The role of butyrate in human colonic epithelial cells: an energy source or inducer of differentiation and apoptosis?

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Colo-rectal cancer is a major health problem in industrialized countries and the high incidence has been linked with the diet (Burkitt, 1971). The colonic epithelium is in direct contact with dietary factors present in the lumen and these factors may affect the pattern of growth, differentiation and cell death within the tissue. Epidemiological studies suggest that a low-fat, high-fibre diet is protective for colo-rectal cancer (Burkitt, 1971; Howe *et al.* 1992) whereas a diet high in red meat and saturated fat and low in dietary fibre and vegetables is associated with an increased risk for colo-rectal cancer. Colon cancers most often develop from premalignant adenomas (sometimes referred to as polyps) which, in turn, are derived from the normal colonic epithelium. Sandler *et al.* (1993) conducted an epidemiological study which showed that diets high in fat and low in carbohydrates and fibre not only increased the risk for colo-rectal cancer, but also for the precursor adenomas. The diet, therefore, may be particularly important for those individuals predisposed to large-bowel cancer and a better understanding of how dietary factors may influence colon cancer risk may lead to dietary intervention.

Dietary fibre is composed of non-polysaccharides such as lignin and the NSP, carbohydrates such as cellulose, pectin and gums which are derived from plant cell walls. Fibre escapes digestion in the stomach and small intestine, as do oligosaccharides and some resistant starches (Silvester *et al.* 1995) but in the large bowel symbiotic bacteria are able to ferment these carbohydrates, releasing the short-chain fatty acids (butyrate, propionate and acetate) as a by-product of this metabolism.

There are a number of ways in which fibre could protect against colo-rectal cancer. It increases transit rate and bulk, thereby potentially decreasing exposure to carcinogens in the diet. It adsorbs carcinogens, modifies intestinal microflora (and may, therefore, alter bile salt and carcinogen metabolism), decreases faecal bile salt excretion and lowers the colonic pH, as well as increasing the concentrations of the short-chain fatty acids in the large bowel (McIntyre *et al.* 1993). The diet is complex, and in epidemiological studies it is difficult to unravel the effects of individual components. For example, diets high in animal fat may also contain carcinogens generated during cooking of meat. Diets low in fruit and vegetable fibre may be low in vitamins which have antioxidant effects (for review, see Rogers *et al.* 1993). Animal studies have helped in this respect in that diets can be more carefully controlled, but the results are variable because of the types of fibre administered (Levin, 1992). Dietary wheat bran results in higher concentrations of butyrate than either oat bran or guar gum (Kashtan *et al.* 1992; McIntyre *et al.* 1993).

BUTYRATE AS AN ENERGY SOURCE AND INDUCER OF DIFFERENTIATION

The production of short-chain fatty acids by the colonic bacteria is of significance *to the*

colonic epithelium, since these by-products can be metabolized by the colonic epithelium to produce ketones, CO_2 and energy, and there is evidence that butyrate may be the preferred energy source of the colonic epithelium over glucose or glutamine (Roediger, 1982). Indeed, butyrate may account for 70% of the total energy consumption of the colonocyte (Scheppach, 1994). *In vitro* studies have shown that in colo-rectal tumour cell lines butyrate inhibits cell proliferation and induces a more differentiated phenotype (Augeron & Laboisse, 1984; Whitehead *et al.* 1986; Deng *et al.* 1992; Gamet *et al.* 1992). Butyrate is used in the treatment of ulcerative colitis, restoring the normal balance of proliferation and differentiation in the colonic crypt (Scheppach *et al.* 1992). It has been considered as a potential therapy for colo-rectal cancer, and it has been suggested that butyrate could be targeted to carcinoma cells using a tumour-specific monoclonal antibody to deliver a measured dose in a liposome capsule (Otaka *et al.* 1989).

