Trefoil Peptide Expression and Goblet Cell Number in Rat Intestine: Effects of KGF and Fasting/Refeeding

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Running head: KGF induction of gut trefoil peptides
Abstract

The trefoil factor family peptides TFF1, TFF2 and TFF3 are important for mucosal protection and restitution. KGF stimulates proliferation and differentiation of epithelial cells with potent effects on goblet cells. To investigate interactions between food intake and KGF, rats were fed ad libitum (control), fasted for 72 h or fasted for 72 h and then re-fed for 72 h, ± KGF (3 mg/kg/day). With fasting, goblet cell number in duodenum increased, TFF3 mRNA in duodenum and jejunum decreased and TFF3 protein did not change or increased. KGF during fasting stimulated colonic growth, normalized TFF3 mRNA in duodenum and jejunum and broadly up-regulated gut goblet cell number and TFF3 protein expression. With fasting/re-feeding, KGF increased small bowel and colonic mucosal growth, goblet cell number and TFF3 protein, but had variable effects on TFF3 mRNA. KGF specifically induced TFF2 mRNA and protein in the duodenum and jejunum with both nutritional regimens. We conclude that nutrient availability modifies rat intestinal goblet cell number, TFF3 mRNA and the gut-trophic effects of KGF in a region-specific manner. KGF enhances TFF2 expression in proximal small bowel and increases goblet cell number and TFF3 protein content throughout the intestine independent of food intake.

Key words: goblet cells, intestinal mucosa, keratinocyte growth factor, KGF, trefoil peptides
Introduction

Together with mucins, trefoil factor family (TFF) peptides are major constituents of the mucus layer that protects the gastrointestinal mucosa from injurious agents (2, 5, 13, 15, 26, 27, 34, 38, 42). TFF1 (formerly pS2) is synthesized primarily in gastric mucosal pit cells (TFF1). TFF2 (formerly spasmolytic polypeptide or SP) is produced by gastric mucous neck cells and duodenal submucosal Brunner’s glands (8, 22, 42). TFF3 (formerly intestinal trefoil factor or ITF) is synthesized by goblet cells throughout small intestine and colon (36, 39). Trefoil peptides are resistant to protease digestion and their cellular localization is ideal for gut epithelial protection (7, 29, 42). In vitro and in vivo studies strongly link trefoil peptides with gastrointestinal mucosal restitution (7, 19, 29, 42). TFF1, TFF2 and TFF3 are up-regulated at sites of gastric and intestinal mucosal injury, where these proteins stimulate gut epithelial cell migration and mucosal repair (16, 20, 25, 33, 44, 46). However, the role of TFF peptides in physiologic and adaptive gut epithelial cell proliferation, apoptosis and turnover remains uncertain (17, 29, 31, 38, 44). Recent studies in TFF2-deficient mice clearly show that TFF2 stimulates gastric mucosal cell proliferation (12); in contrast, epithelial cell proliferation and apoptosis in jejunum was unaltered in mice with targeted transgenic expression of TFF3 in jejunal mucosa (28).

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family (FGF-7) synthesized by stromal cells (18). The presence of KGF and the KGF receptor in the gastrointestinal mucosa suggests the involvement of the endogenous KGF action pathway in gut physiology (10, 18). KGF specifically stimulates the proliferation and differentiation of epithelial cells, including cells of the gastrointestinal mucosa (18, 24). KGF treatment augments both small bowel and colonic mucosal growth, repair and barrier function in models of malnutrition, parenteral nutrition, chemotherapy/irradiation and intestinal inflammation (4, 9, 11,
KGF appears to have particularly trophic effects on intestinal goblet cell expression \textit{in vivo} (9, 18). KGF also increased small intestinal TFF3 mRNA in a rat model of short bowel syndrome (23) and TFF3 protein in murine models of inflammatory bowel disease (4). In undifferentiated colonic HT-29 subclone H2 cells, KGF up-regulated TFF3 mRNA and protein expression and stimulated differentiation into goblet cells through regulation of the goblet cell silencer inhibitor, a goblet-cell specific transcription factor (21).

Gut mucosal growth, function and repair are highly dependent upon nutrient availability (3, 14, 48). Nutritional status also regulates expression of various endogenous intestinal growth factors, including insulin-like growth factor-I and KGF and its receptor (10, 45, 47, 48, 49).

However, little data exist on nutrient regulation of intestinal goblet cells (6, 30, 35, 37) or TFF peptide expression. Our hypotheses were 1) gut mucosal atrophy induced by fasting would be associated with a reduction in the number of goblet cells and decreased TFF expression; 2) KGF would increase gut mucosal growth, goblet cell number and TFF expression; and 3) effects of KGF would be modified by food intake. Therefore, we used rat models of fasting and fasting/refeeding, with or without administration of KGF, to assess mucosal growth, goblet cell number and expression of TFF peptide mRNA and protein in rat small bowel and colon.
Materials and Methods

Animal protocols

Male Sprague-Dawley rats weighing 170-210 g were housed in individual cages in the Emory University Animal Care Facility on a 12-h light, 12-h dark cycle. Animals were acclimatized to laboratory conditions for 3 days with ad libitum water and standard pelleted rat food (Laboratory Rodent Chow 5001, PMI Feeds Inc., St. Louis, MO). Water was provided ad libitum to all rats and animal food intake measured daily. Initial body weight of each of the study groups was not different and all control animals gained the expected amount of body weight over time (Table 1). The animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University and followed the newest Guiding Principles of Research (1).

