FOXM1-mediated downregulation of uPA and MMP9 by 3,3'-diindolylmethane inhibits migration and invasion of human colorectal cancer cells

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Abstract. Although 3,3'-diindolylmethane (DIM) has been suggested to reduce the risk of colorectal cancer, the underlying biological mechanism is not clearly understood. In the present study, we investigated the effect of DIM on the migratory and invasive activities of the human colorectal cancer cell lines DLD-1 and HCT116. DIM significantly inhibited the migration and invasion of colorectal cancer cells as assessed by wound healing and Matrigel invasion assays. The migratory ability of the DLD-1 and HCT116 cells was significantly reduced by DIM at 24 and 48 h. DIM also significantly inhibited the invasion rate of the DLD-1 and HCT116 cells in a dose-dependent manner. The mRNA expression levels of urokinase type plasminogen activator (uPA) and matrix metalloprotease 9 (MMP9) were significantly attenuated, whereas expression of E-cadherin mRNA was significantly enhanced, following DIM treatment. DIM also decreased the protein levels of uPA and MMP9, yet significantly increased E-cadherin protein expression. In addition, DIM significantly reduced the mRNA and protein levels of FOXM1 in the DLD-1 and HCT116 cells. Our results suggest that DIM can influence the cell migratory and invasive properties of human colorectal cancer cells and may decrease the invasive capacity of colorectal cancer through downregulation of uPA and MMP9 mediated by suppression of the transcription factor FOXM1.

Introduction

Colorectal cancer is the third most common cancer worldwide, and its incidence in South Korea has dramatically increased as a result of changes in the economy and lifestyle (1). Although genetic predisposition is an important contributing factor for the occurrence of colorectal cancer, the main mechanisms underlying the pathogenesis of colorectal cancer remain largely unclear. Previous studies suggest that diet plays a significant role in colorectal cancer development and progression (2,3). Moreover, cellular migration and invasion are important features of colorectal cancer progression, and therefore interception of the migration and invasion of colorectal cancer cells could be a potential therapeutic target. Despite improvements in conventional therapies for colorectal cancer, the quality of life of patients with colorectal cancer remains poor, and new therapeutic targets or preventive tools for colorectal cancer are therefore urgently needed.

The cancer preventive role of 3,3'-diindolylmethane (DIM) in several types of cancer cells has been highlighted, and several studies have proposed DIM as a potent natural dietary compound that reduces colorectal cancer risk and performs several antitumor activities (4-11). These functions include inhibition of colorectal cancer cell proliferation by suppression of YAP through an Akt-dependent process, alteration of cell cycle progression, and activation of caspase-8 to induce apoptosis (6,10,11). Movement of cells in the extracellular matrix (ECM) and their adhesion to the ECM are critical processes in the invasion of metastatic colorectal cancer cells. Despite extensive efforts to understand the mechanism underlying adhesion to the ECM during colorectal cancer metastasis, the effects of DIM on the adhesion and migratory properties of colorectal cancer cells have not been elucidated. To clarify the effect of DIM on the metastatic functions of colorectal cancer, we investigated its effect on migration and invasion of cultured colorectal cancer cells. In the present study, we showed that DIM significantly suppressed the cell migratory and invasive properties of colorectal cancer cells and that these effects may be in part mediated through inactivation of FOXM1 and the resultant downregulation of urokinase type plasminogen activator (uPA) and matrix metalloprotease 9 (MMP9).

Materials and methods

Materials. Antibodies specific for E-cadherin, uPA, FOXM1 and GAPDH were purchased from Santa Cruz Biotechnology

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Inc. (Santa Cruz, CA, USA), and the antibody against MMP9 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). DIM was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA).

Cell culture. The DLD-1 and HCT116 cell lines were obtained from the University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM)/F12 medium (both from Gibco, Grand Island, NY, USA) were used for cell culture. Cells were grown in RPMI-1640 (DLD-1) or DMEM/F12 (HCT116) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 mg/ml streptomycin and 100 IU/ml penicillin in a 5% CO_2 humidified atmosphere at 37˚C. The DLD-1 and HCT116 cells were treated with various concentrations of DIM in FBS-free medium.

