Increased induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats fed diets containing purified genistein or genistein-rich soya protein

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The isoflavonoid genistein inhibits mitosis and increases apoptosis in a variety of tumour cell lines in vitro, and may exert anticarcinogenic effects in vivo. To assess its effects on the colon, rats were fed a semi-synthetic control diet, or similar diets enriched with genistein (0.25 g/kg), either as the pure isoflavone or as part of a soya protein isolate, for 7 days before receiving subcutaneous injections of saline or 1,2-dimethylhydrazine (DMH). After 48 h, rats given saline were killed and samples of their small and large intestinal mucosa were obtained for assessment of crypt cell mitosis and apoptosis by visual analysis of isolated intact crypts. Rats given DMH were fed control diet and killed after 48 h for assessment of crypt cytokinetics or maintained for 42 days then killed and their colonic mucosa analysed for aberrant crypt foci (ACF). Two further groups were given control diet before DMH, followed by the genistein or soya-based diet for 42 days before assessment of ACF. Neither genistein nor soya protein isolate had a significant effect on crypt cell mitosis or apoptosis in untreated rats, or on the proliferative response to treatment with DMH. However, consumption of pure genistein or the soya protein isolate before treatment with DMH was associated with a 3-fold ($P < 0.001$) or 2-fold ($P < 0.05$) increase, respectively, in ACF in the distal colon. There was no significant effect of genistein or soya protein isolate given after DMH treatment. We conclude that genistein has no detectable effect on colonic crypt mitosis or apoptosis in the rat in vivo, but that it promotes induction of ACF by an as yet undefined mechanism when fed immediately before treatment with DMH.

Introduction

The isoflavonoids are a sub-group of the flavonoids, a large group of phenolic compounds containing two benzene rings (A and B) linked by a heterocyclic ring (C). Unlike the flavonols, in which the B ring is linked to carbon 2 on the central C ring, the isoflavonoids have a C–B ring linkage at position 3, an arrangement which confers some structural similarity to the oestrogens. Genistein (4',5,7-trihydroxyisoflavone), which occurs in soya beans and is consumed in significant quantities in many parts of Asia, interacts with mammalian oestrogen receptors at concentrations that can occur naturally in human tissues (1). Genistein appears to act as an anti-oestrogen in some models (2,3) and has attracted a great deal of interest as a possible inhibitor of oestrogen-dependent tumour growth (4), but it is also a potent inhibitor of other enzymes involved in cell proliferation, including tyrosine protein kinases (5) and type II DNA topoisomerase (6). Recently, genistein has also been shown to interact directly with the transforming growth factor (TGF) β1 signalling pathway in a manner which could, in turn, inhibit cell proliferation (7).

Much of the interest in genistein as a potential anticarcinogen has been fuelled by its well established antiproliferative effects against a considerable range of tumour cells in vitro. For example, genistein inhibits the growth of hormone-dependent tumours of the breast (8) and prostate (9), as well as leukaemia (10), melanoma (11) and gastric cancer cells (12). Genistein has been shown to block mitosis and stimulate apoptosis in several colorectal cancer cell lines (13) and in cell lines isolated from neonatal rat intestine (14). These latter findings have prompted a number of attempts to demonstrate an anticarcinogenic effect of purified genistein, or of soya products containing isoflavonoids, in animal models. However, the results have been inconclusive or contradictory. McIntosh et al. (15) compared the effects of various protein sources on induction of colorectal neoplasia by dimethylhydrazine (DMH) in rats and observed higher numbers of tumours in animals fed soya protein than in those fed whey or casein. However, Thiagarajan et al. (16) observed a protective effect of soya products against induction of aberrant crypt foci (ACF). These lesions are biomarkers of abnormal crypt cell proliferation which have been shown to correlate with the subsequent development of adenomas and adenocarcinomas in rat distal colon (17). Pereira et al. (18) and Thiagarajan et al. (16) also observed a protective effect of purified genistein against induction of ACF, whilst Rao et al. (19) reported that genistein increased tumour numbers in rat colon after treatment with azoxymethane (AOM).

In the present study we aimed to clarify the effects of genistein on cell proliferation and apoptosis in the rat small and large intestines, and to relate these to the induction of ACF in the distal colon where there is evidence that these lesions are predictive biomarkers of neoplasia (17). Rats were fed semi-synthetic diets containing either soya protein or purified genistein, before or after treatment with DMH. The effects on crypt cell proliferation and apoptosis were determined using visual analysis of isolated intact crypts, a method which is free of the morphometric artefacts associated with the use of sectioned material (20). Crypts were obtained shortly after the final DMH treatment and also concurrently with the sampling of distal colonic tissue for analysis of ACF 42 days later.

