Differential Expression of Mucosal Trefoil Factors and Mucins in Pediatric Inflammatory Bowel Diseases

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In the intestinal mucosa trefoil factors (TFF) and mucins (Muc) - primarily produced by goblet cells - are thought to play a major role in providing barrier function during infection and inflammation. To investigate their role in pediatric Crohn’s disease (CD) and ulcerative colitis (UC) we obtained mucosal biopsies of children with CD, UC and healthy controls and analyzed genetic expression. Levels of TFF2 mRNA were lower in inflamed mucosal samples (terminal ileum (TI) and duodenum) of children with CD, but higher in non-inflamed mucosal samples when compared to healthy controls ($p < 0.05$). Similarly, TFF2 levels in the TI were significantly lower in inflamed UC tissue. Adjustment for goblet cell density revealed slightly less marked, yet significantly different gene expression in IBD and controls. Furthermore, TI expression of TFF2 and Muc2 was inversely correlated with interleukin-8 expression in CD ($p = 0.027$). In Summary, our data demonstrate significant changes in Muc and TFF mRNA expression in pediatric patients with IBD suggesting a role in mucosal healing. Further studies are needed to elucidate a potential use as biomarkers for disease progression.

Inflammatory bowel diseases (IBD), mainly comprised of the two entities Crohn’s disease (CD) and ulcerative colitis (UC) are a world-wide health-care problem with increasing incidence. While CD and UC have distinct clinical features, both disorders are characterized by relapsing inflammation in the gastrointestinal tract. Even though the etiology remains largely unknown, the underlying pathophysiological mechanisms are thought to involve genetic susceptibility, environmental factors, gut microbial composition and altered immune response patterns.

The trefoil factor family (TFF) is a group of peptides abundantly secreted onto the surface of the gastrointestinal tract by goblet cells. These 7–12 kDa small, protease-resistant proteins play an important role in maintaining epithelial integrity of the gastrointestinal tract through regulation of restitution and regeneration of the intestinal epithelium. While the complexity of their biological functions and the molecular mechanisms involved remain to be fully understood, TFF proteins have been shown to play key roles following mucosal injury through inhibition of apoptosis and anti-inflammatory signaling. Similar to TFF’s, hepatocyte growth factor (HGF) and mucins are also involved in healing processes following intestinal epithelial damage. HGF modulates intestinal epithelial cell proliferation and migration, thus accelerating intestinal mucosal repair processes. Mucins are epithelial glycoproteins important for the protection of mucosal integrity through preservation of the epithelial barrier function.

Currently only little information is available on the potential roles and regulations of mucins and HGF in the intestinal mucosa of adult IBD patients and there is a complete lack of data in pediatric IBD. As TFFs, mucins and HGF play important roles in mucosal protection, regeneration and restitution processes following inflammatory damage, we hypothesized that their expression may be altered in pediatric patients with IBD when compared to healthy children.

Results

Patient characteristics and IL8 mRNA levels are shown in Table 1. PCDAI and SES-CD differed significantly between the inflamed and the non-inflamed CD patient group with $p$-values of 0.04 and 0.02, respectively.
Table 1 | Clinical characteristics of the study population [median ± standard deviation (sd)]

|                         | controls | non-inflamed | total    | inflamed | non-inflamed | non-inflamed |
|-------------------------|----------|--------------|----------|----------|--------------|--------------|
| number (n)              | 5        | 5            | 5        | 5        | 21           | 21           |
| gender                  |          |              |          |          | male         | female       |
|                         | 13       | 2            | 3        | 2        | 2            | 2            |
|                         | 8        | 3            | 1        | 2        | 12           | 12           |
| age (years)             | 10.4 ± 6.1 | 13.6 ± 2.2  | 12.4 ± 3.6 | 13.7 ± 2.1 | 13.7 ± 2.1 |
| PCDAI                   | 20.4 ± 14.4 | 20.4 ± 14.4 | 14.3 ± 20.7 | 14.3 ± 20.7 | 14.3 ± 20.7 |
| SES-CD*                | 0.6 ± 0.3 | 0.6 ± 0.3    | 0.6 ± 0.3 | 0.6 ± 0.3 | 0.6 ± 0.3    |
| CU disease extent       | 6 ± 0.6   | 6 ± 0.6      | 6 ± 0.6  | 6 ± 0.6  | 6 ± 0.6      |
| CRP (mg/dl)             | 5.6 ± 3.6 | 5.6 ± 3.6    | 5.6 ± 3.6 | 5.6 ± 3.6 | 5.6 ± 3.6    |
| IL6 mRNA copy numbers   |          | 102 ± 99.5   | 12365 ± 56679 | 1698 ± 1458 | 659 ± 552.7  |
| terminal ileum          |          | 51 ± 121.5   | 13869 ± 26640 | 1815 ± 6297 | 1273 ± 3625  |
| ascending colon         |          |              |          |          |              |              |