Wound healing assay. To detect the effect of DIM on the migration of the HCT116 and DLD-1 colorectal cancer cells, we performed a wound healing assay as previously described (12). Control and DIM-treated colorectal cancer cells were grown to confluency, and a wound was made through the monolayer using a 200-μl pipette tip. Accurate measurements of the wounds were taken during the time course to calculate the migration rate according to the equation: Percentage of wound healing = [(wound length at 0 h) - (wound length at 24 or 48 h)]/(wound length at 0 h) x 100.

Matrigel invasion assay. BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences, San Jose, CA, USA) were used for the *in vitro* cell invasion assay according to the manufacturer's protocol. Briefly, the Matrigel-coated chambers were rehydrated in a humidified tissue culture incubator at 37˚C in a 5% CO_2 atmosphere. Cells ($2.5x10^4$) were suspended in 500 μl medium in each Matrigel-coated Transwell insert and the lower chamber of the Transwell was filled with 500 μl of medium. After incubation, the cultures were washed and stained with a Diff-Quik kit (Sysmex Corp., Kobe, Japan). Cells on the upper side of the insert membrane were removed and cells that migrated to the lower side of the membrane were counted on an inverted microscope (magnification, x100). Five fields were randomly selected, and the invasion rates were calculated as previously described (12).

RNA isolation and real-time PCR. Total RNA was isolated from the DLD-1 and HCT116 cells before and after DIM (100 μM) treatment. Reverse transcription was carried out with a PrimeScript™ RT reagent kit (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed using SYBR Premix Ex *Taq* (Takara Bio Inc.) in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR program was initiated at 95˚C for 30 sec followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The results were normalized to those for GAPDH and were calculated from threshold cycle numbers. Primer sequences were as follows: E-cadherin sense, 5'-GGATTGCAAATTC CTGCCATTC-3' and antisense, 5'-AACGTTGTCCCGGGTG TCA-3'; uPA sense, 5'-AGAATTCACCACCATCGAGA-3' and antisense, 5'-ATCAGCTTCACAACAGTCAT-3'; MMP9 sense, 5'-GACCTCAAGTGGCACCACCA-3' and antisense, 5'-GTGGTACTGCACCAGGGCAA-3; FOXM1 sense, 5'-AC GTCCCCAAGCCAGGCTC-3 and antisense, 5'-CTACTG TAGCTCAGGAATAA-3'; GAPDH sense, 5'-GTCTCCTC TGACTTCAACAGCG-3' and antisense, 5'-ACCACCCTGTT GCTGTAGCCAA-3'.

Western blot analysis. Colorectal cancer DLD-1 and HCT116 cells were treated with DIM at the indicated concentrations for 48 h. Cell lysates were prepared by suspending the cells in lysis buffer (Intron Biotechnology, Seoul, Korea), and western blot analysis was performed as previously described (6,13). Briefly, cell extractions were incubated on ice for 20 min and centrifuged at 13,000 x g for 5 min at 4˚C (6). The protein concentration was determined using a BSA protein assay kit (Pierce, Rockford, IL, USA). Whole lysate was resolved on an SDS-PAGE gel, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) (6).

Statistical analysis. The statistical significance of differences between groups was tested by one-way ANOVA and then later compared among groups with an unpaired Student's t-test. The experimental data are presented as the means ± SEM. A p-value <0.05 was considered to indicate a statistically significant result. All experiments were repeated more than three times.

Results

DIM inhibits the migration of colorectal cancer cells. A wound healing assay was performed to investigate the effect of DIM on colorectal cancer cell migration. This assay tested the effect of DIM on the migration of the DLD-1 and HCT116 cells based on the ability of cells to interact with extracellular matrix in the presence of DIM. In the presence of 20 μM DIM, the migratory ability of the DLD-1 and HCT116 cells maintained in 5% FBS was significantly reduced at 24 and 48 h (Fig. 1A and B). Treatment of the DLD-1 and HCT116 cells with 50 μM DIM in the presence of 10% FBS had an even greater antimigratory effect at 24 and 48 h (Fig. 2). These results indicate that DIM has an antimigratory effect on colorectal cancer cells.

