INFLAMMATORY BOWEL DISEASE

Tumour necrosis factor α and nuclear factor κ B inhibit transcription of human *TFF3* encoding a gastrointestinal healing peptide

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Background and aims: Tumour necrosis factor α (TNF- α) induction of nuclear factor κ B (NF κ B) activation plays a major role in the pathogenesis of inflammatory bowel disease (IBD). Trefoil factor family peptides TFF1, TFF2, and TFF3 exert protective, curative, and tumour suppressive functions in the gastrointestinal tract. In this study, we investigated effects of the TNF- α /NF κ B regulatory pathway by TNF- α on expression of TFFs.

Methods: After TNF- α stimulation, expression of TFF genes was analysed by quantitative real time polymerase chain reaction and by reporter gene assays in the gastrointestinal tumour cell lines HT-29 and KATO III. Additionally, NF κ B subunits and a constitutive repressive form of inhibitory factor κ B (IkB) were transiently coexpressed. In vivo, morphological changes and expression of TFF3, mucins, and NF κ B were monitored by immunohistochemistry in a rat model of 2,4,6-trinitrobenzene sulphonic acid induced colitis.

Results: TNF- α stimulation evoked up to 10-fold reduction of *TFF3* expression in the colon tumour cell line HT-29. Downregulation of reporter gene transcription of *TFF3* was observed with both TNF- α and NF κ B, and was reversible by IkB. In vivo, the increase in epithelial expression of NF κ B coincided with reduced TFF3 expression during the acute phase of experimental colitis.

Conclusions: Downregulation of intestinal trefoil factor TFF3 is caused by repression of transcription through TNF- α and NF κ B activation in vitro. In IBD, perpetual activation of NF κ B activity may contribute to ulceration and decreased wound healing through reduced TFF3.

Inflammatory bowel disease (IBD) is represented by two specific gastrointestinal disorders: ulcerative colitis (UC) and Crohn's disease (CD). Pathological processes accompanying IBD are associated with aberrant expression of many proinflammatory cytokines, including tumour necrosis factor α (TNF- α).¹ Blocking TNF- α by inhibitors² or antibodies is an effective treatment strategy in CD.³ Through a downstream signalling pathway, TNF- α triggers degradation of IkB, the inhibitor of nuclear factor κ B (NF κ B) thereby allowing NF κ B transcription factor to translocate into the nucleus.⁴ Recruitment of this transcription factor results in transcriptional activation of multiple components of the inflammatory response leading to further NF κ B activation.⁵ NF κ B is a dimer of variable subunits from NF κ B/Rel protein family that to date has five known members: p65, c-Rel, RelB, p50, and p52. The ability of different dimers to recognise slightly different DNA targets increases the ability of NF κ B subunits to differentially regulate gene expression.⁶ Blocking the p65 subunit in the 2,4,6-trinitrobenzene sulphonic acid (TNBS) induced mice colitis model abrogates the signs of colitis.⁷ In line with these observations, IBD is generally treated with agents that directly or indirectly inhibit NF κ B activation.⁸

In common with immune cells, intestinal epithelial cells also respond to the largely macrophage generated TNF- α . This leads to NF κ B mediated up or downregulation of some genes⁹⁻¹² involved in the early immune, acute phase, and inflammatory response. Although inhibition of NF κ B is a therapeutic concept, it is not known why perpetual activation of NF κ B leads to IBD.

The family of trefoil factor (TFF) peptides, with its three members TFF1 (pS2), TFF2 (spasmolytic polypeptide), and TFF3 (intestinal trefoil factor/ITF) represents typical secretory products of mucin producing cells. These small molecules are

predominantly expressed in the digestive tract of mammals. Constitutional expression of TFF3 is largely restricted to the goblet cells of the small and large intestine^{13 14} whereas TFF1 and TFF2 are expressed in the foveolar and glandular cells of the stomach and in Brunner's glands of the duodenum.^{15 16} Although the molecular function of TFF peptides in the gut is not completely understood, evidence from in vitro and in vivo experiments suggests a key role in protecting and curing the gastrointestinal mucosa.^{17 18} Oral and systemic application of recombinant TFF peptides promote mucosal defence and wound healing.¹⁹⁻²¹ Mice overexpressing human TFF1²² or rat TFF3²³ in the intestine displayed increased resistance to intestinal damage and ulceration. Mice that lack TFF1²⁴ showed gastric mucosa abnormalities and increased tumorigenesis while mice lacking TFF3²⁵ had a higher susceptibility to gut related injury.

