3,3'-Diindolylmethane suppresses growth of human esophageal squamous cancer cells by G1 cell cycle arrest

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Abstract. 3,3'-Diindolylmethane (DIM), an active metabolite of indole-3-carbinol, is thought to have antitumor effects in experimental animals and induce apoptosis in various cancer cells. However, the biological functions of DIM in human esophageal cancer cells are unknown. Thus, the purpose of this study was to investigate the cytotoxic effects of DIM in human esophageal squamous cell carcinoma (ESCC) cells to elucidate the molecular mechanism of cell death. Three human ESCC cell lines (TT, TE-8 and TE-12) were used to test the response to DIM. MTT, cell cycle and western blot analyses were conducted. DIM significantly inhibited the proliferation of ESCC cells in a dose- and time-dependent manner. The percentage of G1 phase cells increased 48 h after being treated with DIM. DIM also reduced cyclin D1, cyclin E2, cyclin-dependent kinase (CDK) 4 and CDK 6 activities, and increased p15 and p27 levels. Additionally, DIM diminished pro-caspase-9 protein expression levels and induced increased cleaved poly (ADP-ribose) polymerase levels. These results indicate that DIM leads to G1 phase cell cycle arrest and induces apoptosis by activating caspase-9 in ESCC cells.

Introduction

Esophageal cancer is the eighth most common cancer worldwide and ranks sixth in cancer mortality (1). Esophageal cancer is represented by esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). In recent decades, the incidence of EAC has increased, whereas that of ESCC has decreased in the West, and an epidemiological shift from patients with ESCC to those with EAC has been observed in western countries (2-4). Although EAC rates have dramatically increased in western countries, ESCC still predominates worldwide. Most ESCC occurs in developing countries and is thought to be associated with low socioeconomic status, alcohol and tobacco use, malnutrition, and organ dysfunction (3,5). ESCC is typically found in the middle and upper third of the esophagus, whereas EAC is mostly located in the lower third of the esophagus. Because of the location of ESCC, a worse prognosis with a significantly higher overall survival is observed after resection of ESCC (6-8). Thus, a number of treatment options, such as surgery, radiation, and neoadjuvant radiochemotherapy, which includes an early and accurate assessment of clinical response, are performed for patients with ESCC (3). Despite recent progress in multimodality treatment for ESCC, long-term survival of these patients still remains disappointing, with 5-year survival rates of <25%. These dismal prognoses may be due to the poorly understood premalignant state of ESCC and to the lack of an efficient screening strategy for ESCC. Therefore, novel therapeutic approaches must be developed to improve ESCC survival outcomes.

Dietary chemopreventive agents have been a focus of studies, because they can inhibit, delay, or reverse multistate carcinogenesis. 3,3'-Diimdolylmethane (DIM), an active metabolite of an indole-3-carbinol (I3C) derivative from cruciferous vegetables such as broccoli, cabbage, and cauliflower, elicits anti-cancer effects in *in vivo* and *in vitro* models (9-11). Administering DIM reduces the incidence and multiplicity of mammary tumors in rats and mice (12,13), and *in vitro* studies have shown that DIM has anti-proliferative effects in a variety of cancer cell types, including human colon, pancreas, prostate, and breast cancer (14-23). However, the molecular mechanisms of how DIM acts on human esophageal cancer cells have not been investigated. To address these issues, we examined whether DIM could induce cell death in human ESCC cells. We demonstrated that DIM induced apoptosis in esophageal cancer cells by activating caspase-9 followed by G1 phase cell cycle arrest. These effects may be due to reduced cyclin-dependent kinase (CDK) activity.

Materials and methods

Reagents and cell cultures. DIM was purchased from LKT Laboratories (St. Paul, MN, USA). Antibodies to CDK2,

