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SCIENCE DIRECT

Regulatory Peptides 126 (2005) 163–171

REGULATORY PEPTIDES

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Luminal and parenteral TFF2 and TFF3 dimer and monomer in two models of experimental colitis in the rat

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Received 8 July 2004; received in revised form 26 August 2004; accepted 19 September 2004
Available online 18 October 2004

Abstract

Background: Peptides of the trefoil factor family (TFF1, TFF2 and TFF3) are cosecreted with mucus from mucus-producing cells in most organ systems and are believed to interact with mucus to form high-viscosity stable gel complexes. In the gastrointestinal tract, they sustain the mucosal barrier, and both injected and orally administered TFF peptide have protective and healing functions in the gastric mucosa.
Aim: To investigate the possible treatment effect of luminally and parenterally administered TFF peptides in experimental colitis in rats.
Methods: Colitis was induced by administration of 5% dextran sodium sulphate in the drinking water or by one intraperitoneal injection of mitomycin C, 3.75 mg/kg. TFF peptides were administered as subcutaneous injections or directly into the lumen via a catheter placed in the proximal colon. Treatments were saline, TFF2, TFF3 monomer or TFF3 dimer 5 mg/kg twice per day throughout the study [dextran sulphate sodium (DSS)] or from day 4 to 7 (mitomycin C). Colitis severity was scored in a stereomicroscope and histologically.
Results: Luminal treatment with TFF3 in its dimeric form significantly improved the colitis score in both colitis models, whereas TFF2 had positive effect only in DSS-induced colitis. The TFF3 monomer was without any effects in both models. Treatment effect was most pronounced in the middle part of the colon, closest to the tip of the catheter. Injected TFF peptides, especially the TFF3 monomer, aggravated the colitis score in both colitis models.
Conclusions: Intracolonic administration of TFF3 dimer and TFF2 improves experimentally induced colitis in rats. The TFF3 monomer has no effect. Parenteral administration of TFF peptides aggravates the colitis especially the TFF3 monomer.

Keywords: TFF; Mitomycin C; Dextran sulphate sodium

1. Introduction

The trefoil factor family (TFF) consists of three peptides, TFF1, TFF2 and TFF3, which are cosecreted with mucus from mucus-producing cells and which by binding to mucus and by complex formation are able to influence the viscosity of mucus. They contain one or two trefoil domains defined as a sequence of 38 or 39 amino acid residues which by means of three disulphide bonds create a characteristic structure consisting of three loops. TFF2 has two trefoil domains, whereas TFF1 and TFF3 have one, but both are able to form dimers [1–3]. TFF1, previously known as pS2, breast-cancer-associated peptide, and TFF2, previously known as SP, spasmolytic polypeptide, are primarily localized to the stomach [4–6], whereas TFF3, known as intestinal trefoil factor (ITF), is more generally distributed to mucus-secreting cells and glands in all organ systems, including the goblet cells of the intestines [7–13]. Under pathophysiological conditions, all three TFF peptides can be up-regulated, and, in the GI tract, they are found in the ulcer-associated cell line (UACL) cells, which are cells that

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doi:10.1016/j.regpep.2004.09.007

arise in areas with ulceration or inflammation [14–16]. A main function of the TFF peptides is considered to be regulation or modification of the viscosity of mucus-containing exocrine secretions on mucosal surfaces [17–20]. Recent investigations have disclosed that the three TFF peptides differ in respect to tertiary structure and surface charge [21–23]. TFF2 has a very compact structure in comparison to the others, and the highest viscosity is obtained following interaction between mucus and TFF2, whereas TFF3, in its dimeric form, produces a mucus/TFF complex with lower viscosity and elasticity, and TFF3 monomer is without any direct effect on the viscosity [20].

