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Willemsen, L.E.; Koetsier, M.A.; van Deventer, S.J.H.; van Tol, E.A.

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Background: The mucus layer protects the gastrointestinal mucosa from mechanical, chemical, and microbial challenge. Mucin 2 (MUC-2) is the most prominent mucin secreted by intestinal epithelial cells. There is accumulating evidence that subepithelial myofibroblasts regulate intestinal epithelial cell function and are an important source of prostaglandins (PG). PG enhance mucin secretion and are key players in mucoprotection. The role of bacterial fermentation products in these processes deserves further attention.

Aims: We therefore determined whether the effect of short chain fatty acids (SCFA) on MUC-2 expression involves intermediate PG production.

Methods: Both mono- and cocultures of epithelial cells and myofibroblasts were used to study the effects of SCFA on MUC-2 expression and PG synthesis. Cell culture supernatants were used to determine the role of myofibroblast derived prostaglandins in increasing MUC-2 expression in epithelial cells.

Results: Prostaglandin E1 (PGE1) was found to be far more potent than PGE2 in stimulating MUC-2 expression. SCFA supported a mucoprotective PG profile, reflected by an increased PGE1/PGE2 ratio in myofibroblast supernatants and increased MUC-2 expression in mono- and cocultures. Incubation with indomethacin revealed the latter to be mediated by PG.

Conclusions: SCFA can differentially regulate PG production, thus stimulating MUC-2 expression in intestinal epithelial cells. This mechanism involving functional interaction between myofibroblasts and epithelial cells may play an important role in the mucoprotective effect of bacterial fermentation products.

MATERIAL AND METHODS

Cell culture

Monolayers (MC) of intestinal epithelial T84 (passages 57–64) and LS174T (passages 110–120) (ATCC, Manassas, USA) cells or intestinal myofibroblasts CCD-18Co (passages 10–14) were cultured in 24 or 96 well tissue culture plates (Corning BV, Acton, USA). Parallel with MC, cocultures (CC) were set up by culturing epithelial cells directly on a confluent layer of CCD-18Co cells, as previously described. In brief, CCD-18Co cells were seeded in a twofold dilution in the culture plates and grew confluent within one week. T84 and LS174T cells were seeded in a fivefold dilution on top of the CCD-18Co cells, as previously described. In brief, CCD-18Co cells were seeded in a twofold dilution in the culture plates and grew confluent within one week. T84 and LS174T cells were added in a fivefold dilution on top of the CCD-18Co layer (CC) or in a separate plate (MC). Cells were cultured in DMEM/F12 glutamax I (Invitrogen Life Technologies, Carlsbad, USA) with penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen Life Technologies), and 5% heat inactivated fetal bovine serum (Invitrogen Life Technologies).

Abbreviations: IBD, inflammatory bowel disease; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; CC, coculture; MC, monoculture; PG, prostaglandins; SCFA, short chain fatty acids; MUC-2, mucin 2; BLU, light units.
Medium was refreshed every two days. CCD-18Co monolayers were used when grown confluent while CC and MC were used when the epithelial cell layer had grown subconfluent.

**Stimulation of CC and MC with PG or SCFA**

CCD-18Co monolayers or MC/CC T84 or LS174T were incubated for 24 hours with a concentration range (0.025-4.0 mM) of acetic acid, propionic acid, or butyric acid (VWR International, West Chester, USA). To determine the effects of PG on MUC-2 expression, MC/CC T84 were incubated for 24 hours with 0.01–100 ng/ml PGE_1 or PGE_2 (dissolved in 100% ethanol, diluted to 1 mg/ml stocks in phosphate buffered saline, and stored at -80°C, Sigma-Aldrich BV, St Louis, Missouri, USA). In addition, supernatants of CCD-18Co which had been stimulated for 24 hours with butyrate in the absence or presence of 10^-5 M indomethacin (to block prostaglandin production; Sigma-Aldrich BV) were transferred to MC T84. Supernatants and/or cells were collected and PG concentration or MUC-2 expression was determined.