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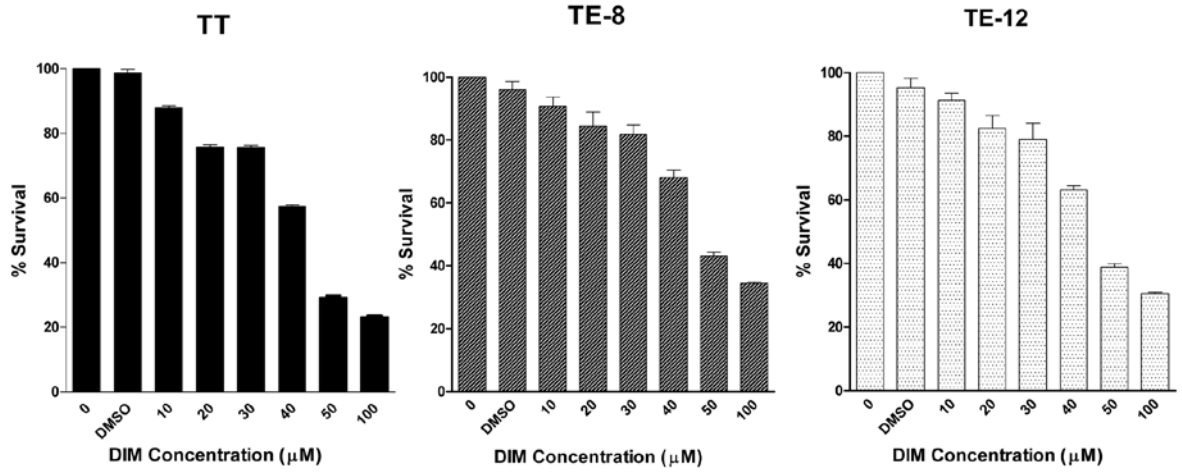

Figure 1. 3,3'-Diindolylmethane (DIM) inhibits cell proliferation in human esophageal squamous cell carcinoma (ESCC) cell lines. The logarithmically growing human ESCC cell lines TT, TE-8 and TE-12 were treated with various concentrations of DIM for 72 h, and cell proliferation was assessed by the MTT assay. Data are presented as percentage of control plates containing no DIM. Each point represents the mean (± SE) of at least three independent experiments with triplicate dishes.

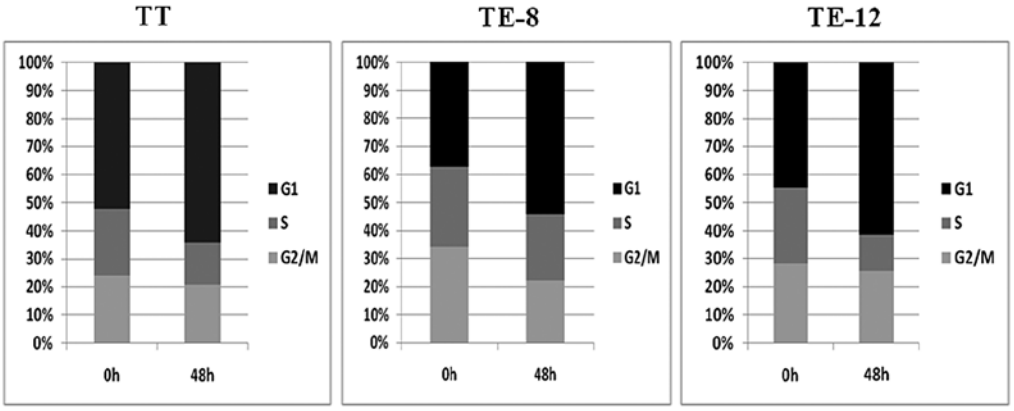

Figure 2. 3,3'-Diindolylmethane (DIM) induces cell cycle arrest at the G1 phase in esophageal squamous cell carcinoma (ESCC) cell lines. TT, TE-8 and TE-12 cell lines were incubated with 40 μM DIM for 0 and 48 h. Cell cycle distribution was calculated as the percentage of cells in the G1, S, and G2/M phases. All experiments were performed three times.

CDK-4, CDK-6, cyclin D1, cyclin D3, cyclin E2, p15, p27, pro-caspase-9 and cleaved poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA, USA). The human ESCC cell lines TT, TE-8 and TE-12 were obtained from Dr Izzo (University of Texas MD Anderson Cancer Center). TT, TE-8 and TE-12 cells were maintained in DMEM-F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (PAA Laboratories, Inc., Pasching, Austria), 100 mg/ml streptomycin, and 100 IU/ml penicillin (Gibco) as a monolayer in 100-mm dishes (BD Biosciences, Sparks, MD, USA) under standard conditions at 37˚C in a 5% CO_2 humidified atmosphere. All experiments were performed with the cell lines at 60-80% confluence.

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TT, TE-8 and TE-12 cells were plated in 96-well plates (SPL, Seoul, South Korea) at $1x10^4$ cells/well. After 24 h, the cells were treated with DIM (10, 20, 30, 40, 50 or 100 μM) for 72 h. Cell viability was assessed with the MTT assay as described previously (24). Experiments were conducted in triplicate, and viability assays were repeated three times to confirm results.