A receptor-like activity to the trefoil peptides has been demonstrated throughout the gastrointestinal tract [24–27]. In several experimental studies, it has been demonstrated that both luminally administered and injected TFF2 and TFF3 in the dimeric form increase the resistance of the mucosa of the stomach and accelerate the healing of gastric ulcers [28–33]. A similar effect might be expected in the intestinal system, but the results of experimental studies have been less consistent and convincing there than in the upper gastrointestinal tract. TFF3 knockout mice had increased sensitivity to dextran sulphate sodium (DSS) colitis [34], and luminal treatment with TFF2 increased the healing of DNBS-induced colitis in rats [35]. Recently, Soriano et al. [36] found positive effects on clinical parameters following both luminal and systemic pretreatment with TFF2 in DSS-induced colitis, but there was no effect on the histological parameters and no effect on existing colitis. In the present study, we have investigated the effect of both systemic and luminal treatment with TFF2 and TFF3 monomer and dimer in two rat models of experimental colitis. In order to obtain adequate luminal treatment, we have introduced an experimental model, in which rats are fitted with a proximal colonic catheter for direct intraluminal administration. We demonstrate an effect of luminal treatment with TFF2 and TFF3 dimer in both rat models, and a highly significant aggravation following systemic treatment, especially following the monomer.

2. Material and methods

2.1. Animals

The experimental studies were approved by the Danish National Committee of Animal Studies. One hundred twenty-eight female Wistar rats weighing approximately 200 g were used in the study. They were maintained throughout the course of the experiment on water and chow (no. 1314, Altromin, Lage, Germany) ad libitum in the animal facilities of the Panum Institute, University of Copenhagen, Copenhagen, Denmark, with temperature (21 °C) and humidity (55%) controlled rooms with a light–dark cycle of 12 h each.

2.2. Induction of colitis

Two models of experimental colitis were used in the study, that induced by dextran sulphate sodium (DSS) and that induced by mitomycin C. DSS colitis was induced by giving 5% DSS (cat. no. 160110, ICN Biomedicals, OH, US) in the drinking water for 10 days. The rats were kept in single cage, and their water intake was monitored each day to secure that there were no differences in DSS intake between the groups. Treatments were given from the day before initiation of DSS exposure until sacrifice on day 10.

Mitomycin C colitis was induced by one single intraperitoneal injection of mitomycin C (cat. No. 100498, ICN Biomedicals), 3.75 mg/kg. Treatment was given for 4 days from day 4 to day 7 inclusive, where the rats were sacrificed.

The rats were sacrificed by an overdose of methohexital (Brietal, Lilly, USA). The abdomen was opened by a midline incision, and the colon was fixed in situ by intraluminal injection of ice-cold 0.1 mol/l phosphate buffer, pH 7.4 with 4% paraformaldehyde to slightly distend the colon to avoid mucosal foldings. After 5 min, the colon, including the anal canal, was taken out, cut open antimesenterially and suspended on a polyethylene plate. After fixation for a further 24 h, the specimens were rinsed in tap water and surface-stained as whole mounts with Alcian Green 3BX for 30 min. The colonic specimens were examined using a Wild Photomacroscope, and the extent of disease in the colon was quantified in a blinded way. Mitomycin colitis results in a granulated surface with small shallow ulcerations, and the DSS colitis in areas with flat erosions, both easily distinguished from the normal mucosa (Fig. 1A–C).

For histological analysis, specimens, approximately 2 -m cut in the longitudinal direction, were taken out in a blinded way from the middle and distal colon. Histological sections of 5 μ were stained with PAS–hematoxylin–aurentia. In DSS colitis, there is primarily epithelial atrophy, formation of erosions and mucosal inflammation, which progress to the other layers (Fig. 1D–H). The severity of colitis was evaluated in a blinded way by means of a histological scoring technique as published by Williams et al. [37].

2.3. Treatment groups

The rats were divided into groups of eight. In half of the rats, treatments were given as subcutaneous injections twice daily, and, in the other half, they were given directly into the colonic lumen via a polyethylene tube which, 5 days prior to the experiment in anaesthetized rats, was inserted into the lumen of the proximal part of the colon. The catheter, measuring 0.8×1.6 mm (in inner and outer diameter) and approximately 15 cm in length was fitted with a 3-mm piece of a catheter measuring 1.4×2.0 mm (inner and in outer diameter) in each end (4 mm from the ends of the catheter). A little incision was made antimesenterially in the proximal colon, and the end of the catheter with the small outer polyethylene tube was

