I3C and ICZ inhibit migration by suppressing the EMT process and FAK expression in breast cancer cells

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Abstract. Indole-3-carbinol (I3C) and indole[3,2-b] carbazole (ICZ) are major bioactive food components in cruciferous vegetables. Although previous studies have demonstrated the anticancer activity of I3C and ICZ in various types of cancer cells, the manner in which indole compounds regulate migration or related epithelial-to-mesenchymal transitions (EMT) has yet to be determined. In this study, we investigated the effects of I3C and ICZ on migration using breast cancer cells (MCF-7 and MDA-MB231). Pre-treatment with I3C and ICZ significantly inhibited the migration of breast cancer cells without cytotoxicity, as measured by monolayer scratch assay. In addition, I3C and ICZ decreased vimentin (a mesenchymal marker) and focal adhesion kinase (FAK) mRNA expression, while increasing E-cadherin (an epithelial marker) expression. Matrix metalloproteinase (MMP)-2 and -9 activity was also reduced by I3C and ICZ. Taken together, we propose that I3C and ICZ pre-treatment inhibits the migration of breast cancer cells through suppression of the EMT process and reduced MMP activity by repressing FAK expression. Our findings suggested that I3C and ICZ are potential compounds for inhibition of breast cancer cell migration.

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide, with an estimated 1.4 million new cases and 458,000 deaths in 2008 (1,2). Breast cancer is the second leading cause of cancer-related mortality in woman, while its incidence in the developing world is on the rise (3). Notably, incidence has increased 6.8% annually during the last 6 years in Korea, leading to an increase in mortality (4).

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Epithelial-to-mesenchymal transition (EMT) is the process whereby epithelial cells transform to a mesenchymal state. EMT is an essential process during embryonic development, wound healing, inflammation and fibrosis as well as tumorigenesis (5-7). During the EMT process, cancer cells lose epithelial markers, such as E-cadherin and occuludins, and acquire mesenchymal markers, such as vimentin, fibronectin, N-cadherin and matrix metalloproteinases (MMPs). MMPs facilitate migration, invasion and metastasis in cancer (5,7-9). Cell migration is involved in tumor invasion and metastasis. Degradation of the extracellular matrix (ECM) by MMPs is understood to be a pre-requisite for cell migration to native or provisional tissue matrix based on studies of several systems (10,11).

Focal adhesion kinase (FAK) is a 125-kDa non-receptor tyrosine kinase localized to sites of integrin clustering, termed focal adhesions (2,12). FAK activation leads to a number of cell processes including cell attachment, migration, invasion, proliferation and survival (12,13). Furthermore, in breast cancer, the FAK gene is amplified and its protein is overexpressed (14).

Recent studies have reported that natural bioactive compounds derived from plants exhibit anticancer effects by suppressing the EMT process (3,15,16). Indole-3-carbinol (I3C) is naturally found in vegetables of the *Cruciferae* family such as broccoli, brussel sprouts and cauliflower (17,18). I3C autolytically breaks down into indole glucosinolates and indole[3,2-b] carbazole (ICZ), an acid-derived condensation metabolite (19,20). Epidemiological studies have reported that high dietary intake of cruciferous vegetables is associated with lower cancer risk. In particular, I3C strongly suppresses development and growth of breast cancer (17,18).

In the present study, the effects of I3C and ICZ on migration in two human breast cancer cell lines, MCF-7 and MDA-MB231 were examined. The mRNA expression of E-cadherin, vimentin, FAK and MMP activity was also investigated.

Materials and methods

Cell culture and I3C and ICZ treatment. MCF-7 and MDA-MB231 breast cancer cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA)

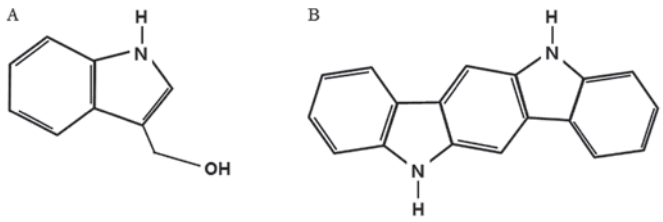

Figure 1. Structures of I3C and ICZ. (A) I3C; (B) ICZ.

and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were then incubated in a standard humidified incubator with 5% CO_2 at 37˚C. I3C and ICZ were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1). Stock solutions of I3C and ICZ were prepared in ethanol and diluted into the growth medium such that the final concentration of ethanol did not exceed 0.1% (v/v).