Study 1: Food deprivation. Weight-matched rats (N=8/group) were given food ad libitum (control) or were fasted for three consecutive days. Animals were not monitored for coprophagia. We have previously shown in rats that a 3-day period of fasting induces gut mucosal atrophy (47). Rats were also given daily intraperitoneal (ip) injections of either recombinant human KGF (Amgen Inc., Thousand Oaks, CA; 3 mg/kg) (Fasted-KGF) or saline (Fasted-Sal). The fed control group was given daily i.p. saline injections. Animals were sacrificed on the morning of day 4. As expected, at sacrifice, fasted animals had lost about 20% of their initial body weight, whereas control animals continued to gain weight (Table 1).

Study 2: Food deprivation and refeeding. Weight-matched rats (N=8/group) were fed ad libitum for 6 consecutive days (control) or were fasted for 3 days, followed by 3 days of ad libitum refeeding. We have previously shown in rats that gut mucosal atrophy induced by 3-days of fasting can be reversed by 3 days of oral refeeding (9, 47). During the entire 6-day protocol, fasted/refed rats were given daily i.p. injections of KGF (3 mg/kg; Refed-KGF) or saline (Refed-Sal); control rats were given i.p. saline. To ensure identical food intake between groups during
re-feeding, the Refed-KGF rats were pair-fed the average daily food intake consumed by the Refed-Sal rats. Animals were sacrificed on the morning of day 7.

Tissue preparation

At the end of the individual study periods, animals were anesthetized with a mixture of i.p. ketamine (100 mg/ml) and xylazine (20 mg/ml) administered at doses of 0.1 to 0.15 ml/100 g body weight. The abdomen was opened by a midline incision, the stomach antrum, ligament of Treitz and ileal-cecal junction were identified and marked. The small intestine and colon were removed sequentially from the peritoneal cavity and the lumen flushed with ice-cold saline to clear intestinal contents. The small bowel and colon were individually suspended from a ring-stand with a constant distal weight and defined intestinal segments excised. Full-thickness sections (2 and 4 cm) were obtained from defined segments of proximal duodenum, proximal jejunum, distal ileum and proximal colon for determination of TFF protein and mRNA content, respectively. Full-thickness samples of stomach antrum were obtained from ad libitum fed rats to use as positive controls in TFF1 and TFF2 mRNA and protein expression studies. All tissue samples were weighed, snap-frozen in liquid nitrogen and stored at -80°C for later analysis. Additional 0.5-cm sections from each intestinal segment were fixed in 4% paraformaldehyde and paraffin-embedded for later morphologic analysis and immunohistochemistry.

Gut mucosal growth indices

Paraffin-embedded sections of duodenum, jejunum, ileum and colon were stained with hematoxylin and eosin. Crypt depth (CD) and villus height (VH) were measured manually by two pathologist co-investigators blinded to study group using a viewing Olympus BH-2 light microscope and calibrated ocular micrometer. A total of 15 to 25 well-oriented crypts and villi from each small bowel segment and 15 to 25 colonic crypts from each colon segment per animal
were measured and averaged.

**Gut mucosal goblet cell number**

Goblet cells were identified by classical morphology in each small bowel and colonic section in all animals. Total goblet cell number was determined along the combined crypt-villus axis of 10 well-oriented crypt-villus units in each small bowel section and in 10 well-oriented colonic crypt units, from the crypt base to the luminal surface. To confirm goblet cell numbers identified by morphology, goblet cells in duodenal and colonic sections from the same animals (N= 5-6/group) were localized using Alcian blue staining (19). Positive staining with Alcian blue indicates the presence of acid mucins within goblet cells and was quantitated as outlined for morphological assessment.

**RNA preparation and Northern blot analysis**

Northern blotting was performed as previously described (48). Total cellular RNA was isolated using TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to manufacturer’s instructions. RNA integrity was confirmed by ethidium bromide staining. The rat TFF1 cDNA was a 135 bp *KpnI/SacI* fragment based on the rat TFF1 cDNA sequence made by polymerase chain reaction (PCR) and subcloned into pGEMT (Promega, Madison, MI) (20). The TFF2 cDNA was a 267 bp *XbaI* fragment of the rat TFF2 cDNA sequence (22), made by reverse transcription polymerase chain reaction (PCR) and subcloned into pGEMT. The TFF3 complementary DNA (cDNA) probe used for hybridization was a 500 base pair (bp) *ApaI/BstXI* fragment of the rat TFF3 cDNA sequence (36), subcloned into pGEMT vector. The cDNAs were labeled with 32P dCTP by random priming (Prime-It®; II Random Primer Labeling Kit, Stratagene, La Jolla, CA) followed by spin column removal of unincorporated nucleotides. Total RNA (25 µg /lane) was loaded onto 1% agarose gels, electrophoresed and transferred to nylon membranes. The membranes were hybridized with the respective TFF probes for 16 to 18 h at
42° C in hybridization solution, as described (48), washed under high stringency conditions and
exposed for various times at -80°C using Kodak XOMAT film with intensifying screens. To
control for RNA loading, all membranes were stripped and re-hybridized with a mouse 18S
cDNA probe (American Type Culture Collection, Rockville, MD). Bands were quantitated by
laser densitometry.

