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Therapeutic Activity of 3,3′-Diindolylmethane on Prostate Cancer in an In Vivo Model

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BACKGROUND. Prostate cancer (PC) is the second leading cancer-related death in men in Western countries. Hence, efficient anti-carcinogenic and therapeutic compounds against PC are badly needed. We have previously shown that 3,3′-diindolylmethane (DIM) has a suppressive effect on the growth of human breast and PC cell lines. The objective of this study was examination of the potential therapeutic effects of DIM in an in vivo model.

METHODS. TRAMP-C2, a mouse PC cell line, was injected into the flank of male C57BL/6 mice. When tumors appeared, mice were injected intraperitoneally with either corn oil (vehicle) or DIM (2.5, 5, or 10 mg per kg body weight) 3-times a week, for 3 weeks, and tumor volumes were measured bi-weekly with calibermeters. Later, the tumors were removed, their final weights and volumes were measured, and tumor sections were tested for histological studies.

RESULTS. DIM had a significant inhibitory effect, caused by diminished tumor growth. Histological examination of tumors from treated groups revealed apoptosis and decreased cell proliferation, compared with the controls. DIM didn’t affect body weights or kidney and liver functioning.

CONCLUSIONS. The inhibitory action of DIM on tumor growth was demonstrated in vivo. Hence, this compound at the concentrations tested may offer an effective and non toxic therapeutic means against tumor growth in rodents, and may serve as a potential natural anti-carcinogenic compound in humans.

KEY WORDS: apoptosis; 3,3′-diindolylmethane; in vivo; prostate cancer; TRAMP-C2 cell lines

INTRODUCTION

Prostate cancer (PC) is the most commonly-diagnosed malignancy accounting for 29% of the newly diagnosed cancers and the second leading cause of male death in Western industrialized countries [1–3]. Mortality from PC results from metastases to the bones and lymph nodes and progression from androgen-dependent to androgen-independent prostatic growth [4]. Androgen withdrawal causes involution of the prostate gland, as a result of inhibition of cellular proliferation and stimulation of apoptosis of the androgen-dependent cells. Although androgen withdrawal remains the only effective therapy for men with advanced disease, in approximately 80% of the patients, progression to the lethal and untreatable stage of androgen-independence eventually occurs [5,6]. Therefore, it is important to explore new strategies for the treatment of PC, that will be effective against both androgen-dependent and -independent cancer cells.

Many Epidemiological studies have shown a strong correlation between diets rich in fruits and vegetables and a lower risk of cancer of various types in people consuming such diets [7,8]. A number of studies have demonstrated a decreased incidence of various cancers (including PC) in humans consuming large amounts of Cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, and cauliflower [9,10]. These

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vegetables contain glucobrassicin, which undergoes hydrolysis by myrosinase upon crushing, or by cooking [11,12]. The main hydrolysis product of glucobrassicin is indole-3-carbinol (I3C). In a low pH environment, I3C is converted into many polymeric products, among which 3,3'-diindolylmethane (DIM) is the main one [11,13]. It was shown that these products have apoptotic properties. The apoptotic effects of I3C and DIM has been previously demonstrated in human breast [14–16] and prostate [17] cancer cell lines. These studies indicated that indole-derivatives induce apoptosis through p53- and bax-independent pathways. Other studies have shown that I3C and DIM have an inhibitory effect on the viability and proliferation of prostate [18], colon [19,20], cervical [21], and endometrial [22] cancer cell lines.

Animal studies have reported that MNU- and DMBA-induced tumors in rats were inhibited by providing the animals with cabbage or broccoli in the diet [23,24]. Other studies have shown that in mice or rats fed indolic compounds there was a significantly lower incidence of both carcinogen-induced [25–28] and spontaneous tumors [29,30], and the time intervals for the appearance of cancer was longer.

Animal models are a crucial element in the development of cancer chemoprevention agents. The core objective of using animal models in this process is preclinical efficacy testing of candidate chemoprevention compounds in in vivo models of cancer development, to provide an important screening test for the selection of agents for clinical trials [31]. The purpose of the current study was to examine the potential beneficial effects of DIM in the treatment of the subcutaneously-transplanted tumor cell line, TRAMP-C2, in C57BL/6 mice.

**MATERIALS AND METHODS**

**Materials**

DIM was purchased from Designed Nutritional Products (USA). Cell culture media and reagents were obtained from Biological Industries (Israel). [3H]-methyl thymidine was purchased from Amersham Biosciences (UK). 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) was purchased from Sigma (Saint Louis, MO). Monoclonal rat anti-mouse Ki-67 antigen was obtained from DakoCytomation (Denmark). Biotinylated anti-rat IgG was obtained from Vector Laboratories (USA). All other chemicals were purchased from Sigma or other local sources.

