Effect of topical butyrate on rectal epithelial kinetics and mucosal enzyme activities

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1. This study aimed to determine the effect of luminal butyrate on proliferative kinetics, a differentiation marker (alkaline phosphatase), and a molecule that controls cell-substratum adhesion (urokinase) in histologically normal human rectal mucosa.
2. Ten subjects with a colonoscopically normal colon (seven had previous adenomas) were given either butyrate or saline enemas for 4 days in a double-blind cross-over manner. Rectal biopsies were taken before and after each course of enemas. Epithelial proliferative kinetics were measured immunohistochemically using antibodies to proliferating cell nuclear antigen. Urokinase and alkaline phosphatase activities were measured spectrophotometrically in biopsy homogenates.
3. Both saline and butyrate enemas were well tolerated and induced no histological change except for a significant increase in crypt length ($P < 0.05$). The number of proliferating cells per crypt also increased significantly after butyrate ($P = 0.018$).
4. Compared with saline enemas, butyrate did not affect kinetic indices nor alkaline phosphatase activities. However, mucosal urokinase activities were significantly lower in butyrate-treated patients ($9.5 \pm 2.0$ i.u./g) than in saline-treated patients ($12.8 \pm 2.0$ i.u./g; $P = 0.045$).
5. Delivering of extra butyrate to the distal colon in healthy subjects may stabilize cell-substratum adhesion in surface epithelium and therefore offer a potential mechanism by which elevating distal colonic luminal butyrate concentrations might be beneficial in patients with colitis or hyperproliferative large bowel epithelium.

INTRODUCTION

Fermentation of carbohydrates in the large bowel lumen yields large amounts of the short-chain fatty acid, butyrate [1]. Butyrate plays essential roles in providing energy for colonic epithelium [2] and in promoting sodium and water absorption [3]. Butyrate has other powerful effects on cell lines in vitro, including inhibition of cell growth, induction of differentiated phenotype and apoptosis, and the stimulation or inhibition of the synthesis of a large variety of proteins [4,5]. Isolated colonic crypt cells also respond to butyrate but the response often does not parallel that found in colon cancer cell lines. For example, butyrate has no effect on the rate of DNA synthesis and does not promote differentiation in isolated normal cells [6] but suppresses it in cells from cancer-bearing colons [7]. Butyrate also inhibits the production of the neutral protease, urokinase (u-PA) [8], its receptor (u-PAR) [9], and interleukin-8 [10]. However, it is uncertain whether the effects of butyrate on normal cells in vivo also occur in the normal colonic epithelium when exposed to butyrate in vivo. Extrapolation from the isolated cell system must be made cautiously since normal epithelial-mesenchymal relationships are disrupted and the trauma of their isolation appears to markedly induce the synthesis and secretion of factors such as u-PA [8], u-PAR [9] and interleukin-8 [10].

In several studies, butyrate has been instilled luminally in rat large bowel which has been starved of carbohydrate fermentation products by unphysiological manoeuvres such as the use of an elemental diet or diversion of the faecal stream away from that segment. These have consistently shown butyrate's trophic effect on colonic epithelium [11,12] although the effects on mucosal hydrolase or u-PA activities have not been reported. Under physiological conditions, however, greater degrees of luminal fermentation do occur and mucosal atrophy is not seen. Modulating luminal butyrate concentrations more than 10-fold by varying the amount and/or type of dietary fibre ingested had, in rats, only small effects, if any, on colonic epithelial proliferative kinetics and hydrolase expression, but significantly altered mucosal u-PA activity; the lowest activities were found in the rats with the highest faecal butyrate concentrations (the rats fed unprocessed wheat bran) [13]. However, these effects do not necessarily specifically reflect differences in butyrate concentration since multiple other luminal characteristics, such as concentrations of other short-chain fatty acids, pH and the spectrum of the bacterial flora, are also affected by the diets.

The effect of direct instillation of butyrate into large bowel lumen on epithelial and mucosal characteristics in humans has only been studied in patients with active ulcerative colitis [14]. Butyrate enemas used twice daily changed the distribution of proliferating cells and favoured reduced proliferative activity. Since inflam-
matory activity was also reduced, it is uncertain whether the effects were secondary to butyrate itself or to butyrate’s effect on reducing inflammation. The aim of the present study then was to use a similar regimen of butyrate enemas in human subjects in whom rectal histology was normal and to measure their effects on rectal epithelial proliferative kinetics, a differentiation marker (alkaline phosphatase), and the activity of u-PA, a molecule that controls cell-substratum adhesion.

**METHODS**

**Subjects studied**

Ten subjects were studied. Their ages ranged from 42 to 70 (mean 56) years and six were female. The subjects had had a colonoscopy within the previous 12 months which had revealed the large bowel free of adenomas or other lesions. Seven of the subjects had previous adenomatous polyps removed, one had previous hyperplastic polyps, and two had diverticular disease alone. They all gave written informed consent before commencement of the study and the protocol was approved by the Board of Medical Research and Ethics Committee of The Royal Melbourne Hospital.