Butyrate has two contrasting roles: as an energy source for normal colonic epithelium and as an inducer of differentiation in malignant cells. Jass (1985) hypothesized that the shift from aerobic to anaerobic metabolism during colo-rectal carcinogenesis may interfere with the oxidation of butyrate. The resultant accumulation of butyrate within the cell cytoplasm may explain why malignant cells are sensitive to the differentiating effects of butyrate. The cells would be less dependent on butyrate as a respiratory fuel. Furthermore, it could be an initial deficiency in levels of butyrate coupled with increased energy requirements of the neoplastic tissue which promote the switch to anaerobic metabolism (Jass, 1985).

APOPTOSIS AND THE MAINTENANCE OF NORMAL CELL NUMBERS IN THE COLON

Since in colo-rectal tumour cells, butyrate induces a differentiated phenotype, we questioned whether it was also capable of inducing apoptosis in these cells as apoptosis may represent the terminal stage of colonic epithelial cell differentiation pathways (discussed on p. 939). Apoptosis is a physiological process by which cells effectively commit suicide. Apoptosis is characterized by morphological changes including loss of cell–cell contact, loss of cell–matrix adhesion, cell shrinkage, loss of microvilli and chromatin condensation (Arends & Wyllie, 1991). The chromatin forms dense crescent shapes lining the nuclear membrane, but the membrane then breaks down and the chromatin mass subsequently fragments. The plasma membrane becomes convoluted and the cell separates into a cluster of membrane-bound 'apoptotic bodies'. *In vivo* these are normally phagocytosed by macrophages or neighbouring cells. In many tissues undergoing rapid turnover of cells apoptosis is involved in the maintenance of tissue homeostasis. In the gut, where the epithelial cells are exposed to dietary carcinogens, cells undergo apoptosis as a means of eliminating damaged cells and, thus, protecting the tissue against neoplastic change. This has been demonstrated in animal models using various mutagenic compounds and also γ-radiation (Potten *et al.* 1992; Clarke *et al.* 1994; Merritt *et al.* 1994). Apoptosis in response to DNA damage may be p53-dependent, occurring rapidly after damage (Clarke *et al.* 1994; Merritt *et al.* 1994), or may be independent of p53 and occur as a later response (Bracey *et al.* 1995). The gastrointestinal tract is exposed to a wide variety of genotoxicants in the diet. These may be natural plant toxins, contaminants, or may be produced during cooking (Chadwick & George, 1992).

In addition, apoptosis may also be in part responsible for the removal of cells at the top of the crypts. The colonic epithelium is a dynamic tissue in a state of continual renewal. Cells proliferate in the lower two-thirds of the normal colonic crypt and cease division as they migrate further up the crypt. The continuous movement of cells up the colonic crypt is tightly linked to differentiation. The fate of the cells as they reach the top of the crypt is a matter of some controversy as the process is poorly understood. Exfoliation could be due to a passive sloughing off of cells, perhaps due to physical forces exerted by cells migrating up from below, or due to an active programmed cell death process. Loss of cell–cell contact is an early stage in the apoptotic process and, therefore, apoptosis may be used as a mechanism by which cells detach from the tissue into the lumen. Since in the colon there is a clear route of exit of apoptotic cells into the lumen, it is possible that such cells are rarely phagocytosed at the top of the crypt. Therefore, the apoptotic cells may not be obvious at the luminal surface.

The first evidence that apoptosis occurs at the top of the crypt came from *in situ* end-labelling studies demonstrating DNA fragmentation towards the luminal surface (Gavrieli *et al.* 1992). However, Bicknell & Cohen (1995) have reported that kilobase DNA fragmentation may also occur during some forms of necrosis. They suggest that morphological analysis for apoptotic cells should confirm the apoptotic nature of the cell death. This evidence has been provided by Hall *et al.* (1994) in a careful study in which they counted morphologically apoptotic cells within colonic crypts and showed that apoptosis occurs chiefly in the upper two-fifths of the crypt. Given that the estimated *in vivo* life of an apoptotic cell is 1 h, the rate of apoptosis could entirely offset the rate of new cell birth in the crypt (Hall *et al.* 1994). A disruption of this balance between cell gain through mitosis and cell loss through programmed cell death may be an important contributory factor in colo-rectal carcinogenesis.