**Dot blotting MUC-2**

We used a dot blot technique to determine MUC-2 expression in cell cultures as mucins are extremely large glycoproteins (over 500 kDa) which makes them difficult to handle in western blotting techniques. We used the anti-HCM (human colon mucin) antibody raised against purified mucins from mucosal scrapings which recognises peptide epitopes of MUC-2 in colonic goblet cells. Our method was validated using preimmune serum (T84 stained negative), negative control cells (CCD-18Co), and bovine serum albumin. Cell samples were collected in laemmli (protein isolation buffer) and protein determination was performed using the DC protein assay (Biorad, Hercules, California, USA) according to the manufacturer’s protocol with minor modifications. Samples (0.3–0.7–1.0 μg/2 μl) were dotted onto nitrocellulose membranes (Schleicher and Schuell, Riviera Beach, USA). Membranes were blocked in TBST/5% Protivar (Nutricia Roetermeer, the Netherlands) followed by one hour of incubation with anti-MUC-2 antibody (kindly donated by Dr Einerhand, Erasmus University, Rotterdam, the Netherlands). After washing, blots were incubated with goat antirabbit-horseradish peroxidase (Santa Cruz Biotechnology Santa Cruz, USA) and for substrate detection ECL (Roche Diagnostics Indianapolis, Indiana, USA) was used. Densitometry was performed using the Lumi-Imager (Roche Diagnostics) and the signal was expressed in light units (BLU). BLUs were also expressed relative to control incubations (%BLU). SCFA incubations were performed in parallel with MC and CC, and MUC-2 expression was analysed within the same dot blot. To compare the stimulatory effect of SCFA on MUC-2 expression in MC and CC, we deducted basal MUC-2 expression levels.

**Measurement of PGE_1 and PGE_2**

Supernatants from CCD-18Co were analysed for PG production using ELISAs for PGE_1 (R&D Minneapolis, USA; 21% cross reactivity with PGE_2) and PGE_2 (Biotrak Amershams Biosciences corp., Piscataway, USA; 4% cross reactivity with PGE_1). Concentrations measured during SCFA incubations were expressed relative to basal secretion. The PGE_1/PGE_2 ratio was calculated to determine a shift towards a more mucoprotective PG profile.

**Viability measurements**

As SCFA have been reported to be cytotoxic at high doses, we measured the viability of CCD-18Co, MC, and CC using a WST-1 assay (Roche Diagnostics). After 24 hours of incubation, the medium was refreshed and cells were incubated with WST-1. After one hour for MC and CC, and three hours for CCD-18Co, 100 μl sample of the supernatant was measured at A450-A655 in a spectrophotometer (Biorad).

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**Figure 1** Prostaglandin stimulation of mucin 2 (MUC-2) expression in monocultures (MC)/cocultures (CC) of T84. (A) Dot blot analyses showed MC T84 cells to be positive for MUC-2 whereas CCD-18Co were negative. (B) Representative dot blot showing the effects of prostaglandins PGE_1 and PGE_2 on MUC-2 expression in CC T84. (C) Densitometric analysis revealed PGE_1 to enhance MUC-2 expression in both MC and CC T84 (1–100 ng/ml; **p<0.002**) whereas PGE_2 only marginally affected MUC-2 expression in CC T84 (10–100 ng/ml; *p<0.01*). MUC-2 expression is presented relative to controls (%BLU).

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Data analysis
All data are presented as mean (SEM). Data were analysed with the univariate ANOVA using SPSS software version 10.

RESULTS
MUC-2 expression of MC/CC T84 after incubation with PG
Protein titration of T84 or CCD-18Co homogenates revealed T84 to be positive for MUC-2, and as expected CCD-18Co were negative in the dot blot analyses (fig 1A). The differential effects of PGE_1 and PGE_2 on MUC-2 expression by CC T84 are shown in a representative dot blot (fig 1B). Densitometry revealed that MUC-2 expression in both MC and CC T84 was stimulated by PGE_1 rather than PGE_2 (fig 1C). MUC-2 expression in both MC and CC T84 was dose dependently increased after 24 hours of incubation with PGE_1 (1–100 ng/ml; p<0.002) whereas PGE_2 only marginally increased MUC-2 expression in CC T84 cultures at the highest doses (10–100 ng/ml; p<0.01).

Effect of SCFA on PGE_1 versus PGE_2
Supernatants of unstimulated CCD-18Co contained more PGE_2 than PGE_1 (633 (371) v 891 (437) pg/ml; p<0.05, n = 6). Butyrate in particular enhanced PGE_1 and reduced PGE_2 concentrations in all dosage groups (fig 2A; p<0.01, p<0.002). Acetate and propionate incubations followed the same trend. As a result, the PGE_1/PGE_2 ratio increased after SCFA incubation (fig 2B; p<0.01, p<0.002). Culture medium levels for PGE_1 and PGE_2 were low or undetectable.

