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ORIGINAL ARTICLE

Irinotecan-induced mucositis is associated with changes in intestinal mucins

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Abstract
Purpose Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy. Irinotecan is used to treat a variety of solid tumours, through the inhibition of DNA topoisomerase I and is linked with severe mucositis and diarrhoea. Mucus production appears to be increased, which may contribute to the development of diarrhoea.

Methods Dark agouti rats were treated with irinotecan, and tissues collected at several time points up to 72 h. Goblet cells and mucin secretion were investigated, as well as mucin expression (Muc2 and Muc4) and kruppel-like factor (Klf) 4 using immunohistochemistry in the gastrointestinal tract. Both goblet cells and cells positive for Muc expression were counted, and analysed statistically using the Mann–Whitney *U* test with Bonferroni correction.
Results Goblet cells decreased significantly after irinotecan treatment. However, mucin secretion increased. Mucin expression changed significantly after treatment. Muc2 and Muc4 decreased significantly in the villi of the jejunum after treatment, Muc2 and Muc4 decreased significantly in the crypts. Muc2 decreased significantly in the colon.
Conclusions Irinotecan causes an increase in mucin secretion and a net decrease in mucin-producing goblet cells, and the expression of Muc2 and Muc4 in the gastrointestinal tract is altered following treatment. Increased mucin secretion is likely to be related to altered mucin expression, and may contribute to chemotherapy-induced diarrhoea.

Keywords Intestine · Mucositis · Cancer chemotherapy · Immunohistochemistry · Mucin

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Introduction

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy. Approximately 40% of patients receiving standard dose chemotherapy and 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation exhibit the abdominal pain, ulceration, bloating, vomiting

and diarrhoea typically associated with mucositis [1–3]. The pathobiology of mucositis is intricate and involves a series of steps, each entailing complex signalling pathways [4].

Irinotecan is used to treat a variety of solid tumours, through the inhibition of DNA topoisomerase I. However, unfortunately it is linked with severe mucositis and diarrhoea [5–10]. The metabolism of irinotecan has been described previously [11]. Irinotecan and SN-38 bind to the topoisomerase I-DNA complex, leading to double strand (ds) breakage and cell death. We have previously shown that irinotecan treatment decreases goblet cells in the large intestine [7]. However, mucus production appears to be increased, which may contribute to the development of chemotherapy-induced diarrhoea (CID) [7]. A more recent study has examined the effects of 5-fluorouracil (5-FU), a chemotherapeutic agent causing mucositis [12], and demonstrated that the mucin content of the jejunum is altered by 5-FU.

Goblet cells are highly polarised exocrine cells that synthesise and secrete mucins [13, 14]. The baseline secretion of mucins consists of the periodic exocytosis of mucin granules [15], effecting the slow continual release of mucins, maintaining a mucus blanket [13], protecting the epithelium from mechanical and chemical stress [14, 16]. The mucus layer also allows transport between the luminal contents and epithelium [16]. The structure of mucins allows the maintenance of the normal intestinal flora, by providing attachment sites for intestinal flora and pathogenic bacteria [14], and simultaneously protecting the mucosa from bacterial overgrowth and/or penetration [13]. Goblet cell differentiation is thought to be regulated by kruppel-like factor (Klf) 4 (previously known as GKLF), an epithelial zinc-finger protein, also implicated in growth arrest and down regulation of cell proliferation [17].

Muc genes (Muc1-17) are regulated by cytokines, bacterial products and growth factors [16]. The biosynthesis of mucins is affected by conditions or agents affecting the differentiation of precursor cells into goblet cells, and those that uncouple the process of protein synthesis (fasting or malnutrition) [16]. The Muc gene family contains members which are dissimilar, with two structurally and functionally distinct subfamilies: secreted gel-forming mucins (Muc2 and Muc5AC) and transmembrane mucins (Muc1, Muc3, and Muc4). Of particular interest for this study are Muc2, and Muc4, representing one transmembrane and one secreted mucin.

The aims of this study, are to determine if the expression patterns of mucins (Muc2 and Muc4), and/or Klf4 alter in the gastrointestinal tract of rats following treatment with irinotecan, as well as to establish if these changes are comparable with changes seen in goblet cells and the incidence of diarrhoea.

Materials and methods

Animals

Animals used in this study were female dark agouti (DA) rats, weighing between 150 and 170 g. Rats were individually housed in Perspex cages at a temperature of 22 ± 1°C and subject to a 14 h light/10 h dark cycle. Approval for the use of animals was granted by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), and The University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2004). Due to the nature of the diarrhoea induced by irinotecan, animals were monitored four times daily and if any animal showed certain criteria (as defined by the Animal Ethics Committees) they were killed. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, cold to touch with no spontaneous movement and a hunched appearance.