**Western immunoblotting**

A rabbit monoclonal antibody against human TFF2 (provided by Dr. Lars Thim, Novo
Nordisk A/S, Malov, Denmark) and a rabbit anti-rat polyclonal TFF3 antibody raised against a
21-residue synthetic peptide from the predicted C-terminal sequence of rat TFF3 were used (12,
36). The anti-human TFF2 antibody has been shown to be specific for TFF2 in rat stomach and
intestine (12). Tissues were suspended in ice-cold lysis buffer containing phosphate-buffered
saline, NP-40, sodium deoxycholate, sodium dodecyl sulfate, EDTA, chymotrypsin, thermolysin,
pronase, papain, and pancreas extract. Samples were homogenized, centrifuged, and supernatants
stored at –70°C. Protein concentration of each preparation was determined by the Bradford
method, performed in duplicate, using bovine serum albumin as a standard. Thirty µg of
solubilized protein/sample were resolved by SDS-PAGE in 16.5% Tris-Tricine gels (Bio-Rad
Inc., Hercules, CA). Identical gels were checked for equality of protein loading using Coomassie
blue staining. Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham,
Arlington Heights, IL). Membranes were blocked for 60 min at 22°C with 5% dry milk, 0.1%
BSA, 1X PBS and 0.05% Tween-20, and incubated with either TFF2 or TFF3 antibody (1:500)
in blocking solution. Bound antibody was detected with anti-rabbit horseradish peroxidase -
conjugated secondary antibody (anti-rabbit IgG, Santa Cruz Biotechnology Inc. Santa Cruz, CA)
and ECL Chemiluminescence Detection system reagents (Amersham Arlington Heights, IL).
Protein bands were quantified by densitometry.
Immunohistochemistry

Deparaffinized intestinal sections were re-hydrated, proteolysed with proteinase K, washed, blocked with 1% gelatin-PBA, and incubated with primary anti-human TFF2 and anti-rat TFF3 antibody (1:500 dilution), respectively. Binding of primary antibody was visualized by incubation with goat anti-rabbit immunoglobulin G (1:500; Vector Laboratories, Burlingame, CA) and use of avidin, biotinylated horseradish peroxidase, and diaminobenzidine tetra-hydrochloride reagents according to the manufacturer's instructions (ABC-Peroxidase Elite, Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin. Tissue sections processed without primary antibody or with overnight incubation of primary antibody with TFF2 or TFF3 protein (10 μM at 4°C) were used as negative controls.

Statistical analysis

The abundance of TFF mRNA and protein was expressed in densitometry units, normalized against values obtained for control animals within each experiment. mRNA data from Northern blotting experiments were normalized to 18S mRNA expression. One-factor analysis of variance (ANOVA) was used to detect significant inter-group differences, in which case individual study groups were compared using the Fisher’s protected least significant difference test. P values < 0.05 were considered statistically significant in all analyses. Data are expressed as mean (SE).
Results

Gut mucosal growth indices.

Fasting for three days induced site-specific effects on gut mucosal growth indices (Table 2). The significant changes were a modest decrease in jejunal and increase in ileal villus height (to 83% and 112% of control values, respectively) and a decrease in jejunal, ileal and colonic crypt depth (to 85%, 85% and 88% of control values, respectively). KGF administration completely prevented the decrease in colonic crypt depth during food deprivation, but did not alter the small bowel mucosal growth indices. Rats in the fasting/re-feeding protocol exhibited site-specific effects on gut mucosal growth parameters and KGF induced both small bowel and colonic mucosal growth responses (Table 3).

Goblet cell number.

Goblet cell number increased in a proximal to distal intestinal gradient in the control, nutrient restricted and re-fed rats (Table 4). Fasting alone increased duodenal goblet cell number by 25% compared to control fed values, but did not modify goblet cell number in the other gut segments. KGF markedly increased goblet cell proliferation in all gut segments during food deprivation (Table 4). In fasted/refed rats, goblet cell number in duodenum and colon increased (to 118% and 125% of control, respectively). In this model, KGF markedly increased goblet cell number in duodenum, jejunum, ileum and colon (Table 4). The magnitude of the goblet cell response to KGF in re-fed rats was quantitatively similar to the response to KGF when given during food deprivation alone.

