사단 **한국식품저장유통학회**
The Korean Society of Food Preservation

3,3'-Diindolylmethane (DIM) decrease adhesion, migration and invasion in human prostate cancer cells

Hyeon-A Kim*

Department of Food and Nutrition, Mokpo National University, Mokpo 534-729, Korea

3,3′-Diindolylmethane (DIM)이 인체 전립선암 세포의 부착, 이동 및 침윤성에 미치는 영향

김현아*

목포대학교 식품영양학과

Abstract

Indole 3-carbinol (I3C), important component of cruciferous vegetables and its major acid-catalyzed metabolite, 3,3'-diindolylmethane (DIM) have been suggested to have an inhibitory effect on the tumor growth and metastasis. This study investigated the effect of DIM on the adhesion, migration and invasion of highly invasive PC3 and DU145 human prostate cancer cell lines. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.0 g/L glucose, 3.7 g/L sodium bicarbonate and 10% fetal bovine and were incubated in a humidified incubator at 37℃ and 5% CO_2. DIM reduced the adhesion of PC3 and DU145 cells in a dose dependent manner. The pretreatment of PC3 cells with DIM reduced the adhesion dose dependantly, but inhibition was less effective than the treatment with DIM during the adhesion assay. The migration and invasion of PC3 and DU145 cells were reduced by DIM dose dependantly, and the inhibition of DIM was less effective in the DU145 cells than in the PC3 cells. The pretreatment of PC3 cells with DIM for 24 hr before the assay reduced invasion of PC3 cells by 37%. These results suggest that DIM inhibits adhesion, migration and invasion of the PC3 and DU145 cells and may be an effective antimetastatic therapy in addition to traditional chemotherapy.

Key words : 3,3'-diindolylmethane (DIM), prostate cancer cells, adhesion, migration, invasion

Introduction

Prostate cancer is the most malignant cancer and 70% of prostate cancer patients develop bone lesions, suggesting that metastases are a significant event contributing to prostate cancer morbidity and mortality (1,2). The process of metastasis has been described as a series of events. The initial step involves detachment of tumor cells from the primary tumor mass. Detached tumor cells adhere to the extracellular matrix (ECM), most likely through cell surface receptors. ECM proteins are degraded by tumor cell produced proteolytic enzymes. Tumor cells can migrate through proteolytically modified ECM and penetrate into the blood or lymph (3,4). Tumor cells that survive in the blood are carried to distant sites where they attach to basement membrane. Motility and invasion into the new target tissue results in the formation of a secondary tumor (4). This event occurs repeatedly during tumor invasion and acquisition of increased adhesiveness, and enhanced motility and invasiveness are likely to contribute to cancer progression (3,5,6). These stages appear to be determined both by characteristics inherent to the tumor cell such as hormone receptor status, production of proteolytic enzymes, expression of cell adhesion molecules and increase in motility (7,8) as well as a number of growth factors, matrix

molecules and cytokines in the metastatic microenvironment (9).

Epidemiological studies have shown that the incidence of prostate cancer is lower in humans who eat large amount of cruciferous vegetables, such as broccoli, cauliflower, cabbage and Brussels sprouts (10,11). Cruciferous vegetables contain indole 3-carbinol (I3C) which is chemically unstable in acid environments and rapidly converted to a variety of polymeric products in the stomach (12-14). Among them, 3,3'-diindolylmethane (DIM) is a major acid catalyzed metabolite. Since I3C readily converted to DIM under a variety of biological conditions, the biological effects of I3C may be attributable to both I3C and DIM (12). I3C has been suggested to reduce of breast tumor growth (15,16) and incidence of lung metastasis in experimental animals (17). DIM has an effect on the inhibition of angiogenesis and the growth of transplantable human breast carcinoma in athymic nude mice (18). DIM was also reported to induce apoptosis and inhibit growth of prostate cancer cells (12,13). However there is a little information if DIM inhibits the metastasis of prostate cancer.

In present study, we examined the inhibitory effects of DIM on the adhesion, migration and invasion of highly invasive PC3 and DU145 human prostate cancer cell lines. These data suggest that DIM may prove to be a therapeutic intervention for prostate cancer progression.

