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# Role of Short-chain Fatty Acids in the Prevention of Colorectal Cancer

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**Short-chain fatty acids (SCFAs: acetate, propionate, *n*-butyrate) arising in the large bowel during bacterial fermentation of dietary fibre and starch have paradoxical effects on colonic epithelial proliferation. While the three major SCFAs stimulate proliferation of normal crypt cells, *n*-butyrate and, to a lesser degree, propionate inhibit growth of colon cancer cell lines. At the molecular level, *n*-butyrate causes histone acetylation, favours differentiation, induces apoptosis and regulates the expression of various oncogenes. To understand the complex effects of SCFAs on carcinogenesis, it is important to study the intermediate stages of the adenoma–carcinoma sequence where a "switch" from stimulation to suppression of cell proliferation must occur.**

**Key words: short-chain fatty acids, butyrate, propionate, acetate, fermentation, dietary fibre, resistant starch, colonic cell proliferation, differentiation**
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## INTRODUCTION

CARBOHYDRATES escaping enzymatic digestion in the small intestine are delivered to the colon where they are fermented by the anaerobic microflora. These include non-starch polysaccharides (the major fraction of dietary fibre), resistant starch, and endogenous polysaccharides from mucus and shed epithelial cells. The most important end products of bacterial carbohydrate breakdown are the short-chain fatty acids (SCFAs: acetate, propionate and *n*-butyrate). SCFAs are rapidly and efficiently taken up by the epithelial cells which line the colonic lumen. Butyrate serves as an energy yielding substrate in the colonocytes and, additionally, affects several cellular functions (proliferation, membrane synthesis, sodium absorption). Propionate and acetate are released by the basolateral membrane to the portal circulation and may have effects far from their production site. The physiology of the fermentation process including SCFA production has been reviewed in depth by Macfarlane and Cummings [1].

The attempt to review comprehensively papers relating SCFAs and protection from colonic carcinogenesis would exceed the scope of this paper. Instead, selected papers are presented as *pars pro toto*.

## COLONIC SCFA CONCENTRATIONS AND CARCINOGENESIS

SCFAs are produced in the proximal colon of hindgut fermenters (including man) in an average molar ratio of acetate: propionate:butyrate equivalent to 60:25:10 mmol/l. This ratio, however, is not constant but is determined by the kind of substrate fermented. Assuming that butyrate protects against cancer formation, fermentable substrates should be examined which are primarily broken down to butyrate.

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It has been shown *in vitro* and *in vivo* that the fermentation of starch yields high levels of butyrate. To study the impact of starch malabsorption on faecal SCFAs, 11 healthy volunteers consumed a controlled diet rich in starch for two 4-week periods. They received the glucosidase inhibitor acarbose in one of the study periods and placebo in the other. The faecal concentration (μmol/g wet weight) of *n*-butyrate (+58%) rose significantly when acarbose was added to the diet. The faecal excretion (mmol/day) of total SCFAs (+95%) and of their constituents acetate (+97%) and *n*-butyrate (+182%) was significantly higher when starch malabsorption was induced by acarbose.

Another approach was used by McIntyre and associates [3]. These authors argued that butyrate concentrations show a falling gradient along the large bowel with highly fermented dietary fibre (guar, oat bran), being low in the distal colon, where benign and malignant tumours are most prevalent. They suggested a "lente" fermented fibre (wheat bran) to maintain faecal butyrate concentration at caecal values. In the rat, they induced tumours with dimethylhydrazine, and assessed the impact of different fibre-containing diets on the number and size of tumours. Significantly fewer tumours were seen in the rats fed wheat bran compared with those fed guar or oat bran, and the total tumour mass was lowest in rats fed wheat bran. The concentration of butyrate in stools correlated significantly and negatively with tumour mass. Thus, the type of fibre which is associated with high butyrate concentrations in the distal large bowel is protective against colorectal cancer in this animal model. In this context, it is interesting that a low butyrate to acetate ratio has been found in enema samples from patients with adenomatous polyps and colon cancer [4].

