

Nutrient regulation of intestinal proliferation and apoptosis

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The intestinal mucosa is an important interface between the animal and its environment. Through this interface the animal acquires water, nutrients and other bioactive compounds, whilst the mucosa provides a barrier against toxins and infective agents. The intestinal epithelium is replaced every 2–6 d (depending on species and intestinal site) and its integrity depends on the production of new cells at a rate equal to that at which cells are lost from the villus tip by extrusion (Madara, 1990) or by programmed cell death (apoptosis; Hall *et al.* 1994). Each villus is clothed by a single layer of epithelial cells which migrate up from six to ten surrounding crypts. Within the crypts, stem cells located at or near the bottom constitute a self-maintaining population which, by asymmetric division, give rise to a new stem cell and to another daughter or 'transit' cell which, in turn, is capable of a limited number (perhaps four to five) of further divisions (Potten & Loeffler, 1990). The stem (and transit) cells are pluripotent, giving rise to all four lineages, including Paneth cells, goblet cells, entero-endocrine cells and columnar absorptive cells (enterocytes). Growth of the intestine during early postnatal ontogenesis requires an increase in the number of crypts, which occurs by crypt fission (Cheng & Bjerknes, 1985). Crypt fission rate post-weaning, which is normally at a relatively low level, can be increased by irradiation (Cairnie & Millen, 1975), small-bowel resection (Totafurno *et al.* 1987) and treatment with mutagens (Park *et al.* 1995), but little is known about the effects of nutritional manipulation.

Regulation of cell proliferation and apoptosis in the intestine

Role of tumour suppressor gene APC in cell proliferation and apoptosis

The accessibility and distinctive tissue architecture of the gut mucosa and the accumulating knowledge of gene defects associated with colo-rectal tumourigenesis (Reale & Fearon, 1996) have helped elucidate the roles of key genes regulating cell number in this epithelium. The tumour suppressor gene *APC* is normally expressed by

non-dividing cells in the upper part of the crypt and on the villus. This, together with the upwards shift in distribution of dividing cells within crypts from individuals with familial adenomatous polyposis in whom one *APC* allele is mutated (Mills *et al.* 1995), led to the suggestion that the *APC* gene product suppressed cell proliferation. Direct evidence was published by Baeg *et al.* (1995), who showed that over-expression of *APC* blocked cell cycle progression from G_0 or G_1 to S phase and was associated with reduced cyclin-dependent protein kinase 2 activity. The *APC* protein is large (2843 amino acid residues), with several distinct domains, is located in the cell cytoplasm and probably functions normally as an homo-oligomer. Whilst its cellular role(s) is not fully understood, it seems likely that *APC* acts as a switch or gate-keeper, receiving extracellular signals via E-cadherin and β -catenin and modulating these before onward transmission via the actin cytoskeleton. Transfection of full length *APC* into HT-29 colo-rectal cancer cells (in which both *APC* alleles are mutated) resulted in considerable diminution of cell growth and several-fold increase in apoptosis (assayed by Hoechst staining of floating cells; Morin *et al.* 1996). How *APC* switches on apoptosis is not known, but it is now apparent that *APC* plays a key role in regulating both proliferation and apoptosis, thereby regulating cell number in the intestine. Recent reports of the absence of any difference in cell proliferation in the macroscopically-normal intestinal mucosa of mice engineered to have a mutation in one of the *Apc* alleles compared with the wild-type controls (Wasan *et al.* 1997; Mathers *et al.* 1998) suggests that as little as 50% of the normal cell complement of the *Apc* protein may be sufficient to ensure normal regulation of proliferation.

Apoptosis: roles of the bcl-2 family genes

Kerr *et al.* (1972) coined the term 'apoptosis' to describe the 'active, inherently programmed' form of cell death which acts as the counter-weight to mitosis in the regulation of cell number in tissue homeostasis. It is activated during embryonic development, allowing the focal removal of unwanted cells, and during therapeutically-induced tumour regression. Apoptotic cells undergo a programme of

Abbreviations: SCFA, short-chain fatty acids.

morphological changes, including (often) separation from adjacent cells, cell shrinkage, chromatin condensation, nuclear fragmentation and the production of discrete membrane-bound apoptotic bodies which are phagocytosed by neighbouring cells without inducing an inflammatory response (Que & Gores, 1996). Internucleosomal DNA cleavage occurs relatively late in the apoptotic pathway to produce mono- and oligomers of about 200 base pairs which yield characteristic ladders on agarose gel electrophoresis.