The trigger for apoptosis at the luminal surface may be one of a number of changes in the local environment. In most tissues cell survival depends on a constant supply of survival signals (Raff, 1992) which may be provided by neighbouring cells or extracellular matrix components in addition to the systemic circulation. In the colon there is the possibility that these may also originate in the lumen.

BUTYRATE INDUCES APOPTOSIS IN COLO-RECTAL TUMOUR CELL LINES

Epithelial cell culture models provide a useful system for studying apoptosis, since in culture, cells undergoing apoptosis also detach from the substrate in a manner analogous to the *in vivo* situation, allowing apoptotic cells to be collected from the culture medium and analysed. Such cell culture systems permit the study of the effects of endogenous and exogenous factors on proliferation, differentiation and apoptosis, and on gene expression. They also permit the study of the events leading to cell detachment. In culture, colo-rectal tumour cells undergo apoptosis spontaneously, and the level of apoptosis varies between individual cell lines. By examining the effects of physiologically-obtainable levels of butyrate (2–4 mM) on cell lines derived from colonic adenomas and carcinomas, we showed that butyrate can induce apoptosis (Hague *et al.* 1993). The apoptotic cells are shed into the culture medium, and the floating cells show the classic apoptotic morphology of condensed chromatin and characteristic DNA fragmentation pattern of multiples of 200 base pairs (Wyllie, 1980). In the seven cell lines tested for their response to sodium

butyrate, all exhibited apoptosis after treatment (Hague *et al.* 1995). However, two of the carcinoma cell lines were relatively resistant to butyrate. A subset of carcinomas, therefore, may be resistant to butyrate-induced apoptosis. It will be important to determine the mechanisms by which this resistance is achieved, in addition to investigating how butyrate induces apoptosis in cells. Heerdt *et al.* (1994) demonstrated elevated alkaline phosphatase (*EC* 3.1.3.1) activity in a carcinoma cell line treated with sodium butyrate. Alkaline phosphatase is a commonly used marker of differentiation. Interestingly, the floating apoptotic cells induced by butyrate had increased levels of alkaline phosphatase activity compared with the adherent cell population, an observation suggesting that apoptosis occurred subsequent to differentiation. We have found similar results in floating apoptotic cells from butyrate-treated adenoma and carcinoma cultures. Furthermore, butyrate also increases the expression of E-cadherin protein, another marker of colonic epithelial cell differentiation, in colo-rectal adenoma cells (A. J. Butt and C. Paraskeva, unpublished results). The finding that butyrate induces apoptosis, apparently via terminal differentiation, provides evidence that for colonic epithelial cells apoptosis may form the culmination of a differentiation pathway and this may be analogous to the *in vivo* situation.

We also examined whether propionate and acetate could induce apoptosis in three colo-rectal tumour cell lines, AA/C1, RG/C2 and PC/JW/FI. Both sodium propionate and sodium acetate induced apoptosis, but to a lesser extent than butyrate (Hague *et al.* 1995). Acetate was the least effective, but did induce apoptosis at concentrations of 40 mM and above. This is in accordance with the differential effects of the three short-chain fatty acids in terms of inhibiting proliferation and inducing differentiation (Whitehead *et al.* 1986; Gamet *et al.* 1992). In the colon, the molar ratios of acetate:propionate:butyrate are 57:22:21, and the concentration of acetate in the colonic contents may reach 60 mmol/kg (Cummings *et al.* 1987). These levels are not constant, but vary according to the substrates fermented (Scheppach *et al.* 1995). It may be, therefore, that the concentrations of acetate required to induce apoptosis would be physiological. Of further interest, in our studies (Hague *et al.* 1993, 1995) and that of Heerdt *et al.* (1994), butyrate-induced apoptosis was manifested as floating apoptotic cells, and no increase in apoptosis was detected in the adherent cells. In contrast to these findings, propionate induces apoptosis which is detectable in the adherent cells. The reason for the later detachment of the cells after treatment with propionate is as yet unknown, but the answer may lie in the different metabolism of those short-chain fatty acids with an odd number of C atoms. Propionate may prove to be a useful tool for the investigation of apoptotic pathways since it permits the study of the earlier stages of apoptosis. Up to 50% apoptosis can be obtained in the adherent cells, and many of these are in the early stages of apoptosis where the nuclear membrane remains intact and the chromatin forms dense crescent shapes lining it.

