Study on the elucidation of pathogenic mechanism underlying 5-flourouracil-induced intestinal mucositis in relation to the composition of "intestinal microbiota"

（5-フルオロウラシル誘起腸炎の病態解明と腸内細菌の関与に関する研究）

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Preface

5-Flourouracil (5-FU) is one of the most commonly used chemotherapeutic drugs in the treatment of cancer. However, about 50-80% of patients treated with 5-FU encounters intestinal mucositis characterized by severe diarrhea. Exact understanding of the sequence of events in the pathogenesis of intestinal mucositis is a crucial point for effective control and treatment. Intestinal microbiota is an important constituent of the gut environment and its balance shares in keeping a healthy gut. 5-FU was reported to cause dysbiosis, which is disturbance in composition and function of the intestinal microbiota. Therefore, in my study, I was interested to confirm the effect of 5-FU on intestinal mucositis, its chronological stages and the composition of microbiota. Then, I tried to manipulate the composition of the disturbed intestinal microbiota under 5-FU treatment using the probiotic (*Bifidobacterium bifidum* G9-1, BBG9-1) administrations. BBG9-1 had significant protective effects on the clinical symptoms and the pathology of intestinal mucositis in the 5-FU-treated mouse model. Based on these results, I suggest that probiotics are useful for the treatment of 5-FU-induced mucositis and diarrhea.

This study is divided into two chapters. First chapter entitled "Apoptosis, dysbiosis and expression of inflammatory cytokines are sequential events in the pathogenesis of 5-FU-induced intestinal mucositis in mice" has been published at ***Basic and Clinical Pharmacology and Toxicology***. Second chapter entitled "Probiotic *Bifidobacterium bifidum* G9-1 attenuates 5-fluorouracil-induced intestinal mucositis in mice via suppression of dysbiosis-related secondary inflammatory responses" has been published in the ***Clinical and Experimental Pharmacology and Physiology***.

This study will make a significant contribution in the treatment of gastrointestinal side-effects caused by cancer chemotherapy.

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Abbreviations

5-FU	5-Flourouracil
DNA	Deoxyribonucleic acid
CFU	Colony forming unit
CIGIS	Chemotherapy-induced gastrointestinal syndrome
CINV	Chemotherapy-induced nausea and vomiting
CID	Chemotherapy-induced diarrhea
CIC	Chemotherapy-induced constipation
GAM	Gifu anaerobic broth
GI	Gastrointestinal
INF-γ	Interferon-γ
i.p	Intraperitoneal
IL-1β	Interleukin-1β
LPS	Lipopolysaccharides
MPO	Myeloperoxidase
NF-κB	Nuclear factor-κB
NOX1	NADPH oxidase 1
OTU	Operational taxonomic unit
PBS	Phosphate buffer saline
PRPs	Pattern recognition receptors
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
YAMC	Young adult mouse colonic epithelium cell

Introduction

Chemotherapy-induced gastrointestinal toxicity

Most cancer patients receive curative or palliative chemotherapeutic intervention throughout the course of treatment [1]. Antimetabolites are the most common group of drugs used for chemotherapy They belong to the first effective discovered chemotherapeutics. They include folic acid, pyrimidine and purine analogues. Their chemical structure is similar to the naturally occurring compounds used in the synthesis of the nucleic acids DNA and RNA. Antimetabolites induce cell death during the S-phase of cell cycle where they are incorporated in the nucleic acids or they inhibit enzymes needed for their production [2]. 5-flourouracil (5-FU) is an uracil analogue converted by multiple alternative biochemical pathways to several cytotoxic forms [3-5]. Nausea and vomiting, anorexia and diarrhea, are commonly encountered side-effects with 5-FU treatment which may be severe and life-threatening [6-8]. 5-FU-induced diarrhea occurs in a dose dependent fashion and may be bloody. Profuse nausea, vomiting and diarrhea can lead to dehydration and hypotension [9]. Although chemotherapy has greatly improved the overall survival in many types of cancer, gastrointestinal side-effects remain the major obstacle which greatly impact the quality of life [1].

The gastrointestinal (GI) tract is a vital organ functioning for digestion and absorption of nutrients and excretion of wastes. It is not only essential for adequate nutrition but also for protection from ingested pathogens, allergens and toxins [10]. It has been reported that the incidence of chemotherapy induced GI tract side-effects is as high as 40% in the patients receiving standard dose chemotherapy and 100% of patients receiving high dose chemotherapy [10-14]. They are major obstacles facing oncologists, because they lead to delay, dose reduction and sometimes discontinuation of treatment [10]. GI tract toxicity is the main limitation in chemotherapy and results in damage to the small intestine, a condition known as GI syndrome [3]. However, the cellular targets underlying mechanisms of chemotherapy induced GI syndrome (CIGIS) are still very controversial [15]. GI tract toxicities present clinically as 4 main presentations namely, chemotherapy-induced nausea and vomiting (CINV), oral mucositis, chemotherapy-induced diarrhea (CID) and chemotherapy-induced constipation (CIC). Although the exact underlying pathology remains unclear, they are heavily associated with the development of mucositis, which is inflammation and ulceration of the GI tract mucosa anywhere from the oral cavity to the rectum [16].

Mucositis does not only decrease the quality of life in most cancer patients because of its associated intense pain, but it is also a high-risk factor for neutropenia and malnutrition. This association renders mucositis a clinically important disease [17]. GI tract mucositis has been reported in up to 80% of patients who have received cancer treatment by using 5-FU [3]. Anticancer drugs induce apoptosis of cancer cells as well as rapidly dividing normal cells [18]. The small intestine, thus, considered to be the most vulnerable to anticancer drugs, because the mucosal replacement cycle is approximately 3-4 days. GI tract mucositis leads to problems that influence the prognosis of patients, such as pain, decreased nutritional intake, increased need of intravenous nutrition and increased risk of systemic infection [19, 20]. Its prevention, early detection and treatment are therefore the most significant [21]. Currently, there is no single satisfactory strategy for the management of intestinal mucositis caused by 5-FU [22]. So, my study aimed first to understand the precise underlying pathophysiological events contributing in 5-FU-induced intestinal mucositis development and secondly, how we can prevent its severe progression from the insight of its underlying mechanisms.

Gastrointestinal mucosa: Histology and physiology

The intestine has a wide range of functions, ranging from digestion and absorption of nutrients to the secretion of important regulatory hormones. Thus, it is functioning as an immune organ and acting as a barrier against many noxious environmental agents. Proper function of the gut is therefore essential for good health and wellbeing [23-28].

The histology of the small intestinal mucosa consists of three basic layers: the inner epithelial layer (villi), the lamina propria and the muscularis mucosa [25]. In between the villi there are the crypts of Lieberkuhn. These represents the proliferative regions of the small intestine where all cells of the absorptive epithelium are generated. These cells migrate up from the base of the crypt to the villi to cover its whole surface [23, 29]. Overlying the luminal surface of the small intestine is the mucus layer. It acts as a protective barrier for the mucosa and is continuously secreted by specialized epithelial cells [23].

Four main types of cells form the epithelial layer of the small intestinal mucosa. They are the absorptive enterocytes, enteroendocrine cells, goblet cells and Paneth cells. The most numerous are the absorptive cells and their main function is the final digestion and absorption of nutrients. The goblet cells synthesize and secrete the mucin which is responsible for making the mucous layer that covers the whole epithelial surface [30]. Enteroendocrine cells produce a wide array of hormones e.g gastrin, secretin and cholecystokinin which modulate the metabolism in the small intestine and its associated

tissues [24]. Lastly, the Paneth cells which are only located at the crypt base, are thought to secrete various antimicrobial peptides, enzymes and proteins into the crypt lumen. By this function, they can keep the sterility of the metabolically active crypt region of the gut [29].

The crypts of Lieberkuhn carries the proliferative zones of the intestinal mucosa. There are approximately four to six actual stem cells in each crypt, counted from the base of the crypt [31]. Each stem cell divides nearly once per day to produce rapidly dividing clonogenic (daughter) stem cells. These daughter stem cells form a population of transit cells that may comprise about six successive cell generations. These clonogenic cells retain many characteristics of the parent cells. The stem cells produce one absorptive and three secretory cell lineages. The absorptive enterocytes, goblet cells and enteroendocrine cells move up from the crypt to the villus. In contrast, the Paneth cells differentiate and move down to the base (cell positions 1–3) of the crypt [32]. If DNA damage is found in actual stem cells, they do not attempt to repair the DNA but go immediately into apoptosis [29, 31]. The differentiated fully functional enterocytes move out of the crypt and spread over the villus surface. Once they reach the apex of the villus, they are exfoliated into the lumen. This process is continuous and, as a result, the whole epithelial surface of the intestine is replaced every 3–7 days. Intestinal epithelial cells are thus highly susceptible for affection by anti-cancer drugs [29].

The cell proliferation and development in the epithelium and its responses to trauma are modulated, to a considerable extent, by specialist cells in the lamina propria or through signals and regulatory factors sent via the lamina propria from distant locations [23]. It has been studied that the intestinal subepithelial myofibroblasts proliferate in response to several cytokines and growth factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in vitro [23] and in vivo [33]. The lamina propria also harbors an extensive network of blood and lymphatic vessels as well as mast cells, macrophages, lymphocytes and other immune cells [34]. Macrophages are found throughout the entire GI tract not only in the mucosal lamina propria, but also in the smooth muscle layers of the gut [35]. These macrophages play an essential housekeeping functions in the gut wall as clearance of apoptotic cells, and tissue remodeling during and after inflammation. They produce a variety of cytokines and other soluble factors that help maintaining tissue homeostasis. They also possess a highly phagocytic and bactericidal activities [36].

The human GI tract represents one of the largest interfaces (250-400 m^2) between the host and the environmental factors in the human body. During life time, about 60 tons of food pass through the human GI tract, along with an abundance of microorganisms from the outside environment which represents a continuous treat to gut integrity [37]. “Intestinal microbiota” is the term used to describe

the collection of bacteria, archaea and eukarya colonizing the GI tract where they co-developed with the host over thousands of years to form a mutually beneficial relationship known as 'symbiotic relationship' [38, 39]. Intestinal microbiota has been estimated to exceed about 1.0×10^{14} cells, approximately 10 times more bacterial cells than human cells and over 100 times the genomic content (microbiome) as the human genome [38, 40]. Together, the vast number of bacterial cells in the body, the host and the microorganisms inhabiting are often referred to as a 'superorganism' [40, 41].

Composition and role of the human "Intestinal microbiota"

Combined data from studies isolated over 2,000 microbiota species from human beings, classified into 13 different phyla, of which about 90% belonged to *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. Among the 13 identified phyla, three phyla contained only one species isolated from humans. For example, *Akkermansia muciniphila* is the only known representative species of the *Verrucomicrobia* phyla. In humans, 386 of the identified species are strictly anaerobic and hence will generally be found in mucosal regions such as the oral cavity and the GI tract [42].

The microbiota offers many benefits to the host, through a range of physiological functions such as strengthening of gut mucosal barrier, energy metabolism and regulation of host immunity [43-45]. However, these processes can be disturbed due to altered microbial composition, known as dysbiosis. Dysbiosis has been reported to be implicated in a large number of intestinal and extra-intestinal diseases. This is because the microbiotas which differ in terms of composition may share some degree of functional redundancy. This hypothesis forms the basis for developing therapeutic strategies to manipulate the microbial community in diseases [46].

Microbiota encodes many carbohydrate digesting enzymes, which enable them to ferment complex carbohydrates structures and yielding metabolites such as short-chain fatty acids (SCFAs) [47]. Three predominant SCFAs are butyrate, propionate and acetate, found in a ratio of 1:1:3 in the GI tract [48]. They are rapidly absorbed by enterocytes and share in the pathways of different cellular processes such as proliferation, differentiation, gene expression, chemotaxis, and apoptosis [49]. Many researchers reported that butyrate possess anti-inflammatory and anticancer activities with a specific distribution gradient within the intestinal mucosal structure, being lowest at lumen side and highest at the crypt bottom [50, 51]. This finding suggested that butyrate has a role in intestinal epithelial cells turnover and homeostasis by promoting cellular proliferation at the crypts' bottom while increasing apoptosis and exfoliation of cells at lumen side [52]. Additionally, butyrate was found to attenuate bacterial translocation and promotes gut barrier function by enhancing tight-junction assembly and mucin

synthesis [52]. Furthermore, SCFAs also play a role in regulating inflammatory response by influencing the production of various kinds of cytokines [49, 50]. For example, they stimulate the release of IL-18, which is involved in the maintenance and repair of epithelial integrity [49]. Intestinal microbiota is also a corner stone in the de novo synthesis of some essential vitamins which cannot be synthesized by the host [53]. For instance, Lactic acid bacteria are the main organisms producing vitamin B12 [53, 54]. *Bifidobacteria* are the main manufacture of folate, involved in pivotal host metabolic processes such as DNA synthesis and repair [55]. Examples of other intestinal microbiota synthesized vitamins include vitamin K, riboflavin, biotin and thiamine [56].

The development of both intestinal mucosal and systemic immune systems, is another important function of microbiota [57]. The epithelial cells' pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), can identify the molecular effectors produced by intestinal microbes. These effectors mediate processes which can ameliorate certain inflammatory gut disorders, differentiate between beneficial and pathogenic bacteria or increase the number of immune cells or PRRs [58-62]. Additionally, the physical presence of the microbiota in the GI tract affects the colonization of many pathogens by acting as a competitor for nutrient sources and attachment sites or by producing antimicrobial substances [63]. Many lines of evidence supported the role of intestinal microbiota in influencing epithelial homeostasis [57]. One interesting observation was that some "Germ-free mice" exhibited impaired epithelial cell turnover that was reversible upon microbiota colonization. In addition, these mice had an extremely thin adherent mucus layer, which was thickened after exposure to some bacterial products as Lipopolysaccharides (LPS) [64].

Given the close symbiotic relationship existing between the intestinal microbiota and the host, it is not surprising to observe a divergence from the normal microbiota composition (dysbiosis) in a plenty of disease states ranging from chronic GI diseases to neurodevelopmental disorders [65, 66]. The application of metabolomics approaches has greatly advanced our understanding of the mechanisms linking the intestinal microbiota composition and its activity to health and disease phenotypes. At a functional level, a potential way to describe a 'dysbiotic microbiota' might be one which fails to provide the host with the full complement of beneficial properties. Whether dysbiosis is a cause or a consequence of the disease, it is therefore likely to exacerbate the disease progression and affect the types of strategies needed to restore symbiosis. Depending on the type and stage of disease, these include the development of microbiome modulators (e.g. antimicrobials, diet, prebiotics or probiotics) mostly aimed at changing the composition of the host microbiota, or of microbial-based solutions to replace some of the defective microbes and their associated benefits (e.g. specific commensal strains, probiotics, defined microbial

communities, microbial-derived signaling molecules or metabolites). Given the contribution of host genetics in many diseases associated with a dysbiotic microbiota, dual therapeutic strategies (e.g. combining immunotherapy and microbiota-targeted approaches) may also be required to restore the environment required to re-establish an effective communication between the host and the targeted microbiota. Success in these strategies is dependent on our mechanistic understanding of how the microbiota affects and is affected by the host at a molecular and biochemical level [67].

