Indole-3-carbinol (I3C) exhibits inhibitory and preventive effects on prostate tumors in mice

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Abstract

Prostate cancer (PC) is the most commonly diagnosed malignancy for men in Western countries. Research showed that cruciferous vegetables containing indole derivatives were involved in cancer prevention. This study was designed to investigate the effect of indole-3-carbinol (I3C) in cell lines and on PC tumor growth in mice when given as a therapeutic and as a preventive treatment. The effect in vitro of I3C on the viability, proliferation and apoptosis of mouse PC cell line TRAMP-C2 and on bovine capillary endothelial (BCE) cells were examined using MTT, BrdU and FACS analyses. The effect of I3C (20 mg/kg body weight) as both a therapeutic and a preventive treatment on the growth of PC cells, inoculated subcutaneously in C57BL/6 mice, was evaluated using tumor volume measurements and immunohistochemistry. I3C decreased the proliferation rate in 3-folds (staining to Ki-67), and promoted apoptosis (staining with caspase 3). I3C, injected intraperitonially (I.P.), significantly inhibited the tumor growth (a 78% decrease in tumor volume) and affecting the angiogenesis process by decreasing the microvessel density (CD31 endothelial marker) and complexity. I3C has a significant inhibitory effect on PC cells in vitro and in vivo, and offers a potential usage as both preventive and therapeutic agent for humans.

Keywords: Indole-3-carbinol; Prostate cancer; Preventive treatment; Therapeutic treatment; TRAMP-C2 cells; Angiogenesis

1. Introduction

Prostate cancer (PC) is the most commonly diagnosed malignancy and second leading cause of cancer-related death for men in Western industrialized countries. Due to its high incidence and mortality, the costs associated with its detection and treatment, and the fact that no consensus exists as to what constitutes the best treatment for any stage of the disease, it is a major issue in public health (Cookson, 2001; Tang and Porter, 1997). If PC is not detected early, or is allowed to reach a more aggressive form of the disease, it may advance to stages characterized by local invasion of the seminal vesicles, followed by metastases primarily to the bones and the lymphatic nodes, usually resulting in death. These metastases are due to successful tumor establishment, which depends also on the angiogenesis process (the development of new capillaries from an existing vascular networks), to provide oxygen and nutrients to rapidly proliferation cells (Folkman, 2000). Since little attention was given to the study of controlling tumor angiogenesis in PC, it is a major area to explore, for the development of preventive and therapeutic
2. Materials and methods

2.1. Materials

Indole-3-carbinol (I3C), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), propidium iodide (PI) and RNase were purchased from Sigma (St. Louis, MO, USA). Cell culture media and reagents were obtained from Biological Industries (Israel). 5-bromo-2-deoxyuridine (BrdU) was from Roche Diagnostics (Germany). Monoclonal rat anti-mouse Ki-67 antigen was obtained from DakoCytomation (Denmark). Monoclonal rat anti-mouse CD31 was obtained from BD Biosciences, Pharmingen (USA). Biotinylated anti-rat IgG was obtained from Vector Laboratories (USA). Cleaved caspase 3 antibody was purchased from Santa Cruz Biotechnology. All other chemicals were purchased from Sigma or from local sources.

2.2. Cell culture

For the in vitro and in vivo studies a TRAMP-C2 mouse cell line (obtained from Prof. R. Apte, Ben-Gurion University of the Negev, Israel), were used (Greenberg et al., 1995). These cells are epithelial, which express androgen receptors and do not have a mutated p53 (Grossmann et al., 2001; Foster et al., 1997). The cells were maintained in high-glucose DMEM with l-glutamine, containing 5% FCS and supplemented with 5% Nu-Serum, insulin (5 μg/ml), penicillin-streptomycin (25 units/ml) and dihydrotestosterone (10⁻¹⁰ M). Bovine capillary endothelial (BCE) cells (a gift from Dr. Juda Folkman, Harvard University, USA) were maintained in DMEM containing 5% FCS, 5% penicillin-streptomycin and 5% Nu-Serum. All cells were cultured at 37 °C and 5% CO₂.

2.3. Cell viability and proliferation assays

The effect of I3C on cell viability and proliferation was determined using the MTT and BrdU assays. The viability of the cells was determined using MTT according to Mosmann (1983). Data were recorded as percentage of cell growth relative to the respective control (cells treated with medium only) for each concentration of I3C.

The BrdU assay for proliferative cells was carried out as described by Porstmann et al. (1985).