Experimental plan

Eighty-one rats were randomly assigned to groups. For each time point there was one group of six rats receiving irinotecan and one group of three control rats receiving no treatment. Rats receiving irinotecan received 0.01 mg/kg subcutaneous atropine (to reduce the cholinergic reaction) immediately prior to administration of 200 mg/kg intraperitoneal irinotecan (This dose has been previously shown to induce diarrhoea [9, 18]). Irinotecan (kindly supplied by Pfizer, Kalamazoo, USA) was administered in a sorbitol/lactic acid buffer (45 mg/mL sorbitol/0.9 mg/mL lactic acid, pH 3.4), required for activation of the drug, at time designated 0 h. Groups of rats were killed by cardiac puncture and cervical dislocation under 3% halothane in 100% O_2 anaesthesia at times 30 and 60 min, 2, 6, 12, 24, 48 and 72 h post irinotecan treatment. The intestines (from the pyloric sphincter to the rectum) was dissected out and separated into the small intestine (pyloric sphincter to ileocaecal sphincter) and colon (ascending colon to rectum). The small intestine was flushed with chilled, sterile distilled water, and a 1 cm sample taken at approximately 25% of the length, was collected for histological procedures. The colon was also flushed with chilled sterile, distilled water. A 1 cm sample of colon, taken at approximately 50% of the length was collected for histology procedures. The stomach was dissected and contents emptied and discarded. A small piece (1 cm × 0.5 cm) of stomach was collected for histology. All samples for histological examination were fixed in 10% neutral buffered formalin, processed and embedded in paraffin.

Diarrhoea assessment

All animals were checked four times daily and diarrhoea recorded according to previous gradings [7, 19]. This was graded as 0 no diarrhoea, 1 mild diarrhoea (staining of anus), 2 moderate diarrhoea (staining over top of legs and lower abdomen) and, 3 severe diarrhoea (staining over legs and higher abdomen, often with continual anal leakage). All diarrhoea assessments were conducted in a blinded fashion by two investigators (A.M.S. and R.M.L.).

Histology

Samples of jejunum and colon were collected and fixed in 10% neutral buffered formalin for routine histological examination. Fixed samples were processed and embedded in paraffin. Sections of 4 μm thickness were stained with routine haematoxylin and eosin (H&E). Briefly, the wax was dissolved with xylene and sections rehydrated before staining in Lillie–Mayer's haematoxylin for 10 min. After differentiating in 1% acid alcohol and bluing in Scott's tap water, sections were counterstained in eosin, dehydrated, cleared and mounted, and then examined using light microscopy and reported on by a professional veterinary pathologist.

Alcian Blue-PAS stain

To assess the composition of mucin Alcian Blue-PAS staining was performed. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were stained in Alcian Blue [1% Alcian Blue 8GX (CI 74240) in 3% glacial acetic acid] for 5 min, then rinsed in distilled water. Sections were oxidised in 1% periodic acid before washing in running water and rinsing in distilled water. Sections were treated for 15 min in Schiff's reagent and washed for 7 min in running water. Slides were dehydrated, cleared and mounted.

Quantitative histology

To determine the effect of irinotecan on mucus secretion, goblet cells were counted. Decreased goblet cells indicated release of mucins from the mucosal surface, and cavitation of mucus cells is a sign of accelerated mucus secretion by compound exocytosis [20]. Therefore, both the number of goblet cells and percentage of cavitated cells were analysed, according to a method previously described [20]. Briefly, a cavitated cell is recognised by apical indentation into the intracellular store of mucus granules. Goblet cells and cavitated cells in crypts and villi that were deemed to be greater than 80% complete were counted under high power magnification, with a total of at least 15 villi/crypts per section analysed. Analysis was conducted in a blinded fashion.

High iron diamine stain

To investigate the distribution of sulphated mucins high iron diamine staining was performed. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were stained for 18–24 h in high iron diamine solution (2.4% *N*,*N*-dimethyl-*meta*-phenylenediamine dihydrochloride, 0.4% *N*,*N*-dimethyl-*para*-phenylenediamine dihydrochloride, 2.8% v/v 10% ferric chloride). Following staining, slides were washed in running water for 2 min. Sections were then counterstained with 1% Alcian Blue in 3% glacial acetic acid for 5 min, then washed before being dehydrated, cleared and mounted. Analysis was conducted in a blinded fashion.

Immunohistochemistry

Sections were cut from paraffin blocks at 4 μm and mounted onto silane-coated glass slides. Sections were dewaxed in xylene and brought to water via a graded series of alcohols. Antigen retrieval was carried out using 10 mmol/L citrate buffer (pH 6.0) and heat, with sections subjected to microwave (mw) irradiation in a domestic microwave with a carousel, microwaved on HIGH (930 W) until boiling, followed by LOW (650 W) for 10 min. Endogenous peroxidases were blocked with 3% H_2O_2 in methanol. Non-specific antibody binding was blocked with either 20% normal goat serum (NGS) (Sigma) (anti-Muc2 and anti-Muc4) or normal horse serum (NHS) (Sigma) (anti-Klf4) in PBS applied at room temperature (RT) for 30 min.

