

# Dietary calcium supplementation increases apoptosis in the distal murine colonic epithelium

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## Abstract

**Background**—Increased dietary calcium might reduce colorectal cancer risk, possibly by reduction of colonic epithelial hyperproliferation, but not all studies have demonstrated this. Little is known about the effects of calcium on colonic apoptosis.

**Aim**—To quantify the effects of increasing calcium on apoptosis and cell proliferation in normal murine colonic crypt epithelium.

**Methods**—Twenty one day old male C57Bl/6 mice were fed either control AIN-76 diet (0.5% calcium wt/wt; n = 10) or the same supplemented with calcium carbonate (1.0% calcium; n = 10) for 12 weeks. Apoptotic cells in proximal and distal segments were counted and expressed as an apoptotic index (AI: frequency of apoptosis/100 longitudinal crypts). The bromodeoxyuridine (BrdU) labelling index was also determined. Differences were analysed by the student's *t* test.

**Results**—In control animals, the AI was significantly higher in the caecum/proximal colon (mean, 28.6; SEM, 2.0) compared with the distal colon (mean, 19.9; SEM, 1.8; *p* = 0.004). In the calcium treated group, the AI in the caecum/proximal colon (mean, 30.6; SEM, 1.7) was similar to controls (*p* = 0.71) but the AI in the distal colon was significantly greater (mean, 32.6; SEM, 1.8; *p* = 0.001) than in control mice and was raised to values similar to those in the proximal colon. Calcium was also associated with reduced crypt cellularity and, in the proximal colon, a downward shift in the crypt position at which apoptosis occurred. There were no significant differences in the BrdU labelling index between groups or between proximal and distal colonic segments in each group.

**Conclusions**—Increased dietary calcium is associated with the induction of apoptosis in normal mouse distal colonic epithelium without affecting cell proliferation. This might contribute to its putative chemopreventive role in colorectal carcinogenesis. Whether this effect is direct or indirect requires further study.

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Despite recent major advances in our understanding of the molecular genetic events that occur during colorectal carcinogenesis, colorectal cancer remains a leading cause of morbidity and mortality in developed coun-

tries. Although 5–15% of patients with colorectal cancer might have an inherited predisposition to the disease, environmental factors are thought to be responsible for most cases of “sporadic” colorectal cancer.<sup>1</sup>

Of the dietary factors believed to be relevant to colonic carcinogenesis, much attention has focused recently on calcium.<sup>2</sup> Epidemiological studies and diet histories have reported an inverse association between dietary calcium intake and the incidence of colorectal cancer.<sup>3–6</sup> In vitro studies of colon cancer cell lines,<sup>7</sup> studies in experimental animals,<sup>8–11</sup> and intervention trials in humans<sup>12–14</sup> also support the concept that dietary calcium supplementation reduces the risk of subsequent colorectal cancer development, although negative studies have also been reported.<sup>15–17</sup>

Chemoprevention of colorectal cancer with calcium is attractive because it is safe, non-toxic, cheap, and seems acceptable to patients. However, the mechanism(s) of the beneficial effects of calcium are unclear. Colonic epithelial hyperproliferation is seen in patients at increased risk of colorectal cancer,<sup>18, 19</sup> and has been suggested to be an early event in the neoplastic process. Several in vitro animal and human studies have shown that supplemental calcium reduces colonic hyperproliferation,<sup>12–14</sup> whereas other authors have not been able to demonstrate this effect.<sup>15, 16, 20</sup>

Apoptosis, or programmed cell death, is important in the maintenance of tissue homeostasis in terms of the balance between cell birth and cell death in many tissues, including the colon. Failure of apoptosis has been shown to occur during colonic carcinogenesis,<sup>21</sup> and might contribute to tumour development and progression. Relatively little is known about the factors regulating apoptosis in the gastrointestinal tract and, to date, the effects of calcium have not been investigated. In our study, we aimed to assess the effects of dietary calcium supplementation on both the apoptotic process and cell proliferation in normal murine colonic epithelium.