2.4. Apoptosis assay

The effect of I3C on apoptosis was evaluated by determining DNA fragmentation of the cells treated with I3C, compared to the untreated group. FACS measures the fraction of nuclei containing a hypodiploid DNA content (sub-G1 phase), which is a characteristic feature of apoptosis. For the measurement, cells were harvested when reaching 40,000 cells/well and then treated for 24–72 h with a concentration of 150 μM I3C. This concentration was chosen since no cytotoxic effects were observed in the LDH assay for culture lysis in both cell lines (data not shown). The cells were then washed with PBS and fixed in 4 ml of cold ethanol (4 °C). DNA staining was achieved by re-suspending the cells in PBS containing propidium iodide (50 μg/ml) and 0.1% RNase. Cells were counted using a flow cytometer (Cell Quest, Becton Dickinson FACScan). The percentage of apoptotic cells was determined for 20,000 cells, and statistically analyzed with Multicycle software (Phoenix Flow Systems). Data are reported as percentage of apoptotic cells out of the untreated respective controls.

2.5. Animals

Six-week-old male C57BL/6 mice, 24.4 ± 3.3 g, (Harlan Laboratories, Israel) were housed in polycarbonate cages (5 mice/cage) and were kept in a room lighted 12 h per day and maintained at 22 ± 1 °C.

2.6. In vivo experiments

The mice were inoculated subcutaneously (S.C.) with 5 × 10⁶ TRAMP-C2 cells. When the tumor size reached 150–200 mm³ (measured by caliper as described by Kang et al., 1999) the mice were randomly distributed into three groups of 15 animals each, and treated as follows: therapeutic treatment group (T.T.), therapeutic treatment control group (T.T.C.) and untreated control group (U.C.). The T.T. group was treated I.P. with I3C dissolved in corn oil (20 mg/kg), three times a week for 14 days starting on day zero. I.P. administration allows I3C to achieve maximal systemic exposure (Garikapasy et al., 2004). The T.T.C. group
was treated only with corn oil and the U.C. group, the negative control group, was not treated at all. A preliminary trial with mice, using 10 mg/kg and 20 mg/kg, based on the study of Kedmi et al., showed that although a dose of 10 mg/kg did show an inhibitory effect on the tumors, the higher dose level was more effective (data not shown). Hence, we decided to use the higher dose, namely 20 mg/kg. Tumor sizes and mice weights were measured thrice a week, and tumor volumes were calculated using the formula \( \text{volume} = \frac{1}{2} \times \text{width}^2 \times \text{height} \times \pi \). In addition, a preventive study (P.T.) was performed, in which only 4 I.P. injections of I3C (20 mg/kg) were given to 20 mice every other day, before inoculating the tumor cells. The preventive group was not subjected to any further treatment with I3C. The preventive treatment control group (P.T.C.) was given corn oil only.

Both the therapeutic and the preventive treatments were terminated on day 14, since some tumors in the control groups started to show necrosis. The mice were sacrificed, tumors were harvested and tumor volumes and weights were measured. In addition, blood samples were collected in order to examine the liver and kidney functions, commonly used to characterize the status of their metabolism (levels of ALT, ASTL, LDH, ALP, L6, URE and CREJ), as well as the liver weights. The in vivo experiments were conducted under the approval and the guidance of the Technion Ethics Committee.

2.7. Immunohistochemistry

Tumors were embedded in OCT (Tissue-Tek; Sakura Finetec USA, Inc.), frozen on dry ice butane and stored at \(-80\) °C. Tumor sections (5 μm) were stained using hematoxylin–eosin. Immunohistochemical analyses of CD31 for endothelial cells and microvessel density, Ki-67 for cell proliferation and caspase 3 for apoptosis were performed on tumor sections using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Detection was carried out using a DAB chromogen, which results in a positive brown staining. Negative control slides were obtained by omitting the primary antibody.

Measurement of microvessel density (MVD) was carried out within the hot spots and calculated as described by Sabo et al. (2001). MVD represents the mean percentage of the microvessel area within the microscopically computerized field.

The proliferation index was defined as the average of 3 field counts within the 5 microscopic fields which showed most advanced proliferation (“hot spots”), calculating the percentage of Ki-67-positive tumor cells out of the total number of counted cells. The results represent the means ± SD.

The number of apoptotic cells was assessed for the average of 3 field counts within the 5 microscopic fields, which showed the most advanced apoptosis (“hot spots”), calculating the number of caspase 3-positive tumor cells out of the total number of counted cells. The results represent the means ± SD.