## SCFAs AND PROLIFERATION OF NORMAL COLONOCYTES

Data on the interaction of SCFAs with colonic epithelial proliferation are mainly derived from *in vitro* work employing intact

colonic tissue or permanent cell lines. Effects of SCFAs on the adenoma–carcinoma sequence are summarised in Figure 1. When the impact of SCFAs on colonic carcinogenesis is addressed, a paradoxical effect on normal and neoplastic epithelial cells becomes evident. While butyrate and, to a lesser degree, propionate reduce proliferation of cancer cells *in vitro*, all three major SCFAs stimulate proliferation in normal colonic epithelium.

Scheppach and associates [5] assessed the effect of SCFAs on proliferation of normal caecal mucosa in biopsy specimens obtained from 45 individuals at routine colonoscopy. Tissues were incubated for 4 h with sodium salts of SCFAs at physiological concentrations or equimolar NaCl (control). Cell proliferation was measured autoradiographically by pulse labelling with [$^3$H]thymidine for 1 h. Caecal crypt proliferation was raised significantly in all incubation experiments with SCFAs. Butyrate (10 mmol/l, increase +89%) and propionate (25 mmol/l, increase +70%) were as effective in stimulating proliferation as the combination of the 3 SCFAs (acetate 60 mmol/l + propionate 25 mmol/l + butyrate 10 mmol/l, increase +103%), although the effect of acetate (increase +31%) was minor. SCFAs stimulated DNA synthesis only in the basal 3 of 5 crypt compartments, which is considered the physiological proliferation zone. Other authors have obtained similar results in rat experiments [6, 7].

As demonstrated by Aghdassi and associates [8], the suppression of normal crypt cell proliferation may impair nutritional recovery in rats with small bowel resection. Animals with 80% small bowel resection were fed a liquid diet enterally for 16 days, with or without metronidazole to reduce fermentation. This antibiotic significantly lowered the total amount of SCFAs in the caecum. Resected rats receiving metronidazole had a significantly lower weight gain, carcass protein, nitrogen balance and mucosal dry weight, protein and DNA, compared with resected rats without metronidazole. This study provides evidence that SCFAs are important luminal trophic factors that may favour intestinal adaptation in rats with massive small bowel resection. It is highly unlikely that the stimulation of a physiological pattern of proliferation can be related to the process of carcinogenesis.

## BUTYRATE AND COLONIC "HYPERPROLIFERATION"

"Normal" proliferation of colonocytes occurs in the basal 60% of the crypts. In the upper 40% of the crypts, however, proliferation stops and colonocytes become fully differentiated, to be extruded after approximately 7 days of upward migration. The expansion of the proliferative compartment to the crypt surface (hyperproliferation, involving the upper 40% of the crypt length) is considered a preneoplastic biomarker. SCFAs stimulate proliferation in the basal crypt compartments *in vitro* without increasing cell labelling in the upper crypt [5]. The effect of butyrate on hyperproliferating colonic epithelium differs clearly from the effect on normal epithelium.

Bartram and associates [9] induced hyperproliferation *in vitro* by incubating biopsy specimens from the human ascending colon with deoxycholic acid. This secondary bile acid has been found to be co-carcinogenic in many animal models. Deoxycholic acid significantly raised the upper crypt labelling index, measured by bromodeoxyuridine immunohistochemistry. When the tissue was co-incubated with deoxycholic acid and *n*-butyrate, the increase of DNA synthesis in the upper crypt was no longer observed. Similar data were obtained in biopsies from the human rectosigmoid colon [10]. In conclusion, butyrate antagonised hyperproliferation induced by deoxycholic acid.