**Cell Culture**

TRAMP-C2, a mouse PC cell line, originating from transgenic C57BL/6 mice, was used in this study. These cells are epithelial, p53 wild-type, express androgen receptor [32], but are considered as androgen-independent [33]. Cells were grown in DMEM high glucose, with l-glutamine and without sodium pyruvate medium, supplemented with 5% FCS, 5% Nu-Serum, 25 U/ml penicillin–streptomycin, 5 μg/ml insulin, and 10^-8 M di-hydrotestosterone. Cells were cultured at 37°C in an atmosphere of 95% air and 5% CO₂.

**Cell Viability**

Cells were plated onto 96-well plates (2 × 10⁴ cells/ml), and on the second day the medium was replaced by a medium containing various concentrations of DIM (10–100 μM) for 24–72 hr. Cell viability was determined using the MTT assay as described previously [34]. In order to exclude the possibility for cytotoxic effects of the indole-derivative on the cells, lactate dehydrogenase (LDH) leakage into the medium was measured in aliquots of the extracellular fluid of each sample, as described previously [35]. This method examines the leakage of the intracellular enzyme, LDH, from the cells, which is a marker of cell lysis [36].

**Cell Proliferation ([3H]-Thymidine Incorporation)**

Cells were plated onto 24-well plates (2 × 10⁴ cells/well) and on the second day treated with varying concentrations of DIM at 37°C, for 24 hr. [3H]-thymidine (1 μCi) was added to each well, 15 hr before the end of the treatment. The medium was removed and the cells were washed 3 times with PBS, following by the addition of 300 μl 0.2 N NaOH to each well and then incubation at room temperature for 20 min. Aliquots (100 μl) were transferred into scintillation vials with 4 ml of Ultima Gold scintillation fluid (Packard) and counted for their radioactivity by a Beckman liquid scintillation counter.

**Analysis of DNA Fragmentation**

Cells were treated with 75 μM DIM for 24–72 hr. At the end of each treatment, cellular DNA was extracted by proteinase K digestion as described previously [17]. Ten microgram DNA were resolved by 1.5% agarose gel electrophoresis at 60 V for 1 hr and examined under UV light.

**Cell Morphology Characterization**

Controls and cells treated with 50 μM DIM for 48 hr were centrifuged onto glass slides by Cytospin (300g for 5 min) and stained with Giemsa using the Diff-Quick kit (Baxter, USA). Cell morphology was evaluated by light microscopy.
Animals

Six-week-old male C57BL/6 mice were purchased from Harlan Laboratories (Israel). The animals were housed in polycarbonate cages (4–6 mice/cage) and were kept in a room lighted 12 hr per day and maintained at 22 ± 1°C. Rodent diet (Koffolk 1949, purchased from Koffolk, Inc., Tel Aviv, Israel) and tap water were given ad libitum. The experiment was approved by the Institute’s “Committee for the Supervision of Animal Experiments.”

In Vivo Tumor Growth Assay

TRAMP-C2 cells were suspended at a concentration of 5 × 10^7 cells/ml medium. 0.1 ml aliquots (5 × 10^6 cells) were injected subcutaneously into the flank of the mice, using a 27-gauge needle. Two weeks after tumor cell transplantation, the mice were treated 3-times a week by IP (intraperitoneal) injection, for 3 weeks (10 doses), with either corn oil (vehicle) or DIM (2.5, 5, or 10 mg/kg) suspended in corn oil. Tumor size was measured biweekly with a calibermeter and the volumes were calculated using the formula length × width ^2 × 0.52 [37]. At the end of the treatment period, the mice were sacrificed, tumors were collected, weighed, measured, and tested for histological studies. Blood samples were also collected from the mice for analysis of liver and kidney functioning.

Histological Studies

Tumors from control and treated groups were kept in formalin and paraffin blocks were prepared. Four micron sections were cut and fixed onto slides for histological stainings.

DAPI Staining

DAPI is a fluorescent compound which specifically binds to the DNA and creates a stable complex providing a 20-times higher fluorescence than DAPI alone, and allows visualization of DNA morphology. DAPI staining of the slides was conducted as described previously [38]. Slides were photographed using a fluorescence microscope at ×200 magnification.

