypt). The administration of Glu significantly inhibited the decrease in cell proliferation induced by 5-FU (3.1±0.3 cells/crypt). This finding suggests that Glu hinders the decrease of cell proliferation caused by 5-FU, maintaining intestinal morphology.

Effect of glutamate on 5-fluorouracil-induced intestinal crypt apoptosis

A marked increase of TUNEL-positive apoptotic cells induced by 5-FU (50 mg/kg) was observed on day 1 (24 h after the initial treatment with 5-FU) (31). As shown in *Fig. 4A* and *4B*, the number of TUNEL-positive apoptotic cells was significantly higher in the 5-FU-treated group (3.3±0.4 apoptotic cells/crypt) than that in the control group (0.5±0.2 apoptotic cells/crypt). On the contrary, pretreatment with Glu (1000

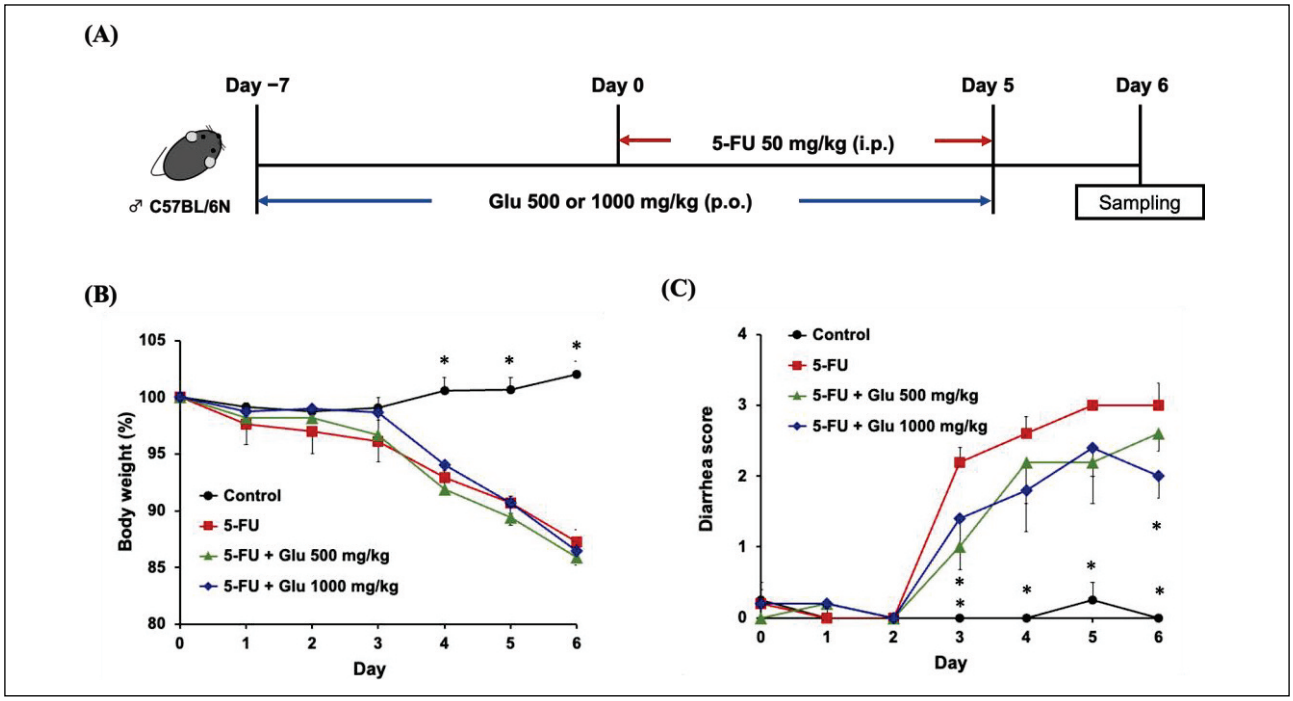

Fig. 1. Effect of glutamate (Glu) on 5-fluorouracil (5-FU)-induced intestinal mucositis. Body weight and diarrhea scores were checked daily during the 5-FU (50 mg/kg) treatment. (A): Experimental schedule for the induction of intestinal mucositis by 5-FU and pretreatment with Glu (500 and 1000 mg/kg). (B): Body weight as a percentage of the body weight on day 0 (day of 5-FU initial treatment). (C): Diarrhea was scored daily using a 5-grade scale (0–4) during the 5-FU treatment.
Values are expressed as mean ±SE (n=5 for each group). Data were analyzed as *$p<0.05$ vs. 5-FU group.