Alcian blue staining detects acid mucin-positive goblet cells and was performed in duodenal and colonic mucosal sections to confirm the morphologic assessment. Results using Alcian blue were statistically similar to data derived from morphologic determination of goblet cell number. The number of Alcian blue-positive cells/10 crypt units in duodenal mucosa was 169±18, 209±3
and 343±13 in control, Fasted-Sal and Fasted-KGF groups, respectively (P<0.05 Fasted-Sal vs control and P<0.01 Fasted-KGF vs control and Fasted-Sal). In colonic mucosa, the number of Alcian blue-positive cells were 294±15, 295±8 and 539±9 in control, Fasted-Sal and Fasted-KGF groups, respectively (P<0.01 Fasted-KGF vs control and Fasted-Sal). In the fasting/refeeding model, the number of Alcian blue-positive cells in duodenal mucosa was 156±4, 184±3 and 397±13 in control, Refed-Sal and Refed-KGF groups, respectively (P<0.08 Refed-Sal versus control and P<0.01 Refed-KGF vs control and Refed-Sal). In colon, the number of Alcian blue-positive cells was 303±7, 358±16 and 578±3 in control, Refed-Sal and Re-fed-KGF groups, respectively (P<0.05 Refed-Sal vs control, P<0.01 Refed-KGF vs control and Refed-Sal).

**TFF1 and TFF2 expression.**

The expected 0.5 kb TFF1 mRNA transcript was demonstrated in stomach (not shown), but TFF1 mRNA was not detected in duodenum, jejunum, ileum or colon in any of the study groups, even after 8 days of auto-radiographic exposure. The 0.6 kb rat TFF2 mRNA transcript was evident in stomach after 3 hours of exposure (Figure 1, Panel A, lane 1). TFF2 mRNA was not detected after 48 hours of exposure in duodenum (Figure 1, Panels A and C) or jejunum (Figure 1, Panels C and D) in control, fasted, or fasted/refed animals given saline. However, KGF treatment induced expression of TFF2 mRNA in duodenum and jejunum in both nutritional models (Figure 1). Up-regulated TFF2 gene expression with KGF was particularly marked in the duodenum of food-deprived rats (Figure 1A, lanes 4 and 7). TFF2 mRNA was not detected in ileum or colon after 8 days of exposure (not shown).

TFF2 immunoreactivity was consistently detected in duodenal sub-mucosal Brunner’s glands in all study groups (Figure 2, Panels A and B). In jejunum, faint expression of TFF2 protein was present but cellular localization was variable between animals; low-level staining was observed in goblet cells, along the apical border of villus epithelial cells and in secreted mucus (not
shown). TFF2 was not observed in the duodenal mucosa of saline-treated rats; however, KGF administration in both nutritional models ectopically induced TFF2 protein expression in goblet cells of the duodenum and, to a lesser extent, the jejunum (Figure 2, Panels C and D). TFF2 staining after KGF treatment appeared heaviest in the crypt and lower villus region, with decreased staining in the mid- to upper villus goblet cells.

Using Western blotting, we detected a \( \approx 16 \text{ kD} \) band in duodenal and jejunal tissue identical in size as purified TFF2 protein and the band observed in rat stomach (Figure 3). Food deprivation alone did not alter TFF2 protein abundance in duodenum and jejunum; however, KGF given in this setting increased TFF2 abundance in duodenum, and, to a lesser extent, jejunum compared to controls (Figure 3). In the fasting/refeeding protocol, KGF did not significantly alter TFF2 protein abundance in either duodenum (control 100±5, Fasted-Sal 121±4, Fasted-KGF 137±24, respectively; NS) or jejunum (control 100±14, Fasted-Sal 153±19, Fasted-KGF 140±29, respectively; NS).

**TFF3 expression.**

Fasting decreased steady-state expression of TFF3 mRNA in duodenum and jejunum (to 55% and 30% of control values, respectively) but did not alter TFF3 mRNA values in ileum or colon (Figure 4A). KGF treatment during food deprivation prevented the decrease in duodenal and jejunal TFF3 mRNA, but did not alter TFF3 mRNA expression in ileum or colon. In contrast to changes in duodenal and jejunal TFF3 mRNA, fasting increased TFF3 protein levels in jejunum and did not alter levels in the other intestinal segments (Figure 4B). KGF treatment in this nutritional model also increased TFF3 protein abundance in duodenum, ileum and especially colon (to 135%, 263% and 1578% of control values, respectively) and tended to increase values in jejunum (NS).

Fasting/refeeding did not modify intestinal TFF3 mRNA levels compared to continuously fed...
control animals (Figure 5A). KGF treatment in this nutritional setting increased TFF3 mRNA abundance in duodenum, ileum and colon (to 238%, 686% and 244% of control values), but did not alter values in jejunum. TFF3 protein levels in fasted/refed rats were similar to those of fed controls (Figure 5B). KGF administration in this model increased TFF3 protein expression by 2- to 4-fold in duodenum, jejunum, ileum and colon compared to control values (Figure 5B).

