Allyl-isothiocyanate causes mitotic block, loss of cell adhesion and disrupted cytoskeletal structure in HT29 cells

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Epidemiological evidence indicates that Brassica vegetables protect against colorectal cancer. Brassicas contain glucosinolates, the breakdown products of which exhibit antiproliferative effects against cancer cells. We have examined the effects of allyl-isothiocyanate (AITC), a major breakdown product of the glucosinolate sinigrin, on proliferation and death of colorectal cancer cells. HT-29 colorectal cells were exposed to AITC for 24 h and the number of adherent and detached cells determined. Both populations were analysed for cell-cycle characteristics and examined by light and electron microscopy for features of apoptosis and mitosis. Evidence of apoptosis was also determined by flow cytometric analysis of Annexin V staining in the detached population of cells. AITC-treated cells were also stained for α-tubulin. Treatment caused cells to round up after 7 h of exposure and subsequently detach. At 24 h these cells were blocked in mitosis. Detached AITC-treated cells showed no signs of apoptosis as assessed by morphological features or by Annexin V staining but they did show evidence of disrupted tubulin. AITC inhibits proliferation of cancer cells by causing mitotic block associated with disruption of α-tubulin in a manner analogous to a number of chemotherapeutic agents.

Introduction

Epidemiological and experimental studies suggest that diets rich in Brassica vegetables protect against colorectal cancer (1–3). The tissues of Brassica vegetables are a good source of many potentially protective dietary factors including folic acid, phenolics, carotenoids, selenium and vitamin C (4–6), but one group of chemical constituents, the glucosinolates, are specific to these vegetables (7,8). Isothiocyanates (ITCs) are a major group of breakdown products of glucosinolates, produced during food preparation, during digestion and by colonic bacteria (9,10).

ITCs induce phase 2 enzymes (11–13), thereby blocking the action of potential carcinogens. However, rodent studies show that pure glucosinolates and ITCs, given after chemical induction of cancer, reduce the incidence of pre-cancerous lesions (14,15). This suggests that the modulation of carcinogen metabolism is not their only mode of action. In a previous study we showed that oral treatment of rats with the glucosinolate sinigrin led to an increase in apoptosis in colonic crypts within 48 h of exposure to the chemical carcinogen 1,2-dimethylhydrazine (DMH) (14). Sources of sinigrin and its metabolite allyl-isothiocyanate (AITC) in the diet include horseradish, cabbage and Brussels sprouts. Recently, we reported that oral administration of raw Brussels sprouts also led to a significant enhancement of apoptosis in the colorectal crypts of rats treated previously with DMH (16). It is becoming increasingly well recognized that a low level of apoptosis, rather than increased mitosis, is a good predictor of risk of colorectal neoplasia in both animal models (17) and in humans (18).

Many ITCs have a cytotoxic effect against tumour cell lines (19–26), and AITC is an effective, and apparently selective, inducer of cell death in both colorectal cancer cells (21,27) and prostate cells (28). This induction of cell death is dose-dependent (27) and has been linked to a block in cell cycle at G2/M in both Hela cells (19) and prostate cells (28). The aim of the present study was to investigate how AITC affects cell-cycle progression and survival in the colorectal cell line HT29, using flow cytometric analysis of cell-cycle distribution and Annexin V staining. We gained further insight into mechanism of action by assessing morphological changes, using both light and electron microscopy, and examination of the spindle-protein α-tubulin. Our study suggests that the effect of AITC is to cause a mitotic block, a phenomenon frequently associated with disruption of the mitotic spindle, and α-tubulin condensation.

