Temporal changes in TFF3 expression and jejunal morphology during methotrexate-induced damage and repair

C. J. Xian, G. S. Howarth, C. E. Mardell, J. C. Cool, M. Familiari, L. C. Read, and A. S. Giraud. Temporal changes in TFF3 expression and jejunal morphology during methotrexate-induced damage and repair. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G785–G795, 1999.—Trefoil factor TFF3 has been implicated in intestinal protection and repair. The present study investigated the spatiotemporal relationship between TFF3 expression and morphological changes during intestinal damage and repair in a rat model of methotrexate-induced small intestinal mucositis. Intestinal tissues from rats with mucositis were collected daily for 10 days. Mucosal damage was characterized by an initial decrease in cell proliferation resulting in crypt loss, villus atrophy, and depletion of goblet cells, followed by hyperproliferation that lead to crypt and villus regeneration and mucous cell repopulation. TFF3 mRNA levels increased marginally during histological damage, and the cell population expressing TFF3 mRNA expanded from the usual goblet cells to include some nongoblet epithelial cells before goblet cell repopulation. TFF3 peptide, however, was depleted during histological damage and normalized during repair, mirroring the disappearance and repopulation of goblet cells. Although there is no temporal relationship between TFF3 levels and crypt hyperproliferation, confirming the nonmitogenic nature of TFF3, the coincidental normalization of TFF3 peptide with repopulation of goblet cells and mucin production after proliferative overshoot suggests that TFF3 may play a role in the remodeling phase of repair.

mucositis; intestinal trefoil peptide; intestinal mucosal injury; regeneration

INTESTINAL TREFOIL FACTOR (ITF, also known as TFF3) and the other two known mammalian members of the trefoil peptide family—ps2 (also known as TFF1) and spasmolytic polypeptide (SP, also known as TFF2)—share a three-loop (or “trefoil”) structural motif (for reviews, see Refs. 23, 27–29, 33, and 34). Formation of the three intrachain loops in trefoil peptides, secured by three disulfide bonds from six cysteine residues, results in rigidity of the structural motif and stability against acid hydrolysis and proteolysis in the gut (12–35). In mammals, trefoil peptides are produced predominantly in the gastrointestinal tract, generally with a regional specificity. TFF1 (ps2) and TFF2 (SP) are mainly produced by mucous cells of the gastric mucosa and biliary-pancreatic ducts; however, TFF3 (ITF) is predominantly synthesized by the goblet cells in the small and large intestine (22, 30, 32). Trefoil peptides are secreted in association with mucins into gut lumen and are concentrated within the mucus layer (15).

Because of their stability against gastric acid and luminal proteolysis as well as their abundance in the gut lumen, trefoil peptides are well suited to exert functions at the luminal-mucosal interface. However, the functional effects and mechanisms of action of trefoil peptides in the gut are not completely clear. Evidence from in vitro and in vivo experiments suggests that trefoil peptides may play a key role in protecting the gastrointestinal mucosa from various insults (4, 6, 8), probably by preserving the integrity of the epithelial barrier by promoting the formation of a continuous gel with mucin glycoproteins on the mucosal surface (16). Previous studies have also shown that trefoil peptides enhance mucosal repair by stimulating cell restitution immediately after damage (10, 20). In support of these maintenance and reparative functions of trefoil peptides in the gut are studies demonstrating overexpression of trefoil peptides at sites of gastrointestinal ulceration and inflammation (26, 39), an increased resistance to intestinal damage in transgenic mice overexpressing TFF1 (21), and a higher susceptibility to gut-related injury in mice that lack either TFF1 (18) or TFF3 (19) genes.

We attempted to further our understanding of the role of trefoil peptides in intestinal mucosal injury and repair by examining the spatiotemporal relationship between expression of TFF3, the major trefoil peptide expressed in the intestine, and the small intestinal mucosal damage and repair processes in a rat model of methotrexate-induced mucositis. Methotrexate, as a chemotherapeutic agent, exerts its toxicity to intestinal epithelial cells by directly inhibiting DNA synthesis, leading to a reduction of mitosis in the crypts and shortening of the villi (3, 38). This model of intestinal mucositis is characterized histologically by crypt loss, villus fusion and atrophy, gross capillary dilatation, a mixed cellular infiltrate, and a rapid recovery (13, 31). These features resemble the gut mucositis experienced as a common side effect by patients undergoing chemotherapy or radiotherapy (3). In the present study, a complete time course of methotrexate-induced intestinal damage and repair in rats was used to investigate the timing and location of TFF3 protein and mRNA...
expression in the intestinal tissues, as related to histo-
pathological and cellular changes.

