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Xylan-regulated delivery of human keratinocyte growth factor-2 to the inflamed colon by the human anaerobic commensal bacterium *Bacteroides ovatus*

Zaed Z R Hamady,1,2 Nigel Scott,3 Mark D Farrar,1 J Peter A Lodge,2 Keith T Holland,1 Terence Whitehead,4 Simon R Carding1,5

**Abstract**

**Background** Human growth factors are potential therapeutic agents for various inflammatory disorders affecting the gastrointestinal tract. However, they are unstable when administered orally and systemic administration requires high doses increasing the risk of unwanted side effects. Live microorganism-based delivery systems can overcome these problems although they suffer from the inability to control heterologous protein production and there are concerns regarding biosafety and environmental contamination.

**Methods** To overcome these limitations we have developed a new live bacteria drug-delivery system using the human commensal gut bacterium *Bacteroides ovatus* engineered to secrete human growth factor-2, which plays a central role in intestinal epithelial homeostasis and repair of gastrointestinal mucosa. 1

**Results** In response to xylan BO-KGF produced biologically active KGF both in vitro and in vivo. In DSS treated mice administration of xylan and BO-KGF had a significant therapeutic effect in reducing weight loss, improving stool consistency, reducing rectal bleeding, accelerating healing of damaged epithelium, reducing inflammation and neutrophil infiltration, reducing expression of pro-inflammatory cytokines, and improving stool consistency, reducing rectal bleeding, accelerating healing of damaged epithelium, reducing inflammation and neutrophil infiltration, reducing expression of pro-inflammatory cytokines, and accelerating production of goblet cells. BO-KGF and xylan treatment also had a marked prophylactic effect limiting the development of inflammation and disruption of the epithelial barrier.

**Conclusion** This novel, diet-regulated, live bacterial drug delivery system may be applicable to treating various bowel disorders.

Human growth factors such as keratinocyte growth factor-2 (KGF-2) are essential for epithelial cell proliferation and preserving the integrity of the intestinal mucosa.1 They are promising future therapies for inflammatory bowel disease (IBD).1,4 In animal models, KGFs reduce the severity and extent of gut mucosal injury5-7 and KGF-1 has been successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of mucositis.8-9 In all of these studies, however, KGF was successfully used in pre-clinical models of 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Experimental colitis

GCAGATCTGGGAGATATCGGCAATG; and XYLm-m, GTCTATAGCTCTTTTATATCTCTTATATCG that are flanked by NdeI and BamHI restriction sites (bold). The promoter was cloned upstream of the kfg-2 sequence and was transferred to B. ovatus V975 by conjugation using a suicide shuttle vector15 to create BO-KGF. BO-KGF viability was determined by exposure to room O2 at 22°C for 5 days removing samples daily and plating on Brain Heart Infusion (BHI)—haemin agar plates supplemented with tetracycline (2 μg/ml) in an anaerobic chamber. Colonies were counted manually at 48 h and corrected for dilution factor to obtain the colony forming units (CFU)/ml.

Animals and induction of colitis
Six- to 8-week-old male C57BL/6 mice (Harlan Laboratories, Bircester, UK) housed in a conventional animal facility were fed standard laboratory chow (xylan content ~3%). Twenty-four hours prior to treatment mice were fed a xylan-reduced (~0.7%) diet (Special Diet Services, Witham Essex, UK). In the treatment protocol, acute colitis was induced by adding 2.5% (w/v) DSS (molecular weight, 35–45 kDa; MP Biomedicals, Irvine, California, USA) to drinking water for 5 days, which was then changed to either water alone or water supplemented with xylan (Sigma-Aldrich, Poole, UK). Bacteria (2×107 in 0.2 ml phosphate-buffered saline (PBS)) were administered by oro-gastric gavage on alternate days from day 5. Groups of mice (n=8 each) received either BO-KGF or wild-type BOV975 with control groups comprising normal healthy animals and animals that received DSS and were treated with PBS or, dexamethasone (5 μg/g s.c. once daily). In the prevention protocol BO-KGF and xylan administration was coincident with DSS treatment and was continued for 5 days. Two groups of mice (n=8 each) received BO-KGF, a third group received BO V975 with a fourth group receiving PBS alone by oro-gastric gavage at day 0 and on alternate days for 5 days. Xylan was added to the drinking water of one group of animals receiving BO-KGF and BOV975. Normal healthy control mice received normal drinking water throughout the experiment. At autopsy, colonic length was measured and samples taken for RNA extraction, myeloperoxidase (MPO) assay16 and histopathology.

