Olfactomedin-4 is a glycoprotein secreted into mucus in active IBD☆

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Mucins;
Mucus

Abstract

Background: Olfactomedin-4 (OLFM4) is a glycoprotein characteristic of intestinal stem cells and apparently involved in mucosal defense of the stomach and colon. Here we studied its expression, regulation and function in IBD.

Methods: The expression of OLFM4, mucins Muc1 and Muc2, the goblet cell differentiation factor Hath1 and the proinflammatory cytokine IL-8 was measured in inflamed or noninflamed colon in IBD patients and controls. OLFM4 protein was located by immunohistochemistry, quantified by Dot Blot and its binding capacity to defensins HBD1-3 was investigated. The influence of bacteria with or without the Notch blocker dibenzazepine (DBZ) and of several cytokines on OLFM4 expression was determined in LS174T cells.

Results: OLFM4 mRNA and protein were significantly upregulated in inflamed CD (4.3 and 1.7-fold) and even more pronounced in UC (24.8 and 3.7-fold). OLFM4 expression was correlated to IL-8 but not to Hath1. In controls immunostaining was restricted to the lower crypts but in inflamed IBD it expanded up to the epithelial surface including the mucus. OLFM4 bound to HBD1-3 without profoundly inactivating these defensins. In LS174T-cells OLFM4 mRNA was significantly augmented after incubation with Escherichia coli K12, Escherichia coli Nissle and Bacteroides vulgatus. DBZ downregulated OLFM4 expression and blocked bacterial induction whereas IL-22 but not TNF-α was stimulatory.

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1. Introduction

In both inflammatory bowel diseases (IBD) the chronic inflammation is mediated by an immune response directed against commensal bacteria, possibly triggered by a disproportionate immune response toward these microbes that damages the mucosa. On the other hand, there is increasing evidence for a primary role of a defective mucosal barrier in Crohn's disease (CD) and ulcerative colitis (UC). Bacteria from the lumen massively contaminate the mucus layer which is normally sterile in the bottom stratum. Some bacteria are epithelial adherent or may even invade the subepithelial space and thus trigger an immune response.

The mucosal barrier is a multilayer structure composed of the mucus layer and its origin, the epithelium. In the large intestine the key secretory cells are the goblet cells. They produce various mucins forming the mucus layer which is acting as a physical and chemical barrier against commensals and pathogens. The colonic epithelium also produces antimicrobial peptides which are ultimately secreted into the mucus. The more important colonic peptides, the defensins, are characterized by a broad antimicrobial activity against a variety of gram-positive and gram-negative bacteria preventing luminal microbes to enter the lower mucous layer and attack the epithelium.

This intestinal protective barrier mediated by mucus and defensins is disturbed in IBD. In CD, the expression of the antimicrobial peptides is compromised enabling the bacteria to invade the mucosa and thus trigger inflammation. In case of colonic involvement, CD is linked to a diminished expression of HBD1 independent of inflammation. In UC, the mucus layer is thinner than normal and may even be missing. This is accompanied by a diminished mucin synthesis which is apparently related to a failure in the differentiation of intestinal stem cells toward goblet cells. This differentiation is governed by the key transcription factor Hath1 which is correlated with mucin synthesis.

Olfactomedin-4 (OLFM4) is an olfactomedin domain-containing protein which was found preferentially in the human gastrointestinal tract. The function of OLFM4 in the digestive tract is probably complex. OLFM4 may be an important part of the gastrointestinal mucosal surface and therefore play a role in its defense. For instance, OLFM4 is known to create large polymers stabilized by disulfide bonds similar to mucins. Moreover, it was demonstrated to interact with cell surface cadherin and lectins facilitating cell adhesion. A role of OLFM4 in epithelial defense was concluded from an upregulation in a mouse primary gastric epithelial cell line GSM06 incubated with Helicobacter pylori, as well as in H. pylori infected patients in vivo. Finally, in addition to LGR5 also OLFM4 was found to be a small and large intestinal marker for crypt stem cells in humans.

However, little is known about its relevance in the colon. Shinozaki et al. found OLFM4 transcripts to be significantly upregulated in the epithelium in active vs. inactive UC but its precise function remained unclear. In the present study we attempted to better define the role of OLFM4 in the pathogenesis of IBD and suggest that this peptide acts as an inflammation induced mucus component binding defensins.