Sections were incubated with either rabbit anti-Muc2 (Santa Cruz Biotechnology, California, USA) for 1 h at RT, or mouse anti-Muc4 (Zymed Laboratories, California, USA) or goat anti-Klf4) primary antibodies diluted in 5% NGS or NHS, overnight at 4°C. Sections were incubated sequentially with an appropriate secondary antibody (Vector Laboratories, USA) and 5 μL/mL Vectastain ABC kit solution (Vector Laboratories, USA). For visualisation of bound antibodies, sections were incubated with 3′3-diaminobenzidine (DAB) (Zymed) for up to 5 min, or until a brown precipitate appeared on positive control sections. Nuclei were counterstained with haematoxylin. Sections were examined using light microscopy. Negative controls were carried out by incubating with 5% serum instead of the primary antibody solution. Quantitative assessment of sections was carried out by counting the stained cells in villi/crypts that were deemed to be at least 80% under high power magnification, with at least 15 complete villi/crypts counted per section. Analysis was conducted in a blinded fashion.

Statistical analysis

Quantitative results were statistically analysed using the Mann–Whitney *U* test. For adjustments for multiplicity, the significance levels of the *P* values were determined according to the Bonferroni correction.

Results

Diarrhoea

Cholinergic diarrhoea was observed in 23% of treated rats 2 h after treatment, despite treatment with atropine (Fig. 1). Mild diarrhoea was seen in 23% of experimental rats between 2 and 6 h and by 12 h, 30% of rats had mild diarrhoea and 5% of rats had moderate diarrhoea. At 24 h this had increased to 39% of rats having mild diarrhoea, and 12% having moderate diarrhoea. At 48 h 20% of rats had mild diarrhoea. Late onset diarrhoea was apparent 72 h after treatment with 33% of treated rats having mild diarrhoea. No control rats had diarrhoea at any time point investigated.

Histology

Pathological changes caused by irinotecan in the jejunum and colon have been previously described [9]. There were no histological changes in control rats. Between 2 and 72 h after irinotecan treatment, uneven widespread apoptosis of basal crypt enterocytes was observed in the jejunum (Fig. 2). Extensive damage was observed from 2 h after treatment. The most severe damage was seen at 6 h following chemotherapy, consistent with that seen in previous studies [9]. The severity of damage decreased from 12 to 72 h after treatment. Changes in the colon included uneven, but widely disseminated apoptosis of enterocytes in the mid and basal regions of crypts at 6 h after treatment. Dilated crypt lumina were lined with attenuated epithelium from 48 to 72 h. Occasional debris was observed in the lumen from 48 to 72 h after treatment (more severe at 72 h) with condensation of the stroma also observed at 72 h (Fig. 2).

Goblet cell composition and distribution

Stained mucins in the jejunum decreased in both the crypts and villi from 90 min after chemotherapy, with few stained mucins present by 48 h (Fig. 3). Mucins became more sulphated in treated rats. Mucin composition did not alter significantly in the colon after irinotecan. However, high iron diamine staining showed mucins in the colon changed from being predominantly carboxylated (blue/green) to sulphated (brown) (Fig. 3).

Effect of irinotecan on mucin discharge

Goblet cell counts in untreated rats showed 9.9 ± 0.3 (mean $\pm$ SEM) cells/crypt in the jejunum, with $16.7 \pm$