As TNF- α and NF κ B deregulation is the hallmark of IBD, we investigated whether they affect TFF gene expression. In addition to two well characterised cell lines, we chose a rat model of TNBS induced colitis, simulating features similar to those found in human UC.^{26 27}

Our data indicated that in the HT-29 colon cell line, TNF- α , through activation of NF κ B transcription factor, downregulated the TFF3 gene by transcriptional repression. In the rat

Abbreviations: CD, Crohn's disease; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IL, interleukin; IR, immunoreactivity; ITF, intestinal trefoil peptide; IkB, inhibitor of nuclear factor κ B; NF κ B, nuclear factor κ B; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; TFFs, trefoil factors (TFF1, TFF2, TFF3); TNBS, 2,4,6-trinitrobenzene sulphonic acid; TNF- α , tumour necrosis factor α ; UC, ulcerative colitis.

colitis model, NF κ B activation was associated with decreased TFF3 expression in inflamed epithelia.

MATERIALS AND METHODS

Cell culture, RNA extraction, and reverse transcription

The gastric adenocarcinoma cell line KATO III was cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The colon adenocarcinoma cell line HT-29 was cultivated in Waymouth medium with Glutamax and 10% FCS.²⁸ For endogenous *TFF* expression, cells were seeded in 24 well plates. After 24 hours, cells were incubated in serum free medium and stimulated by addition of 20 ng/ml TNF- α (Biomol). The effect of TNF- α on expression of *TFFs* was tested in the range 2.5–40 ng/ml and was dose responsive. For our experiments, we used the minimal concentration (20 ng/ml) where TNF- α exerted a significant effect on *TFF* expression. Total RNA was prepared after 24, 48, 60, and 72 hours using RNA-pure (Peqlab). Isolated RNA was treated with RQ1 RNase free DNase (Promega) followed by cDNA synthesis using oligo-T15 primer and MMLV reverse transcriptase (Promega).

Real time quantitative PCR

Polymerase chain reaction (PCR) was performed with SYBR Green on an i-Cycler iQ (Bio-Rad). Cycle conditions were 95°C, 57°C, and 72°C for 20, 40, and 10 seconds, respectively. Relative quantification values were obtained from the threshold cycle number of three independent experiments measured in triplicate according to the manufacturer's instructions.

The following PCR primers for trefoil peptides and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used:

TFF1-f: 5'-TTTGAGCAGAGAGGAGGCAATG-3'

TFF1-r: 5'-ACCACAATTCTGTCTTTCACGGGG-3'

TFF2-f: 5'-CCCATAACAGGACGAAGTGC-3'

TFF2-r: 5'-GCACTGATCCGACTCTTGCT-3'

TFF3-f: 5'-CTTGCTGTCTCCAGCTCT-3'

TFF3-r: 5'-CCGGTTGTGCACTCCTT-3'

GAPDH-f: 5'-TGCACCACCAACTGCTTAG-3'

GAPDH-r: 5'-GATGCAGGGATGATGTTC-3'

The effect of TNF- α treatment on GAPDH expression was not significant at any time point, indicating that GAPDH could be used as a stable housekeeping gene in this experiment.²⁹

Transient transfection and luciferase assay

Luciferase reporter plasmid pGL3 (Promega) was used to construct *TFF* recombinants using the 5'-flanking region of human *TFF1* (position -1100 to +38), *TFF2* (position -821 to +61), and *TFF3* (position -867 to +63), as described previously.²⁸ Transient transfection was performed using the cationic polymer transfection reagent ExGen 500 (MBI Fermentas). Six hours after transfection, cells were shifted to serum free medium or serum free medium containing 20 ng/ml TNF- α (Biomol). Expression of genes of interest was measured 48 hours later by the Dual-Luciferase Reporter Assay system (Promega). Results were calculated according to the manufacturer's protocol and compared with non-stimulated controls. Each experiment was independently performed a minimum of five times. For cotransfection experiments, 320 ng of the reporter plasmid (containing *TFFs* promoter) and 96 ng of the following expression plasmids coding for NF κ B subunits were used: pMT2T-p50, pMT2T-p65, or CMV-pUHD as control vector. NF κ B subunit expression in pMT2T vector is controlled by adenovirus major late promoter and simian virus 40 enhancer.^{30,31} A degradation resistant mutant I κ B expression plasmid used for specific inhibition of NF κ B activity³² was obtained from Dr Kube, Tübingen,