To sum up, it is quite clear from the above-mentioned functions, the importance of a healthy "Intestinal microbiota" ecosystem, and its disturbance in cases of dysbiosis might greatly affect the whole intestinal homeostasis, influencing the onset or progression of different intestinal or extra-intestinal diseases.

Pathophysiology of chemotherapy-induced intestinal mucositis

According to Sonis [3, 18, 20], the development of chemotherapy-induced mucositis involves a complex and dynamic range of biological events. It often passes through five stages namely initiation, primary damage response, signal amplification, ulceration, and healing stages. Chemotherapy-induced mucositis does not only involve the intestinal mucosal epithelial cells, but also the submucosa and the supporting connective tissues.

I The initiation stage: It occurs immediately after the administration of the chemotherapeutic drug. Chemotherapy directly damages the DNA and mitochondria by generating reactive oxygen species (ROS) [68-71]. A more pronounced damage in the epithelial cells are believed to occur by ROS because they are considered to be important downstream mediators in the process of tissue damage [68, 69].

II The primary damage response stage: The DNA damage together with ROS generation trigger a cascade of complex biological events through the activation of various transcription factors [18]. Nuclear Factor-κB (NF-κB) is among the most related transcription factors to mucositis,

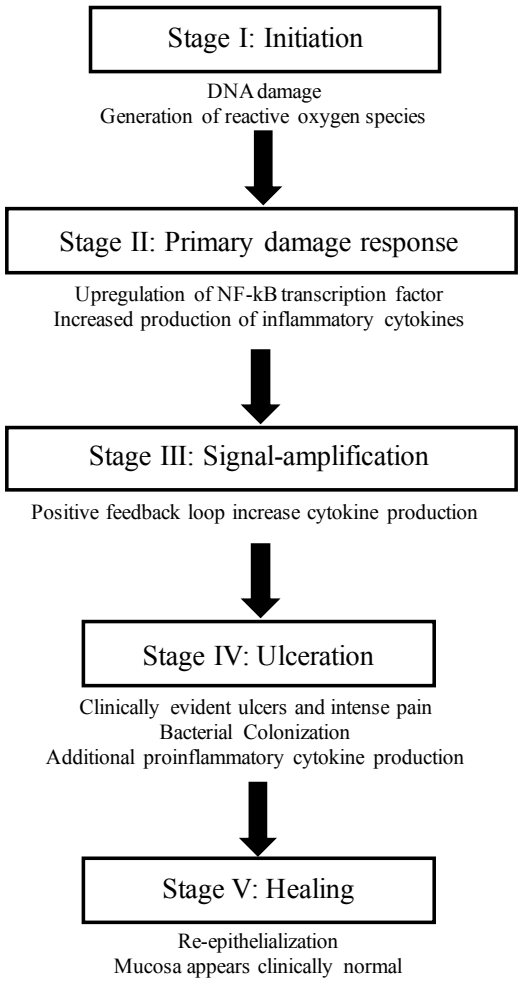

Figure 1. Different stages of mucositis development.

where its activation stimulates other genes' transcription related to mucositis progression [72]. These genes encode proinflammatory cytokines (eg, IL-1β, IL-6, TNF-α) and antioxidant enzymes [72, 73]. The submucosa starts to be affected where the release of inflammatory cytokines results in secretion of destructive metalloproteases by submucosal fibroblasts [74-76].

III The signal-amplification stage: the initial promotion of transcription factors further upregulates a wide range of inflammatory cytokines, leading to concomitant activation of other signaling pathways, magnifying the initial signals toward more tissue destruction [18].

IV The ulceration stage: here, the clinical manifestations of mucositis starts to appear because the mucosal integrity is lost and painful ulcers are formed [18]. The exposure of submucosa enables the entry of resident intestinal microorganisms and bacterial colonization. Inflammatory cytokines production is further induced by this secondary infection [20, 77]. Significant pain is the most prominent symptom, with a higher risk of systemic infection [12].

V The final healing phase: It occurs after cessation of cancer therapy, where re-epithelialization starts following the signals from submucosal extracellular matrices and mesenchymal tissue [20, 77]. At the clinical level, normal mucosal appearance is restored at this stage [18].

Although Sonis described the fundamental mechanisms involved in the pathophysiology of chemotherapy-induced mucositis [3, 18, 20], they were applied over all areas of the GI tract. Considering the histologic differences from the oral mucosa to the rectum, a more precise elucidation of the underlying mechanisms of chemotherapy induced mucositis in the small intestine need to be addressed.

Management modalities of chemotherapy-induced mucositis

Chemotherapy-induced mucositis is a multi-stage and multi-factorial disorder, where its nature, severity and duration depends on the type, duration and intensity of chemotherapy. Thus, a palliative treatment targeting only one aspect of mucositis is unlikely to be successful. As a result, a multiple therapeutic approach is required, and to be effective, a precise detailed understanding of the underlying mechanisms of mucositis is essential. The clinical treatments presently available cannot prevent mucositis, but at least can limit its severity and duration, especially in combination regimens [78].

Currently used drugs are mainly preventive or palliative for the clinical symptoms of 5-FU-induced intestinal mucositis. For chemotherapy-induced nausea and vomiting, serotonin antagonists as ondansetron and granisetron, are the drugs of choice [79]. Guidelines recommended the use of either ranitidine or omeprazole with or without folic acid supplements, for ameliorating the epigastric pain

encountered with 5-FU treatment [80, 81]. The CID is the commonest GI tract side effect of 5-FU administration and should be properly treated because it can be life-threatening with severe complications [80]. This CID is extensive and complex, resulting from many mechanisms which can be secretory, osmotic, malabsorptive, exudative and dysmotility [81]. Beside the aggressive fluid management, guidelines recommend the administration of loperamide, an opioid-receptor agonist which acts on the μ-opioid receptors in the myenteric plexus in the intestine, which function to decrease the intestinal motility by directly affecting the smooth muscle layer of the intestinal wall. Octeriotide, a somatostatin analogue, is another choice recommended as twice subcutaneous injection if loperamide failed to control the diarrhea associated with standard or high-dose 5-FU treatment [80-89]. The main aims for treating CID are, decreasing the volume of diarrhea, aggressive treatment of dehydration and preventing neutropenia and possible infections in case of prolonged diarrhea [78].

Numerous therapies to ameliorate intestinal mucositis are thus under development, because the current guideline treatment is basically a palliative one with no actual treatment of the underlying mucositis mechanism. Recently, studies focus on growth factors, cytokines and hormones which can manipulate immune responses or epithelial cell apoptosis or proliferation [78]. Their exact mechanisms of action remain unclear; however, their efficacy depends on the timing of treatment in relation to therapy and target tissue. An increase in the scavenging potential of the tissue may prevent damage to DNA or cellular components by ROS. They may block the initial inflammatory responses to chemotherapy and thereby limit the damage to the mucosa. Reduced damage to the epithelium minimizes the opportunity for pathogens to colonize or invade the mucosa. The preservation of progenitor cells, combined with reduced rates of cell loss, would facilitate rapid restoration of the epithelial structure. Some palliative treatments appear to modify or stimulate subepithelial fibroblasts. This may aid restitution and repair in the epithelial layer [18].

From the previously mentioned importance of intestinal microbiota in the intestinal environment and the new approaches targeting the underlying mechanisms not just a palliative treatment, I was interested to investigate their exact role in the pathogenesis of 5-FU-induced intestinal mucositis. This was done by experimental simulation of Sonis model of chemotherapy-induced intestinal mucositis [18] followed by trials to check on the intestinal microbiota constituents and observing the effect of its manipulation in mice.

Chapter I: Apoptosis, dysbiosis and expression of inflammatory cytokines are sequential events in the pathogenesis of 5-FU-induced intestinal mucositis in mice.

1. Background

In previous studies, we have reported that apoptosis is the main initial event during induction of intestinal mucositis [90]. The ROS produced by NADPH oxidase 1 (NOX 1) were the key mediators of the process of apoptosis [91]. However, some reports showed that 5-FU induced intestinal mucositis not only induce apoptosis but also causes recruitment of neutrophils with further release of inflammatory cytokines causing abnormal inflammation [92-95]. During the progress of intestinal mucositis by 5-FU, dysbiosis has also been reported but the exact sequence of events was not fully investigated [88]. The pathways by which intestinal-microbiota can stimulate the release of more inflammatory cytokines are still questioned. So, the first aim is to investigate the chronological events through which 5-FU induce mucositis in term of apoptosis, dysbiosis and inflammatory cytokines.

2. Materials and Methods

2.1 Animals

Animal studies were performed in compliance with the ARRIVE guidelines [96]. The protocols were approved by the committee on the Ethics of Animal Research of Kyoto Pharmaceutical University (Permission Number: 18-17-007). Male C57BL/6J mice 22–26 g and 8–9 weeks old (SLC Co., Shizuoka, Japan) were acclimated to standard laboratory conditions with a 12-hr light/dark cycle and temperature 22 ± 1°C and were maintained in plastic cages with free access to food and water. Experiments were carried out using six to eight mice per group under un-anaesthetized conditions.

2.2 Induction and assessment of intestinal mucositis

Animals were given 50 mg/kg 5-FU by intraperitoneal (i.p.) injection once daily for 6 days (days 0–5), while control animals received saline. 5-FU, was obtained from Sigma-Aldrich (St. Louis, MO, USA), dissolved in physiological saline, and prepared immediately before injection at 0.1 mL/10 g body-weight. Disease severity was assessed daily by body-weight measuring and stool consistency scoring (0–4: 0 = normal, 1 = soft but still formed, 2 = very soft, 3 = diarrhea and 4 = severe diarrhea, watery stool with severe perianal staining) [97]. On days 1, 2, 4 and 6 following exposure to 5-FU, animals were killed under deep anesthesia, and the jejunum was collected and immersed overnight in 10% neutralized formalin. Tissue samples were excised, embedded in paraffin, sectioned at 4-μm and stained with haematoxylin/eosin. Measurement of the villus height (from the top of the villus to the villus–crypt junction), and crypt damage (surviving crypts per millimeter and surviving cells per crypt by counting the number of nuclei in each crypt) were performed using a light microscope at magnifications of 100× and 1000× respectively (Model BX-51; Olympus, Tokyo, Japan) fitted with a digital camera system (DS-Ri1; Nikon, Tokyo, Japan). Five intact and well-oriented villi and crypts per sample were measured and averaged. Data were collected by two investigators blinded to experimental groups.

2.3 Measuring the myeloperoxidase (MPO) activity

Jejunum tissues were rinsed with cold phosphate-buffered saline (PBS), weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Wako). The homogenized samples were subjected to three freeze-thaw cycles, then centrifuged at 2,000 × g for 10 min at 4°C. MPO activity was determined by adding 5 µl of supernatant to 95 µl of 10 mM phosphate buffer (pH 6.0) and 50 µl of 1.5 M o-dianisidine hydrochloride (Sigma-Aldrich) containing 0.0005%

(w/v) hydrogen peroxide. Changes in absorbance at 450 nm were recorded using a microplate reader (VERSAmax; Molecular Device, Sunnyvale, CA, USA). Sample protein content was estimated spectrophotometrically (Pierce protein assay kit; Pierce, Rockford, IL, USA), and MPO activity was obtained from the slope of the reaction curve according to the following equation: Specific activity (μmolH_2O_2/min/mg protein) = (OD/min) / {OD/min H_2O_2} × mg protein [98]. MPO is the most abundant enzyme stored in the azurophyllic granules of neutrophilic granulocytes. Evaluating MPO activity is crucial to understand its effect on inflammation [98].

2.4 Determination of proinflammatory cytokines mRNA expression in the small intestine by real-time RT-PCR

Animals were killed by CO_2 inhalation on days 0 (without 5-FU treatment). 0.5 (12 h), 1, 3 and 5 after initial 5-FU administration; and the jejunum tissues were removed, washed with cold PBS and immersed in RNAlater (Ambion, Austin, TX, USA) at 4°C until use. Total RNA was extracted from whole jejunum layer using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions and reverse-transcription (RT) was performed using RevaTra Ace-alpha with random hexamers (Toyobo, Osaka, Japan). Expression of target genes was quantified on a Thermal Cycler Dice Real Time System (Takara), using SYBR Premix ExTaq (Takara) and Perfect Real-Time reagents (Takara) for β-actin (primer set ID: MA050368), TNF-α (primer set ID: MA097070) and IL-1β (primer set ID: MA025939). Expression levels for each mRNA were standardized to that of β-actin mRNA, calculated using the comparative C_T ($\Delta\Delta C_T$) method, and normalized to mean value of day 0 or to control animals not treated with 5-FU at each time point.

2.5 Analysis of apoptosis

Animals were killed 24 and 48 hours after initial 5-FU administration (days 1 and 2, respectively), and jejunum samples were fixed with 10% neutralized formalin, embedded in paraffin and cut into 4-μm thick sections. Apoptosis of enterocytes in the small intestine was detected by terminal deoxyneucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an In-Situ Apoptosis Detection Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. For each sample, the number of TUNEL-positive apoptotic cells from 10 crypts was counted and averaged at a magnification of 1,000 × under a light microscope (model BX-50; Olympus).

2.6 Extraction of bacterial DNA from faecal samples

Faecal samples were suspended in PBS and centrifuged at 14,000 × g, and pellets were suspended in 100 mM Tris–HCl pH 9.0, 40 mM EDTA and 1% sodium dodecyl sulphate. The suspension was mixed with buffer-saturated phenol and 0.1 mm glass beads and shaken at 4000 rpm for 10 sec. in Micro Smash MS-100 (TOMY, Tokyo, Japan). After centrifugation at 14,000 × g for 5 min., the supernatant was collected, extracted with phenol–chloroform and precipitated with isopropanol. The resulting DNA pellet was washed with 70% ethanol, dried and dissolved in 10 mM Tris–HCl pH 8.0 and 1 mM EDTA.

2.7 Sequencing and analysis of bacterial 16S rDNA

The structure of the intestinal microbial community was analyzed based on the V3–V4 region in 16S rRNA, following published methods [99]. Amplified 16S rDNA V3–V4 fragments were purified with AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). The Illumina Nextera XT Index Kit (Illumina, San Diego, CA, USA) with dual 8-base indices was used to allow multiplexing. Purified barcoded libraries were then quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). Subsequently, multiplexed library pools (10 pM) were spiked with 12.5% PhiX control DNA (10 pM) to improve base calling, and sequenced with MiSeq Reagent Kit v2 chemistry (Illumina) using 2 × 250-bp paired-end runs on a MiSeq platform (Illumina). Reads were then demultiplexed and cleared of index bases in MiSeq Reporter (Illumina), filtered (QV ≥ 30) and merged using CLC Genomics Workbench (CLC Bio, Aahus Denmark). 16S rDNA sequences were analyzed by homology using Local RDP Classifier (World Fusion, Tokyo, Japan) and assigned to genera at 50% confidence using Metagenome@KIN (World Fusion).

