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3,3′-Diindolylmethane Induces Apoptosis in Human Cancer Cells

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3,3′-Diindolylmethane is a dimer of indole-3-carbinol formed both *in vivo* and *in vitro.* In this study, human cancer cells MCF-7 (with wild-type p53), T47-D (mutant p53), and Saos-2 (deficient in p53 gene), were used to examine the anticancer activities of 3,3′-diindolylmethane. The dose-dependent growth inhibitory effect was found in all these cell lines. Exposure of the cells to 50 μM solution of 3,3′-diindolylmethane for 48 h, apoptosis (programmed cell death) was evidenced by the characteristic morphology of cell nuclei under fluorescence microscope and the DNA ''ladder'' in agarose gel electrophoresis. The percentage of apoptotic cells in each cell line was found to be 12% for MCF-7, 14% for T47D and 13% for Saos2 cells. Exposure of MCF-7 cells to 100 μM 3,3′-diindolylmethane for 24 h, 19% of apoptotic cells were detected by flow cytometry analysis. The lowest dose required for induction of apoptosis in MCF-7 cells was found to be 10 μM after 72 h incubation. Western blot showed that wild-type p53 protein was unchanged after MCF-7 cells had been exposed to 50 μM 3,3′-diindolylmethane for 8 h. This study provides evidences that 3,3′-diindolylmethane induces apoptosis in human cancer cells and that the induction of apoptosis is independent of p53 pathway.

In the ancient herbal literature cabbage is claimed to have therapeutic effects for various kinds of cancer (1). A number of previous studies have demonstrated decreased risk of various cancers in subjects with a high consumption of cruciferous vegetables, such as cabbage, Brussels sprouts, cauliflower and broccoli (2, 3). Mice that were fed dried cabbage showed a decreased incidence of pulmonary metastases after intravenous injection with mammary tumor cells (4). It was suggested that indole-3-carbinol is the major indole derivative hydrolyzed from glucobrassicin present in cruciferous vegetables (5). The biological effects of indole-3-carbinol have been extensively studied in many experimental protocols. These include one or more of the following activities: 1) induction of xenobiotic drug metabolic enzymes; 2) induction of conjugating enzymes; 3) induction of C_2 hydroxylation of estrone thus decreasing the major carcinogenic estrone metabolite, 16α-hydroxyestrone; 4) inhibition of carcinogen-DNA binding and scavenging free radicals; 5) decrease of nuclear estrogen receptor levels in MCF-7 cells (6-11). Indole-3-carbinol has also been found recently to possess the ability to inhibit the growth of multidrug resistant tumors transplanted into animals (12).

In a low pH environment, indole-3-carbinol may be converted into many polymeric products, among which 3,3′-diindolylmethane is a major one (13, 14). An *in vivo* study revealed that 3,3′-diindolylmethane was the major species when indole-3-carbinol had been fed in the diet (15). The anticancer activity of indole-3-carbinol has been attributed, in part, to 3,3′-diindolylmethane (13, 14).

Recent studies have shown that indole-3-carbinol inhibits the growth of human breast cancer cells (16) and mouse mammary epithelial cells transfected with *ras* oncogene *in vitro* (17). It

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has been reported that in tissue culture medium, indole-3-carbinol can be self-condensed to 3,3′-diindolylmethane (18). In this study we demonstrate that 3,3′-diindolylmethane suppressed human cancer cell growth by inducing apoptosis.

MATERIALS AND METHODS

Chemicals. 3,3′-diindolylmethane was synthesized from indole-3-carbinol according to the method of Leete and Marion (19). Cell culture media and reagents were obtained from Biological Industries, Beit Haemek, Israel.

Cell culture. Human cancer cell lines with different p53 status were used in this study. Human breast cancer MCF-7 (wild-type p53), T47D (mutant p53) cells and human osteosarcoma Saos2 (deficient in p53 gene) cells were maintained in medium I (DMEM medium containing 7.5% fetal calf serum, 100 μg/ml of streptomycin, 100 U/ml of penicillin, 0.25 μg/ml amphotericin, 2 mM of L-glutamine and 0.25 U/ml of insulin) at 37°C in a humidified 5% CO_2 incubator. 3,3′-diindolylmethane was dissolved in dimethyl sulfoxide (DMSO) and added to medium II [RPMI 1640 medium (without phenol red) containing 10% fetal calf serum, 100 μg/ml of streptomycin, 100 U/ml of penicillin, 0.25 μg/ml amphotericin, 2 mM of L-glutamine, 0.25 U/ml of insulin]. The final concentration of DMSO in the experimental medium (medium II) did not exceed 0.2 % (v/v) and the control only contained an equal percentage of DMSO in medium II.

