Abstract. Gastric cancer is the fourth most common cancer and is one of the leading causes of cancer-related mortality worldwide. Forkhead box M1 (FOXM1) is overexpressed in gastric cancer, suggesting that it is important in gastric cancer oncogenesis. However, no studies have investigated the role of 3,3'-diindolylmethane (DIM), a component of cruciferous vegetables, in the regulation of FOXM1 and its signaling pathway in gastric cancer. Here, we report for the first time that DIM effectively downregulated Akt/FOXM1 in gastric cancer cells. Combination treatment with DIM and paclitaxel significantly and dose-dependently inhibited the proliferation of SNU638 cells when compared to treatment with DIM or paclitaxel alone. Colony formation of SNU638 cells was significantly attenuated by treatment with DIM and paclitaxel, and DIM potentiated the inhibition of colony formation in SNU638 cells by paclitaxel when compared to treatment with a single agent. Treatment with DIM plus paclitaxel substantially increased apoptosis as indicated by increased levels of cleaved polyADP-ribose polymerase (PARP) and cleaved caspase-9 protein. DIM dose-dependently sensitized gastric cancer cells through downregulation of FOXM1 and potentiated the effects of paclitaxel. FOXM1 effector genes such as CDK4, p53 and cyclin D1 were downregulated in gastric cancer cells by combination treatment with DIM and paclitaxel. In addition, DIM significantly and dose-dependently inhibited phosphorylation of Akt and potentiated paclitaxel-induced inhibition of Akt function in gastric cancer cells. Therefore, our results indicate that DIM effectively potentiates the efficacy of chemotherapeutic agents such as paclitaxel by downregulation of the Akt/FOXM1 signaling cascade in gastric cancer cells. Our findings suggest that DIM enhances the therapeutic efficacy of paclitaxel in gastric cancer and is a potential clinical anticancer agent for the prevention and/or treatment of gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer and is considered one of the most deadly cancers worldwide (1-4). Although the incidence of gastric cancer has decreased recently, the mortality rate remains high due to late diagnosis and a lack of understanding of molecular pathogenesis. In South Korea, the survival of gastric cancer patients has increased due to early detection. The 5-year survival rate for stage I gastric cancer is >90% in Korea (4,5). However, in the West, the 5-year survival rate is generally <50% for stage II and 20% for stage III cancer (4,6,7). Thus, early diagnosis of gastric cancer and preventive treatment are urgently needed to control the high mortality of this disease.

Diet is suggested to be crucial in gastrointestinal cancer development and progression (8). Epidemiological studies show a strong inverse correlation between vegetable intake and the risk of cancer including gastric cancer (9-15). Turati et al (12) and Guercio et al (16) reported that high allium vegetable consumption reduces gastric cancer risk, and a high intake of cruciferous vegetables was found to be inversely associated with the risk of gastric cancer in humans in a study by Wu et al (15). The compound 3,3'-diindolylmethane (DIM) is a component of cruciferous vegetables that inhibits tumor growth through induction of apoptosis in various types of cancer cells (17-20). Our previous study demonstrated that DIM suppressed gastric cancer cell growth via activation of the Hippo signaling pathway (17). DIM was found to inhibit gastric cancer cell growth by modulating the aryl hydrocarbon receptor (21). However, the function of DIM in gastric cancer cells has not been clearly elucidated.

Forkhead box M1 (FOXM1) is an oncogenic FOX transcription factor (22,23). Increased expression of FOXM1 has been noted in a variety of aggressive human cancers (24,25), suggesting that FOXM1 is important in oncogenesis. In gastric cancer, FOXM1 is highly expressed and strongly correlated with poor prognosis associated with drug resistance (26-29). Although FOXM1 is important in gastric cancer, no studies have investigated DIM and the regulation of FOXM1 in gastric cancer.
cancer. Moreover, the molecular mechanisms by which DIM contributes to Akt/FOXM1 signaling pathway regulation in gastric cancer are not fully understood. We tested whether DIM potentiated the efficacy of chemotherapeutic agents such as paclitaxel in gastric cancer cells. Our results showed that DIM inhibited Akt/FOXM1 signaling, which led to the chemosensitization of gastric cancer cells and potentiation of the efficacy of chemotherapeutic agents such as paclitaxel. Our findings also demonstrated that the Akt/FOXM1 pathway is a novel molecular target of DIM, and that targeting this pathway by combination treatment with DIM and paclitaxel is a new strategy for gastric cancer treatment.

