All compounds have shown previously that allyl isothiocyanate (AITC), a constituent of cruciferous vegetables, significantly inhibits survival of PC-3 and LNCaP human prostate cancer cells in culture, whereas proliferation of a normal prostate epithelial cell line is minimally affected by AITC even at concentrations that are highly cytotoxic to the prostate cancer cells. The present studies were designed to test the hypothesis that AITC administration may retard growth of human prostate cancer xenografts in vivo. Bolus i.p. injection of 10 μmol AITC, three times per week (Monday, Wednesday and Friday) beginning the day of tumor cell implantation, significantly inhibited the growth of PC-3 xenograft (P<0.05 by two-way ANOVA). For example, 26 days after tumor cell implantation, the average tumor volume in control mice (1025 ± 205 mm³) was ~1.7-fold higher compared with AITC-treated mice. Moreover, the results of the present study indicate that AITC administration inhibits growth of PC-3 xenografts in vivo by inducing apoptosis and reducing mitotic activity.

Abbreviations: AITC, allyl isothiocyanate; Cdc25, cell division cycle 25; Cdk1, cyclin-dependent kinase 1; ITCs, isothiocyanates.

These authors contributed equally.

Sanjay K.Srivastava1, Dong Xiao1, Karen L.Lew, Pamela Hershberger, Demetrius M.Kokkinakis, Candace S.Johnson1, Donald L.Trump1 and Shivendra V.Singh1

Departments of Pharmacology, Pathology and Medicine, and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

1Present address: Roswell Park Cancer Institute, Buffalo, NY, USA

2To whom correspondence should be addressed at: 2.32A Hillman Cancer Center Research Pavilion, University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, 5117 Center Avenue, Pittsburgh, PA 15213, USA

Email: singhs@msx.upmc.edu

We have shown previously that allyl isothiocyanate (AITC), a constituent of cruciferous vegetables, significantly inhibits survival of PC-3 and LNCaP human prostate cancer cells in culture, whereas proliferation of a normal prostate epithelial cell line is minimally affected by AITC even at concentrations that are highly cytotoxic to the prostate cancer cells. The present studies were designed to test the hypothesis that AITC administration may retard growth of human prostate cancer xenografts in vivo. Bolus i.p. injection of 10 μmol AITC, three times per week (Monday, Wednesday and Friday) beginning the day of tumor cell implantation, significantly inhibited the growth of PC-3 xenograft (P<0.05 by two-way ANOVA). For example, 26 days after tumor cell implantation, the average tumor volume in control mice (1025 ± 205 mm³) was ~1.7-fold higher compared with AITC-treated mice. Moreover, the results of the present study indicate that AITC administration inhibits growth of PC-3 xenografts in vivo by inducing apoptosis and reducing mitotic activity.
Materials and methods

Materials

AITC was purchased from Aldrich (Milwaukee, WI). Antibodies against Bax, BID, Bcl-X<sub>L</sub>, cell division cycle (Cdc)25C and cyclin-dependent kinase 1 (Cdk1) were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against cyclin in situ peroxidase (Cdc15), antibodies against Cdc25B were from BD Transduction (San Diego, CA), and the antibodies against cyclin B1 and actin were from Oncogene Research Products (Boston, MA). PC-3 cells were obtained from American Type Culture Collection (Rockville, MD). Male athymic mice (6–10 weeks old) were purchased from Harlan Sprague Dawley (Madison, WI).

Cell culture and colony formation assay

Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn’s modification) supplemented with 7% (v/v) non-heat inactivated fetal bovine serum and 10 ml/1 PSN Antibiotic Mixture ( Gibco BRL, Grand Island, NY). PC-3 cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The experiments described in this paper were conducted using PC-3 cells that were passaged <25 times. The cytotoxic effect of AITC was assessed by colony formation assay as described by us previously (17) with slight modifications. Briefly, 2 × 10<sup>4</sup> cells were plated in 6-well plates, and allowed to attach overnight. Subsequently, the medium was replaced with fresh complete medium containing desired concentrations of AITC or equal volume of DMSO, and the plates were incubated for 10 days at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. AITC or DMSO containing medium was replaced every 48 h. The colonies were fixed in ethanol and stained with a Giemsa solution (Sigma, St Louis, MO). Colonies containing >50 cells were counted under an inverted microscope. The IC<sub>50</sub> value was determined from a plot of percentage of survival versus AITC concentrations.