Materials and methods

**Animals and diets**

Semi-synthetic diets were prepared as indicated in Table 1 with either casein (acid casein; Nestlé Nederland BV, Amsterdam) or a soya isolate (Protein...
Table 1. Composition of diets (g/kg)

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<tr>
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<th>Control</th>
<th>Genistein</th>
<th>Soya protein</th>
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<tr>
<td>Soya proteina</td>
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<td>0</td>
<td>208</td>
</tr>
<tr>
<td>Caseinb</td>
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<td>438</td>
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<td>200</td>
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</tr>
<tr>
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<td>100</td>
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<tr>
<td>Vitamin mixd</td>
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<td>2</td>
<td>20</td>
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<tr>
<td>ni-Methionine</td>
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<td>2</td>
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<tr>
<td>Genistein</td>
<td>0</td>
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aSoya protein isolate containing 87% (w/w) of protein with 1.23 mg genistein/g protein and <2 mg Ca²⁺/g protein.
bAcid casein (moisture 10%, free acidity ≤0.02% and fat ≤1.5%).
cMineral mix: (g/kg) CaHPO₄, 325; NaHPO₄, 185; CuCO₃, 205; KCl, 175.75; MgSO₂·H₂O, 100; ZnCO₃, 0.75; FeSO₄·7H₂O, 3.6; CuSO₄·5H₂O, 0.375; KIO₃, 0.025; MnSO₄·H₂O, 4.5.
dVitamin mix: (g/kg) nicotinic acid, 3.0; cyanocobalamin (B12) in mannitol, 2.5; D-pantothenic acid (calcium D-pantothenate), 2.0; thiamin hydrochloride, 0.5; riboflavin (B2), 0.5; pyridoxine, 0.5; pteroylmonoglutamic acid (folic acid), 0.5; niacin, 0.5%: vitamin K, 0.1. All components mixed and ground, passed through a 250 µm sieve, then added to 90 g of choline bitartrate and 890.85 g of starch. Rovimix E-50 (7.5 g), Rovimix A-500 (1.25 g) and Rovimix D-3500 (0.75 g) were added to the above and the whole mixed.

Technologies International, St Louis, MO, USA) as the protein source. The level of lipid (200 g/kg) was selected to be more typical of human diets than of a conventional rat diet. The concentration of genistein (Apin Chemicals Ltd, Abingdon, UK) in the soya-based diet was equivalent to that in the pure genistein-based test diet (0.25 g/kg). Seventy male Wistar rats (~150–180 g) were obtained from a commercial supplier and housed singly in polycarbonate cages with wire bottoms and tops, in air-conditioned small animal rooms having an ambient temperature of 21°C, 55% humidity and a 12 h–12 h light–dark cycle. They were allowed free access to water and fed a commercially prepared pelleted diet for 1 week, randomly allocated into the experimental groups described below and then maintained on the control diet for a further week before the experiment was started.

Experimental design

To assess the acute effects of diets containing genistein on crypt cell proliferation in the colon, three groups of eight rats were fed the phytoestrogen-free control diet, the purified genistein diet or the soya protein diet, respectively, for 7 days, before receiving a sham injection (0.9% NaCl) subcutaneously into the neck scruff. All animals were killed 48 h later and tissues were collected for analysis of mitosis and apoptosis. To investigate the effects of previous exposure to genistein on crypt cell proliferation and apoptosis induced by DMH, three groups of 13 rats were given phytoestrogen-free control diet, the purified genistein diet or the soya protein diet, respectively, for 7 days before a subcutaneous injection of DMH into the neck scruff (30 mg/kg). All animals were switched back to the control diet immediately after this first DMH injection, and five from each group were killed after 48 h, a time point at which, as we have previously observed, crypt cell apoptosis remains elevated and susceptible to manipulation by diet (21). The remaining eight rats in each group were maintained on the control diet, receiving a second DMH injection after 7 days, and were killed 42 days later for analysis of ACF. A further two groups of eight animals were fed the control diet before and between DMH injections, transferred to the genistein or soya protein diet after DMH treatment and killed after 42 days. In all cases food was removed at the time of DMH injections and restored 18 h later.

Recovery and preparation of tissues

Following deep anaesthesia with sodium barbitual (Euthatal; Rhone Merieux, Harlow, UK) and cervical dislocation, laparotomy was performed and abdominal organs removed. The mass and length of the small intestine and colon, and the mass of the caecum (full), spleen, liver and testes were recorded. Segments of whole intestine (~1 cm) from the proximal jejunum and distal ileum (10% and 95% of the overall small intestinal length, respectively), mid caecum, proximal and distal colon were transferred to ethanol:acetic acid (75:25) for subsequent examination and quantification of mitotic and apoptotic figures.