Materials and methods

Cell culture and AITC treatment

HT29 colorectal cancer cells obtained from ECACC at passage 135 were grown in DMEM (Sigma-D5671, Gillingham, UK) supplemented with l-glutamine (2 mM), penicillin (50 μg/ml), streptomycin (50 μg/ml) and 10% fetal calf serum (Sigma). Cultures were incubated in 5% CO2 at 37°C. HT29 colorectal cancer cells obtained from ECACC at passage 135 were split 1:8 every 7 days and media changed every 3 days. AITC (Sigma), was added to ethanol (1 μM) and then serially diluted incomplete DMEM media to the desired working concentration (12 μM) immediately prior to the addition to cells. Cells were harvested from log-phase cultures and resuspended in 3 ml of warm DMEM medium at 1 × 10^6 cells/25 cm² flask. Flasks were then incubated at 37°C for 72 h to achieve logarithmic growth. Two hours prior to challenge with AITC the cells were removed, replaced with 3 ml of fresh medium and returned to the incubator. Ethanol was added to control flasks to a final concentration (0.1%) identical to that of treated flasks. For subsequent experiments this step was removed as no effect of ethanol was detected.

Determination of cell number

Seven and 24 h after exposure to AITC, adherent cells and those that had detached from the flask surface were harvested separately. The incubating medium was centrifuged and the number of detached cells per flask assessed after re-suspending the cell pellet in a known volume of PBS. The adherent population was removed by trypsinization (0.25% trypsin; 0.02% EDTA in PBS, pH 7.4. Sigma), centrifuged and counted after re-suspending the cell pellet in a known volume of PBS. Cell numbers were determined using a haemocytometer.

Cell-cycle analysis

The cellular DNA content was assessed using the method of Clarke et al. (29). Cell-cycle analysis by flow cytometry was performed on cells 24 h after challenge with AITC (12 μM) as described above. After washing twice in

Abbreviations: AITC, allyl-isothiocyanate; DMC, 1,2-dimethylhydrazine; ITC, isothiocyanates; PI, propidium iodide.
PBS containing 1 mM EDTA, samples of cells were re-suspended in 80% v/v ice-cold ethanol at a density of $5 \times 10^3$ cells/ml and fixed on ice for 30 min. Cells were then washed twice in PBS before being re-suspended in 1 ml of staining solution, consisting of PBS containing 30 μg/ml ribonuclease A, 0.1% v/v Triton X-100 and 50 μg/ml propidium iodide (PI) (all Sigma). Cells were stained for 30 min at room temperature and analysed by flow cytometry. Data were analysed on logarithmic scales for forward angle and right-angle light scatter, and on a linear scale for red fluorescence. Non-cellular material was excluded from the analysis by gating on the forward angle and right-angle light-scattering characteristics of either adherent or detached cells. Red fluorescence intensity was used for the determination of position within the cell cycle, using Phoenix Multicycle for Windows cell-cycle analysis software (Coulter Electronics, Luton, UK). Cells with a DNA content of less than 2 n were designated as apoptotic, whilst those ranging from 2 to 4 n were designated as being in G1, S or G2/M phases of the cell cycle. Non-adherent cells from three flasks were pooled to give sufficient numbers for analysis. The adherent cells from each flask were analysed separately.

Annexin V-FITC labelling of externalized phosphatidyl serine

Externalization of phosphatidyl serine (PS) and the assessment of membrane integrity in the detached population of cells were assessed using the ApoDetect V-FITC kit (Zymed Laboratories, San Francisco, CA) designed for the quantification of these apoptotic biomarkers. Twenty-four hours after challenge detached cells were harvested by first removing the medium from all flasks to universal tubes. To ensure complete removal of detached cells the remaining adherent cells were then washed with 3 ml PBS, which was then added to the removed media. Any detached cells present in the medium and PBS washings were spun down in a centrifuge at 1000 r.p.m. for 5 min. The resulting cell pellet was washed in 1 ml PBS prior to staining. Sub-samples of $5 \times 10^3$ cells were re-suspended in 100 μl binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) and 10 μl Annexin V-FITC (10 μg/ml) were added. Cells were allowed to stain in the dark, at room temperature, for 10 min before the addition of 800 μl of binding buffer to each sample. Cells were then pelleted and re-suspended in 500 μl of the binding buffer, and 20 μl of PI (20 μg/ml) solution was added. Cells were then analysed for red and green fluorescence by flow cytometry. Both red and green fluorescence, as well as forward angle and right-angle scatter, were analysed on a logarithmic scale. Green fluorescence defines cells in which PS was present on the outer membrane, reflecting loss of membrane asymmetry. This is an earlier stage in apoptosis than the characteristic morphological changes considered as ‘the gold standard’ for apoptosis. PI can only enter cells when membrane integrity is compromised, such as occurs in necrosis, giving rise to red fluorescence staining of the chromatin. Necrosis may occur as a consequence of apoptosis in cell culture systems where no phagocytic removal of cells can occur, as would take place in vivo. Thus, live cells were defined as those showing no fluorescence, and apoptotic cells as those showing only green fluorescence. Cells exhibiting both green and red fluorescence were designated late apoptotic/necrotic and red cells were regarded as necrotic.