2.8 Quantitative PCR for bacterial 16S rDNA

Quantitative real-time PCR was performed on an ABI Prism 7000 SDS (Thermo Fisher Scientific, Waltham, MA, USA), using published primer sets (Table 1) [100, 101] and POWER SYBR Green PCR Master Mix (Thermo Fisher Scientific). Standard curves were constructed from serial dilutions of DNA from type strains. Targets were amplified over one cycle at 50°C for 2 min. and one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 1 min at 61.5°C for Akkermansia muciniphila or at 60°C for all others.

Table 1. Phylum-specific primers and type strains.

Target	Primer name	Sequence	Type strain
Firmicutes	Firm928F	TGAAACTYAAAGGAATTGACG	*Lactobacillus acidophilus JCM1132*
	Firm1040R	ACCATGCACCACCTGTC	
Bacteroidetes	Bact798F	CRAACAGGATTAGATACCCT	*Bacteroides fragilis JCM11019*
	Bact967R	GGTAAGGTTCCTCGCGTAT	
y-Proteobacteria	yPro1080F	TCGTCAGCTCGTGTYGTGA	*Escherichia coli JCM1649*
	yPro1202R	CGTAAGGGCCATGATG	
Actinobacteria	Act920F	TACGGCCGCAAGGCTA	*Bifidobacterium longum JCM1217*
	Act1200R	TCRTCCCCACCTTCCTCCG	
Verrucomicrobia (Akkermansia muciniphila)	AKmucF	CAGCACGTGAAGGTGGGGAC	*A. muciniphila BAA-835*
	AKmucR	CCTTGCGGTTGGCTTCAGAT	

2.9 Administration of ampicillin or aztreonam on 5-FU-induced intestinal mucositis

Intestinal mucositis was induced in mice (6-8 mice/group, 4 groups) using 5-FU and accompanied by administration of the broad-spectrum antibiotic ampicillin (250mg/kg) and the selective gram-negative antibiotic aztreonam (50 mg/kg) [102, 103] and the same parameters were measured. Antibiotic administered per os was done twice daily for 6 days, with control animals receiving carboxymethylcellulose. Ampicillin and aztreonam were obtained from Wako (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan), respectively. The two antibiotics were suspended in carboxymethylcellulose (Nacalai Tesque, Kyoto, Japan) and prepared immediately before oral administration at 0.1 mL/10 g body-weight.

2.10 Determination of proinflammatory cytokines mRNA expression in cell culture by real-time RT-PCR

Young adult mouse colonic epithelium (YAMC) cells, isolated from either the small intestine or colon of young adult mice and cultured in conditioned medium from a human colon carcinoma cell line, were kindly provided by Dr. Robert Whitehead (Vanderbilt University, Nashville, TN, USA) [104], were cultured to confluence at 33°C in RPMI1640 supplemented with heat-inactivated 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, insulin–transferrin–selenium premix (Wako) and 5 U/mL murine IFN-γ (Invitrogen, Grand Island, NY, USA). Cytokine expression was assessed after cells in a 12-well microplate were directly exposed to 10^{3}–10^{6} μM 5-FU for 6 hours. YAMCs were washed with ice-cold PBS, and total mRNA was extracted from YAMC cells using Sepasol-RNA I Super G (Nacalai Tesque. Inc.) and cDNA was synthesized using primers, probes and PrimeScript RT Master Mix (TaKaRa Bio Inc., Shiga, Japan). Pre-designed primers and probes for the genes encoding β-actin (primer set ID: MA050368), TNF-α (primer set ID: MA097070) and IL-1β (primer set ID: MA025939) were purchased from TaKaRa Bio Inc. Quantitative RT-PCR was performed in the Thermal Cycler Dice

Real Time System (TaKaRa), using SYBR Premix ExTaq II (TaKaRa). The levels for mRNA expression for genes encoding TNF-α and IL-1β were standardized to that of β -actin mRNA, calculated using the comparative $\Delta\Delta$Ct method, and normalized to mean value of control cells not treated with 5-FU.

2.11 Statistical analysis.

Data are reported as mean ± S.E.M. from six to eight animals per group. Data were analyzed in GraphPad Prism 6.0 h (GraphPad Software, La Jolla, CA, USA). Parametric data were analyzed by Student's t-test and one-way analysis of variance followed by Holm–Sidak's multiple comparison test, whereas nonparametric data were analyzed by Mann–Whitney U-test and Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparison test. $p < 0.05$ was considered statistically significant.

3. Results:

3.1 Effect of 5-FU administration on body-weight, stool score and intestinal histology

Repeated administration of 50 mg/kg 5-FU caused significant weight loss on day 5 after initial exposure to 5-FU, with mean body-weight dropping to 83.6% of control animals on day 6 (Fig. 2A). 5-FU also caused clinical diarrhea beginning on day 3, with mean diarrhea score 2.8 ± 0.2 on day 6 (Fig. 2B). Histologically, the drug caused severe intestinal mucositis characterized by shortened villi, decreased number of crypts and crypt cells (Fig. 2C). Significant histological changes were observed from day 2, with mean villus height (Fig. 2D), number of crypts (Fig. 2E) and number of crypt cells (Fig. 2F) on day 6 decreasing to 48.7%, 31.4% and 33.1% of those of control animals, respectively.

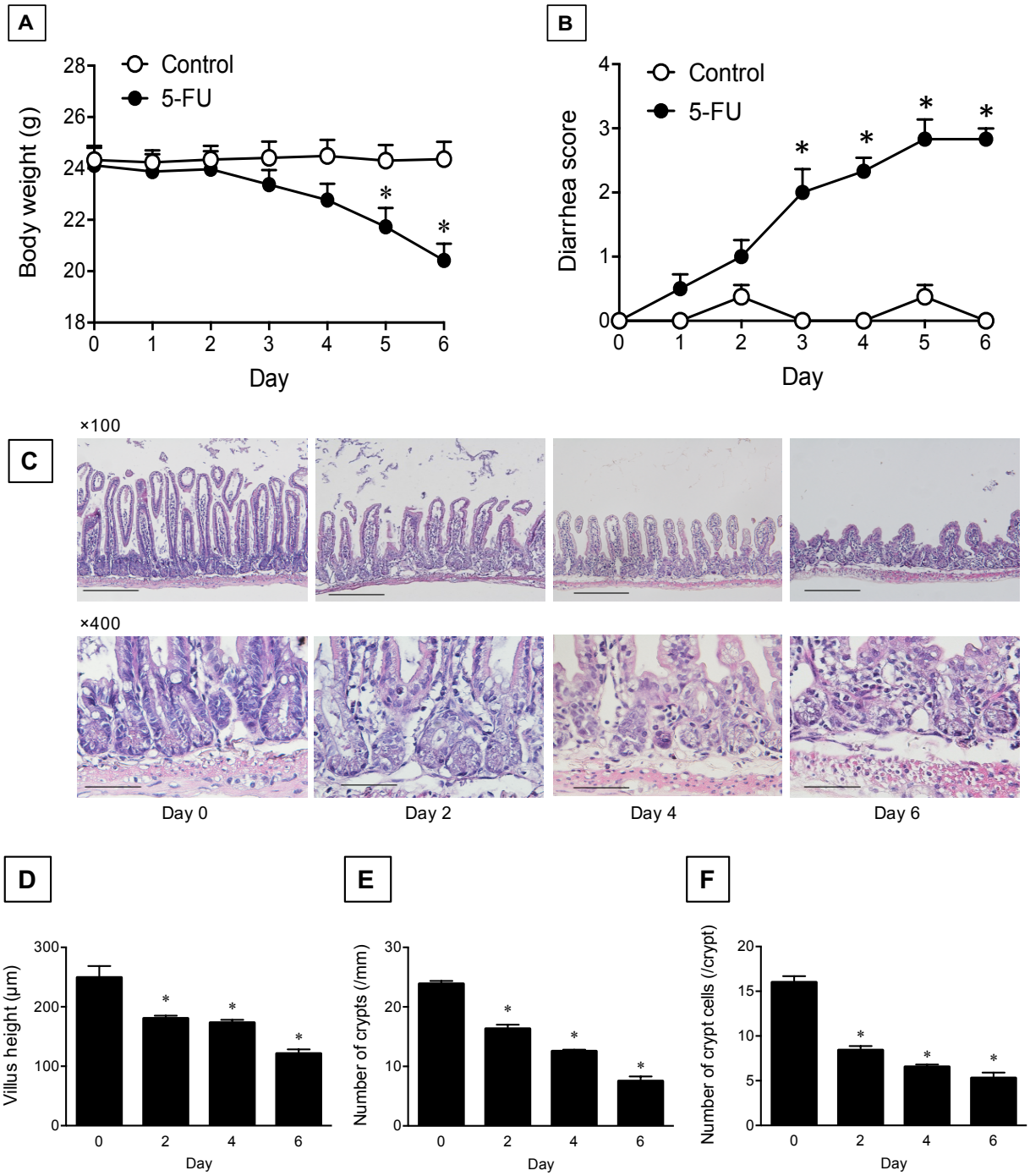

Figure 2. Body-weight, diarrhea and histological changes in mice intraperitoneally injected with 50 mg/kg 5-fluorouracil (5-FU) once daily for 6 days (days 0–5). Body-weight was measured daily (A), while diarrhea was scored daily using a 5-point scale (0–4) as described in Materials and Methods (2). The jejunum was obtained on days 0 (control; n = 8), 2 (n = 8), 4 (n = 8) and 6 (n = 6), stained with haematoxylin/eosin and imaged at 100× (top panels) and 400× (bottom panels, C). The height from the top of the villus to the villus–crypt junction (D), the number of crypts per millimetre (E) and cells per crypt (F) were measured under light microscopy. Data are mean ± S.E.M. *p < 0.05 versus control (not treated with 5-FU). These figures are cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):162, Figure 1(A-F).**

3.2 Intestinal myeloperoxidase activity, cytokine expression, apoptosis and cell proliferation during 5-FU treatment

Repeated administration of 5-FU significantly increased the intestinal MPO activity 9.5 folds on day 4 (Fig. 3A). Similarly, 5-FU up-regulated the expression of TNF-α on day 1, with a further spike in expression on day 4 to 9.7 ± 2.7 times in comparison with untreated mice (Fig. 3B). Similarly, IL-1β expression jumped 7.6 ± 0.6 times on day 4. The drug also induced apoptosis in intestinal crypts (Fig. 3C) on day 1, with 82.5 ± 11.0 apoptotic cells/mm in treated mice and 1.3 ± 0.6 apoptotic cells/mm in control animals (Fig. 3E). However, the number of apoptotic cells in treated animals diminished to 21.8 ± 0.6 cells/mm on day 2 (Fig. 3E). Finally, the drug suppressed cell proliferation in intestinal crypts (Fig. 3D), decreased on day 1 to 71.1% in comparison with control animals (Fig. 3F).

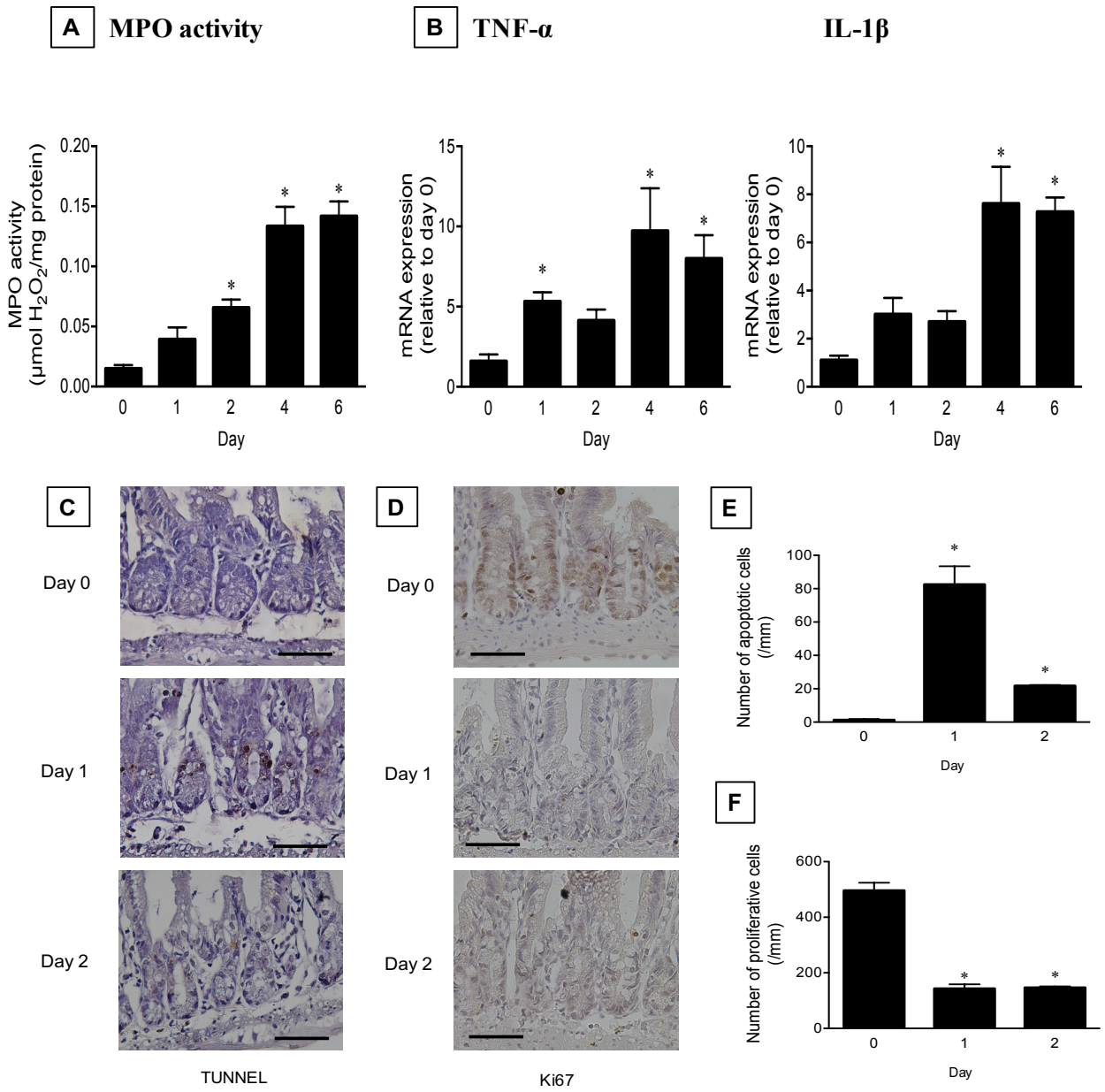

Figure 3. Myeloperoxidase activity (MPO), TNF-α and IL-1β mRNA expression, apoptosis and cell proliferation in the small intestine in mice intraperitoneally injected with 50 mg/kg 5-fluorouracil (5-FU) once daily for 6 days (days 0–5). The jejunum was collected on days 0 (control; n = 8), 1 (n = 8), 2 (n = 8), 4 (n = 8) and 6 (n = 6). MPO was determined using o-dianisidine (A), while TNF-α and IL-1β mRNA expression was quantified by real-time RT-PCR (B). Expression was normalized to β-actin mRNA, and to the mean value in control (5-FU- untreated) mice. Apoptosis in intestinal crypts was assessed using TUNEL assay (C, 400×) and quantified using a light microscope on days 0 (control), 1 and 2 (E). Similarly, cell proliferation in intestinal crypts was determined immunohistochemically using antibodies against Ki-76 (D, 400×) and quantified under a light microscope on days 0 (control), 1 and 2 (F). Data are represented as mean ± S.E.M. *$p < 0.05$ versus control (not treated with 5-FU). These figures are cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):163, Figure 2(A-F)**.