MATERIALS AND METHODS

Time course of methotrexate-induced intestinal mucositis. Intestinal mucositis was induced in 120-g body wt male Sprague-Dawley rats using methotrexate as described previ-
ously (13). Briefly, methotrexate at 2.5 mg/kg was subcutane-
ously administered to rats in the suprascapular region once daily for 3 consecutive days. One group of ad libitum-fed normal rats without methotrexate injection (n = 8) were used as control animals (day 0). Daily for 10 days, groups of rats (n = 8) were injected intraperitoneally with bromodeoxyuridine (BrdU) (an analog of thymidine that is incorporated into DNA during the S phase of the cell cycle) at 50 mg/kg and were killed 1 h later. Two groups of pair-fed rats (n = 4) without methotrexate injection but consuming daily amounts of food similar to the methotrexate-injected animals were killed on day 5 and day 8 for comparison with their methotrexate-injected counterparts. Two 3-cm-long tissue specimens from the proximal jejunum, the region of the intestine with maximal damage, were freshly collected, with the proximal segment immediately snap-frozen in liquid nitrogen (for RNA and protein extraction) and stored at −80°C and with the distal segment fixed in methacarn fixative for 2 h. Each fixed intestinal specimen was dehydrated by passage through graded alcohol, cut into four equal lengths, and embedded in paraffin wax. Paraffin-embedded, transverse sections (4 µm thick, each with 4 intestinal segments) from these processed tissues were used for histological analysis, cell proliferation study, TFF3 immunohistochemistry, and in situ hybridiza-
tion.

Semiquantitative histopathological analysis of intestinal damage and repair. Hematoxylin- and eosin-stained tissue sections from the proximal jejunum were examined with a light microscope. An overall score of intestinal damage severity was semiquantitatively assessed as described (13), using criteria that included villus fusion and stunting (atrophy), disruption of brush-border and surface enterocytes, reduction in numbers of mitotic figures, crypt loss/architectural disruption, disruption or distortion of crypt cells, crypt abscess formation, infiltration of polymorphonuclear cells and lympho-
cytes, and dilatation of lymphatics and capillaries.

Periodic acid-Schiff staining and goblet cell counting. As an additional means to assess intestinal damage, goblet cell numbers were derived from the 4-µm paraffin sections of the proximal jejunal specimens. To highlight the goblet cells, sections were stained for mucins by the periodic acid-Schiff (PAS) technique (5). Counting PAS-stained goblet cells in both villi and crypts was performed using computer-aided video image analysis. For each specimen, a total of 20 microscopic fields of crypts and 20 fields of villi under a ×20 objective were measured per animal. For each field, the total area of crypts (including areas of epithelium, crypt lumen, and stroma) or the total area of villus epithelium only (excluding lumen and stroma) was traced to provide an area of measurement, and the number of goblet cells within that field was counted. Goblet cell counts per unit area were then derived from the two values.

TFF3 Western blotting. To demonstrate potential changes in TFF3 protein expression, Western blotting was performed on total protein samples isolated from proximal jejunal tissues using RNA-DNA-protein separation reagent (Progen Industries, Brisbane, Australia). First, the protein concentra-
tion was quantitated using Bradford reagent (Sigma, St. Louis, MO) with BSA (Sigma) as a standard. Equal amounts of protein (200 µg) from each sample, 200 ng of rat recombi-
nant TFF3 (used as a standard, a generous gift from Dr. Lars Thim, Novo Nordisk), and 2 µg of a biotinylated broad range of protein molecular weight markers (Bio-Rad, Hercules, CA) were treated with a reducing sample buffer, separated on a 12.5% SDS-PAGE minigel, and electroblotted onto a 0.2-µm nitrocellulose filter. The filters were probed with a rabbit anti-rat TFF3 antiserum (32) at 1:12,500 dilution, detected with a swine anti-rabbit biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents (Dako, Carpinteria, CA). Immunoreactivity was developed as enzyme-chemiluminescence signal using the enhanced chemiluminescence Western blotting system (Amersham, Buckinghamshire, UK). The specificity of this anti-TFF3 antiserum has been demonstrated previously by RNAs and Western blotting, revealing no cross-reaction with other trefoil peptides (9).