Analysis of crypt cell apoptosis and proliferation

The frequencies of apoptotic and mitotic cells within intact microdissected crypts were determined using morphological criteria as previously described (21,22). Briefly, samples fixed in ethanol:acetic acid (75:25) were rehydrated in 50% ethanol and distilled water, hydrolysed in 1 M HCl for 7 min at 60°C, rinsed, stained with Feulgen’s reagent (15 mM basic fuschin, 45 mM magnesium metabisulphite, 5% 1 M HCl in distilled water) for 30 min, rinsed in distilled water and stored in 45% aqueous acetic acid overnight. Under a dissecting microscope, the muscle layers were teased from the mucosa and thin strips of crypts were micro-dissected and lightly compressed under a cover slip to separate and flatten the crypts. Ten randomly chosen intact crypts per animal were viewed under a light microscope (magnification ×400). Apoptotic cells were identified by the presence of condensed chromatin and spherical apoptotic bodies containing nuclear material as described by Kerr et al. (23) and were clearly distinguishable from nucleated blood cells. All cells in prophase, metaphase, anaphase, and telophase were classified as mitoses.

Aberrant crypt foci

To count ACF, the entire colon was divided into proximal and distal halves each 7–8 cm in length. These were cut longitudinally and placed in fixative (ethanol:acetic acid; 75:25) in glass vials, keeping the samples as flat as possible, then rehydrated and bulk-stained with Feulgen’s reagent as described above, and mounted on a slide under cling film to maintain a flat surface. The mucosal surface of the distal colon was scanned for ACF under a light microscope (magnification ×100) and the frequency and type of lesion was recorded (Figure 1). By focusing the microscope on the mucosal surface, enlarged and slightly elevated lesions were readily identifiable by comparison with the normal adjacent mucosa. Lesions were classified as single enlarged crypts or foci containing two or more abnormal crypts with thickened epithelial linings and enlarged luminal openings (22).

Statistics

Data were analysed either by one-way analysis of variance (with Tukey’s method to assess significance of differences between individual means) or, where indicated in the text, by two-way analysis of variance. All calculations were carried out using Minitab software (State College, PA, USA). Data are expressed as means with standard errors.

Results

Food consumption and growth

Rats fed the genistein or soya protein for 1 week did not consume significantly more or less food than the control group. Injection with DMH transiently suppressed food intake in all groups to a similar extent, but final body mass was not affected by diet or DMH administration. Feeding diets containing either pure genistein or soya protein before DMH treatment had no effect on the pattern of weight gain over the following 42 days, but the same diets fed after DMH tended to retard this, although not significantly (Figure 2a). Soya-based diet, when fed after the DMH treatment, also acutely depressed the food conversion efficiency (Figure 2b). However, food conversion
efficiencies in the final week of the experiment were not significantly different across the groups.

**Organ weights**

Neither the genistein- nor the soya protein-supplemented diets had any significant effects on small intestinal or colonic fresh mass and length, or on the fresh weights of the full caecum and the spleen. Animals fed the soya protein diet and receiving a single DMH injection had livers 14.5% lighter than those of similarly treated animals on the control diet ($P < 0.05$), but this difference was no longer statistically significant when liver weights were normalized for body mass.

**Crypt cell mitosis and apoptosis**

Incorporation of genistein into the diet had no effect on mitosis in the small or large bowel, and there was no detectable interaction with DMH 48 h after injection (Figure 3a). Treatment with DMH was, however, associated with a small reduction in mitosis in the colon. Acute increases in crypt cell apoptosis occurred in the small and large intestines of all groups 48 h after treatment with DMH, and two-way analysis of variance confirmed a significant effect of treatment with DMH (Figure 3b). There were no statistically significant differences in crypt cell mitosis or apoptosis at either site among the groups 42 days after treatment with DMH (Figure 4). **Aberrant crypts**

There was a significantly higher incidence of both ACF (Figure 5a) single aberrant crypts (Figure 5b) in the distal colons of animals fed the genistein-enriched ($P < 0.001$) and soya-enriched ($P < 0.05$) diet for 1 week before treatment with DMH than in controls fed casein diets only, but no effect of diet in animals fed genistein or soya protein after DMH treatment.