3.3 Effect of co-administration of ampicillin or aztreonam with 5-FU on body-weight, stool score and intestinal histology

Twice-daily co-administration of 250 mg/kg ampicillin or 50 mg/kg aztreonam mitigated weight loss (Fig. 4A) and diarrhea (Fig. 4B) induced by 5-FU. On day 5, the antibiotics significantly restored 5-FU-induced weight loss. On the other hand, the antibiotics did not prevent mild diarrhea on day 1, but significantly reduced 5-FU-induced severe diarrhea on day 6. Similarly, the antibiotics significantly diminished villi shortening (Fig. 4C, D) and crypt degradation (Fig. 4C, 4E) due to 5-FU administration on day 6. Loss of crypt cells due to 5-FU (Fig. 4C, 4F) was also significantly recovered on day 6.

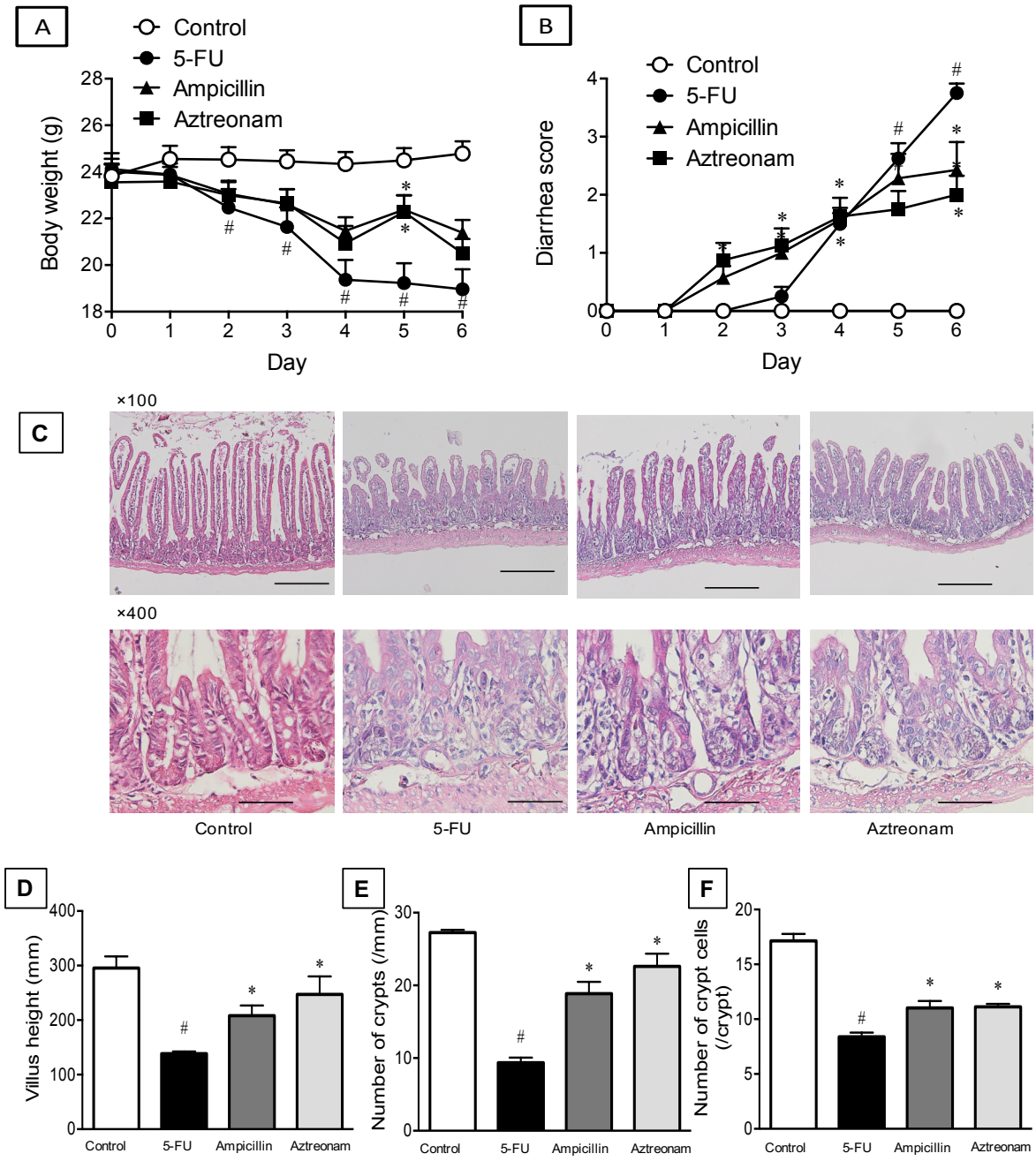

Figure 4. Effect of co-administered antibiotics on weight loss, diarrhea and intestinal mucositis due to 5-fluorouracil (5-FU). Animals received an intraperitoneal injection of 50 mg/kg 5-FU once daily for 6 days (days 0–5), with 250 mg/kg ampicillin (n = 6) and 50 mg/kg aztreonam (n = 6) co-administered per os twice daily. Body-weight was measured daily (A), while diarrhea was scored daily using a 5-point scale (0–4) as described in Materials and Methods (B). Jejunum tissues collected on day 6 were stained with haematoxylin/eosin and imaged at 100× (top panels) and 400× (bottom panels, C). The height from the top of the villus to the villus–crypt junction (D), the number of crypts per millimeter (E) and cells per crypt (F) were measured by light microscopy. Data are mean ± S.E.M. #$p < 0.05$ versus untreated animals from control (not treated with 5- FU, n = 8); *, versus animals treated with 5-FU only (n = 7). These figures are cited from ***Basic Clin Pharmacol Toxicol.* 2017;121(3):164, Figure 3(A-F).**

3.4 Effects of co-administeration of ampicillin or aztreonam with 5-FU on biochemical indices

Twice-daily co-administration of ampicillin and aztreonam significantly blocked the 5-FU-associated increase in MPO activity (Fig. 5A). Likewise, up-regulation of TNF-α and IL-1β expression due to 5-FU was significantly suppressed on day 6 (Fig. 5B). In contrast, the antibiotics did not affect 5-FU-induced apoptosis (Fig. 5C, 5D) and up-regulated TNF-α expression on day 1 (Fig. 5E).

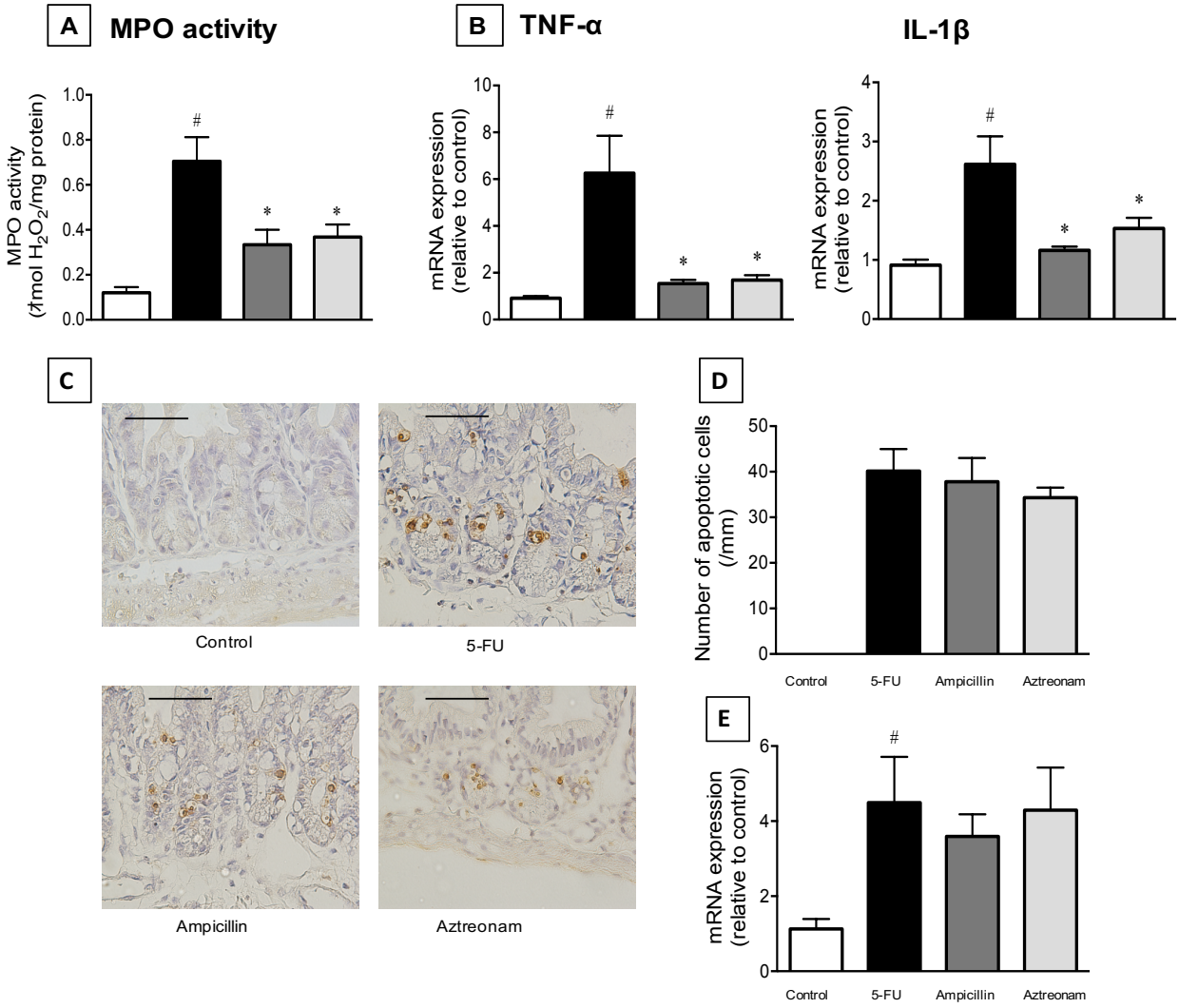

Figure 5. Effect of co-administered antibiotics on 5-fluorouracil (5-FU)-induced increase in intestinal myeloperoxidase (MPO) activity, TNF-α and IL-1β mRNA expression and apoptosis. Animals received 50 mg/kg 5-FU by intraperitoneal injection once daily for 6 days (days 0–5), with 250 mg/kg ampicillin (n = 6) and 50 mg/kg aztreonam (n = 6) simultaneously administered per os. The jejunum was obtained on day 6. MPO activity was measured with o-dianisidine (A), while TNF-α and IL-1β expression was quantified by real-time RT-PCR (B). Expression was normalized to β-actin, and to the mean value in control mice not treated with 5-FU. Apoptosis in intestinal crypts was assessed on day 1 using TUNEL (C, 400×) and quantified on a light microscope (D). Intestinal TNF-α expression was also quantified on day 1 (E). Data are mean ± S.E.M. #$p < 0.05$ versus control (not treated with 5-FU, n = 8); *$p < 0.05$ versus animals treated with 5-FU only (n = 7). These figures are cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):165, Figure 4(A-E).**

3.5 Effect of 5-FU and co-administration of antibiotics on intestinal microbiota in mice

A total of 11 phyla were detected in mice microbiota, of which *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* were dominant. These phyla comprised 99.5% of the microbiota in control animals, 99.0% in animals exposed to 5-FU and 98.6% in animals treated with ampicillin and 5-FU (Figure 6). In control animals, the ratio of *Firmicutes* to *Bacteroidetes* was 1.52, with abundance of 56.3% and 37.1%, respectively. Repeated administration of 5- FU decreased the abundance of *Firmicutes* to 30.7%, but increased that of *Bacteroidetes* to 51.7%, resulting in a ratio of 0.59. In contrast, daily co-administration of ampicillin increased the abundance of *Firmicutes* to 73.6% and decreased the abundance of *Bacteroidetes* 9.6%, resulting in an increased ratio of 7.6. 5-FU treatment also increased the abundance of *Verrucomicrobia* 3.3 times in comparison with control animals. However, this response was completely blocked by ampicillin, which reduced *Verrucomicrobia* abundance 0.78 times relative to control animals. The observed changes in intestinal microbiota were confirmed by quantitative PCR using phylum-specific primers. We found that 5-FU treatment decreased both *Firmicutes* and *Bacteroidetes* to 53.8% and 75.3% of levels in control animals, respectively. We note that the decrease was larger in the former than in the latter (Table 2). Interestingly, *Verrucomicrobia* significantly increased 3.1 times after exposure to 5-FU, while daily co-administration with ampicillin strongly reduced the abundance of all dominant phyla.

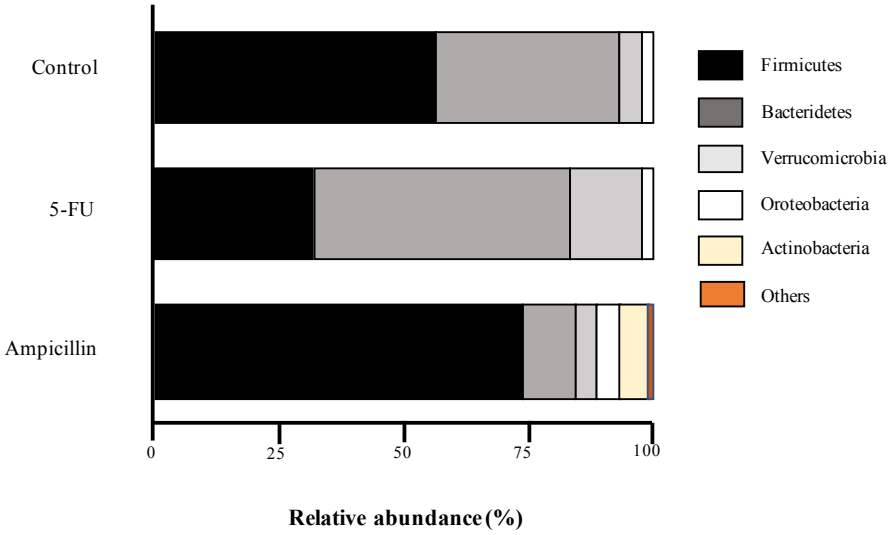

Figure 6. Effect of antibiotics on 5-fluorouracil (5-FU)-induced changes in intestinal microbiota. Animals were administered 50 mg/kg 5-FU by intraperitoneal injection once daily for 6 days (days 0–5), with 250 mg/kg ampicillin co-administered per os. Caecum faeces were collected on day 6, and the relative abundance of enterobacterial phyla was analyzed by next-generation sequencing of bacterial 16S rDNA. This figure is cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):165, Figure 5.**

Table 2. Effect of co-administered ampicillin on 5-FU-induced changes in mouse bacterial flora.