Immunoreactive TFF3 protein was readily detected within goblet cells in all bowel segments. In ileum, TFF3 protein was localized within goblet cells and along the overlying mucosal surface, reflecting secretion into the lumen (Figure 6). Consistent with the Western blot data shown in Figure 5, ileal mucosa TFF3 protein abundance and cellular expression was similar in fasted/refed and control rats (Figures 6A and 6B). However, KGF treatment in this model increased ileal goblet cell number and TFF3 immunoreactivity approximately 2-fold (Figure 6C). TFF3 staining was eliminated with overnight adsorption of primary antibody with TFF3 peptide or omission of primary antibody (not shown). TFF3 protein was localized to colonic goblet cells and was unaltered by fasting/refeeding (Figures 7A and 7B). KGF treatment increased both goblet cell number and TFF3 immunoreactivity in colon (Figure 7C).
Discussion

Nutritional status markedly influences gut mucosal growth and function, but surprisingly little is known about the effects of nutritional factors on intestinal goblet cells or their secreted proteins (6, 33, 41). Little is known regarding the influence of nutrient intake on gut mucosal responses to recombinant growth factors, including KGF (45, 48, 49). To our knowledge, this is the first study to evaluate effects of diet and KGF on concomitant gut goblet cell and TFF peptide expression and to determine whether KGF regulates TFF1 and TFF2 expression.

Gut mucosal growth indices. Enteral nutrient availability induced segment-specific effects on small intestinal and colonic mucosal growth, consistent with previous studies (9, 45, 47). In the absence of exogenous nutrients (FD model), KGF did not increase small bowel crypt depth or villus height, but prevented the decrease in colonic crypt depth. When given during fasting followed by re-feeding, KGF increased duodenal, jejunal and ileal villus height and duodenal and colonic crypt depth. We did not investigate the time course of response to KGF or administer KGF to control fed rats. However, KGF has been shown to increase small bowel mucosal growth as early as one day after treatment in fed rats (18). Also, we previously showed that KGF administration during re-feeding induced greater duodenal and ileal mucosal growth when animals were refed at 25% of ad libitum intake (9). Thus, the level of nutrient intake appears to influence KGF gut-trophic responses in small bowel, with the presence of some luminal food being important. In contrast, this study and our previous work in different nutritional models demonstrate that KGF potently stimulates colonic growth independent of the level of nutrition (9). Additional studies to determine responses to KGF in parenterally fed animals, in which luminal food is absent, would be of interest (32).

Goblet cell number. Intestinal goblet cell number and crypt depth/villus height during altered nutrient availability alone were generally dissociated (please see data shown in Tables 2-4). Of
interest, goblet cell number increased in the duodenum after fasting alone or fasting/re-feeding. To our knowledge, this is the first demonstration of increased intestinal goblet cell number during altered nutrient availability. These data are consistent with another study showing increased mucus production in proximal small bowel after a 48-hr fast in rats (37). Earlier investigations indicated that chronic protein depletion or protein-energy undernutrition decreased goblet cell number or mucin synthesis in rodent and piglet proximal small intestine, respectively (30, 35). Differences between our results and these latter studies may relate to the type or duration of malnutrition or the animal species utilized. Colonic goblet cell number increased in the fasting/re-feeding model but was unaltered with fasting alone. Different local or systemic nutrition-related factors govern expression of the goblet cell lineage in the duodenum compared to colon. Increased goblet cell expression in these gut segments may represent a protective adaptation to diminished nutrient availability. KGF increased goblet cell number uniformly throughout the small and large intestine and this effect was unrelated to nutrient availability. In vivo studies to determine whether malnutrition, enteral versus parenteral feeding, or KGF alters goblet cell differentiation, proliferation, apoptosis and production of specific mucins (6, 43) would be of interest.

**TFF1 and TFF2 responses.** As expected, TFF1 and TFF2 mRNA was abundant in rat stomach tissue by Northern blotting but were not detected in intestine, with or without oral intake. KGF treatment did not induce TFF1 mRNA in any bowel segment or TFF2 mRNA in ileum and colon. Thus, KGF stimulation of trefoil peptides is both region- and TFF-specific. It is possible that a more sensitive assay, such as PCR or in situ hybridization, may have enabled detection of these mRNAs in the intestine.

We confirmed cellular localization of TFF2 protein in duodenal submucosal Brunner’s glands, as previously reported by others (22, 33, 34, 39, 44). However, we show for the first
time that KGF up-regulates TFF2 expression in rat proximal small bowel, with ectopic
eexpression in goblet cells and secretion of the peptide into the lumen. Ectopic local up-
regulation of TFF2 was previously shown to occur in ulcer-associated cell lineages and in goblet
cells at sites of gut mucosal injury (20, 25, 33, 46), suggesting a role for TFF2 in bowel mucosal
restitution. Of interest, local KGF mRNA in gut mucosa is up-regulated at sites of injury in
inflammatory bowel disease and with mucosal ulceration (4, 49). Our results showing that
exogenous KGF increased goblet cell TFF2 suggest that local up-regulation of TFF2 in areas of
mucosal inflammation may be due, in part, to increased local levels of KGF.

**TFF3 responses.** The broad pattern of upregulated TFF3 protein after KGF injection
occurred concomitantly with increased TFF mRNA in most gut regions. However, in ileum and
colon after food deprivation (Fig 4) and in jejunum after fasting/refeeding (Fig 5), TFF3 mRNA
levels were unchanged despite an increase in TFF3 protein abundance. In addition, with fasting
alone, TFF3 mRNA decreased in duodenum and ileum, while protein levels at these sites were
maintained. Taken together, these data suggest that TFF3 expression is controlled at a post-
transcriptional level in a region-specific manner and differently depending on nutrient supply or
KGF administration.