TFF3 immunostaining. To examine any potential site-
specific changes of TFF3 protein in the intestine during the damage/repair time course, immunohistochemical detection of TFF3 was performed on paraffin sections of the proximal jejunum. After nonspecific binding sites were blocked with 5% pig serum, sections were incubated overnight at 4°C with the above rabbit anti-TFF3 serum at 1:600 dilution in Tris-buffered saline (TBS, pH 7.4) containing 1% BSA. After sections were washed, staining was visualized with a swine anti-rabbit biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents (Dako) and 3,3′-diaminobenzidine (DAB) substrate (Sigma). A normal rabbit serum was used as a negative control.

TFF3 RNase protection assay. An RNase protection assay was used to quantitate the changes in TFF3 gene expression during the mucositis time course, according to a previously described method (9, 11, 17). Total RNA was extracted using the RNA-DNA-protein separation reagent from the specimens of proximal jejunal tissues used for protein extraction. Antisense-[32P]UTP-labeled riboprobes for TFF3 and glyceralde-
hyde-3-phosphate dehydrogenase (GAPDH) (used as an internal loading control) were made, respectively, from a rat TFF3 cDNA (423 bp/Bluescript, a generous gift from Profes-

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In situ hybridization was performed on 4-µm paraffin sections of the proximal jejunum mounted on 3-aminopropyltriethoxysilane-coated glass slides. Sections were dewaxed, hydrated, and treated with 0.2 N HCl for 20 min at room temperature. Sections were then permeabilized with 10 µg/ml proteinase K (Sigma) for 15 min at 37°C. After postfixation for 5 min with 2% paraformaldehyde, sections were neutralized for 10 min with 0.2% glycine in PBS (pH 7.4). After washing and dehydration, sections were covered with 25 µl of hybridization mix (preheated for 5 min at 85°C) containing 0.5 µg/ml sense (used as a negative control) or antisense probe, 50% formamide, 10% dextran sulfate, 0.05% Triton X-100, 500 µg/ml herring sperm DNA (Boehringer Mannheim), 0.05% polyvinylpyrrolidone, and 5× SSC (750 mM NaCl and 75 mM sodium citrate, pH 7.0). Sections were covered with glass coverslips and incubated in a humidified chamber for 18 h at 58°C. Unhybridized probes were washed off with 2× SSC for 30 min at room temperature, 2× SSC for 1 h at 65°C, and 0.1× SSC for 1 h at 65°C. After nonspecific binding sites were blocked with 10% normal sheep serum for 30 min, the sections were incubated for 30 min at 37°C with an alkaline phosphatase-coupled sheep anti-DIG IgG (Boehringer Mannheim) and developed by incubating in nitro blue tetrazolium-X-phosphate substrate for 18 h in the dark (Boehringer Mannheim). For negative controls, apart from the use of the sense probe in place of the antisense probe in the hybridization described above, sections that had been pretreated with RNase A (Boehringer Mannheim) (100 µg/ml in 2× SSC) for 1 h at 37°C were also used for hybridization with both sense and antisense probes. All of these controls consistently generated a low background.

BrdU immunostaining and labeling index. BrdU labeling was used as an index of cell proliferation in the intestinal tissues. Immunostaining of BrdU was performed on 4-µm paraffin sections of the proximal jejunum. Deparaffinized sections were incubated for 20 min in ice-cold 0.3% H2O2 in methanol to quench any endogenous peroxidase activity. Sections were then washed and treated with 1 M HCl for 8 min at 60°C to partially denature the double strand DNA. After sections were blocked with 10% normal rabbit serum for 30 min in TBS, sections were then incubated at room temperature for 1.5 h with a mouse anti-BrdU monoclonal IgG (Dako) at 1:100 dilution. Labeling was visualized with a rabbit anti-mouse biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents (Dako) and with DAB substrate (Sigma). A normal mouse monoclonal IgG (Dako) was used as a negative control.