Disease activity index
The disease activity index was based upon the sum of the scores assigned to weight loss, stool consistency (diarrhoea) and faecal blood. Diarrhoea was scored as normal, 0; loose stools, 1; or watery diarrhoea, 4. Blood in the stool was scored as none, 0; occult bleeding, 1; slight bleeding, 2; small amounts of blood and pink—red coloration, 3; or gross bleeding, 4. Weight loss was scored as none, 0; 1–5%, 1; 5–10%, 2; 10–15%, 3; or >15%, 4.

Histology
The colon was sampled at 1, 2 and 3 cm from the anal verge. Tissues were fixed in 10% (w/v) formalin, paraffin-embedded, sectioned and stained with haematoxylin & eosin (H&E). Specimens were scored microscopically with the total colitis score being the sum of the five sub-scores; the extent of crypt injury, lesions, acute and chronic inflammatory infiltrate and goblet cell depletion. The cumulative histology score is based upon the averaged scores of individual animals. Two pathologists read all samples independently in a blinded manner. For goblet cell quantification sections of colon were stained with alcin blue and the nuclei counterstained with nuclear fast red. Three fields from each section were counted from three sections per animal, averaging the values to obtain the average number of goblet cells per crypt.

Immunohistochemistry
Sections of colon were sequentially incubated with monoclonal rat anti-Ki67 antibody (DAKO, Glostrup, Denmark), biotin-conjugated polyclonal rabbit anti-rat IgG (Vector, Peterborough, UK), streptavidin hors eradish peroxidase conjugate and 3,3’-diaminobenzidine chromogen (DAKO). Sections were counterstained with haematoxylin. The average number of Ki-67 positive cells per crypt was obtained by counting three fields from each section, averaging the values obtained from three sections per animal.

KGF-2 quantification
For in vitro assays bacteria were cultured in the absence or presence of xylan (0.05% w/v) for 4 or 24 h after which the supernatant was assayed by ELISA developed using commercial capture (R&D Systems, Oxford, UK) and detection (ProSci Inc., Poway, CA, USA) antibodies. For in vivo KGF-2 production, C57BL/6 mice were fed a xylan-reduced diet for 24–48 h prior to administering BOV975 or BO-KGF by oro-gastric gavage. Xylan (50 mg/ml) was added to drinking water and 24 h later the colonic contents were removed, weighed and mixed with 500 μl PBS containing protease inhibitors prior to ELISA. Samples were assayed in triplicate and recombinant KGF-2 (R&D Systems) was used to generate a standard curve.

KGF-2 bioassay
KGF-2 bioactivity was assessed using primary normal human keratinocytes (NHK) (Invitrogen, Paisley, UK) in an epithelial cell proliferation assay based on 5-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, NHK were cultured in 96-well plates containing serial dilutions (0–40 ng/ml) of recombinant KGF-2 (Sigma) or BO-KGF culture supernatants in the presence or absence of 2 μg/ml neutralising anti-KGF-2 antibody (MBL International) for 4 days after which MTT (Sigma-Aldrich) was added. Cells were incubated for further 4 h and the supernatants removed. The crystal formazan product was dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich) and OD570 was measured. The amount of KGF-2 in each dilution was estimated from a linear regression equation of optical density (OD) values against standards.

Detection of BO-KGF in mice faeces
Mice maintained on a xylan-containing diet were administered a single dose of 2×108 CFU BO-KGF. Fresh faeces were collected into sterile containers, weighed, homogenised in BHI medium and centrifuged. Serial dilutions of the supernatants were spread onto BHI–haem plates supplemented with tetracycline (5 μg/ml), rifampicin (55 μg/ml), kanamycin (50 μg/ml) and vancomycin (200 μg/ml) and CFU counted 48 h later.