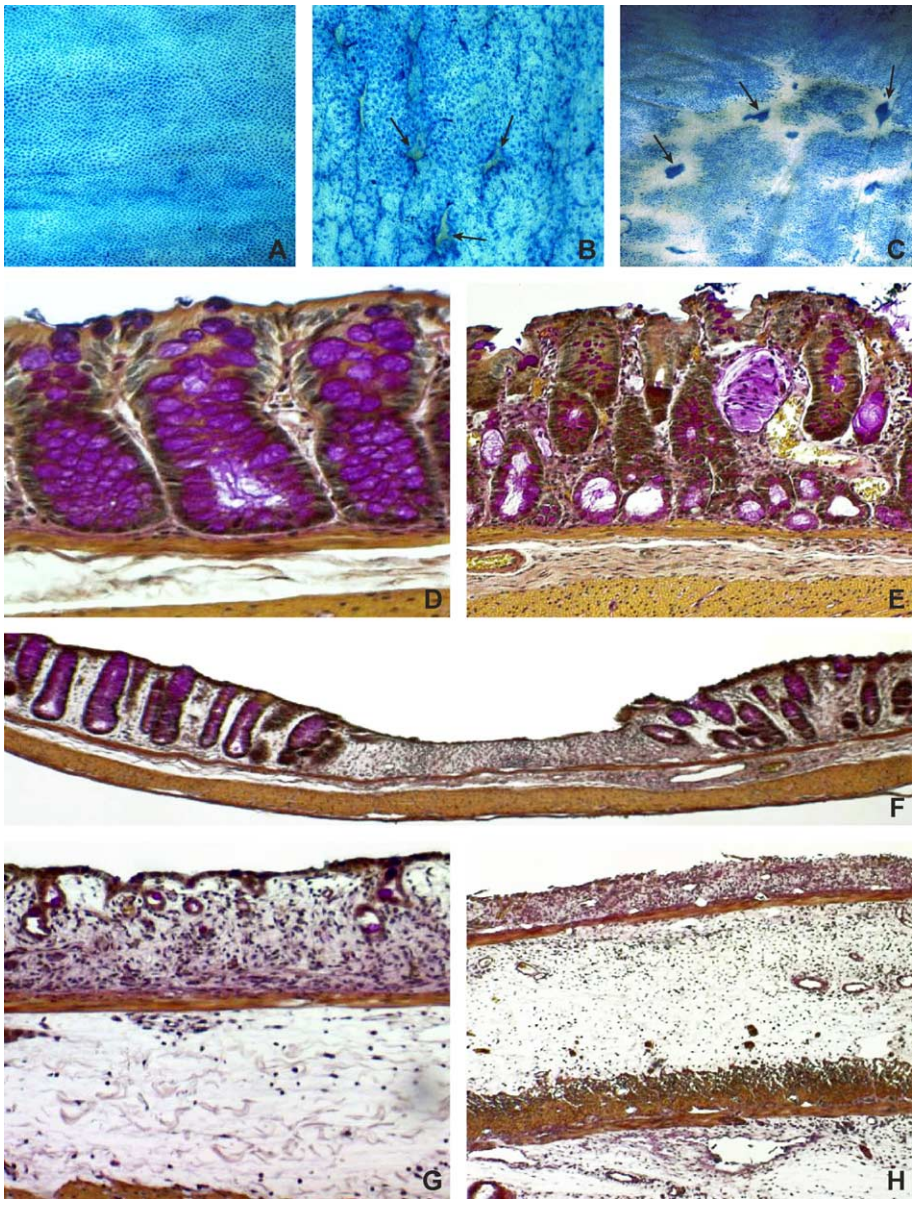

Fig. 1. (A–C) Stereomicroscopy. (A) The normal colonic mucosal surface as observed in the stereomicroscope following whole mount staining with Alcian green. The crypt openings are seen as dark green dots (accumulation of goblet cells) regularly distributed on the surface. (B) Mitomycin-induced colitis. Irregular granulated surface with small shallow ulcerations (arrows). (C). Dextran-induced colitis with superficial (dark green) erosions (arrows) in the middle of areas with epithelial degeneration without goblet cells. Original magnifications: A, ×12; B, ×8; C, ×16. (D–H) Histology. (D) Histology of the normal mucosa with tall columnar epithelium in the crypts and plenty of goblet cells. Mitomycin-induced colitis with inflammation in the mucosa, mucus accumulations, epithelial degeneration and increased height of the mucosa. (E) Dextran-induced colitis, a superficial erosion surrounded by almost normal mucosa. (F) Dextran-induced colitis in more advanced stages, with basal 2/3 crypt damage and involvement of the (G) submucosa and (H) transmural inflammation with both crypts and surface epithelium lost. PAS–Hematoxylin–Aurentia. Original magnifications: D, ×150; E and G, ×80; F, ×50; H, ×60.