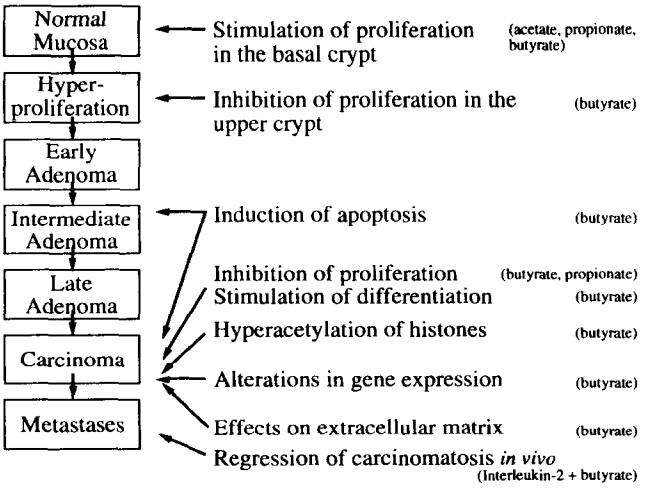


**Figure 1. Effects of short-chain fatty acids (SCFAs) on colonic epithelial cells at different stages of the adenoma–carcinoma sequence.**

## BUTYRATE AND ADENOMA CELL LINES

Limited data are available concerning potential effects of SCFAs on intermediate stages of the adenoma–carcinoma sequence. In view of the contrasting action of SCFAs on normal and neoplastic colonic cells, this information is especially important. To date, it is unknown where the "switch" from stimulation to suppression of proliferation occurs.

In a recent paper by Hague and associates [11], the induction of apoptosis by butyrate (1–4 mmol/l) was studied in two adenoma cell lines (RG/C2 and AA/C1). Butyrate increased the number of apoptotic cells characterised by internucleosomal DNA fragmentation. In contrast, transforming growth factor $\beta_1$, which is thought to have an important role in the control of growth in colonic epithelium, did not induce apoptosis. The adenoma cell line RG/C2 did not contain wild-type *TP53*, therefore this tumour suppressor gene is not required to mediate signals for the induction of apoptosis in colonic tumour cells. Butyrate also induced apoptosis in colon carcinoma cells (HT-29, SW-620), which has been confirmed by Heerdt and colleagues [12]. A disruption of the balance between cell gain through mitosis and cell loss through programmed cell death (apoptosis) is thought to be an important event in carcinogenesis.

## BUTYRATE, PROPIONATE, AND PROLIFERATION OF COLON CANCER CELL LINES

Abundant literature has accumulated on butyrate inhibition of the growth of human colon cancer cell lines. In these isolated tumour cells, butyrate suppresses proliferation at concentrations between 1 and 5 mmol/l, without impairing cell viability; at higher levels cytotoxic effects occur. In contrast with these findings, normal colonic tissue tolerates butyrate concentrations of 10–60 mmol/l. The growth-limiting action of butyrate is observed in lines from many colon carcinomas (SW-620, SW-480, CaCo-2, HT-29, HRT-18, LIM-1215, SK-CO-1) and malignancies from other organs (e.g., breast, pancreas).

In the original paper by Kim and associates [13], human colonic adenocarcinoma cell lines (SW-620, SW-480) were incubated with sodium butyrate (5 mmol/l) for 8 days. Doubling times were increased between 1.18 and 7.6-fold while cell viability was unaffected. The removal of butyrate from the medium resulted in the resumption of rapid growth. Gross morphological alterations including cell enlargement, process formation, and cellular flattening occurred during culture with butyrate.

Gamet and colleagues [14] demonstrated in the HT-29 adenocarcinoma cell line that, similar to butyrate (2–5 mmol/l), propionate (2–10 mmol/l) also inhibited growth. The addition of acetate (2–10 mmol/l) to the medium had no effect on cell proliferation. The antiproliferative action of butyrate and propionate was associated with an inhibition of ornithine decarboxylase, a key enzyme in polyamine synthesis. These data suggest that propionate, together with butyrate, may partially account for the protective effect of some dietary fibres with regard to carcinogenesis.