BrdU labeling index was derived from counting the numbers of positively labeled cells and the total number of crypt epithelial cells. For each animal, 50 well-orientated full crypts were selected for analysis under a light microscope with a ×40 objective. The number of BrdU-labeled cells and the total number of crypt epithelial cells on the left half of the crypt were counted and used to calculate the mean BrdU labeling index (%) for that animal. Although methotrexate caused significant mucosal damage in the proximal small intestine, at least 50% of mucosal area remained sufficiently intact for reliable measurements of BrdU labeling index, even in tissues collected on day 5, when crypt disruption was maximal.

Statistical analysis. Results of the RNase protection assays (integrated optical density ratios between TFF3 and GAPDH), goblet cell density (goblet cell number/mm² crypt area or villus epithelium), and BrdU labeling (%) between the groups of animals treated with methotrexate and untreated normal controls were compared with one-way ANOVA.

RESULTS

Acute features of methotrexate-induced small bowel damage and repair. The current study aimed to examine the expression of TFF3 during small intestinal damage and subsequent repair and to correlate the expression with histopathological changes. Methotrexate injections induced considerable histopathological changes (Fig. 1, A–D) in the proximal small intestine, the most sensitive region of rat gut. Damage was apparent on day 3 (Fig. 1B) and reached maximal severity on day 5 (Fig. 1C), with histological features of crypt loss, villous atrophy, fusion or shortening, and inflammatory cell infiltrate. Intestinal damage then declined in severity on day 6, followed by a rapid recovery on day 7 (Fig. 1D). In the repair phase, the crypts and villi were elongated on day 7 compared with day 0 normal controls (Fig. 1D), followed by a gradual normalization over the next 3–4 days (not shown). Semiquantitative scoring of damage severity over the time course (Fig. 2A) illustrates that the methotrexate-induced damage is acute, similar to findings reported previously (13, 31). Pair-fed controls (receiving no methotrexate injections but a similar food intake to their methotrexate-injected counterparts) revealed no significant changes in villus height and crypt depth compared with normal controls (not shown), indicating that the histological changes apparent in methotrexate-injected animals did not result from a reduction in food intake.

The methotrexate-induced mucositis was also characterized by an acute time course of depletion and repopulation of goblet cells in the intestinal mucosa. Goblet cells in the proximal jejunum were identified by PAS staining (Fig. 1, E–H), and the density of goblet cells in both villi and crypts was measured (Fig. 2B). Over 98% of all goblet cells were PAS stained during the intestinal injury/recovery time course, indicating that the change in PAS-stained cell measurement reflected the change in goblet cell number rather than an alteration in mucin synthesis itself. Quantitative analysis of the goblet cell population in both crypts and villi throughout the methotrexate-induced damage and repair time course revealed a time-dependent change in goblet cell density in both the villus epithelium and crypt mucosa (Fig. 2B). On days 1 and 2 after methotrexate injection, there was no reduction in goblet cell population in either crypts or villi compared with normal controls (Fig. 2B). Compared with normal controls (Fig. 1E), the goblet cell numbers in the villi began to decline on day 3 (Figs. 1F and 2B), reaching the lowest level on day 5 (Figs. 1G and 2B). In the crypts, the loss of goblet cells was more rapid, with the number dramatically reduced on day 3 to near the lowest level observed on day 5 (Figs. 1F and 2B). In both crypts and villi, the goblet cell numbers began to increase on day 6, rapidly returning to normal levels on day 7 (Figs. 1H and 2B) and then were maintained on day 8 (Fig. 2B).