**Protocol**

The subjects were instructed to ingest a diet low in fibre and resistant starch for the duration of the study. Three days after starting the diet, rectal biopsies were taken 5–7 cm from the anal verge without sigmoidoscopy, using standard colonoscopic biopsy forceps. The patients were then randomized to receive an enema of either normal saline or sodium butyrate (for composition, see below) twice daily for 4 days. Neither the patient nor the investigator was aware of the nature of the enema. The enemas were self-administered. At the end of that time a further set of rectal biopsies were taken in the morning approximately 2 h after the last enema. The subjects then crossed over to receive an enema of either normal saline or sodium butyrate (for composition, see below) twice daily for 4 days. Biopsies were taken at a similar time of the day about 2 h after the last enema. The subjects also filled out a diary card stating the times of day the enemas were administered and when the bowels opened as well as any adverse events or other effects experienced during the treatment. Six patients received the saline enema first and four the butyrate enema first.

Four biopsies were taken on each occasion. Two were placed immediately in Methacarn fixative and, after 1 h, transferred to 100% ethanol and stored at −20 °C. The other two biopsies were placed in 1 ml of ice-cold Tris–mannitol buffer (50 mM D-mannitol, 2 mM Trizma base in dH₂O, pH 7.4) and stored at −20 °C. The biopsies were coded in order to blind the investigator to the timing of the biopsy.

**Enema composition**

Butyrate enemas comprised sodium n-butyrat at a concentration of 80 mM at pH 7.0. Osmolality was corrected to 290 mOsmol/l using normal saline and water for irrigation. Saline enemas comprised isotonic sodium chloride at pH 7.0. The total volume of each enema was 60 ml.

**Epithelial proliferative kinetics**

Thin sections of paraffin-mounted, Methacarn-fixed biopsies were cut (2 μm thick) and stained using an immunoperoxidase technique with antibodies to the proliferating cell nuclear antigen (PCNA, Sigma, Castle Hill, NSW, Australia) as recently described and validated by us [15]. Briefly, longitudinally cut crypts were identified and the number of cells in each crypt column counted. The number of crypt columns examined ranged from 28 to 58 (mean = 40). Cells staining strongly positive for PCNA were identified and their position in the crypt column recorded. From these data, the following indices were determined: crypt column height, the number of PCNA-positive cells per crypt, the labelling index calculated by dividing the number of positive cells by the total number of cells in the crypt columns (mean 3480 cells counted), and the distribution of positive cells according to five equal quintiles, quintile 1 being the base of the crypt, and quintile 5 at the surface end of the crypt, expressed as percentage of positive cells in that quintile. All sections were evaluated by one experienced scientist who was blinded to the timing of the biopsy. The performance of the latter in terms of variances of component errors in cell counting has recently been described in detail [15].

**Enzymic activities**

Before assay, mucosal samples in Tris–mannitol buffer were thawed and mechanically homogenized at 4 °C. Triton X-100 was added to a final concentration of 0.1%. Myeloperoxidase activity was measured within 5 min of homogenization spectrophotometrically using guaiacol as substrate [16]. The results were expressed as change in absorbance ‘units’/min per mg of protein of the homogenate. Alkaline phosphatase activities were assayed in aliquots of the homogenate spectrophotometrically using p-nitrophenol as substrate as previously described [17]. u-PA activity was measured in mucosal homogenates by the colorimetric method of Coleman and Green [18]. The assay has previously been shown to be specific for u-PA and the results not affected by the addition of antibody specific for tissue plasminogen activator [19]. Because of the presence of plasminogen in the assay, the activities of both pro-u-PA and u-PA are measured. Enzyme activities were expressed relative to the mucosal protein content which was measured using bovine γ-globulin as standard [20].

**Statistical evaluation**

Results are expressed as means ± S.E.M. unless otherwise stated. Paired data were compared using a two-tailed paired t-test. Because of the study’s cross-
over design, the order in which the enemas were applied was taken into consideration but found to have no influence on the results. The number of bowel actions over the 4-day period during saline or butyrate enema treatment was compared using the Wilcoxon signed rank test. A $P$ value of 0.05 or less was considered statistically significant.

RESULTS

Compliance to and tolerability of butyrate and saline enemas

All subjects were able to retain the enemas for at least 1 h. Compliance, as assessed by daily diary cards, was very good with only one subject failing to use the final butyrate enema due to spillage. Compared with their normal bowel habit, all but one subject experienced reduced frequency of bowel actions after commencement of the low fibre diet. Over the 4-day treatment period, the median (range) number of times the subjects opened their bowels was 7 (1-11) during use of saline enemas, which was significantly more frequent than that during butyrate enema treatment [4 (2-7); $P = 0.0431$. Only one patient reported an adverse event, comprising the sensation of being 'bound up' together with tiredness during butyrate enema therapy only. The number of bowel actions during butyrate enema therapy in this patient was 4 compared with 10 during saline enema therapy.