Growth inhibition. Cells were seeded into T-25 tissue culture flasks in medium I. On the second day, the medium was replaced by medium II containing different concentrations of 3,3′-diindolylmethane. The medium was changed with a fresh one every other day. After 5 days, attached cells were trypsinized and the number of cells in each flask was determined using a hematocytometer. Each experiment was repeated 3 times and each treatment was in triplicate. The 50% inhibitory concentration (LC_{50}) was determined from plot of relative number of cells (% of control) *versus* the logarithm of the compound concentration.

Cell morphology. MCF-7 cells were exposed to 50 μM 3,3′-diindolylmethane in medium II for 48 h. At the end of the experiment, cells (both floating and attached) were washed with phosphate-buffered saline (PBS), fixed in 70% ethanol and then stained with 10 μg/ml propidium iodide (Sigma). Alterations in cell nuclei were photographed under a fluorescence microscope.

Determination of DNA fragmentation in agarose gel. At the end of each treatment, cells (both floating and attached) were centrifuged at 500 $\times$ g. After being washed with PBS, cells were lysed in a buffer containing 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% SDS and 200 μg/ml proteinase K at pH 7.5, kept in a water bath at 55°C overnight. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform only. RNase A (7,000 U/ml) (Sigma) was added and the mixture was then incubated at 37°C for 2 h. The samples were then extracted again with phenol:chloroform:isoamyl alcohol followed by chloroform. DNA was precipitated in two volumes of ethanol in the presence of 0.3 M sodium acetate. After recovery by centrifugation, the DNA pellet was washed with 70% ethanol, dried in air and suspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, at pH 8.0). DNA samples were run on 1.5% agarose gel at 60 Volts and visualized by ethidium bromide staining under UV light.

Flow cytometry analysis. After treatment, 1-2 $\times$ 10^6 cells were trypsinized and washed twice with ice-cold PBS. Cells were washed with 1 ml phosphate-citric acid buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid, at pH 7.8) and stained with 1 ml solution containing 0.2% Triton X-100, RNase A (7,000 U/ml), 50 μg/ml propidium iodide. The stained cells were held at room temperature for 20 min in the dark and then kept at 4°C in the dark until measured. Samples were analyzed using FACScan (Becton Dickson, San Jose, CA) at a laser setting of 36 mW and an excitation wavelength of 488 nm.

Western blot. Equal numbers of cells were plated in 6-cm dishes. On the third day, cells were treated with 50 μM 3,3′-diindolylmethane. After 8 h, cells were lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl (pH 6.8), 5 mM EDTA, 150 mM NaCl, 2% SDS, 1 mM iodoacetamide, 1 mM PMSF and 5% β-mercaptoethanol. Protein concentrations of the cell lysates were quantified by the method of Bradford (20). 40 mg of protein from each sample was separated by 12% SDS-polyacrylamide slab gel electrophoresis and electrophoretically transferred onto a nitrocellulose membrane filter. The membrane was subjected to immunoblotting using the Enhanced Chemiluminescence (Boehringer Mannheim) detection system according to the manufacturer's instructions. Wild-type p53 monoclonal antibody 421 (kindly provided by Prof. V. Roter, Weizmann Institute, Israel) was used as the primary antibody. After additional incubation in ECL reagents, the membrane was exposed to Kodak X-ray films.