Light and transmission electron microscopy

Cells were seeded and challenged with AITC for 24 h as described above for cell-cycle analysis. Samples of control (untreated) and AITC-treated (12 μM) cells were taken from the re-suspended detached and adherent populations prior to centrifugation and prepared for transmission electron microscopy. Cells were fixed in 3% glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 M cacodylate buffer (pH 7.2), for 1 h at room temperature. After three washes in 0.1 M cacodylate buffer (pH 7.2) pelleted cells were embedded in molten low-melting point agarose (Type VII, A-4018, Sigma), which was then chopped into small pieces. These pieces were post-fixed in 2% aqueous osmium tetroxide and dehydrated through an ethanol series. After three washes in 100% ethanol, the pieces were transferred to acetone, then infiltrated and embedded in Spurr resin (Agar Scientific) (30). The sections (~75 nm thick) were cut, collected on copper grids and stained sequentially with uranyl acetate (saturated in 50% ethanol) and Reynolds’s lead citrate. Sections were examined and photographed in a JEOL 1200 EX/B transmission electron microscope at 60 kV. For the determination of the number of cells in each phase of the cell cycle, sections (1 μm thick) from the same block were stained with 1% toluidine blue in 1% sodium borate and examined at ×40 under a light microscope (Olympus BX60 microscope and images recorded with AcQuis Software, Cambridge, UK). Eight random views per treatment were assessed for features of chromosome condensation associated with mitosis and apoptosis.

Assessment of cytoskeletal changes by fluorescent staining of α-tubulin

The integrity of α-tubulin in adherent and detached cells 24 h after treatment with AITC was assessed on cells grown in 25 cm² flasks as described above. Adherent and detached cells were harvested as described previously and 200 μl of each cell suspension were sited onto glass slides using a cytospin centrifuge (MegaFuge 1.0R, Heraeus) at 600 r.p.m. for 8 min. We chose to use this approach so that attached and detached cells could be processed in an identical manner to ensure a proper comparison. Microtubules were visualized using the method of Haggarty (31). Cells were fixed with 0.2% glutaraldehyde and then stained with a mouse anti-α-tubulin antibody (Molecular Probes, OR, USA, A-1126) followed by Alexa-Fluor® 488 goat anti-mouse conjugate (Molecular Probes, A-11001). Chromatin was labelled with 0.1% ethidium bromide. Cells were viewed using an Olympus BX60 microscope with red and green filters and images recorded with AcQuis Software.

Statistical analysis

Unless stated otherwise, experiments were run in triplicate and results compared in Excel by two-tailed unpaired t-test. In the case of flow cytometric analysis the statistical analysis within the curve-fitting programme was used to determine significance.

Results

Determination of cell number

Challenging HT29 cells with AITC (12 μM) resulted in the shedding of cells from the surface of the flask into the surrounding medium 24 h after treatment. Cell detachment was not apparent 7 h after challenge, but inspection through an inverted microscope revealed that adherent cells treated with AITC were rounded up: an appearance normally seen in cells undergoing mitosis. At 24 h, results from five separate experiments showed that under control conditions 2.2% (SEM = 0.7%) of cells became detached from the flask surface, whereas treatment with AITC resulted in an average of 25.2% (SEM = 8.6%) of cells becoming detached. When the ratio of detached cells to total cell number in treated flasks was expressed relative to the ratio in control flasks, it was apparent that an average of 17.2 (SEM = 6.7) times as many cells became detached in response to AITC treatment for 24 h. Over the same period the number of adherent cells in AITC-treated flasks was 53.9% of control cell number (SEM = 12.6%).