inserted and secured with 6-0 silk sutures. The incision in the intestinal wall was closed also with 6-0 silk sutures. The catheter was led subcutaneously to the neck region, and, through a little incision, the last 10 mm was allowed to protrude from the surface. The end of the catheter was secured by silk 6-0. The correct localization and function of the catheter was confirmed by administration of 0.5-ml barium sulphate via the catheter with an X-ray taken immediately following the application of contrast medium (Fig. 2).

The treatments were given twice a day as subcutaneous injections consisting of 0.5 ml isotonic saline or TFF2, TFF3 dimer or TFF3 monomer 1 mg in 0.5 ml H2O. The same doses were given twice a day directly into the colonic lumen via the colonic catheter.

TFF2, TFF3 dimer and monomer were produced and purified as previously described [38,39].

The results are shown as mean±S.E.M.. Comparison between groups was performed by two-way analysis of variance (ANOVA) followed by Fisher's protected least

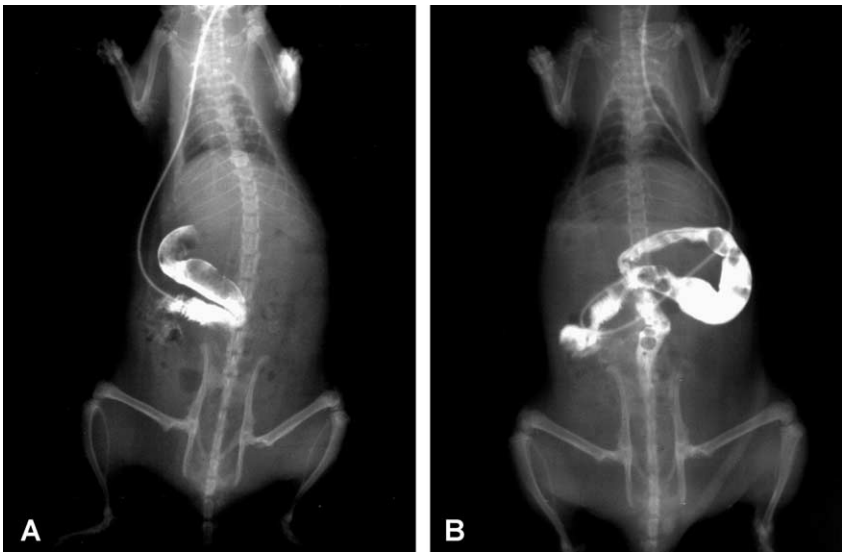

Fig. 2. X-ray examination of a rat fitted with an intracolonic catheter immediately after introduction of (A) 0.5- and (B) 2-ml contrast medium via the catheter. After 0.5 ml, mainly the right colon is visualized, whereas a volume of 2 ml is distributed to the entire colon.

significant difference post hoc analysis. Probability values of $p<0.05$ were considered significant.

3. Results

3.1. Luminally administered TFF

3.1.1. Mitomycin-induced colitis

Evaluated from the stereomicroscopic examination, luminal treatment with the TFF3 dimer resulted in a significant ($p<0,05$) reduction in the extent of disease in the colon from 72%±7 in the controls to 50%±7, whereas TFF2 and TFF3 monomer had no effect (Fig. 3A). This was confirmed at the histological scoring, where TFF3 dimer resulted in reduced activity both in the middle and in the distal colonic segment, 3.2±0.9 and 3.7±1.1, respectively, in comparison to 4.9±0.9 and 5.7±0.7 in the controls, whereas TFF2 and TFF3 monomer were without effect (Fig. 4A). Thus, only luminal TFF3 dimer had a positive effect in mitomycin-induced colitis.