RESULTS

Growth inhibition. Human cancer cell lines, MCF-7, T47D and Saos2 with different p53 status, were exposed to 0.1-50 μM 3,3′-diindolylmethane for 5 days. The medium was replaced by a fresh one every other day. At the end of the experiment, attached cells were trypsinized and the number of cells was determined by a hematocytometer. Fig. 1 shows the dose-dependent growth inhibitory effect caused by 3,3′-diindolylmethane on the different cell lines. The IC_{50}s

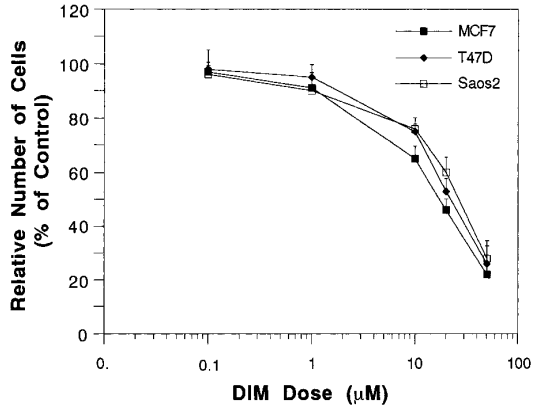

FIG. 1. Dose-dependent growth inhibitory effect of 3,3′-diindolylmethane (DIM) on MCF-7, T47D and Saos2 cells. Exponentially growing cells were incubated in the absence or presence of 3,3′-diindolylmethane for 5 days as described under ''Materials and Methods''. The results are expressed as means ± standard deviation of three independent experiments, in which each treatment was in triplicate.

were as follows: 17 μM for MCF-7, 24 μM for T47D and 30 μM for Saos2 cells. At a concentration of 50 μM 3,3′-diindolylmethane, MCF-7 cells showed a 78% growth inhibition, while T47D and Saos2 cells showed 74% and 72%, respectively.

Induction of apoptosis in MCF-7, T47D and Saos2 cells. The significant influence of 3,3′-diindolylmethane on the growth of these cells led us to investigate whether the effect of this compound was a result of triggering programmed cell death, since at the relatively higher concentrations some dead cells were observed. The typical morphological changes of MCF-7 cell nuclei after 48 h incubation with 50 μM 3,3′-diindolylmethane is shown in Fig. 2B. The propidium iodide staining shows the apoptotic nuclei, either condensed or fragmented. After treatment of MCF-7, T47D and Saos2 cells with 50 μM 3,3′-diindolylmethane for 48 h, the genomic DNA from both floating and attached cells was subjected to agarose gel electrophore-

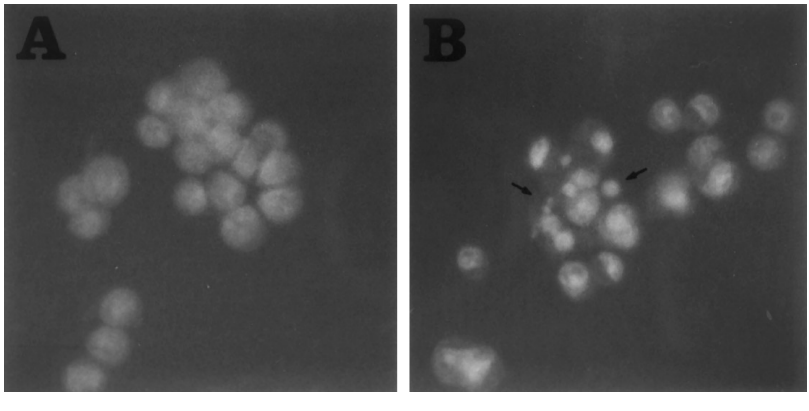

FIG. 2. Morphological alterations of MCF-7 cell nuclei after incubation with 3,3′-diindolylmethane. Cells were incubated in the presence or absence of 50 μM 3,3′-diindolylmethane for 48 h. Cells (both floating and attached) were washed with PBS, fixed in ethanol and stained with propidium iodide, as described under ''Materials and Methods''. The morphological changes in cell nuclei were observed under a fluorescence microscope. A: untreated control; B: some treated MCF-7 cells show a marked apoptotic nucleus (condensed or fragmented as indicated by arrows).

