Effects of Brassica vegetable juice on the induction of apoptosis and aberrant crypt foci in rat colonic mucosal crypts in vivo

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Glucosinolates are sulfur-containing glycosides found in the Brassica vegetables. Their breakdown products include isothiocyanates, which are produced following exposure to the endogenous plant enzyme myrosinase. Isothiocyanates are pungent, biologically active compounds that suppress carcinogenesis in vivo, and induce apoptosis in vitro. We have shown previously that oral administration of the isolated glucosinolate sinigrin induces apoptosis, and suppresses aberrant crypt foci in the colonic mucosa of rats treated previously with 1,2-dimethylhydrazine (DMH). In this study we explored the effects of both raw and thermally processed Brussels sprout tissue on the modulation of crypt cell apoptosis and mitosis, and the frequency of aberrant crypt foci in the colon. Freeze-dried raw and microwave-cooked Brussels sprouts contained high levels of intact glucosinolates, but they were absent from freshly prepared sprout juice. Oral administration of uncooked Brussels sprouts, whether as a juice, or as a freeze-dried powder, was associated with significantly enhanced levels of apoptosis and reduced mitosis in the colonic crypts. However, this effect was confined to rats previously injected (48 h) with DMH, in which levels of apoptosis and mitosis following DNA damage were already high. There was no effect of treatment in control animals. There was also little evidence of these effects when intact glucosinolates were administered in blanched sprout tissue, which lacked active myrosinase. We conclude that glucosinolate breakdown products derived from Brassica vegetables can exert a profound effect on the balance of colorectal cell proliferation and death in an animal model of colorectal neoplasia.

Introduction

There is good evidence that several classes of compounds found in fruits and vegetables exhibit anticarcinogenic effects mediated by a number of different mechanisms (1). Assuming that the multi-step carcinogenic pathway begins with initiation, in which unrepaired or misrepaired DNA damage is replicated, followed by promotion, which facilitates the transformation and clonal expansion of preneoplastic cells and neoplastic cells, there are a number of stages at which the possibility of anticarcinogenic activity can be envisaged. Consumption of Brassica vegetables, which are members of a genus in the family Cruciferae, is associated with reduced risk of cancer in human populations (2), and they and their constituents have been shown to inhibit the formation of tumours at various sites after treatment of rats with model carcinogens (3). One mechanism by which suppressing agents might act is by inducing previously initiated cells to follow the apoptotic pathway, causing their deletion from the tissue and thus preventing clonal expansion of the lesion (4).

The Brassica vegetables contain between 15 and 20 different glucosinolates (5,6). These sulfur-containing glycosides are sequestered within the intact vegetable tissue, but are released by physical damage, and exposed to the enzyme thioglucoside glucohydrolase (myrosinase). The resulting hydrolysis and further degradation leads to biologically active products including the isothiocyanates (7). The glucosinolate sinigrin is present in many Brassica vegetables, but it occurs in particularly high concentrations in Brussels sprouts (Brassica oleracea var. gemmifera). The sinigrin breakdown product allyl isothiocyanate (AITC) has been reported to induce glutathione S-transferase activity in the liver and small intestine of the rat (8). We have shown previously that AITC selectively induces cell death in the undifferentiated phenotype of the HT29 human tumour cell line (9), and that dietary sinigrin induces increased apoptosis in the colorectal crypt of rats treated previously with 1,2-dimethylhydrazine (DMH) (10). These observations led us to propose that AITC may thereby act as a suppressor of colorectal carcinogenesis. In the present study we conducted three experiments to explore the effects of raw and thermally processed Brussels sprout tissue on the modulation of crypt cell apoptosis and mitosis, and the appearance of preneoplastic lesions in the colon of rats treated previously with DMH.

Materials and methods

Vegetable preparations

Brussels sprouts (commercial varieties, 'Stephene', 'Ajax' and 'Cyrus') were grown by Novartis Seeds (Enkhuizen, Netherlands) and shipped directly to the laboratory. After delivery the sprouts were stored at 4°C and used within 72 h. For the preparation of juice, raw sprouts were cut in half by hand, and passed through a domestic centrifugal juice extractor (Kenwood JE500, Kenwood, UK). The liquid obtained was filtered through double-layered muslin prior to gavage. Blanched sprouts were prepared by placing whole sprouts in a domestic microwave cooker (Model, R-2V16M, Sharp, UK) for 6 min on full power (650 W). For preparation of dietary supplements, fresh or blanched sprouts were frozen whole at –40°C, then freeze-dried, before being ground to a powder in an electric coffee grinder (Moulinex, France).