	Firmicutes	*Bacteroidetes*	*Proteobacteria*	*Actinobacteria*	*Verrucomicrobia (Akkermansia muciniphila)*
Control	2383.8 ± 168.8	743.2 ± 83.3	19.5 ± 2.5	6.1 ± 0.5	14.2 ± 4.1
5-FU	1283.1 ± 225.1 *	560.3 ± 62.3*	24.0 ± 2.7	3.9 ± 0.6*	44.6 ± 12.9*
Ampicillin	1.7 ± 0.7 * *	0.05 ± 0.01**	0.03 ± 0.01 **	0.06 ± 0.02 **	0.01 ± 0.00 **

5-FU, 5-fluorouracil.

Animals were administered 50 mg/kg 5-FU by intraperitoneal injection once daily for 6 days (days 0-5), with 250 mg/kg ampicillin co-administration *per os* twice daily. Caecum faeces were collected on day 6, and bacterial 16S rDNA was quantified by PCR. Data are mean ± S.E.M. from six animals per group.

*$p < 0.05$ *versus* control (not treated with 5-FU).

**$p < 0.05$ *versus* animals treated with 5-FU only.

This table is cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):166, Table 2.**

3.6 Effect of 5-FU on proinflammatory cytokine expression in YAMC cells

5-FU up-regulated TNF-α m RNA expression in YAMC cells in dose-dependent fashion (Fig. 7A). TNF-α was significantly up-regulated upon exposure to 10 μM of 5-FU, with expression increasing 3.5 times at 1000 μM. In contrast, 5-FU did not promote the expression of IL-1β (Fig. 7B).

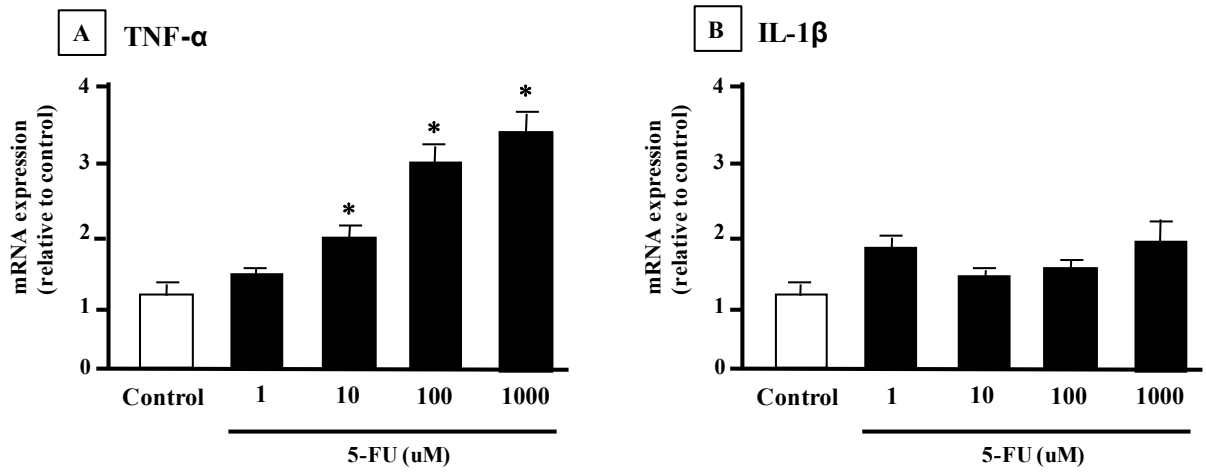

Figure 7. Effect of 5-fluorouracil (5-FU) on expression of TNF-α (A) and IL-1β (B) mRNA in Young adult mouse colonic epithelium cells (YAMC) exposed for 6 hours to 1–1000 μM 5-FU. Data are mean ± S.E.M. from five independent experiments. *$p < 0.05$ *versus* control (not treated with 5-FU). These figures are cited from ***Basic Clin Pharmacol Toxicol.*** **2017;121(3):166, Figure 6.**

4. Discussion

Despite the broad general knowledge regarding chemotherapy-induced intestinal mucositis, the exact pathogenesis is still not fully understood [78, 105, 106]. In our study, we try to clearly identify the precise sequential events underlying the pathogenesis of mucositis caused by 5-FU as one of the most commonly used agents incriminated in the development of mucositis in cancer patients.

On day 1, the only remarkable observations were the initial burst of the TNF-α inflammatory cytokine and the significant detection of apoptotic cells accompanied by reduction in the proliferative cells in the intestinal crypts. It is well known that activated macrophages are the main source of TNF-α production among inflammatory cells [107, 108]. However, on day 1, almost no signs of inflammation were detected histologically which could explain the initial detectable rise in TNF-α. Several reports investigating the pathology in intestinal diseases, had claimed that intestinal epithelial cells by themselves represent a cellular source of TNF-α. This ability allows the intestinal epithelial cells to initiate and introduce early inflammatory signals to the surrounding epithelial and inflammatory cells in the lamina propria [109-111]. These reports are in line with our *in vitro* results. We showed that TNF-α mRNA expression in YAMC cells was significantly elevated after exposure to ascending concentrations of 5-FU for 6 hours. This was not the case with IL-1β which was not elevated. Some other reports suggested that the continuous exposure and cross-talking between the intestinal epithelial cells and the intestinal microbiota under physiological conditions created a proinflammatory function in the intestinal epithelial cells especially Paneth cells [112, 113]. The TNF-α released from the intestinal epithelial cells were found to act on nearby epithelial cells initiating apoptosis, and on the adjacent inflammatory cells in lamina propria creating a positive feedback response which might explain the second surge of inflammatory cytokines on day 4 [109-111]. The Paneth cells are one of the major sources of TNF-α. This can explain why the apoptotic cells were mostly localized in the intestinal crypts, the main site of Paneth cells [112].

On the following day, the number of TUNEL-positive apoptotic cells start to decrease reaching lowest levels on day 2, which indicates that apoptosis is an initial event, even if temporary in the pathogenesis of 5-FU-induced intestinal mucositis [90, 97, 99]. The occurrence of apoptosis was previously confirmed in other studies during the first 24 hours after exposure to 5-FU due to several suspected mechanisms. First of all, we have previously reported that activation of caspase-3 during 5-FU treatment is the main downstream effector by cleaving the majority of cellular substrates and thereby initiating apoptosis [114, 115]. The localization of TUNEL-positive apoptotic cells was matching with the

observed pattern of the immunopositive cells for cleaved caspase-3, the active form of caspase-3. This has been shown to occur through alteration in the pre- and anti-apoptotic proteins such as bax and bcl-2 [95]. Caspase-3 is also known to stimulate the release of TNF-α which was confirmed to be elevated on day 1 [116, 117]. This extrinsic TNF-α-induced apoptosis via caspase-3 in intestinal epithelial cells was also observed in Jin et al [118]. Secondly, it has been reported that activation of NF-κB is involved in 5-FU-induced intestinal mucositis as a key transcriptional regulator of inflammatory responses including TNF-α expression in intestinal epithelial cells. The NF-κB pathway is thus likely to upregulate TNF-α expression in response to 5-FU from day 1 [119, 120]. ROS generated from DNA damage, is known to activate transcriptional factors and results in a series of processes defined as "acute tissue reactions" that exerts direct damage on many cell types. NF-κB is among these factors [68, 69]. Another expected apoptotic pathway is mediated by death receptors belonging to the TNF receptor superfamily namely receptors for Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL). However, this pathway has been implicated in apoptosis associated with inflammatory bowel diseases (IBD) and not 5-FU induced intestinal mucositis. Further studies are needed to elucidate such role. Furthermore, some studies reported that augmentation of NOX1-derived ROS production induced by 5-FU was contributed to intestinal crypt cell apoptosis [121].

The anti-cancer mechanism of 5-FU entails blocking of DNA synthesis by inhibiting topoisomerase II enzyme in rapidly proliferating cancer cells. As a result, the rapidly growing progenitor cell population at the bottom of intestinal crypts are particularly affected. Thus, leading to mitotic inhibition, disruption of cell-to-cell interactions and impaired epithelial integrity. Therefore, hypo-proliferation was observed as early as apoptosis in the intestinal crypts as a result of direct cytotoxicity of 5-FU on intestinal cells [122].

From these observations, it can be concluded that loss of epithelial integrity by induction of apoptosis and suppression of progenitor cells proliferation by 5-FU direct cytotoxic effect are the initial steps in the occurrence of intestinal mucositis with absence of observed systemic clinical affection at same time point. Administration of ampicillin and azterionam, however, did not show any effect on apoptosis and upregulation of TNF-α on day 1. Apart from Sonis model of chemotherapy-induced mucositis, our results clearly elucidated the occurance of apoptosis as a very early event in the pathogenesis of 5-FU-induced intestinal mucositis

On the following days, it was clear that repeated administration of 5-FU results in severe intestinal mucositis reaching its peak on day 5. This mucositis is histologically characterized by significant

shortening of the intestinal villi and significant destruction of the intestinal crypts' morphology. These findings agree with previous reports about the damaging effect of 5-FU administration in experimental mucositis in mice [6]. Furthermore, the onset of significant weight loss and increase in diarrhea score on day 4 was in parallel with the underlying significant histological changes and the second upregulation of TNF-α together with the significant increase in IL-1β and MPO activity. This coincidence is consistent with a previous study that closely linked the change in systemic symptoms with the severity of the intestinal mucositis [6-8]. Apart from TNF-α, IL-1β has recently been reported to be released mainly from the damaged epithelial cells after DNA damage through an unknown mechanism. IL-1β shares in the pathology of mucositis by disruption of the epithelial tight junctions [123].

The loss of intestinal epithelial barrier by the afford mentioned mechanisms, exposes the underlying submucosa to intestinal microbiota with loss of the mucus protective layers. This interaction exposes the immune system to the bacterial population together with influx of inflammatory immune cells releasing more inflammatory cytokines. This interaction was translated as a significant rise in the grade of inflammation by elevation of TNF-α, IL-1β and MPO activity and more destruction of intestinal villi and crypts morphology on day 4. Unlike the results on day 1, the administered antibiotics greatly affected the grade of intestinal inflammation on the following days.

Microbiome-targeted studies aim to focus on the structure and function of the intestinal microbiome map in multiple disease conditions [124]. So, in this study, I tried to identify the microbial change occurred after 5-FU administration using 16S rDNA high sequencing-based approaches. Our study identified a total of 11 phyla in mice microbiota, of which *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* were dominant. *Firmicutes* and *Bacteroidetes* account for more than 90 % of the control microbiota in a ratio of 1:1.52. Quantitavive PCR was further used to identify the main strains within each phylum. It was found that *lactobacillus acidophilus* is the main strain in *Firmicutes*, *baecteroides fragilis* in *Bacteroitedes*, *enterobacteriaceace* in *Proteobacteria*, *bifidobacterium longus* in *Actinobacteria* and *A.mucinophila* in *Verrucomicrobia* respectively (Table 2). The results showed that 5-FU treatment reduced the overall diversity of gut microbiota in mice with altered microbial composition compared to those in the control group, a condition known as dysbiosis. Repeated administration of 5-FU has decreased the abundance of *Firmicutes*, the gram-positive bacteria, and increased the abundance of *Bacteroidetes* and *Verrucomicrobia*, the gram-negative bacteria. In other words, 5-FU reduced the abundance of obligate anaerobic bacteria and expanded the abundance of facultative anaerobic bacteria especially the phylum *Proteobacteria* [125].

Several studies revealed that destruction of the intestinal epithelial barrier and the disturbed absorptive function of the intestinal mucosa after chemotherapy affected almost all metabolic pathways including carbohydrate, amino acids, lipids, nucleotides and energy metabolism [126,127]. These processes are not only related to the host, but they form a "Dialogue" between the host and the associated intestinal microbiota. The interrelated connection was disturbed by chemotherapy and resulted in loss of many beneficial processes.

To start with, *lactobacilli*, the major constituent of *Firmicutes*, is a well-known probiotic used to improve the gut ecosystem [128]. So, the depletion of *lactobacilli* may be associated with induction of gut inflammatory response. Moreover, reduction of *bifidobacterium*, one of the *Actinobacteria*, affects the level of butyrate in mucosa leading to decreased production of SCFAs which are well known to maintain the homeostasis in the intestinal epithelium [129]. The depletion of *bifidobacterium* has also been reported to affect the intestinal tight-junction proteins and plays a role in intestinal permeability [130].

On the other hand, the overall population of microbiota were previously reported to modulate activation of NF-κB pathways, the main transcriptional factor in inflammatory cytokines production. *Bacteroidetes* are well known to be enteropathogenic bacteria which increased in abundance by exposure to 5-FU. *A.mucinophila*, which belongs to *Verrucomicrobia*, is well known to degrade mucin protein in the intestinal mucus protective barrier, another way by which 5-FU promotes gut inflammation through dysbiosis. The *Proteobacteria* phylum basically compromises about 0.1% or less and suggests a healthy gut microbial. So, its dramatic increase in abundance can be considered as another important dysbiotic change induced by chemotherapy administration [131].

Dysbiosis is not only a matter of intestinal microbial community number change per se, but the change in the function of this community is capable of driving a detrimental distortion of microbe-host homeostasis that can further augment the intensity of intestinal inflammation with chemotherapy [131]. And because the microbiota has been involved in the pathogenesis of chemotherapy-induced intestinal mucositis, we precisely tried the co-administration of ampicillin, a broad-spectrum antibiotic, and aztreonnam, a gram-negative selective antibiotic, on the induced mucositis aiming to correct dysbiosis. The ampicillin strongly reduced the abundance of all bacteria, thus preventing the drastic changes in the structure of enterobacterial community after 5-FU administration. Although reduction in the total intestinal microbial load is usually considered as a side effect of antibiotic therapy in healthy individuals, the relative correction of the dysbiosis induced by 5-FU is considered to be the required target. This is

confirmed by the significant effect of ampicillin on most of the experimental parameters. The second upregulation of TNF-α level and the rise in IL-1β and MPO activity from day 4 were significantly lower in the ampicillin-treated groups in comparison to the 5-FU-treated groups. The histologic examination further supported the ampicillin protective effect by preservation of the intestinal villi length and the crypt morphology. All these data were reflected clinically as prevention of the significant body weight loss and increase in diarrhea scores detected in the 5-FU-treated group.