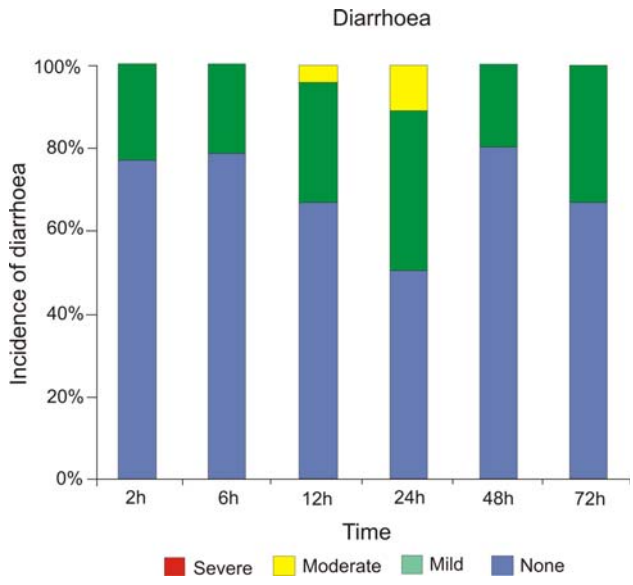

Fig. 1 Incidence of diarrhoea

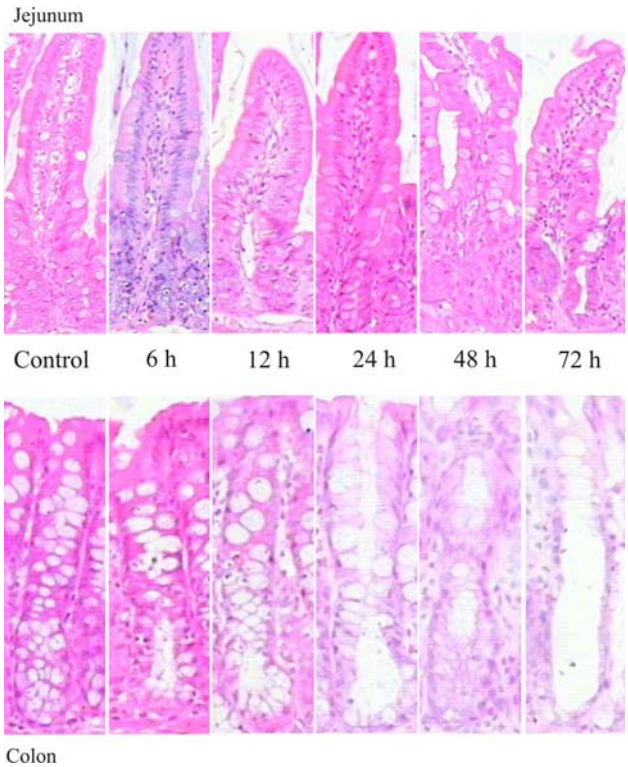

Fig. 2 Histopathology. Widespread damage was observed between 6 and 72 h after treatment in the jejunum, including basal enterocyte apoptosis. Damage in the colon was also observed and included apoptosis, attenuated epithelium lining dilated crypts, occasional debris and condensation of the stroma. Original magnification ×10

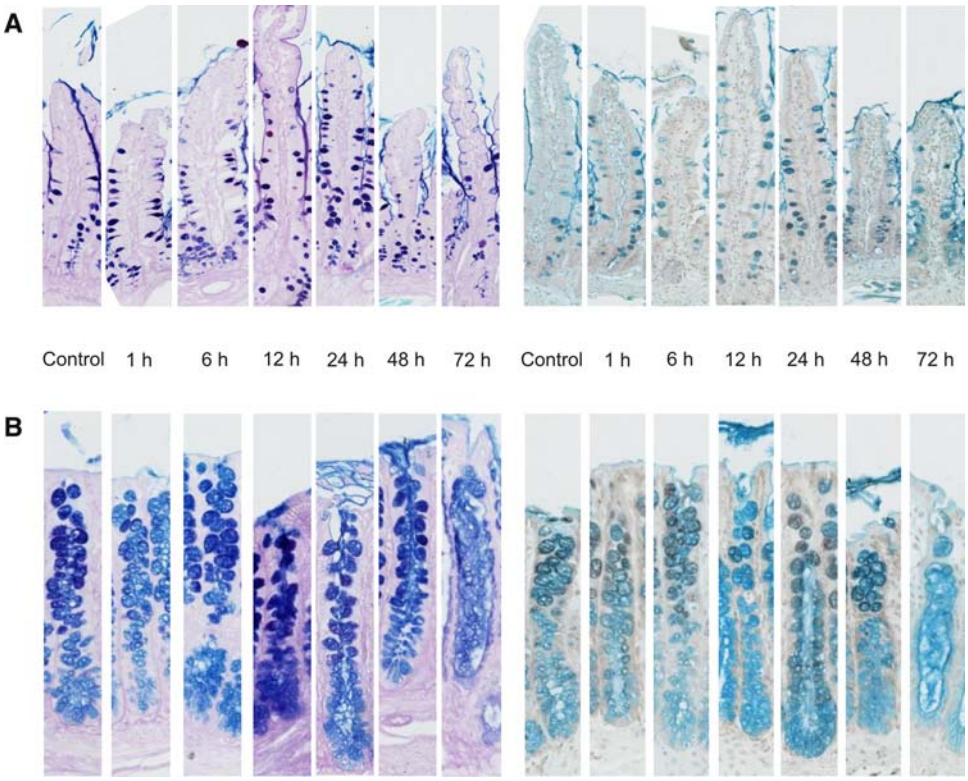

Fig. 3 Alcian blue-PAS staining and high iron diamine staining of sections of **a** jejunum and **b** colon. Original magnification ×20

2.9% deemed to be cavitated, and 13.2 ± 0.8 cells/villus, with 34.3 ± .2.7% deemed to be cavitated. Total goblet cells in the crypt decreased significantly after irinotecan from 48 to 72 h ($P < 0.0001$). Cavitated goblet cells (as a percentage of total goblet cells) increased significantly at 48 h ($P < 0.0001$). Total goblet cells in the villus increased significantly 24 h after treatment ($P < 0.02$), and cavitated goblet cells increased significantly at 72 h ($P < 0.003$) (Fig. 4).

Total goblet cells in the colon of untreated rats numbered 36.9 ± 1.0 cells per crypt, with 7.9 ± 0.7% cavitated. The total number of goblet cells decreased significantly 48–72 h after treatment ($P < 0.0004$). Cavitated goblet cells (as a percentage of total goblet cells) increased significantly from 12 to 72 h ($P < 0.0001$) (Fig. 4).

Expression of mucins

Muc2

Muc2 was expressed by most goblet cells in the jejunum and colon (Fig. 5). There number of Muc2 positive cells significantly decreased at 12 h ($P < 0.003$) after treatment in the jejunal villi, with a significant decrease seen in the crypts at 24–48 h ($P < 0.001$). A significant decrease was seen in the colon between control and 24–72 h ($P < 0.0007$).

Muc4

Muc4 was expressed by most goblet cells in the jejunum and colon (Fig. 6). In the jejunum, a decrease (not significant) in Muc4 positive cells was observed in the villi from 48 to 72 h ($P < 0.004$). Significant decreases were seen in the crypts at 1 h ($P < 0.003$), 6 h ($P < 0.003$), and 48–72 h ($P < 0.004$). Differences between group mean in the colon were not significant.

