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Short communication

Expression of trefoil factor genes in the duodenum and colon of dogs with inflammatory bowel disease and healthy dogs

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ABSTRACT

Trefoil factors (TFF) are small peptides produced by goblet cells, which are crucial for epithelial restitution. In humans with inflammatory bowel disease (IBD), TFF expression is up-regulated as part of an unspecific repair mechanism. The goal of this study was to assess TFF gene expression in the gastrointestinal tract from dogs with IBD compared to healthy controls. Preliminary assessment by PCR revealed TFF1 and 3 expression in the small and large intestine, whereas TFF2 was amplified only in the stomach. Subsequent RT-qPCR (with relative quantification against 3 reference genes) on endoscopic duodenal (IBD $n = 22$, healthy controls $n = 18$) and colonic (IBD $n = 12$, controls $n = 11$) biopsies revealed that TFF1 expression was significantly up-regulated in the duodenum from IBD dogs (Mann–Whitney $p = 0.001$), whereas TFF3 expression was significantly lower in IBD colon compared to controls (t-test $p = 0.018$).

This study demonstrates evidence for dysregulation of TFF gene expression in canine IBD. Up-regulation of TFF1 could signify ectopic expression as a compensatory repair-mechanism, whereas down-regulation of TFF3 could contribute to defective epithelial barrier function, respectively. Whether this is a cause or consequence of IBD could not be established.

1. Introduction

The mucus layer coating the gastrointestinal tract represents one of the front lines of innate immune defences of the host. This mucus is composed of mucin glycoproteins and trefoil factor (TFF) peptides secreted by goblet cells. In healthy humans, TFF1 and 2 have been found to be expressed mostly in the stomach, whereas TFF3 is most abundant in the small and large intestinal mucus layers (Taupin and Podolsky, 2003). When the gastrointestinal epithelium is injured, rapid and coordinated auto- and cross-induction of TFFs at the edge of the injury occurs through the presence of TFF response elements in TFF gene promoters (Podolsky, 2000). Reshaping, stretching and migration of adjacent epithelial cells occurs to seal the defect in the barrier (Podolsky, 2000) – a process known as restitution (Giraud, 2000). TFFs also prevent apoptosis of epithelial cells through cell detachment (Dignass et al., 1994; Taupin et al., 2000). A lack of TFFs (as for example in rodent models of targeted deletion of the TFF3 gene) results in increased sensitivity to colonic injury by dextran sodium sulphate due to an inability to repair the epithelium (Podolsky, 2000).

Dysregulation of TFF expression has been demonstrated in human inflammatory bowel disease (IBD), where both TFF1 and TFF2 have been found to be ectopically expressed in the intestine (Poulsom et al., 1993; Shaoul et al., 2004;

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Wright et al., 1993). TFF3 expression is up-regulated on the mRNA level both in Crohn's disease (Shi et al., 2010) and ulcerative colitis (Longman et al., 2006). TFFs are linked to the function of innate immune receptors, especially toll-like receptor (TLR) 2 and 4 (Kim and Ho, 2010; Podolsky et al., 2009). TLRs have been implicated in the tolerogenic responses towards commensal microbiota in health, but also seem to play an important role in the pathogenesis of IBD in humans (Cario, 2010) and dogs, where TLR2 expression has been found to be associated with disease severity (McMahon et al., 2010) and single nucleotide polymorphisms in TLR5 were shown to convey an increased risk of IBD (Kathrani et al., 2011), especially in German Shepherd dogs (Kathrani et al., 2010). In goblet cell lines and normal intestinal epithelial cells derived from mice or humans, stimulation with the TLR2/1 ligand Pam_3CSK_4 induced the expression of TFF3 (Podolsky et al., 2009). In addition, oral administration of Pam_3CSK_4 protects mice from DSS colitis by decreasing cell apoptosis and hastening repair of zonulin-1 tight junction complexes; whereas TLR2-deficient mice show decreased colonic production of TFF3 and are thus more susceptible to DSS colitis (Podolsky et al., 2009).

As dysregulation of TLRs has been demonstrated in dogs with IBD in several studies (Burgener et al., 2008; McMahon et al., 2010), and especially TLR2 – which is likely to have a direct link with TFF production and epithelial restitution – is up-regulated in canine IBD tissues (McMahon et al., 2010), we were interested in the expression profile of TFFs in the healthy and diseased canine intestine. These peptides have only recently been characterised in small animals (Campbell and Jabbes, 2008), and to our knowledge, no study has assessed their expression in the dog so far. Our hypothesis was that trefoil factors are differentially expressed in different canine gastrointestinal tissues and a difference in TFF expression exists between healthy and IBD dogs.