*SES-CD = Simple Endoscopic Score.
*MAEO endoscopic index: 0 = Normal or inactive disease, 1 = Mild disease (erythema, decreased vascular pattern, mild friability), 2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions), 3 = Severe disease (spontaneous bleeding, ulceration).

Discussion

This is the first study to analyze TFFs and their role in GI healing processes in pediatric IBD. It also analyzes the role of GI tissue inflammation and disease activity on TFF expression.

Comparing GI genetic expression of TFF peptides in healthy children and IBD patients, we found the most distinct degree of dysregulated expression in inflamed ileum and biopsies. In inflamed CD and UC, we observed increased expression of TFF2 and MUC2 in IBD patients compared to healthy controls.

TFF2-deficient and TFF-deficient rats showed increased susceptibility to GI injury and inflammation. This may explain the observed correlation between TFF expression and goblet cell density in IBD patients. TFF2, which is produced by intestinal goblet cells, is known to promote healing and regeneration processes.

Furthermore, TFF2 and MUC2 are known to correlate with the degree of inflammation and to inversely correlate with the expression of IL-8. Similarly, the MAYO clinical disease index differed significantly between CD patients and healthy controls.

In patients with acutely inflamed CD, we observed significantly higher mRNA levels of TFF2 and MUC2 in the mucosa of the terminal ileum (TI) when compared to healthy controls (Fig. 1). This is consistent with data from an experimental rat model in which TFF2 and MUC2 expression were significantly higher in inflamed tissues compared to non-inflamed tissues.

Goblet cell vesicles are known to play a role in mucosal healing and regeneration processes. TFF2-deficient rats showed reduced mucosal goblet cell density. This raises the question of whether differences in gene expression of TFFs and MUC2 in IBD patients could be explained by differences in goblet cell density. Further studies are needed to investigate this hypothesis.

In conclusion, this study provides new insights into the role of TFFs in pediatric IBD and demonstrates the potential of TFFs as biomarkers for mucosal healing and regeneration processes. Further research is needed to clarify the clinical relevance of these findings and to investigate the potential of TFFs as therapeutic targets for IBD treatment.
the suspicion that the altered expression pattern is rather the consequence of ongoing inflammatory cell damage rather than the cause of the inflammation itself. Overall, the complexity and heterogeneity of both trefoil peptides and mucin glycoproteins make it difficult to decipher their various roles in pathophysiological processes as cause for or consequence of IBD. Further studies are needed to identify their immunological functions and possible interacting partners.

We observed significantly higher levels of TFF1 in the TI in CD when compared to healthy controls and patients with active UC. This TFF1 neo-expression in IBD was also observed before in both adult patients with severe CD and in an experimental dog model\(^{12,13}\) and is probably a compensatory repair mechanism to counteract the decreasing mucosal barrier function due to inflammatory tissue damage. This potentially qualifies TFF1 as a marker to discriminate between CD and UC in ambiguous cases of acute IBD with TI involvement. Finally, we observed high levels of HGF in CD when compared to UC and healthy controls which is in accordance with the concept that CD is a process affecting the entire intestinal wall whereas UC only affects the mucosa.