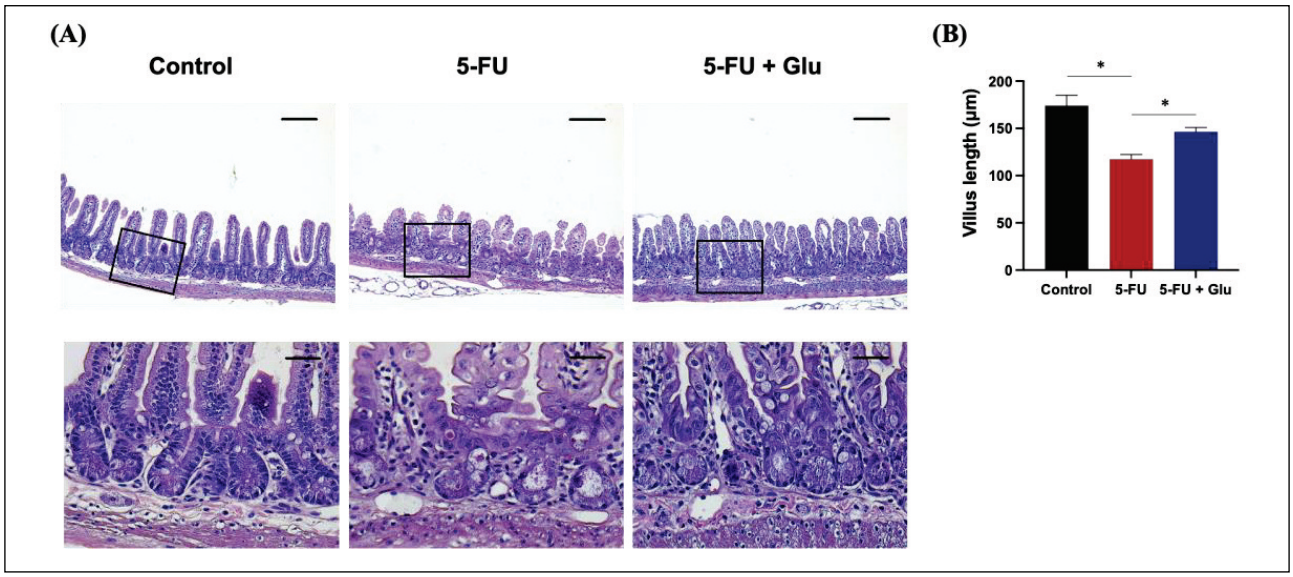

Fig. 2. Effect of Glu on 5-FU-induced histological changes in the small intestine. Ileum tissue was removed 24 h after the end of 5-FU (50 mg/kg) treatment for 6 days. Glu (1000 mg/kg) was administered twice daily for 13 days, starting 7 days before the initial treatment with 5-FU. (A): Hematoxylin and eosin (H&E) stained sections and (B): the length from the top of the *villus* to the *villus*-crypt junction as measured using light microscopy. Three of the most representative *villi* for each mouse were used for measurements. Values are expressed as mean ±SE (n=5 for each group). Data were analyzed as **p*<0.05 vs. 5-FU group. Scale bar, 200 μm (upper) and 50 μm (lower).