DIM inhibits the invasion of colorectal cancer cells. We further investigated the effect of DIM on colorectal cancer cell invasion using a Matrigel invasion assay. We found that DIM significantly inhibited the invasion of the DLD-1 and HCT116 cells in a dose-dependent manner; 20 μM DIM significantly suppressed the invasion rates by 20 and 40% in the DLD-1 and HCT116 cells, respectively, whereas 50 μM DIM suppressed the invasion rates of both cell lines by 70% (Fig. 3). These data suggest that DIM significantly inhibits the migration and invasion rates of colorectal cancer cells.

Effect of DIM on the expression of migration-related mRNAs and proteins in colorectal cancer cells. To further study the effect of DIM on the migration and invasion of colorectal cancer cells, we performed RT-PCR and western blot analyses to investigate the underlying molecular events. Loss of E-cadherin expression is used as an indicator of

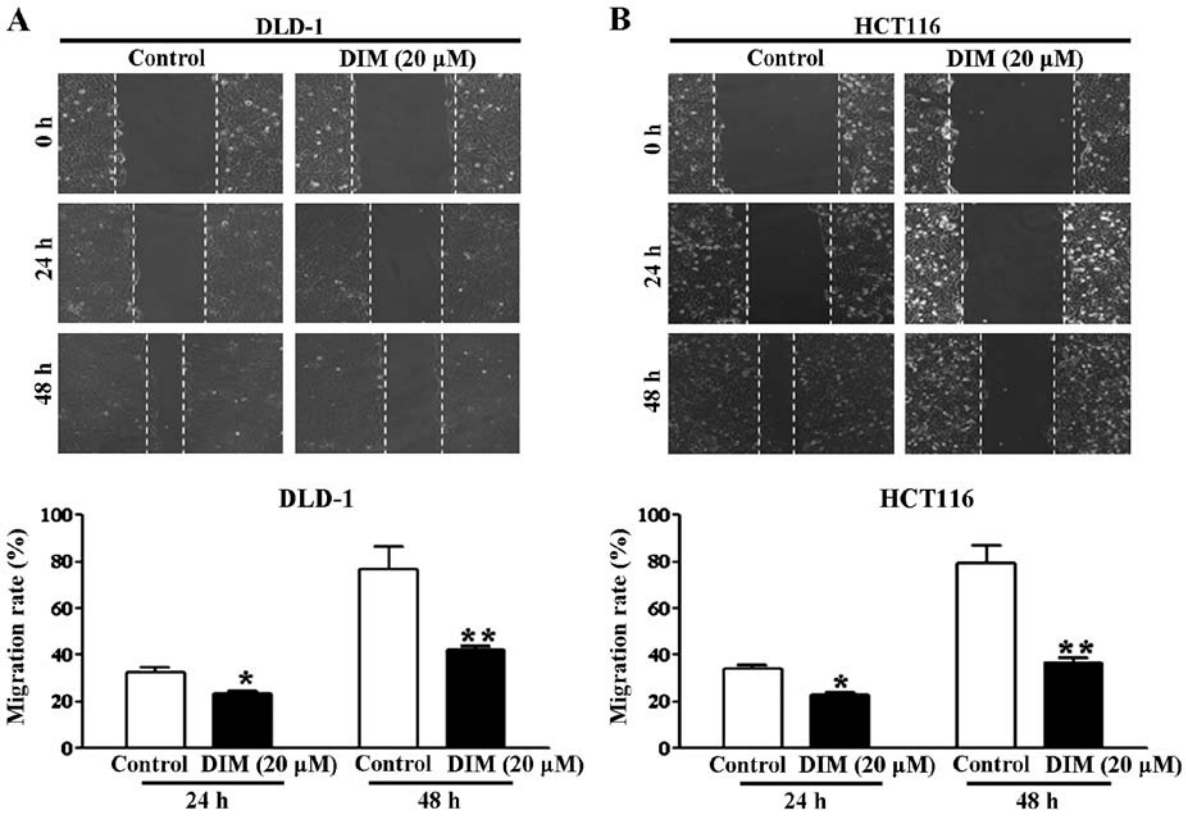

Figure 1. Effect of DIM on the migration rates of the colorectal cancer cells assessed by a wound healing assay. (A) DLD-1 and (B) HCT116 cells were treated with 20 μM DIM with 5% FBS and incubated for 24 and 48 h. Both the DLD-1 and HCT116 cells showed a significant antimigratory effect at 24 and 48 h. *p<0.05 and **p<0.01 compared to the control. DIM, 3,3'-diindolylmethane; FBS, fetal bovine serum.