Flow cytometric analysis of cell cycle and Annexin V-FITC labelling

Cell-cycle analysis, undertaken 24 h after challenge with AITC showed a shift in the cell-cycle phase distribution in response to treatment. In the adherent population of cells treated with AITC, fewer cells were in the G1 population and significantly more (P < 0.05) in G2/M (Figure 1) when compared with untreated controls. Data obtained for detached cells collected 24 h following treatment with AITC showed that practically all cells were in the G2/M phase of the cell cycle. Apoptosis was not detected as a sub G0 peak during cell-cycle analysis, even in the control detached cells. However, as we have reported previously, under these conditions the sub G0 population cannot be defined by curve fitting analysis as no discrete population can be selected, even when, as in the present study, apoptosis is clearly detectable by Annexin V or TUNEL staining (29).

Staining to detect the presence of externalized PS by Annexin V revealed that very few apoptotic, damaged or necrotic cells were present in the detached population (Figure 2). The majority (90%) of detached cells remained unstained, suggesting that they were intact, with an undamaged membrane and no PS translocation. These values were significantly higher (P < 0.05) than in untreated control cells (35%).

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**Histology**

The use of electron microscopy allows for unequivocal identification of cells in interphase, metaphase and apoptosis, as shown in Figure 3A-C. By examining identically produced semi-thin resin sections under a light microscope it was possible to quantify the numbers of cells with these different phenotypes. This analysis revealed that in the adherent population of untreated control cells 98% of cells were in interphase and 1% in mitosis (Figure 4A).

Twenty-four hours after AITC exposure (Figure 4B) this figure had not changed significantly, as 96% were in interphase and 3% in metaphase. The detached population of untreated control cells (Figure 4C) consisted of cells in either apoptosis (57%) or necrosis (34%). In contrast, the detached population of AITC-treated cells was composed almost entirely of apparently healthy cells (Figure 4D). The majority of these cells were in metaphase (89%) with only a small number (5%) in apoptosis.

**Cytoskeletal structure**

α-Tubulin fluorescent staining revealed that the cytoskeletal structure of a small percentage of adherent cells treated with AITC was disrupted (Figure 5B), with staining restricted to small areas of globular α-tubulin on the periphery of the nucleus. A similar pattern of disrupted tubulin was also detectable in the detached cells (Figure 5C), and in this case a majority of cells were affected. In control populations a small percentage of cells had clearly visible spindle apparatus (Figure 5A) but no such intact structures were seen in either the adherent or detached AITC-treated populations.

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**Fig. 1.** Cell-cycle analysis of both adherent and detached populations of the HT29 cell line 24 h after challenge with AITC (12 μM). Data show the percentage of cells in G0/G1 (black column), S (shaded column) and G2/M (clear column) (expressed as mean and standard error, n = 3 for adherent cells; asterisks indicate significant differences between treatment and control for each phase of the cell cycle). The data for detached cells represent pooled samples to give sufficient cells for analysis.

**Fig. 2.** Flow cytometric analysis of detached population of HT29 cells, 24 h after challenge with AITC (12 μM), following staining with Annexin V and PI. The graph shows the percentage of cells designated as: healthy (PI⁻/AnV⁻) (black column), early apoptotic (PI⁻/AnV⁺) (hatched column, left diagonal down), late apoptotic/necrotic (PI⁺/AnV⁻) (hatched column, right diagonal down), necrotic damaged (PI⁺/AnV⁺) (clear column). (PI = propidium iodide, AnV = Annexin V.) Values are expressed as means and SD from three experiments and significant difference (P < 0.05) between test and control for each category indicated by asterisks.