Real-time PCR
RNA was isolated from colonic tissue using the SV Total RNA Isolation System (Promega, Southampton, UK). First-strand cDNA was synthesised using oligo d(T) primer with M-MuLV reverse transcriptase (New England Biolabs, Hitchin, UK). Real-time quantitative PCR was performed using an iCycler (Bio-Rad, Hemel Hempstead, UK) with SYBER Green Jump Start Taq ready mix (Sigma-Aldrich). A melting curve was obtained to verify the presence of a single amplicon. Primers: tumour necrosis factor α (TNFα) (sense: ACCGGCATGGATCTCACAAGAC, anti-sense: GTCGGTGAGGAGGACATGATG); IL1β (sense: CAGCCAGCACCTACTCTCTAC, anti-sense: TGTCACCTCAGCTCTGAGTC); IL6 (sense: CCGAGAGGAGACTCATCAG, anti-sense: TACCGATTTCCACGAGAC); transforming growth factor β1 (TGFβ1) (sense: GACTCTCACCCTGCAAGAC,
anti-sense: GACTGGCGAGCCTAGTTTG); KGF-2 (sense: ACATTGTGCCTAGGCTTTTC, anti-sense: ACCATGTCCTGACCAAGAC); intestinal trefoil factor (sense: CTCTTCTTGTAGTGAACCG, anti-sense: CTCCTTCCTTCTGAGC); b-actin (sense: CTTCTTCTTGGTATGGAATCC, anti-sense: GTAATCTCCTTCTGAGC). Quantification of mRNA was performed using the standard curve method using b-actin products to normalise cDNA samples and for comparing levels of cytokine gene expression.

Statistical analysis
Data are expressed as mean ± SEM. Statistical significance was determined using one-way analysis of variance or the Kruskal–Wallis test if the data were skewed as determined by the Shapiro–Wilk test. An associated p value of less than 0.05 was considered significant.

RESULTS
Utilisation of the xylanase promoter for the controlled production of human KGF-2 by B. ovatus
The B. ovatus xylanase promoter sequence was cloned upstream of the B. fragilis enterotoxin secretion signal sequence and the coding sequence of the mature human kfg-2 gene into B. ovatus (V975) genomic DNA using the suicide vector pBT2 to obtain BO-KGF. In culture BO-KGF secreted KGF-2 protein (>300 pg/ml) within 4 h of exposure to xylan (0.05% w/v) the levels of which increased to ~700 pg/ml after 24 h (p=0.019) and were >20-fold higher than that produced in the absence of xylan (<30 pg/ml, p<0.001) (figure 1a). BO-KGF produced KGF-2 was biologically active as shown by the dose-dependent induction of proliferation of primary human keratinocytes (NHK). Inhibition of NHK proliferation by an anti-KGF-2 antibody established that the epithelial cell growth-promoting activity in BO-KGF culture supernatants was due to KGF-2.

Figure 1
Utilisation of the xylanase promoter for the controlled production of human KGF-2 by Bacteroides ovatus. (a) Levels of KGF-2 in culture supernatant of B. ovatus wild type BO V975 and recombinant BO-KGF strains cultured in the absence (BO-KGF-X) or presence of 0.05% (w/v) xylan for 4 h (BO-KGF+4X) or 24 h (BO-KGF+24X) were determined by ELISA. The data represents the mean (± SEM) of triplicate values obtained from two experiments. *p<0.05 comparing BO-KGF+24X to BOV975+X and BO-KGF-X groups. (b) Serial dilutions of culture supernatants of BO-KGF grown in the presence of 0.05% (w/v) xylan were added to NHK human keratinocytes in the presence or absence of a neutralising anti-KGF-2 antibody (aKGF2Ab). NHK proliferation was quantified by MTT assay 4 days later by measuring absorbance at 570 nm (OD570). Data points are mean (± SEM) values of triplicate samples from one of two experiments. (c) Amounts of KGF-2 in the colonic washout of C57BL/6 mice were determined by harvesting the colonic contents 24 h after oral administration of 2×10^8 CFU BOV975 or BO-KGF and assaying by ELISA. All mice were maintained on a xylan-reduced diet with normal drinking water (-X) or water supplemented with 30, 50 or 70 mg/ml xylan. Non-treated (Control) mice served as an additional control group. Data points are mean (± SEM) values of triplicate samples from two experiments. (d) Viability of wild-type and recombinant strains of B. ovatus after exposure to environmental O₂ was determined by culturing BO-KGF anaerobically in routine growth media (RGM) with 0.1% (w/v) glucose and 0.05% (w/v) xylan for 24 h and then exposing to the room oxygen. Samples were then removed at regular intervals up to 120 h for plating on BH–haemin plates supplemented with tetracycline in an anaerobic cabinet. Colonies were counted 48 h later. Data shown are mean (± SEM) CFU values from one of three experiments. (e) Viability of BO V975 (dashed line) and BO-KGF (solid line) in C57BL/6 mice faeces was determined by administering 2×10^8 CFU of bacteria by oral gavage on day 0 and collecting fresh stool samples on subsequent days with BO CFU determined as described in the Materials and Methods section.
Experimental colitis