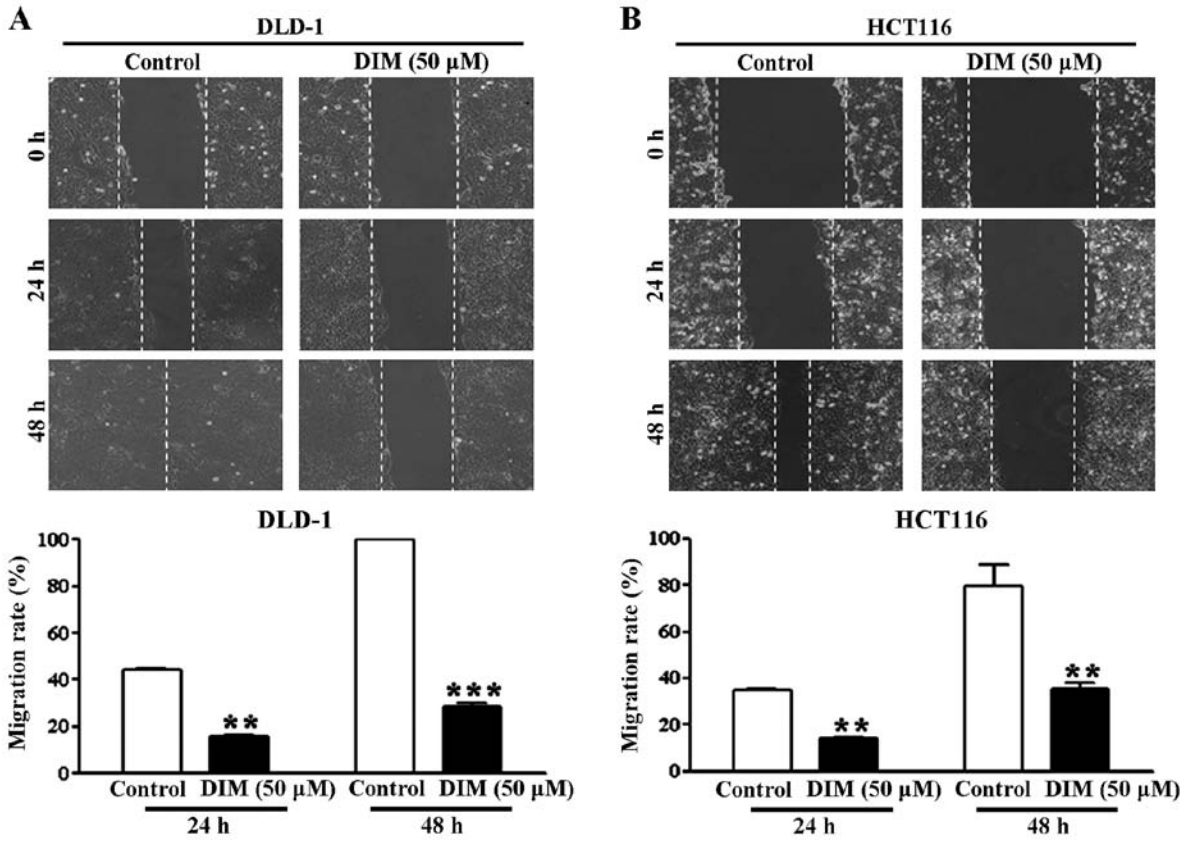

Figure 2. Effect of DIM on the migration rates of colorectal cancer cells by wound healing assay. (A) DLD-1 and (B) HCT116 colon cancer cells were treated with 50 μM DIM in the presence of 10% FBS. Treatment with 50 μM DIM showed a significant antimigratory effect at 24 and 48 h in both cell lines. **p<0.01, ***p<0.001 compared to the control. DIM, 3,3'-diindolylmethane; FBS, fetal bovine serum.