**Fig. 3.** Transmission electron microscopy of cells in interphase (A), metaphase (B) and apoptosis (C). (Scale bar = 2 μm.)
Discussion

We have shown previously that both sinigrin, and Brassica vegetables rich in sinigrin, suppress mitosis and selectively amplify apoptosis in rats that have recently undergone treatment with the carcinogen DMH, but have no detectable effects on control untreated animals (14,16). ITCs have been reported previously to induce apoptosis in a variety of different tumour cell lines (21,28,32–35). In the present study, 7 h after initial exposure to AITC, cells begin to ‘round up’, a phenomenon usually associated with mitosis. At this point there was no significant detachment of cells, but subsequently cells became detached from the surface of the flask and floated in the medium. Rounding-up and detachment of HT29 cells after ITC challenge has been reported by others. In the study of Gamet-Payrastre et al. (34) treatment with sulforaphane, an ITC found in broccoli and a potent inducer of phase II enzymes, caused a degree of cell-cycle arrest and apoptosis in cells 48 h after challenge. The cell-cycle block was shown to be in the G2/M phase. Hasegawa et al. (19) also found that the ITC phenethyl-ITC, benzyl-ITC and AITC inhibit cell-cycle progression of HeLa cells at the G2/M phase. Because cell detachment was such a prominent response to AITC treatment we chose to investigate responses in both the adherent cells and the population of cells, which had detached from the substratum over the 24-h period following exposure to AITC. Although a small but significant increase in the numbers of cells in G2/M was observed in the adherent population, analysis of the detached population, 24 h after challenge with AITC, revealed that nearly all cells were in the G2/M phase of the cell cycle. An increase of cells in G2/M may be caused either by faster cell proliferation or a block in the cell cycle at this point. However, we report data in this paper that confirm our previously published results showing that AITC causes a reduction in cell number 24 h after exposure (27). Thus, the increase in the percentage of cells in G2/M seen here suggests a cell-cycle block rather than increase in cell proliferation.

In the present study we were unable to detect an increase in apoptosis in the attached population of cells treated with AITC when compared with untreated controls, and although about half of untreated detached cells were apoptotic, whether defined histologically (57%) or by Annexin V staining (45%), only 5 and 7%, respectively, of the AITC-treated detached cells were apoptotic when assessed by these two methods. This contrasts with previously published results using other ITCs such as sulforaphane (34) or phenethyl ITC (24,33,36) where apoptosis was reported, assessed either histologically or by DNA laddering. However, in the present

Fig. 4. Analysis of semi-thin sections by light microscopy. Ninety-eight per cent of the control adherent population were in interphase (A) and after 24 h exposure to AITC (B) this figure had not changed significantly. The untreated detached population consisted mostly of apoptotic or necrotic cells (C). In contrast the AITC-treated detached population consisted almost entirely of healthy cells in mitosis (D). (Scale bar = 10 μm.)
study, the number of detached cells following AITC treatment was ~17-fold greater than in control conditions. Therefore, if we consider the level of apoptosis in the total cell population (attached and detached), the number of apoptotic cells is ~17 \times \frac{7}{45} = 2.6\text{-fold} greater after treatment with AITC than in controls. This is consistent with the 2-fold increase in apoptosis seen in animal studies using the parent glucosinolate, sinigrin or Brussels sprouts (14,16). Consistent with this observation is the fact that, as we have reported previously (27), caspase activity is increased at 24 h in the detached population of cells treated with AITC, indicating the onset of apoptosis. However, prior application of caspase inhibitors did not inhibit detachment (27) suggesting that induction of caspase activity is not the prime cause of detachment and reduction in cell number.