2. Material and methods

2.1. Patients

The diagnosis of CD and UC was based on classical clinical, radiological and endoscopic findings, Endoscopic biopsies were immediately snap-frozen in liquid nitrogen. All patients gave their written informed consent and the study was approved by the ethical committee of the University of Tübingen (Germany).

For real-time PCR analysis biopsies from the colonic sigma were obtained in a total of 160 individuals, who underwent routine colonoscopy for various indications, such as colon cancer screening, IBD, diarrhea or obstipation. Thirty-three of these biopsies were classified as healthy controls, were from CD patients (36 noninflamed and 36 inflamed samples) and 55 had the diagnosis of UC (28 noninflamed and 27 inflamed samples). All samples were collected at the Robert Bosch Hospital (Stuttgart, Germany) and the intensity of the flare was clinically evaluated in these patients using the Colitis Activity Index (CAI) for UC and the Crohn's disease activity index (CDAI) for CD.

Immunostaining was performed in formalin-fixed or Carnoy-fixed paraffin-embedded colonic tissue. A total of 18 formalin-fixed colonic resections (6 controls, 6 inflamed CD and 6 inflamed UC samples) and 10 Carnoy-fixed rectal biopsies (5 inflamed CD and 5 inflamed UC samples) were investigated. For Dot Blot analysis, sigma biopsies of 4 controls, 4 inflamed CD and 4 inflamed UC patients, as well as mucin extracts obtained by colonoscopic brushings from 3 controls, 3 inflamed CD and 3 inflamed UC samples were collected. Brushings were performed by gently scrubbing the rectal mucosa with an endoscopic brush, removing the endoscope from the rectum and, outside the patient, the brush was cut with scissors and snap frozen in liquid nitrogen.

2.2. RNA isolation and reverse transcription

The frozen tissues were mechanically disrupted and total RNA was isolated using TRizol reagent (Invitrogen, Karlsruhe, Germany). RNA quality was checked with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). 500 ng of total RNA was reverse transcribed with AMV reverse transcriptase according to the supplier's protocol.
2.3. Protein preparation

Frozen sigmoid biopsies were pulverized with a pestle in liquid nitrogen. Proteins were extracted under gentle agitation for 90 min in 100 μl homogenization buffer (50 mM Tris HCl, 250 mM sucrose, 1 mM EDTA, pH 7.6). Extracts were centrifuged for 20 min (13200 g, 4 °C) and the supernatants were immediately snap-frozen in liquid nitrogen. Mucus extracts were incubated for 2 h in 300 μl 5% acetic acid following a centrifugation for 10 min (7000 g, 4 °C). Then the supernatants were extracted and dried in a vacuum concentrator. Pellets were diluted in 80 μl 0.01% acetic acid and immediately snap-frozen in liquid nitrogen. Protein content in biopsies and mucus extracts was measured using a Bicinchoninic Acid Protein Assay (Smith) and standard curves were produced by serial dilution of the correctly generated plasmids. The mRNA data were normalized to ß-actin mRNA.

2.4. Quantitative real-time reverse transcriptase PCR

For mRNA quantification, real-time PCR was carried out in a SYBR Green fluorescence temperature cycler (LightCycler®, Roche Diagnostics, Mannheim, Germany). Single-stranded cDNA (or gene-specific plasmids as controls) corresponding to 10 ng of RNA conducted as a template with specific oligonucleotide primer pairs (Table 1) as described previously.13 All primers were tested for specific binding to the sequence of interest using BLAST. Plasmids for each product were generated with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the supplier’s protocol. PCR-amplified DNA fragments were confirmed by sequencing. Internal standard curves were produced by serial dilution of the correctly sequenced plasmids. The mRNA data were normalized to ß-actin mRNA.