Determination of cell viability by MTT assay. Cells were cultured in a 96-well plate at a density of $1x10^5$/ml. After 24 h, cells were treated with various concentrations of I3C and ICZ and incubated for 48 h. An MTT solution [0.5 mg/ml phosphate-buffered saline (PBS)] was then added to each well, and cells were incubated for 4 h. The supernatants were carefully removed, and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm on a microplate reader. The cell viability was determined relative to untreated control cells.

Wound healing assay. Cells were grown to confluence on 35-mm culture dishes. The monolayer was then scratched with a sterile pipette tip and was washed with PBS to remove cellular debris. Cells were then grown in RPMI-1640 containing 5% FBS in either I3C or ICZ (0, 1, 2.5, 5 or 10 μM). After 48 h, cell migration was observed under a microscope and photographed.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from breast cancer cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse-transcribed using the AccessQuick™ RT-PCR system (Promega, Madison, WI, USA). The primer sequences used for amplification of the sense and antisense strands were: FAK, 5'-GCGCTGGCTGGAAA AAGAGGAA-3' and 5'-TCGGTGGGTGCTGGCTGGT AGG-3'; E-cadherin, 5'-ACATTGTCACCTCGCAGAC-3' and 5'-GCGGATTGTAGAAGTCTTGG-3'; vimentin, 5'-TGG CACGTCTTGACCTTGAA-3' and 5'-GGTCATCGTGATGC TGAGAA-3'; GAPDH, 5'-CGGAGTCAACGGATTTGGT CGTAT-3' and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. PCR was carried out under the following conditions: 30 cycles for FAK at 94˚C for 30 sec, 65˚C for 90 sec and 72˚C for 30 sec; 30 cycles for E-cadherin and vimentin at 94˚C for 45 sec, 58˚C for 45 sec, and 72˚C for 45 sec; and 25 cycles for GAPDH at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The PCR products were separated on 1.5% (w/v) agarose gel. Data analyses were carried out using a 7500 system with the SDS software version 1.3.1 (Applied Biosystems Inc., Foster City, CA, USA).

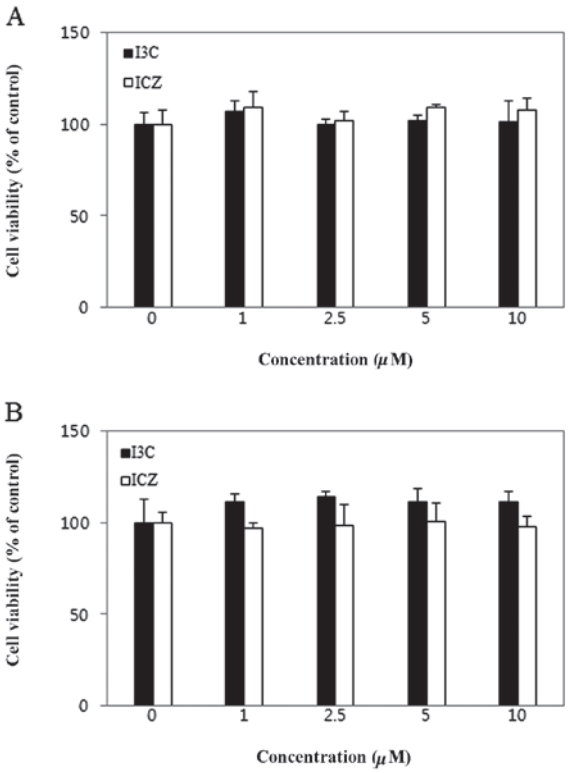

Figure 2. Effects of I3C and ICZ on cell viability in breast cancer cells. (A) MCF-7; (B) MDA-MB231. Cell viability was measured using MTT assay, as described in Materials and methods. The results are presented as the mean ± standard deviation (SD) of at least three independent experiments, each performed in triplicate. I3C, indole-3-carbinol; ICZ, indole[3,2-b] carbazole.