## Materials and methods

### ANIMALS

After a one week run in period, 20 male C57Bl/6 mice aged 21 days (mean, 19 g; SEM, 1; Harlan Olac Ltd, Bicester, UK) were randomly allocated to receive either the control diet or the same diet supplemented with calcium carbonate (see below) for 12 weeks. Mice were housed in groups of five each cage in the biomedical research facility, University of Edinburgh, and maintained in conditions of controlled temperature and humidity, with a 12 hour light–dark cycle. Animals had free access to diets and distilled, deionised water, were

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inspected daily, and were weighed weekly. During week 10 of the study, animals were placed in individual metabolic cages and 48 hour food intake was recorded. The study was carried out under a Home Office approved project licence and mice were inspected regularly by the university veterinary department.

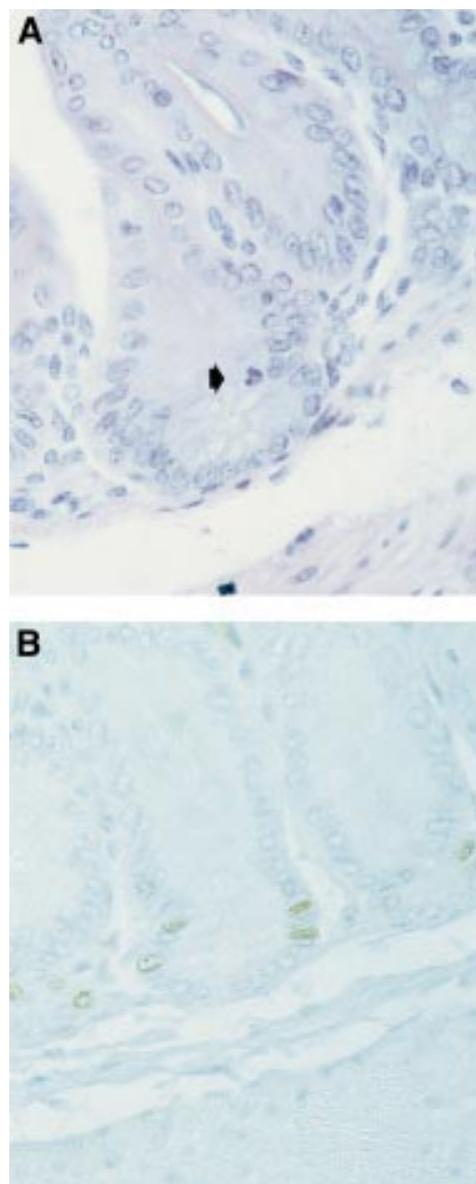
#### DIETS

Control mice were fed a semisynthetic, fibre free diet based on AIN-76,<sup>22</sup> obtained from Special Diet Services Ltd, Witham, Essex, UK and designed for optimum rodent growth. The calcium content is 0.5% wt/wt. In both groups the concentrations (% weight) of sucrose (53%), casein (21%), corn starch (15%), and corn oil (5%) were the same. For the calcium treated group, the basal diet was supplemented with calcium carbonate (Sigma, Poole, Dorset, UK) to achieve a final concentration of 1.0% wt/wt. Calcium carbonate was chosen because of its solubility, high bioavailability, palatability, and our desire to keep the phosphorus content identical in both diets. The concentrations of phosphorus (0.39%) and vitamin D3 (400 000 IU/g) were the same in both groups.