In mice maintained on a xylan-containing (5% xylan) diet that received oral doses of BO-KGF, KGF-2 was detected in the colonic contents at levels (16.4 ng/g) significantly higher than in mice administered BO-KGF and maintained on a xylan-reduced (<0.7% xylan) diet (p=0.01) (figure 1c). The low levels of KGF-2 detected in BO-KGF-treated mice maintained on a xylan-reduced diet probably reflect induction by residual xylan in the diet. However, this was not significantly different from that in control non-treated mice (p=0.87). The highest levels of KGF-2 production were induced with 30 mg/ml of xylan (figure 1c). At higher xylan concentrations KGF-2 levels were reduced, reflecting the suppressive effect of high concentrations of xylan on xylan promoter activation.15

Biological safety and containment of BO-KGF

A significant advantage of using B. ovatus as a drug delivery vehicle is its sensitivity to oxygen, which provides an in-built biosafety feature that is either lacking11 or necessitates additional genetic modification in recombinant strains of L. lactis.19 Upon exposure to environmental oxygen, 50% of BO-KGF survived at 24 h with <5% surviving beyond 96 h exposure (figure 1d). By comparison, the growth of L. lactis (MG1614) was unaffected under these conditions with a 103- to 104-fold increase in CFU seen within 24 h (V Wong, SR Carding, unpublished data, 2009).

Treatment of acute colitis by BO-KGF

BO-KGF was tested in the acute murine DSS colitis model, which is a well characterised, simple and reproducible model of intestinal inflammation that is independent of lymphocyte-mediated responses and in which the clinical severity can be quantified and new therapeutic agents evaluated.20 Although no mouse model mimics all aspects of human IBD, this model is well suited to our purpose since DSS primarily affects epithelial cells and inhibits their proliferation21 enabling the efficacy of epithelial growth factors delivered by B. ovatus to be evaluated. A concentration of 2.5% (w/v) DSS was empirically determined as optimal for inducing acute colitis in 100% of 6-8-week-old male C57BL/6 mice that resulted in no mortality. DSS was added to the drinking water for 5 days after which mice were given normal drinking water or, water supplemented with 30 mg/ml xylan. Since neither parental nor recombinant strains of B. ovatus were able to colonise the mouse colon (figure 1e) bacteria were administered by oro-gastric gavage on alternate days starting at day 5 until the endpoint at day 11. The dose of bacteria was based on a previous study22 and pilot experiments in which 2×108 CFU was found to result in the highest levels of bacteria in vivo 24 h after administration.

Xylan regulated KGF-2 production controlled colitis both clinically and pathologically. Weight loss was significantly less in BO-KGF-treated animals given xylan (11–15%) than in animals given BO-KGF but no xylan (21–23%, p=0.005) or BO V975 and xylan (23–24%, p=0.001) and, in DSS-treated animals that received no bacteria (22%, p=0.006) (figure 2a). Disease activity index scores were also significantly lower in mice treated with BO-KGF and xylan (p=0.005) compared with other treatment.