Figure 1 Effect of tumour necrosis factor α (TNF- α 20 ng/ml) on trefoil factor (TFF1, TFF2, TFF3) mRNA expression, measured by quantitative real time polymerase chain reaction in the intestinal tumour cell line HT-29 (A) and the gastric tumour cell line KATO III (B). *TFF* expression was measured 24, 48, 60, and 72 hours after TNF- α stimulation, and was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed relative to the appropriate time point of the untreated control (%). Δ c_t (threshold cycle) values were determined by subtracting the difference in c_t levels between the *TFF* genes and the GAPDH gene after incubation with TNF- α or with no TNF- α (control) for the indicated times. Remaining *TFF* expression is calculated by the formula $2^{-[\Delta c_t - \Delta c_t(\text{control})]}$ and expressed relative to the appropriate untreated time point control. *Statistically significant (99% confidence interval).

Germany. A luciferase reporter vector carrying six times the consensus binding sequence of NF κ B (6 $\times$ NF κ B) in the TK minimal promoter was obtained from Dr Schulze-Osthoff, Marburg, Germany.

Statistical analysis

Data were analysed by one way ANOVA and, if indicated, differences between groups were analysed by appropriate parametric or non-parametric tests with 95% confidence interval. Data are presented as mean (SD) values of at least three separate experiments.

Rat model of TNBS induced colitis

The experiment was approved by the local ethics committee. TNBS 20 mg in 0.25 ml of 50% ethanol were injected following the protocol of Morris and colleagues.²⁶ During the experiment, animals had free access to water and food. Six rats were sacrificed on day 0 (control), 4, 11, and 21 after TNBS

Figure 2 Effect of tumour necrosis factor α (TNF-α) stimulation on trefoil factor (TFF1, TFF2, TFF3) *TFF* reporter gene expression in the intestinal cell line HT-29 and the gastric cell line KATO III. *TFF* reporter gene activities 44 hours after TNF-α (20 ng/ml) stimulation are expressed relative to unstimulated controls. *Statistically significant (95% confidence interval).

Figure 3 Gene activation of a nuclear factor κB (NFκB) responding reporter gene (6xNFκB-Luc) by tumour necrosis factor α (TNF-α) functionally demonstrates NFκB activation in HT-29 and KATO III cell lines. *Statistically significant (95% confidence interval).

injection. After autopsy, specimens were taken from the margin area of the treated colon segment. Samples were washed in ice cold phosphate buffered saline, fixed for 24 hours in 5% paraformaldehyde, and embedded in paraffin wax.

Histology and immunohistochemistry

Consecutive colon sections (2 μm) were stained with haematoxylin-eosin for pathomorphological examination, with periodic acid-Schiff (PAS) for mucins, and with antibodies against TFF3 and the p65 subunit of NFκB. Antigen unmasking for immunohistochemistry was performed by heat treatment (99°C) for 10 minutes in 10 mM sodium citrate, pH

6. Slides were incubated overnight at 4°C in a humid chamber with anti- TFF3 rabbit serum³³ or with anti-NFκB to detect the activated form of the p65 subunit (Santa Cruz Biotechnology, California, USA). Specific antibody binding was visualised by biotin conjugated sheep antirabbit IgG (Dako), followed by a streptavidin-biotin-horseradish peroxidase complex (Dako) and diaminobenzidine.

Microscopy was done using a light microscope (E-1000, Nikon) with differential interference contrast in combination with a video camera A113C IR-F (Basler) and a True Colour Analysis System LUCIA 4.60 (Nikon).

RESULTS

TNF-α downregulates endogenous TFF3 and TFF3 reporter gene expression

Following TNF-α induction for 24, 48, 60, and 72 hours, quantitative real time PCR revealed a permanent reduction in TFF3 mRNA in the colon cancer line HT-29 (fig 1A). Normalised to the housekeeping gene GAPDH, TNF-α evoked a threefold reduction in *TFF3* expression after 24 hours and a 10-fold reduction after 48 hours in HT-29. After 72 hours, the TFF3 mRNA level began to rise, probably because TNF-α, given in a single dose, was either degraded or used up by the cells. *TFF1* and *TFF2* expression were not significantly affected. TNF-α treatment did not influence cell viability, as determined by trypan blue staining.