#### HISTOLOGICAL ANALYSIS OF APOPTOSIS

One hour before death, mice were injected intraperitoneally with 5'-bromodeoxyuridine (BrdU; 50 mg/kg body weight; Sigma). All animals (over 12 weeks of age) were killed under CO<sub>2</sub> asphyxiation. All animals were injected and killed between 12:00 and 14:00 hours to minimise the effects of diurnal variation on crypt kinetics. The entire colon was removed, from anal verge to caecum, opened longitudinally, pinned flat on to cork, and rinsed thoroughly with 0.9% saline to remove faecal contents. The colonic length was measured, the colon divided into proximal and distal halves, and each half rolled lengthwise upon itself. These "Swiss roll" preparations were fixed in Carnoy's solution for 24 hours and then stored in 70% alcohol. After processing, tissues were embedded in paraffin wax, individually coded, and 3 µm sections cut and stained with haematoxylin and eosin for counting of apoptotic cells. Only intact, U-shaped crypts that extended to the gut lumen were accepted as satisfactory for counting. In cases where fewer than 100 satisfactory crypts were present, a second section was cut from a deeper part of the tissue, at least 100 µm from the initial section. Apoptotic cells were identified by light microscopy at a magnification of ×400, according to predefined criteria,<sup>23</sup> and by one experienced observer (MJA), who was blinded to the origin of each tissue section. These criteria were: cell shrinkage, loss of contact, cellular fragmentation, and condensation of chromatin around the nuclear outline or into small spheres after nuclear fragmentation (fig 1).<sup>23, 24</sup> Adjacent clusters of apoptotic fragments were counted as one apoptotic cell. Crypts adjacent to lymphoid follicles or aggregates were not scored because apoptotic lymphoid cells (rather than epithelial cells) may be present in these locations.

To determine the optimal number of crypts to be scored for apoptosis, a running mean count



**Figure 1** (A) Photomicrograph showing an apoptotic cell in the proliferative compartment near the base of a crypt in the distal colon of a calcium treated mouse (arrow). The apoptotic cell appears within a vacuole and contains several spheres of condensed chromatin. Haematoxylin and eosin stained, ×630 magnification. (B) Photomicrograph of four bromodeoxyuridine labelled cells in the proliferative compartment of a crypt in the distal colon of a calcium treated mouse. Immunoperoxidase staining, ×630 magnification.

was made on multiple specimens, counting the frequency of apoptosis in up to 250 crypts. This revealed that counts based on 100 crypts gave a representative and reproducible score of the frequency of apoptosis. Thereafter, the total number of apoptotic cells or bodies/100 crypts was measured for each specimen and the result expressed as an apoptotic index (AI). Apoptotic counts were also made using the HOME (highly optimised microscopic environment) system and found to be nearly identical to counts made with standard microscopes.<sup>23</sup> The total number of nuclei in each hemicypt and the crypt position of each apoptotic cell were recorded. For this, the location of each apoptotic cell was recorded and expressed as per cent distance

Table 1 Mean (SEM) animal weights in grams

	Week 0	Week 12
Control group	19.5 (0.4)	23.3 (0.4)
Calcium supplemented group	19.9 (0.3)	24.1 (0.3)

Differences between the two groups were not significant.

along the crypt length, relative to the number of nuclei in each hemicrypt (where 0 = crypt base and 100 = crypt surface).

Sections for BrdU counting were stained as described previously,<sup>25</sup> using a mouse monoclonal antibody to BrdU (Becton Dickinson, San Jose, California, USA) at a dilution of 1/500 and a streptavidin–biotin, peroxidase-conjugated rabbit antimouse antibody at 1/400 for detection of staining. Sections were counterstained with haematoxylin, dehydrated, cleared, and mounted in synthetic resin (fig 1). For analysis, 100 intact, longitudinal, U-shaped crypts were counted in each proximal and distal colonic segment and the results expressed as total labelling index (LI).

#### STATISTICAL ANALYSIS

Data were analysed using a statistical software package (Minitab v11.2, Minitab Inc., Pennsylvania, USA) on an IBM PC. Results were confirmed to be normally distributed by Ryan-Joiner test and then differences between groups were analysed by the two sample student's *t* test. A value of  $p < 0.05$  was considered significant. To examine the influence of several possible explanatory variables (animal weight, percentage weight gain during the study, and measured daily food intake) on the response variables (AI and BrdU labelling index), multiple regression analysis was performed. Stepwise regression analysis was then performed to extract any significant independent predictors.