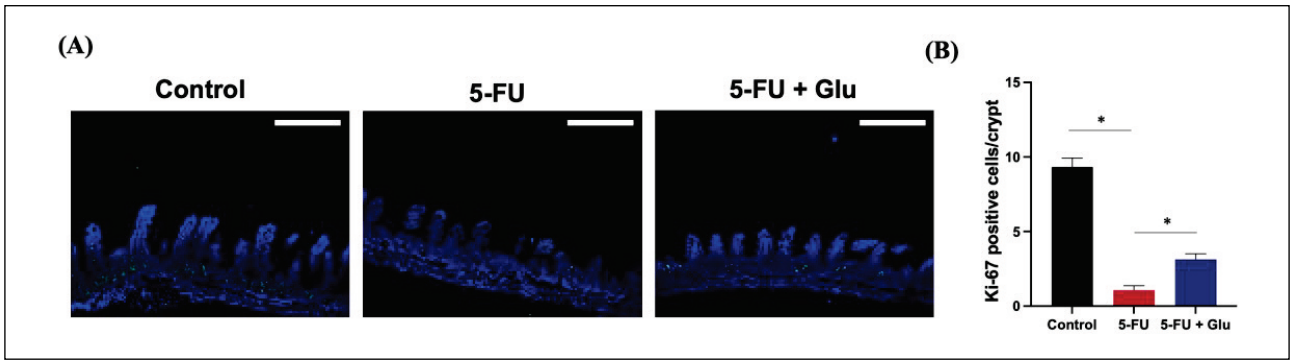

Fig. 3. Effect of Glu on the 5-FU-induced decrease in intestinal proliferation. Ileum tissue was removed 24 h after the end of 5-FU (50 mg/kg) treatment for 6 days. Glu (1000 mg/kg) was administered twice daily for 13 days, starting 7 days before the initial treatment with 5-FU. (A): Proliferative cells detected by immunostaining with Ki-67 antibody (green). (B): Number of Ki-67 positive cells counted in three of the most representative crypts from each section. Values are expressed as mean ±SE (n=5 for each group). Data were analyzed as *p<0.05 vs. 5-FU group. Scale bar, 250 μm.

mg/kg) significantly decreased the number of apoptotic cells induced by 5-FU (1.8±0.4 apoptotic cells/crypt). These findings suggest that Glu inhibits the induction of apoptosis by 5-FU.

Effect of glutamate on intestinal barrier dysfunction caused by 5-fluorouracil

FD-4 was administered p.o. 4 h before blood sample collection on day 6 to investigate intestinal permeability. Compared with the control group (0.4±0.1 μg/mL), the FD-4 concentration in the plasma of the 5-FU group (3.7±0.5 μg/mL) was significantly elevated (p<0.05). Although Glu (1000 mg/kg) treatment reduced the plasma FD-4 concentration (3.1±0.3 μg/mL) compared to that of the 5-FU (50 mg/kg) treatment, the decline was not significant (p>0.05; *Fig. 5A*).

Consistent with the results of the FD-4 concentrations in the plasma, infiltration of FD-4 in the small intestine was observed. 5-FU treatment increased FD-4 infiltration in the submucosal layers in the 5-FU group compared to that in the control group (*Fig. 5B*). However, a decrease in the infiltration of FD-4 in the ileum was observed in the 5-FU+Glu group compared to that in the 5-FU group.

To further clarify the effect of Glu on 5-FU-induced barrier dysfunction, we performed an in vitro assay using IEC-6 cells. TEER values measured in IEC-6 monolayers with or without Glu pretreatment after 24 h of 5-FU treatment revealed that 5-FU treatment for 24 h significantly decreased TEER values, wherein Glu pretreatment maintained higher TEER value than that in control (*Fig. 5C*). Taken together, the *in vivo* experiments suggest that Glu partially prevents the 5-FU-induced intestinal barrier dysfunction, wherein *in vitro* Glu improves the in 5-FU-induced alterations in cell permeability. These results suggest that Glu strengthens the barrier function in the small intestine.