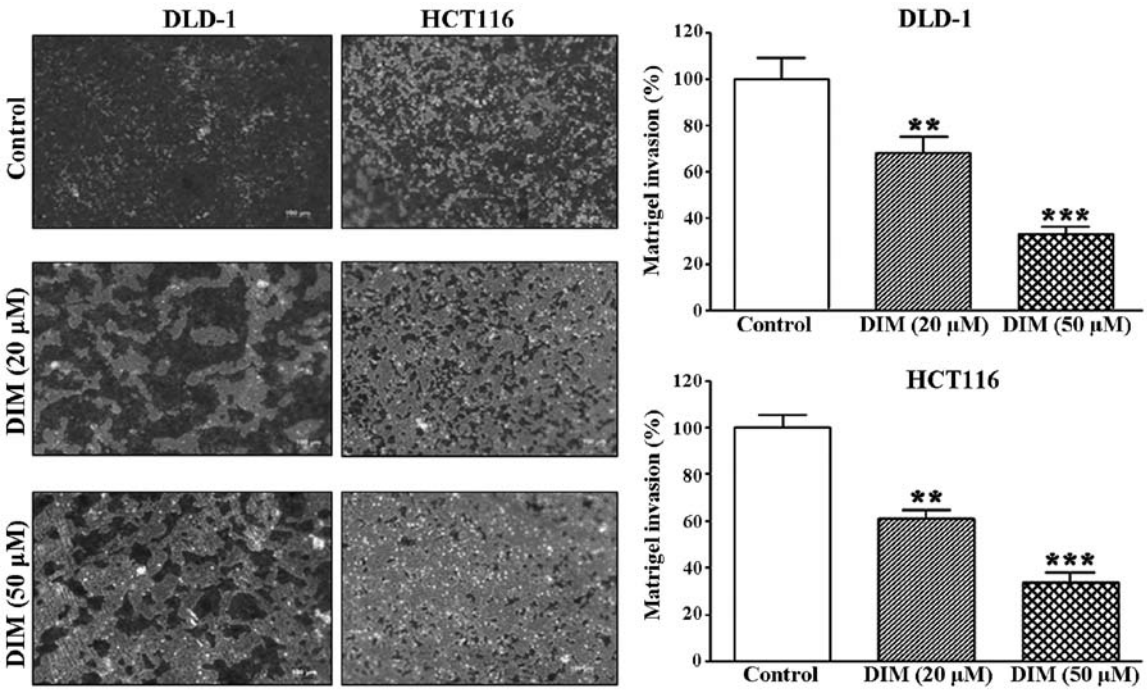

Figure 3. Effect of DIM on the invasion rates of colorectal cancer cells by Matrigel invasion assay. DIM significantly inhibited the invasion rates of the DLD-1 and HCT116 cells at concentrations of 20 and 50 μM. **p<0.01, ***p<0.001 compared to control. DIM, 3,3'-diindolylmethane.