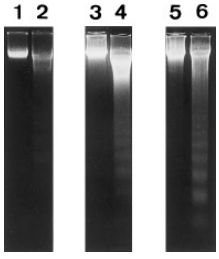

FIG. 3. DNA fragmentation following treatment with 3,3′-diindolylmethane in MCF-7, T47D and Saos2 cells. Exponentially growing cells were plated into T-75 flasks. Cells were incubated in RPMI 1640 medium in the presence or absence of 50 μM 3,3′-diindolylmethane for 48 h. DNA was prepared as described under ''Materials and Methods'' and separated on a 1.5% agarose gel. Lanes 1 and 2 are DNA samples from Saos2 cells. Lane 1: untreated control; Lane 2: 50 μM, 48 h; Lanes 3 and 4 are those from T47D cells. Lane 3: control; Lane 4: 50 μM, 48 h; Lanes 5 and 6 are those from MCF-7 cells. Lane 5: control; Lane 6, 50 μM, 48 h.

sis. Apoptosis due to this treatment was recognized by the appearance of a typical DNA fragmentation ladder detected by gel electrophoresis in all three cell lines (Fig. 3). Apoptosis was further quantified under a fluorescence microscope. The percentage of apoptotic cells (the number of apoptotic cells out of 100 total cells counted) in each cell line was 12% for MCF-7, 14% for T47D and 13% for Saos2 cells. Fig. 4 shows the time-dependent effect in inducing apoptosis after treatment of MCF-7 cells with 100 μM. 10% of subdiploid DNA content was found after 6 h, while 19% was found after 24 h. Fig. 5 shows induction of apoptosis in MCF-7 cells by 10 μM 3,3′-diindolylmethane. After 72 h incubation, the DNA ladder was evidenced by agarose gel electrophoresis.

P53 is not induced in MCF-7 cells. MCF-7 cells contain wild-type p53. To study if wild-type p53 was induced in the presence of 3,3′-diindolylmethane in MCF-7 cells, we further studied the expression of wild-type p53 protein by Western blot analysis using monoclonal p53 antibody. In this study, Saos2 cell line was used as a negative control as it does not possess the p53 gene. Fig. 6 shows that wild-type p53 was not induced in MCF-7 cells after treatment with 50 μM 3,3′-diindolylmethane for 8 h.

DISCUSSION

In this study we have demonstrated that diet-derived indole-containing compound, 3,3′-diindolylmethane, induces apoptosis in human cancer cells. It is thought that the rate of tumor cell growth is a balance between the rate of proliferation and the rate of cell death. Apoptosis is usually regulated by the deprivation of the survival factors such as growth factors (e.g. EGF, IGF-1, estrogens, IL2), extracellular matrix, oncogenes (e.g. ras, v-Raf), differentiation-related genes (e.g. *bcl*-2) or viral genes (e.g. SV40 T antigens) (21). In contrast to the survival factors, both p53, a tumor suppressor gene, and *bax*, one of the genes belonging to the *bcl*-2 family, increase the susceptibility of cells to apoptosis (22, 23). Although synthesis of a new protein (or proteins) is most likely to be required for apoptosis to occur, gene expression is not universal in induction of apoptosis (24). It is not clear from this study if 3,3′-diindolylmethane induces apoptosis requiring the synthesis of a new protein or proteins which are lethal to cells. However, the result that apoptosis was induced in the cell lines with different p53 status and the observation that p53 was not induced in MCF-7 cells, which contain wild-type p53 protein, indicate that wild-type p53 was not required for the effect caused by 3,3′-diindolylmethane.

In this study, the lowest concentration required for induction of apoptosis in MCF-7 cells was found to be 10 μM for 3,3′-diindolylmethane. An animal study has shown that when 2,000 ppm indole-3-carbinol was fed to trouts, about 70 μM was found in the trout liver (15).