The different chronologic outcomes of ampicillin treatment on day 1 and day 4, being negative in the former and positive in the later, further speculates the sequence of the underlying stages in the pathogenesis of 5-FU-induced intestinal mucositis. Ampicillin has no role in preventing apoptosis, hypoproliferation and 5-FU direct toxic effect on day1 which seem to be the so early steps in mucositis. However, ampicillin effectively ameliorated the secondary inflammatory responses on day 4 through its effect on the intestinal microbiota content. Destruction of the epithelial cells together with loss of the symbiotic relationship between the epithelial cells and the intestinal microbiota may have triggered a change in the microbiota composition with the subsequent dysbiosis. Although the underlying mechanism of dysbiosis by 5-FU is still unclear, the disruption of the intestinal mucosal barrier is likely to be the triggering cause.

Based on all these date, we can suggest that crypt cell apoptosis, hypoproliferation, dysbiosis and expression of inflammatory cytokines are the sequential events underlying the pathogenesis of 5-FU induced intestinal mucositis, where TNF-α-mediated apoptosis and disruption of the epithelial barrier having a crucial role. Antibiotic administration could be an effective way in correction of the 5-FU-induced dysbiosis and thereby suppressing the secondary inflammation (Figure 8).

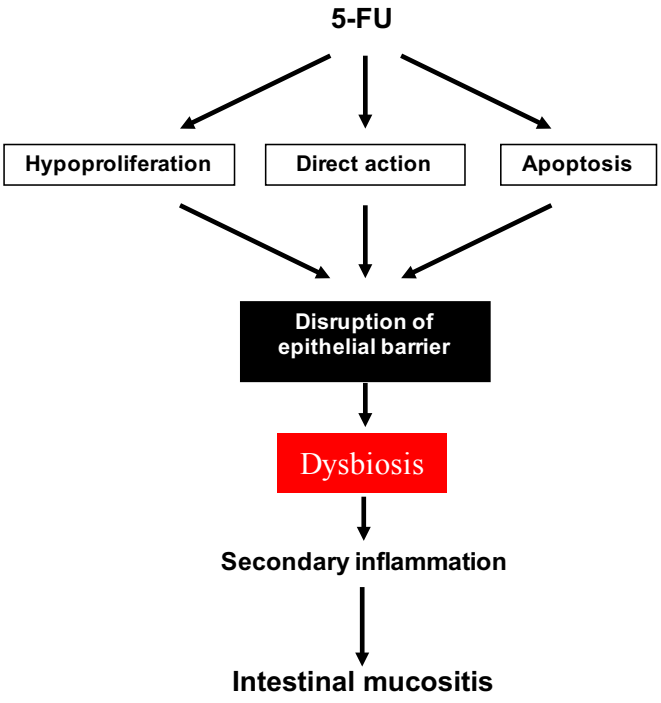

Figure 8. Pathogenesis of intestinal mucositis due to 5-Fluorouracil

Chapter II: Probiotic *Bifidobacterium bifidum* (BBG9-1) attenuates 5-fluorouracil-induced intestinal mucositis in mice via suppression of dysbiosis-related secondary inflammatory responses.

1. Background

It has been shown that the pathogenesis and progression of chemotherapy-induced intestinal mucositis is being greatly affected by the gut microbiota [132]. Dysbiosis is greatly related to 5-FU-induced intestinal mucositis [133]. It has been reported that probiotics had beneficial effects on regressing the GI tract symptoms related to chemotherapy particularly diarrhea [134-136]. As mentioned in the introduction, probiotics are live bacteria or bacterial components that contribute to human health via various metabolic processes [137, 138]. Most commonly known probiotics are lactic acid bacteria such as *lactobacilli* and *bifidobacteria*. *Bifidobacterium* is a major constituent of intestinal microbiota and is currently being used for treatment of diarrhea and constipation [139, 140].

From our results and conclusions about dysbiosis in Chapter I, in this study, we hypothesized that *bifidobacterium bifidum* G9-1 (BBG9-1) may improve the dysbiotic effect of 5-FU induced intestinal mucositis on intestinal microbiota.

2. Material and Methods

2.1 Animals

This study was performed in strict accordance with the ARRIVE guide-lines for reporting experiments involving animals [96]. The protocols were approved by the Committee on the Ethics of Animal Research of Kyoto Pharmaceutical University (Permission Number: 16-13-010). Male ICR (CD1) mice weighing 36-44 g and 9 weeks old (SLC Co., Shizuoka, Japan) were acclimated to standard laboratory conditions with 12- hour light–dark cycles and a temperature of 22 ± 1°C. Experiments were performed using six un-anaesthetized mice per group.

2.2 Induction and assessment of intestinal mucositis

Intestinal mucositis was induced through 5-FU administration following the same protocol and assessments as in chapter I. *Bifidobacterium bifidum* G9-1 (BBG9-1), which was isolated from infant faeces, was obtained from the Culture Collection of Biofermin R&D Center. It was cultured at 37°C for 18 hours in Gifu anaerobic broth (GAM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.7% glucose and 0.1% Tween 80. The bacteria were washed twice with PBS, and the pellets obtained after low-speed centrifugation were stored at –80°C until use. BBG9-1 (10^7-10^9 CFU) was co-administered orally once daily for 9 days, starting 3 days before the onset of 5-FU treatment, with control animals receiving saline.

2.3 Measuring the MPO activity

MPO activity was determined following the same protocol as described in chapter I.

2.4 Determination of proinflammatory cytokines mRNA expression in the small intestine by RT-PCR

Cytokine mRNA expression was extracted from the jejunum tissues following the same protocol described in chapter I.

2.5 Analysis of apoptosis

Apoptosis was detected in crypt intestinal cells using the same protocol described in chapter I.

2.6 Extraction of bacterial DNA from faecal samples

The extraction of bacterial DNA from caecum contents was conducted in accordance with a previously described method in chapter I [141].

2.7 Illumina library generation and DNA sequencing

Analysis of the bacterial 16S rRNA gene V3-V4 region was conducted in accordance with the previously described method in chapter I [99].

2.8 DNA sequence analysis

De-multiplexing and removal of indices were performed using the MiSeq Reporter software (Illumina). The resulting set of de-multiplexed sequences was processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [142] to remove low-quality sequences and chimeras, construct operational taxonomic units (OTUs), and assign taxonomic groups. In brief, 30 000 raw reads were randomly obtained and merged by fastq-join with default settings. Next, 5000 high-quality 16S reads, with an average quality value >25, were randomly chosen from all filtered reads for each sample and then checked for chimeras. OTUs were constructed with total high-quality reads by clustering the 16S reads with a 97% identity threshold. Representative reads from each OTU were then mapped to the 16S rRNA gene database using UCLUST with ≥97% identity. Taxonomic comparisons of gut microbiota were conducted at the phylum and genus level. Weighted UniFrac distance analysis, a phylogenetic tree-based metric, was used to measure differences in the overall bacterial gut microbiota structure [143].

2.9 Statistical analysis

Data are reported as mean ± SEM. Data were analyzed using GraphPad Prism 6.0 h (GraphPad Software, La Jolla, CA, USA). Parametric data were analyzed by one-way analysis of variance (ANOVA) followed by a Holm-Sidak's multiple comparison test, whereas nonparametric data were analyzed by a Mann-Whitney *U* test and Kruskal-Wallis test followed by Dunn's multiple comparison test. $P<.05$ was considered statistically significant.

3. Results

3.1 Effect of BBG9-1 on 5-FU-induced body weight loss, diarrhea, and shortening of the small intestine

Intestinal mucositis was induced in mice by repeated administration of 5-FU for 6 days. BBG9-1 was administered orally once daily for 9 days, beginning 3 days before the onset of 5-FU treatment. Repeated administration of 5-FU caused a significant body weight loss and increase in diarrhea score reaching 1.8 ± 0.5 on day 6 (Fig. 9A, B). Daily administration of BBG9-1 attenuated 5-FU-induced body weight loss in a dose-dependent manner, and a significant effect was observed at a dose of 10^9 CFU/mouse. Moreover, daily administration of BBG9-1 tended to reduce the severity of diarrhea, though this was not statistically significant. The length of the small intestine was found to be reduced at 24 hours after the end of 5-FU treatment. This reduction was also prevented by daily administration of BBG9-1 in a dose-dependent manner, and a significant effect was observed at a dose of 10^9 CFU/mouse (Fig. 9C).

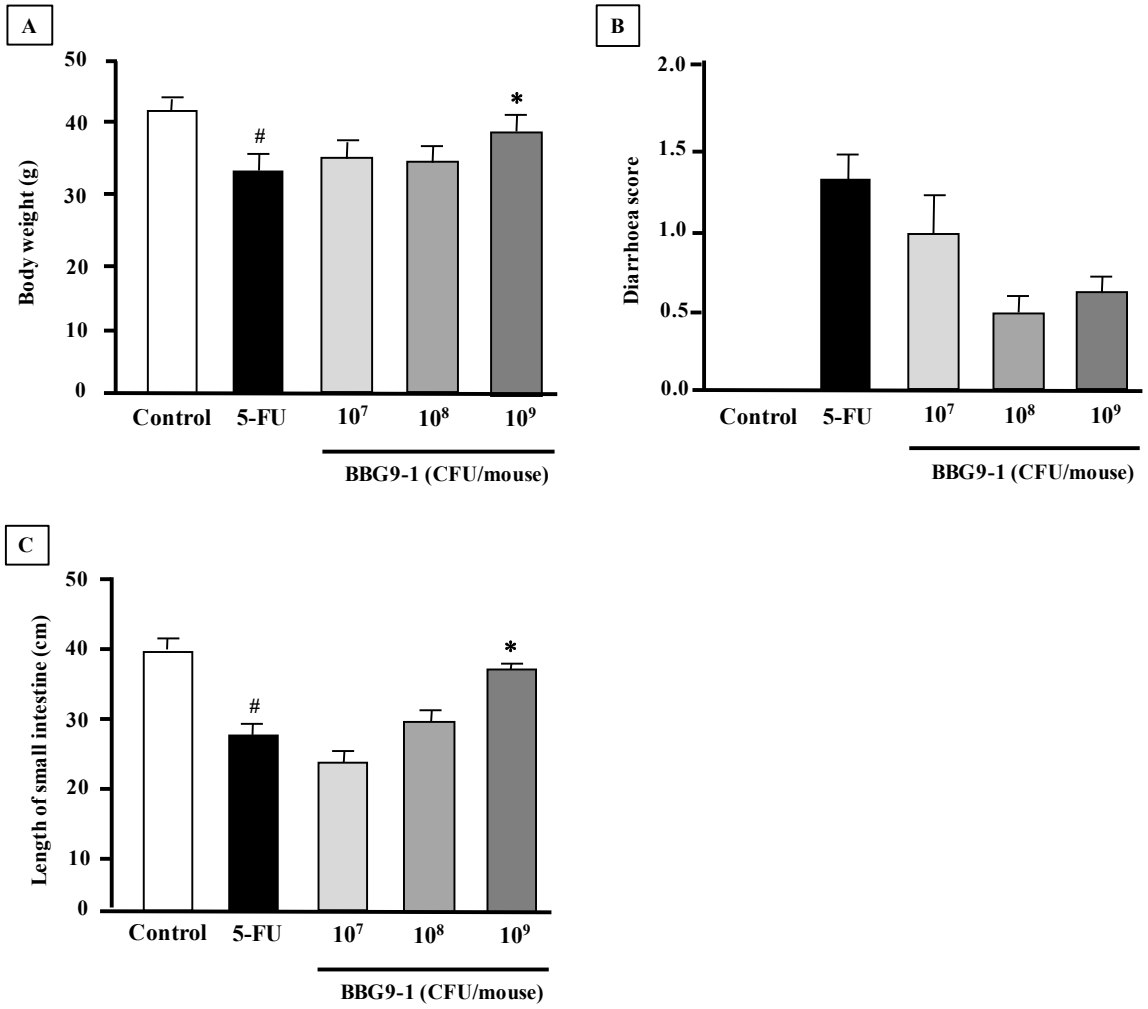

Figure 9. Effect of BBG9-1 on 5-FU-induced body weight loss, diarrhea, and shortening of the small intestine. Animals received 5-FU (50 mg/kg) intraperitoneally once daily for 6 days (days 0-5) and BBG9-1 (10^7-10^9 CFU/mouse) orally once daily for 9 days, starting from 3 days before the onset of 5-FU treatment. (A) Body weight was measured on day 0 and 5 and is indicated as final body weight on day 5 (g), whereas (B) diarrhea was scored on day 5 using a 4-grade scale as described Material and Methods. (C) The small intestine was removed and measured 24 hours after the end of 5-FU treatment. Data are presented as the mean ± SEM for six animals. #P<0.05 as compared to the control group (5-FU-untreated); *P<0.05 as compared to the 5-FU group (5-FU-treated alone) (parametric ANOVA with Holm-Sidak's test for body weight loss and length of small intestine; nonparametric Kruskal-Wallis with Dunn's test for diarrhea score). These figures are cited from ***Clin Exp Pharmacol Physiol*** **2017; 44(10):1018, Figure 1(A-C)**

3.2 Effect of BBG9-1 on 5-FU-induced intestinal mucositis

Repeated administration of 5-FU caused severe intestinal mucositis, histologically characterized by shortening of villus height and destruction of crypts (Fig. 10A). Villus height was reduced by 29.1%, the number of crypts was reduced by 33.0%, and the number of cells in each crypt was reduced by 40.0%, compared to those of control (5-FU-untreated) animals, by 24 hours after the end of 5-FU treatment (Fig. 10B). Daily administration of BBG9-1 attenuated the shortening of villi and the decrease in the number of cells in the crypts, with a significant effect being observed at a dose of 10^9 CFU/mouse. The decrease in the number of crypts was also suppressed by daily administration of BBG9-1, especially at a dose of 10^9 CFU/mouse, but this was not statistically significant.