The results of Kim and associates [13] and Gamet and associates [14] were confirmed in our laboratory (Richter F, unpublished) when HT-29 cells were incubated with SCFAs. Butyrate (2 mmol/l) and, to a lesser extent, propionate (5 mmol/l) inhibited proliferation. Branched-chain fatty acids (iso-butyrate, iso-valerate, 1–2 mmol/l), which arise during the fermentation of proteins or peptides, had no effect on growth of this colon cancer cell line. These data support the hypothesis that protein and carbohydrate fermentation may affect the colonic environment in different ways.

## BUTYRATE AND MARKERS OF DIFFERENTIATION

There is good evidence that butyrate does not inhibit growth of colon cancer cells simply by cytotoxic action. On the contrary, this fatty acid induces markers of differentiation at the same time as it inhibits proliferation. In the presence of butyrate, tumour cells assume a phenotype more like the original non-neoplastic tissue. Other markers of differentiation induced by butyrate include alkaline phosphatase and other hydrolases, carcinoembryonic antigen [15] and membrane-associated glycoproteins and glycolipids [16].

Gum and colleages [17] studied the effect of butyrate on the induction of alkaline phosphatase in the human colonic tumour cell line LS-174-T. Culture of these cells in the presence of butyrate (2 mmol/l) caused the activity to increase from <0.0001 unit/mg of protein to >0.7 unit/mg of protein over an 8-day period. This induction proceeded in a non-linear manner, with a lag time of 2–3 days occurring before enzymatic activities began to rise. Northern blot analysis indicated that treatment of these cells with butyrate caused >20-fold induction of a 2700-base messenger RNA that hybridised to a cDNA probe for placental-like alkaline phosphatase (PLAP). These results indicate that a placental-like protein and messenger RNA are induced by butyrate in LS-174-T cells, with a time course consistent with cellular differentiation preceding induction.

More recently, the same group [18] examined the *PLAP* gene promotor in LS-174-T cells, using transient transfection experiments. Chimaeras from various lengths of the *PLAP* promotor clone were made, and transfected cells were studied in the presence or absence of butyrate (2 mmol/l). The region between nucleotides -363 and -170 was found to represent a strong negative control element within the *PLAP* promotor (total length 512 nucleotides). The effects of this negative control element were significantly reduced in the presence of sodium butyrate. Thus, the region which is functionally involved in *PLAP* gene transcriptional regulation, as judged by promotor deletion experiments, appears to be crucial for the regulation of the *PLAP* gene and its induction by butyrate.

It should be emphasised that the differentiating action of butyrate is observed in tumour cells, but not in non neoplastic colonocytes cultured under identical conditions [19]. This paradox could possibly be solved by investigating intermediate stages of the adenoma–carcinoma sequence.

## EFFECT OF BUTYRATE ON HISTONES

Attempts have been made to investigate the effects of butyrate on proliferation and differentiation of neoplastic cells at the molecular level. Early trials have focused on acetylation, phosphorylation and methylation [20] of DNA–histone complexes in various cell lines.

Whitlock and associates [21] incubated HeLa cells for 16 h in the presence of sodium butyrate (1–10 mmol/l). The extent of histone acetylation was estimated by quantitative densitometry of the H4 region of the stained gel. The amount of $^{32}P$ in H3 was determined by autoradiography and quantitative densitometry. At a concentration of 1–5 mmol/l, butyrate increased the extent of histone acetylation dose-dependently by inhibiting histone deacetylase activity. The changes in H3 phosphorylation closely parallelled changes in histone acetylation, suggesting a relationship between the two modifications.

The consequence of hyperacetylation could be a release of bonds between DNA and histones. This may result in an increased accessibility of DNA, not only to nucleases, but also to various factors involved in the control of gene expression. At present, the importance of these butyrate effects is uncertain.