Gelatin zymography. The MMP activity was measured using gelatin zymography. Cells ($5x10^5$) were seeded in a 6-well plate and incubated for 24 h. Then, cells were treated with I3C and ICZ (0, 1, 2.5, 5 or 10 μM) and conditioned medium was collected. Equal volumes of conditioned medium were separated by electrophoresis on 10% SDS-polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, the gels were washed with 2.5% Triton X-100 to remove SDS and were then incubated in developing buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 150 mM NaCl, 0.02% NaN_3, pH 7.5) at 37˚C for 18 h. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Pierce Biotechnology, Rockford, IL, USA) and de-stained. Gelatinase activity was visualized as clear bands.

Statistical analyses. The experimental results are presented as the means ± standard deviation (SD). The significance of treatment effects was assessed using Tukey's multiple range tests following one-way ANOVA using the SAS software (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. The cytotoxicity of I3C and ICZ on breast cancer cells was measured using an MTT assay. As shown in Fig. 2, I3C and ICZ did not affect viability of MCF-7 and MDA-MB231 breast cancer cells at concentrations ranging from 1 to 10 μM.

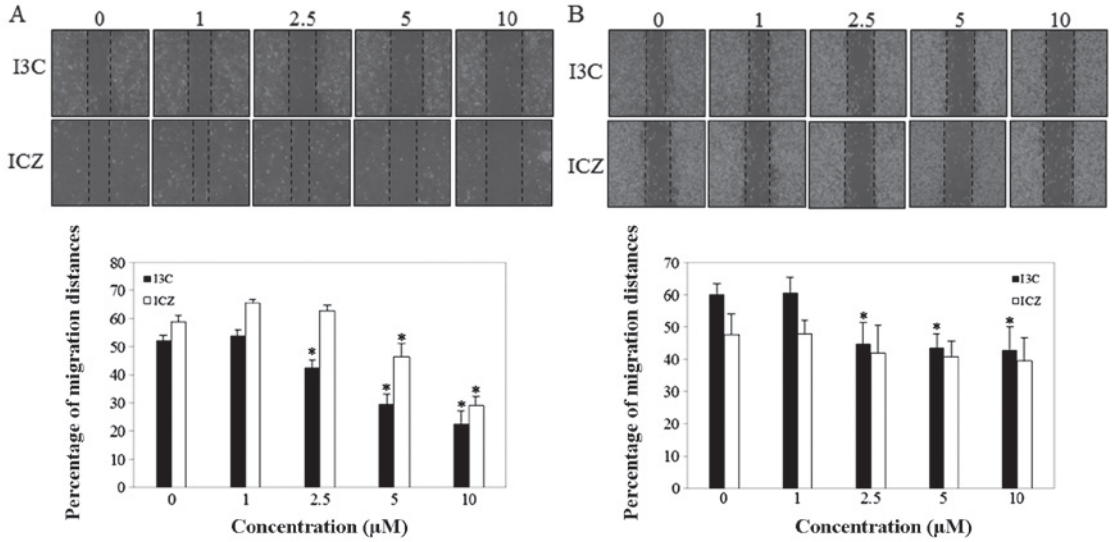

Figure 3. Effect of I3C and ICZ on migration in breast cancer cells. (A) MCF-7; (B) MDA-MB231. Confluent cells were carefully wounded using sterile pipette tips and re-cultured with or without I3C and ICZ, as described in Materials and methods. After 48 h, cells were photographed under a microscope. The results are presented as the mean ± standard deviation (SD) of at least three independent experiments, each performed in triplicate. *Significant difference (P<0.05), as determined by Tukey's multiple range tests. I3C, indole-3-carbinol; ICZ, indole[3,2-b] carbazole.