**Materials and methods**

**Cell lines and experimental reagents.** The gastric cancer cell line SNU638 was obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). Primary antibodies for Akt, p-Akt, p-GSK-3β, cleaved-PARP, cleaved-caspase-9 and caspase-3 were obtained from Cell Signaling Technology (Beverly, MA, USA), and antibodies to FOXM1 and GAPDH were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). DIM was purchased from LKT Laboratories (St. Paul, MN, USA), and paclitaxel was dissolved in dimethyl sulfoxide (DMSO) (both from Sigma Chemical Co., St. Louis, MO, USA).

**Cell proliferation assay.** SNU638 cells were seeded in 96-well plates. After 24 h, the cells were treated with DIM (0, 50, 75 or 100 µM), followed by 72 h of growth with or without paclitaxel (0, 50, 75 or 100 nM). Cell growth was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as previously described (30,31). MTT (50 µl) was added to the culture medium of growing cells at the indicated time points, and the cells were incubated for a further 3 h. DMSO (200 µl) was added and the absorbance was measured at 540 nM using a model Epoch microplate reader (BioTek, Winooski, VT, USA). Three independent experiments were performed in triplicates.

**Soft agar colony formation assay.** Colony formation assays were performed as previously described (17,19). Cells were seeded at 5x10⁴ cells/plate with or without DIM and paclitaxel, grown until visible colonies appeared and observed under microscopy to count colonies. Experiments were performed >3 times.

**Western blot analysis.** SNU638 cells were treated with DIM (0, 50, 75 or 100 µM), followed by 72 h of growth with or without paclitaxel (0, 50, 75 or 100 nM). Cells with or without DIM and paclitaxel were harvested and suspended in lysis buffer (Intron Biotechnology, Inc.) as previously described (17,19,31). The protein concentration was determined using BSA protein assay kits (Pierce, Rockford, IL, USA). Whole lysates were resolved on SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA), and probed with specific primary antibodies for cleaved-PARP, cleaved-caspase-9, caspase-3, p-Akt, Akt, FOXM1, cyclin D1, CDK4, p53 and p-GSK-3β and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology). Immunodetection was performed with an enhanced chemiluminescent kit (Amersham, Arlington Heights, IL, USA).

**Statistical analysis.** Experiments were repeated >3 times. Data are expressed as mean ± SE. Comparisons between groups were evaluated using the Student's t-test or one-way ANOVA where appropriate. A p-value <0.05 was considered to indicate a statistically significant result.

**Results**

**Inhibition of cell growth by DIM and paclitaxel.** SNU638 gastric cancer cells were treated with the indicated doses of DIM and paclitaxel for 72 h. DIM and paclitaxel dose-dependently inhibited gastric cancer cell viability at 72 h. As shown in Fig. 1, treatment with 75 µM DIM or 25 nM paclitaxel caused 30-50% growth inhibition of SNU638 cells. However, a combination of DIM and paclitaxel resulted in 70-80% growth inhibition of the SNU638 cells at 72 h, suggesting a greater inhibitory effect of the combination treatment. Our results revealed that a combination of DIM with a low-dose of paclitaxel had substantially greater inhibition of cancer cell growth compared with either agent alone.
Inhibition of colony formation by DIM and paclitaxel. We further investigated the antitumor effects of DIM and paclitaxel using colony formation assays. As shown in Fig. 2, treatment with 50 µM DIM and 25 nM paclitaxel significantly inhibited the colony formation of the SNU638 cells. Moreover, the combination treatment with DIM and paclitaxel at the indicated doses resulted in greater inhibition of the colony formation of the SNU638 cells than treatment with a single agent (Fig. 2).