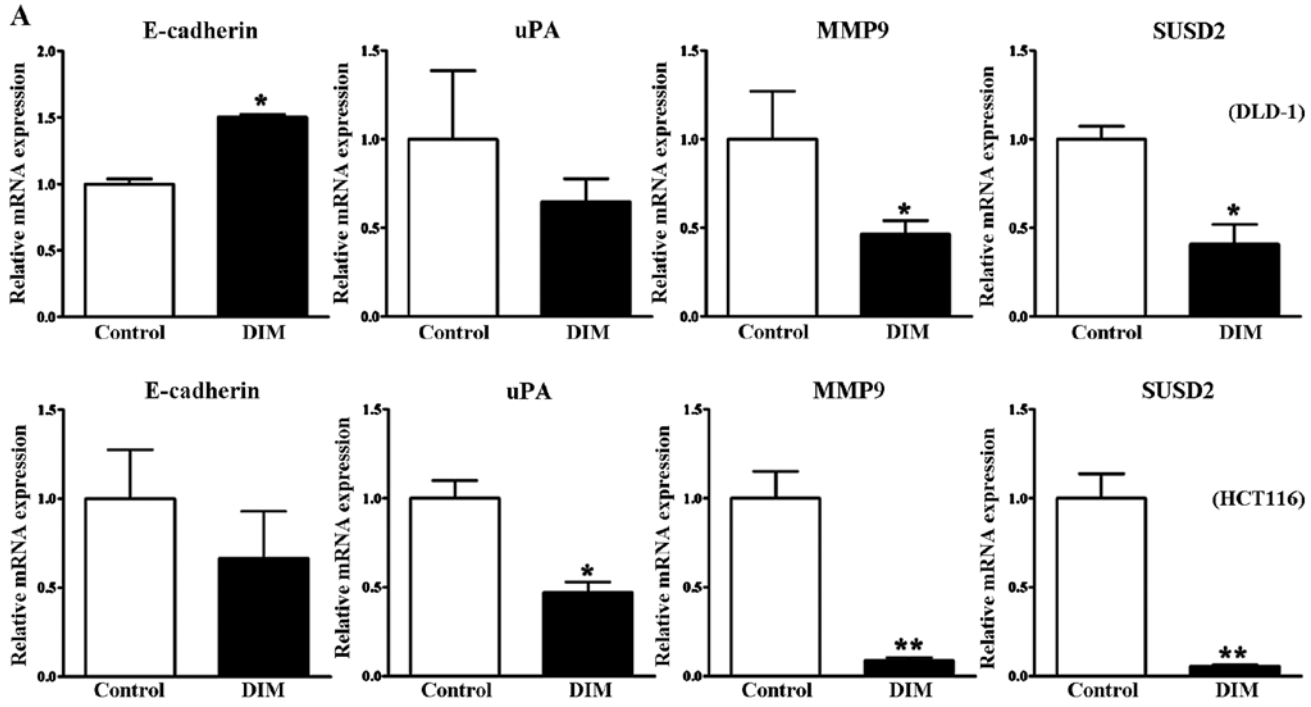

Figure 4. Expression of metastasis marker genes in colorectal cancer cells after DIM treatment. (A) mRNA levels of E-cadherin, uPA, MMP9 and SUSD2 in the DLD-1 and HCT116 cells were measured by real-time PCR after treatment with DIM (100 μM) for 48 h. Data are expressed as mean (± SE). Values were normalized to GAPDH. *p<0.05, **p<0.01 compared to the control.

invasive epithelial cancers. In the present study, DIM treatment induced a significant increase in the E-cadherin mRNA level in the DLD-1 cells, yet there was no significant change in the HCT 116 cells (Fig. 4A). However, E-cadherin protein levels were significantly increased following DIM treatment in both the DLD-1 and HCT116 cells in a dose-dependent manner (Fig. 4B). Therefore, our data indicated that DIM increased E-cadherin expression and thus suppressed the invasiveness and progression of colon cancer cells. We further found that treatment with 100 μM DIM significantly decreased the mRNA levels of genes related to invasion and cancer progression such as uPA, MMP9 and SUSD2 in DLD-1

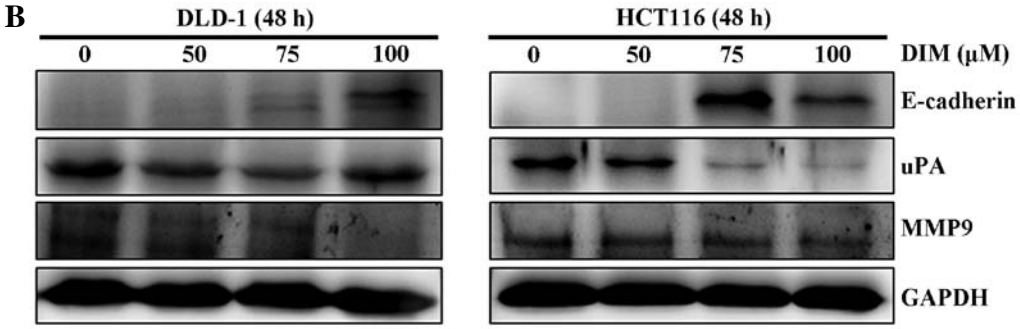

Figure 4. Continued. (B) Western blot analysis of the effect of DIM on E-cadherin, uPA and MMP9 protein expression in the DLD-1 and HCT116 cells. The protein levels of E-catenin, uPA and MMP9 were measured after DIM (0, 50, 75 and 100 μM) treatment for 48 h. GAPDH was used as an internal control. DIM, 3,3'-diindolylmethane.