KGF-stimulated TFF3 production was localized to goblet cells and abundant secreted TFF3
was present in the gut lumen. TFF3 mRNA and/or protein levels in some gut segments varied
independently from changes in goblet cell number during altered nutrition (e.g. duodenum in
both models and in colon with FD/re-feeding). Discordance between goblet cell number and
TFF3 production was previously observed in mice with partial ablation of intestinal goblet cells
in which chemical mucosal injury increased goblet TFF3 expression (19). Increased goblet cell
number with KGF treatment was invariably associated with increased TFF3 protein abundance in
all gut segments. Previous studies show that KGF increases muc2 mRNA levels in HT29
subclone H2 cells (21). Given the emerging roles for mucins such as muc2 in intestinal mucosal
growth, protection and barrier function (43, 44), in vivo studies to determine whether KGF or
specific dietary factors differentially regulate goblet cell production of specific mucins would be
of interest (43).

Changes in local TFF2 or TFF3 expression and mucosal crypt depth and villus height did not
generally correlate in the current study. However, our study was not designed to test whether
intestinal growth responses to diet or KGF are mediated by changes in goblet cell number or
local TFF production. Studies of gut growth responses to nutritional alterations and KGF in
TFF2 and TFF3 knock-out and transgenic mice are needed to address these questions.

**Conclusions.** In rat intestine, nutrient availability modulated goblet cell number, small
intestinal TFF3 mRNA abundance and trophic effects of KGF, but these effects were region-
specific. KGF markedly increased goblet cell number and TFF3 expression throughout the
intestine despite short-term lack of food intake. In addition, KGF induced ectopic TFF2
expression in proximal small bowel goblet cells. Further studies are necessary to determine the
mechanisms involved in the nutritional and KGF modulation of goblet cells and their trefoil
peptide products. In addition, more information on the specific roles of goblet cells and TFF
peptides in mediating actions of nutrients and KGF in the gut are needed. Increased goblet cell
number, TFF2 and TFF3 expression may mediate some of the beneficial effects of KGF
observed previously (4, 9, 11, 24). Our results extend these studies and suggest that KGF may
have potential therapeutic roles in disorders associated with malnutrition, including intestinal
inflammation and short gut syndrome.
Acknowledgements:

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References


Table 1. Changes in rat body weight with fasting and refeeding.

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<td>216 (2)</td>
<td>238 (3)</td>
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<tr>
<td>Fasted-Sal</td>
<td>218 (2)</td>
<td>172 (3)*</td>
<td></td>
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<tr>
<td>Fasted-KGF</td>
<td>219 (2)</td>
<td>174 (2)*</td>
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<tr>
<td>Control</td>
<td>222 (1)</td>
<td>250 (4)</td>
<td>273 (4)</td>
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<tr>
<td>Refed-Sal</td>
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<tr>
<td>Refed-KGF</td>
<td>224 (3)</td>
<td>172 (2)*†</td>
<td>221 (3)*†</td>
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Data as mean (SEM). Body weight expressed in grams. Control = Rats fed ad libitum for 3 days (fasting study) or 6 days (fasting/refed study).

Fasted-Sal = Fasted for 3 days + saline. Fasted-KGF = Fasted for 3 days + KGF (3 mg/kg/day). Refed-Sal = Fasted for 3 days/refed for 3 days + saline.

Refed-KGF = Fasted for 3 days/refed for 3 days + KGF (3 mg/kg/day).

N=8/group. * P<0.05 vs control; † P<0.05 vs Fasted-Sal.
Table 2. Intestinal mucosal growth indices after fasting: KGF prevents decreased colonic crypt depth but does not alter small bowel mucosal growth

<table>
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<tr>
<th></th>
<th>Duodenum</th>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>510 (19)</td>
<td>460 (16)</td>
<td>176 (7)</td>
<td>--</td>
</tr>
<tr>
<td>Fasted-Sal</td>
<td>459 (17)</td>
<td>384 (13)*</td>
<td>198 (7)*</td>
<td>--</td>
</tr>
<tr>
<td>Fasted-KGF</td>
<td>469 (17)</td>
<td>389 (22)*</td>
<td>208 (5)*</td>
<td>--</td>
</tr>
<tr>
<td><strong>Crypt depth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>200 (3)</td>
<td>128 (4)</td>
<td>112 (3)</td>
<td>196 (4)</td>
</tr>
<tr>
<td>Fasted-Sal</td>
<td>198 (7)</td>
<td>109 (4)*</td>
<td>95 (6)*</td>
<td>173 (4)*</td>
</tr>
<tr>
<td>Fasted-KGF</td>
<td>182 (9)</td>
<td>114 (5)*</td>
<td>93 (2)*</td>
<td>201 (9)*</td>
</tr>
</tbody>
</table>