2.5. Immunohistochemistry

A monoclonal antibody directed against OLFM4 (N212) was produced by W.Y. in the Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Science, Hiroshima, Japan; proof for its specificity has previously been published.35 Immunostaining for OLFM4 was performed using a two-step immunoperoxidase technique (EnVision™, Dako, Glostrup, Denmark) as described previously.36 Antigen retrieval was performed by heating for 30 min in a steamer (pH 9). Then, sections were incubated for 1 h with the primary anti-OLFM4 antibody diluted 1:100 in TBST (20 mM Tris-Base (pH 7.4), 0.14 M NaCl, 0.1% Tween 20). Visualization was performed using a detection kit as outlined by the supplier (Dako, Glostrup, Denmark: horse-radish-peroxidase (HRP)-labeled secondary antibody, detection with 3′-diaminobenzidine tetrahydrochloride). Sections were counterstained with hematoxylin. The grade of inflammation was blindly evaluated in H & E stained sections by an experienced pathologist blinded to the immunohistochemical and molecular biological results.

2.6. Dot Blot analysis

The specificity of the anti-OLFM4 antibody (N212) was tested in Western Blot experiments using human sigma biopsies. We found a clear signal at 57 kDa in inflamed UC samples which is less intense in inflamed CD and uninflamed controls (data not shown). Due to the limited protein amounts obtained by a single biopsy, we decided to switch to the Dot Blot technique which needs less total protein amounts as compared to the Western Blot. Therefore, 10 μg of total protein was transferred to 0.45 mg pore size nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) and blocked with 5% skimmed milk powder in TBST for 1 h. The membranes were washed and incubated for 1 h with the primary anti-OLFM4 antibody (diluted 1:1000 in 5% skimmed milk powder in TBST). Then, the membranes were washed again and treated for 1 h with the secondary HRP-conjugated goat anti-mouse immunoglobulin G antibody (Immuno Research Laboratories, West Grove, PA, USA; diluted 1:5000 in 5% skimmed milk powder in TBST). Protein detection was performed using the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Chalfont St Giles, UK). Signals were visualized with a chemiluminescence camera charge-coupled device LAS-1000 (Fuji, Tokio, Japan). Densitometric analysis was performed with AIDA 2.1 software (Raytest, Straubenhardt, Germany).

After 24 h primary and secondary antibodies were removed from the membranes by incubation for 30 min in Restore™ Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA). Then, Dot Blot analysis was performed for ß-actin on the same membranes. The primary ß-actin antibody (Sigma, Deisenhofen, Germany) and also the secondary HRP-conjugated goat anti-mouse immunoglobulin G antibody (Immuno Research Laboratories, West Grove, PA, USA) were diluted 1:5000 in 5% skimmed milk powder in TBST. Detection of ß-actin was performed as described above. In sigma biopsies, OLFM4 protein content was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide primer pairs used for PCR measurements.</th>
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<tr>
<td>Product</td>
<td>Forward primer (5′- &gt; 3′)</td>
</tr>
<tr>
<td>ß-actin</td>
<td>GCCAAACCGGAGAAGATGA</td>
</tr>
<tr>
<td>IL-8</td>
<td>ATGACTTCCAGCTGGCGGTGCG</td>
</tr>
<tr>
<td>OLFM4</td>
<td>TGCCATTTGCCGAGAATCGTGGCTCT</td>
</tr>
<tr>
<td>Muc1</td>
<td>AGACGTGACGGTGGATAGT</td>
</tr>
<tr>
<td>Muc2</td>
<td>ACCCGCATTGTACCCCTCT</td>
</tr>
<tr>
<td>Hath1</td>
<td>CGAGAGAGCATCCGGGAATGTAGC</td>
</tr>
</tbody>
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normalized to β-actin by dividing the densitometric intensity (LAU) of OLFM4 through the LAU of β-actin in the same biopsy. Notably, OLFM4 and β-actin, were exposed for the same time period (5 min).