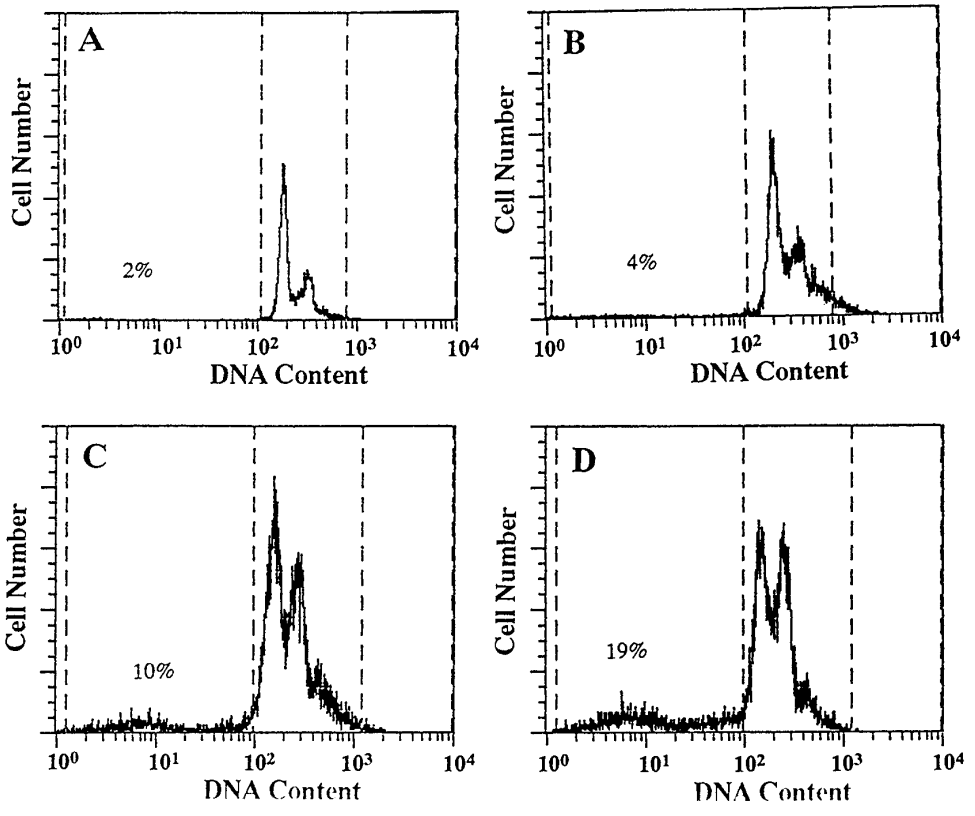

FIG. 4. Representative histograms of DNA analysis by flow cytometry after treatment of MCF-7 cells with 100 μM 3,3′-diindolylmethane. A: untreated control; B, C, D are samples treated for 3, 5, 24 h, respectively. The subdiploid DNA content represents apoptotic cells. The data were from representative experiments (n = 3). X^2 analysis showed that the percentage of apoptotic cells in C or D, was significantly higher than that of A ($p < 0.01$).

A recent study revealed that indole-3-carbinol at a dose level of 100 or 50 mg/day, 5 times a week, was not toxic to Sprague-Dawley rats and these treatments decreased the incidence of chemically-induced tumors (25). In a human trial, 500 mg/day/person of indole-3-carbinol was not found to be toxic in a continuous three-month study (26). The lowest dose required for induction of apoptosis by 3,3′-diindolylmethane and the relatively low toxicity of indole-3-

FIG. 5. Induction of DNA fragmentation by 10 μM 3,3′-diindolylmethane. MCF-7 cells were incubated without or with 10 μM 3,3′-diindolylmethane for different time intervals. DNA was extracted as described under ''Materials and Methods'' and separated on a 1.5% agarose gel. Lane 1: untreated control; Lane 2: 24 h; Lane 3: 48 h; Lane 4: 72 h.

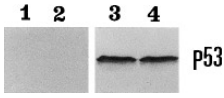

FIG. 6. Lack of correlation between 3,3′-diindolylmethane-induced apoptosis and the expression of wild-type p53 protein. The expression of p53 was determined by Western blot measured by ECL, as described under ''Materials and Methods'', using equal amounts of lysates from Saos2 and MCF-7 cells. Lanes 1 and 2: Saos2 cells, which are deficient in p53 gene, were used as negative control. Lanes 3 and 4: MCF-7 cells. Lane 1 and 3: untreated controls; Lanes 2 and 4: 50 μM 3,3′-diindolylmethane for 8 h.

carbinol suggest that higher serum and tissue concentrations of 3,3′-diindolylmethane might be pharmacologically achievable in cancer adjuvant chemotherapy by either 3,3′-diindolylmethane or indole-3-carbinol. Yet, further confirmatory studies are still needed.

In conclusion, this is the first study which provides evidences that the diet-derived indole-derivative, 3,3′-diindolylmethane, induces apoptosis in human cancer cells *in vitro.* Induction of apoptosis by this compound is through a wild-type p53 independent pathway, although the mechanism by which 3,3′-diindolylmethane induces apoptosis needs to be further addressed.

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