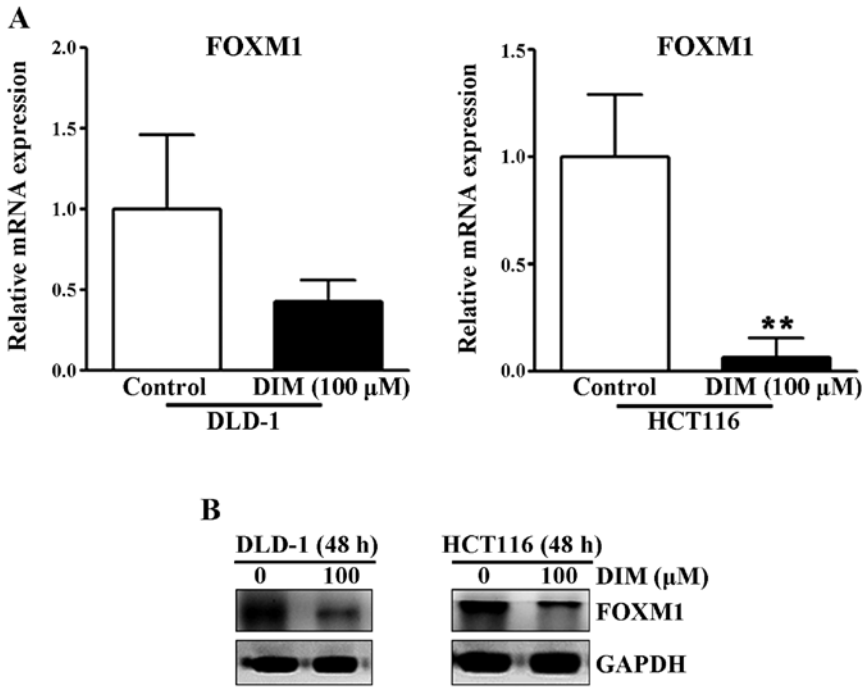

Figure 5. Expression of FOXM1 in colorectal cancer cells after DIM treatment. (A) The mRNA level of FOXM1 in the DLD-1 and HCT116 cells was measured by real-time PCR after treatment with DIM (100 μM) for 48 h. Data are expressed as the mean (± SE). Values were normalized to GAPDH. **p<0.01 compared to the control. (B) Western blot analysis of the effect of DIM on FOXM1 protein expression in the DLD-1 and HCT116 cells. The FOXM1 protein level was measured after DIM (100 μM) treatment for 48 h. GAPDH was used as an internal control. DIM, 3,3'-diindolylmethane.

and HCT-116 cells (Fig. 4A). The protein levels of uPA and MMP9 were also downregulated in a dose-dependent manner after DIM treatment (Fig. 4B). These results suggest that DIM inhibits the migration and invasion of colorectal cancer cells by targeting uPA and MMP9.

Effect of DIM on FOXM1 protein and mRNA expression levels in colorectal cancer cells. The transcription factor forkhead box M1 (FOXM1) is an important regulator of cell differentiation and proliferation (14). Since FOXM1 is highly activated in most human cancers and is overexpressed in a number of aggressive human carcinomas (14), we examined whether DIM alters the expression levels of FOXM1 in colorectal cancer cells. As shown in Fig. 5, DIM significantly reduced the mRNA and protein levels of FOXM1 in the DLD-1 and HCT116 cells at 48 h, suggesting that DIM significantly inhibits the migration and invasion rates of colorectal cancer cells by inactivation of FOXM1.

Discussion

DIM is considered to be a promising natural cancer preventive agent. Although DIM has been proven to impart antitumor effects on numerous cancers both *in vitro* and *in vivo* (4-9,11,13,15-19), its antimetastatic effects in colorectal cancer and the underlying mechanism have not been fully elucidated. In the present study, we demonstrated that DIM inhibits the migration and invasion of colorectal cancer cells by inactivation of FOXM1 and subsequent downregulation of uPA and MMP9.