2.7. HBD1-3/OLFM4 binding assay

To investigate the possibility that OLFM4 binds to the major colonic defensins, we developed an HBD1-3/OLFM4 binding assay. Therefore, 96 well plates (Nunc, Roskilde, Denmark) were coated for 3 times in triplicates overnight at 4 °C with 5 μg HBD1, HBD2 or HBD3 (Peptanova, Sandhausen, Germany) or 5 μg bovine serum albumin (BSA, Sigma, Deisenhofen, Germany) per well in 100 μl coating buffer (50 mM NaHCO₃/Na₂CO₃, pH 9.6). As a control, 100 μl coating buffer alone was used. The next day the wells were washed with TBST and blocked with 5% skimmed milk powder in TBST for 1 h. After repeated washing with TBST, the wells were incubated overnight at 4 °C with 0, 2 or 6 μg OLFM4 (Sino Biological, Beijing, China) in 100 μl 5% skimmed milk powder in TBST. Then, wells were washed again and incubated with the anti-OLFM4 antibody diluted 1:500 in 5% skimmed milk powder in TBST for 1 h. After repeatedly washing, wells were incubated with a HRP-labeled secondary antibody (Dako, Glostrup, Denmark) for 30 min. Again, wells were washed and thereafter incubated with 200 μl 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma, Deisenhofen, Germany) for 10 min. Photometric visualization was carried out with Wallac Victor™ 1420 Multilable Counter (Waltham, MA, USA) at 405 nm wave length.

2.8. Flow cytometric assay

Antimicrobial activity was measured with a flow cytometric test as described previously. Briefly, suspensions of Escherichia coli ATCC 25922 were grown overnight in Schaedler Broth (BD, Sparks, MD, USA) diluted 1:6 with sterile distilled water at 37 °C. Subsequently 1.5 × 10⁶ mid-logarithmic-phase bacteria/ml in Schaedler Broth 1:6 in a final volume of 100 μl were incubated with 5 μg BHD1, HBD2 or HBD3 (Peptanova, Sandhausen, Germany) and 2 or 6 μg recombinant human OLFM4 (Sino Biological, Beijing, China) at 37 °C. Since OLFM4 is diluted 1 μg/ml in OLFM4-solvent buffer (0.2 μm filtered solution of PBS, pH 7.4, 3.2% glycerol, 8% trehalose, 8% mannitol) by the company, bacteria were also incubated with 5 μg HBD1-3 and 2 or 6 μg OLFM4 solution buffer alone (obtained by the company) for control experiments. Three independent experiments were performed in triplicates. After 90 min, 1 μg/ml of the membrane potential sensitive dye DiBAC₄(3) (bis-(1,3-dibutylbarbituric acid) trimethine oxonol; Invitrogen, Carlsbad, CA, USA) was added. After 10 min of incubation, the samples were centrifuged for 10 min at 4500 g and the bacterial pellets were resuspended in 300 μl phosphate buffered saline (pH 7.4). With a FACSCalibur™ flow cytometer (BD, Sparks, MD, USA) 10000 events of each sample were analyzed for light scattering and green fluorescence. Antimicrobial activity was determined as percentage of fluorescent depolarized bacteria.

2.9. Cell culture experiments

The colon adenocarcinoma cell line LS174T (American Type Culture Collection, Manassas, USA) was cultivated in Dulbecco’s modified Eagle medium (DMEM, Gibco Life Technologies, Eggenstein, Germany) completed with 10% fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 1% non essential amino acids (Gibco Life Technologies, Eggenstein, Germany), 1% penicillin/streptomycin (Gibco Life Technologies, Eggenstein, Germany) and 1% sodium pyruvate (Gibco Life Technologies, Eggenstein, Germany) in a humidified atmosphere at 37 °C and 5% CO₂. For experiments, cells were seeded for at least 3 times in triplicates into 12-well culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) at a density of 0.65 × 10⁶ per well. At about 70% confluence, cells were washed with phosphate-buffered saline (Gibco Life Technologies, Eggenstein, Germany) and incubated in FCS- and antibiotic-free DMEM for 12 h.