Klf4

Klf4 was expressed in the jejunum and colon. Klf4 was expressed in enterocytes in the apical region of villi, and in the apical region of colonic crypts. In the villi, there was a significant difference between group means of counted cells, with decreases observed from 30 to 90 min, and again from 6 to 12 h after treatment. In the colon there was also a significant difference between group mean. An increase was detected at 24 h after irinotecan treatment (Fig. 7).

Discussion

Irinotecan-induced mucositis manifesting as diarrhoea is a severe dose-limiting side effect, with large cost to health

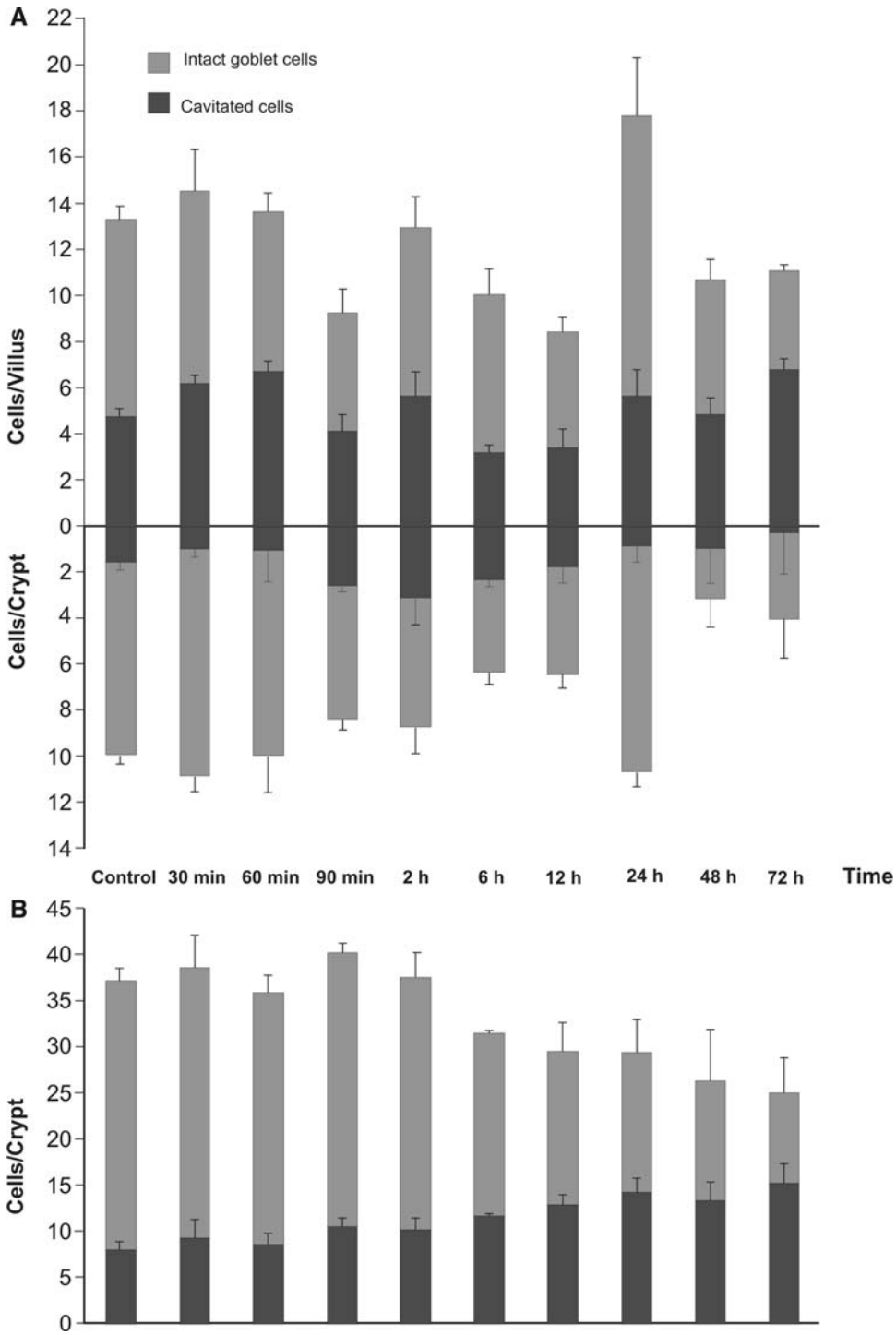

Fig. 4 Goblet cell counts of intact and cavitated cells. **a** Crypts and villi of jejunum (mean ± SD). **b** Crypts of colon (mean ± SD)

services, and is often life-threatening [21]. Several mechanisms have been proposed for the development of irinotecan-induced diarrhoea, ranging from changes in the architecture and absorption rates of the large intestine [8, 22], to increases in intestinal β-glucuronidase levels caused by changes in intestinal bacteria [10, 23]. This study demonstrates for the first time that mucin secretion may also be involved in the development of mucositis and subsequent diarrhoea, with a significant increase in cavitated cells after treatment, and altered mucin expression.