Effect on proliferative kinetics

Histology of all biopsies was normal and no features of inflammation were seen. As shown in Figure 1A, the use of enemas per se (irrespective of whether they contained saline or butyrate) resulted in significantly longer crypts. Likewise, the number of PCNA-positive cells per crypt tended to be greater after the use of enemas (Figure 1B), but this only reached statistical significance in association with butyrate enemas. However, the labelling index was unaffected (Figure 1C) and the distribution of PCNA-positive cells across the 5 quintiles was similar (Figure 2). When the effect of butyrate was compared with that of saline enemas,
Effect on enzyme activities

Myeloperoxidase activity was very low (<0.8 units/min per mg of protein) in all biopsies which corresponded to the lack of inflammation evident histologically. Alkaline phosphatase activities were similar across the groups (Figure 3). In contrast, u-PA activities differed across the groups (Figure 3). u-PA activities were significantly lower after treatment with butyrate enemas compared with those after treatment with saline enemas ($P = 0.045$). u-PA activities after butyrate treatment also tended to be lower than those before enema therapy but failed to reach statistical significance ($P = 0.074$). Saline enemas had no effect on u-PA activity compared with pretreatment levels ($P = 0.61$) The order in which the enemas were given had no influence upon the results.

**DISCUSSION**

Previous studies of the direct effects of butyrate or a combination of short-chain fatty acids on the colonic mucosa in humans and experimental animals have used one of three models: atrophic colon due to luminal butyrate deficiency after faecal diversion or elemental diet [11,12], hyperproliferating colon due to bile salt injury [21], or inflamed colon [14]. In these models, the control comparator comprises abnormal mucosa and this creates difficulties in interpreting the physiological effects. For example, in butyrate deficiency, the mucosa is atrophic mainly because of the dependence of the epithelium on butyrate as an energy source [2]. It is therefore difficult to distinguish effects of enhanced energy supply per se from the effects directly mediated by butyrate. Similarly, in colitis models, distinguishing the effects of the direct action of butyrate from those indirectly mediated by reduction in mucosal inflammation is not readily achieved. An alternative approach is to identify the effects of butyrate in vivo by delivering more butyrate to the distal colon via the ingestion of slowly fermented fibres such as wheat bran [13]. This dietary change is, however, associated with multiple other effects on luminal contents, such as changes in pH and in the concentrations of other short-chain fatty acids. It introduces, therefore, too many variables to be able to confidently dissect the effects of butyrate alone. The approach applied in the present study — to examine the effect of supplementing butyrate delivery to a histologically normal rectum (albeit mostly in patients with previous adenomas) — has not previously been reported. An attempt was made to reduce luminal butyrate levels by the use of a low residue diet. This did constipate most of the subjects but the rectal mucosa showed no histological evidence of energy deficiency since the crypts were of normal length. Thus the experimental design addressed the issue of whether a modest increase in delivery of butyrate to the normal rectal mucosa induced changes.

Butyrate has been shown to cause a variety of effects on epithelial proliferative kinetics. It suppresses cell proliferation [4] and induces apoptosis [5] in colon cancer cell lines. Treatment of normal colonic mucosa in organ culture with butyrate results in shortening of crypts with an increased relative number of proliferating cells [22]. *In vivo*, luminally instilled butyrate increases epithelial proliferation and crypt length in atrophic mucosa [11] but decreases proliferation in inflamed mucosa [14]. Despite these varied but substantial effects, rectal butyrate supplementation under
the conditions of the present study did not affect epithelial population kinetics. These findings mimic butyrate’s effect on isolated normal crypt cell populations in which no change in the rate of DNA synthesis nor DNA content was observed [6].

The use of an enema per se did, however, induce longer crypts, most probably by increasing proliferative activity to support the expanded population. The underlying mechanisms are unclear but may involve a ‘cleansing action’ of either enema in washing away toxic substances that might contribute to cell death in the surface epithelium. Reduction of such injury may lead to prolongation of the life span of cells and subsequent lengthening of crypts. Alternatively, the effect may represent the result of ‘physical stimulation’ of the colonic epithelium by luminal contents as previously reported in mice [23].