Ki-67 Staining

Examination of cell proliferation in the tumors was conducted using an immunohistochemical reaction of slides with anti-mouse Ki-67 antigen antibody, according to the manufacturer instructions. Ki-67 is a large nuclear protein, preferentially expressed during all active phases of the cell cycle (G1, S, G2, and M), but absent from resting cells (G0). Slides were photographed using a light microscope at ×200 magnification. The proliferation index of the cells was determined in the central and peripheral areas of the tumors. The index was calculated as the ratio of Ki-67-positive tumor cells to all counted tumor cells ×100. The results presented are the means ± SD (standard deviation), calculated according to the cell number in three different fields at ×400 magnification.

Statistical Analysis

The MTT experiment was repeated twice, each with eight replicates, and the thymidine experiment was repeated twice, each in triplicate. Hence, the data are presented as means ± SE (standard error). The in vivo experiment was performed with nine mice in each group and the data are presented as means ± SD. The differences between the controls and treated groups were analysed using Student’s t-test and two-way ANOVA and the levels of significance were noted.

RESULTS

Effect of DIM on Cell Viability and Proliferation

Before performing the in vivo experiment, the effect of DIM on viability and proliferation of the mouse PC cell line TRAMP-C2, was examined in vitro. Cells were treated with DIM (10–100 μM) for 24–72 hr. Cell viability was determined by the MTT assay and cell proliferation was evaluated by the [3H]-thymidine incorporation assay, as described under “Materials and Methods.” The results indicated that DIM had a significant inhibitory effect (P < 0.001) on the viability (Fig. 1A) and proliferation (Fig. 1B) of the cells, and that this effect was time- and dose-dependent. In order to exclude the possibility for cytotoxic effects of the indole on the cell line, we performed the LDH assay, which is one of the most widely-used methods for measuring cellular lysis [36]. We observed that treatment of the cells with DIM (10–100 μM) did not cause a statistically-significant increase in LDH levels in the condition medium, compared with controls (data not shown). We, therefore, assume that no toxic effects were manifested, as judged by this enzyme activity, at the above concentrations.

Effect of DIM on Apoptosis Induction

The significant decrease in cell viability and proliferation occurring after exposure to DIM led us to investigate whether the effect of this compound was mediated through the induction of apoptosis. Following treatment of the cells with DIM (75 μM) for 24, 48, and 72 hr, DNA was extracted from the cells and loaded on agarose gel, as described under “Materials and Methods.” DNA ladder was observed after 24 hr of

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treatment with 75 \, \mu M \text{DIM}, \text{which became more pronounced with time (Fig. 2A).}

Apoptotic cells were also characterized by morphological changes, such as cell shrinkage and the generation of apoptotic bodies. Cells were treated with \text{DIM (50 \, \mu M)} \text{for 48 hr, followed by Giemsa staining. The results in Figure 2B show that the treated cells exhibited typical apoptotic morphology, condensation, and fragmentation of the nucleus, in comparison to untreated cells.}

\textbf{In Vivo Tumor Growth Assay}

The therapeutic activity of \text{DIM} was investigated in male C57BL/6 mice, in which tumors were initiated by transplanting TRAMP-C2 cells (which was derived from a primary tumor in the prostate of C57BL/6 mouse) subcutaneously, into the flank of a mouse. After initial detection of tumors, animals were treated \text{IP} with either corn oil (control) or \text{DIM (2.5, 5, or 10 mg/kg)}, 3-times a week, for a period of 3 weeks. Tumor
volumes were measured twice a week as described under “Materials and Methods.” Our results indicate (Fig. 3) that treatment with DIM caused a decrease in the growth of the tumors in comparison to the control group. While the tumor volumes in the control group increased by approximately 9-folds over the treatment period, those of the DIM-treated groups (5 and 10 mg/kg) were increased only by 5-folds. Starting from 2 weeks since the first treatment, the differences between the control group and the DIM-treated groups (5 and 10 mg/kg) were statistically significant ($P < 0.05$).

At the end of the experiment, the animals were sacrificed and their tumors were weighted and measured. Figure 4 shows that there was a statistically significant difference between the final tumor weights (Fig. 4A) and volumes (Fig. 4B) in the control group, in comparison to all DIM-treated groups. Tumor volumes and weights were at least 2-times smaller and lighter in the treated groups in comparison with those of the control group.

In order to better understand how DIM exerts its in vivo effects, tumors were collected, fixed in formalin, and sections were cut for several histological stainings. In order to examine apoptotic characteristics in the tumor cells, the slides with tumor specimens were stained with DAPI. The results presented in Figure 5 show that there were differences in the morphology of cells from the control group and the treated groups. Cells in the treated group are shown to be condensed, with a condensed nucleus, while cells in the control group are large and their nucleus is un-condensed. These results may indicate the occurrence of an apoptotic process.