In vivo xenograft assay

Mice were fed AIN-76A semipurified diet (ICN Biomedicals, Aurora, OH) for 8 days prior to the start of the experiment. The animals were maintained on this diet throughout the duration of the experiment. PC-3 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA), and a 0.1 ml suspension containing 10<sup>6</sup> cells was injected subcutaneously on both left and right flank of each mouse. Mice were randomized into two groups of five mice per group (two tumors per mouse). The experimental group of mice received bolus i.p. injection of 10 μmol AITC in 0.1 ml cottonseed oil three times per week (Monday, Wednesday and Friday) beginning the day of tumor cell implantation. Control mice received an equal volume of vehicle (cottonseed oil) three times per week. We used cottonseed oil as a vehicle for AITC administration not only because this ITC compound is insoluble in aqueous solution but also because cottonseed oil has been used previously for administration of other ITCs to mice (18). AITC dose of 10 μmol was selected because (i) it was within the range (3–12 μmol) of concentrations used previously for other ITCs (1,2,18,19) and corresponded to the IC<sub>50</sub>, for AITC in PC-3 cells (~15 μM), and (ii) 20 μmol AITC (three times per week) exhibited toxicity to nude mice (Srivastava et al., unpublished observations). Body weights of the mice of both groups were determined periodically to assess non-specific toxicity of AITC. Tumor measurement began once each mouse had palpable tumor. Tumor size was determined three times per week using a caliper. Tumor volume was calculated as described by us previously (20). The experiment was terminated 26 days after tumor cell implantation because tumors of control mice began to show signs of possible necrosis. The tumor tissues of control and AITC-treated mice were harvested, washed with ice-cold phosphate-buffered saline and divided into two pieces. One piece was processed for histology and immunohistochemistry, whereas the second portion was used for western blotting.

Histological analysis of mitosis and apoptosis

Tumor tissues were fixed in 10% neutral-buffered formalin overnight. Subsequently, the tissues were dehydrated, embedded in paraffin and sectioned (6 μm) every 100 μm intervals. Sections were stained with hematoxylin and eosin and mitotic activity was defined as the number of cells undergoing mitosis per high-power view (400×). For apoptosis, paraffin-embedded sections (6 μm) were de-paraffinized and immunostained using ApoTag plus peroxidase in situ apoptosis detection kit (Intergen, NY) according to manufacturer’s instructions. The sections were counterstained with methyl green and brown color apoptotic bodies were counted at 400× magnification.

Western blot analysis

Tumor tissues from control and AITC-treated mice were minced and suspended in a lysis solution described by us previously (16). Tumor tissues were homogenized using a Polytron, and the homogenate was centrifuged at 14,000 g for 15 min. The supernatant fraction was collected and used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (21). The proteins were transferred onto PVDF membrane. After blocking for 1 h with 10% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was probed for 1 h at room temperature with the desired primary antibody. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were visualized using enhanced chemiluminescence kit (NEL Life Science Products, Boston, MA). Each membrane was stripped and re-probed with antibodies against actin to correct for differences in protein loading.

Statistical analysis

Statistical significance of difference in tumor volume and body weight between control and AITC-treated mice was assessed by two-way ANOVA. Statistical significance of differences in apoptotic and mitotic cells and protein expression between control and AITC-treated tumors were determined by Student’s t-test.

Results

Cytotoxic effect of AITC on PC-3 cells was determined by colony formation assay, and the results are shown in Figure 1A. The colony formation by PC-3 cells was significantly inhibited in the presence of AITC with an IC<sub>50</sub> of ~2.2 μM (Figure 1A).

Figure 1B shows the effect of AITC treatment on growth of PC-3 tumor xenografts in nude mice. Each mouse of both the groups had measurable tumor on day 7 after tumor cell implantation. AITC treatment resulted in a statistically significant inhibition (P < 0.05 by two-way ANOVA) of PC-3 xenograft growth, and the growth inhibitory effect of AITC was evident in terms of tumor volume (Figure 1B) as well as wet tumor weight (data not shown). For example, on day 21, the average tumor volume in control mice (640 ± 95 mm<sup>3</sup>, n = 10) was ~1.8-fold higher compared with AITC-treated mice. Similarly, the average tumor volume in control group was ~1.9-fold higher than that in AITC-treated group (464 ± 58 mm<sup>3</sup>) on day 24. These results indicated that the growth of PC-3 xenograft in nude mice was retarded upon AITC administration.