To determine whether the reduction in TFF mRNA level was due to repression of transcriptional initiation, we used firefly luciferase reporter genes controlled by the 5'- flanking regions of the corresponding *TFF* genes. These constructs were previously shown to resemble endogenous *TFF* gene expression in a variety of gastrointestinal cell lines.²⁸ Compared with non-stimulated controls, TNF-α caused downregulation of all three *TFF* reporter genes in the HT-29 and KATO III cell lines (fig 2). A statistically significant reduction was noted for the *TFF3* reporter gene in HT-29 (8.5%), suggesting that the observed inhibition of endogenous TFF mRNA (fig 1) is due to repression of transcriptional initiation.

To prove that TNF-α mediates activation of NFκB, we transfected cells with a vector containing 6xNFκB binding sites in a promoter controlling expression of firefly luciferase reporter gene (fig 3). Indeed, TNF-α stimulated its transcription (11-fold in HT-29, sixfold in KATO III), indicating a functional TNF-α inducible NFκB signalling pathway in both cell lines. These results, as well as the fact that NFκB is a key player in signalling inflammatory processes and governing the immune response, led to a detailed investigation of this transcription factor. A search in the Transfac 4.0 database for putative NFκB binding sites revealed several motifs in the 5'-flanking region of all three human *TFF* genes (table 1) sharing 70–90% identity to the consensus NFκB binding site.

Transiently expressed NFκB (p50/p65) affects TFF3 expression

To demonstrate that NFκB transcription factor is indeed responsible for *TFF3* downregulation, we transfected the

Table 1 Putative nuclear factor κB (NFκB) binding sites in trefoil factor (TFF) promoters

TFF1 promoter		TFF2 promoter		TFF3 promoter	
Position	Identity (%)	Position	Identity (%)	Position	Identity (%)
-93	90	-257	80	-97	80
-231	90	-297	80	-226	80
-284	90	-421	90	-382	80
-609	90	-541	80	-644	80
-735	90	-655	80	-811	70
-890	90				

Consensus NFκB binding sequence: GGGA/G(C/A/T)TT/CT/CCC.

Figure 4 Transient nuclear factor κ B (NF κ B) expression affects trefoil factor *TFF3* transcription. Cotransfection of plasmids constitutively expressing NF κ B subunits p50, p65, and p50/65 downregulated expression of the *TFF3* reporter gene in the HT-29 cell line. TFF reporter gene activity was expressed relative to controls transfected with *TFF* reporter plasmid and empty pUHD vector. *Statistically significant (95% confidence interval).

HT-29 cell line with plasmids overexpressing NF κ B subunits p50 and p65, separately and in combination. These subunits can dimerise with each other or with other members of the NF κ B/Rel protein family already present in cells, translocate to the nucleus, and affect gene transcription. Reporter plasmids carrying *TFF* gene promoters were cotransfected with expression vectors encoding the NF κ B subunits. *TFF3* expression was significantly reduced on overexpression of the p50 and p65 subunits, and by a combination of both subunits (fig 4). It is interesting to note that cotransfection of p50 and p65 NF κ B subunits had a greater, but not significant, downregulating effect on *TFF1* and *TFF2* expression in comparison with TNF- α stimulation, probably because overexpressed NF κ B subunits are more effective than TNF- α alone.

I κ B neutralised NF κ B mediated *TFF3* repression

To prove that *TFF3* downregulation is due to NF κ B activation, we inhibited NF κ B activation using its specific inhibitor I κ B. We used a constitutive repressive form of I κ B, also called

super-repressor, that resists proteolytic degradation.³² We verified that the I κ B expression vector inhibits activation of NF κ B responding reporter gene (fig 5A). To test the efficiency of constitutively expressed I κ B, 6 $\times$ NF κ B-luc reporter plasmid was transiently cotransfected with or without I κ B expression vector and stimulated with TNF- α to induce the NF κ B pathway. NF κ B activation by TNF- α resulted in pronounced transcription of a NF κ B responding reporter gene. Its stimulation by TNF- α was abrogated in the presence of I κ B, strongly indicating that in the HT-29 cell line TNF- α acts through the NF κ B signalling pathway and that this effect can be neutralised by I κ B.