3.1.2. Dextran-induced colitis

The percentage of colon affected was reduced following luminal treatment both with TFF2 and TFF3 dimer, from 50%±9 to 23%±7 and 25%±5, respectively, or by 49% and 44% (Fig. 3B). At the histological scoring, there was a significant effect in the middle part of the colon of both TFF2 and TFF3. Following TFF2, the score was reduced by 43%, and following TFF3 dimer by 77% (Fig. 4B). The monomer was without any effect. In the distal part of the colon, the histological score in the controls was more than double that, in the middle part (3,6±0,8 vs. 1,7±0,2) and in this part, there was no effect of any of the treatments.

Thus, luminal treatment with both TFF2 and TFF3 dimer reduced the disease activity primarily in the middle part of the colon in dextran-induced colitis, whereas the monomer was without any effect.

3.2. Subcutaneously administered TFF

3.2.1. Mitomycin-induced colitis

The percent of colon involved was increased compared to the controls following both TFF2 and the TFF3 monomer (50%±12 vs. 72%±10 and 100%, respectively), whereas the TFF3 dimer was without any effect (Fig. 3A). At the histological examination, there was no effect of TFF2 or the TFF3 dimer either in the middle or in the distal part of the colon, but the disease activity was significantly aggravated following the monomer, 7.5±0.4 and 7.7±0.6, respectively, in comparison to 4.0±0.4 and 4.0±0.6 in the controls (Fig. 4A). Thus, there were no beneficial effects of treatment with subcutaneous TFF peptides in mitomycin-induced colitis but a considerable aggravation following treatment with the monomer.

3.2.2. Dextran-induced colitis

Following subcutaneous administration, all three TFF peptides resulted in increased extent of disease in the colon (Fig. 3B). In the controls 51%±7 of the colonic surface was affected by colitis compared to 91%±6 after TFF2, 72%±7 after TFF3 dimer and 100% after the monomer.

At the histological examination, the score for the TFF2 group was more than doubled in both the middle and the distal segment, whereas the group treated with TFF3 monomer had increased activity in the middle segment, and the group treated with the dimer had slightly increased activity in the distal segment (Fig. 4B). Thus, in dextran-induced colitis, there were no beneficial effects of injected