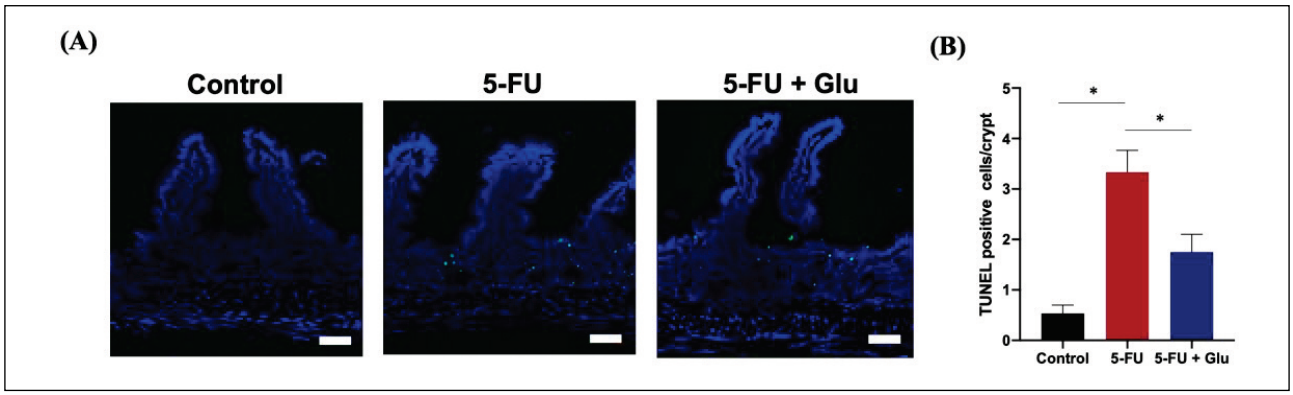

Fig. 4. Effect of Glu on 5-FU-induced intestinal crypt apoptosis. Ileum tissue was removed 24 h after the end of 5-FU (50 mg/kg) treatment for 1 day. Glu (1000 mg/kg) was administered twice daily for 8 days, starting 7 days before treatment with 5-FU. (A): Apoptotic cells detected using TUNEL staining (green). (B): TUNEL-positive cells were counted in three of the most representative crypts from each section. Values are expressed as mean ±SE (n=4–5 for each group). Data were analyzed as **p*<0.05 vs. 5-FU group. Scale bar, 100 μm.