In the present study, we found that there was a significant reduction in cellular migration of colorectal cancer

cells (DLD-1 and HCT116) following treatment with 20 and 50 μM DIM. Treatment with 50 μM DIM with 10% FBS of the colorectal cancer cells resulted in a more pronounced inhibition of migration than treatment with 20 μM DIM with 5% FBS. DIM at concentrations of 20 and 50 μM also significantly inhibited the invasion rates of the DLD-1 and HCT116 cells in a Matrigel invasion assay. The inhibition of cancer cell metastatic function by DIM has been reported by many researchers in various types of cancer including prostate, breast, ovarian, thyroid and nasopharyngeal cancers (15-23). A previous study on the antitumor effects and chemopreventive role of DIM in colorectal cancer proposed that DIM inhibited colorectal cancer cells by inactivation of YAP and induction of G1 and G2 cell cycle arrest (6,8). However, the effects of DIM on invasion and metastasis in colorectal cancer cells have not previously been described, and this is the first study to show attenuation of the adhesion and migratory properties of colorectal cancer cells by DIM.

As our data showed that DIM regulated colorectal cancer cell invasion and metastasis, we further investigated whether DIM altered the expression levels of metastasis marker proteins in colorectal cancer cells. Indeed, DIM significantly induced expression of E-cadherin, an indicator protein of invasive function in epithelial cancer cells, in both the DLD-1 and HCT116 cells. Our findings suggest that induction of E-cadherin expression by DIM treatment represents loss of epithelial invasive function in colorectal cancer cells. We also found that DIM decreased the protein levels of uPA and MMP9 and significantly downregulated mRNA levels of uPA, MMP9, and SUSD2 in the DLD-1 and HCT116 cells. Our results are consistent with those of previous studies in other cancer cells. For example, inhibition of invasion in prostate cancer by DIM was previously shown to be mediated by MMP9 and uPA, which regulated the bioavailability of vascular endothelial growth factor (18). Downregulation of uPA by DIM has also been reported to contribute to the inhibition of cell growth and migration of breast cancer cells (15). Therefore, our results strongly indicated that inhibition of uPA and MMP9 by DIM attenuates the invasion and metastasis of colorectal cancer cells.

Forkhead box M1 (FOXM1) is a member of the FOX family of transcriptional factors and a cell cycle regulator that is essential for cell cycle progression (24). FOXM1 has been reported to play an important role in the promotion of cancer cell proliferation and to be involved in malignant tumor development in a variety of cancers, including lung, liver, ovarian and breast cancer (24-30). Moreover, overexpression of FOXM1 is associated with tumor invasion and metastasis and predictive of poor prognosis in many cancer types, including ovarian, esophageal, lung, liver and bladder cancer (31-35). Chu *et al* reported that overexpression of FOXM1 stimulated migration/invasion and is correlated with a poor prognosis of colorectal cancer (36). Activation of FOXM1 increases colorectal cancer progression and metastasis by activation of uPA receptor expression (37). Therefore, it is obvious that abnormal regulation of FOXM1 plays a critical role in tumor development and metastasis of colorectal cancer.

Since elevated expression of FOXM1 has been observed in human colorectal cancer (36,37), we investigated whether DIM altered the expression levels of FOXM1 in colorectal cancer cells. We found that DIM significantly suppressed the mRNA and protein levels of FOXM1 in two different colorectal cancer cells (DLD-1 and HCT116). These observations are in agreement with previous studies demonstrating that DIM effectively downregulated FOXM1 in various breast cancer cell lines (38-40). Downregulation of FOXM1 has been found to inhibit the expression of metastasis factors that are involved in the degradation of the ECM, such as uPA and MMP9, in breast cancer cells (41). In the present study, we found that the inhibition of migration and metastasis by DIM was accompanied by downregulation of FOXM1, uPA and MMP9. Therefore, the antimetastatic effects of DIM in colorectal cancer cells may be associated with inhibition of FOXM1, which in turn may induce downregulation of uPA and MMP9 expression. However, further studies are needed to elucidate how DIM specifically controls the expression of FOXM1 and its target genes.

In conclusion, DIM inhibited the cell migratory and invasive properties of colorectal cancer cells, most likely through downregulation of uPA and MMP9 mediated by suppression of the FOXM1 transcription factor. Our results suggest the potential application of FOXM1 downregulation by DIM as a novel approach for the treatment of aggressive colorectal cancer.

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