Induction of apoptosis by DIM and paclitaxel. To elucidate the apoptotic effects of DIM and paclitaxel on SNU638 cells, we measured levels of the apoptotic factors: cleaved-PARP, cleaved-caspase-9 and caspase-3. As shown in Fig. 3A, treatment with 50 µM DIM or 25 nM paclitaxel alone dose-dependently increased cleaved-PARP and cleaved caspase-9 proteins, whereas expression of caspase-3 was significantly decreased. The combination treatment of DIM and paclitaxel at the indicated doses significantly induced cleaved-PARP and cleaved caspase-9, while caspase-3 was significantly suppressed (Fig. 3B). These results revealed that the combination of DIM and paclitaxel induced greater apoptotic effects in the SNU638 cells than the single agents alone, supporting DIM enhancement of paclitaxel-induced cell growth inhibition in gastric cancer cells.

Downregulation of FOXM1 levels by DIM and paclitaxel. FOXM1 is an important transcription factor for cancer cell growth (22). Therefore, we examined whether DIM and paclitaxel have a functional impact on FOXM1 expression in gastric cancer oncogenesis. After treatment of the SNU638 cells with DIM or paclitaxel alone, FOXM1 expression decreased dose-dependently at 72 h (Fig. 4A). Expression of FOXM1 was reduced after treatment with a combination of DIM and paclitaxel compared to treatment with either
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agent alone (Fig. 4B). Levels of the cyclin-dependent kinase inhibitor p53 which is a downstream gene affected by FOXM1, increased dose-dependently after DIM or paclitaxel treatment, and combination treatment with these two drugs increased the levels of p53 at 72 h (Fig. 4B). CDK4 and CDK6 protein significantly and dose-dependently decreased after a 72 h treatment with DIM or paclitaxel (Fig. 4A), and the combination treatment significantly downregulated CDK4 protein level (Fig. 4B). These results revealed that the combination of DIM and paclitaxel induced more marked downregulation of FOXM1, which induced cell cycle inhibition in the SNU638 cells, when compared with treatment with each single agent alone. These findings support that DIM effectively potentiates paclitaxel cytotoxicity.

Downregulation of Akt by DIM and paclitaxel. To study the functional relevance of alteration of Akt expression in gastric cancer cells mediated by DIM or paclitaxel, we measured p-Akt, Akt and p-GSK-3β protein in the SNU638 cells. DIM significantly and dose-dependently decreased the p-Akt level,
while the Akt protein level was unchanged (Fig. 5A). Paclitaxel also significantly and dose-dependently inhibited expression of p-Akt at 72 h (Fig. 5A). Furthermore, the combination treatment with DIM (50 μM) and paclitaxel (25 nM) resulted in significantly greater reduction in p-Akt protein at 72 h compared with treatment with either agent alone (Fig. 5B). In addition, the p-GSK-3β protein level was significantly and dose-dependently increased by DIM or paclitaxel treatment, and treatment with the combination of the two drugs induced higher expression of p-GSK-3β protein in the gastric cancer cells than did either agent alone.

Discussion

The present study was performed to investigate the molecular mechanisms by which DIM contributes to Akt/FOXM1 signaling pathway regulation and to determine whether DIM potentiates the efficacy of chemotherapeutic agents such as paclitaxel in gastric cancer cells. We determined the effects of DIM on Akt/FOXM1 and its signaling pathway in gastric cancer cells. Our results showed that DIM can function as a FOXM1 suppressor in gastric cancer cells. Inactivation of Akt by DIM resulted in the downregulation of FOXM1 and caused suppression of the FOXM1 downstream signaling pathways of the cell cycle and apoptotic induction. In addition, we found that DIM effectively potentiated the efficacy of chemotherapeutic agent paclitaxel by downregulation of the Akt/FOXMI signaling cascade in gastric cancer cells. Therefore, our results suggest that DIM enhances the therapeutic efficacy of paclitaxel in gastric cancer and is a potential clinical anticancer agent for prevention and/or treatment of gastric cancer.