## Results

#### ANIMAL GROWTH AND DIETS

Both diets were well tolerated and no toxicity was observed in either group. There were no significant differences in initial or final animal weights between the groups (table 1). Mean percentage weight gain during the study was 19.5% in control mice and 21.1% in calcium supplemented mice. No significant differences in daily food intake (control group: mean, 4.1 g; SEM, 0.9; calcium group: mean, 4.4 g; SEM, 0.6) were observed but spillage of powdered feed and contamination with urine made accurate measurements difficult, leading to wide variations in recordings (see discussion).

#### APOPTOTIC INDEX

Figure 2 summarises the results. In control animals, the AI was significantly greater in the proximal colon (mean, 28.6; SEM, 2.0) compared with the distal colon (mean, 19.9; SEM, 1.8;  $p = 0.01$ ). In mice receiving the calcium supplemented diet, the AI was similar in both proximal (mean, 30.6; SEM, 1.7) and distal colonic segments (mean, 32.6; SEM, 1.8;  $p = 0.43$ ). There was no significant difference in the proximal colonic AI between control and calcium treated groups ( $p = 0.46$ ) but the AI in

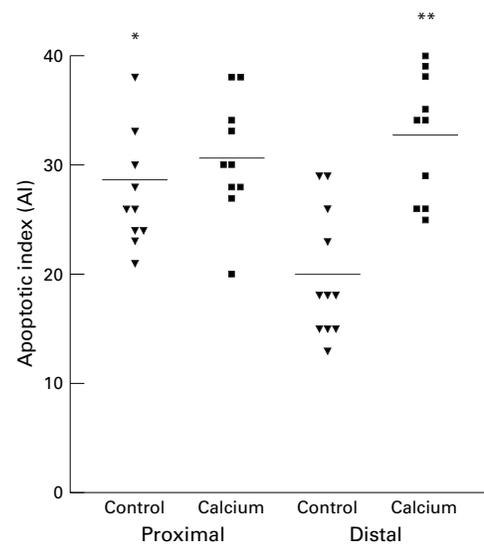


Figure 2 Apoptotic index (expressed as total number of apoptoses for each 100 intact crypts) in proximal and distal colonic segments of control (black triangles) and calcium treated (black squares) mice. \* $p = 0.01$  compared with control distal colon. \*\* $p = 0.001$  compared with control distal colon.

distal colonic segments was significantly greater in calcium treated mice than in controls ( $p = 0.001$ ).

To examine the possibility that the effects of calcium on AI could be confounded by differences between the groups in animal weight, per cent weight gain, or daily food intake, stepwise multiple regression analysis was performed. In control mice, no association was found between any of these variables and the AI in either the proximal or distal colon. In the calcium supplemented group, no association was found between weight or weight gain and the AI, but food intake was a significant independent predictor of AI in the distal colon (t ratio = 6.60;  $p = 0.02$ ). In the proximal colon, none of these factors was an independent predictor of AI, although a trend towards an inverse association between food intake and AI was noted (t ratio = -3.88;  $p = 0.06$ ).

Table 2 shows the crypt length, as measured by the number of nuclei in each hemicrypt. From these results it can be seen that there was no significant difference between proximal and distal colonic segments in either control mice ( $p = 0.07$ ) or calcium treated mice ( $p = 0.80$ ). There were, however, significantly more nuclei in each hemicrypt in both the proximal ( $p < 0.001$ ) and distal ( $p = 0.01$ ) colon of control animals compared with calcium treated animals.

Table 2 also shows the median crypt position of apoptotic cells in proximal and distal colons from each group. In control mice, the differences between the proximal (mean, 39.5; SEM, 1.9) and distal colon (mean, 37.1; SEM, 2.8) were not significant ( $p = 0.49$ ). In the calcium supplemented mice there was a minor but significant difference in mean crypt position between proximal (mean, 31.6; SEM, 1.0) and distal (mean, 35.8; SEM, 1.4) parts of the colon ( $p = 0.03$ ). The mean position at which apoptotic bodies were recorded was significantly

Table 2 Number of nuclei in each hemicrypt and relative position of apoptotic cells

	Nuclei/hemicrypt	Crypt position
<i>Control group</i>		
Proximal	24.3 (0.73)*	39.5 (1.9)‡
Distal	22.6 (0.46)†	37.1 (2.8)
<i>Calcium supplemented group</i>		
Proximal	20.9 (0.37)	31.6 (1.0)
Distal	21.0 (0.36)	35.8 (1.4)¶

Results are expressed as mean (SEM).