Changes in TFF3 peptide expression closely associated with intestinal damage and repair. ITF or TFF3, known to be involved in mucosal defense and ulcer
production in the recovered intestine of day 5 the damaged jejunum in severe mucosal histological damage (crypt loss, villus shortening, and fusion) and a near depletion of goblet cells in partial histological damage of the jejunum and a significant loss of goblet cells in the intestine of stained cells per unit area (mm²) of traced epithelium in the villi or of 6 (means severity scores for 4 animals at each time point. In accordance with the profile of histopathological changes (Figs. 1, A–D) and density changes in goblet cell population (Fig. 1, E–H), this staining pattern was maintained on days 1 and 2 after the commencement of methotrexate injections (data not shown). However, the staining was diminished on day 3 (Fig. 4B) when intestinal damage was considerable (Fig. 1B), and the goblet cell number was significantly reduced (Fig. 1F) and absent on days 4 (data not shown) and 5 (Fig. 4C) when the histological damage severity was maximal (Fig. 1C) and the goblet cell number was the lowest (Fig. 1G). TFF3 staining was again visible on day 6 (not shown) when damage severity declined, and goblet cells reappeared and returned to normal levels on day 7 (Fig. 4D) when the intestinal structure (Fig. 1D) and goblet cell population (Fig. 1H) appeared normal and then remained unaltered on days 8–10 (not shown). This result indicates that depletion of TFF3 accompanied the intestinal damage and particularly the disappearance of goblet cells induced by methotrexate, and, conversely, normalization of TFF3 peptide correlated with subsequent mucosal repair, particularly the repopulation of goblet cells.

TFF3 mRNA marginal upregulation during intestinal damage and expanded expression before goblet cell repopulation. To examine whether the changes in TFF3 were also apparent at the mRNA level, a RNase protection assay was employed to measure the changes in TFF3 gene transcript over the time course of intestinal damage and repair. These measurements were conducted on total RNA samples from the tissue specimens used to extract the total protein for the Western blot analysis. Figure 3C illustrates the result of a representative protection assay for TFF3 compared with the expression of the GAPDH gene, which was employed as an internal loading control. densitometric analysis (Fig. 3D) of TFF3 mRNA expression, standardized with

Fig. 1. Features of hematoxylin and eosin staining (A–D) and goblet cell periodic acid-Schiff (PAS) staining (E–H) in proximal jejunum of rats during methotrexate-induced intestinal damage and repair. A and E: normal jejunal histology and goblet cells (as highlighted by PAS staining, magenta in color) in the normal intestines. B and F: partial histological damage of the jejunum and a significant loss of goblet cells in the intestine of day 3 rats. C and G: severe mucosal histological damage (crypt loss, villus shortening, and fusion) and a near depletion of goblet cells in the damaged jejunum in day 5 rats. D and H: near normal histology and repopulation goblet cells with mucin production in the recovered intestine of day 7 rats. Scale bar in H = 100 µm (applies to A–H).
GAPDH expression, revealed a pattern of change reciprocal to that demonstrated in TFF3 protein as described above. Although the changes in TFF3 gene expression did not reach a statistically significant level on any day after methotrexate injection compared with day 0 control (P > 0.05), the mRNA level tended to decrease initially on day 1, returning to normal levels on day 2, and tended to be upregulated from days 3 to 5 during the damage phase and before the goblet cell repopulation; levels then declined starting from day 6 to near or below normal levels on days 8 and 10 after the normalization of the goblet cell population.

TFF3 mRNA in situ hybridization was used to localize the changes in its level over the damage/repair time course. Negative controls, including the use of a sense probe and the predigestion of RNA in tissue sections by incubating with RNase A (at 50 µg/ml for 30 min at 37°C), consistently gave negative staining (not shown). In normal intestine, TFF3 mRNA expression was confined to goblet cells in both villi and crypts (Fig. 4E). On days 1 and 2 during methotrexate treatment, no obvious changes were observed in TFF3 mRNA staining in the intestine (not shown). On day 3 (Fig. 4F), day 4 (not shown), and particularly day 5 (Fig. 4G), however, the intensity of mRNA staining appeared higher in the remaining goblet cells compared with that of the day 0 controls. Furthermore, on day 5, TFF3 mRNA staining was also present in some non-goblet-featured epithelial cells (Fig. 4G). From day 6 to day 10, TFF3 mRNA staining returned to normal (Fig. 4H showing day 7 only). These results from the in situ hybridization as well as from the RNase protection assays indicate that there was a marginal increase in TFF3 mRNA expression during the intestinal damage and an expansion in the cell population expressing TFF3 mRNA just before the goblet cell repopulation.