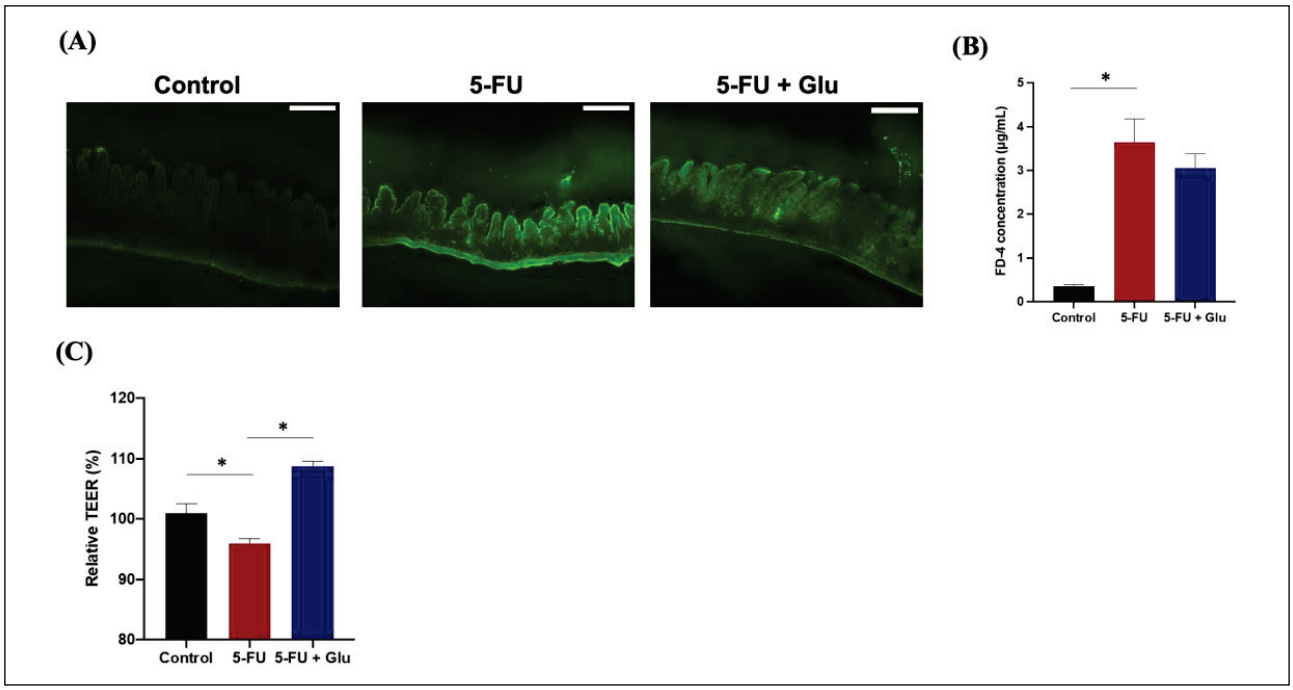

Fig. 5. Effect of Glu on the 5-FU-induced increment in intestinal permeability. Blood samples and ileum tissue were collected 24 h after the end of 5-FU (50 mg/kg) treatment for 6 days. Glu (1000 mg/kg) was administered twice daily for 13 days, starting 7 days before the initial treatment with 5-FU. Changes in TEER values in IEC-6 monolayers were measured 24 h after 5-FU treatment. (A): Intestinal permeability evaluated by measuring fluorescein isothiocyanate-dextran (FD-4) concentration in the plasma (B) and observing infiltrated FD-4 using fluorescence microscopy. (C): The TEER value determined on the day of 5-FU treatment was set as 100%. Values are expressed as mean ±SE (n=5–6 for each group or n=3 for independent experiments). Data were analyzed as **p*<0.05 vs. 5-FU group. Scale bar, 250 μm.

DISCUSSION

In this study, we demonstrate the effects of Glu on 5-FU-induced intestinal mucositis. The pretreatment duration and dose of Glu used in this study were based on our previous study and Animal Equivalent Dose (AED), respectively. In our previous study, we have shown that pretreatment with 1% MSG for 5 days recovered non-steroidal anti-inflammatory drug (NSAID)-induced small intestinal lesions in rats (32). Therefore, in this study, we followed the scheme of 7 days of pretreatment to ensure the intestine was in good condition before administering the aggressive anticancer medicine, 5-FU. Glu constitutes up to 8–10% of amino acid content in the human diet, with a daily intake of about 10–20 g/day in adults (20). Conversion of these doses using the AED calculation formula (33; shown in equation 1) revealed that the doses used in this study (1000 mg/kg/day ×2 doses) are equivalent to the recommended daily intake of humans (with an average weight of 60 kg; 10–20 g/day).

AED (mg/kg) = Human dose (mg/kg) × K_m ratio [equation 1];

(K_m ratio; human K_m/mouse K_m; human K_m=37, mouse K_m=3)

Similarly, the clinical dose of 5-FU depends on the regimen of the cancer treatment; 5–15 mg/kg/day is repeatedly administered to humans. According to the AED calculation, the

equivalent doses of 5-FU for humans (5–15 mg/kg/day) are 61.5–184.5 mg/kg for mice. Based on this calculation, the selected dose of 5-FU (50 mg/kg dose) corresponds to a low dose of 4.1 mg/kg in humans.

Diarrhea is frequently observed in patients with cancer undergoing chemotherapy, and it can be life-threatening (34). Furthermore, in a previous study, we have shown that 5 FU at 50 mg body weight (i.p.) induces body weight loss and diarrhea in mice (5). Although the detailed mechanism of the development of diarrhea induced by 5-FU is complicated and not fully understood, 5-FU may damage the intestinal mucosa, leading to apoptosis of the crypt cells and mucin hypersecretion, resulting in diarrhea (5). This study demonstrated that pretreatment with Glu did not significantly affect body weight loss but inhibited diarrhea induced by 5-FU treatment. Although bodyweight loss and diarrhea are common symptoms of chemotherapeutic-induced mucositis, studies have shown that diarrhea and mucositis occur *via* independent mechanisms (35). Interactions between epithelial cells and the microbiota greatly affect nutrition and health through the metabolism of dietary components (18). Changes in the microbiota (5, 36) and water transport proteins (37, 38) also result in diarrhea in chemotherapeutic-induced mucositis. Moreover, dietary proteins or amino acids profoundly affect the profile and function of the gut microbiota (39, 40). In particular, poly-gamma-glutamate, an anionic polymer of Glu, has been shown to modulate the microbiota by increasing the abundance of Lactobacillales and reducing the abundance of Clostridiales (41). Together with these studies, it can be inferred that the alleviative effect of Glu on 5-FU-induced intestinal mucositis is related to the regulation of intestinal microbiota.