To test the effect of NF κ B inhibition on *TFF3* expression, HT-29 cells were cotransfected with *TFF3* reporter plasmid and different plasmids constitutively expressing the NF κ B subunits (p50, p65, p50/p65). In addition, to inhibit NF κ B plasmid expressing I κ B was added (fig 5B). Cotransfection with the NF κ B subunits showed significant downregulation of *TFF3* expression (fig 5B). After transient expression of I κ B together with p50, p65, and p50/65, *TFF3* downregulation was neutralised. With this experiment we confirmed that *TFF3* downregulation is controlled by NF κ B transcription factor.

Association of *TFF3* repression with NF κ B recruitment in experimental colitis

The TNBS hapten induces focal inflammation, oedema, and ulceration accompanied by loss of the typical epithelial structure, decreased number of goblet cells, and infiltration of immune cells.^{26–27} After rapid development of inflammation with complete loss of parts of the treated mucosa (days 2–4), complete regeneration occurs over the next 15–20 days. In normal colonic tissue (fig 6A–C), all mucin carrying cells of the upper two thirds of the crypt (fig 6B) exhibited distinct *TFF3* immunoreactivity (IR) (fig 6A) whereas expression of activated NF κ B was confined to a few cells of the lamina propria (fig 6C).

Four days after TNBS administration (fig 6D–F), lesions of variable severity were found in all animals. Irregular crypts displayed a reduction in the number of goblet cells, especially in the upper third and at the surface. Weak *TFF3* IR was detectable in a small fraction of the remaining goblet cells at the luminal surface (fig 6D). Activated NF κ B was expressed in macrophages and in other stromal cells, and in a number of epithelial cells, mainly in the middle and upper third of the crypts (fig 6F).

Figure 5 Specific nuclear factor κ B (NF κ B) inhibitor I κ B abrogates NF κ B mediated downregulation of trefoil factor *TFF3* in the HT-29 cell line. (A) Tumour necrosis factor α (TNF- α) significantly (*) stimulated transcription of the NF κ B responsive 6 $\times$ NF κ B-luc reporter gene. Cotransfection of plasmid constitutively expressing the NF κ B inhibitor I κ B inhibited TNF- α induced 6 $\times$ NF κ B-luc responsive gene expression. (B) Transient expression of NF κ B subunits (p50, p65, p50/p65) significantly (*) reduced *TFF3* reporter gene expression. Coexpression of a plasmid constitutively expressing the NF κ B inhibitor I κ B neutralised *TFF3* downregulation induced by expression of the NF κ B subunits.

Figure 6 Immunoreactivity (IR) of trefoil factor TFF3 (A, D, G, J), nuclear factor κ B (NF κ B) p65 (C, F, I, L), and periodic acid-Schiff (PAS) staining for mucins (B, E, H, K) in a rat model of experimental colitis. (A–C) 2,4,6-Trinitrobenzene sulphonic acid (TNBS) induction, day 0 (control): the intact colonic mucosa displays arrayed crypts with regularly distributed goblet cells with normal mucin production (B) and pronounced TFF3 staining in (A) in the upper two thirds of the crypt. NF κ B (C) is confined to the stroma cells of the lamina propria (arrow). (D–F) TNBS induction, day 4: serious damage to the colonic epithelium is associated with a lower number of goblet cells and mucin production (E). Weak TFF3 staining is restricted to a few goblet cells of the surface mucosa (D). NF κ B staining (F) is pronounced in the infiltrating cells of the lamina propria (arrow) and in some epithelial cells (arrowheads). (G–I) TNBS induction, day 11: the distribution of the goblet cells as well as PAS staining (H) is irregular but increased compared with the acute inflammation state (E). TFF3 (G) staining is still reduced in many of the PAS positive goblet cells. NF κ B (I) is present in the infiltrating cells (arrows) as well as in the crypt luminal epithelium (arrowheads), especially in areas with both low TFF3 IR and a low number of goblet cells. (J–L) TNBS induction, day 21: epithelial and goblet cells are regularly distributed and mucin production is restored (K). TFF3 (J) expression pattern has also recovered and is detectable in almost the same intensity as in control samples (A). NF κ B (L) is barely detectable in the epithelium.