Flow cytometry cell cycle analysis. Cells were plated in 100-mm dishes at $1x10^6$ cells/dish in DMEM/F-12. The cells were treated for 48 h with various concentrations of DIM. After trypsinization, the nuclei were stained with propidum iodide (Sigma Chemical, St. Louis, MO, USA) as described previously (14,24). The percentage of cells in the different cell cycle phases was measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analyzed using the Becton-Dickinson software (Lysis II, Cellfit).

Western blotting. Cells were scraped in medium and suspended in lysis buffer (Intron Biotechnology, Inc., Seoul, South Korea). Extracts were incubated on ice for 20 min and centrifuged at 13,000 x g for 5 min. The protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Whole lysate (20 μg) was resolved on a 10 % SDS-PAGE gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting and then probed with ~0.1 μg/ml of rabbit anti-human

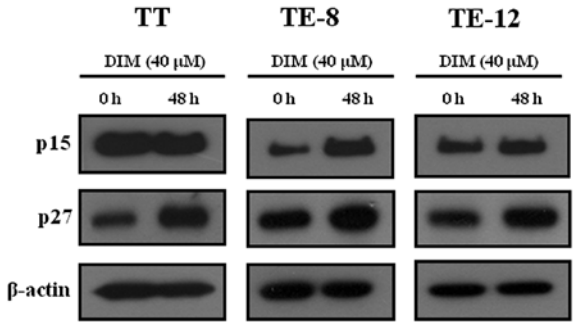

Figure 3. 3,3'-Diindolylmethane (DIM) increases p15 and p27 protein levels in esophageal squamous cell carcinoma (ESCC) cell lines. TT, TE-8 and TE-12 cells were harvested at 48 h after incubation with 40 μM DIM. Cell lysates were analyzed by western blotting with the indicated antibodies. β-actin was used as an internal control.

CDK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-human CDK4, mouse anti-human CDK6, mouse anti-human cyclin D1, mouse anti-human cyclin D3, rabbit anti-human cyclin E2, rabbit anti-human p15, rabbit anti-human p27, rabbit anti-human pro-caspase-9, and rabbit anti-human cleaved PARP antibodies (Cell Signaling Technology). The membrane was then washed with TBS-T (10X TBS and 0.1% Tween-20) and incubated for an additional 1 h with HRP-linked anti-rabbit and anti-mouse antibodies (Cell Signaling Technology). Protein bands were visualized with the Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL, USA).

Statistical analysis. Results are expressed as means ± SE. The Student's t-test was used to test for significant differences. P-values <0.05 were considered significant.

Results

Effect of DIM on cell viability. As shown in Fig. 1, DIM significantly suppressed viability in a dose-dependent manner in TT, TE-8 and the TE-12 ESCC cell lines. The IC_{50} of DIM was approximately 40 μM (Fig. 1), suggesting that DIM has potent antitumor effects.

Effect of DIM on cell cycle arrest. To determine whether DIM regulates cell cycle progression in human ESCC cells, fluorescence-activated cell sorting analysis was performed to characterize the cell population. The cell cycle fractions were measured after a 48 h treatment with 40 μM DIM and were compared with that of cells incubated in medium alone. As shown in Fig. 2, the percentage of G1 cells increased significantly ($P<0.05$) in the TT, TE-8 and TE-12 ESCC cell lines at 48 h after DIM treatment compared to those not treated with DIM (TT, 48.66±2.73 to 58.33±1.75; TE-8, 36.19±1.91 to 48.83±2.77; TE-12, 38.95±2.76 to 47.56±4.66, respectively). These results indicate that DIM induced G1 phase arrest in human ESCC cells after a 48 h DIM treatment.

Effect of DIM on cell cycle regulatory protein expression. Because CDK inhibitors inhibit CDK activity, we next determined whether there were alterations in cell cycle regulatory proteins such as p15 and p27 after treatment with 40 μM DIM. The levels of p15 and p27 proteins, which negatively regulate