2. Materials and methods

2.1. Study population

Duodenal ($n = 22$) and colonic ($n = 12$) endoscopic biopsies were obtained during the routine workup of dogs with chronic enteropathies at the Queen Mother Hospital for Animals of the Royal Veterinary College, London. The diagnosis of IBD was made based on appropriate clinical signs (vomiting and/or diarrhoea, weight loss) of at least 3 weeks' duration, exclusion of other causes of chronic gastrointestinal signs and the presence of lymphoplasmacytic and/or eosinophilic inflammation on histopathological review of duodenal biopsies. Details on the dogs' signalement can be found in Table 1. Written owner consent was obtained for all dogs included into the study. Additionally, the study was approved by the Royal Veterinary College Ethics and Welfare Committee. One mucosal biopsy from duodenum and colon, respectively, was placed immediately into RNAlater (Ambion, Huntingdon, UK) and stored at −80 °C until further use. Duodenal ($n = 18$) and colonic ($n = 11$) biopsies were also obtained from healthy Beagle dogs as control tissues and stored in the same manner as described above.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from tissues using the mammalian total RNA extraction kit (Sigma–Aldrich, Gillingham, UK). Twenty-five mg of tissue were homogenised into 0.5 ml lysis solution, using the Mixer Mill MM300 (Retch, Leeds, UK). Subsequently, an in-solution DNase digestion step (Turbo DNase, Ambion, Austin, USA) was performed. cDNA was synthesised using oligo$(dT)_{15}$ primers and the Improm II reverse transcriptase enzyme (Promega, Southampton, UK). Controls were performed without reverse transcription. None of the samples showed evidence of amplifiable genomic DNA with this assay.

2.3. Standard polymerase chain reaction

Internal primers for TFF1, 2 and 3 (Table 1) were designed based on the available canine gene sequence using Primer3 software (Rozen and Skaletsky, 2000). Polymerase chain reactions (PCR) were performed with the immolase polymerase (Bioline, London, UK) according to the manufacturer's instructions using the following temperature steps: 95 °C for 7 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, finalised by a final extension step at 72 °C for 7 min. Reactions were carried out in 25 μl, with a primer concentration of 800 pmol and $MgCl_2$ concentration of 2.5 mM for all experiments. Amplification of a single PCR product of the correct size was confirmed on 1.5% agarose gels.

2.4. Generation of positive controls for quantitative PCR

To obtain positive controls suitable for subsequent quantitative (q)PCR analysis, we cloned parts of the canine sequences for TFF1 and TFF3 and three reference genes previously shown to be most stably expressed in canine endoscopic intestinal samples (Peters et al., 2007), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), succinyl-dehydrogenase (SDHA) and Tata-box binding protein (TBP). For TFFs, the same primers as for standard PCR were used, whereas for the reference genes, primers were designed as described above to create a product between 400 and 800 bp (Table 1) as described before (Schmitz et al., 2012). Experimental setup and cycling conditions were the same as for standard PCR described above. Amplified fragments were again visualised on a 1.5% agarose gel and purified using the Wizard SV Gel and PCR clean-up system (Promega). Purified fragments were cloned into the pGemT-Easy vector (Promega) following the manufacturer's instructions. Plasmid DNA was extracted from several colonies using the PureYield Plasmid Miniprep system (Promega), sequenced (Source Bioscience, Cambridge, UK) and confirmed against published sequences (Table 1).

2.5. Quantitative polymerase chain reaction

For the TFFs the same primers were used as in standard PCR. For the reference genes, published primers were tested for qPCR and found to be suitable (McMahon et al., 2010) (Table 1). Each qPCR reaction was performed in 20 μl, contained 200 nM of each primer and 1 μl of cDNA

Table 1
Signalement and final diagnosis of the dogs suffering from inflammatory bowel disease used in the present study. FRD: food responsive disease (elimination or single protein diet); SRD: steroid-responsive disease; ARD: antibiotic-responsive disease (metronidazole in all cases); PLE: protein-losing enteropathy; D: duodenum; C: colon; F: female; Fn: female neutered; M: male; Mn: male neutered.