The body weights of the control and treated mice were determined periodically to assess non-specific toxicity of AITC. The average body weights of the control and AITC treated mice did not differ significantly by two-way ANOVA suggesting that AITC administration did not cause weight loss (Figure 1C). The mice in AITC-treated group appeared healthy and did not show any other sign of non-specific toxicity, such as food and water withdrawal and impaired movement.

We have shown previously that reduced survival of PC-3 cells following exposure to AITC is associated with G2/M arrest and apoptosis induction (16). To determine whether AITC-mediated in vivo growth inhibition of PC-3 xenografts was caused by increased apoptosis and/or reduced mitotic activity, the tumors from control and AITC-treated mice were harvested at the termination of the experiment for analysis of cells undergoing apoptosis and mitosis. As shown in Figure 2, histological analysis revealed a statistically significant decrease in the number of cells undergoing mitosis in tumors of AITC-treated mice compared with control tumors. Adjacent sections from the same tumor were analyzed for apoptotic bodies using TUNEL assay, which showed a significantly higher count of apoptotic bodies in tumors of AITC-treated mice compared with controls (Figure 2).

To gain insights into the mechanism for increased apoptosis in tumors of AITC-treated mice, the tumors of both groups were analyzed for the expression of Bcl-2 family of proteins.
that are known to regulate programmed cell death (22,23). Western blots for the expression of Bcl-2, Bcl-XL, Bax and BID in tumors of control and treated mice are shown in Figure 3. Densitometric scanning of the immunoreactive bands, followed by correction for differences in protein loading, indicated an ~70% reduction in Bcl-2 expression in the tumors of AITC-treated mice compared with controls ($P < 0.05$ by Student’s $t$-test). On the other hand, the expression of Bcl-XL did not differ significantly between tumors of control and AITC-treated mice. Similar to Bcl-XL, the expression of Bax was not affected by AITC treatment. On the other hand, AITC treatment resulted in cleavage of BID, which is known to promote apoptosis (24). These results indicated that increased apoptosis in tumors of AITC-treated mice may be due to down-regulation of Bcl-2 and increased cleavage of BID.

To gain insights into the mechanism for reduced mitotic activity in tumors of AITC-treated mice, the expression of cyclin B1, Cdk1, Cdc25B and Cdc25C was compared by western blot analysis, and the results are shown in

Fig. 2. Histological analysis of mitotic activity and apoptotic bodies in tumors of control and AITC-treated mice. Tumors from control and AITC-treated mice were harvested 26 days after tumor cell implantation, and processed for scoring of mitotic activity and apoptotic bodies as described in ‘Materials and methods’. Three separate tumors from control and AITC-treated mice were analyzed. At least five separate randomly selected fields on each slide were analyzed. Data are mean ± SE. *Significantly different compared with control, $P < 0.05$ by Student’s $t$-test.

Fig. 3. Western blot analysis for expression of Bcl-2, Bcl-XL, BID and Bax using tumor lysates from control and AITC-treated mice. The blots were stripped and re-probed with antibodies against actin to correct for differences in protein loading.
when compared with control tumors (scanning of the immunoreactive bands revealed a reduction in expression of cyclin B1, Cdc25B and Cdc25C, respectively, in the tumors of AITC-treated mice compared with control tumors. Densitometric scanning of the immunoreactive bands revealed a reduction of ~44, 45 and 90% in expression of cyclin B1, Cdc25B and Cdc25C, respectively, in the tumors of AITC-treated mice when compared with control tumors (P < 0.05 by Student’s t-test). Interestingly, the expression of Cdk1 was significantly higher in the tumors of AITC-treated mice relative to control tumors (Figure 4).

Figure 4. Western blot analysis for expression of cyclin B1, Cdk1, Cdc25B and Cdc25C in tumors of control and AITC-treated mice. The blots were stripped and re-probed with antibodies against actin to correct for differences in protein loading.