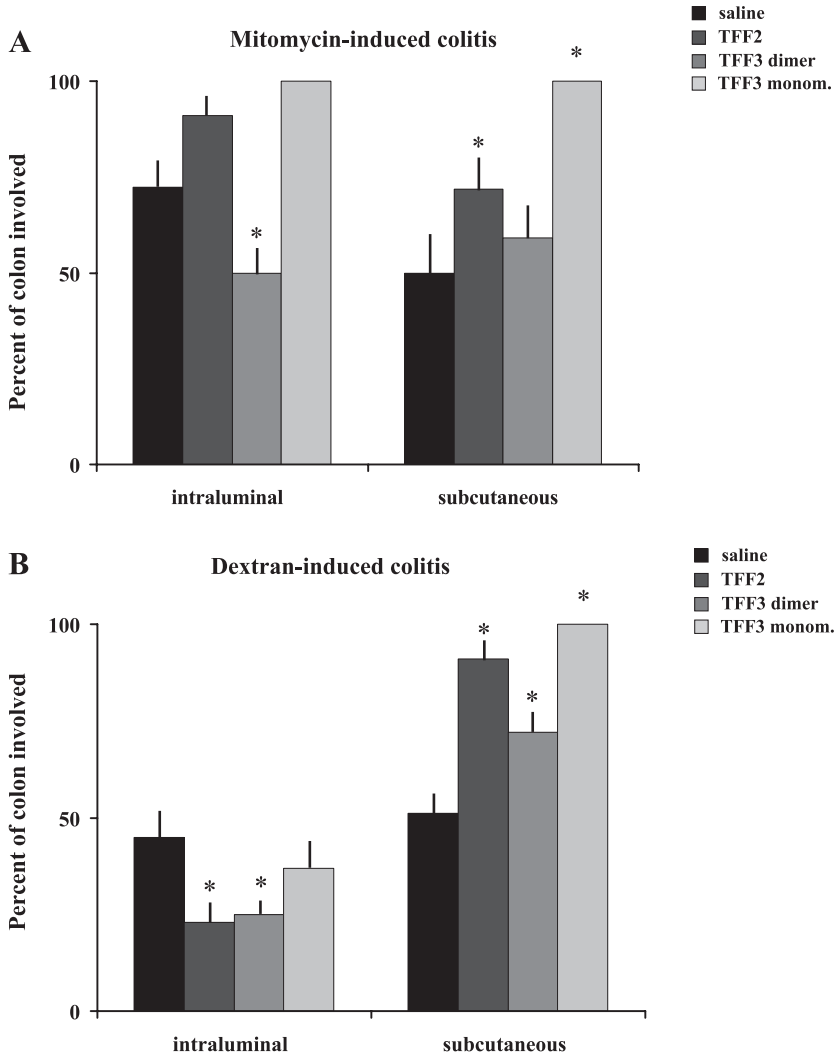

Fig. 3. Effect of systemic and intraluminal TFF2, TFF3 dimer and TFF3 monomer on the extent of disease in the colon evaluated from stereomicroscopic examination. (A) In mitomycin-induced colitis, the TFF3 dimer given intraluminally (left) reduces the extent of disease, whereas systemic treatment (right) with TFF2 or TFF3 monomer increase the extent of disease. (B) In DSS-induced colitis, luminal treatment (left) with both TFF2 and TFF3 dimer reduce the extent of disease, whereas systemic treatment (right) results in increased extent following all three TFF peptides. Values represented are mean±S.E.M. *p<0.05 vs. the control group.

TFF peptides but a significant aggravation, especially following treatment with TFF2.

4. Discussion

In as much as the etiology of the inflammatory bowel diseases, Crohn's disease and ulcerative colitis, is unknown, and probably not the same for the two diseases, it is not possible in animal models to mimic precisely human diseases [40]. In the present study, we have investigated the effect of TFF peptides in two different models of inflammatory bowel disease, the commonly used DSS model [41] and the mitomycin C model.

In the former, dextran sodium sulphate is administered in the drinking water, and the primary effect is damage to the surface epithelium from the luminal side. Therefore, this model seems most appropriate to disclose an increase in the protective ability of the mucosal barrier following a treatment. The treatment with TFF peptides therefore was given from the day before initiation of DSS administration and throughout the study.

Mitomycin C is a cytotoxic compound which induces an inflammatory response primarily localized in the mucosa as the result of a single intraperitoneal injection [42]. The surface epithelium remains rather intact with few ulcerations. The colitis has its maximum after 4–7 days and heals spontaneously within 2–3 weeks. The remaining GI tract is unaffected. In this model, the colitis is not induced from the luminal side, and the surface epithelial barrier is almost intact, and the model seems appropriate to detect an anti-inflammatory effect. Treatment is given from day 4, where the cytotoxic effect has declined, to day 7, where there still is pronounced inflammation.