Figure 2  Therapeutic effect of xylan-induced BO-KGF treatment. DSS was administered to adult mice via drinking water for 5 days after which it was withdrawn and replaced with either normal drinking (X) or water containing 30 mg/ml xylan (+X). At days 5, 7 and 9 post-DSS administration mice were gavaged with 2×108 CFU of wild type Bacteroides ovatus (BO V975) or KGF-producing B. ovatus (BO-KGF). Control groups (n=8 animals) received no bacteria (DSS Alone), steroids (+Steroid) or were normal healthy animals (Normal). (a) Body weight was determined prior to (0) and daily after DSS administration for up to 11 days. *p<0.05 comparing BO-KGF +X with BO-KGF-X, BOV975 +X and DSS Alone groups. (b) Disease activity index (DAI) scores were based on cumulative scores for weight loss, stool consistency and faecal blood presented at day 11. **p<0.01 comparing BO-KGF +X with DSS Alone, BOV975 +X and BO-KGF-X groups. †p=0.031 comparing steroid treatment with DSS Alone. (c) Colonic length was measured from the ileocecal junction to the anal verge at necropsy. Data are expressed as mean (+SEM). **p<0.01 comparing BO-KGF +X group with DSS Alone, BOV975 +X, BO-KGF-X, and p<0.05 to steroid treated group. (d) The amount of TGFβ1 mRNA in the colonic mucosa of groups of mice described in (a) was determined by real-time PCR and by standardising values against β-actin. Data are expressed as mean (+SEM) of RNA samples obtained from six animals in each group. †p=0.001 comparing BO-KGF +X with DSS Alone and BOV975 +X. p<0.01 comparing steroid treatment with DSS Alone and BOV975 +X. TGF, transforming growth factor.
groups. The improvement in the disease activity was better than in steroid-treated animals although it was not statistically significant (figure 2b). Reduction in colon length, which is an independent measure of inflammation,23 correlated well with the improved clinical results in BO-KGF and xylan treated animals. The colons of animals treated with BO-KGF and xylan (7.5 cm ± 0.14) were significantly longer than those in animals treated with BO-KGF alone (6.8 cm ± 0.22), BOV975 and xylan (6.7 cm ± 0.2), or those in the DSS alone group (6.5 cm ± 0.16) (p<0.001) (figure 2c). Furthermore, expression of the pro-fibrogenic cytokine TGFβ1 in the colonic mucosa of colitic mice treated with BO-KGF and xylan was significantly lower than in non-treated mice and in mice treated with BOV975 and xylan (p<0.01) (figure 2d).

Histopathology showed that xylan controlled BO-KGF treatment reduced epithelial damage and inflammatory infiltrate scores in acute colitis by 45% compared to non-treated mice (p=0.001) and by 57% compared to BO-KGF alone (p=0.005). This therapeutic effect of BO-KGF and xylan was as effective as steroid treatment (figure 3a, b). In addition, MPO activity, a marker of neutrophil infiltration and activity, was reduced by more than 65% in BO-KGF and xylan treated animals compared to non-treated colitic animals (p<0.001). It was also reduced by >50% compared to animals treated with BO-KGF in the absence of xylan (p=0.001) and in animals treated with BOV975 and xylan (p<0.001) (figure 3c).

BO-KGF also had a beneficial effect on other biomarkers of disease and inflammation and in particular, expression of the...
Experimental colitis

pro-inflammatory cytokines TNFα, IL1β and IL6 which are mechanistically linked to DSS-induced inflammation.\textsuperscript{20} Compared to non-treated mice expression of all three cytokines were all significantly lower after BO-KGF and xylan treatment ($p<0.001$) (figure 3d). TNFα and IL1β mRNA levels were also lower in BO-KGF and xylan-treated animals compared to animals treated with BO V975 and xylan ($p<0.01$). IL6 expression levels were lower in BO-KGF and xylan-treated animals compared to mice treated with BO-KGF alone ($p<0.001$). These effects were equivalent to that of steroid treatment. Expression of goblet cell-derived intestinal trefoil factor (ITF)-3, a potent inducer of epithelial cell restitution,\textsuperscript{24} was also upregulated by BO-KGF and xylan treatment (figure 3d).

Xylan-controlled KGF-2 secretion by BO-KGF promotes epithelial mitogenesis and mucin production

Xylan-controlled KGF-2 delivery had a positive effect on colonic epithelial cell proliferation and generation of mucin-producing goblet cells. Compared to non-treated animals or animals receiving BO-KGF alone there was a 30–40\% increase in the number of proliferating (Ki-67\textsuperscript{+}) cells per crypt in animals treated with BO-KGF and xylan ($p=0.037$ and 0.005, respectively) (figure 4a, b). This increase in epithelial mitogenic activity was not attributed to increased endogenous KGF production as KGF-2 mRNA expression in the colonic mucosa of BO-KGF treated animals was unchanged and comparable to that of non-treated animals (figure 4c). The number of mucin-containing goblet cells was significantly higher in the colon of mice treated with xylan and BO-KGF compared to either non-treated mice, animals treated with BO-KGF in the absence of xylan or healthy mice ($p<0.001$) (figure 5a, b).