Data as mean (SEM). Intestinal villus height and crypt depth were determined as outlined in Materials and Methods. Control, Fasted-Sal and Fasted-KGF groups are as described in Table 1. N=8/group. * P<0.05 vs Control; † P< 0.05 vs Fasted-Sal.
Table 3. Trophic effects of KGF in small bowel and colonic mucosa with nutrient repletion after fasting

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Villus height (µm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>573 (11)</td>
<td>571 (16)</td>
<td>205 (8)</td>
<td>--</td>
</tr>
<tr>
<td>Refed-Sal</td>
<td>596 (11)</td>
<td>520 (14)*</td>
<td>177 (7)*</td>
<td>--</td>
</tr>
<tr>
<td>Refed-KGF</td>
<td>683 (28)*†</td>
<td>628 (13)*†</td>
<td>239 (7)*†</td>
<td>--</td>
</tr>
<tr>
<td><strong>Crypt depth (µm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>183 (4)</td>
<td>147 (6)</td>
<td>133 (5)</td>
<td>193 (6)</td>
</tr>
<tr>
<td>Refed-Sal</td>
<td>196 (6)</td>
<td>160 (3)*</td>
<td>121 (6)</td>
<td>197 (4)</td>
</tr>
<tr>
<td>Refed-KGF</td>
<td>253 (16)*†</td>
<td>108 (3)*†</td>
<td>123 (5)</td>
<td>234 (10)*†</td>
</tr>
</tbody>
</table>

Data as mean (SEM). Intestinal villus height and crypt depth were determined as outlined in Materials and Methods. Control, Refed-Sal and Refed-KGF groups are as described in Table 1. N=8/group. * P<0.05 vs Control; † P< 0.05 vs Refed-Sal.
Table 4. Nutrient and KGF regulation of goblet cell number in small bowel and colonic mucosa

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>159 (17)</td>
<td>227 (12)</td>
<td>240 (12)</td>
<td>305 (8)</td>
</tr>
<tr>
<td>Fasted-Sal</td>
<td>199 (4)*</td>
<td>227 (3)</td>
<td>246 (3)</td>
<td>295 (6)</td>
</tr>
<tr>
<td>Fasted-KGF</td>
<td>368 (7)*†</td>
<td>430 (16)*†</td>
<td>465 (14)*†</td>
<td>560 (12)*†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>164 (15)</td>
<td>228 (6)</td>
<td>252 (7)</td>
<td>285 (8)</td>
</tr>
<tr>
<td>Refed-Sal</td>
<td>194 (3)*</td>
<td>237 (5)</td>
<td>259 (3)</td>
<td>357 (10)*</td>
</tr>
<tr>
<td>Refed-KGF</td>
<td>410 (13)*†</td>
<td>450 (7)*†</td>
<td>503 (4)*†</td>
<td>576 (0)*†</td>
</tr>
</tbody>
</table>

Data as mean (SEM). Goblet cell number was determined as outlined in Materials and Methods, expressed as total goblet cells/10 crypt-villus units. Control, Fasted-Sal and Fasted-KGF groups are as described in Table 1. Control, Refed-Sal and Refed-KGF groups are as described in Table 2. N=8/group. *P<0.01 vs Control; † P< 0.01 vs Fasted-Sal or Refed-Sal.
**Figure 1.** **KGF increases TFF2 mRNA expression in rat duodenum and jejunum.** TFF2 mRNA was determined by Northern blotting in full-thickness portions of stomach antrum, duodenum and jejunum from control, Fasted and Refed rats, as described in Materials and Methods. Panel A, lane 1 shows the 0.6 kb TFF2 mRNA transcript in stomach antrum of an ad libitum fed rat (Stom; 3 hour exposure). Panel A shows a representative duodenal experiment (48 hour exposure) from two sets of rats studied as follows: C = rats fed ad libitum for 3 days with daily i.p. saline (control) (lanes 2 and 5); Fast-Sal = Fasted for 3 days with daily i.p. saline (lanes 3 and 6); and Fast-KGF = Fasted for 3 days with daily i.p. KGF (3 mg/kg/day) (lanes 4 and 7). Panel B shows a representative jejunal experiment from two sets of rats studied as outlined for Panel A.
Panel C shows a representative duodenal blot (48 hour exposure) from two sets of rats studied as follows: C = rats fed ad libitum for 6 days with daily i.p. saline (control) (lanes 1 and 4); Refed-Sal = Fasting for 3 days, followed by re-feeding for 3 days with daily i.p. saline (lanes 2 and 5); Re-fed-KGF = Fasted/refed with daily i.p. KGF (lanes 3 and 6). Panel D shows a representative jejunal blot from two sets of rats studied as outlined for Panel C. TFF2 mRNA was not detected in ileum or colon in any of the study groups outlined above, even after 8 days of exposure (not shown). N=5/individual study group.
Figure 2. KGF ectopically induces TFF2 protein expression in rat duodenal mucosa.