DIM, a non-toxic dietary chemopreventive agent, has been investigated for preventing, inhibiting and reversing the progression of various types of cancer (32). Many studies have suggested that DIM is strongly associated with tumor growth suppression mediated by several signaling pathways in human carcinomas (9,17-20,24,32). We found that DIM significantly inhibited gastric cancer cell growth in a dose-dependent manner and potentiated the antitumor effects of paclitaxel in gastric cancer cells. Colony formation was also significantly attenuated by treatment of SNU638 cells with DIM and paclitaxel, and DIM notably potentiated paclitaxel's colony formation inhibition in SNU638 cells compared to treatment with a single agent. In addition, treatment with DIM plus paclitaxel significantly increased apoptosis as indicated by increased levels of cleaved PARP and caspase-9 proteins. These observations are similar to the findings of previous studies demonstrating that DIM enhances taxotere-induced growth inhibition of breast cancer cells (24). Therefore, our findings provide strong evidence in support of the enhancement by DIM of paclitaxel-induced cell growth inhibition and apoptosis in gastric cancer cells.

Accumulating evidence shows that activation of FOXMI is associated with the development of human cancers (33-35). FOXM1 is a FOX transcription factor that regulates a number of cell cycle regulators (33,34,36,37). Several studies have shown that FOXM1 is overexpressed in tumors including lung, liver and breast cancers and is associated with prognosis (38-40). In addition, suppression of FOXM1 by genetic or pharmaceutical approaches significantly inhibited the proliferation and migration of cancers in in vitro and in vivo studies (41-44). FOXM1 is highly expressed in gastric tumor tissue compared to normal gastric epithelium and is associated with the prognosis of gastric cancer patients (27). In addition, Qian et al demonstrated that FOXM1 promotes gastric cancer cell proliferation through activation of twist 1 (45). FOXM1 was shown to be overexpressed in human gastric cancers in previously published studies (27,45), thus it appears to be an attractive potential target for prevention and therapeutic intervention in gastric cancer. It is of interest to examine how DIM effects FOXM1 regulation in gastric cancer cells. We found that DIM sensitized gastric cancer cells through dose-dependent downregulation of FOXM1 and potentiated the effects of paclitaxel, which significantly suppressed FOXM1 expression in gastric cancer cells. FOXM1 and its effector genes, CDK4, p53 and cyclin D1, were significantly downregulated in the gastric cancer cells by combination treatment with DIM and paclitaxel. Our results confirm the hypothesis that inactivation of FOXM1 is one of the molecular methods by which drug combinations potentiate cell cycle arrest and inhibition of proliferation of gastric cancer cells.

Since FOXM1 is a downstream gene in the Akt signaling pathway that modulates cell survival and metastasis (22,23), we examined whether DIM affects the Akt signaling pathway that is associated with FOXM1 in gastric cancer cells. DIM significantly and dose-dependently inhibited phosphorylation of Akt, which is the active form of Akt and significantly increased phosphorylation of GSK-3β with paclitaxel in gastric cancer cells. DIM significantly potentiated paclitaxel-induced inhibition of Akt function and induced activation of GSK-3β in gastric cancer cells. In agreement with our findings concerning gastric cancer cells, studies have demonstrated that DIM modulates PI3K/Akt signaling, which induces cell cycle arrest and apoptosis in various types of cancers (19,32,46-48). Other studies have shown that FOXM1 is regulated by FOXO3a, a downstream gene in the PI3K/Akt/FOXO signaling pathway (22,49). When PI3K/Akt signaling is activated, FOXO3a is inactivated and does not suppress expression of FOXM1. Although we did not measure FOXO3a expression, we assume that DIM inhibits activation of Akt signaling, which leads to increased accumulation of FOXO3a. Thus, increased FOXO3a may inhibit FOXM1 expression in gastric cancer cells. Therefore, our findings suggest that inactivation of Akt by DIM promotes inhibition of FOXM1 expression, further potentiating paclitaxel-induced inactivation of Akt and FOXM1 in gastric cancer cells. However, the underlying mechanisms by which Akt mediates FOXO3a and FOXM1 by DIM in the nucleus and cytosol need to be further explored.

In conclusion, we demonstrated the molecular mechanism by which DIM suppresses gastric cancer cell growth by inhibition of FOXM1 function via the Akt signaling pathway. Combination treatment with DIM and paclitaxel is a potential strategy for treating gastric cancer patients, although further in-depth studies and testing of combinations of these two drugs are required to support this therapy for gastric cancer.

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References