Lack of association between changes in TFF3 peptide level and cell proliferation. To examine any potential mitogenic role for TFF3 in regeneration of the crypt epithelium, the time course of crypt cell production was...
examined in concert with the analysis of TFF3 protein expression. Analysis of proliferating cell BrdU labeling in the proximal jejunum reveals that methotrexate induced a significant decrease in crypt cell proliferation on day 3 (Figs. 5, A and B, and 6) but an upregulation on days 5 and 6 (Figs. 5C and 6) followed by a normalization on day 7 (Figs. 5D and 6). Comparison between the histopathological (Fig. 2A) and cell proliferation (Fig. 6) time courses indicated that a decrease in crypt cell proliferation (on day 3) preceded the histological damage (which was maximal on day 5), and, conversely, crypt cell hyperproliferation (on days 5 and 6) preceded epithelial regeneration. Comparison between the time courses of crypt cell proliferation (Fig. 6) and intestinal TFF3 protein production (Fig. 3, A and B) shows that the overshoot in crypt cell proliferation was not preceded by an upregulation of TFF3 peptide (on and before day 5); conversely, no increase in cell proliferation was followed after the normalization of TFF3 peptide production (on and beyond day 7). On day 5, although the TFF3 peptide was absent, crypt cell proliferation was upregulated. On day 6, crypt cell proliferation was upregulated, although the TFF3 level had recovered. These data indicate that TFF3 was unlikely to be acting as a mitogen during the regenera-

Fig. 5. Bromodeoxyuridine (BrdU) labeling in proximal jejunum in normal and methotrexate-damaged rats. Epithelial BrdU labeling (darkly labeled nuclei), confined in crypts in the normal intestine (A), was dramatically reduced on day 3 (B), upregulated on day 5 but now with some distribution also in lower villus epithelium (C), and normalized on day 7 (D). Scale bar in D = 100 µm (applies to A–D).

Fig. 6. Cell count measurements of BrdU labeling in the proximal jejunum in rats from the methotrexate damage/repair time course. BrdU labeling was expressed as percentage of the number of positively labeled cells over the total numbers of epithelial cells of the crypts (means ± SE, n = 4 animals). *Significantly different from day 0 control (P < 0.001).
tion of the intestine following methotrexate-induced damage.

**DISCUSSION**

We have demonstrated that the small intestinal manifestations of the toxicity of the DNA synthesis-inhibiting chemotherapy agent methotrexate (3, 38) in the rat reflect an acute and transient process of mucosal damage and regeneration, confirming previous findings (31). This process was characterized by an initial decrease in crypt cell proliferation, which precedes crypt loss, villus shortening and atrophy, and goblet cell depletion. The repair phase commenced when crypt cell proliferation had returned to normal levels and was accelerated during the period of marked upregulation in crypt cell production. The overshoot of cell proliferation resulted in crypt elongation, an increase in villus height, and goblet cell repopulation. Intestinal regeneration continued after day 7 with normalization of crypt depth and villus height to basal levels over the subsequent 4–5 days.

We have shown that a depletion of TFF3 protein coincided with the severity of histopathological damage, with the TFF3 peptide absent during the time period of maximal histological damage. Mirroring the TFF3 protein depletion more closely, however, was the time course of change in the goblet cell population in the proximal small intestine. TFF3 is produced and secreted into the intestinal lumen by goblet cells in association with mucin glycoproteins (27). During the damage phase, goblet cells were reduced in number or completely depleted, as was TFF3 peptide. During the repair phase, the repopulation of goblet cells was accompanied by a return of TFF3 immunoreactivity. Localization of TFF3 peptide to goblet cells and the close association between the changes of TFF3 peptide and goblet cell number during the damage/repair time course indicate that the change in TFF3 peptide may be secondary to the changes in goblet cell population. Alternatively, the depletion of TFF3 peptide may have been responsible for the lack of mucin production. However, the latter possibility can be eliminated, since it has been shown that, in TFF3 knockout mice, mucin production by goblet cells is still preserved (19), indicating that TFF3 is not essential for mucin production. Because the reduction of TFF3 protein production was most likely secondary to the depletion of goblet cells, the depletion of TFF3 may have been a late rather than an early event of methotrexate-induced damage. Our time course measurements suggest that methotrexate-induced intestinal damage and the depletion of goblet cells occur as consequences of a decrease in cell proliferation earlier in the time course. However, whether the diminished expression of mucin glycoproteins and TFF3 in the intestine may have contributed to the intestinal damage awaits further study. The close spatial and temporal association between TFF3 peptide production and goblet cell number suggests that TFF3 and mucins may act in concert to maintain intestinal integrity. Intestinal goblet cells secrete TFF3 and mucin glycoproteins to form a continuous gel that covers the intestinal epithelial layer and contributes to mucosal protection (2, 27, 33). In support of this, Kindon et al. (16) demonstrated that TFF3 and intestinal mucin glycoproteins interacted to protect colonic epithelial cell monolayers against toxin- or bile acid-induced damage.