The proto-oncogene *bcl-2* encodes a 26 kDa membrane-associated protein which protects cells against apoptosis. The *bcl-2* homologues *bcl-X_L* and *MCL-1* are also cell death suppressors, whilst other members of this family, including *Bax*, *Bcl-X_s*, *Bak* and *Bad*, are cell death promoters. Competing dimerization by the protein products of this gene family is probably responsible for determining whether apoptosis is switched on or off (Lu *et al.* 1996). Homodimers of *bcl-2* protect cells against apoptosis, while heterodimerization of *bcl-2* and *Bax* switches on programmed cell death (Fig. 1). Kamada *et al.* (1995) provided strong evidence for the role of *bcl-2* in maintaining intestinal mucosal integrity by generating mice lacking the *bcl-2* gene using homologous recombination in embryonal stem cells. Homozygous *bcl-2*-deficient mice had short, bizarre-shaped intestinal villi with few mitotic cells and clusters of apoptotic cells.

Hall *et al.* (1994) reported observing apoptotic cells in the stomach, small-intestinal and colonic epithelium of rodents and human subjects. The numbers of apoptotic cells observed were small, but Hall *et al.* (1994) argued that this was as expected because apoptosis is a rapid process (apoptotic bodies may be cleared in 1–2 h). Their calculations suggested that apoptosis might account for the bulk of cell loss in the intestine and, thus, is a central feature of the regulation of cell number in adult tissues. This is a novel suggestion since it has been accepted wisdom that most cell loss from the intestine occurs by cell shedding or exfoliation from the villus tip (small intestine) or luminal surface (colon). In support of the conventional view, Madara (1990) developed an elegant model to explain how cellular extrusion could occur without loss of mucosal integrity. Resolution of this controversy would be assisted

by the development of more robust methods of quantifying apoptosis, especially if this included a measurement of rate as distinct from state (the analogy is with the stathmokinetic approach to the measurement of crypt cell proliferation rate; Goodlad & Wright, 1982).

Dietary modulation of cell proliferation and apoptosis

Intestinal cell proliferation responds rapidly to changes in the amount and nature of the diet. Starvation of rats for 72 h reduced the number of mitotic cells per colonic crypt from 6.8 (SD 0.2) to 5.3 (SD 0.3), whilst after 24 h refeeding this had increased to 8.0 (SD 0.3) mitotic cells per crypt (Konishi *et al.* 1996). Massive small-bowel resection leads to hypertrophy of both the muscle layers and mucosa of the residual intestine. Not surprisingly, this is associated with a large, sustained (up to at least 7 d) increase in crypt cell proliferation, and Helmrath *et al.* (1997) have reported recently that apoptotic index (no. of apoptotic cells per crypt secretion) was increased within 12 h of surgery with further increases up to day 3. This high rate of apoptosis (about seven times that in the sham-operated controls) was maintained to day 7 post-surgery, presumably as a means of removing excess cells in the establishment of a new steady state. Excessive rates of epithelial apoptosis resulting from exposure to gluten in genetically-susceptible individuals appear to contribute to the villous atrophy, which results in a characteristically flat small-bowel mucosa despite a fourfold increase in crypt cell proliferation in active coeliac disease (Moss *et al.* 1996). Consumption of gluten-free diets returned small-intestinal apoptotic scores (no. of apoptotic cells per 100 total epithelial cells) to normal (Moss *et al.* 1996). Lewin & Weinstein (1996) have suggested that apoptosis may be de-regulated in other intestinal disorders characterized by 'flat' lesions, e.g. those induced by chemotherapy or folate or vitamin B₁₂ deficiencies. Apoptosis may also be important in the normal maturation of the intestine after birth. Culture of human fetal small intestine with the exogenous nucleotide AMP resulted in suppression of cell proliferation, induction of differentiation and the appearance of apoptotic cells on the villi (Tanaka *et al.* 1996). This was accompanied by an increase in *Bax* mRNA but no change in *bcl-2*.

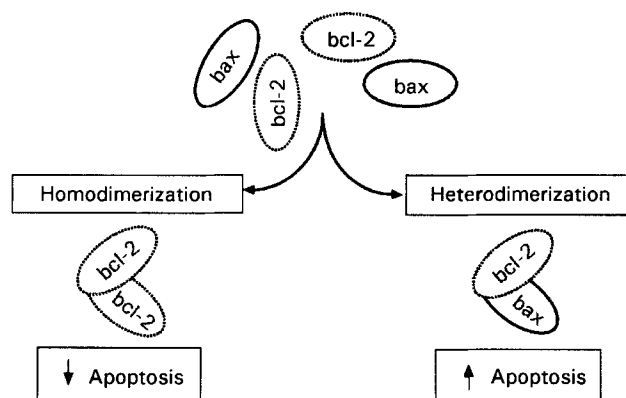


Fig. 1. Competing dimerization of *bcl-2* family proteins determines whether apoptosis is switched off or switched on.