Relative crypt position refers to the percentage distance along crypt length: 0, crypt base; 100, crypt surface.

\*p = 0.001 v calcium distal colon.

†p = 0.01 v calcium distal colon.

‡p = 0.003 v calcium proximal colon.

¶p = 0.03 v calcium proximal colon.

lower down the crypts of calcium treated mice in the proximal colon only ( $p = 0.002$ ), compared with controls. In summary, calcium treatment was associated with an increase in AI in the distal but not proximal colon. In the proximal colon of the calcium treated group there was also a minor but significant shift towards the crypt base as the mean position at which apoptotic cells were recorded.

#### BROMODEOXYURIDINE LABELLING INDEX

Figure 3 shows the results of the BrdU labelling index. For both control and calcium supplemented mice, the LI was greater in the proximal colon than distally. The difference in control mice (proximal colon: mean, 223; SEM, 18.4; distal colon: mean, 184; SEM, 15.8) was not significant ( $p = 0.12$ ). In the calcium treated group, there was a trend towards a greater LI in the proximal colon but the difference did not reach significance (proximal: mean, 219; SEM, 19.1; distal: mean, 175; SEM, 12.5;  $p = 0.07$ ).

There was no significant difference in the LI of the proximal colons between the two groups ( $p = 0.91$ ). Similarly, no difference was seen in the distal colonic LI between the two groups ( $p = 0.66$ ). Stepwise regression analysis did

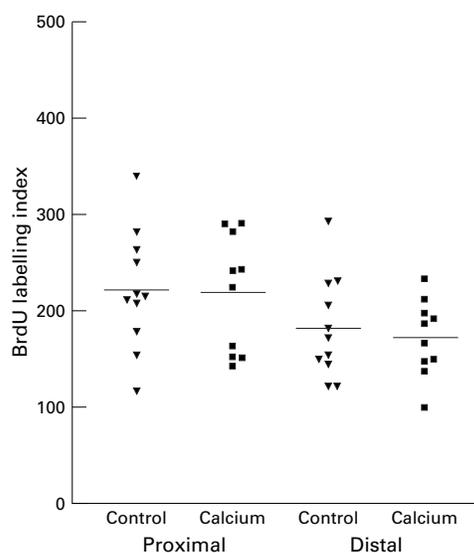


Figure 3 Bromodeoxyuridine (BrdU) labelling index (total number of labelled cells in each 100 intact crypts) in proximal and distal colonic segments of control (black triangles) and calcium treated (black squares) mice. No significant differences were seen between groups or between the proximal and distal colon in either group.

not reveal any significant association between weight, weight gain, or food intake and LI in either region of the colon in either group.

#### Discussion

Maintenance of colonic epithelial cell populations is a dynamic balance between cell birth and cell death<sup>26</sup> and disturbance of this, resulting from either excessive cell proliferation or defective apoptosis, might contribute to colonic tumorigenesis.<sup>27</sup> Many studies have emphasised the role of abnormal cell proliferation, but failure of apoptosis has only recently been appreciated, and few studies have examined the effects of dietary components on intestinal apoptosis. Our study was undertaken to assess the effects of dietary calcium supplementation on apoptosis and cell proliferation in normal murine colon.