On day 11 (fig 6G–I), crypt branching indicated the beginning of recovery. The resulting irregular mucosal architecture was associated with an overall increase in the number of goblet cells compared with day 4 (fig 6H). Also, the start of recovery of TFF3 expression in a fraction of goblet cells of the crypt was noted (fig 6G). NF κ B was strongly expressed in both epithelial cells and infiltrated lymphocytes. Interestingly, localisation of NF κ B in the epithelium was predominantly detected in regions of low TFF3 and mucin expression (fig 6I).

On day 21 (fig 6J–L), the mucosal architecture was completely rebuilt with regularly sized and distributed crypts. The healing process was associated with an increase in PAS positive mucin producing cells (fig 6K) and recovery of TFF3 peptide in goblet cells (fig 6J), reaching levels found in control animals. Recovery of the normal epithelial architecture was accompanied by a strong reduction in NF κ B expression in both epithelial and stromal cells (fig 6L). Taken together, these results suggest NF κ B recruitment in association with a strong reduction in TFF3 expression during the acute phase of colitis.

DISCUSSION

Several lines of evidence imply that cytokine (TNF- α and interleukin (IL)-1) induction leading to NF κ B activation plays

a major role in the pathogenesis of IBD. Antibodies against TNF- α and inhibitors of its production are currently widely used drugs in IBD.³ In addition, drugs that directly or indirectly inhibit NF κ B are promising therapeutic tools in IBD.^{34–36} Sulphasalazine, one of the most effective agents for IBD, inhibits TNF- α induced NF κ B activation via inhibition of I κ B phosphorylation.⁸

Trefoil peptides play an important role in maintenance of epithelial integrity, protection, and wound healing.^{20–37} During experimental mucositis, the temporal change in TFF3 mRNA is associated with the commitment of the epithelial cells to differentiate into goblet cells.³⁸ Moreover, intestinal trefoil factor (TFF3) confers colonic epithelial resistance to apoptosis and stimulates cell migration.^{21–39} Finally, several reports show that both luminal and systemic application of recombinant TFF peptides as well as overexpression of transgenic TFF3 promote mucosal defence and heal experimental colitis.^{20–23–25–40–41}

In the present study, we demonstrated that TNF- α induced downregulation of TFF3 transcription is mediated by NF κ B. The strong reduction of TFF3 peptide expression in a rat model of colitis was associated with inflammation and NF κ B recruitment. These results are consistent with many reports demonstrating NF κ B signalling in gastrointestinal epithelia.^{4–5} A

relatively late effect on TFF3 expression (44–48 hours after TNF- α stimulation) indicates a possible indirect effect of NF κ B activation on TFF3 expression. Recently, we showed that TFF3 downregulation also occurs on stimulation of HT-29 with IL-1 β , another strong activator of NF κ B.⁴²

Although for simplicity NF κ B is often referred to as if it were a single entity, in reality it is a complex mixture of homo- and heterodimers all with distinct characteristics and biological properties.⁴³ These homo- or heterodimeric complexes vary in their DNA binding specificity and as such can differentially regulate gene expression. NF κ B exists in the cytoplasm in an inactive form by virtue of its association with a class of inhibitory proteins called I κ Bs.^{4,6} I κ Bs display a preference for specific NF κ B complexes. Each I κ B has the potential to participate in distinct regulatory pathways affecting activation of different NF κ B complexes and at the end can influence transcription of different genes. The existence of a multigene family of NF κ B proteins and a multigene family of I κ B proteins, together with possible post-translational modifications could, in part, account for the complex regulatory potential displayed by NF κ B.⁴⁴ Additionally, it is known that NF κ B can interact with other transcription factors such as AP-1 and C/EBP. Both factors belong to the bZIP class of DNA binding proteins characterised by leucine zipper structure and adjacent basic DNA binding domain. The bZIP region has been reported to directly interact with the Rel homology domain of NF κ B.^{45–46} Putative AP-1 and C/EBP binding sites are located within the 5'-flanking region of all TFF genes.⁴² Alternatively, binding of NF κ B may compete with the binding of NF-1 for interaction with a coactivator such as p300/CBP.⁹