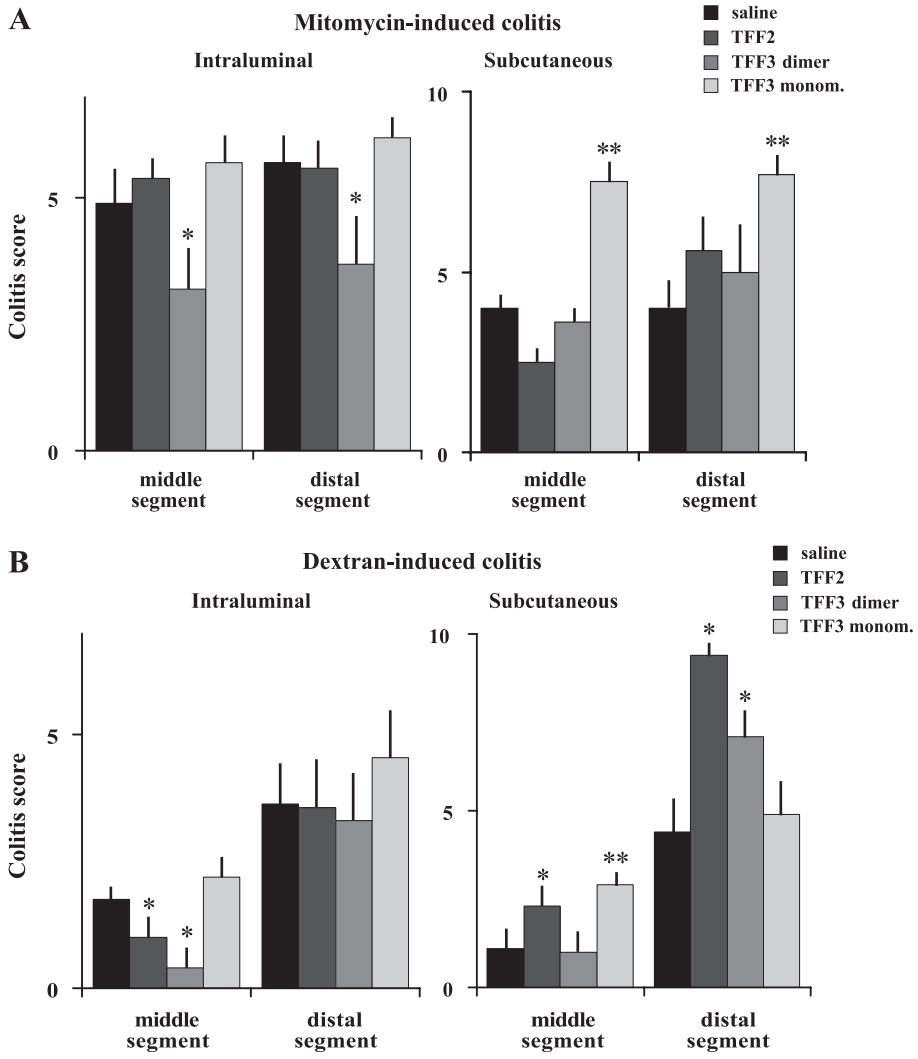

Fig. 4. Effect of systemic and intraluminal TFF2, TFF3 dimer and TFF3 monomer on the histologic colitis score in the in middle and distal colon. In mitomycin-induced colitis (A) the TFF3 dimer given intraluminally (left) reduces the histologic score both in the middle and distal segment whereas the monomer and TFF2 are without effects. Systemic treatment (right) with TFF3 monomer results in considerably increased score, whereas the dimer and TFF2 are without effects. In DSS-induced colitis (B) luminal treatment (left) with TFF2 and TFF3 dimer decrease the colitis score in the middle segment but there is no effect in the distal segment. Systemic treatment (right) results in significantly aggravated score in both segments following TFF2, and in the middle segment following TFF3 monomer and in the distal segment the dimer. Values represented are mean±S.E.M. *p<0,05; **p<0,01 vs. the control group.

The TFF peptides are very resistant to degradation by proteases, and orally administered TFF peptide passes intact through the stomach and small intestine. In the cecum, however, they are degraded, probably by bacteria, and the oral route is therefore not possible for treatment of the colonic mucosa [33]. The rats given oral treatments are therefore equipped with a catheter led subcutaneously from the neck region to the proximal part of the colon, and the TFF peptides are introduced directly into the colonic lumen through this catheter.

We found that the TFF peptides had positive effects only when administered from the luminal side. TFF3 in its dimeric form worked in both colitis models, whereas TFF2 had effect only in DSS-induced colitis. TFF3 in the monomeric form had no effect at all. When the middle segment and the distal segment were compared at the histological examination, we found that, in the DSS model, there was treatment effect only in the middle segment of the colon and not in the distal segment. This probably reflects the degradation of the TFF peptides in the colonic lumen [33], not leaving sufficient concentrations of peptide to pass to the distal segment, which is most distant from the tip of the catheter. Thus, the three forms of TFF peptides investigated did not have the same effect when administered luminally. It has been increasingly evident that the trefoil peptides, despite similarities, including the one- or two-trefoil domains, have variations in their biological and structural characteristics. TFF2 has a very compact structure and fixed orientation [21], whereas the two-monomer units of TFF1 have no fixed orientation and are separated by a flexible linker which allows several conformations [23]. The dimeric form of TFF3 has a shorter linker sequence, and the

molecule is therefore more compact in comparison to TFF1, but the structural characteristics are still very different from those of TFF2 [43], and the molecule seems to possess characteristics intermediate between TFF1 and TFF2 [44].