Immediately after preparation, samples of juice and raw and blanched freeze-dried tissue were collected and stored at –20°C. Glucosinolates were extracted from freeze-dried material and converted to desulphoglucosinolates. Glucosinolates, and their breakdown products extracted from sprout juice, were analysed by liquid chromatography–mass spectrometry (LC–MS) using the methods of Rose et al. (11).

Animals and diets

Male Wistar rats (~130 g on arrival) were obtained from a licensed animal supplier and housed in a purpose-built, air-conditioned animal facility with an ambient temperature of 21°C and a 12 h–12 h light–dark cycle. The UK Home Office approved routine animal care and all experimental procedures. The rats were allowed to acclimatize for 9 days to the new conditions during which they consumed ad libitum, a semi-synthetic basal diet containing casein (200 g/kg), corn oil (80 g/kg), corn starch (310 g/kg), sucrose (310 g/kg), cellulose (40 g/kg) and quantities of micronutrients appropriate for growing rats. Experimental diets were prepared by adding freeze-dried powdered

Abbreviations: ACF, aberrant crypt foci; AITC, allyl isothiocyanate; DMH, 1,2-dimethylhydrazine; JNK, c-Jun N-terminal kinases.
Ten days after arrival, the rats were randomly distributed into two equal-sized treatment groups, one received injections of the colon-specific carcinogen DMH (30 mg/kg body wt) whilst the other received sham injections of saline.

**Experimental protocols**

Ten days after arrival, the rats were randomly divided into two groups of 20, containing equal numbers of DMH-treated and saline-treated animals. After 23 and 45 h, 20 test animals (10 DMH and 10 saline) were gavaged with 2 ml freshly prepared sprout juice. Control animals (10 DMH and 10 saline) received no gavage. The basal semi-synthetic diet was available ad libitum throughout the study. Forty-eight hours after the second injection all animals were killed, and a sample of the mid-colon was removed and fixed for analysis of apoptotic and mitotic levels in the crypt epithelium as described below.

**Experiment 2.** Thirty-six rats received a single injection of DMH or saline and were then randomly distributed into three dietary groups of 12, each containing six DMH and six saline-treated rats. The groups received 20 g/day of basal unsupplemented diet, basal diet supplemented with raw sprouts or basal diet supplemented with blanched sprouts. Forty-eight hours after injection, all animals were killed and a full thickness sample of the mid-colon was removed and fixed for analysis of apoptotic and mitotic levels in crypt epithelial cells.

**Experiment 3.** Forty-two rats received two injections of DMH or saline at 5 day intervals, and were then allocated to three dietary groups of 14 (seven DMH and seven saline). The three groups received, respectively, 20 g/ day of basal unsupplemented diet, basal diet supplemented with raw sprouts or basal diet supplemented with blanched sprouts for 28 days. All groups were then returned to basal diets for a further 14 days, after which they were killed, the whole colon removed, fixed and stained for the assessment of aberrant crypt foci (ACF) as described below.

**Uptake and metabolism of glucosinolates.** To provide qualitative confirmation that glucosinolate breakdown products were absorbed from the experimental diets, a separate feeding study was performed. Three groups of nine rats each were transferred from basal semi-synthetic diets to the experimental diets used in Experiment 3, and three animals from each group were killed after 1, 3 and 7 days. The lumen content of the mid-colon (<0.5 ml) was collected from the bladder of each animal, frozen and stored at -40°C prior to analysis for urinary metabolites. Samples were centrifuged at 3000 g for 5 min and 50 ml of the supernatant was injected directly into an LC–MS system for the determination of ITC-N-acetylcycteine conjugates as described by Rose et al. (11).

**Assessment of crypt cell apoptosis, mitosis and the frequency of ACF in the whole colon.** Immediately after death the abdomen was opened by a ventral incision and the intact colon was removed by severing at the caeco-colonic junction. The colon was freed of its mesenteric attachments, cut into thin sections and flushed with phosphate-buffered saline (pH 7.4). For the assessment of cellular kinetics after 48 h, full thickness samples (~0.5 cm) were removed from the mid-colon (~55% of total length), fixed and stored in acetic acid:ethanol (25:75) prior to analysis. For the assessment of ACF the whole colon was stored under the same conditions. The frequency of cells undergoing apoptosis and mitosis was determined in intact micro-dissected crypts, using and modified criteria as described previously (10).