Many putative binding sites of NF κ B are present in the upstream region of all three human TFF genes. In the TFF3 promoter, four putative NF κ B binding sites displaying 80% identity (two mismatches) to the consensus site were found (table 1). Mutating these putative NF κ B binding sites would be necessary to discover which is responsible for NF κ B binding and thus regulation of TFF3 expression. In addition, binding of NF κ B to chromatin in vivo would give a more realistic picture than demonstrating in vitro binding to double stranded oligonucleotides by EMSA. A NF κ B binding site with two mismatches was reported to downregulate human papilloma virus HPV16 gene expression although the affinity of NF κ B for this site was 250-fold lower compared with affinity for the consensus NF κ B binding site.⁹ The discrepancy between in vitro and in vivo DNA binding has also been observed with other transcription factors, including c-Myc.⁴⁷

Although NF κ B is largely considered to be a transcriptional activator of a variety of genes involved in early immune, acute phase, and inflammatory responses,³¹ it can also directly repress transcription of some genes.^{9–11, 48–49} The background of repression has still to be elucidated.

Taken together, our in vitro data suggest downregulation of TFF3 expression in HT-29 as a consequence of NF κ B activation by TNF- α , and our in vivo data confirm reduced TFF3 expression in the rat model of colitis. Bearing in mind that TFF3 promotes mucosal defence, it is intriguing to ask whether its strong reduced expression may in vivo causally contribute to epithelial dismorphology and ulceration.

In a rat model of TNBS induced colitis, we demonstrated that NF κ B expression in both immune and epithelial cells paralleled a marked reduction in TFF3 expression (fig 6). Consistent with our data, Xian and colleagues³⁸ showed that TFF3 expression in the intestine is decreased in methotrexate induced damage. Itoh and colleagues⁵⁰ also reported a decrease in TFF3 production during the early stage (1–4 days after induction) of experimentally induced ulcerative damage with TFF3 upregulation during the recovery phase. Tran and colleagues²⁰ showed that TFF3 expression is unaltered during hapten/ethanol induced colitis in the rat, suggesting that inflammatory and ulcerogenic influences counteract each other. We observed a similar time course of marked depletion

of TFF3 production associated with a reduction in the number of goblet cells. Our time course measurements suggested that TFF3 reduction may be an early event in TNBS induced colitis. Clearly, the observed reduction in the number of goblet cells did not fully account for the strong reduction in TFF3 expression as we noticed mucin producing cells with decreased and with no TFF3 immunoreactivity on days 4 and 11. Therefore, the strong reduction in TFF3 expression during the acute phase of experimental colitis might provoke cellular changes already attributed to TFF3, such as redifferentiation, apoptosis, decrease in cell migration, and decreased barrier function,⁵¹ all of which may eventually contribute to ulceration.

However, in an apparent contradiction to our results, upregulation of trefoil peptides was observed in an ulcer associated cell lineage^{52–55} and in an experimental rat model.⁵⁶ In the latter, a cryoprobe was used to damage the rat stomach, and TFF2 and TFF3 mRNA were upregulated in discrete cells of the regenerating glands at the ulcer margin. This damage may not reflect the actual situation during TNBS induced colitis. More likely, the observed upregulation of TFF2 and TFF3 may be part of the acute phase response to cope with the harsh damage, necrosis, and threat of epithelial integrity in the stomach. In gastric and intestinal cell lines, we found that osmotic forces, ethanol, as well as acute phase gene regulators HNF-3 and GATA-6 induced upregulation of TFF genes.^{57–59} Recently it was reported that duodenal TFF3 expression was low in association with chronic inflammation and villus atrophy in coeliac disease patients, and that recovery following removal of gluten from the diet was accompanied by recovery in TFF3 expression.⁶⁰ In humans, upregulation of trefoil genes TFF1 and TFF2 but not TFF3 was observed in IBD.⁵² As TFF1 and TFF2 are not expressed under normal conditions in the intestine (except in Brunner's gland of the duodenum), their upregulation could be easily detected and has been initially noted in cancer and in patients with IBD.⁶¹ Later, TFF1 and TFF2 upregulation was confined to the ulcer associated cell lineage, restricted to the nearby mucosa of the duodenal ulcers.^{52–55} Our in vitro and in vivo data imply that TNF- α causes NF κ B mediated transcriptional repression of the TFF3 gene and thereby reduces the steady state level of this peptide. This regulatory mechanism may partially explain the impairment of wound healing in IBD. Further studies are required to address the biological implications of TFF3 downregulation in vivo. Activation of TFF genes may eventually generate opportunities for treatment of gastrointestinal diseases.

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