We found that apoptosis in control mice occurred more frequently in the caecum and proximal colon than distally. Reasons for this might include regional differences in enteric flora, bacterial fermentation of fibre, and luminal concentrations of short chain fatty acids, bile acids, and other dietary components. We measured only the frequency of observed apoptotic cells and not the rate at which apoptotic cells appeared. Although the results could conceivably be explained by regional differences in the duration of the apoptotic process, there is little or no evidence for variation in the duration of apoptosis in normal tissues, including colonic epithelium.<sup>23</sup> A proximal–distal gradient in proliferative activity has also been reported in both normal rodent and human colonic epithelium.<sup>28, 29</sup> The importance of these regional variations in colonic crypt kinetics is unknown but the proximal and distal colon show considerable differences in structure, histology, function and, probably, regulation of colonocyte growth and differentiation.<sup>26</sup> Our data support this concept and are consistent with the known distribution of colorectal neoplasia, both in carcinogen induced rat models and in humans. They also highlight the importance of considering different colonic segments separately in experimental studies of carcinogenesis.

Differences in energy intake and weight gain might affect cell proliferation in animals, but possible effects on apoptosis are unknown. The nutrient densities of both groups were identical in our study and no differences in final weights or weight gain were seen. Although data on individual energy intake were incomplete, regression analysis shows no significant correlation between these parameters and the BrdU labelling index in either proximal or distal colon in either group of mice.

Calcium treatment had no effect on the total AI in proximal colonic epithelium, but was associated with a pronounced increase in distal colonic AI, which was raised to values similar to those seen in the proximal colon of both groups. In stepwise multiple regression analysis, we found measured food intake to be a significant independent predictor of AI in the distal colon of calcium treated mice. Thus, it is possible that the observed increase in AI might

be related to overall food intake and not calcium supplementation per se. However, because of our study design, the effects cannot be separated because calcium was administered by mixing with the diet. Thus, variations in food intake must also reflect changes in calcium ingestion by the animals, and it is equally plausible that the results are directly related to calcium supplementation. Furthermore, differences in food intake between the groups were small and the groups were well matched for initial weight, final weight, and growth during our study, making differences in energy intake an unlikely explanation for the observed results. Further studies, possibly by administering calcium in drinking water, will be needed to answer this question conclusively.

Furthermore, the observed effects of calcium on the AI could be direct or indirect, resulting from precipitation of toxic or cytolytic faecal bile acids or fatty acids. We did not measure stool concentrations of these compounds because this effect of calcium supplementation has already been well characterised.<sup>30–32</sup> Further studies will be necessary to determine whether there is a threshold concentration at which calcium produces these effects and whether calcium deficient diets (resembling the diet of a large proportion of humans) have the opposite effect. Our finding of increased apoptosis in the distal colon only is also consistent with epidemiological studies reporting that low calcium diets are associated with an increased risk of distal but not proximal tumours,<sup>5</sup> and animal studies where calcium supplementation reduced the incidence of azoxymethane induced tumours only in the distal colon.<sup>33–34</sup>

Calcium supplementation was also associated with significantly reduced crypt cellularity, as measured by the number of nuclei in each crypt column. This effect was noted in both proximal, and to a lesser extent, distal colon. The luminal concentrations and effects of short chain fatty acids and faecal bile acids on crypt cell kinetics differ between the proximal and distal bowel, and it is possible that binding and precipitation of these substances by calcium was responsible for the regional differences. Alternatively, a direct effect of calcium cannot be excluded.

These effects of calcium on apoptosis could conceivably contribute to its chemopreventive actions by increasing the death rate of proliferating cells, including those carrying mutations relevant to tumorigenesis. Expansion of the proliferative compartment of the colonic crypt towards the lumen (“phase 2” defect) is a feature of neoplastic development,<sup>35</sup> and varying the calcium content of the diet can modulate this.<sup>36</sup> A downward shift in the region of apoptotic activity in the crypt might contribute to the beneficial effects of calcium on these proliferative abnormalities.

We were unable to confirm a proximal–distal gradient in cell proliferation in normal colon, as noted by others.<sup>15–28–29</sup> A trend towards an increased BrdU labelling index was seen in the proximal colon of both animal groups, but this failed to reach significance. Calcium supplementation did not significantly affect the LI in

either proximal or distal colonic segments in our study. These findings are in contrast to those of several other studies,<sup>8–37</sup> but not all authors have demonstrated an effect of calcium on cell proliferation in rodents.<sup>38</sup> Likewise, demonstrating an antiproliferative effect in humans has been much more difficult.<sup>16–17–20</sup> Taken together, the results of animal and human studies suggest that calcium might have other chemopreventive effects, separate from, or in addition to, its effects on cell proliferation.