Then, cells were incubated with E. coli K12, E. coli Nissle, Bacteroides vulgatus, Symbioflor G1/G2/G3, Lactobacillus fermentum and acidophilus, as well as Bifidobacterium longum, breve and adolescentis for 6 and 24 h. All E. coli strains and B. vulgatus were cultivated under aerobic, Lactobacilli and Bifidobacteria under anaerobic conditions as previously described. Bacteria were killed per heat inactivation in a water bath at 65 °C for 1 h. Then, bacteria were washed with PBS and adjusted to a density of 3 × 10⁸ cells/ml with FCS- and antibiotic-free DMEM. To investigate the possible role of the Notch signaling pathway in regulating OLFM4, cells were incubated for 6 and 24 h with the γ-secretase inhibitor dibenzazepine (DBZ, Axon Medchem, Groningen, Netherlands) in a concentration of 1 μM (in 0.1% DMSO in DMEM) in the absence or presence of E. coli Nissle. LS174T cells were also treated with 10 ng/ml TNF-α (Sigma, Deisenhofen, Germany), 100 ng/ml IL-22 (Sigma, Deisenhofen, Germany), 10 ng/ml IL-4 (Sigma, Deisenhofen, Germany) and 10 ng/ml IL-13 (Sigma, Deisenhofen, Germany) for 6, 12 and 24 h. At the end of experiments cells were washed with PBS and mRNA was isolated using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the supplier’s protocol.

2.10. Statistics

Statistical analyses were performed and all graphs were generated with the GraphPad Prism version 4.0 software using the Mann–Whitney test. In case of the PCR measurements in human sigma biopsies, Bonferroni correction was also done. Spearman’s rank analysis was performed for nonparametric correlation between the different subgroups of the quantitative real-time PCR results. Values of p < 0.05 were considered to be statistically significant. Data are presented in box and whiskers (means).

3. Results

3.1. OLFM4 and mucins are differentially expressed in inflamed IBD

In colonic biopsies transcripts of the proinflammatory cytokine IL-8 (Fig. 1A) were equally enhanced in inflamed CD
(14-fold, \( p < 0.001 \)) and UC (38-fold, \( p < 0.001 \)) compared to the respective noninflamed samples. In controls and noninflamed IBD samples IL-8 expression was comparably low. The grade of histological inflammation was similar in both inflamed IBD entities (inflammation score for controls: 1.0, for CD: 7.2 and for UC: 7.0). The CAI was 2.6 for the noninflamed UC group and 12.1 for the inflamed UC patients. The CDAI was 188 in case of noninflamed colonic CD and 237 in inflamed colonic CD patients.

OLFM4 mRNA (Fig. 1B) was significantly induced in inflamed CD (4.3-fold, \( p < 0.001 \)) and UC (24.8-fold, \( p < 0.001 \)) vs. noninflamed biopsies. This upregulation was clearly significant compared to noninflamed controls and noninflamed IBD samples.
higher in inflamed UC than in inflamed CD samples (5.8-fold, p = 0.04). Again, controls and noninflamed samples were in the same range. Immunostaining for OLFM4 in normal human colonic tissues was confined to the lower third of crypts but expanded during inflammation up to the epithelial surface (Fig. 2). The OLFM4 protein content normalized to β-actin as determined by Dot Blot analysis (Fig. 3) was also clearly augmented in inflamed CD (1.7-fold, p = 0.03) and UC (3.7-fold, p = 0.03) as compared to controls. Again, this induction was numerically more pronounced in inflamed UC as compared to inflamed CD (ns).

Muc1 transcripts (Fig. 1C) were also significantly augmented in inflamed CD (1.9-fold, p < 0.001) and UC (1.8-fold, p = 0.002) samples as compared to controls. In contrast to OLFM4, this induction was less significant in inflamed UC than in inflamed CD biopsies (p = 0.03). Controls and noninflamed samples exhibited a comparable expression. Muc2 mRNA levels did not show significant differences between the 5 subgroups (data not shown). Hath1 expression (Fig. 1D) was significantly lower in inflamed vs. noninflamed UC (0.7-fold, p = 0.02) but not CD. Transcripts of OLFM4 correlated significantly with IL-8 (Spearman r: 0.68, p < 0.001, Fig. 1E) and Muc1 (r: 0.57, p < 0.001) but not with Hath1 (r: −0.17, ns, Fig. 1F).