Cellular localization of TFF2 protein in duodenal mucosa was determined using a rabbit monoclonal antibody against human TFF2 peptide in control, Fast-saline treated and Fast-KGF treated rats, as described in Materials and Methods. Panel A shows TFF2 staining localized to duodenal Brunner’s glands (black arrow) and no staining in crypt cells (white arrow) in a control rat. Similar cellular localization of TFF2 was present in fasted rats given saline (Panel B). KGF induced TFF2 expression in duodenal crypt and villus goblet cells in fasted rats (Panels C and D). Panel C shows TFF2 staining in Brunner’s glands (black arrow) and heavy staining in the crypt region (white arrow). Panel D shows KGF induction of TFF2 in villus cells. Secreted TFF2 protein is also evident in the lumen.
Figure 3. KGF increases TFF2 protein expression in duodenum and jejunum during food deprivation. TFF2 protein expression was determined by Western blotting in full-thickness portions of stomach antrum, duodenum and jejunum from control, fasted and fasted/re-fed rats, as described in Materials and Methods. Black bars = rats fed ad libitum for 3 days with daily i.p. saline (control). White bars = Fasting for 3 days with daily i.p. saline (Fast-Sal). Grey bars = Fasting for 3 days with daily i.p. KGF (3 mg/kg/day) (Fast-KGF). Data (mean±SE) are expressed as percent of control values. Representative autoradiograms are shown above the quantitative histograms. In the autoradiogram corresponding to duodenum (left panel), lane 1 contained purified recombinant TFF2 protein alone, lane 2 contained control rat stomach protein and lanes 3-5 duodenal protein from control, Fast-Sal.
and Fast-KGF groups, respectively. Tissue sections processed without primary antibody were used as negative controls. N=8/group. * P<0.05 versus control.
A

0.4 kb

Duodenum Jejunum Ileum Colon

TFF3 mRNA (% control/18S)

B

6.5 kD

Duodenum Jejunum Ileum Colon

TFF3 protein (% control)
Figure 4. KGF prevents the decrease in TFF3 mRNA expression induced by food deprivation in duodenum and jejunum and up-regulates TFF3 protein in all intestinal segments. TFF3 mRNA (0.4 kb) was determined by Northern blotting, normalized to mouse 18S mRNA expression, and TFF3 protein (6.5 kD) was measured by Western immunoblotting in full-thickness intestinal portions, as described in Materials and Methods. Black bars = Sprague-Dawley rats fed ad libitum for 3 days with daily i.p. saline (control). White bars = Fasted for 3 days and with daily i.p. saline (Fast-Sal). Grey bars = Fasted for 3 days with daily i.p. KGF (3 mg/kg/day) (Fast-KGF). Data (mean±SE) are expressed as percent of control values. Representative autoradiograms are shown above the quantitative histograms. N=8/group. * P < 0.05 versus control; † P < 0.05 versus Fast-Sal.
A

**TFF3 mRNA** (% control/18S)

- Duodenum
- Jejunum
- Ileum
- Colon

B

**TFF3 protein** (% control)

- Duodenum
- Jejunum
- Ileum
- Colon

**Duodenum**

**Jejunum**

**Ileum**

**Colon**
Figure 5. KGF up-regulates TFF3 mRNA and protein in the small bowel and colon during food deprivation/re-feeding. TFF3 mRNA (0.4 kb) was determined by Northern blotting, normalized to mouse 18S mRNA expression, and TFF3 protein (6.5 kD) was measured by Western immunoblotting in full-thickness intestinal portions, as described in Materials and Methods. Black bars = Rats fed ad libitum for 6 days with daily i.p. saline (control). White bars = Fasted for 3 days, followed by re-feeding for 3 days with daily i.p. saline (Re-fed-Sal). Grey bars = Fasted/re-fed with daily i.p. KGF (3 mg/kg/day) (Re-fed-KGF). Data (mean±SE) are expressed as percent of control values. Representative autoradiograms are shown above the quantitative histograms. N=8/group. * P < 0.05 versus control; † P < 0.05 versus Re-fed-Sal.
Figure 6. KGF increases goblet cell number and TFF3 protein expression in ileum during fasting/re-feeding. Ileal mucosal TFF3 protein expression within goblet cells was similar in ad libitum fed control rats (Panel A) and rats fasted for 3 days, then re-fed for three days (Panel B). KGF treatment in this model increased ileal goblet cell number and TTF3 protein abundance (Panel C). TFF3 staining localized in ileal goblet cells is indicated by arrows in Panels A and C. No staining was observed in ileal sections processed without primary antibody or following overnight incubation of primary antibody with specific TFF3 peptide (not shown). Similar results were observed in 4 other sets of animals under these conditions.
Figure 7. KGF increases goblet cell number and TFF3 protein expression in colon during fasting/re-feeding. Colonic mucosal TFF3 protein expression was similar in control rats (Panel A) and FD/re-fed rats (Panel B). KGF treatment in this model increased colonic goblet cell number and TTF3 protein abundance (Panel C). The arrow in Panel A shows TFF3 staining localized in a colonic goblet cell. The white arrow in Panel C shows goblet cell TFF3 staining and the black arrow shows secreted TFF3 protein within the colonic lumen. No staining was observed in colonic sections processed without primary antibody or following overnight incubation of primary antibody with specific TFF3 peptide (not shown). Similar results were observed in 4 other sets of animals under these conditions.