The flow-cytometric method used in this study for the analysis of the cell-cycle distribution was unable to distinguish between the G2 and M phases of the cell cycle. However, the evidence provided by electron microscopy showed that the majority of the detached population of AITC-treated cells were in metaphase. Metaphase-arrest has not been reported for ITCs before, but it is a common characteristic of compounds that interfere with microtubule formation (37,38). Microtubules are essential structural molecules needed for cell division, but once cells enter mitosis the spindle must be disassembled to allow separation of the daughter cells. Compounds that interfere with assembly or disassembly of microtubules have been shown to induce metaphase arrest and apoptosis in a variety of cancer cells (37). These compounds fall into two categories: those that block polymerization of tubulin to form microtubules, such as vincristine, vinblastine and colchicines, and those that promote polymerization and stabilize tubulin, such as the therapeutic drug Paclitaxel (39). In the present study, fluorescent labelling of \( \alpha \)-tubulin revealed well-formed mitotic apparatus in the small proportion of adherent control cells undergoing mitosis. In contrast, intact spindles were not seen in either adherent or detached AITC-treated cells. However, areas of disrupted \( \alpha \)-tubulin were visible around the periphery of the nucleus, 24 h after treatment with AITC, with the numbers of such cells being higher in the detached population when compared with attached cells, as would be predicted from the increased numbers of cells seen in metaphase. It should be noted that in AITC-treated cells the disrupted tubulin had neither the appearance of depolymerized tubulin nor the classical appearance of condensed tubulin. This may be a consequence of the method of preparation, and the precise details of the tubulin abnormalities therefore require further investigation. Our preliminary results indicating tubulin disruption by AITC are supported by recently reported findings in mammary cells showing disruption of tubulin by sulforaphane (40). However, in this case the effect of sulforaphane at 15 \( \mu \text{M} \) was described as ‘mild’ and concentrations of 100–300 \( \mu \text{M} \) were required to get a clear response. At higher doses these authors show tubulin condensation.

Cell death induced subsequent to metaphase arrest has recently been described as mitotic catastrophe (41), which has various manifestations, including formation of micronuclei with individual nuclear envelopes. Such multiple nuclear membranes were not seen by electron microscopy in this study. However, this may have been a matter of timing. The apparent preferential sensitivity to AITC of tumour cells compared with differentiated cells (23) may be due to deficiencies in the prophase checkpoint by a mechanism analogous to that proposed for Paclitaxel (41). The mechanism underlying AITC-mediated tubulin disruption and metaphase arrest, and its resemblance to known tubulin-disrupting chemotherapeutic drugs such as paclitaxel or colchicines, requires further investigation.

Fig. 5. Fluorescent micrographs showing HT29 cells after immunofluorescent staining for \( \alpha \)-tubulin, and counter-staining with ethidium bromide. Under control conditions a small proportion of attached cells contained intact spindles (A), but a similar fraction showed disrupted tubulin after exposure to 12 \( \mu \text{M} \) AITC for 24 h (B). Most cells that became detached following treatment with AITC showed varying degrees of disruption to the cytoskeleton (C). The large arrow identifies a mitotic cell, and the narrow arrows show cells typical of those only seen following AITC treatment. (Scale bar = 10 \( \mu \text{m} \).)
In conclusion, Brassica vegetables containing high concentrations of sinigrin, the parent glucosinolate of AITC, may protect against colorectal cancer in part through modulation of post-initiation events. The most likely mechanism is that glucosinolates are broken down to biologically active products in food preparation, during digestion, or in the large intestine by bacteria. These biologically active products are taken up into colorectal cells, either from the colonic lumen or from the circulation. We suggest that, in the crypt epithelial cells, the active compounds disrupt tubulin, either directly or indirectly, and block dividing cells in metaphase. In tumour cells cultured in vitro, cells round-up and detach, but do not immediately enter apoptosis; rather they remain as intact cells for several hours. In vivo, damaged cells would enter apoptosis and would subsequently be engulfed by phagocytic T cells rather than proceed to necrosis as is seen in cell culture (42). This could give rise to the selective amplification of apoptosis in animals previously treated with DMH as we have reported previously (14, 16). Such a mechanism would parallel many characteristics of microtubule interfering compounds such as Paclitaxel (37).

References

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