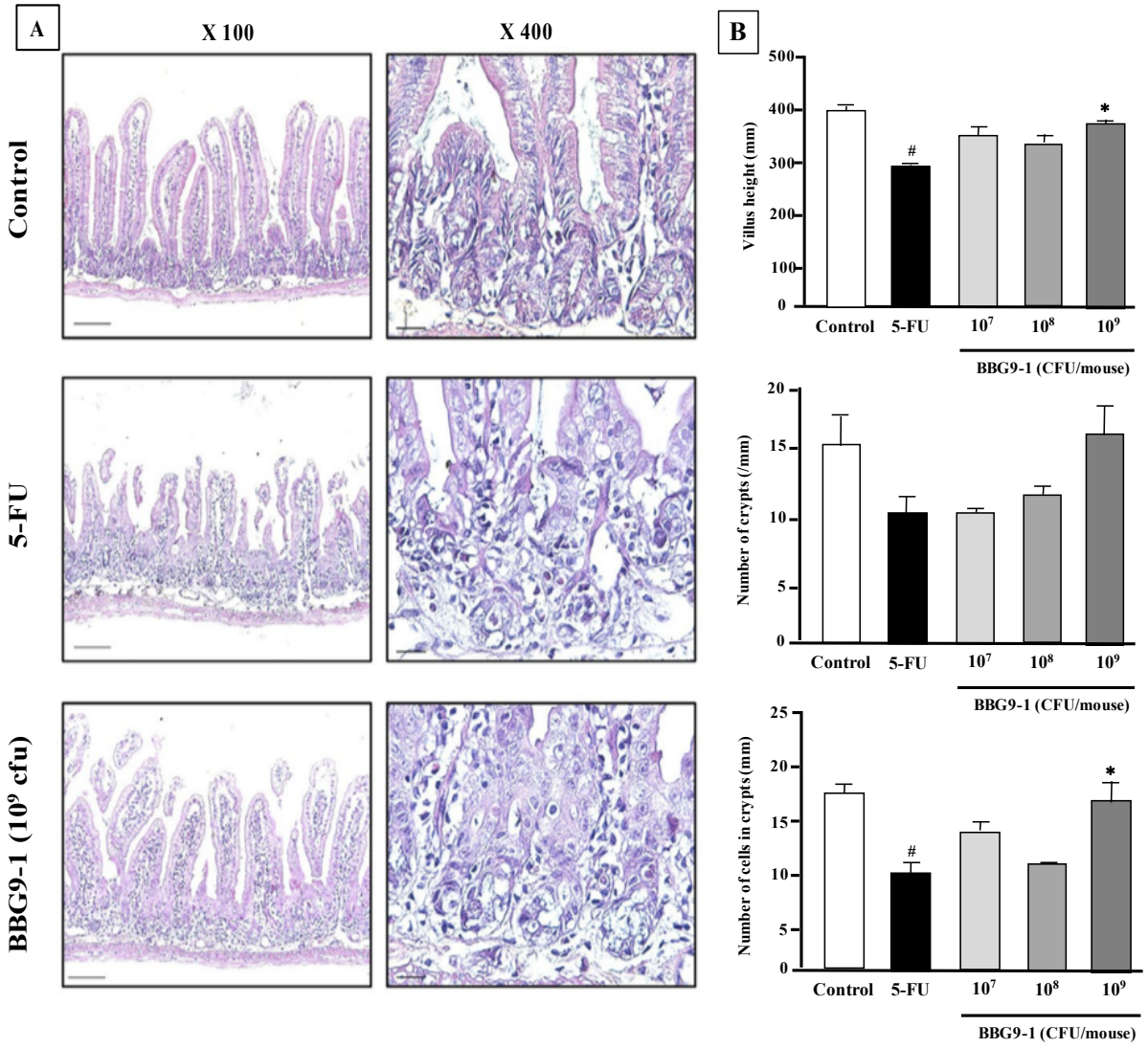

Figure 10. Effect of BBG9-1 on 5-FU-induced intestinal mucositis. Animals received 5-FU(50mg/kg) intraperitoneally once daily for 6 days (days 0-5) and BBG9-1 (10^7-10^9 CFU/mouse) orally once daily for 9 days, starting from 3 days before the onset of 5-FU treatment. (A) The small intestine was removed 24 hours after the end of 5-FU treatment, stained with haematoxylin/eosin, and imaged at 100× (left panels) and 400× (right panels). (B) The villus height (from the top of the villus to the villus–crypt junction), the number of crypts per millimeter, and the number of cells per crypt were measured using light microscopy. Data are shown as mean ± SEM for six animals. #P<0.05 as compared to the control group (5-FU-untreated); *P<0.05 as compared to the 5-FU group (5-FU-treated alone) (parametric ANOVA with Holm-Sidak's test). These figures are cited from ***Clin Exp Pharmacol Physiol*** **2017;44(10):1019, Figure 2(A, B)**

3.3 Effect of BBG9-1 on 5-FU-induced inflammatory responses

The marked increases in intestinal MPO activity (Fig. 11A) and TNF-α (Fig. 11B) and IL-1β (Fig. 11C) mRNA expression were detected on day 6 following the onset of 5-FU treatment. These inflammatory responses were significantly attenuated by daily administration of BBG9-1 (10^9 CFU/mouse).

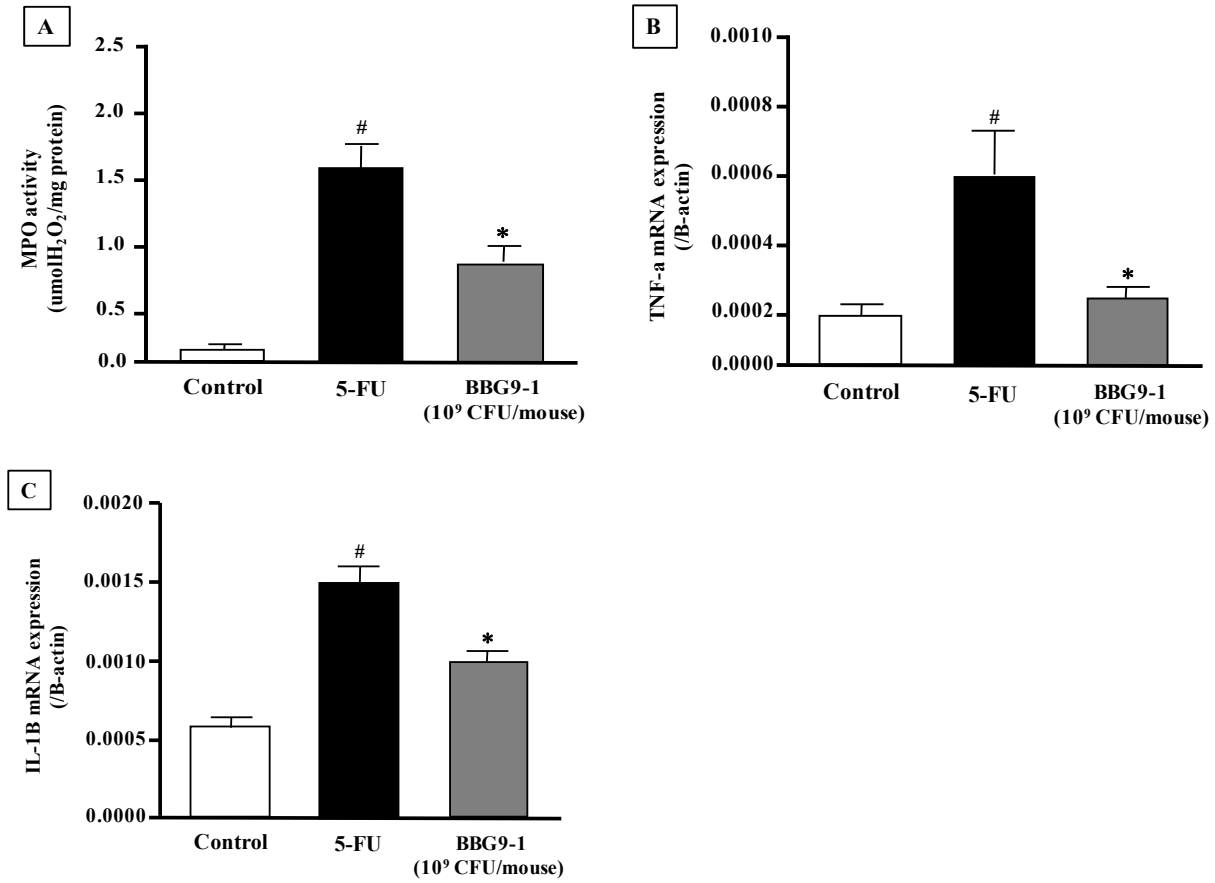

Figure 11. Effect of BBG9-1 on 5-FU-induced increases in intestinal myeloperoxidase (MPO) activity and TNF-α and IL-1β expression. Animals received 5-FU (50 mg/kg) intraperitoneally once daily for 6 days (days 0-5) and BBG9-1 (10^9 CFU/mouse) orally once daily for 9 days, starting from 3 days before the onset of 5-FU treatment. The small intestine was removed 24 hours after the end of 5-FU treatment. MPO activity was determined using *o*-dianisidine (A), whereas the expression of TNF-α and IL-1β mRNA was quantified by real-time RT-PCR and normalised to that of β-actin (/β-actin) (B, C). Data are shown as mean ± SEM for six animals. #P<0.05 as compared to the control group (5-FU-untreated); *P<0.05 as compared to the 5-FU group (5-FU-treated alone) (parametric ANOVA with Holm-Sidak's test). These figures are cited from ***Clin Exp Pharmacol Physiol*** **2017;44(10):1020, Figure 3(A-C)**

3.4 Effect of BBG9-1 on 5-FU-induced apoptosis in intestinal crypts

TUNEL-positive apoptotic cells were detected in intestinal crypts at 24 hours after the first administration of 5-FU (Fig. 12A). However, pre-treatment with BBG9-1 failed to suppress the induction of crypt cell apoptosis (Fig. 12B).

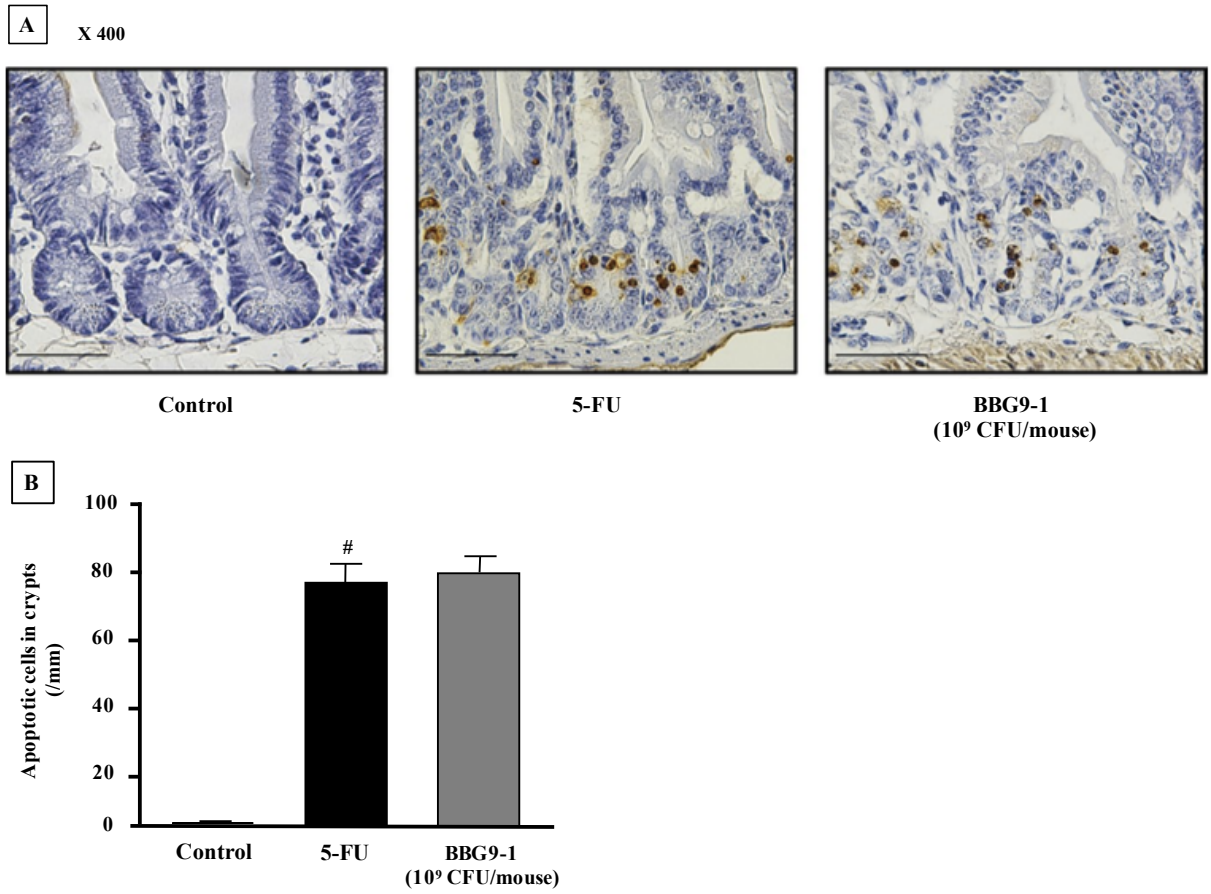

Figure 12. Effect of BBG9-1 on 5-FU-induced apoptosis in intestinal crypts. Animals received 5-FU (50mg/kg) intraperitoneally, and the small intestine was removed 24 hours later. BBG9-1 (10^9 CFU/mouse) was administered orally for 3 days, starting from 3 days before 5-FU injection. (A) Apoptosis was assessed be performing TUNEL assays (400×) and (B) quantified using a microscope. Data are shown as mean ± SEM for six animals. #P<0.05 as compared to the control group (5-FU-untreated) (parametric ANOVA with Holm-Sidak's test). These figures are cited from ***Clin Exp Pharmacol Physiol*** **2017;44(10):1021, Figure 4 (A, B)**

3.5 Effect of BBG9-1 on 5-FU-induced alterations in intestinal microbiota structure

Next, we examined the effect of BBG9-1 on alterations to the intestinal microbiota structure with 5-FU-induced intestinal mucositis. The intestinal microbiota was recently implicated in the pathogenesis of chemotherapy-associated intestinal mucositis. Weighted UniFrac-based principal coordinate analysis (PCoA) revealed differentia clustering of the intestinal microbiota structures between control (5-FU-untreated) and 5-FU-treated mice (Fig. 13A). The average weighted UniFrac distance also indicated that the distance between control and 5-FU-treated mice was significantly larger than the distance within control mice (Fig. 13B). These results indicate that repeated administration of 5-FU induces alterations in intestinal microbiota structure. In contrast, PCoA plots indicated that the microbiotas of BBG9-1-treated animals were broadly distributed between the clusters for control and 5-FU-treated mice. Similarly, the average UniFrac distance between control and BBG9-1-treated animals was significantly smaller than that between control and 5-FU treated animals. Further, the distance between 5-FU-treated and BBG9-1-treated animals was significantly larger than that within the 5-FU- treated. These results suggest that daily administration of BBG9-1 attenuates 5-FU-induced alterations in the intestinal microbiota structure.