No.	Age (months)	Gender	Breed	Site of biopsy	Final diagnosis
1	52	F	Labrador	D, C	FRD
2	171	Mn	Bichon Frise	D, C	FRD
3	51	Mn	Boxer	D, C	FRD
4	131	Fn	Weimaraner	D, C	FRD
5	41	Mn	Crossbreed	D	FRD
6	62	M	Boxer	D	FRD
7	41	Fn	GSD	D	FRD
8	113	Fn	Cairn Terrier	D	FRD
9	60	M	Labrador	D	FRD
10	125	M	Border Terrier	D	FRD
11	124	M	Shi Tzu	D	FRD
12	44	Fn	Dalmatian	D, C	FRD, PLE
13	178	M	Poodle	D	FRD, PLE
14	106	Mn	Bedlington Terrier	D	ARD
15	60	Fn	Border Collie	D, C	ARD
16	102	Mn	Border Terrier	D, C	ARD
17	120	Fn	Golden Retriever	D, C	ARD
18	18	Mn	Dogue de Bordeaux	D, C	ARD
19	77	Fn	Schnauzer	D, C	ARD, PLE
20	82	Mn	Rough Collie	D, C	SRD
21	30	Mn	Miniature Schnauzer	D	SRD
22	78	Fn	Greyhound	D, C	SRD, PLE

in addition to SsoFast Evagreen Supermix (Bio-Rad, Hemel Hampstead, UK). The reaction involved an enzyme activation step at 95 °C for 5 min, followed by 40 cycles of 94 °C for 15 s, annealing temperature (see Table 2) for 10 s, elongation at 72 °C for 10 s and an additional melting step of 80 °C for 5 s before each plate reading (to melt primer dimers). Each reaction was carried out in triplicate and reaction efficiency was determined for each gene using 10-fold dilutions (10^7–10^1 molecules μl^{-1}) of plasmids containing the cloned amplicon. Melting curves were generated to ensure a single amplicon had been produced. Gene expression was quantified by averaging the triplicate absolute measurements for each biological sample for the TFF and reference genes, following normalisation of the expression of each target gene to the geometric mean of the three reference genes.

2.6. Statistical analysis

Statistical analysis was performed using Graph Pad Prism software package (Version 5, Graph Pad Software Inc., La Jolla, California, USA). Data were tested for normal distribution using histograms and the D'Agostino and Pearson omnibus normality test. Comparison of gene expression across tissues in both groups of dogs and between disease subgroups (food responsive disease [FRD] vs. antibiotic-responsive disease [ARD] vs. steroid-responsive disease [SRD]) was performed using the Kruskall–Wallis test. If significant differences across all groups were observed, a Dunn's multiple comparison was performed as a post hoc test. For pair-wise comparisons, Mann–Whitney *U* tests or *t*-tests were performed depending on normal distribution of data.

3. Results and discussion

In the present study, we aimed to characterise the expression of TFF genes in the canine gastrointestinal tract and compare the mRNA expression levels between healthy dogs and dogs with IBD. The distribution between FRD, ARD and SRD within the study group can be found in Table 1. Using standard PCR, differential expression of TFF1, 2, and 3 could be shown throughout the gastrointestinal tract irrespective of disease status. Whereas TFF1 and TFF3 could be detected by standard PCR in intestinal tissues from healthy and IBD dogs, there was no amplification

Table 2
Characteristics of primers used for polymerase chain reactions. All were used at an annealing temperature of 55 °C.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	NCBI accession number	Size of PCR product	Standard (S) PCR, quantitative (Q) PCR, for plasmids (P)
TFF1	GGAGCACAGGGTGATCTACG	AACAGCAGCCCTTGTCCTTA	NM_001002992.1	161	S, Q, P
TFF2	GAAGCAAGAGTCGGAGCAGT	TTGAGGATTGGGAAGAAGCA	NM_001002991.1	150	S
TFF3	CAGCTTGGCAGTGGCTTAC	TGCATTCTGTGTCCTGCAAC	NM_001002990.1	182	S, Q, P
GAPDH	GATTGTCAGCAATGCCTCCT	CGGTTGCTGTAGCCAAATTC	XM_003435649.1	534	P
GAPDH	GGAGAAAGCTGCCAAATATG	ACCAGGAAATGAGCTTGACA	XM_003435649.1	194	Q
SDHA	GGACAGAGCCTCAAGTTTGG	GGCATCCTTCCGTAATGA	XM_535807.3	412	P
SDHA	GGACAGAGCCTCAAGTTTGG	GGCATCCTTCCGTAATGA	XM_535807.3	92	Q
TBP	GGGACCGCAGCAGATTACTA	GCTCCCGTACACACCATCTT	XM_863447.1	788	P
TBP	GGGACCGCAGCAGATTACTA	GCCATAAGGCATCATTGGAC	XM_863447.1	267	Q