Materials and Methods

Cells and culture condition

The PC3 and DU145 human prostate cancer cell lines were originally derived from bone and brain metastases, respectively, and obtained from ATCC (Rockville,MD). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg), supplemented with 3.0 g/L glucose, 3.7 g/L sodium bicarbonate and 10% fetal bovine and incubated in a humidified incubator at 37℃ and 5% CO_2. Cultures used in subsequent experiments were at <20 passages.

3,3'-Diindolylmethane stock solution

3,3'-Diindolylmethane (DIM, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in DMSO to prepare DIM stock solution. The final concentration of DIM stock solution was 0.1 M. The final concentration of DMSO in the culture medium was 0.1% (v/v).

Cell adhesion assay

Plates (12-wells) were coated with 30 ㎍ of Matrigel (Becton Dickinson, Bedford, MA). To block nonspecific binding sites, Matrigel coated culture plates were rehydrated with serum free DMEM containing 0.1% BSA for 90 min at 37℃ and then washed with same medium. PC3 and DU145 cells are trypsinized and resuspended in serum free DMEM containing 0.1% BSA. PC3 cells were seeded at 2×10^5/well and DU145 cells were seeded at 1×10^5/well in the presence of DMSO. 1, 10, or 50 µM DIM and incubated for 90 min at 37℃ and 5% CO_2. At the end of 90 min, the medium was removed and the cells were washed gently two times with PBS to remove unattached cells. The attached cells were harvested by trypsinization, resuspended and counted. In some experiment, cells were seeded into Matrigel coated wells in the presence of DMSO or 50 µM of DIM and incubated for 15, 30, 60 or 90 min at 37℃ and 5% CO_2. To examine the effect of pretreatment of DIM, PC3 cells were treated with DMSO, 1, 10 or 50 µM of DIM and incubated for 24 hr at 37℃ and 5% CO_2 before the adhesion assay. The pretreated cells were used for adhesion assay without DMSO or DIM during the assay. Each assay was performed in triplicates and repeated in three independent experiments. Values were expressed as the average of triplicate experiment.

Cell motility assay

Motility was assessed using a scratch wound assay (19). Cells were seeded into 6 well cell culture plates at a concentration of 2×10^5 cells and cultured in 10% FBS DMEM to near confluence. Confluent monolayer was carefully wounded using a sterile pipette tip and washed cellular debris gently with PBS. The wounded monolayer was incubated in 10% FBS DMEM containing 16 ㎍/mL fibronectin and DMSO, 1, 10 or 50 µM of DIM for 24 or 48 hr. Migrating cells were examined under 10X by phase contrast microscope and photographed.

Invasion assay

The ability of cells to migrating across Matrigel barrier (invasion) was determined by the modified Boyden chamber. Briefly, Boyden chambers were assembled using 8 ㎛ Falcon transwell inserts (Becton Dikinson, Bedford, MA) as the upper chamber and the 12 well plates as the lower chamber. Matrigel was applied to the insert at 25 ㎍ and left overnight to dry in a laminar flow fume hood. The insert was re-hydrated

with serum free DMEM for 90 min at 37℃ and 5% CO_2. Following rehydration, Matrigel coated inserts were washed with the same medium. Exponentially growing prostate tumor cells were harvested and resuspended in 0.1% BSA DMEM. PC3 cells were seeded at $2×10^5$ and DU145 cells were seeded at $1×10^5$. DMSO, 1, 10 or 50 μM of DIM was carefully added to the upper chamber. Twenty microgram of fibronectin (for PC3 cells) or 10% FBS (For DU145 cells) was added as a chemoattractant in the lower chamber. The Boyden chamber was incubated for 24 hr at 37℃ and 5% CO_2. At the end of incubation the cells in the upper chamber were removed gently. Cells that had transverse the Matrigel and attached to the lower surface of the insert were fixed with 10% formalin and stained with 0.5% crystal violet. Inserts were examined under 10X or 20X by phase contrast field microscopy and photographed. To examine the effect of pretreatment of DIM, PC3 cells were treated with DMSO, 1, 10 or 50 μM of DIM and incubated for 24 hr at 37℃ and 5% CO_2. The invasion assay was performed without DMSO or DIM. Values for invasion were expressed as the number of migrated cells per microscopic field. Four fields were counted. Each assay was performed in triplicates and repeated in three independent experiments. Values were expressed as the average of triplicate experiments.