All crypts were analysed by a single individual who, at the time of the analysis, was not aware of the groups from which the samples were obtained. Fixed samples of colon were rehydrated by passage through 50% ethanol and pure distilled water, hydrolysed in 1 M HCl at 60°C for 7 min, and then stained with Feulgen’s reagent. Small rows of crypts were micro-dissected under a low-power microscope, placed under a coverslip and slightly flattened to display individual crypts. The length of each crypt was estimated by comparison with a calibrated eyepiece graticule, and the numbers of mitotic profiles and apoptotic nuclei per crypt were counted. All nuclei in prophase, metaphase, anaphase or telophase were classified as mitoses. Apoptotic nuclei appear as dense spherical or crescent shaped bodies under these conditions, or as more dispersed arrays of small, irregular granules (10,12). A total of 10 intact isolated crypts were counted per animal.

To determine the numbers of ACF in the colon of each rat, whole fixed colons were stained with Feulgen’s reagent, slit open and folded out to form a sheet that was gently flattened between two microscope slides. The mucosal surfaces were examined under a microscope at X40 total magnification. ACF were identified as crypts with thickened walls and enlarged, often multiple crypt openings (10,12). The total numbers of ACF were counted for individual animals and the data for each group were expressed as means and standard errors of the mean.

**Statistical analysis**

In all experiments significance of differences between individual means was assessed by one-way ANOVA combined with Tukey’s test for individual comparisons, using the Minitab statistical package (Release 12; Mintab, State College, PA).

**Results**

**Glucosinolate content of sprout supplements**

Table I shows the quantities of individual glucosinolates present in the powdered Brussels sprouts fed to animals during Experiments 2 and 3. Both fresh and blanched whole sprout tissue contained a similar mixture of intact glucosinolates, amongst which sinigrin, progoitrin and glucosinap were predominant. As expected from the known activity of the endo-
genous enzyme thioglucoside glucohydrolase, no intact glucosinolates were detected in the juice prepared for gavage. The breakdown products were not analysed in detail, but it was noted that butenyl isothiocyanate was the main isothiocyanate present and free allyl isothiocyanate, the principle breakdown product of sinigrin, was not detectable in significant quantities.

**Experiment 1.** The modulation of mitosis and apoptosis in colorectal mucosa of rats after gavage with raw sprout juice

There was no significant effect of DMH treatment on food intake or final body weights of the rats. Figure 1 shows the average number of apoptotic and mitotic figures in crypt cells of animals receiving DMH or saline injections, and the effect of consuming raw sprout juice subsequent to injection. Treatment with DMH significantly (P < 0.01) increased levels of mitosis from 1.40 ± 0.08 to 3.11 ± 0.35 events per crypt. This elevated level was diminished (1.85 ± 0.19) if animals received raw sprout juice after carcinogen treatment (P < 0.01), but no effect was seen if animals received raw juice in the absence of DMH (1.34 ± 0.12). Similarly DMH treatment increased levels of apoptosis to 0.35 ± 0.05 events per crypt and gavaging with sprout juice further increased levels to 1.25 ± 0.28 events per crypt. This value was significantly greater than all other groups (P < 0.01). Gavage with sprout juice in the absence of DMH had little effect on basal levels of apoptosis (0.03 ± 0.02 and 0.01 ± 0.01).

**Experiment 2.** The modulation of mitosis and apoptosis in colorectal mucosa of rats after consumption of diets supplemented with raw or blanched sprouts

Food consumption in DMH-treated rats fed raw sprouts (25.4 g/48 h) was significantly lower than in saline-treated rats fed raw sprouts (36.5 g/48 h; P < 0.05). In DMH-treated rats fed cooked sprouts the food intake was 25.1 g/48 h, compared with 39.6 g/48 h in the equivalent group treated with saline. There were no other differences in food intake amongst the groups.

As in the previous experiment, apoptotic nuclei were rare in the colonic crypts of control rats (0.02 ± 0.018), but were significantly more frequent in DMH-treated animals (1.17 ± 0.36; Figure 2). Consumption of raw sprout tissue significantly (P < 0.01) increased this already elevated level of apoptosis to 3.07 ± 0.24 apoptotic events per crypt. Consumption of raw tissue in the absence of DMH treatment had no significant effect on the basal level of crypt cell apoptosis. The level of apoptosis in DMH-treated animals given blanched sprout tissue (1.98 ± 0.53) was higher than in the DMH-treated controls but not significantly so. There was no effect of blanched sprouts on basal levels of apoptosis. DMH treatment increased mitosis from 1.72 ± 0.44 to 2.22 ± 0.59 in control animals, but consumption of raw or blanched sprouts did not have a significant effect on levels of mitosis seen in untreated animals (1.67 ± 0.19 and 2.17 ± 0.19).
Discussion