Effects of NSP on intestinal cell proliferation

It is well established that plant structural carbohydrates play an important role in the development of the rumen mucosa during weaning (Tamate *et al.* 1962). More recently, evidence has accumulated that certain dietary NSP increase cell proliferation in rodents (Jacobs & Lupton, 1984; Goodlad *et al.* 1987; Mathers *et al.* 1993; Gee *et al.* 1996; Key *et al.* 1996). The reason for this hyperproliferation is not clear, and some possible factors are summarized in Fig. 2. Soluble NSP sources which increase gut lumen viscosity, e.g. guar gum, may result in delayed digestion and absorption, the transfer of nutrients to the distal intestine (as indicated by up-regulation of brush-border hydrolases in the terminal ileum; Mathers *et al.* 1992) and increased crypt cell proliferation (Pell *et al.*

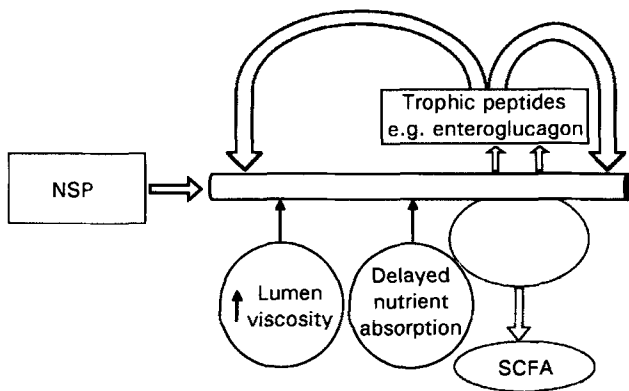


Fig. 2. Conceptual model of potential factors influencing crypt cell proliferation when NSP are consumed. SCFA, short-chain fatty acids.

1992). This may be mediated by the increased release of trophic peptides, e.g. enteroglucagon from the L-cells of the distal small bowel and colon (Goodlad *et al.* 1987). In addition, NSP are fermented in the caecum and colon, producing short-chain fatty acids (SCFA) which have been shown to be trophic for both the rumen epithelium (Sakata & Tamate, 1978) and the rodent intestinal mucosa (Sakata, 1987).

We tested the hypothesis that lumen viscosity is an important factor modulating crypt cell proliferation by feeding rats semi-purified diets containing (as the only NSP source) cellulose (known to have no effect on lumen viscosity or cell proliferation) or guar-gum preparations of contrasting molecular mass and, therefore, viscosity (Lynn *et al.* 1994). With all guar-gum preparations, intestinal epithelial cell proliferation was increased throughout the small and large bowels, with greater increases for the more viscous products. Gee *et al.* (1996) showed that hydroxypropylmethylcellulose, which increases lumen viscosity but is not fermented, had no effect on crypt cell proliferation in the distal ileum. Similarly, there was no effect with the non-viscous disaccharide lactitol, which is not digested in the small bowel, but is rapidly fermented in the large bowel and which stimulated enteroglucagon release. Guar gum, which is also readily fermented, was the most potent stimulator of enteroglucagon release and the only one of the test carbohydrates to increase cell proliferation. This suggests that cell proliferation may be modulated by an interaction between lumen viscosity and SCFA production and that enteroglucagon alone is insufficient to cause a trophic effect (Gee *et al.* 1996).

Short-chain fatty acids, cell proliferation and apoptosis

Direct infusion of SCFA into the distal intestine of rats fed on elemental diets stimulated large-bowel epithelial proliferation rate (Sakata, 1987), whilst isolation of the large bowel from its normal ileal supply of substrate and, therefore, prevention of fermentation and SCFA production, suppressed proliferation (Sakata, 1988). Supplementation of parenteral nutrition with SCFA caused hypertrophy of the residual intestine following massive small-bowel resection (Koruda *et al.* 1988). This evidence supports the

suggestion that SCFA are lumen trophic factors for the rodent intestine (Sakata, 1987) as they are for the rumen (Sakata & Tamate, 1978).

Since most of the studies in this area have been carried out using models in which the basal level of SCFA production is suppressed, it could be argued that the additional SCFA merely return proliferative rates to normal, i.e. that this is an homeostatic response to a normal gut lumen metabolite (Fig. 3). We examined this possibility by feeding rats on diets based on white or wholemeal breads (similar to human 'Western' diets) which would be expected to provide normal background levels of SCFA production. These diets were supplemented with graded amounts of cooked haricot beans (*Phaseolus vulgaris*) which are good sources of fermentable carbohydrates (NSP, resistant starch and oligosaccharides) and resulted in several-fold increases in SCFA production, with the greatest increase for butyrate (Key *et al.* 1996). Despite the very large increases in SCFA, there was no evidence that this had any effect on duodenal cell proliferation. When the basal diet contained wholemeal bread there was no effect of enhanced SCFA supply on cell proliferation in the caecum or colon, but with the white bread-based diets, addition of haricot beans increased caecal cell proliferation. We concluded that evidence that SCFA are responsible for enhanced cell proliferation above normal levels is not convincing (Key *et al.* 1996). In those instances where enhanced SCFA supply is accompanied by increased cell proliferation, the increase may be:

- from a hypoproliferative state towards normal (Fig. 3);
- a transient phenomenon accompanying tissue hypertrophy; or
- an homeostatic response to increased cell loss by exfoliation or apoptosis.