Statistical analysis

It was performed using Sigma Stat software. Results were expressed as the mean±SD of three independent experiments, and statistical comparisons were based on one-way ANOVA, followed by Duncan's multiple range test. A p value<0.05 was considered statistically significant.

Results and Discussion

The process of metastasis consists of a series of interactions between tumor cells and their environment. The ability of malignant tumor cells to invade host tissues contributes to their locally destructive nature and can play an important role in their metastatic spread (4-6). Therefore, inhibition of adhesion, motility or invasion may be therapeutic target of metastasis. We chose two human prostate cancer cell lines, PC3 and DU145, derived from bone and brain metastases, respectively, and investigate the effect of DIM on the adhesion, migration and invasion. It was reported that DIM at a concentration of 150 μM did not cause a significant increase in the LDH levels compared with the control in the LDH assay which is the most widely used for measuring cellular lysis (20). We, therefore, assume that no toxic effects were manifested at the concentration used in this study.

Effect of DIM on the adhesion of PC3 and DU145 cells

The initial invasive action of metastatic cells requires tumor cells and the ECM interaction. Adhesion assays performed for 90 min, over 85% of the tumor cells attached to Matrigel coated wells. Treatment of 1 μM of DIM reduced adhesion of PC3 cells by 6%. At the higher concentration of 10 μM and 50 μM of DIM, PC3 cell adhesion to Matrigel coated wells was reduced by 17.2 and 65.5% respectively (Fig. 1).
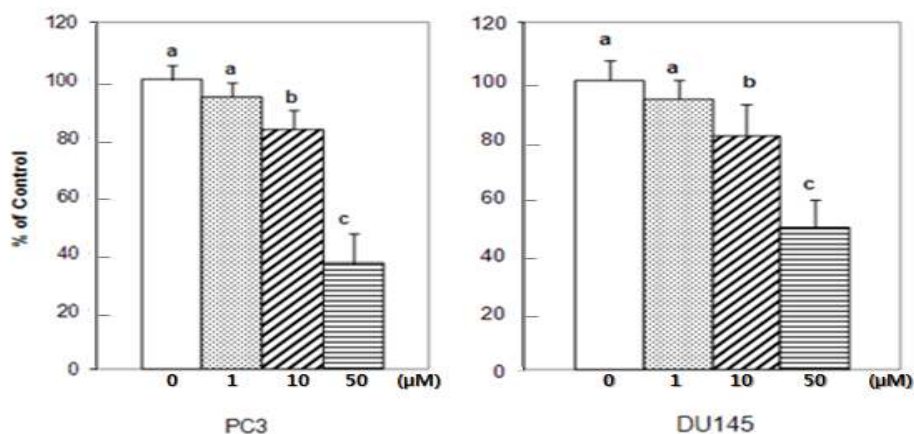

Fig. 1. Effect of DIM on the adhesion of PC3 and DU145 cells to the basement membrane

Cells were added to Matrigel coated plates and incubated for 90 min in present of 1, 10 or 50 μM of DIM. Attached cells were counted. Data were expressed as mean±SD. Stand error bars represent three independent experiments and each experiment was triplicated. Means with the same letter are not significantly different by Duncan's multiple range test (p<0.05).