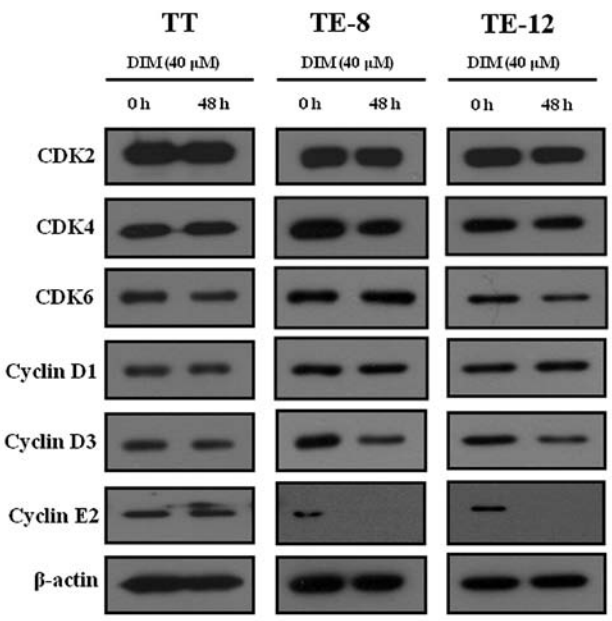

Figure 4. 3,3'-Diindolylmethane (DIM) reduces the levels of cyclin dependent kinase (CDK)2, CDK4, CDK6, cyclin D3 and cyclin E2 in esophageal squamous cell carcinoma (ESCC) cell lines. TT, TE-8 and TE-12 cells were harvested at 48 h after incubation with 40 μM DIM. Cell lysates were analyzed by western blotting with the indicated antibodies. β-actin was used as an internal control.

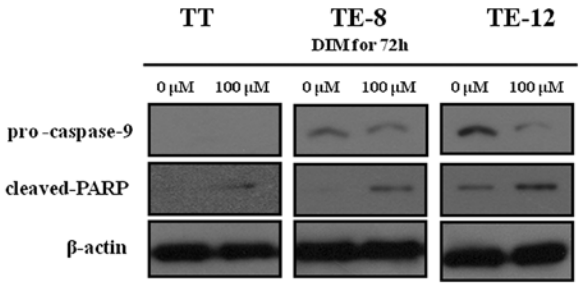

Figure 5. 3,3'-Diindolylmethane (DIM) reduces the level of pro-caspase-9 and increases the level of cleaved poly (ADP-ribose) polymerase (PARP) in esophageal squamous cell carcinoma (ESCC) cell lines. TT, TE-8 and TE-12 cells were harvested at 72 h after incubation with 100 μM DIM. Cell lysates were analyzed by western blotting with the indicated antibodies. β-actin was used as an internal control.

various cyclin/CDK complexes, increased at 48 h after DIM treatment (Fig. 3). G1 progression and G1/S transition are regulated by D-type cyclins in vertebrate cells, which bind to and activate CDK4 and CDK6 as well as cyclin E and cyclin A, which activate CDK2, respectively. Therefore, the effect of DIM on CDK2, CDK4, CDK6, cyclin D1 and cyclin E protein levels were examined to understand the regulation of these cell cycle regulatory proteins in human ESCC cells. Treatment of the cancer cells with 40 μM DIM resulted in downregulation of CDK4 and CDK6 protein levels at 48 h, whereas CDK2 protein expression appeared to be only mildly diminished. Additionally, cyclin D3 decreased, but cyclin D1 expression was not altered in the TT, TE-8 and TE-12 ESCC cell lines. Furthermore, the expression of cyclin E, essential for CDK2 activation during the G1/S transition and S phase progression, decreased significantly at 48 h (Fig. 4). In general, TE-8 and

TE-12 cells had significantly altered cyclin D and E levels following DIM treatment compared to those in the TT cell line, suggesting that DIM induced G1 cell cycle arrest in human ESCC cells through changes in cell cycle regulatory proteins.

Effect of DIM on apoptotic regulatory protein expression. We further examined pro-caspase-9 and cleaved PARP after DIM treatment in the TT, TE-8 and TE-12 ESCC cell lines. The pro-caspase-9 protein levels were inhibited in response to DIM treatment at 72 h in the TE-8 and TE-12 ESCC cell lines but no differences in the pro-caspase-9 protein in TT cell lines were observed (Fig. 5). However, a cleaved form of the PARP protein, a hallmark of apoptosis, was upregulated in all ESCC cell lines exposed to DIM for 72 h (Fig. 5), indicating that DIM induced apoptotic cell death in human ESCC cells.

Discussion

The present study demonstrated that DIM is an effective inducer of apoptosis in human ESCC cells by upregulating several apoptotic proteins. The observed antitumor effects may be due to decreased tumor proliferation and increased tumor cell death following G1 phase cell cycle arrest. Our findings show, for the first time, that DIM could be an efficacious therapeutic agent for preventing ESCC.