The involvement of mucins has not previously been examined in the mucositis setting. This study has shown for the first time that mucin expression alters in the intestine after treatment with irinotecan, suggesting a link between mucin expression and mucositis. This may represent a reduction in mucin production, which in turn results in less

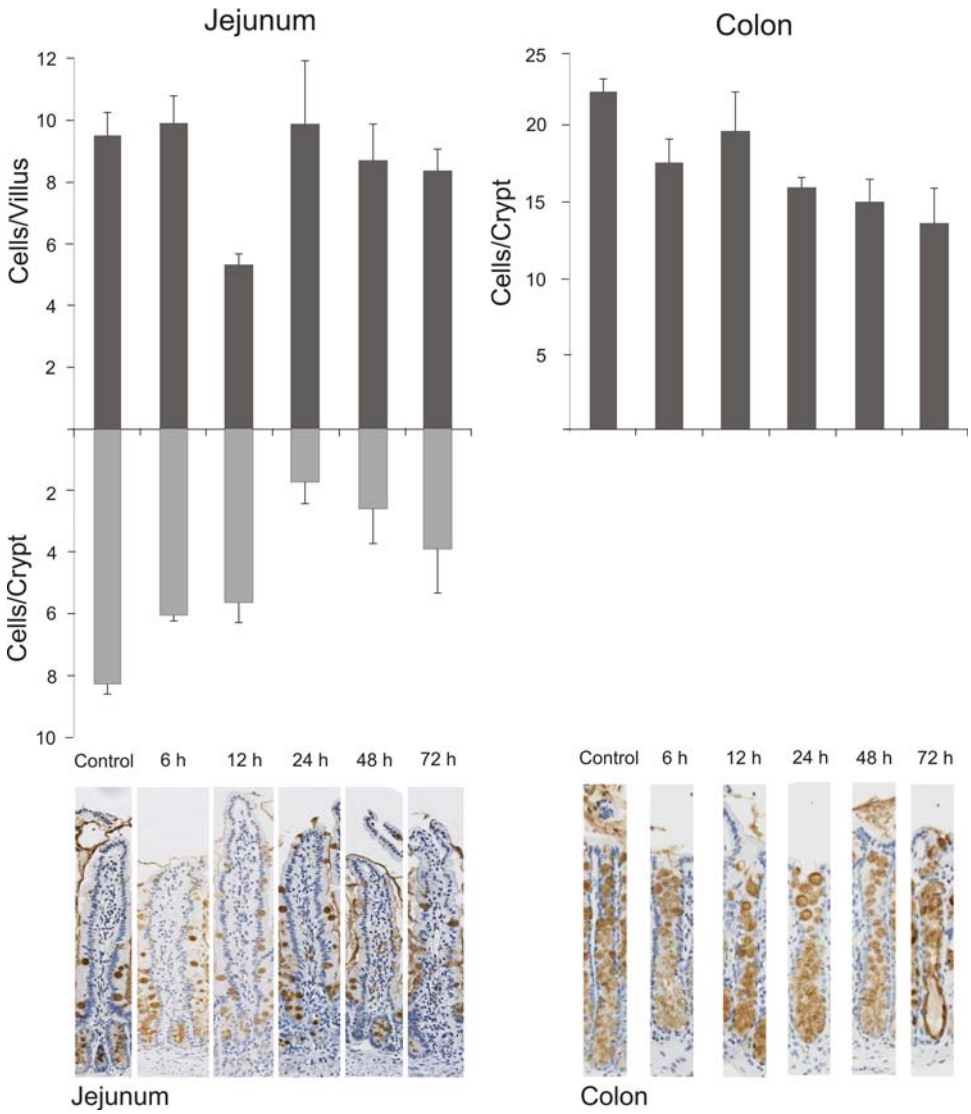

Fig. 5 Muc 2 immunostaining. Counts of stained cells per crypt/villus in the jejunum and crypts in the colon (mean ± SD). Representative images of stained goblet cells in crypts/villi in the jejunum and crypts in the colon. Original magnification ×20

mucin storage in goblet cells. Less mucin storage may cause the decreased staining of goblet cells seen in this study. An alternative theory is that the goblet cells could be damaged, resulting in decreased mucin production. Both scenarios could contribute to a compromised protective mucous layer, exposing the epithelium to damage from noxious agents present in the lumen, and bacterial degradation.