DU145 cell adhesion was also reduced by DIM. DIM at concentration of 50 μM reduced adhesion of DU145 cells by 50% (Fig. 1). Several studies have indicated that prostate cancer cell lines express different number of protein family- eg. integrins- that are known to mediate interactions between cells and ECM proteins (21-24). I3C has been reported to inhibit the adhesion, migration and invasion of breast cancer cells by modulating the expression of a series of signaling proteins associated with adhesion, invasion and migration (25). When adhesion assay was done for 15 min with DIM, 50 μM of DIM inhibited adhesion of PC3 cells by 95% (Fig. 2). PC3 cells were pretreated with DIM for 24 hr before adhesion assay and adhesion assay was performed without DIM. Pretreatment of PC3 cells with 50 μM of DIM reduced adhesion by 27%. Sung and Feldman (26) reported that pretreatment of vitamin D3 for 72 hr reduced adhesion of PC3 and DU145 cells about by 30% and this effect of vitamin D3 is through modulation of cell surface adhesion molecules. However, in the present research, inhibitory effect of DIM during the adhesion assay (for 90 min) was higher than that of pretreatment of DIM for 24 hr (Fig. 1 vs 3). This suggests that DIM may reduce adhesion of PC3 cells by inhibiting both interaction between cancer cells and Matrigel coated membrane and expression of proteins which play an important
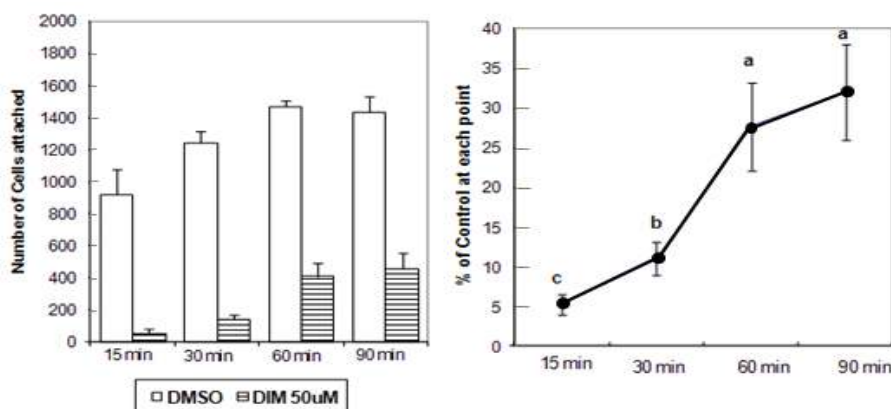

Fig. 2. Effect of DIM on the adhesion of PC3 cells to the basement membrane.

Cells were added to Matrigel coated plates and incubated for 15, 30, 60 or 90 min in present of 50 μM of DIM. Attached cells were counted. Data were expressed as mean±SD. Stand error bars represent three independent experiments and each experiment was triplicated. Means with the same letter are not significantly different by Duncan's multiple range test (p<0.05).
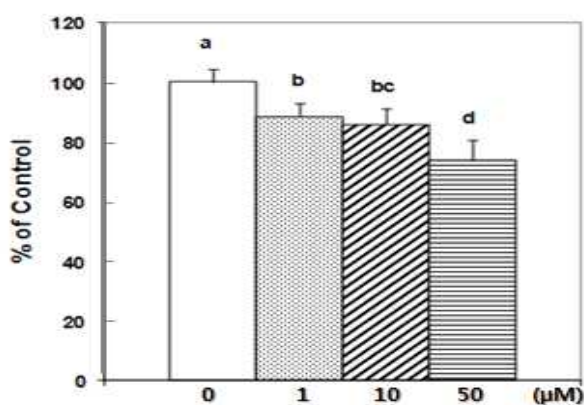

Fig. 3. Effect of pretreatment with DIM on the adhesion of PC3 cells.

PC3 cells were preincubated with DIM or DMSO for 24 hr prior to assay. Pretreated cells were added to Matrigel coated plates and incubated for 90 min without DIM or DMSO. Attached cells were counted. Data are expressed as mean±SD. Stand error bars represent three independent experiments and each experiment was triplicated. Means with same letter are not significantly different by Duncan's multiple range test (p<0.05).

role in adhesion.

Effect of DIM on the migration of PC3 and DU145 cells

DIM reduced migration of prostate cancer cells. Wound assay with PC3 cells was performed for 48 hr, since PC3 cells treated with DMSO did not cover the whole wound area for 24 hr. In our motility model, DIM reduced migration of prostate cancer cells dramatically. Migration of PC3 cells was reduced even by 1 μM, and 50 μM of DIM inhibited migration of PC3 cells more strongly. DIM at concentration of 10 μM and 50 μM reduced migration of DU145 cells (Fig. 4). Tumor metastasis involves extensive interaction of the invasive cancer cells with host and such interactions promote degradation of the ECM by specialized proteolytic enzymes produced by cancer cells (27,28). In vitro capacity of cells to migrate correlates with their invasive behavior *in vivo* (29).