3.2. OLFM4 is secreted into the mucus and binds to β-defensins HBD1-3

Since OLFM4 is upregulated in inflamed IBD and staining was found up to the epithelial surface, we searched for OLFM4 protein in human mucus following Carnoy-fixation. Indeed, rectal IBD biopsies showed a positive immunostaining for OLFM4 in the crypt lumen (Fig. 4A) as well as in the surface mucus (Fig. 4B), implying the secretion of OLFM4 by epithelial cells into the mucus. This observation was confirmed in rectal mucus extracts, which were obtained by colonoscopic brushings and analyzed with the Dot Blot technique (Fig. 4C): OLFM4 protein was found in the mucus of patients with inflamed CD and even more pronounced in patients with inflamed UC, whereas noninflamed mucus controls were almost negative. As expected, β-actin was only marginally detectable in mucus showing that these extracts were almost free of cell detritus.

The defensins HBD1-3 are positively charged and also secreted into the mucus whereas OLFM4 has a negative charge. Therefore, we tested the ability of recombinant OLFM4 to bind HBD1-3 preabsorbed to plastic wells. In contrast to no binding to BSA, OLFM4 preferentially bound to preabsorbed HBD3 N HBD2 and N HBD1 (Fig. 5). Next, we checked the antimicrobial activity of HBD1-3 in the absence and presence of OLFM4. The coincubation of defensins with OLFM4 at 2 and 6 µg/ml was associated with a limited reduction of the antimicrobial activity in case of HBD1 from 66% to 58% (2 µg/ml, ns) and 47% to 39% (6 µg/ml, ns), in case of HBD2 from 69% to 54% (2 µg/ml, ns) and from 57% to 41% (6 µg/ml, ns) and in case of HBD3 from 71% to 65% (2 µg/ml, ns) and from 55% to 51% (6 µg/ml, ns).

3.3. Bacteria and IL-22 induce OLFM4 expression in LS174T cells

The mucin producing colon adenocarcinoma cell line LS174T was incubated with heat killed E. coli K12, E. coli Nissle, B. vulgatus, Symbioflor G1/G2/G3, L. fermentum and acidophilus, as well as B. longum, breve and adolescentis. An incubation for 24 h with E. coli K12 (2.8-fold induction, p = 0.009), E. coli Nissle (2.5-fold, p = 0.02) and B. vulgatus (1.9-fold, p = 0.02) led to a significant increase of OLFM4 expression in these LS174T cells. In contrast, OLFM4 was unaffected by heat killed Symbioflor G1/G2/G3, L. fermentum and acidophilus, as well as B. longum, breve and adolescentis (data not shown).

In addition, LS174T cells showed a significant time-dependent increase of OLFM4 expression following a treatment with 100 ng/ml IL-22 (3.4-fold after 6 h, p = 0.04; 5.3-fold after 12 h, p = 0.01; 9.1-fold after 24 h, p = 0.01). In
contrast, incubation with TNF-α (0.8–1.4-fold increase, ns), IL-4 (0.7–1.0-fold, ns) and IL-13 (0.7–1.3-fold, ns) did not influence OLFM4 expression.

3.4. OLFM4 is regulated by the Notch pathway

To elucidate the mechanism by which bacteria can influence the level of OLFM4 expression, we investigated the involvement of the Notch pathway in the regulation of OLFM4. Accordingly, LS174T cells were treated with heat inactivated E. coli Nissle in the presence or absence of γ-secretase inhibitor dibenzazepine (DBZ, Fig. 6). In the absence of bacteria DBZ treatment resulted in a significant downregulation of OLFM4 transcripts to 11% after 24 h (p=0.004). The coinubation with E. coli Nissle and DBZ for 24 h nearly completely blocked the 2.4 fold induction by E. coli Nissle (p=0.004).

4. Discussion

OLFM4 is still an enigmatic, ambiguous protein. On the one hand, the protein marks intestinal stem cells, on the other hand it also seems to be important in host defense during gastric and colonic infection and inflammation. In particular, the regulation and function of OLFM4 in the colon are still not completely understood.
The current study shows that OLFM4 transcripts and also protein are significantly induced in colonic IBD during inflammation. Moreover, OLFM4 expression correlates significantly with that of the proinflammatory cytokine IL-8, but not with the goblet cell differentiation factor Hath1. This implies that OLFM4 expression is triggered by inflammation and not by differentiation, in contrast to mucins. The relative induction of OLFM4 is clearly higher in active UC compared to active CD. In principle, this observation is consistent with the observation of Shinozaki and his group, who found OLFM4 mRNA to be elevated in active vs. inactive UC.