In the current study, we have demonstrated that the expression of TFF3 is not positively correlated with crypt cell proliferation, suggesting that TFF3 is not a mitogen for intestinal crypt cells. Instead, our results demonstrate that cell proliferation was high when epithelial TFF3 expression was low, indicating that the presence of TFF3 peptide could even be inhibitory to cell proliferation. This notion is consistent with a previous study in TFF3 knockout mice showing that the crypt cell proliferative compartments were expanded compared with wild-type mice (19). This finding is also in accordance with previous findings that revealed that recombinant TFF3 does not stimulate the proliferation of nontransformed gastrointestinal epithelial cells (10).

Observations in TFF3 gene knockout mice have demonstrated that TFF3 has a role in normal crypt cell migration and maturation of the intestinal epithelium (19). Furthermore, in vitro and in vivo evidence suggests that trefoil peptides, including TFF3, play an important role in reestablishing mucosal integrity after injury by stimulating epithelial cell restitution (10, 20). Elucidating a possible role for TFF3 in stimulating repair after methotrexate-induced damage requires further studies, which could include application of recombinant peptide either luminally or parenterally. In this context, we have recently shown that luminal application of TFF2 in the colon accelerates epithelial healing after induction of hapten-induced colitis (37). However, the coincident timing of normalization in TFF3 immunoreactivity and crypt/villus regeneration after the proliferative overshoots on days 5 and 6 indicates that the reappearance of TFF3 peptide may play a role in the stimulation of cell migration during mucosal repair. Consistent with the current study, we have previously demonstrated a decrease in TFF3 protein production during acetic acid-induced ulcerative damage to the gastric mucosa, with TFF3 production returning to basal levels only at the time when the macroscopic healing was complete (9). This latter study also revealed an upregulated expression of TFF3 in and around ulcer sites in the gastric tissues in the later stages of repair after the completion of reepithelization (9), suggesting that TFF3 has an ongoing role in reparative events, such as maturation and positioning, which occur well after the initial reepithelization phase following the initial insult. Similarly, in an acetic acid-induced rat model of colitis, TFF3 expression was shown to be downregulated during the acute phase of colitis and then upregulated during the recovery phase, suggesting its possible involvement in cell differentiation such as the formation of goblet cells (14).