2.8. Fractural analysis of microvessels (MFD)

The box-counting method is widely used for calculating the image fractural dimensions, as described by Sabo et al. (2001). The MFD values range from 1 to 2.

2.9. Data analysis

The MTT assay was repeated three times, each with eight replicates. The BrdU and the FACS assays were repeated thrice, each in triplicate. Hence, data for these assays are given as means ± SE (standard error), whereas data for the in vivo experiment are presented as means ± SD. For the FACS assay, differences between the control groups and the groups treated with I3C were analyzed using Student’s t-test. Results for the BrdU and MTT assays were analyzed using only ANOVA, whereas in the case of the various immunohistochemical data both ANOVA and the Bonferroni post hoc test were used. Levels of significance were indicated by the number of asterisk signs.

3. Results

3.1. Effect of I3C on cell viability and proliferation in vitro

Treating TRAMP-C2 and BCE cells with I3C led to a decrease in cell viability, as shown by the MTT assay (Fig. 1), and proliferation, as shown by the BrdU assay (Fig. 2), compared to the untreated cells. These effects were time- and dose-dependent and were different between the two cell lines. As can be seen from the MTT assay for TRAMP-C2 cells (Fig. 1A), the effect of treatment time on cell viability was already visible after 24 h, and after 48 and 72 h the effect became more pronounced \((p < 0.001)\). However, there was no statistically significant difference between the latter two time intervals in this respect. The effect of I3C on the viability of the BCE cells (Fig. 1B) was smaller, even at the highest dose after 72 h, compared to TRAMP-C2 cells, which reached less than 10% viability with a dose of 300 μM after only 48 h. Generally, the effect of time in TRAMP-C2 cells after 48 and 72 h was significantly higher than that observed for BCE cells \((p < 0.05)\). In the BrdU assay for TRAMP-C2 cells (Fig. 2A), the decrease in DNA synthesis, even after 24 h, was significantly higher than the one observed for BCE

![Fig. 1. Effect of indole-3-carbinol (I3C) on the viability of TRAMP-C2 cells (A) and BCE cells (B). Cell viability was determined by the MTT assay, after incubating the cells with different concentrations (50–400 μM) of I3C for 24–72 h, as described under Section 2. All data presented are the means ± SE and expressed as percentages of the respective controls.](image-url)
cells (Fig. 2B) even after 72 h ($p < 0.01$). In the case of both TRAMP-C2 and BCE cells, no statistically significant difference in the effect of the last two time intervals (48 and 72 h) was observed.

3.2. Effect of I3C on apoptosis induction

The flow cytometric analysis indicated the appearance of the hypodiploid sub-G1 phase in cells treated with I3C, thus verifying its apoptotic effect. These results suggested that I3C induced apoptosis and that this effect depended on cell type and duration of exposure (Fig. 3). As can be seen for TRAMP-C2 cells (Fig. 3A), the number of apoptotic cells as percentages of the respective controls after 72 h was significantly larger than that observed for BCE cells after 72 h ($p < 0.01$).

3.3. In vivo experiments

The preventive and therapeutic effects of I3C, on tumor formation and inhibition respectively were studied in male mice (Figs. 4–7).

On the 11th day after cell inoculation, it was evident that while all groups, with the exception of the one receiving the preventive treatment, exhibited an average tumor size of 170 mm$^3$, the average tumor size in the latter group was 30 mm$^3$. The findings shown in Fig. 4 indicate that the treatment with I3C before and after transplanting of prostate cancer cells caused, at the end of the experiment (day
14), a 78% decrease in tumor volume, compared to the controls (\(p < 0.001\)). These significant differences were already observed 6 days after the therapeutic treatment (\(p < 0.01\)) compared to the respective controls.

As shown in Fig. 5, there is a significant difference (\(p < 0.05\)) in tumor weight between the preventive treatment (P.T.), compared to its respective control (P.T.C.). A significant difference (\(p < 0.01\)) was also evident for the therapeutic treatment (T.T.) compared to its controls (T.T.C., U.C.).