Whilst SCFA (and especially butyrate) may be trophic for the intestinal mucosa in certain circumstances *in vivo*, butyrate is a potent inhibitor of DNA synthesis and cell proliferation *in vitro* and is associated with induction of cellular differentiation (Young & Gibson, 1995). These

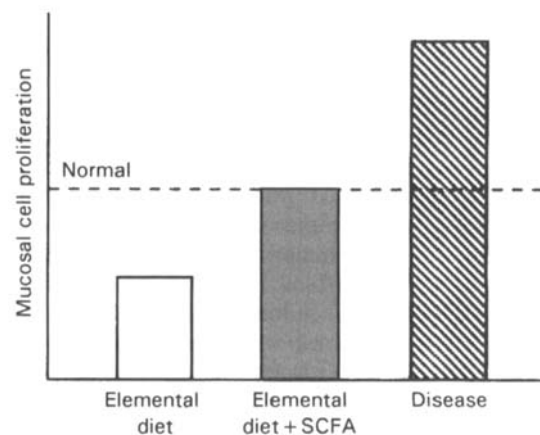


Fig. 3. Hypothesis: hypoproliferative mucosal epithelium in animals fed on elemental diets is returned towards normal by short-chain fatty acids (SCFA). There is no convincing evidence that extra SCFA production causes hyperproliferation characteristic of disease states.

changes in gene expression may be a result of hyperacetylation of histones and enhanced methylation of DNA, but it is unlikely that these global modifications of chromatin proteins and chromatin structure can explain the effects of butyrate since this SCFA switches off and switches on expression of only a limited number of genes (Kruh *et al.* 1995). Incubation of adenoma and colo-rectal tumour cell lines with physiological concentrations of SCFA induced apoptosis, with butyrate being the most effective agent (Hague *et al.* 1995). In contrast, Hass *et al.* (1997) have reported recently that the absence of butyrate from the medium promoted apoptosis in guinea-pig proximal colonic mucosa incubated in Ussing chambers. This was associated with increased expression of *Bax* but no change in *bcl-2*. In an accompanying editorial, Hague *et al.* (1997) have argued that since butyrate is a primary fuel for colonocytes *in vivo* (Roediger, 1982), rapid apoptosis after transfer into culture may be a result of energy deprivation despite the presence of glucose in the incubation solution.

Implication for colo-rectal cancer risk

Increased cell division may be an early event in the development of many human cancers because it increases the risk of accumulation of errors (mutations) in the newly-replicated DNA (Preston-Martin *et al.* 1990). Greater numbers of mitotic cells or an upwards expansion of the proliferative zone within the crypts in macroscopically-normal tissue is associated with greater risk of colo-rectal cancer (Mills *et al.* 1995; Wargovich, 1996). I have suggested previously that the changes in cell proliferation which accompany increased intake of NSP and other fermentable carbohydrates are likely to be physiological not pathological (Fig. 3). Rates of cell proliferation are much greater in the small-bowel mucosa than in the colon, yet tumours in the small intestine are very much rarer than in the large bowel. This might be explained on the basis that it is only mutations in the stem cells which matter since transit cells (Potten & Loeffler, 1990) have a limited capacity for further division. Hickman *et al.* (1995) have argued that the site difference in cancer incidence may arise because apoptosis of stem cells is rare in the colon when compared with the small intestine. There may be 'insufficient altruistic apoptosis' in the colon (Hickman *et al.* 1995) in the face of a greater mutagenic load. If dietary modifications increased cell proliferation on a sustained basis, this might increase the risk of accumulation of mutations because of the greater number of mitotic events, but only if this increased proliferation was the result of a greater number of asymmetric divisions of stem cells as distinct from more symmetric divisions of transit cells. Because of the great difficulty in distinguishing stem cells from other dividing cells in the intestine (Potten & Loeffler, 1990), it is not yet known where the primary increase in cell numbers arises in response to dietary manipulation. Finally, despite the evidence that butyrate may increase cell proliferation *in vivo* under certain conditions, it is unlikely that this has any adverse effect on colo-rectal cancer risk, since butyrate enemas afforded protection against large bowel cancer in a mutagen-treated rat model (D'Argenio *et al.* 1996).

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