In healthy controls, we found OLFM4 staining to be located primarily in the lower third of the colonic crypt, suggesting that in healthy gut this glycoprotein is not a relevant protective factor in the surface epithelium and mucus. However, during active inflammation OLFM4 immunostaining expanded up to the epithelial surface in IBD samples. Shinozaki et al. detected OLFM4 mRNA signals confined to the crypt epithelial cells by in situ hybridization, whereas Clevers and his group found colonic OLFM4 mRNA in humans even restricted to crypt base columnar cells again by in-situ hybridization. It seems possible that in controls the protein expression is retained also in stem cell derived daughter cells colonizing the lower third of the crypt, whereas during inflammation the epithelial cells expressing the protein migrate rapidly up to the surface, where it is secreted. This was evident from the presence of OLFM4 protein in the mucus of Carnoy-fixed IBD biopsies, as well as in rectal IBD mucus obtained by endoscopic brushings. Notably, Dot Blot of these mucus extracts was almost free of β-actin implying that the mucus was not significantly contaminated by cell detritus. The high amino acid sequence similarity between OLFM4 and olfactomedin, the first member of the olfactomedin domain-containing family found to be expressed in the extracellular mucus matrix of olfactory neuroepithelium in bullfrogs, also underlines the fate of OLFM4 as a secretory protein.

Moreover, we measured the expression of OLFM4 in relation to the two crucial mucins Muc1 and Muc2. In contrast to unchanged Muc2, Muc1 was induced in both diseases, although somewhat less significant in UC. This is consistent with prior observations showing a compromised mucin synthesis in UC. Interestingly, this induction pattern contrasts with OLFM4 expression, which showed a higher induction in UC. It is therefore possible that OLFM4 acts as a mucin substitute complementing the mucus layer during inflammation and bacterial attack.

Since OLFM4 is negatively and HBD1-3 positively charged, binding is probably electrostatic. Both defensins and OLFM4 are located in the mucus, therefore it is plausible that they interact and bind to each other also in vivo in order to concentrate the antimicrobial activity in the mucus. Notably, OLFM4 led only to a minor reduction of the antimicrobial activity of HBD1, HBD2 and HBD3. Thus, OLFM4 appears to function as a glycoprotein binding but not profoundly inactivating defensins. However, it should be noted that recombinant OLFM4 differs from native OLFM4 with respect to its glycosylation.

In addition, we observed OLFM4 transcripts to be induced in LS174T cells after incubation with heat killed E. coli K12, E. coli Nissle and B. vulgatus. Although this is the first description in colonic cells, the principle that specific bacteria may enhance OLFM4 expression was previously demonstrated in the mouse primary gastric epithelial cell line GSM06 incubated with H. pylori. Even more pronounced was the induction by IL-22, a susceptibility gene in UC, also compared to the other cytokines tested. The induction of OLFM4 as a mucus glycoprotein is consistent with the recent observation of an IL-22 mediated increase in goblet cell counts and mucin synthesis in experimental animals. This cytokine also increases the innate immunity of several tissues by the induction of antimicrobial peptides such as HBD2 and HBD3. Accordingly, IL-22 was demonstrated to protect mice from colitis, probably by enhancing the mucus/defensin barrier.

Gene expression profiling experiments found OLFM4 to be a target gene of the Notch pathway and thus cell differentiation, proliferation, and immune response to inflammation. We confirmed this observation by cell culture experiments showing that treatment of LS174T cells with the γ-secretase inhibitor DBZ led to a significant downregulation of OLFM4 transcripts. Moreover, the E. coli Nissle mediated induction was also blocked by DBZ, pointing out that the bacterial triggered OLFM4 induction depends on the Notch pathway.

In summary, OLFM4 possesses several “mucin-like” properties (negatively charged polymerizing glycoprotein, secreted into mucus, binding to defensins) and is extensively upregulated in inflamed IBD mucosa where it expands up to the surface epithelium and is secreted into the mucus. The induction may be mediated by bacteria via the Notch pathway and through IL-22. OLFM4 is suggested to have a functional protective role in IBD by binding defensins in the mucus.

Conflict of interest

All Authors have no conflict of interest.
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