Mucosal alkaline phosphatase activity was unaffected by butyrate supplementation. Alkaline phosphatase has previously been used as a marker of differentiation [6,7] because of its gradient of expression from crypt to surface [24]. However, whether measurement of mucosal alkaline phosphatase activity is a sensitive or even valid marker of differentiation remains uncertain (P. R. Gibson, R. Nov, M. Fielding, A. McIntyre, C. F. Finch, O. Rosella, J. M. Mariadason, D. H. Barkla and G. P. Young, unpublished work). While butyrate potently stimulates cell differentiation in cell lines [4], there is no evidence that butyrate exerts this effect on normal colonic epithelial cells. Normal crypt cells studied in vitro neither change their expression of alkaline phosphatase nor change the rate of glycoprotein synthesis on exposure to butyrate [6]. Furthermore, in colonic mucosa in organ culture, butyrate leads to a loss of mucosal alkaline phosphatase activity, probably due to loss of mature surface epithelial cells by the induction of apoptosis [22].

In contrast to the lack of a butyrate-specific effect on epithelial kinetics or alkaline phosphatase activity, butyrate enemas did significantly reduce mucosal u-PA activity independently of an effect of the enema itself. This effect was similar to that observed in rats ingesting wheat bran where mucosal u-PA activity was significantly reduced compared with that in rats fed diets containing soluble or no fibre [13]. Wheat bran leads to considerable elevation of distal colonic luminal butyrate concentrations [26]. Butyrate-mediated reduction of mucosal u-PA activity also mimics the effect we have previously observed of butyrate on u-PA expression and secretion by isolated colonic crypts [8].

The disparity of effects of butyrate on two different end-points, epithelial proliferation and mucosal u-PA activity, might reflect either or both of two possibilities. Firstly, the mechanisms by which butyrate acts on these might differ. Butyrate-mediated stimulation of epithelial proliferation is best seen in butyrate-starved cells. Thus, stimulation is seen only when mucosa is atrophic due to nutrient depletion. Recent evidence has shown that butyrate inhibits cell proliferation by inhibition of histone deacetylase [27]. In cells not dependent upon butyrate for its energy supply and not usually exposed to butyrate, such as cell lines, this effect is likely to predominate. In normal colonic epithelium, histone deacetylase may be less sensitive to butyrate-mediated suppression due to chronic exposure to butyrate. The mechanism by which butyrate inhibits u-PA synthesis is not known but, like alkaline phosphatase [27], may not be due to inhibition of histone deacetylase. The disparity of findings, therefore, might reflect different mechanisms of action of butyrate, each having different concentration curves.

Secondly, differences in butyrate delivery to the target cells may be responsible. It is likely that luminally instilled butyrate is being effectively delivered to the surface epithelium. Since this is a major site of u-PA production in the mucosa (P. R. Gibson, I. Birchall, O. Rosella, V. Albert, C. F. Finch, D. H. Barkla and G. P. Young, unpublished work), butyrate will have the best chance of exerting its modulatory effects on that enzyme. In contrast, proliferating cells are present in the lower portion of the colonic crypt which, by virtue of the unidirectional flow of mucus towards the lumen, would be exposed to lower concentrations of butyrate. In support of this view is the observation that butyrate does inhibit abnormal proliferation when the proliferative compartment is expanded into the upper crypt, as observed in bile-acid-injured colon [21] and in ulcerative colitis [14].

The biological significance of reduced mucosal u-PA activity is uncertain. In the healthy subjects in the present study, a change in mucosal u-PA activity did not correlate with a change in epithelial kinetic indices. However, reduction of mucosal u-PA activity might be important in colitis or hyperproliferating epithelium induced in rats by the ingestion of diets containing resistant starch or soluble fibre, where mucosal u-PA activity is elevated (P. R. Gibson, I. Birchall, O. Rosella, V. Albert, C. F. Finch, D. H. Barkla and G. P. Young, unpublished work and [29]). Its reduction in the surface epithelium might stabilize cell-substratum adhesion and thereby reduce the rate of epithelial death. This would, in turn, lead to a reduction in the rate of epithelial proliferation. In support of this contention, mucosal u-PA activity in the distal colon of rats directly correlates with proliferative indices (P. R. Gibson, I. Birchall, O. Rosella, V. Albert, C. F. Finch, D. H. Barkla and G. P. Young, unpublished work). In colitis, the effect of butyrate on u-PA activity might be one mechanism by which it reduces inflammatory activity when given by enema [14].

In conclusion, rectal supplementation of butyrate in humans with a histologically normal rectum has no discernible effect on rectal epithelial proliferative kinetics beyond that induced by an enema itself nor on differentiation, but inhibits mucosal u-PA activity. The findings mimic those observed with isolated normal colonic crypt cells. The results demonstrate a potential mechanism — stabilizing cell-substratum adhesion in surface epithelium — by which elevating distal...
colonic luminal butyrate concentrations might be beneficial in patients with colitis or hyperproliferative large bowel epithelium.

REFERENCES

11. Reference deleted.
12. Reference deleted.
13. Reference deleted.