Cell proliferation in the tumors was examined using Ki-67 antibody. Our results (Fig. 6) indicated that the number of proliferating cells in slides from the control group is larger than in the slides from groups treated with 5 or 10 mg DIM (proliferating cells are indicated by arrows). The proliferation index of the cells in the peripheral areas of the tumors was calculated and the decrease observed in the treated cells was statistically significant in comparison to the controls. The means $\pm$ SD values observed were as follows: proliferation index of the control was $45 \pm 2.5\%$; in the 5 mg treated group $37 \pm 1.9\%$ ($P < 0.05$); and in the 10 mg treated group $33 \pm 1.3\%$ ($P < 0.001$). The proliferation index of the cells in the central areas of the tumors was also calculated, but no significant differences were found (data not shown). The treatment with DIM (2.5, 5, or 10 mg/kg) did not affect body weights, or kidney and liver functioning, as compared to the controls (data not shown). This may suggest that DIM at the above concentrations is non-toxic and safe for use.
DISCUSSION

PC is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in men in Western industrialized countries. The increasing incidence of PC has led to intense investigations searching for efficient therapeutic or anti-tumorigenic compounds against this type of cancer. We have previously shown that indolic compounds, such as I3C and DIM originating from cruciferous vegetables, have a suppressive effect on the growth of human breast [14,15] and prostate [17] cancer cell lines. In the present study we have shown that DIM also has a suppressive effect on the PC cell line TRAMP-C2, derived from C57BL/6 mice. DIM causes a significant decrease ($P < 0.001$) in cell viability, as its concentration increases (Fig. 1A). Moreover, the lower cell viability is accompanied by inhibition of cell proliferation through a decrease in DNA synthesis (Fig. 1B). This effect was mediated through the induction of apoptosis, as was indicated by DNA fragmentation (Fig. 2A) and characteristic morphological changes (Fig. 2B), which are a hallmark of the apoptotic process [39,40].

Rodent models play an essential role in the investigation and development of effective prevention and intervention strategies for PC. In the present study we have demonstrated that DIM has a potential beneficial effect in the growth of the subcutaneously transplanted tumor line TRAMP-C2 in C57BL/6 mice in vivo. Mice treated with 5 or 10 mg/kg DIM showed a significantly smaller tumor volumes and weights in comparison with those of the controls (Figs. 3 and 4). Histological examination of slides from the tumors revealed (Fig. 5) a possibility for the occurrence of an apoptotic process, since, tumors from treated groups, showed condensed cells and nuclei. Also, immunohistochemistry with Ki-67 antibody showed that there is a significant decrease in the proliferation of cells located in the peripheral areas of the tumors from the DIM treated groups (5 or 10 mg) in comparison with the control (Fig. 6). Therefore, the mode of action of DIM in the inhibition of tumor cells development in vivo involves a decrease in cell proliferation and induction of apoptosis. The difference in the proliferative index of the cells in the center of the tumor in comparison with the peripheral cells, may be related to the angiogenesis

Fig. 5. DAPI staining of tumors sections. In order to explore apoptotic characteristics of the cells, 4 μm sections from the tumors were stained with DAPI as described under “Materials and Methods.” Arrows point to cells having typical apoptotic morphology. The Figures shown are representatives of the results observed (× 200 magnification).
process in the tumor. Nevertheless, more tests are needed in order to elucidate the pathway by which DIM exerts its action. Treatment with DIM didn’t affect body weights and kidney and liver functioning in any of the mice groups. Therefore, using this compound at the concentrations tested may be safe. Recently, Chen et al. [41] showed that DIM (5 mg/kg every other day) also inhibited DMBA-induced mammary tumor growth and volumes in Sprague–Dawley rats. A number of other in vivo studies have shown strong correlation between consumption of cruciferous vegetables or their indolic compounds with a decreased incidence of both carcinogen-induced [25–28] and spontaneous tumors [29,30]. The results of our study, in addition to the above-mentioned studies, suggest that cruciferous vegetables and their indolic compounds, I3C and DIM, may constitute an important anti-carcinogenic and therapeutic food derivative against this type of cancer, offering natural compounds with minimal toxic effects in the treatment of PC. Nevertheless, more experiments are needed in order to examine the optimal concentrations of DIM, which may serve as an efficient means for the prevention, as well as treatment of tumors.

Fig. 6. Proliferation of cells from the tumors. In order to explore proliferation of the cells, 4 μm sections from the tumors were incubated with a specific antibody as described under “Materials and Methods.” Arrows point to proliferating cells. The figures shown are representatives of the results observed (× 200 magnification).

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