Discussion

Prostate cancer is the second leading cause of cancer-related deaths among men in the US (26). The incidence of prostate cancer has steadily increased over the years, and accounts for ~28% of all cancers in American men (27). While molecular mechanisms underlying the onset and/or progression of human prostate cancers are poorly defined, race, age, androgen secretion and metabolism, and diet are the identifiable risk factors associated with this malignancy (27–30). Clinical management of human prostate cancer has been challenging mainly due to limited treatment options (31). Thus, identification of agents that can delay onset and/or progression of human prostate cancers could have a profound impact on clinical management of this deadly malignancy. Recent epidemiological studies have suggested that increased intake of cruciferous vegetables may be protective against prostate cancer risk (32,33). Despite compelling epidemiological correlation, however, no attempts have yet been made to identify the anticancer agents in cruciferous vegetables.

We have demonstrated previously that AITC is highly effective in suppressing proliferation of PC-3 cells in culture (16). Growth suppressive effect for sulforaphane, a natural analog of AITC, has also been documented in LNCaP cells (34). Based on the results of our cellular studies (16), we hypothesized that AITC may be effective in retarding growth of PC-3 cells in vivo. The present study was undertaken to systematically test this hypothesis. We found that the growth of PC-3 xenografts in nude mice is significantly retarded upon AITC administration. To the best of our knowledge, the present study is the first published report on in vivo anticancer activity of a natural ITC compound in a human prostate cancer xenograft model.

The results of the present study indicate that AITC-mediated in vivo growth inhibition of PC-3 tumor xenografts is associated with an increase in apoptosis as well as a reduction in cells undergoing mitosis in the tumor mass. These observations are consistent with cellular studies where treatment of PC-3 cells, as well as LNCaP cells with AITC resulted in apoptosis induction and G2/M phase cell cycle arrest (16). Mechanisms for AITC-induced apoptosis and cell cycle arrest based on our observations in cultured PC-3 cells (16) and PC-3 xenografts (present study) are summarized in Figure 5. The present study reveals that increased apoptosis in the tumors of AITC-treated mice is associated with down regulation of Bcl-2. These results are consistent with our observations in cultured PC-3 cells (16). Similarly, in agreement with our observations in PC-3 cells (16), the expression of Bcl-XL or Bax in vivo is not altered upon AITC administration (present study). It is interesting to point out that apoptosis induction by sulforaphane in HT29 human colon cancer cell line has been shown to be associated with over-expression of Bax but independent of a change in Bcl-2 expression (13). While reasons for this discrepancy are not yet clear, it is possible that the mechanism for ITC-induced apoptosis may be cell line or ITC compound specific.

The tumors from AITC-treated mice exhibited cleavage of p23 BID protein to a p15 fragment. BID cleavage in human leukemic HL-60 cells exposed to 10 μM PEITC, another analog of AITC, has been observed previously (14). The proapoptotic protein BID upon processing by caspase-8 to a p15 fragment is shown to initiate loss of mitochondrial membrane permeability (24) (Figure 5). Thus, it is reasonable to postulate that AITC treatment may activate caspase-8, but studies are needed to validate this possibility.

We have shown previously that a 24 h exposure of PC-3 cells to 10 or 20 μM AITC results in accumulation of cells in G2/M phase (16). The present study revealed a statistically significant decrease in cells undergoing mitosis in the tumor mass from AITC-treated mice compared with control tumors. Eukaryotic G2/M progression is regulated by Cdk1–cyclin B1 kinase complex, which is maintained in an inactive form due to phosphorylations at Thr14 and Tyr15 of Cdk1 (25) (Figure 5). Dephosphorylation, and hence activation of Cdk1 is mediated by dual specificity phosphatases Cdc25B and Cdc25C (25). Present study indicates that the expression of cyclin B1, Cdc25B and Cdc25C is reduced significantly in the tumors of AITC-treated mice compared with that of control mice. The expression of cyclin B1, Cdc25B and Cdc25C is also reduced upon treatment of cultured PC-3 cells with AITC (16). Thus, it seems reasonable to postulate that reduced mitotic activity in AITC-treated tumors may be due to accumulation of inactive Cdk1–cyclin B kinase complex.

In conclusion, the results of the present study indicate that AITC, a constituent of cruciferous vegetables, significantly inhibits growth of PC-3 xenografts in vivo by inducing apoptosis and decreasing proportion of cells undergoing mitosis. The present study is the first published report on in vivo activity of an ITC analog against human prostate cancer.
Acknowledgements

This investigation was supported in part by USPHS grants CA55589 and CA101753 (to S.V.S.), awarded by the National Cancer Institute.

References