Apoptosis is an orderly mechanism of cellular self-destruction that can lead to the selective deletion of damaged cells from tissues. There is increasing evidence that apoptosis plays a major role as a mechanism of defence against cancer, and that disruption of the apoptotic pathway can increase the susceptibility of tissues to neoplastic change (13). Thus, sulindac, which inhibits the growth of colorectal polyps in humans (14), has been shown to enhance apoptosis both in vitro (15), and in human rectal epithelium (16). Samaha et al. (17) have confirmed that the ability of several chemopreventive substances to inhibit chemical carcinogenesis in animal models is positively associated with the apoptotic index of treated tumours in vivo, and they have proposed that the level of apoptosis measurable in a target tissue can be used as a biomarker for the evaluation of compounds with chemopreventive potential. Conversely the neoplastic transformation of a tissue has been shown to be associated with a progressive loss of susceptibility to apoptosis (18). One question that arises from this developing area of research is whether naturally occurring food constituents might also modulate the tendency for apoptosis to occur in the dividing cell population of the colorectal crypt, and thereby influence the vulnerability of the mucosa to the development of neoplasia (4).

The breakdown products that result from hydrolysis of glucosinolates by myrosinase (thioglucoside glycosylhydrolase; EC 3.2.3.1) are mainly isothiocyanates and nitriles. A proportion of these breakdown products enter the epithelial cells of the gastrointestinal mucosa, undergo metabolism and transfer to the circulation, and then further metabolism in the liver, yielding products that are excreted in urine. Up regulation of Phase 2 enzyme activity in gastrointestinal and hepatic tissues by phenethyl isothiocyanate reduces the conversion of procarcinogens to active forms, and enhances the detoxification and excretion of carcinogenic substances (19,20). This blocking activity may account for the ability of glucosinolates and Brassica vegetable tissue to reduce the effectiveness of carcinogens during the initiation stages of experimental carcinogenesis (21). However, isothiocyanates have also been shown to suppress carcinogenesis during the post-initiation stage, after metabolism of the carcinogen is complete. Induction of apoptosis amongst initiated cells in target tissues is one possible mechanism of such suppression. In previous studies we have established that allyl isothiocyanate (AITC), one of the principal breakdown products of the glucosinolate sinigrin, is selectively cytotoxic against undifferentiated HT29 cells (22,23). Induction of apoptosis by breakdown products of glucobrassicin and glucoraphanin has been reported by Gamet-Payrastre et al. (24,25), and by Yu et al. (26), and for other food-borne isothiocyanates by Chen et al. (27).

In a previous study (10), we showed that oral administration of the glucosinolate sinigrin reduced the number of aberrant crypt foci (ACF) induced in the rat colon by multiple doses
of DMH. Because the glucosinolate was given only after treatment with DMH the anti-neoplastic effect must have been brought about by suppression of the developing lesion, rather than by any blockade of initiation. Our observation that the level of apoptosis induced in the colonic crypt by DMH was significantly higher in animals given sinigrin compared with controls led us to propose that the reduction in ACF resulted from a more effective deactivation of initiated cells from the damaged mucosal crypts. This hypothesis is consistent with studies from other laboratories showing that the chemopreventive substances sulindac, curcumin and phenylethyl-3-methyl cafffeate increased the rate of apoptosis in rat colonic adenocarcinomas induced by azoxymethane, whereas an aromatic isothiocyanate found to be pro-carcinogenic in the same animal model (6-phenylhexylisothiocyanate) did not cause any increase in apoptosis compared with control animals (17).

In the present study we demonstrated that both raw and blanched Brussels sprouts contained high levels of intact glucosinolates including sinigrin (Table I). As expected from the known action of myrosinase, preparation of the juice was associated with a nearly complete loss of the endogenous glucosinolates in the sprouts. Similarly, upon activation of intact myrosinase present in raw freeze-dried sprout tissue, isothiocyanates (predominantly butenyl isothiocyanate) were produced. The detection of the N-acetylcyisteine conjugates 3-methylsulfinylpropyl, 3-methylsulfinonylbutyl, 2-propenyl, 3-butenyl, 1-cyano-2, 3-epithiopropane and 1-cyano-3,4-epithiobutane in urine confirmed that the glucosinolate breakdown products liberated from raw or cooked sprout tissue were absorbed, metabolized and excreted in urine. Unexpectedly, allyl isothiocyanate was virtually undetectable in the sprout juice prepared for gavage. It is not clear at present whether this very volatile compound was rapidly lost from the juice, or whether it was rendered undetectable by some other mechanism such as binding to proteins. This finding does however suggest that other glucosinolate breakdown products besides isothiocyanates may contribute to the induction of apoptosis by Brassica juice.