In this study, we have described a disparity between TFF3 peptide and mRNA expression. Although the level of jejunal TFF3 peptide localized in the goblet
cells was decreased in association with the depletion of goblet cells and histological damage, the TFF3 mRNA tended to be upregulated particularly at the time of maximal intestinal damage, preceding the upregulation of TFF3 peptide during the normalization of the mucosa. The explanation for this discrepancy awaits investigation. Although it is possible that the modest increase in synthesis of TFF3 mRNA during the damage process produces TFF3 peptide that is rapidly secreted in a constitutive manner without storage, this possibility is unlikely, since, during the TFF3 mRNA upregulation with tissue damage apparent, the goblet cell number was low, suggesting a low cellular mass that can produce TFF3 peptide. A more likely possibility for this discrepancy could be that the transcription and translation of TFF3 are not parallel, with translation occurring later than transcription. Supporting this possibility was the observation that the appearance of mRNA of trefoil peptides was 2 days earlier than that of their peptides in the developing rat intestine (11). By in situ hybridization, we have shown that TFF3 mRNA expression was confined to goblet cells in normal intestine, consistent with previous studies (7, 22, 30), and that the remaining goblet cells in the damaged intestinal mucosa particularly on day 5 displayed more intense mRNA staining. Furthermore, on day 5 before the repopulation of goblet cells, the cell population expressing TFF3 mRNA was expanded to include some nongoblet-featured epithelial cells. Although it has been documented that both TFF3 mRNA and peptide are mainly confined to the goblet cells in the mature villus epithelium and the crypts (7, 22, 30), differential expression of TFF3 mRNA and peptide has also been reported in the crypts of the small bowel. It has been shown that a high level of TFF3 mRNA was also expressed by some undifferentiated epithelial cells in the crypt despite the absence of its translation into peptide in these nongoblet-featured immature cells (22). Consistent with our current finding that some non-goblet-featured epithelial cells also expressed TFF3 mRNA before goblet cell repopulation in this methotrexate-induced mucositis model, TFF3 mRNA has been reported to be present in poorly differentiated epithelium migrating over lesions after intestinal resection surgery (25), during the recovery phase in acetic acid-induced colitis (36), or in gastric mucosal damage (1) in the rat and in chronic inflammation in man (24). Perhaps it is this lack or delay of translation of TFF3 mRNA into TFF3 peptide in these undifferentiated cells that replenish the depleted TFF3 pool during intestinal damage that could explain the discordance between TFF3 peptide and mRNA expression described in this study.

It has been suggested that the early expression of TFF3 mRNA in the immature cells may be an early marker of commitment to differentiate into goblet cells (22). Indeed, in the developing rat gut, mRNA expression of trefoil peptides including TFF3 precedes mucous cell differentiation (11). It is therefore possible that the dramatic reduction in TFF3 peptide in association with intestinal damage and the depletion of goblet cells in this methotrexate-induced mucositis could provide a positive feedback signal to the regenerating crypt to upregulate TFF3 mRNA in some undifferentiated cells. These undifferentiated cells could then use the upregulated TFF3 mRNA as an early marker for the commitment of goblet cell differentiation to replenish its population and to produce TFF3 peptide. Indeed, repopulation of goblet cells coincided with the reappearance of TFF3 peptide following the initiation of recovery from damage.

In summary, methotrexate-induced small bowel mucositis in the rat is an acute and transient process of mucosal damage and repair, characterized by an initial decrease in crypt cell production, leading to crypt loss, villus atrophy, and depletion of goblet cells, followed by crypt cell hyperproliferation. The upregulated proliferation of crypt epithelial cells precedes crypt and villus regeneration, repopulation of mucous cells, and normalization of mucosal structure. This study has shown that the TFF3 mRNA expression level marginally increases during mucosal damage and cell population expressing TFF3 mRNA, including nongoblet cells before the goblet cell repopulation, expands; however, this study also clearly demonstrated a depletion of TFF3 peptide during the phase of intestinal damage, particularly goblet cell depletion, and its normalization during the phase of mucosal regeneration and goblet cell repopulation. We have also revealed a lack of temporal correlation between TFF3 peptide expression and crypt cell hyperproliferation during the regenerative phase, confirming the nonmitogenic nature of TFF3. However, the upregulated and expanded expression of TFF3 mRNA preceding the goblet cell repopulation and the coincidental normalization of TFF3 peptide levels with that of goblet cell repopulation and mucin production after the crypt cell proliferative overshoot or during the remodeling phase suggest that TFF3 may play a role in later events of intestinal repair. The current study has highlighted a potentially important role for TFF3 in the maintenance of intestinal integrity and in intestinal repair; further understanding of epithelial responses (including regulation of gene expression of intestinal peptides or growth factor systems) to intestinal mucosal injury is needed. Further studies, which could include the use of recombinant TFF3 peptide or an antagonist in vivo, need to be conducted to test whether TFF3 plays an essential role in preventing mucosal damage induced by methotrexate and in enhancing intestinal repair after damage.

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Address for reprint requests and other correspondence: C. J. Xian, Child Health Research Institute, 72 King William Rd., North Adelaide 5006, South Australia, Australia.

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