Effect of DIM on the invasion of PC3 cells and DU145 cells

To successfully penetrate the Boyden chamber, cells must successfully adhere, degrade and transverse the Matrigel coated insert(30). DIM reduced invasion of PC3 cells and DU145 cells. DIM at concentration of 10 µM reduced invasion of PC3 cells by 70% and 50 µM of DIM by 90% (Fig. 5). DIM at concentration of 50 µM reduced invasion of DU145 cells by 70% (Fig. 5). Pretreatment of PC3 cell with DIM for 24 hr before assay reduced invasion of PC3 cell by 37% (Fig. 6). Similar to adhesion assay, pretreatment with DIM for 24 hr was less effective than treatment of PC3 cells during invasion assay. This suggests that DIM may affect the process of adhesion, degradation and transverse the Matrigel coated insert, in addition to expression of the molecules which need for the process such as adhesion molecules and proteolytic enzymes.

In the present study, we detected similar trends in response of PC3 and DU145 cells to DIM. But there was also small cell line difference in responses to DIM. For example, although adhesion to Matrigel of both cell lines showed similar response to DIM, migration and invasion by PC3 cells seemed more sensitive to DIM. It is interesting when we consider the response of proliferation curve of both cell lines to DIM. It was reported that the IC_{50} values for the cells treated with DIM were 40 µM for the PC3 cells and 20 µM
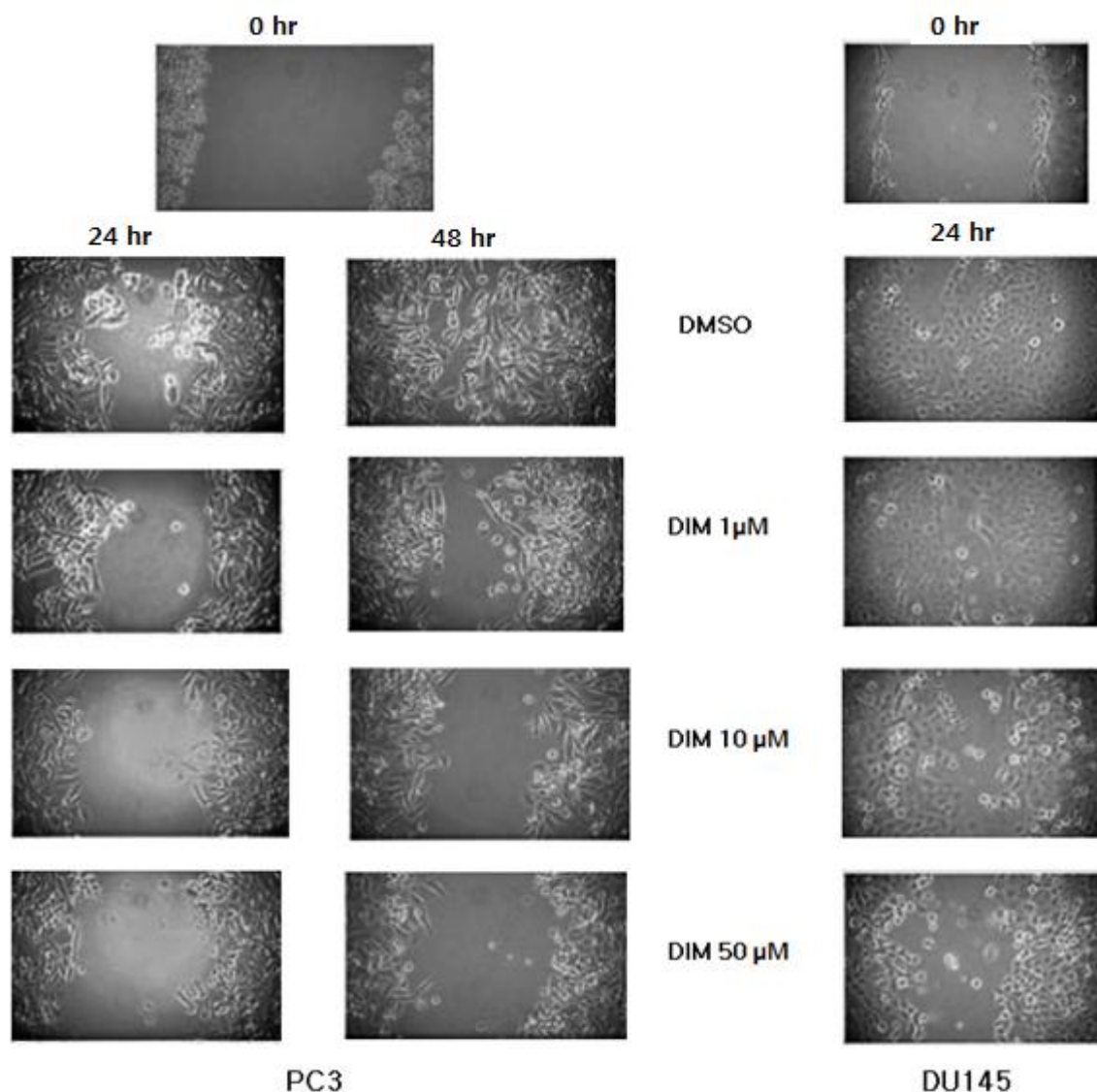