We have previously reported that 5-FU-induced TUNEL-positive apoptotic cells in crypts were mainly observed 24 h after 5-FU treatment (5, 31). Although it has been reported that Glu may regulate cell proliferation in the small intestine (42), the effect of Glu on the induction of apoptosis has not been explored. This study shows that Glu inhibited the shortening of villus length caused by 5-FU related to cell proliferation and apoptosis. Based on previous reports, the mechanism of ameliorative effects of Glu against 5-FU-induced mucositis is thought to be associated with the activation of mTOR signaling. 5-FU induces downregulation of Akt/mTOR pathways, wherein Glu increases metabotropic glutamate receptors (mGluR) (43) and activates mGlu through phosphorylation of mTOR (44). Moreover, Glu has been reported to attenuate lipopolysaccharide (LPS) induced intestinal injury by regulating mTOR and suppressing TLR4 and NOD signaling pathways in weanling pigs (45). These studies indicate that Glu might be able to activate mTOR signaling against 5-FU-induced intestinal mucositis. It is reported that some new factors are involved in process of proliferation and apoptosis of intestinal cells have beneficial effects on cancer cells (46). Additionally, Glu is absorbed from the luminal side of the intestine through a sodium-dependent transporter and metabolized into other amino acids, and dietary supplementation of a composite of amino acids has been shown to suppress intestinal apoptosis (47, 48). However, it is currently unknown whether Glu directly or indirectly regulates 5 FU-induced intestinal apoptosis; therefore, further research is required to elucidate the underlying mechanisms.

Long-term chemotherapy changes the gut microbiota and increases intestinal permeability, resulting in what is known as a leaky gut (49). In this study, we confirmed significant FD-4 infiltration, which implies that 5-FU causes an increase in intestinal permeability. A dysfunctional intestinal barrier facilitates bacteria translocation and inflammation, which might be implicated in cancer progression. Therefore, inhibition of leaky gut caused by chemotherapeutic agents could protect against not only intestinal mucositis but also anticancer effects. Glu upregulates the expression of tight junction proteins (21) and attenuates intestinal barrier injury induced by lipopolysaccharides (50). While chemotherapy typically causes intestinal barrier dysfunction by altering (mainly reducing) the expression of tight junction proteins, the alteration depends on the type and dosage of chemotherapy (51, 52). The findings of this study suggest that Glu improves the barrier dysfunction caused by 5-FU in the small intestine. Salmenkari reported that angiotensin-converting enzyme inhibitors and angiotensin receptor blockers alleviate intestinal inflammation associated with experimental colitis (53). Since whether such pharmacological agents protect against chemotherapeutic agent-induced intestinal mucositis is not considered, testing the effect of such pharmacological agents on 5-FU-induced mucositis is of our great interest for our further study of the role of Glu.

In summary, our study demonstrated that repeated administration of 5-FU shortened the villi, disrupted intestinal crypts, increased apoptosis in the crypts, and reduced intestinal barrier function. Pretreatment with Glu for 7 days significantly suppressed the histological changes, decreased intestinal cell proliferation loss, and apoptosis induced by 5-FU. Therefore, daily administration of Glu may help suppress the intestinal damage caused by chemotherapeutic agents such as 5-FU.

Funding: This research was funded by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, grant number 18K06692 (K.A.); the Research Promotion Program for Acquiring Grants-in-Aid for Scientific Research from Ritsumeikan University (K.A.); and a research grant from Ritsumeikan Advanced Research Academy (S.J.).

Acknowledgments: We thank Dr. Shusaku Hayashi from Toyama University for his technical advice on immunohistochemical staining. We also thank Prof. Dr. Chieko Koike from Ritsumeikan University for acquisition using a confocal laser scanning microscope.

Conflicts of interest: None declared.

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Received: June 9, 2022
Accepted: August 30, 2022

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