Other studies using irinotecan in rats have looked briefly at goblet cells and noted increases in mucin secretion [7, 24]. This study extends these studies by showing increasing cavitated cells from 24 to 72 h in the colon, indicating increasing mucin secretion [20]. This increase correlates with the incidence of diarrhoea, indicating that the increase in mucin secretion in the colon may be contributing to the diarrhoea. The incomplete effectiveness of atropine in this study indicates a neurotransmitter other than acetylcholine (ACh) may be mediating the increased mucin secretion. Apical mucin and chloride secretion is achieved by the mechanical stimulus of villous cells, by non-neuronal mechanisms to provide non-specific protection of the mucosa, protecting against the passage of food and protecting the epithelium from damage. Neuronal modulation is yet to be conclusively demonstrated [25]. However, goblet cells are specialised secretory cells endowed with a variety of receptors coupled to intracellular signalling pathways that regulate exocytotic machinery, and located in the vicinity of enteric nerves, enteroendocrine cells and immune cells, suggesting neurotransmitters, hormones and inflammatory mediators are potentially involved [26]. Efforts towards understanding the regulation of mucus secretion from goblet cells have been made, but the knowledge about chemical transmitters regulating mucus secretion remains limited [25]. Recent studies have shown vasoactive intestinal peptide (VIP, a neurotransmitter) increases mucus secretion in the intestine, as well as serotonin (a neurotransmitter, also known as 5-HT), prostaglandin E2 (PGE_2),

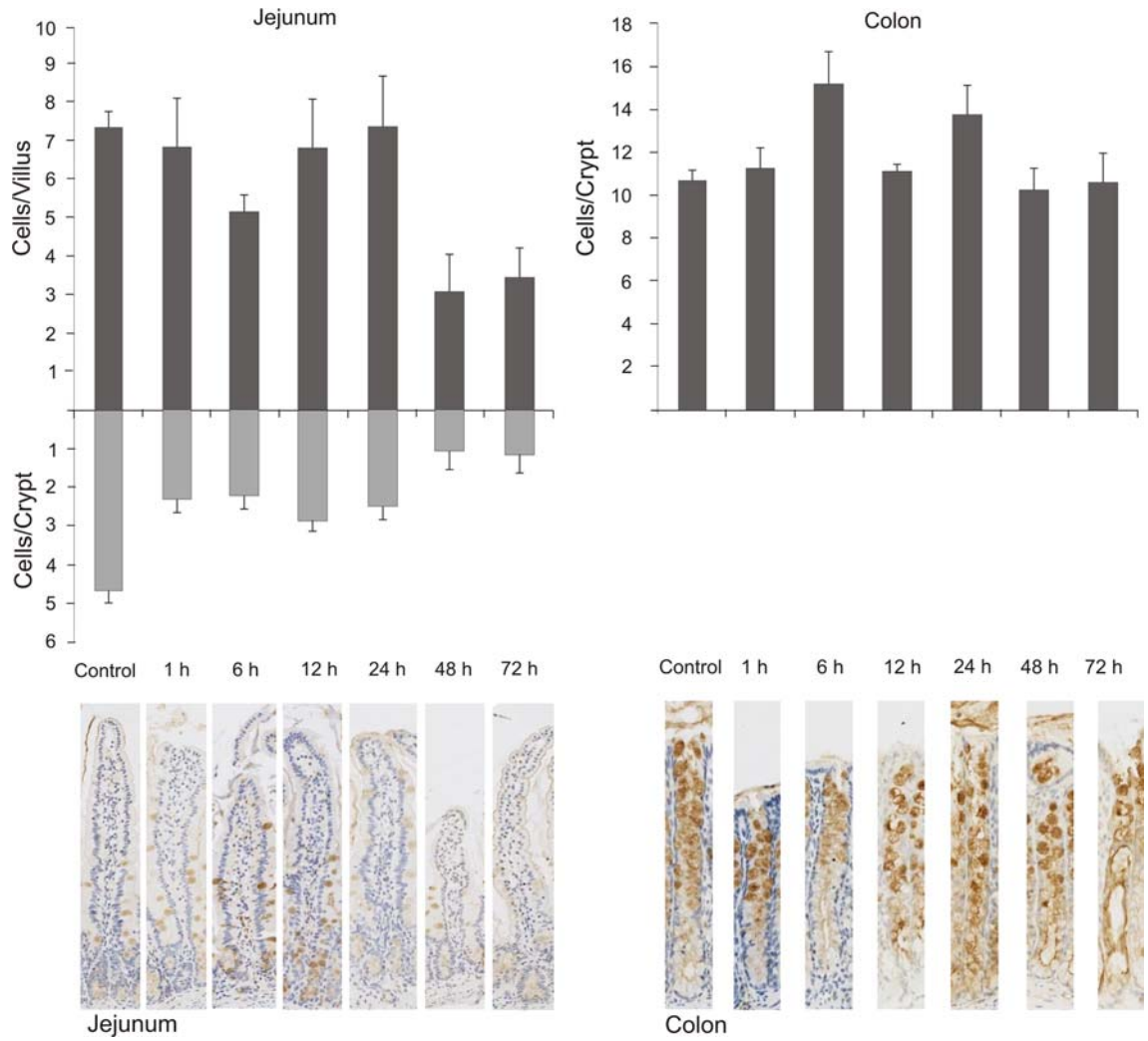

Fig. 6 Muc 4 immunostaining. Counts of stained cells per crypt/villus in the jejunum and crypts in the colon (mean ± SD). Representative images of stained goblet cells in crypts/villi in the jejunum and crypts in the colon. Original magnification ×20

interleukin-1β (IL-1β, a pro-inflammatory cytokine), and sodium nitroprusside (SNP, a nitric oxide generator) [26, 27] .