Fig. 4. Effect of DIM on the migration of PC3 and DU145 cells.

Cells were seeded into 6 well cell culture plates and cultured in 10% FBS DMEM to near confluence. Confluent monolayer was carefully wounded and washed cellular debris gently with PBS. The wounded monolayer was incubated in 10% FBS DMEM containing 16 µg/mL fibronectin and DMSO, 1, 10 or 50 µM of DIM for 48 hr.

for the DU145 cells (20). In summary, the current study has established that DIM can diminish prostate cancer cell adhesion, motility and invasion, and suggest that DIM may be an effective therapy in addition to traditional chemotherapy.

Examination of the in vivo effects of DIM on prostate cancer metastasis will help to determine whether administration of DIM is truly a promising treatment to decrease invasive and metastatic disease.

을 농도 의존적으로 억제하였다. 24시간동안 DIM으로 PC3 세포를 전 처리한 후 부착실험을 한 결과 농도 의존적으로 억제하였다. 그러나 암세포가 부착 시 DIM의 암세포 부착 억제가 전처리에 의한 부착 억제보다 효과적이었다. DIM 은 인간 전립선암세포의 이동과 침윤도 억제하였으며 24시 간 동안 PC3 세포를 DIM으로 전처리를 했을 때도 침윤 억제효과를 나타내었다. 또한 DIM의 억제효과는 세포주에 따라 다소 다른 경향을 보여 PC3 세포에 대한 억제효과가
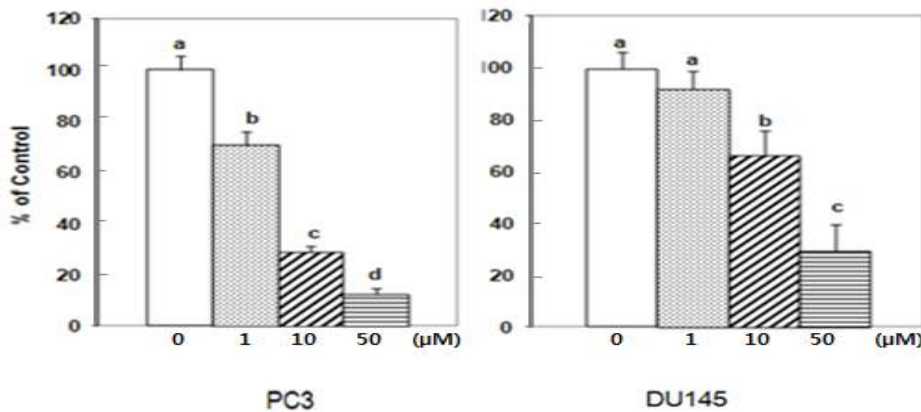

Fig. 5. Effect of DIM on the invasion of PC3 and DU145 prostate cancer cells.