**Figure 1 | Gene expression of TFFs and mucins in the intestinal mucosa.** Expression levels were normalized against the median of GAPDH, b-actin and RPL19. Data is expressed as median with interquartile range, minimum and maximum. * and † indicate p-values < 0.05 and ** indicates p-values < 0.01, respectively.

**Figure 2 | Gene expression adjusted for goblet cell density.** Left: Goblet cells (GC) density was analysed in relation to total numbers of enterocytes counted in PAS stained tissue sections. The dashed line represents the mean goblet cell number in healthy controls. Middle and right: Expression levels were adjusted for GC density and normalized against the median of GAPDH, b-actin and RPL19. Data is expressed as median with interquartile range, minimum and maximum. * and † indicate p-values < 0.05 and ** indicates p-values < 0.01, respectively.
Methods

Over a period of 18 months 21 pediatric subjects were recruited from three centers in Germany (Wuppertal) and the UK (London and Cambridge). Diagnosis of CD and UC was based on clinical, radiological, endoscopic, and histopathological findings in accordance with the Porto criteria and Montreal classification. Lower and upper gastrointestinal (GI) endoscopy was performed by experienced pediatric gastroenterologists and biopsies were collected from the mucosa of duodenum, terminal ileum and ascending colon. Patients with CD and UC were sub-divided into groups of patients with active inflammation and without active inflammation. This differentiation was based on histological findings and mRNA expression levels of the inflammatory cytokine interleukin-8 (IL-8). Patients without macroscopic or histopathologic abnormalities and with no evidence for underlying GI pathology served as controls.

Biopsy samples were immediately placed in RNAlater (Qiagen, Hilden, Germany) and stored at −80°C until processing. RNA was extracted using the RNeasy extraction kit (Qiagen) and RNA integrity was verified with agarose gel electrophoresis. Next, 500 ng of RNA was reverse-transcribed and DNAse-treated utilizing the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RNA integrity was verified with agarose gel electrophoresis. The expression levels of TFF1 and TFF2 were determined by quantitative real-time PCR (Invitrogen, Burlington, Canada). Amplification of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene served as a control. mRNA expression levels were determined using a TaqMan assay (TaqMan Gene Expression Assay kit, Applied Biosystems, Foster City, CA, USA) and analyzed using the ABI Prism 7700 SDS detection system. Data was normalized to GAPDH expression and calculated as 2−ΔΔCt.

Results

Expression of mucin 2 (dark red) is substantially reduced in the terminal ileum of patients with active Crohn’s disease (A) when compared to patients in clinical remission (C). In ulcerative colitis, patients with active disease show lower expression of trefoil factor 1 (light brown) in the terminal ileum (B) when compared to UC patients in clinical remission (D). Microscopic magnification is ×100 in all slides.

Figure 3 | Immunohistochemistry of terminal ileum specimens of patients with CD and UC. Expression of mucin 2 (dark red) is substantially reduced in the terminal ileum of patients with active Crohn’s disease (A) when compared to patients in clinical remission (C). In ulcerative colitis, patients with active disease show lower expression of trefoil factor 1 (light brown) in the terminal ileum (B) when compared to UC patients in clinical remission (D). Microscopic magnification is ×100 in all slides.

Author contributions

K.H. and V.B. have performed gene expression experiments and analyzed the data. K.H. wrote the manuscript. M.Z. and R.H. helped recruiting patients and collecting tissue samples. A.K., A.M., and R.K. made the illustrations and C.T.F. and J.C. made the photographs. M.H. and M.C. wrote the manuscript. M.Z. and R.H. had helped recruiting patients and collecting tissue samples. K.H. and V.B. have performed gene expression experiments and analyzed the data. K.H. wrote the manuscript. M.Z. and R.H. helped recruiting patients and collecting tissue samples. A.K., A.M., and R.K. made the illustrations and C.T.F. and J.C. made the photographs. M.H. and M.C. wrote the manuscript.

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specimens. S.W. and M.Z. critically reviewed the manuscript. S.V. and D.G. performed immunohistochemistry. A.J. and J.P. took part in the main study design, data analyses and supervision of the study.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

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