Immunohistochemistry with Ki-67 antibody showed that the three control groups (T.T.C., P.T.C. and U.C.), in as far as the percentage of proliferative cells is concerned, showed a similar proliferation index (49.2 ± 15.9), as shown in Fig. 6A. There were no significant differences between the group receiving the preventive treatment (P.T.) and the group receiving the therapeutic treatment (T.T.), (16.3 ± 7.1). The number of prolerative cells in the control groups was significantly larger than for the groups treated with I3C (\(p < 0.001\)). Representative CD31-stained microscopic fields from tumors with low and high MVD values are given in Fig. 7. A significantly higher MVD index was noted in the three control groups (Fig. 7A) (MVD = 8.08% ± 2.41), whereas in the preventive and therapeutic treatment groups (Fig. 7B) the MVD index was lower, (MVD = 3.91% ± 1.49). For measuring a parameter of architectural microvascular complexity we evaluated the MFD. A significantly higher vascular complexity was noted in the control groups (MFD = 1.44 ± 0.05), in comparison with the preventive and therapeutic treatment groups (MFD = 1.31 ± 0.09, \(p < 0.001\)). The number of apoptotic cells, measured by caspase 3 cleavage, was significantly smaller in the control groups (0.63 ± 0.15), whereas in the treatment groups it was 1.94 ± 0.41, \(p < 0.01\).

Blood tests for the assessment of liver and kidney functions and liver weights, and also the growth rate of the mice, indicated that I3C does not have any adverse effects at the levels used in this study.

4. Discussion

Since the incidence of PC in recent years has considerably increased it appears very important to investigate potential therapeutic means and, more importantly, preventive treatments (Jemal et al., 2003). We have previously shown the suppressive effect of DIM, a major in vivo metabolite of I3C, and I3C itself on the growth of breast and prostate cancer cell lines, respectively, under both in vitro and in vivo conditions (Ge et al., 1996; Ge et al., 1999).

In this study we showed, in vitro, using the MTT assay, that I3C decreased the viability of PC cell line TRAMP-C2. Kedmi et al. reported a similar inhibitory effect of I3C on the viability of LNCaP cells, which, like TRAMP-C2 cells, possess p53 wild type and express androgen receptors (AR) (Kedmy et al., 2003). A suppressive effect has also been noticed in normal BCE cells. However, when compared to TRAMP-C2 cells this effect was smaller 48 and 72 h after the exposure to I3C. The results point to the fact that,
for both cell types, the time and dose decreased cell viability. Normal endothelial cells, from the same source as TRAMP-C2 cells, were not available in this research; however, the BCE cells used as a model for investigating the growth of vascular endothelial cells, are of a key importance in the angiogenesis process.

The BrdU assay showed that I3C significantly inhibited cell proliferation (by 80%). This inhibition was achieved using 400 μM I3C, after 48 h. This effect was about 50% greater than in the BCE cells, for the measured concentrations and time intervals. In both cases, the effect was time- and dose-dependent, and was mediated through the induction of apoptosis, as measured by FACS. The results indicate that there was a significant difference between the two cell types in the severity of the effect of I3C on apoptosis. The percentage of apoptotic cells in the case of TRAMP-C2 cells was 2-fold higher after 72 h, compared to BCE cells. The results of this assay strengthen the assumption that I3C decreases cell viability and proliferation rate, by inducing apoptosis. This assumption is also supported by an earlier study, carried out in our laboratory, showing that the induction of apoptosis in TRAMP-C2 cells was manifested by DNA fragmentation (Kedmi et al., 2004). A similar apoptotic effect of I3C was observed after 48 h on CaSki (epidermoid cervical carcinoma cells), which also originated from transgenic mice (Chen et al., 2001). Furthermore, the addition of I3C to prostate and breast cancer cells in culture has been found to induce arrest of cell cycle (Cover et al., 1998).

Following the in vitro studies, we tested the efficacy of I3C in vivo on prostate tumor inoculated in mice. The in vivo assay was designed with a view to examining the potential activity of I3C when given as both a preventive and a therapeutic treatment. Mice treated with 20 mg/kg I3C before and after receiving the cell inoculation (groups P.T. and T.T., respectively), showed similar tumor volumes from the 6th day after the therapeutic treatment, compared to the three control groups. The therapeutic treatment proved to be as effective as the preventive one, although – initially – the two treatment groups did not exhibit similar tumor volumes. From day 10 till the end of the experiment (day 14) the difference between the tumor volumes of both the therapeutic and the preventive treatment groups were about one third of the tumor volume observed for the control groups. All the controls (P.T.C., T.C. and U.C. groups) did not differ from each other in this respect. The observed differences in tumor volume are also manifested in the results of the tumor weight measurements. Srivastava et al. showed that when a dose of 250 μg I3C/100 μl was applied topically twice a week for 28 weeks, prior to the skin cancer promotion stage, I3C inhibited the development of tumors (Srivastava and Shukla, 1998). In order to gather more information regarding the mode of action of the suppression of tumor growth, one of our first aims was to examine the possibility that I3C was involved in reducing the proliferation rate of the tumor cells in vivo, in comparison with the in vitro results shown above. Immunohistochemical staining to Ki-67 antibodies revealed that in the treated groups there was a significant decrease in the proliferation index of the cells, located in the peripheral areas of the tumor, compared to the control groups. The results may explain the decrease in tumor growth in the treated groups. The lower the number of prostate tumor cells measured by staining to Ki-67 the less aggressive the cancer is (Pollack et al., 2004). Earlier studies made in our laboratory showed a decrease in proliferation of TRAMP-C2 cells when mice were treated with 5–10 mg DIM/kg as a therapeutic treatment only.