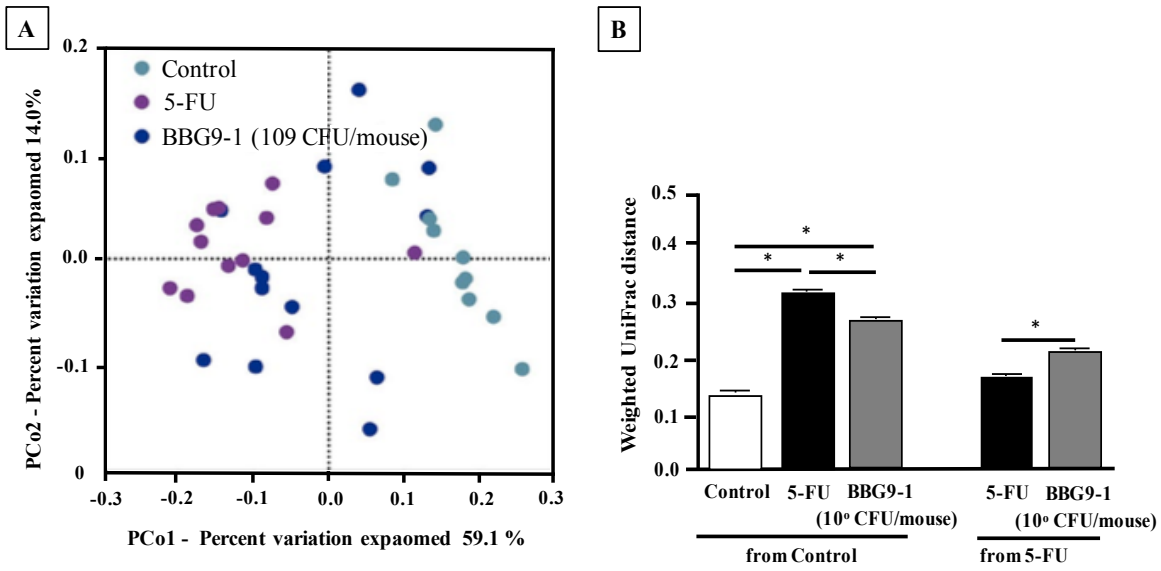

Figure 13. Effect of BBG9-1 on 5-FU-induced changes in the structure of the intestinal microbiota analyzed by weighted UniFrac distance. Animals received 5-FU (50 mg/kg) intraperitoneally once daily for 6 days (days 0-5) and BBG9-1 (10^9 CFU/mouse) orally once daily for 9 days, starting from 3 days before the onset of 5-FU treatment. Caecum contents were collected 24 hours after the end of 5-FU treatment and analyzed by next-generation sequencing of bacterial 16S rDNA. (A) The communities of intestinal microbiota were clustered by principal coordinate analysis of the weight UniFrac distance matrix. PCo1 and PCo2 explained 59.1% of the variation on the x-axes and 14.0% of the variation on the y-axes. 10 (■, control), 12 (■, 5-FU), and 13 (■, BBG9-1) animals. (B) Average weighted UniFrac distances from control and 5-FU-treated animals were determined. Data are presented as the mean ± SEM for 10 (control), 12 (5-FU-treated), and 13 (BBG9-1) animals. **P*<0.05 as compared to the 5-FU group (5-FU-treated alone) (nonparametric Kruskal-Wallis with Dunn's test). These figures are cited from ***Clin Exp Pharmacol Physiol*** **2017;44(10):1022, Figure 5(A, B)**

3.6 Effect of BBG9-1 on 5-FU-induced alterations in relative abundances of intestinal microbiota at the phylum level

A total of eight phyla were detected in the intestinal microbiota, of which *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, A*cctinobacteria*, and *Deferribacteres* were predominant. In control (5-FU-untreated) animals, *Firmicutes* was the most abundant phylum in the intestinal microbiota, representing 76.1 ± 1.7% of OTUs (Fig. 14). Repeated administration of 5-FU significantly decreased the relative abundance of *Firmicutes* to 40.3 ± 3.1%. In contrast, the relative abundance of *Bacteroidetes* was markedly increased by 5-FU treatment, increasing from 6.8 ± 1.1% to 43.6 ± 3.3%. Daily administration of BBG9-1 significantly restored both the higher abundance of *Firmicutes* and the lower abundance of *Bacteroidetes*. The relative abundances of *Proteobacteria* and *Actinobacteria* were also significantly altered by 5-FU treatment, but these responses were not affected by BBG9-1 administration.

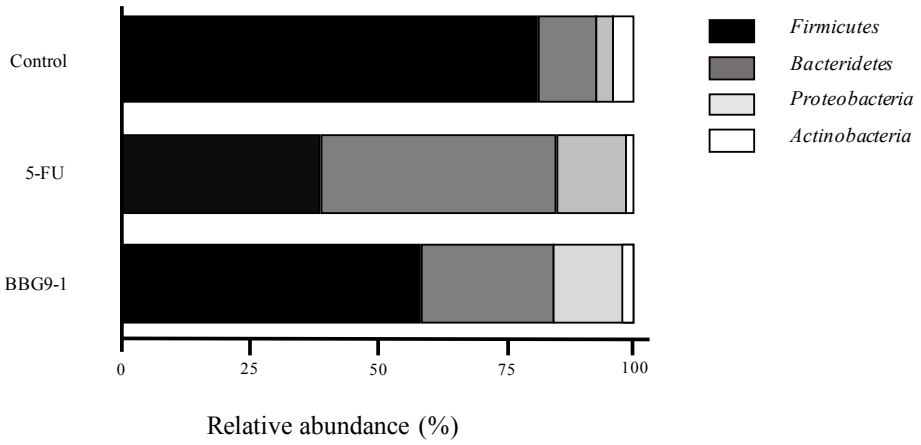

Figure 14. Effect of BBG9-1 on 5-FU-induced changes in the relative abundance of intestinal bacteria. Animals received 5-FU (50 mg/kg) intraperitoneally once daily for 6 days (days 0-5) and BBG9-1 (10^9 CFU/mouse) orally once daily for 9 days, starting from 3 days before the onset of 5-FU treatment. The caecum contents were collected 24 hours after the end of 5-FU treatment, and the relative abundance of each bacterial phylum was analyzed by next-generation sequencing of bacterial 16S rDNA.

4. Discussion:

Manipulation of intestinal microbiota to achieve a better balance in cases of chemotherapeutic treatment and reduction of its side effects, presents a significant research challenge. So, in line with the experiments in chapter I, the effect of oral administration of 3 different doses of BBG9-1 (10^7, 10^8, and 10^9 CFU/mouse respectively) for 9 days starting 3 days before 5-FU injection on the pathogenesis of intestinal mucositis, was investigated and compared with the control (5-FU untreated) and the 5-FU-treated groups.

The current findings coincided with our previously reported results [6-8]. Repeated administration of 5-FU for 5 days was associated with significant body weight loss and increase in diarrhea score. These systemic findings were correlated macroscopically by decrease in the intestinal length measured on day 6. Furthermore, histological examination revealed sever intestinal mucositis characterized by significant shortening of the intestinal villi and destruction of the intestinal crypts. Apoptotic cells were again detected in intestinal crypts from day 1. In terms of chemical examination, there was a significant increase in the inflammatory cytokine expressions of TNF-α, IL-1β and MPO activity. Additionally, the weighted UniFrac-based PCoA analysis indicated that intestinal microbiota clustering structure was greatly altered by 5-FU injection for 5 days. In other words, the mean weighted UniFrac distance within the control and the 5-FU-treated samples was significantly larger in the 5-FU-treated than in the control groups. These results add onto our observations in chapter I, that 5-FU administration alters the structure of intestinal microbiota giving rise to dysbiosis. Additional confirmation was done by analyzing the intestinal microbiota at the phylum level using the next-generation sequencing of bacterial 16S rDNA. The 5-FU group showed a significant decrease in the relative abundance of *Firmicutes* and *Actinobacteria* (mostly gram-positive) coupled with significant increase in the *Bacteroitedes* and *Proteobacteria* (mostly gram-negative), respectively.

The daily administration of BBG9-1 attenuated the body weight loss in a dose dependent manner, achieving significance at the dose of 10^9 CFU/mouse. However, this was not the case with diarrhea score, where the decrease was evident but not significant even with the highest dose of BBG9-1. Still the effect on body weight and diarrhea score by BBG9-1 is correlated with a significant macroscopic and histological protection against the 5-FU induced mucositis in a dose dependent manner. The length of the small intestine and the measured intestinal villi height and crypt morphology were corrected by the 10^9 CFU/mouse dose of BBG9-1 in relation to the control and the 5-FU-treated groups. Similar to chapter I, BBG9-1 administration has no effect on the number of TUNEL-positive apoptotic cells in the

intestinal crypts after 24 hours. However, the level of inflammatory cytokine expression of TNF-α, IL-1β and MPO activity on day 4 were significantly reduced in the BBG9-1 treated groups in a dose dependent manner. Thus, BBG9-1 cannot prevent the early induction of apoptosis but can effectively suppress the inflammatory responses during the pathogenesis of 5-FU induced intestinal mucositis. The similarity between these results and our previous results in chapter I after antibiotic administration suggests a possible preventive effect of BBG9-1 on microbiota-related secondary inflammatory response.

BBG9-1 partly restored the 5-FU induced changes in the clustering of intestinal microbiota. Moreover, the weighted UniFrac distance between the control and the 5-FU-treated animals was significantly reduced, indicating that BBG9-1 is likely to ameliorate 5-FU-induced dysbiosis. On the phylum level, the dose of 10^9 CFU/mouse of BBG9-1 was found to inhibit the 5-FU induced decrease in *Firmicutes* and increase in *Bacteroitedes*. Unlike ampicillin, altered relative abundance of *Actinobacteria* and *Proteiobacteria* was not corrected by BBG9-1 even at the highest dose.

Intestinal microbiota maintains a symbiotic relationship with the gut mucosa and imparts various metabolic, immunological and protective functions, where *Firmicutes* and *Bacteroitedes* are the main predominant constituent of a healthy intestinal microbiota [126]. An important group of receptors is thought to play a role in the communication between the human body and the resident microbiota: The TLR [59-62]. The TLRs are present on the outer membrane of the epithelial cells. Bacteria are recognized by the extracellularly located part of TLR leading to activation of NF-κB. In turn activation of NF-κB results in the development of an inflammatory response. So, microbiota takes a part in the genesis and modulation of inflammatory responses. So far, multiple members of TLR family have been described in mammals; 11 subtypes (TLR-1 to -10) [58]. TLR-2 is activated by peptidoglycans part of the cell wall of gram-positive bacteria, while TLR-4 is activated by LPS, a substance of gram-negative bacteria [59-62]. After binding to TLRs, the bacteria are processed and bacterial parts are transported intracellularly where they bind to other receptors which modulate the inflammatory responses. Thus, the healthy intestinal mucosa is characterized by a low-grade inflammation [62]. The resident microbiota ensures a constant presence of LPS and bacterial DNA, which in turn regulates the expression of TLR-2 and TLP-4. This creates a continuous basal activation of downstream pathways, resulting in a low-grade physiological inflammation [147]. The evident decrease in gram-positive bacteria (*Firmicutes*) and increase in the gram-negative bacteria (*Bacteroitedes*) is therefore responsible for the imbalance in TLR-2 and 4 stimulations and results in severe mucosal inflammation. Administration of BBG9-1 at a dose of 10^9 CFU/mice, which belongs to the *Actinobacteria* phylum, significantly corrected the relative

abundance of *Firmicutes* and *Bacteroitedes* in comparison to the control group [147, 148].

BBG9-1 oral administration, did not only correct the dysbiosis per se, but it seems to share in the correction of 5-FU induced mucositis through other mechanisms. First of all, BBG9-1 derives nutrients from dietary carbohydrates fermentation leading to formation of SCFAs as butyrate, propionate and acetate which are rich energy sources for the host [48-51]. Butyrate is not only considered a source of energy, but it attenuates inflammation, reduces intestinal permeability and stimulates the activation of immune effector molecules. It also plays a role in the composition of the mucus layer, as butyrate is capable of increasing mucin synthesis as well [52-56]. Moreover, butyrate stimulates migration of epithelial cells thereby enhancing mucosal healing [57]. Additionally, the release of SCFs has a hand in decreasing intestinal permeability by increasing the epithelial cell viability [57].

Secondly, administration of BBG9-1 as a strain from *bifidobacteria*, is associated with an enhanced expression of proteins forming the tight junctions and has shown to decrease the intestinal permeability [57]. Lastly, BBG-1 keeps a check on the overgrowth of pathogenic strains by inducing local immunoglobulins (Igs). It was reported that *bifidobacteria* increase IgA synthesis [147]. The gut microbiota activates intestinal dendritic cells that stimulates plasma cells in mucosa to express secretory IgA (sIgA) which in turn coat the intestinal microbiota. The sIgA are predominant of sIgA2 subclass which is more resistant to degradation by bacterial proteases [147, 148]. These mechanisms restrict the translocation of microbiota from the intestinal lumen to the circulation, hereby decreasing the chance of systemic infections from the ulcerated intestinal mucosa [147, 148].

The exact nature and relevance of the relationship between chemotherapy-induced mucositis and intestinal microbiota is a subject to ongoing research. We propose that changes in the commensal intestinal microbiota influence the 3rd phase of mucositis induced by chemotherapy in Sonis' model. In this way, the intestinal microbiota might also influence the severity of mucositis encountered in the 4th ulcerative phase. Our results showed that BBG9-1 attenuated the severity of mucositis and so, we can further hypothesize that microbiota can also attenuates mucositis by influencing the healing phase.

Although the protective role of commensal intestinal microbiota in human disease is increasingly being taken into consideration, research concerning the relationship between intestinal microbiota and chemotherapy-induced mucositis is still scarce. We aimed to study this relation and from our results we concluded that BBG9-1 attenuates 5-FU-induced intestinal mucositis. This beneficial effect could be accounted for by the suppression of secondary inflammatory responses through improvements of

dysbiosis during 5-FU treatment. Thus, BBG9-1 might be clinically useful for the prevention of intestinal toxicity during cancer chemotherapy. This should not only increase the quality of life in patients with mucositis but could also positively influence treatment intensity probably by decreasing the morbidity and mortality in cancer patients.

Conclusion:

From Chapters I and II, I determined the temporal changes observed in 5-FU-induced murine intestinal mucositis; 1) transcriptional upregulation of TNF- α, increased apoptotic cell death and decreased cell proliferation in the intestinal tissues, 2) disruption of the epithelial barrier, 3) intestinal dysbiosis, and 4) secondary inflammatory responses. Antibiotics could ameliorate intestinal mucositis through suppression of the secondary inflammatory responses, not the early responses. I demonstrated that oral administration of a probiotic bacterium, BBG9-1, could also suppress the 5-FU-induced intestinal inflammatory responses suggesting that dysbiosis should be involved in exacerbation of intestinal mucositis during cancer chemotherapy. This study should contribute to the development of novel therapeutic approaches for chemotherapy-induced intestinal mucositis. Thereby, not only increasing the quality of life in patients with mucositis but also positively influencing treatment intensity probably by decreasing the morbidity and mortality in cancer patients.

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