BO-KGF limits the development of acute colitis

Xylan-regulated KGF-2 production by BO-KGF had a prophylactic effect on DSS-induced inflammation. Administering BO-KGF and xylan at the inception of DSS exposure had a significant beneficial effect on weight loss ($p<0.05$) compared to non-treated animals and animals receiving BO-KGF alone or BOV975 and

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Figure 4  BO-KGF and xylan treatment enhances colonic epithelial cell proliferation. (a) Colonic epithelial cell proliferation was assessed by staining sections of colon with anti-Ki-67 antibodies. Colonic tissue was obtained from healthy animals (Normal) and from animals administered DSS alone (DSS Alone), or DSS and treated with xylan and BOKGF (BO-KGF +X) or with BO-KGF alone (BO-KGF-X) as described in the legend of figure 2 ($n=8$ per group). Magnification, $\times 200$. (b) Ki-67\textsuperscript{+} cells were enumerated by counting antibody-reactive cells in the crypts of at least three fields from each section and averaging the values obtained from three sections for each tissue sample. Data are expressed as mean $\pm$ SEM. *$p<0.05$ comparing BO-KFG + X with DSS Alone and BO-KGF-X groups. (c) Endogenous KGF-2 mRNA expression in the colonic mucosa of the groups of mice in (a) was determined by real-time PCR and standardising values against \(\beta\)-actin. Data are expressed as mean ($\pm$SEM) values obtained from RNA samples of five animals in each group.
xylan (Figure 6a). Rectal bleeding was also significantly reduced (p<0.05), stool consistency was improved and colon shortening was reduced (p<0.05) compared to other treatment groups (data not shown). MPO activity in BO-KGF and xylan-treated animals was 70% less than in non-treated animals and in animals treated with BO-KGF alone, and was 60% less than that seen in animals treated with BOV975 and xylan (p<0.01) (Figure 6b).

Consistent with BO-KGF and xylan treatment limiting colitis development inflammatory cell infiltration and crypt damage were significantly reduced compared with non-treated animals (p<0.01), and animals treated with BOV975 and xylan (p<0.05) or, BO-KGF alone (p<0.05) (Figure 6c). Expression of TNFα, IL1β and IL6 were also significantly lower in the colonic mucosa of BO-KGF- and xylan-treated animals compared to non-treated animals and in animals treated with BO-KGF alone (p<0.01) (Figure 6d). The levels of these cytokines were also lower than that in animals treated with BO V975 and xylan although these differences did not reach statistical significance. Collectively, these findings demonstrate that xylan-induced production of KGF-2 by BO-KGF effectively limits the development of DSS-induced colitis.

**DISCUSSION**

Here a recombinant strain of *B. ovatus* producing biologically active KGF under the control of the dietary plant polysaccharide xylan was evaluated for its prophylactic and therapeutic effect in a mouse model of acute colitis. Continuous administration of xylan to BO-KGF-treated mice resulted in a significant improvement of DSS-induced colitis; reducing weight loss, improving stool consistency, reducing rectal bleeding, accelerating healing of damaged colonic epithelium, reducing neutrophil infiltration and expression of pro-inflammatory cytokines as well as promoting production of mucin-rich goblet cells in colonic crypts. BO-KGF and xylan treatment also had a significant prophylactic effect, limiting the development of intestinal inflammation both clinically and histopathologically. To our knowledge, this is the first description of the use of a genetically engineered human commensal anaerobic bacterium for the delivery of therapeutic biologics and of a system by which heterologous protein production is regulated in vivo by a natural dietary factor.

*Bacteroides* are dominant among commensal anaerobes and are found within the mucin layer coating the colonic mucosa making them ideal for therapeutic protein delivery directly to the injured epithelium. While the pathway of uptake and action of BO-KGF-delivered KGF-2 remains to be determined it is likely that the epithelium acquires KGF-2 produced by BO-KGF directly via diffusion, pinocytosis or perhaps by KGF receptors expressed on the basolateral surface of colonic epithelial cells made accessible by the disrupted epithelial barrier in the inflamed colon. The possibility that some of the effect of KGF produced by BO-KGF in the inflamed colon results from its release within the mucosa and lamina propria due to bacterial translocation across...
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damaged or denuded regions of epithelium cannot be excluded. The prebiotic effect of xylan may also contribute to the therapeutic effect of BO-KGF and xylan treatment (synbiotics), explaining its modest beneficial effects in combination with wild-type B ovatus in reducing inflammatory cytokine expression in the inflamed colon. The ability of B ovatus to protect SCID mice from DSS colitis is consistent with this bacterium possessing probiotic properties.