The impact of I3C on the endothelial cells (BCE) in vitro led to the hypothesis that I3C may play a role in tumor angiogenesis. Tumor angiogenesis is a dynamic process, mediated by vascular growth factors and their receptors (Yancopoulos et al., 2000; Carmeliet and Jain, 2000). Complex modulating interactions between tumor cells, matrix metalloproteinases and endothelial cells, establish the final density and complexity of the tumor microvasculature. The data presented show that there is a decrease in the endothelial marker CD31 staining in the treated
groups, compared to the controls. Intratumoral MVD index was significantly lower (about 50%) in groups receiving the I3C treatments, compared to the control groups. The MVD values provide an indication for the risk of development of metastases, as well as for the prediction of the patient’s survival (Herbst et al., 1998). The tumor vessel net in the control tumor samples was longer and twisted, unlike those observed in the I3C treated groups. Hence, we decided to compute the MFD values, since this method serves as a common parameter in tumor research. The more complex vascular pattern defined by increasing method serves as a common parameter in tumor research. Hence, we decided to compute the MFD values, since this method provides an indication for the risk of the patient’s survival (Herbst et al., 1998). The tumor index was significantly lower (about 50%) in groups receiving the I3C treatments, compared to the control groups. Other studies found that HUVEC cells were affected by DIM, which inhibited proliferation, invasion, migration and tube formation (Chang et al., 2005). After examining the impact of I3C on cell proliferation and vascularization both in vitro and in vivo, we explored its apoptotic effect using staining with anti-caspase 3 antibody. Caspase 3 is believed to be the main executioner protease in the apoptotic cascade. The number of apoptotic cells in the treated groups was three times higher than in the control groups.

We assume that one of the feasible ways through which I3C causes a decrease in the tumor volume is by inducing apoptosis, as was observed in our FACS analysis (in vitro). It appears logical to attribute the inhibition in growth of TRAMP-C2 cells (which are androgen-dependent) to the decrease in the number of nuclear androgen receptors by I3C, as established in a previous investigation for the influence of I3C on estrogen receptors in MCF-7 cells (Ge et al., 1999). This assumption is supported by a recent study, showing the inhibitory effect of I3C on AR expression and down-regulation of androgen responsiveness in human PC cells (Hsu et al., 2005). Since the transition to metastatic disease is generally ended with androgen-independent cell population, the question whether I3C can also be used in this case, as a treatment, appears to be a reasonable possibility. Unpublished data from our own laboratory support the earlier report by Kedmi et al. showing that DIM and Ascorbigen, both derivatives of I3C, caused an inhibitory in vitro effect on androgen-independent prostate cancer cell lines DU145 and PC3 (Kedmy et al., 2003). Also Garikapasy and co-workers showed the anti-carcinogenic and anti-metastatic properties of I3C in androgen-independent PC when administered I.P. (Garikapasy et al., 2004).

DIM is the main active acid condensation product of I3C and much is known about its anti-cancer activity. Our study indicates that even I3C itself, which was considered to be a less potent compound than DIM, had a significant effect on prostate tumor growth, not only when given as a therapeutic treatment to mice but also as a prophylactic agent. This effect was related to the ability of I3C to reduce the tumors proliferation index, to induce apoptosis and, above all, to inhibit the angiogenesis process.

Since a dietary intervention study provides the strongest possible evidence for an effect of the diet on the risk of acquiring certain cancers, it seems that consumption of larger daily amounts of cruciferous vegetables, or taking encapsulated I3C-containing pills, may be recommended as a feasible approach to prostate cancer chemoprevention (Chan et al., 1998; Giovannucci et al., 1995). Moreover, the same approach also appears to constitute an effective therapeutic treatment as well.

As is stressed in Section 3, no abnormalities in liver and kidney functions, or in growth curve, were observed in the treated groups, probably indicating that the I3C treatments, at the levels employed in this study, did not bring about untoward effects, as judged by the parameters used, thus attesting that I3C is probably safe.

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References