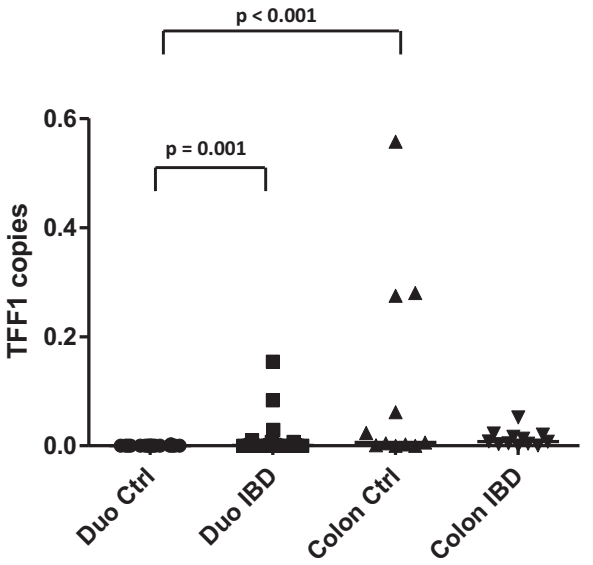

Fig. 1. Trefoil factor 1 expression in duodenal (duo) and colonic (colon) biopsies of healthy control dogs (ctrl) and dogs with inflammatory bowel disease (IBD). Absolute copy numbers relative to the mean of three reference genes are shown.

of TFF2 in any of these samples at different annealing temperatures and $MgCl_2$ concentrations. A temperature gradient (from 50 °C to 60 °C) performed on cDNA from a gastric sample, however, showed good amplification, indicating tissue-specific expression of these genes. Hence, it was decided to focus on intestinal TFF1 and TFF3 expression only for the qPCR assays. Overall, TFF1 expression was negligible compared to TFF3 expression in examined tissues (compare Figs. 1 and 2), showing that TFF3 is the main TFF produced in the canine intestine. TFF1, but not TFF3 expression was significantly different between healthy and diseased dogs (Kruskall–Wallis $p < 0.0001$; Fig. 1, and Kruskall–Wallis $p > 0.05$, Fig. 2, respectively). In

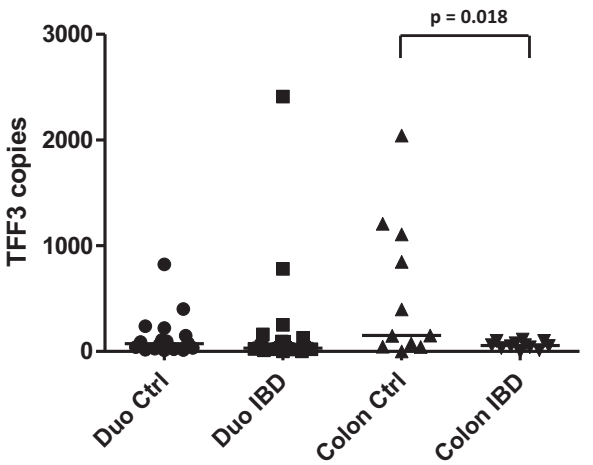

Fig. 2. Trefoil factor 3 expression in duodenal (duo) and colonic (colon) biopsies of healthy control dogs (ctrl) and dogs with inflammatory bowel disease (IBD). Absolute copy numbers relative to the mean of three reference genes are shown with the horizontal bar representing the median.