Nitric oxide may be implicated in the control of gastrointestinal mucin secretion [26]. Research has shown an increase in inducible nitric oxide synthase (iNOS), and subsequently nitric oxide (NO) during 5-FU-induced mucositis [28]. Chemotherapy agents generate reactive oxygen species (ROS) which directly damage cells and stimulate transcription factors, including nuclear factor kappa-B (NF-κB), in turn up-regulating genes resulting in the production of pro-inflammatory cytokines (including tumour necrosis factor [TNF], IL-β, and IL-6) [28]. More recently the same cytokines have been shown to be involved in chemotherapy-induced mucositis using the same dose of irinotecan as this study [29].

Another possibility is that the disruption of actin in damaged cells in the intestine, including goblet cells, may contribute to increased mucin secretion. Actin is present along the apical surface of goblet cells, and is distributed differently from adjacent absorptive cells [30]. Studies have shown that actin filament disruption does not alter the morphology of goblet cells, but does cause the acceleration of mucin granules through goblet cells to the apical surface, where they are then secreted. This is indicative that a loss of actin results in the acceleration of baseline (slow, sustained release of mucins to counteract loss in mucus layer) mucus secretion [30].

Klf4 (an epithelial zinc-finger protein) is thought to be involved in the differentiation of goblet cells [17]. Klf4 has also been implicated in down regulation of cell proliferation and growth arrest [30]. Studies have shown that Klf4-null mice have mature goblet cells in the colon reduced 90% relative to total epithelial cells [17]. This study shows that Klf4 is altered in the stomach, jejunum and colon after treatment with irinotecan, suggesting that changes in the differentiation of goblet cells from multipotent stem cells could be in part responsible for

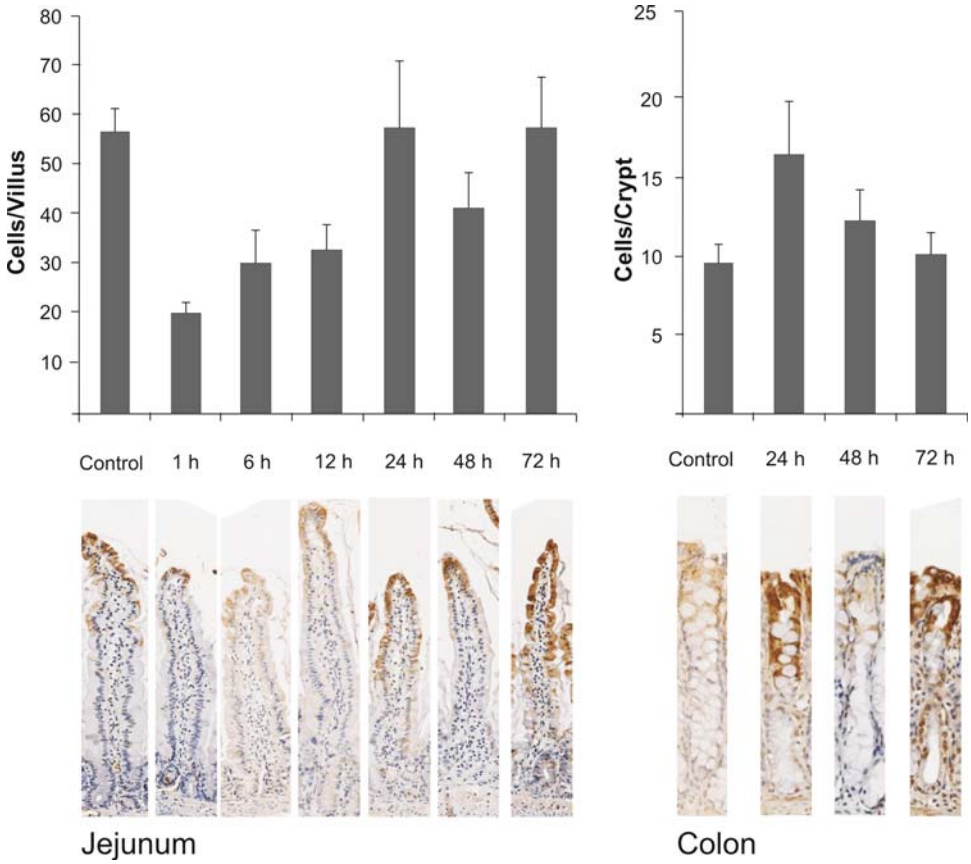

Fig. 7 Klf4 immunostaining. Counts of stained cells per villus in the jejunum and crypt in the colon (mean ± SD). Representative images of stained enterocytes in jejunal villi and colonic crypts. Original magnification ×20

changes in mucin expression and secretion. However, little research has been conducted on Klf4, and the exact extent of its function is yet to be elucidated, let alone its involvement in mucositis. Further studies to explore the potential significance between Klf4 and mucositis are warranted.

In conclusion, this study has shown for the first time that irinotecan causes an increase in mucin secretion and a net decrease in mucin-producing goblet cells, and the expression of Muc2, Muc4 and Klf4 in the GIT is altered following treatment. Increased mucin secretion is likely to be related to altered mucin gene expression, and may contribute to chemotherapy-induced diarrhoea.

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Conflict of interest statement None.

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