**Discussion**

The mucosal epithelial cells of the small and large intestine are among the most highly proliferative tissues of the body. The rate of cell turnover is tightly regulated by endogenous
control mechanisms, but it can also be modified by diet. For example, in rats, crypt cell mitosis is stimulated by certain non-starch polysaccharides (24) and suppressed by polyunsaturated fatty acids of the n-3 series (22). The main route of cell loss from the intestinal mucosa is by exfoliation into the lumen, but apoptosis also occurs within the crypt, and this probably plays an important role in the preservation of tissue integrity and the prevention of neoplasia (25). Non-steroidal anti-inflammatory drugs such as Sulindac cause regression of precancerous colonic lesions in animals (26) and human findings are consistent with recent biological properties are exclusively benign. The complex, potentially genotoxic effects of genistein observed in vitro is attributable to its ability to inhibit mammary gland cell proliferation and stabilize the DNA-topoisomerase-cleaved complex (32). This event can lead to double-strand breaks, aberrant repair and lethal mutations occurring during treatment with DMH or a reduced level of DNA repair or apoptosis during the recovery phase. We cannot firmly distinguish between these possibilities in the present study.

One widely accepted mechanism for increased susceptibility of a tissue to DNA damage is an increased rate of mitosis (34), but as there was no evidence of any effect of genistein on crypt cytokinetics this effect is probably irrelevant to the present study. Much of the cytotoxic effect of genistein, genistein-enriched or soya protein-enriched diets, before or after DMH, for 42 days. Data are means with standard errors. Rats receiving genistein or soya before treatment with DMH had significantly greater numbers of ACF than other groups *P < 0.05; **P < 0.001.

The oestrogenic effects of isoflavones and the strong epidemiological evidence for a protective effect of soya products against breast cancer, have generated considerable interest in genistein and there is perhaps a presumption that its biological properties are exclusively benign. The complex, potentially genotoxic effects of genistein in vitro are often overlooked. There is little or no epidemiological evidence that soya isoflavones are protective against colorectal cancer (38) and previous animal studies on the effects of genistein on colorectal neoplasia have produced evidence for both anti-carcinogenic (16) and pro-carcinogenic (19) effects in animal with the lack of any anti-proliferative or pro-apoptotic activity in the mucosa, but there was a significant and unexpected increase in ACF in animals fed genistein or soya protein before treatment with DMH, and this requires explanation. The protocol used in the present study was designed to explore the effects of timing of exposure to genistein on the outcome of induction of neoplasia with DMH. When the genistein and soya diets were given before DMH treatment, the isoflavonoid and the carcinogen were able to act simultaneously on the colonic crypt cells, whereas when genistein was available only after metabolism of DMH was complete, it could not have exerted any effects during the phase of induction of DNA damage. This type of protocol has been used previously to study anticarcinogens and, in particular, to distinguish between blocking and suppressing agents (30), but in the present study it provided evidence of a pro-carcinogenic effect of genistein exposure before and during initiation of DNA damage by DMH.

DMH is an alkylating carcinogen that causes neoplastic changes in colonic mucosa at a level proportional to the quantity of methylated DNA adducts induced in colonic crypt cells (31). ACF are neoplastic lesions which appear to precede the development of tumours, via the adenoma–carcinoma sequence, in the distal colon of rats treated with DMH (17). They frequently contain K-ras mutations characteristic of genomic damage by alkylating agents (32,33), so the numbers of ACF reflect the level of unrepaired DNA damage induced in the mucosa after exposure to DMH. An elevated level of tumorigenic mutations might reflect increased DNA damage occurring during treatment with DMH or a reduced level of DNA repair or apoptosis during the recovery phase. We observed no suppression of ACF by genistein in the present study. Much of the cytotoxic effect of genistein observed in vitro is attributable to its ability to inhibit mammary gland cell proliferation and stabilize the DNA-topoisomerase-cleaved complex (32). This event can lead to double-strand breaks, aberrant repair and lethal mutations occurring during treatment with DMH or a reduced level of DNA repair or apoptosis during the recovery phase. We cannot firmly distinguish between these possibilities in the present study.

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models of colorectal neoplasia. In this study we have shown a clear enhancement of ACF induction by both genistein and soya protein which appears to act only on the initiation stage of colorectal tumour induction. The implications of these findings for human health are unclear, but the effects of genistein on rapidly proliferating tissues exposed to genotoxic carcinogens deserve further study and the potentially genotoxic effects of genistein should be taken into account by those considering the overall biological activity of this compound.

Acknowledgement
This work was supported by the Office of Science and Technology through the Core Strategic Grant of the BBSRC and by the European Commission COST Action 916.

References