An important consideration in the use of recombinant bacterial expression systems is gene transfer and containment. While this is important for expression systems relying on extrachromosomal plasmids for expression of heterologous genes, it is less of an issue with BO-KGF in which the KGF gene is integrated into the bacterial chromosome using a suicide vector. The anaerobic nature of B ovatus also provides an inbuilt biosafety feature that is either lacking or necessitates additional genetic modification in the recombinant strains of L lactis used in animals and now patients.

The benefit of delivering KGF-2 orally via B ovatus versus systemically via a needle is exemplified by comparing the amounts required to improve histopathology scores in DSS colitis. Daily hypodermal administration of 20–100 μg recombinant KGF-2 for 7 days reduced colonic histopathology score by <50%.6 7 This is less than that achieved by BO-KGF (65%) delivering 3–5 ng of KGF-2 in vivo over a 24 h period. Although we have not compared the outcome of oral delivery of KGF-2 using naked protein versus BO-KGF, based on the susceptibility of naked proteins to extremes of pH and proteases during transit through the gastrointestinal tract we anticipate that BO-KGF will be a more effective means of drug delivery. The ability to localise lower doses of recombinant protein reduces the risk of side effects associated with systemic delivery.

The increase in epithelial proliferation and goblet cell production in BO-KGF and xylan-treated animals can be attributed to the direct action of KGF and specifically to KGF produced by BO-KGF rather than an increase in endogenous KGF production. The increase in intestinal trefoil factor production in these animals suggests KGF acts indirectly by increasing the production of other epithelial growth factors. Since KGF-2 is not known to affect haematopoietic and immune cells, the reduction in expression of pro-inflammatory cytokines mechanistically linked to DSS colitis in BO-KGF- and xylan-treated animals may be explained by improved epithelial barrier function and reduced exposure of underlying cells to enteric antigens. Alterations in chemokine production and expression of pattern recognition receptors by colonic epithelial cells during repair may also contribute to limiting further inflammatory cell infiltration and activation. The reduced expression of TGFβ1 in the colonic mucosa of BO-KGF and xylan-treated animals is noteworthy as levels of this cytokine are used as a biomarker for disease activity in IBD patients and is therefore another indicator of the successful treatment of the disease as described in patients.

In summary, we have utilised a prominent member of the human colonic microbiota for the controlled delivery of an epithelial growth factor for the effective therapeutic and prophylactic treatment of acute colitis. This approach may in the future be of use in treating various gut disorders including colitis as an additive or an alternative to current regimens in patients.

Figure 6  Prophylactic treatment with BO-KGF and xylan limits the development of colitis. At day 0 DSS was added to normal drinking water or to water containing xylan and continued for 5 days. At days 0 and 2, groups of mice (n=8 each) were gavaged with 2×10⁸ CFU BOV975 or BO-KGF. Control groups comprised animals that received no bacteria (DSS Alone) and normal healthy animals (Normal). The experiment was terminated at day 6. (a) Body weight was determined daily between day 0 and day 6.  *p<0.05 comparing BO-KGF+X with other groups. (b) Levels of MPO activity represent averaged values (±SEM) from eight animals. **p<0.01 comparing BO-KGF+X with DSS Alone, BOV975 and BO-KGF-X groups. (c) Pathology scores were based on cumulative scores (figure 2 legend); n=8 animals/group. Data are expressed as mean (±SEM). **p<0.01 comparing BO-KGF+X with the other groups. (d) The amount of TNF-α, IL1β and IL6 mRNA in the colonic mucosa at day 6 was determined by real-time PCR and by standardising values against β-actin. Data are expressed as mean (±SEM) of RNA samples obtained from five animals in each group. *p<0.01, *p<0.05 comparing BO-KGF+X with DSS Alone, BOV975+X and BO-KGF-X groups.
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