Cells were treated with DMSO vehicle or DIM for 24 hr during assay. Data are expressed as mean±SD. Stand error bars represent three independent experiments and each experiment was triplicated. Means with the same letter are not significantly different by Duncan's multiple range test (p<0.05).
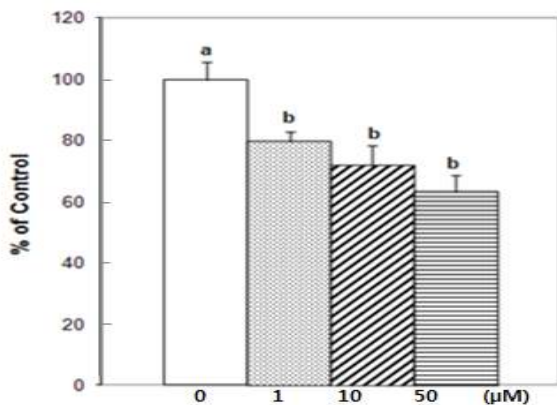

Fig. 6. Effect of pretreatment with DIM on the invasion of PC3 cells.

Cells were treated with DMSO vehicle or DIM for 24 hr prior to assay. DMSO or DIM was not added during invasion assay. Data are expressed as mean±SD. Stand error bars represent three independent experiments and each experiment was triplicated. Means with the same letter are not significantly different by Duncan's multiple range test (p<0.05).

DU145 세포에 대한 억제효과보다 큰 것으로 관찰되었다. DIM은 150 μM 까지 세포 독성이 관찰되지 않은 것으로 보고되고 있으므로 본 연구결과 DIM은 세포 독성이 없는 수준에서 인간 전립선암 세포인 PC3와 DU145 세포의 부 착, 이동, 침윤을 효과적으로 억제하여 전립선암 전이 억제 제로 이용될 수 있을 것으로 사료된다.

References

1. Chiarodo A (1991) National cancer institute roundtable on prostate cancer: future research directions. Cancer Res, 51, 2498-2505

2. Hong MY, Seeram NP, Heber D (2008) Pomegranate polyphenols down-regulate expression of androgen receptor. J Nutr Biochem, 19, 848-855

3. Festuccia C, Bologna M, Gravina GL, Guerra F, Angelucci A, Villanova I, Millimaggi D, Teti A (1999) Osteoblast conditioned media contain TGF-β and modulate the migration of prostate tumor cells and their interactions with extracellular matrix component. Int J Cancer, 81, 395-403

4. Song G, Ohashi T, Sakamoto N, Sato M (2006) Adhesive

요 약

본 연구는 3,3'-diindolylmethane(DIM)이 인간전립선암 세포인 PC3 세포와 DU145 세포의 부착, 이동, 침윤에 미치 는 영향을 살펴보았다. DIM은 PC3와 DU145 세포의 부착

force of human hepatoma HepG2 cells to endothelial cells and expression of E-selectin. Mol Cell Biomech, 3, 61-68

5. Festuccia C, Guerra F, D'Acenzo S, Guinciuglio D, Albini A, Bologna M (1998) *In vitro* regulation of pericellular proteolysis in prostatic tumor cells treated with bombesin. Int J Cancer, 75, 418-431

6. Weber GF (2008) Molecular mechanism of metastasis. Cancer Lett, 270, 181-190

7. Kohn EC, Liotta LA (1995) Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res, 55, 1856-1862

8. Shaikh FM, Seales EC, Clem WC, Hennessy KM Zhuo Y, Bellis SL (2008) Tumor cell migration and invasion are regulated by expression of variant integrin glycoforms. Exp Cell Res, 314, 2941-2950

9. Yoneda T, Sasaki A, Mundy GR (1994) Osteolytic bone metastasis in breast cancer. Breast Cancer Res Treat, 32, 73-84

10. Cohen JH, Kristal AR, Stanford JL (2000) Fruit and vegetable intakes and prostate cancer risk. J Natl Cancer Inst, 92, 61-68

11. Kolonel LN, Hankin JH, Whittemore AS, Wu AH, Gallagher RP, Wilkens LR, John EM, Howe GR, Dreon DM, West DW, Paffenbarger RS (2000) Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. Cancer Epidemiol Biomarkers Prev, 9, 795-804