Considering the mucus/TFF interaction, we have previously reported that the addition of TFF2 to mucin solutions results in significantly increased viscosity and elasticity and forms large complexes, whereas TFF3 dimer produces small complexes and increased viscosity and elasticity to only a minor degree and that the monomer is almost without any effect [20]. Considering these data for mucus/TFF interaction, it is remarkable that TFF2 in the present study is not superior to TFF3 dimer in the colitis models (actually, TFF3 is the best). The lack of effect of the TFF3 monomer is in agreement with the findings that the monomeric form of TFF1 is less active in protection of the gastric mucosa than the dimer [45] and that TFF3 monomer has very little effect on the viscoelasticity of mucus [20].

Injected and luminally administered TFF peptides have comparative protective and healing effects in the stomach, and injected TFF2 has even been demonstrated to work in lower dosages [28,31–33]. Injected TFF peptides bind to mucus-producing cells in the gastric mucosa and seem to end up in the mucus layer in the same way as endogenous TFF peptide [25]. A similar mechanism might be expected in the intestinal system as comparable binding sites have been described both in the small intestine and the colon [25,46]. In the present study, we found, however, that there were no beneficial effects of any of the TFF peptides investigated in any of the two colitis models. On the contrary, the TFF3 monomer and, to a minor degree, TFF2 significantly aggravated colitis induced by mitomycin C, and all three forms significantly aggravated DSS-induced colitis. Thus, we demonstrated effects following injection of the TFF peptides, finding not only that the compounds are not inert but that they also, in this context, are not beneficial. These findings are in agreement with our previous demonstration of aggravation of duodenal ulcers following treatment with injected TFF2 [33]. The TFF3 monomer, which only interacts very little with mucus and which has only little effect in gastric mucosal protection [45], has the most pronounced negative effect.

In contrast with our findings, FitzGerald et al. [47] recently demonstrated a positive effect following subcutaneous administration of TFF1 in its dimeric form in rats with DSS-induced colitis. The effect was potentiated by simultaneous administration of epidermal growth factor. In the present study, we tested TFF2 and TFF3, and the difference in results may be explained by variation in characteristics between the three TFF peptides. However, the dosages in the two studies are very different as we used the same dosage, 1 mg/rat (5 mg/kg), that was demonstrated to heal gastric ulcers following both parenteral and luminal administration. For comparison, Babyatsky et al. [28] needed a dosage of 15 mg/rat TFF2 given intraperitoneally to achieve a protective effect against indomethacin-induced ulceration. FitzGerald et al. used a dose 50 times smaller than the one used in our study, 100 μg/kg or 25 μg per rat. Another possible explanation for the different effect in DSS-induced colitis could be that this much smaller dose might be sufficient to stimulate cell migration, an effect of TFF peptides, which has been demonstrated in vitro [32,47] but too small to interact with mucin which require rather high local concentrations of TFF peptide [20]. The increased cell migration might be beneficial, whereas the TFF/mucin interaction might have negative effect in the present colitis models.

The findings emphasize that the biological and pharmacological effects of the trefoil peptides still remain to be finally clarified. The demonstration in vitro of other effects of the TFF peptides rather than interaction with mucins, especially stimulation of cell migration and reduced apoptosis (for review see [48,49]), implicates the presence of a receptor to the TFF peptides, preferably with basolateral localization. TFF binding sites to mucous cells in the gastrointestinal tract have been demonstrated both in vivo and in vitro [24,50], and two TFF2 binding proteins have been identified from intestinal crypt epithelium, but a functional receptor still remains to be finally identified. We have no explanation for the aggravating effect of injected TFF peptide in the colon in the present study and in the duodenum in our previous study, but the presence of any reaction implies some kind of interaction with the intestinal mucosa that might be dependent on the presence of a receptor-like activity.

In conclusion, we have demonstrated positive effect of luminal treatment with TFF2 and the TFF3 dimer in DSS-induced colitis and of the latter in colitis induced with mitomycin C, whereas TFF3 in its monomeric form was without any effect. On the contrary, injected TFF peptides aggravated both forms of colitis—especially the monomer, which in other contexts is considered to be a biologically inactive form.

Acknowledgments

The Biotechnology Center for Cellular Communication and the Danish Medical Research Council supported the study. The technical assistance of Jette Schousboe and the photographic assistance of Grazyna Poulsen are warmly acknowledged.

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