PG levels, as measured in butyrate containing culture medium spiked with PG, did not differ from spiked medium controls. Moreover, none of the incubations compromised cell viability, as determined by WST analysis (data not shown).

Figure 2  Short chain fatty acids (SCFA) increased the prostaglandin PGE_1/PGE_2 ratio produced by CCD-18Co. (A) Butyrate increased PGE_1 and decreased PGE_2 concentrations (*p<0.01, **p<0.002); acetate and propionate followed the same tendency. Prostaglandin production is presented relative to controls (% control). (B) SCFA increased the PGE_1/PGE_2 ratio (n = 5; *p<0.01, **p<0.002), resulting in a preferred mucoprotective profile.

Figure 3  Increased mucin 2 (MUC-2) expression in cocultures (CC) of T84 compared with monocultures (MC) of T84. Basal MUC-2 expression increased when T84 cells were cocultured with CCD-18Co, the latter however was not apparent using LS174T cells (n = 4, *p<0.05). BLU, light units.
MUC-2 expression of MC and CC T84 or LS174T after SCFA incubation

T84 and LS174T cells were cultured directly on CCD-18Co monolayers and MUC-2 expression was determined. Basal MUC-2 expression was significantly higher in CC T84 compared with MC T84 while MUC-2 expression in CC LS174T was not increased compared with MC (fig 3; p<0.05).

SCFA enhanced MUC-2 expression more in CC compared with MC in both cell lines, except for propionate in MC/CC T84 (p<0.05). Propionate and acetate effectively induced MUC-2 expression in MC and CC of both cell lines (fig 4A, B; p<0.01, p<0.002). Butyrate stimulated MUC-2 expression in MC/CC T84 and CC LS174T but not in MC LS174T (p<0.01, p<0.002). As it is likely that intestinal epithelial cells are exposed to higher levels of SCFA than subepithelial myofibroblasts, we used a broader concentration range in our epithelial mucin expression studies. WST data revealed no toxicity of these SCFA concentrations in both MC and CC (data not shown).

MUC-2 expression of MC T84 after incubation with CCD-18Co supernatant

SCFA stimulated MUC-2 expression to a higher extent in CC compared with MC. Therefore, we tested whether mucin expression in cocultures was regulated by CCD-18Co derived PG by blocking PG production with indomethacin. MUC-2 expression in MC T84 incubated with supernatants of CCD-18Co stimulated with butyrate in the presence or absence of indomethacin are shown in fig 5A. Densitometric analysis showed butyrate to enhance MUC-2 expression in MC T84 (fig 5B; 1 mM, p<0.002). T84 incubation with CCD-18Co supernatants increased MUC-2 expression at lower butyrate

Figure 4  Short chain fatty acids (SCFA) increased mucin 2 (MUC-2) expression in monocultures (MC) and cocultures (CC) of T84 and LS174T. (A, B) SCFA stimulation of MUC-2 expression in the presence of CCD-18Co was found to be more pronounced compared with epithelial monolayers alone (p<0.05). Acetate and propionate dose dependently increased MUC-2 expression in MC/CC T84 (n = 4) and MC/CC LS174T (n = 3) (p<0.01, **p<0.002). Butyrate was effective in MC/CC T84 and CC LS174T (p<0.01, p<0.002) but did not enhance MUC-2 expression in MC LS174T cells. MUC-2 expression was presented after deduction of basal expression levels of either MC or CC. BLU, light units.
by CCD-18Co and T84 derived prostaglandins as indomethacin (indo).

Stimulation of MUC-2 expression by butyrate was found to be mediated by monocultures of T84 incubated with supernatants of CCD-18Co that had been stimulated with butyrate in the presence or absence of indomethacin. (B) Densitometric analysis of four different experiments. Stimulation of MUC-2 expression by butyrate was found to be mediated by CCD-18Co and T84 derived prostaglandins as indomethacin (indo) blocked this effect (0.5–1 mM; ♦ p<0.01). CCD-18Co supernatants (sup) increased MUC-2 expression at lower butyrate concentrations (0.5–1 mM; ♦♦ p<0.002) compared with MC T84 incubated with butyrate (1 mM; ♦♦♦ p<0.002).