Oral administration of uncooked Brussels sprouts, whether as a juice, or as intact, freeze-dried tissues, was associated with a statistically significant enhancement of DMH-induced apoptosis and a suppression of mitosis in the dividing cells of the colonic crypts. In animals fed raw sprout tissue for 28 days after DMH treatment, there was a reduction in the numbers of DMH-induced ACF. This reduction was similar in magnitude to that observed previously by us in rats fed sinigrin after treatment with DMH (10), although in the present study the difference did not reach statistical significance. There was no evidence of any effect when animals were fed blanched sprout tissue, which lacked active myrosinase.

The bioavailability and metabolism of glucosinolates and their breakdown products is not fully understood, but isothiocyanates have been shown to be readily absorbed from the proximal gut in rodents (28) and in human beings (29). Some intact glucosinolates may be absorbed in the small intestine but they do not appear to be biologically active. A significant fraction probably reaches the colon, where the compounds are degraded to isothiocyanates and other breakdown products by bacteria expressing myrosinase activity (30–32). In the present study, oral administration of freshly prepared juice was an effective inducer of apoptosis in the colon, as was consumption of intact glucosinolates combined with active myrosinase in the uncooked sprout tissue. This suggests that uptake via the small intestine, and hence delivery of metabolites to the colonic epithelial cells via the circulation, may be the most effective route of exposure. In contrast, consumption of intact glucosinolates in the blanched sprouts had little effect on levels of apoptosis and proliferation in colonic crypt cells. Several recent studies have demonstrated the reduced bioavailability of glucosinolates breakdown products from the cooked tissues of Brassica vegetables when compared with raw vegetables (29,33). Clearly there is a pressing need for a greater understanding of the uptake, metabolism and delivery of these compounds to target tissues.

As observed previously, the enhancement of apoptosis in the colonic crypt epithelium occurred only in those animals that had been treated with DMH and were therefore subject to high levels of DNA damage. The apoptotic cells were localized near the base of the crypt, and their increased frequency was associated with a reduction in mitosis. These findings imply that glucosinolate breakdown products do not simply cause direct cytotoxic damage to the mucosa, but may act by reducing the threshold for programmed cell death amongst proliferating cells carrying DMH-induced genomic damage. Chen et al. (27) reported that phenylmethylisothiocyanate (PMITC), and phenylethylisothiocyanate (PEITC) both induced prolonged activation of c-Jun N-terminal kinases (JNK; stress-activated protein kinase) in human leukaemia Jurkat T-cells. This subfamily of the mitogen-activated protein kinases (MAP-kinases) are involved in regulating cellular responses to a variety of extra cellular stimuli. In earlier work the same group showed that JNK activation is associated with both mitosis and radiation-induced apoptosis in Jurkat cells, and the duration of JNK activation appears to be a crucial determinant of whether cells enter cell division or programmed cell death (34). If this mechanism is functional in the intestinal crypt, then the effect of isothiocyanates may be to cause increased or prolonged expression of JNK in stem cell progeny carrying unrepaired DNA damage, thereby favouring apoptosis at the expense of mitosis. The cumulative effect of such a shift in the balance of cell birth and death would be to reduce the survival of cells carrying mutations favouring neoplastic transformation.

The present study has established that substances derived from a commonly eaten green vegetable, for which there is epidemiological evidence of a protective effect against colorectal cancer (35), can exert a profound effect on the balance of colorectal cell proliferation and death in an animal model of colorectal neoplasia. Although the use of a complex vegetable extract reduces the precision with which the active constituents can be identified, it does illustrate that human food can exert important physiological effects associated previously with isolated substances administered at pharmacological levels. The relevance of the observed mechanism to the protective effects of Brassica vegetables against cancers of the human alimentary tract remains to be established; this will require further detailed studies on the pharmacokinetics and cellular metabolism of glucosinolate breakdown products in humans.

Acknowledgements

The authors are grateful to Simon Deakin and Valerie Russell for expert animal care. This study was supported in part by the central strategic grant of the BBSRC, and partly by the European Union, as part of a shared cost action under the FAIR Programme (CT97 3029).

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Received November 5, 2002; revised December 11, 2002; accepted December 12, 2002