healthy control dogs, TFF1 expression was higher in the colon than in the duodenum (Mann–Whitney $p < 0.001$). When comparing IBD cases to controls, duodenal TFF1 was significantly up-regulated (Mann–Whitney $p < 0.001$), whereas colonic TFF3 was significantly down-regulated (t-test $p < 0.05$). When comparing TFF1 or TFF3 expression across groups separated by response to treatment, there was no significant difference in the expression of TFF1 in the duodenum ($p = 0.5584$) or colon ($p = 0.5616$) and no difference in the expression of TFF3 in the colon ($p = 0.8808$). However, duodenal TFF3 expression was significantly higher in dogs with FRD compared to ARD and SRD ($p = 0.0101$). According to our results, the distribution of TFF in the intestine of dogs seems different to that in humans: TFF2 appears to be exclusively expressed in the canine stomach, whereas both TFF1 and TFF3 are expressed at low levels in the duodenum and at higher levels in the colon, possibly matching the increased number of goblet cells in the large intestine. In a chronic inflammatory condition like IBD however, TFF1 is up-regulated in the duodenum (possibly ectopically), whereas TFF3 is down-regulated in the colon. This fits well with the observation that in chronic inflammatory enteropathies, the canine small intestine tends to show some goblet cell metaplasia with increased numbers of goblet cells in the duodenum, which is believed to be a response to intestinal injury (S. Priestnall, RVC, personal communication). As enterocytes are lost and the cell turnover increases, intestinal epithelial stem cells produce goblet cells before producing new enterocytes and thus the numbers of these cells can be significantly increased (Ikeda et al., 2002). The histological picture in the large intestine tends to be more variable; however, a decrease of goblet cells seems to be more consistent with IBD (S. Priestnall, personal communication), which would match our findings of decreased TFF3 expression.

These results could also be interpreted as an unspecific response to injury of the intestinal epithelium. It is possible that the up-regulation of TFF1 in the duodenum represents a response to more severe injury compared to the colon or a compensatory repair-mechanism in inflammation in general. As this is purely speculative at this stage; future studies should include a group of dogs suffering from acute gastrointestinal inflammation or specific infections to try to correlate TFF expression with the histological picture, especially with regards to the number of goblet cells.

A lack of TFFs (as seen here with TFF3 in the colon) could contribute to defective epithelial barrier function, causing "leakiness" of tight junctions and delayed repair of epithelial cell damage. Increased intestinal permeability could then lead to an increased antigenic load from microbes or food antigens, which could perpetuate faulty immune recognition of microbe-associated molecular patterns, thus leading to increased intestinal inflammation.

Assuming that canine TFF expression can be induced by TLR2 activation – as demonstrated for humans and mice (Podolsky et al., 2009) – this could fit with the observation that TLR2 is up-regulated in the duodenum of IBD dogs (Burgener et al., 2008; McMahon et al., 2010). Why the situation in the colon is different remains largely unclear, especially as most studies in dogs have so far been

restricted to investigations of the small intestine. The only study that investigated TLR expression in the canine colon (Burgener et al., 2008) has shown that colonic TLR2 is up-regulated in food-responsive and steroid-responsive IBD, which might not fit with the current observation that TFF3 was down-regulated in the colonic tissue studied here. Also why changes seem to be limited to one specific TFF alone (TFF1 in the duodenum and TFF3 in the colon) cannot be explained with the present study. Further studies need to address the mechanism behind the differential regulation of TFFs in detail. In addition, it would be ideal if duodenal and colonic samples from the same dogs could be assessed simultaneously. Unfortunately, this was not always possible in the current study. Also, it would be interesting to see if normal levels of TFF can be restored after treatment for IBD and resolution of clinical signs.

Due to the nature of the study, we were unable to correlate our findings with histopathological scorings or numbers/changes in goblet cells in the small and large intestine, respectively. However, especially assessing numbers of goblet cells might be a difficult task in future studies, as it has been recognised that measurement of goblet cells is not straight-forward and that the number of such cells may be artificially decreased by discharge of mucus during the biopsy process (Washabau et al., 2010). For that reason, assessment of goblet cell numbers was not incorporated into the final version of the guidelines for the evaluation of gastrointestinal biopsies in companion animals (Day et al., 2008).

In conclusion, this is the first study describing the expression of TFF genes in gastrointestinal segments of healthy dogs. We were able to demonstrate that TFF3 seems to be the main trefoil factor expressed in the intestine, whereas expression of TFF1 is considerably lower and TFF2 seems to be restricted to the stomach. In dogs with IBD, TFF expression is altered, which likely adds to the chronic inflammatory picture in this disease by modulating intestinal permeability and repair mechanisms. The reasons for a differential change of TFF1 and TFF3 expression in intestinal segments of dogs with IBD remains unclear, but should be addressed in future investigations of TFF gene or protein expression.

Conflict of interest statement

The authors declare no conflict of interest.

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