Care must also be taken to distinguish between studies of “normal” (for example, 0.5% wt/wt) versus “low” (for example, 0.1%) calcium intake and those that compare normal versus “high” intake (for example, 1.0%). Whereas calcium deficient diets might result in hyperproliferation,<sup>8</sup> which can be reversed by replacing the calcium content, this is different from supplementing a normal calcium diet with further calcium—raising the content higher may not influence proliferative status any further. AIN-76, as recommended by the American Institute of Nutrition for optimum rodent growth,<sup>22</sup> contains 0.5% calcium by weight (1.4 mg/kcal (4.18 kJ)). This is equivalent to a human intake of approximately 3000 mg/day, considerably higher than the recommended daily allowance of 800 mg for adults,<sup>39</sup> but similar to doses used in trials of polyp prevention in humans. The calcium supplemented diet contained 1.0% calcium by weight, in keeping with several other studies of calcium in rodents.<sup>8–32–40</sup> However, this dose is greater than that used in studies on people, and it is unclear whether this degree of supplementation would be palatable or safe in humans. We did not monitor serum calcium concentrations in our mice, but no toxicity was observed, and other authors have demonstrated lack of toxicity using this value of calcium or greater.<sup>9–41</sup> Further studies to examine whether lower amounts of calcium supplementation also affect colonic apoptosis are planned, as are studies of the effects of a calcium deficient diet.

In addition to the precipitation of luminal bile acids and fatty acids,<sup>42</sup> direct effects of calcium on colonic crypt cells have been proposed, including interactions with calmodulin, cAMP, ornithine decarboxylase, and tyrosine kinase.<sup>2</sup> Low concentrations of intracellular calcium stimulate cell proliferation in vitro and cells stop cycling as the concentration rises. Cell surface calcium (polyvalent cation) “receptors” have been reported on colonocytes.<sup>43</sup> Whitfield *et al* have proposed that a luminal concentration gradient of calcium exists, with maximum concentrations at the crypt surface.<sup>34</sup> As cells move up the crypt they might encounter increasing calcium concentrations, leading to cessation of cycling and induction of differentiation. Although speculative, this hypothesis is supported by evidence that colorectal cancer cells in vitro can, unlike normal cells, continue to cycle in the presence of high concentrations of extracellular calcium.<sup>34–44</sup>

Our results are of relevance to humans because colorectal neoplasia is a leading cause of death in most developed countries. Colonoscopic screening to detect and remove polyps

reduces cancer risk but is expensive, invasive, and not without risk. Chemoprevention has therefore been proposed as an alternative strategy, either by dietary means or using aspirin or other non-steroidal anti-inflammatory drugs. Although these drugs show promise, their mode of action is unclear and toxicity concerns exist. Dietary supplementation with calcium is attractive because it is cheap, palatable, and probably non-toxic. Furthermore, experimental evidence is supported by the largest prospective randomised trial of calcium supplementation to date, which demonstrated a significant reduction in recurrence of colonic adenomas (adjusted risk ratio, 0.81) in subjects treated with calcium carbonate (3 g/day, 1200 mg elemental calcium).<sup>40</sup>

In conclusion, our results show that, in normal murine colon, the AI is significantly greater in the caecum and proximal colon than in the distal colon. Dietary calcium supplementation in mice is associated with increased apoptosis in distal colonic epithelium, a downward shift in the crypt position at which apoptotic cells were observed, and shorter crypts in the proximal colon. The possibility that the observed results are related to overall food intake rather than calcium cannot be excluded from our study. In contrast, calcium had no effect on cell proliferation. The chemopreventive actions of calcium supplementation on colorectal neoplasia might depend, at least in part, on its ability to increase the susceptibility of colonocytes to apoptosis, thereby increasing the probability of death of those cells bearing potentially carcinogenic mutations.

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