12. Li Y, Li X, Sarkar FH (2003) Gene depression profiles of I3C and DIM-treated PC3 human prostate cancer cells determined by cDNA microarray analysis. J Nutr, 133, 1011-1019

13. Kedmi MN, Yannai S, Fares FA (2004) Induction of apoptosis in human prostate cancer cell line, PC3, by 3,3'-diindolylmethane through the mitochondrial pathway. British J Cancer, 91, 1358-1363

14. Chinnakannu K, Chen D, Li Y, Wanf Z, Dou QP, Reddy GPV, Sarkar FH (2009) Cell cycle-dependent effects of 3,3'-diindolylmethane on proliferation and apoptosis of prostate cancer cells. J Cell Physiol, 219, 94-99

15. Bradlow HL, Michnovicz JJ, Telang NT, Osbourne MP (1991) Effect of dietary indole3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. Carcinogenesis, 12, 1571-1574

16. Auborn KJ, Fan S, Rosen EM, Goodwin L, Chandraskaren A, Williams DE, Chen D, Carter TH (2003) Indole 3-Carbinol is a negative regulator of estrogen. J Nutr, 133, 2470S-2475S

17. Meng Q, Qi N, Chen DZ, Yuan R, Goldberg ID, Rosen EM, Auborn K, Fan S (2000) Suppression of breast cancer invasion and migration by indole-3-carbinol : associated with up-regulation of BRCA1 and E-cadherin/catenin complexes. J Mol Med, 78, 155-165.

18. Chang X, Tou JC, Hong C, Kim HA, Riby JE, Firestone G, Bjeldanes LF (2005) 3,3'-Diindolylmethane inhibits angiogenesis and the growth of transplantable human breast carcinoma in athymic mice. Carcinogenesis, 26, 771-778

19. Goodman SL, Vollmer HP, Birchmeier W (1985) Control of cell locomotion: perturbation with an antibody directed against specific glycoproteins. Cell, 41, 1029-1038

20. Kedmi MN, Yannai S, Haj A, Fares FA (2003) Indole 3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. Food Chem Toxicol, 41, 745-752

21. Zheng DQ, Woodard AS, Fronaro M, Tallini G, Languino LR (1999) Prostatic carcinoma cell migration via avb3 integrin is modulated by a focal adhesion kinase pathway. Cancer Res, 59, 1655-1664

22. Hywood-Reid PL, Zipf DR, Springer WR (1997) Quantification of integrin subunits on human prostatic cell lines-comparison of nontumorigenic and tumorigenic lines. Prostate, 31, 1-8

23. Rabinovitz I, Nagle RB, Cress AE (1993) Integrin av eosion expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype and in vitro and in vivo. Clin Exp Metastasis, 13, 481-491

24. Witkowski CM, Rabinovitz I, Nagle RB, Affinito KS, Cress AE (1993) Characterization of integrin subunits, cellular adhesion and tumorigenicity of four human prostate cell lines. J Cancer Res Clin Oncol, 119, 637-644

25. Weng JR, Tsai CH, Kulp SK, Chen CS (2008) Indole 3-carbinol as a chemopreventive and anti-cancer agent. Cancer Letter, 262, 153-163

26. Sung V, Feldman D (2000) 1, 25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration. Mol Cellular Endocrinol, 164, 133-143

27. Huang Y, Kim MS, Ratovitski E (2008) Midkine promotes tetraspanin-integrin interaction and includes FAK-Stat1 alpha pathway contributing to migration/ invasiveness of human head and neck squamous cell carcinoma cells. Biochem Biophys Res Commun, 377, 474-478

28. Kawasaki G, Yanamoto S, Yoshitomi I, Yamada S, Mizuno A (2008) Overexpression of metastasis-associated

MTA1 in oral squamous cell carcinomas: correlation with metastasis and invasion. Int J Oral Maxillofac Surg, 37, 1039-1046

29. Mohler JL (1993) Cellular motility and prostate metastasis. Cancer Metastasis Rev, 6, 474-480

30. Cos S, Fernández R, Güézmes A, Sánchez-Barceló EJ (1998) Influence of melatonin on invasive and metastatic properties of MCF-7 human breast cancer cells. Cancer Res, 58, 4383-4390