**Figure 5** Mucin 2 (MUC-2) stimulation by butyrate is mediated by PG. (A) Representative dot blot of MUC-2 expression in monolayers of T84 incubated with supernatants of CCD-18Co that had been stimulated with butyrate in the presence or absence of indomethacin. (B) Densitometric analysis of four different experiments. Stimulation of MUC-2 expression by butyrate was found to be mediated by CCD-18Co and T84 derived prostaglandins as indomethacin (indo) blocked this effect (0.5–1 mM; ♦ p<0.01). CCD-18Co supernatants (sup) increased MUC-2 expression at lower butyrate concentrations (0.5–1 mM; ♦♦ p<0.002) compared with MC T84 incubated with butyrate (1 mM; ♦♦♦ p<0.002).

Doses, representing the additional effect of CCD-18Co derived soluble mediators (0.5–1 mM; p<0.002). The butyrate effect was found to be mediated by PG derived from both CCD-18Co and T84 as indomethacin completely blocked stimulation of MUC-2 expression (0.5–1 mM; p<0.01). In additional analyses, we confirmed that indomethacin reduced PG concentrations in butyrate stimulated CCD-18Co culture supernatants (PG1, p<0.02; PG2, p<0.02; n = 5). Enhanced MUC-2 expression in MC T84 incubated with butyrate, acetate, or propionate was also abrogated by indomethacin (data not shown).

**DISCUSSION**

Bacterial fermentation products may play an important role in mucoprotection, being an energy source for intestinal epithelial cells and stimulating mucin secretion. Butyrate enemas have been found to reduce clinical symptoms in patients with ulcerative colitis, and high butyrate concentrations in the colon are protective against colon cancer. Also, PG are implicated in sustaining mucosal integrity, as revealed by the observation that PG derived from subepithelial myofibroblasts and epithelial cells have been reported to produce PG that may support mucoprotection by enhancing epithelial MUC-2 expression. Our concept of exposure of subepithelial myofibroblasts to bacterial fermentation products is biologically relevant as following epithelial absorption SCFA are found in the bloodstream.

To study MUC-2 expression, we used colonic carcinoma cell lines T84 (columnar crypt epithelium) and LS174T (goblet cell type). When cocultured with subepithelial myofibroblasts, T84 expressed more MUC-2 compared with MC T84. Cell to cell contact, extracellular matrix, and soluble factors such as transforming growth factor β are known to induce epithelial differentiation which might cause T84 cells to increase basal MUC-2 expression. Basal MUC-2 expression of MC LS174T was not enhanced compared with MC LS174T, possibly because LS174T cells are not capable of further differentiation.

SCFA dose dependently induced MUC-2 expression in MC MC T84 and LS174T. SCFA induce mucin exocytosis in the rat colon through activation of cholinergic nerves. SCFA infusion in these studies was one hour or less. We observed that, independent of cholinergic activation, prolonged incubation with SCFA induced MUC-2 expression in epithelial cells, thus skewing the balance towards mucoprotection. Our finding is also supported by a study in which butyrate was found to increase mucin synthesis in colonic biopsies.

In general, SCFA enhanced MUC-2 expression more potently in MC compared with MC. Monolayer LS174T cells were even unresponsive to butyrate incubations while cocultures were effectively stimulated. These data strongly support a role for subepithelial myofibroblasts in the regulation of MUC-2 production by epithelial cells.

Butyrate most effectively stimulated PG1 production by CCD-18Co cells and when transferring these CCD-18Co supernatants to MC T84, MUC-2 expression was further increased compared with incubation of epithelial cells with butyrate alone. The butyrate effects mediated through CCD-18Co supernatant or directly by T84 (data not shown) were abrogated by indomethacin. This implies that enhanced mucin synthesis by SCFA is mediated by PG derived from both subepithelial myofibroblasts and intestinal epithelial cells. The latter is supported by the fact that mucosal epithelial cells constitutively express COX-1 and indeed epithelial cells have been reported to produce PG.

In conclusion, SCFA increased the PG1/PGE2 ratio produced by subepithelial myofibroblasts and PG1 was found to be superior to PGE2 in enhancing MUC-2 expression in epithelial cells. SCFA stimulated epithelial MUC-2 expression was mediated by PG derived from subepithelial myofibroblasts and epithelial cells, as proved by our indomethacin inhibition experiments. The present study therefore suggests that bacterial fermentation products may have beneficial effects on gut health by supporting mucosal barrier integrity.
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Authors’ affiliations

LE M Willemsen, M A Koetsier, E A F van Tol, Numico Research BV, Wageningen, the Netherlands
SJ H van Deventer, Department of Experimental Internal Medicine, Academic Medical Centre, Amsterdam, the Netherlands

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