Several studies have indicated that I3C, a bioactive phytochemical from vegetables of the family Cruciferae, inhibits carcinogenesis in experimental animals and also inhibits the growth of human cancer cells (25,26). Emerging evidence also suggests that DIM, which is an acid stable I3C dimeric product and the predominant bioactive compound, possesses anticarcinogenic effects that reduce growth and induce apoptosis in various cancer cells (11,17,21,26-30). However, no studies have reported successful therapeutic targeting of ESCC by DIM. In this study, we found that DIM induced a dose-dependent inhibition of growth in TT, TE-8 and TE-12 cells. The results support the antitumor effects of DIM on ESCC cells.

The cell cycle controls cell growth, proliferation, and development with highly organized and tightly regulated processes. DIM induces G1 and/or G2 cell cycle arrest in human prostate, colon, and breast cancer cells (31-34). In our study, the cell cycle analysis revealed that DIM induced G1 cell cycle arrest in TT, TE-8 and TE-12 after exposure to 40 μM DIM for 48 h, suggesting that DIM negatively regulates the G1 cell phase and cell proliferation in ESCC cells.

CDKs are key regulatory proteins, that induce the transition from one cell cycle phase to another, and are regulated by several factors such as inhibitory phosphorylation. For example, CDKs are inhibited by the CDK inhibitors p15, p16, p21 and p27 (35,36). In our study, DIM induced a significant increase in p15 and p27 protein expression in the ESCC cell lines after a 48 h treatment. Cyclin D isoforms induce progression in the G1 cell cycle through CDK2, CDK4 and CDK6 (35). Additionally, cyclin E promotes G1 phase progression, which is required for cell survival and cell proliferation (37,38). In the current study, DIM drastically reduced the levels of CDK2, CDK4, and CDK6 in TT, TE-8 and TE-12 cells after exposure to 40 μM DIM for 48 h. Notably, DIM did not considerably reduce cyclin D1 expression, but cyclin D3 and cyclin E2 protein expression was diminished significantly under the same conditions. DIM-mediated G1 cell cycle arrest of ESCC cells appeared to be accompanied by a reduction in CDK2, CDK4 and CDK6 expression; thus, our results demonstrated a direct connection between cell cycle regulation by DIM treatment and interruption in the expression of G1 cell cycle proteins in ESCC cells.

Induction of apoptosis is critical to inhibit the progression of carcinogenesis. Several studies have revealed that DIM induces apoptosis in several cancer cell lines. Apoptosis is a process of programmed cell death, and numerous pathways and signals lead to apoptosis. Caspases are proteolytic enzymes and are the typical executers of apoptosis. Caspase-9 is activated by binding to Apaf-1 in a multisubunit complex called the apoptosome in mammalian cells (36). The release of cytochrome c from mitochondria leads to the formation of apoptosomes containing Apaf-1 and caspase-9. Activation of caspase-9 is followed by activation of downstream caspases, such as caspase-3, eventually resulting in cell death (11,26,36). We observed that DIM induced apoptosis in TT, TE-8, TE-12 cells. In particular, DIM induced a decrease in pro-caspase-9 protein expression in the TE-8 and TE-12 cell lines. Moreover, cleaved-PARP increased significantly in the TT, TE-8 and TE-12 cells after exposure to 100 μM DIM for 72 h. These observations are similar to those of previous studies demonstrating that DIM activates caspases and enhances PARP cleavage in colon and breast cancer cells (11, 26). Therefore, our results clearly show that DIM increased caspase-9 activation and PARP cleavage. This study is the first to link DIM treatment and increased esophageal cancer cell-specific mortality. Activation of caspase-9 and PARP may be one of the important apoptosis mechanisms induced by DIM in ESCC cells.

In conclusion, DIM inhibited proliferation of human ESCC cells (TT, TE-8, TE-12 cells) via G1 phase cell cycle arrest, which was coupled with reduced CDK4 and CDK6 protein levels. Furthermore, DIM induced apoptosis by activating caspase-9. Therefore, our findings suggest that DIM inhibited growth of esophageal cancer cells by cell cycle arrest and apoptosis. Moreover, our findings and the previously acknowledged role of DIM provide a strong rationale for therapeutic targeting of DIM in ESCC. Further *in vivo* validation studies will be needed to determine whether DIM could be used as a potential therapeutic agent for treating ESCC.

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