APOPTOSIS, p53 AND Bcl-2

It is of note that the three short-chain fatty acids, butyrate, propionate and acetate, induce apoptosis in cell lines which have no p53 protein, or which have mutant p53 only (Hague *et al.* 1993, 1995). p53, therefore, is not required for the induction of apoptosis by the short-chain fatty acids. This is further evidence that p53-dependent apoptosis in response to DNA damage occurs by a separate pathway to that occurring via terminal differentiation. To further our understanding of the mechanisms by which butyrate induces apoptosis, we are investigating its effects on the expression of the Bcl-2 family of proteins.

Bcl-2 was discovered as a result of the 14;18 translocation in follicular lymphoma (Tsujimoto *et al.* 1985). In this case overexpression of Bcl-2, due to its abnormal control by the immunoglobulin heavy-chain enhancer, produces B cells with an extended survival capacity. Bcl-2 is now known to be protective against apoptosis in many diverse tissue types. In the colon, Bcl-2 is largely confined to the base of the crypts (Hockenbery *et al.* 1991; Hague *et al.* 1994). In contrast, Bax, the death promoter, is expressed largely in the upper half of the crypt, along with death protectors characteristic of fully-differentiated cell types, Bcl-x and Mcl-1 (Krajewski *et al.* 1994*a,b*, 1995). The gradient of distribution of proteins of the Bcl-2 family up the colonic crypt is indirect evidence that programmed cell death is an integral mechanism by which the crypt length is kept constant. We are using our *in vitro* model system to determine the effects of butyrate and other differentiation agents on the expression of the Bcl-2 family of proteins. We have found decreased levels of Bcl-2 protein in cells exposed to butyrate under conditions where apoptosis is induced; however, there is no change in the level of the Bax protein (A. Hague and C. Paraskeva, unpublished results). This decrease in Bcl-2:Bax may be involved in promoting apoptosis in response to sodium butyrate.

Having shown that butyrate is capable of inducing apoptosis *in vitro* in both premalignant and malignant tumour cells of colonic origin, we need to develop a greater understanding of the effects of butyrate on normal colonic epithelial cells. For example, although butyrate is considered to be an energy source for normal colonic epithelium, we do not know whether differentiation and apoptosis are the end-point of butyrate metabolism in normal colonic epithelial cells. Of importance to the consideration of butyrate in a therapeutic regimen is the question as to whether there is a differential response, i.e. are tumour cells more sensitive to butyrate-induced apoptosis than normal cells? Are normal cells more capable of metabolizing butyrate to produce energy? If tumour cells are more sensitive to butyrate-induced apoptosis than normal cells, this may have important implications for dietary intervention or therapy involving butyrate. It should also be considered, however, that although butyrate can induce apoptosis, there may be conditions in which cells lose their responsiveness to butyrate (Berry & Paraskeva, 1988; Williams *et al.* 1990). This is consistent with the observation that two of the carcinoma cell lines we studied were resistant to butyrate-induced apoptosis (Hague *et al.* 1995). In some circumstances butyrate could, therefore, act as a tumour promoter by permitting clonal expansion of more malignant cell types. Whether an agent acts as an anti-tumour agent or a tumour promoter may depend on the genetic background of the cells and whether there are also specific mutagens in the diet (Paraskeva, 1992).

In summary, our finding that butyrate induces apoptosis in colo-rectal tumour cell lines could, in part, explain the protective nature of short-chain fatty acids. However, further work is required to determine the role of butyrate in the normal colon and whether under certain conditions high levels of butyrate can do more harm than good.

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