## EFFECTS OF BUTYRATE ON GENE EXPRESSION

While the action of butyrate on histones is considered non-specific, there is recent evidence that this fatty acid may affect gene expression in a highly specific manner. This subject has recently been reviewed by Kruh and associates [22]. The butyrate effects may be summarised as follows: (a) The synthesis of a limited number of proteins is induced, which includes alkaline phosphatase [17], glycoproteins, hormone receptors and ion-binding metallothioneins. (b) Butyrate suppresses cancer-specific properties in tumour cells, which recover normal molecular characteristics; little is known about the genes that may be involved. (c) Butyrate inhibits proliferation of colon cancer cells, probably by causing an arrest at the early G1 phase. This could, at least partly, result from the effect of butyrate on the expression of genes involved in the control of the cell cycle, including oncogenes. Butyrate has been shown to reduce the expression of *CMYC* (3T3 fibroblasts, CaCo-2 cells), *CSRC* (SW-620 cells), *CMYB* (LIM-1215 cells) and *CRAS* (HT-29 cells), while inducing the expression of *CFOS* (3T3 fibroblasts). Generally, the regulation of these oncogenes in the described manner is associated with a higher degree of differentiation and, inversely, with cell growth [23].

Foss and colleagues [24] assessed the association of butyrate-induced (2 mmol/l) differentiation of human colon carcinoma cells (SW-620), with the expression of src-related tyrosine protein kinases. In butyrate-treated cells, significantly diminished protein kinase activities and the abundance of $pp60^{c\text{-}src}$ and $p56^{lck}$ were found to parallel the butyrate-induced phenotypic alterations. These data indicate that a higher degree of differentiation of SW-620 cells is associated with a down regulation of src-related kinases.

## BUTYRATE AND TUMOUR INVASIVENESS

Another target of butyrate to affect carcinogenesis is the extracellular matrix. One factor controlling tumour invasion may be cell surface bound urokinase, which activates plasminogen to plasmin. There is a wide variety of substrates for plasmin including fibrin (plasmatic fibrinolysis), laminin, fibronectin and proteoglycan (extracellular matrix). Urokinase is secreted by normal [25] and neoplastic [26] colonocytes, and remains at the cell surface bound to receptors. The penetration of malignant cells to the substratum may be facilitated by urokinase.

Gibson and associates [27] measured secreted and cell-associated levels of urokinase and plasminogen activator inhibitor 1 in colonic crypt cells. Butyrate (0.001–4 mmol/l) caused a concentration-dependent inhibition of both secreted and cell-associated urokinase content. Acetate and propionate had minimal effects. Butyrate also stimulated plasminogen activator inhibitor 1 secretion. Levels of transcripts for urokinase and the inhibitor changed with butyrate exposure in parallel with the levels of secretion of the respective proteins. Cells from the cancer group showed significantly reduced inhibitor secretion and abnormal responses to butyrate (greater inhibition of urokinase secretion and no stimulation of inhibitor secretion).

## BUTYRATE IN COMBINATION WITH INTERLEUKIN 2 FOR RAT COLON CANCER PERITONEAL CARCINOMATOSIS

In a remarkable *in vivo* study by Perrin and colleagues [28], peritoneal carcinomatosis was induced in the rat using PROb colon cancer cells. Established carcinomatosis was treated with intraperitoneal injections of interleukin 2, sodium butyrate and a combination of the two. While both monotherapies were ineffective in reducing tumour mass, the combination resulted in a 60% overall survival rate, and included cases of complete cure. Accompanying *in vitro* work revealed that sodium butyrate enhanced the expression of major histocompatibility complex class I molecules. After butyrate treatment, PROb cells became more sensitive to lymphokine-activated killer cells. The authors concluded that butyrate may act by increasing immunogenicity of colon cancer cells.

## CONCLUSION

It is likely that colonic carcinogenesis is an example of how endogenous (genetic) and exogenous (nutritional) factors interact. According to Vogelstein's model [29], genetic alterations in the colonic mucosa accumulate over one or two decades which lead up to the formation of a malignant tumour. There is, however, evidence that nutritional factors determine the speed of progression in the adenoma–carcinoma sequence. Protective factors (SCFAs and others) may outweigh the detrimental effects of accelerating factors (e.g., secondary bile acids). Future research should be focused on the molecular mechanisms whereby nutrition affects carcinogenesis in the human large bowel. This approach could form the basis for prevention stategies.


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