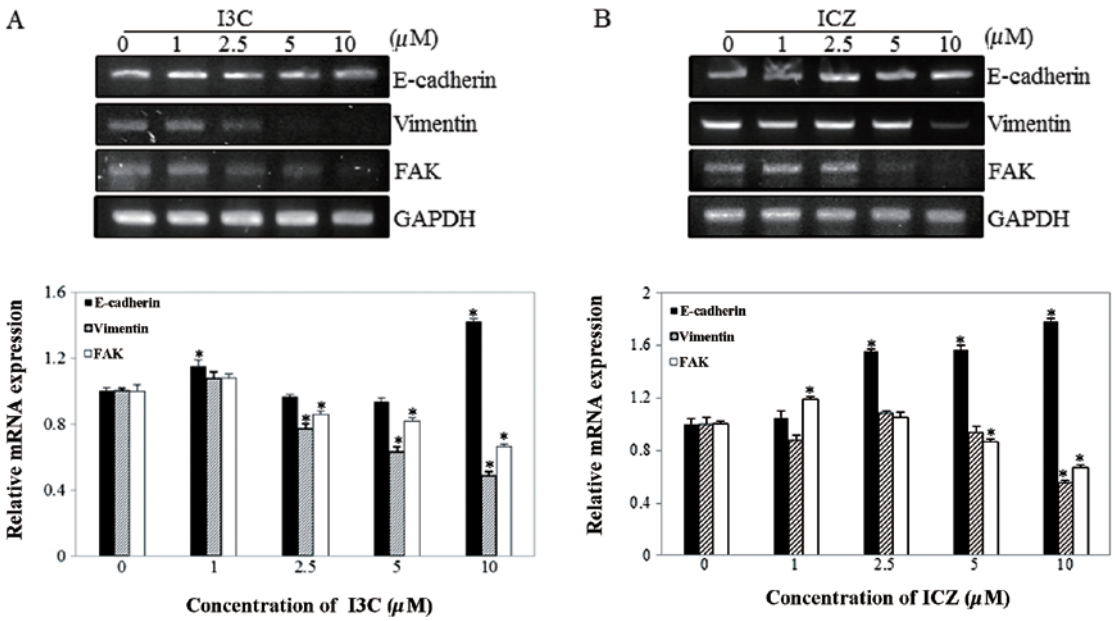

Figure 4. Effects of I3C and ICZ on mRNA expression of E-cadherin, vimentin and FAK in MCF-7 cells. (A) I3C; (B) ICZ. Cells were treated with I3C and ICZ for 24 h. mRNA expression was measured by RT-PCR, as described in Materials and methods. The results are presented as the mean ± standard deviation (SD) of at least three independent experiments, each performed in triplicate. *Statistically significant difference (P<0.05) as determined by Tukey's multiple range tests. I3C, indole-3-carbinol; ICZ, indole[3,2-b] carbazole; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effect of I3C and ICZ on migration. To examine the effect of I3C and ICZ on human breast cancer cell migration, a wound-healing assay was performed. As shown in Fig. 3A, I3C and ICZ at 5 and 10 μM effectively inhibited the MCF-7 cell migration after 48 h in a dose-dependent manner compared to untreated control cells. However, I3C and ICZ only slightly inhibited migration in MDA-MB231 (Fig. 3B). Thus, the subsequent experiments were performed using MCF-7 cells.

Effect of I3C and ICZ on mRNA expression of E-cadherin, vimentin and FAK. Aggressive breast cancers exhibit phenotypic characteristics of EMT (8). Therefore, to determine whether or not I3C and ICZ inhibit the EMT process, mRNA expression of E-cadherin and vimentin was examined. As shown in Fig. 4, E-cadherin mRNA expression significantly increased (1.42- and 1.78-fold) with 10 μM I3C and ICZ, respectively, compared to untreated control cells. In addition, vimentin mRNA expression dramatically decreased (by 52 and 45%) at 10 μM I3C and ICZ, respectively, compared to untreated control cells. FAK has been known to be involved in cell interactions with ECM proteins for migration and enhancement of cell spreading through tyrosine phosphoryla-

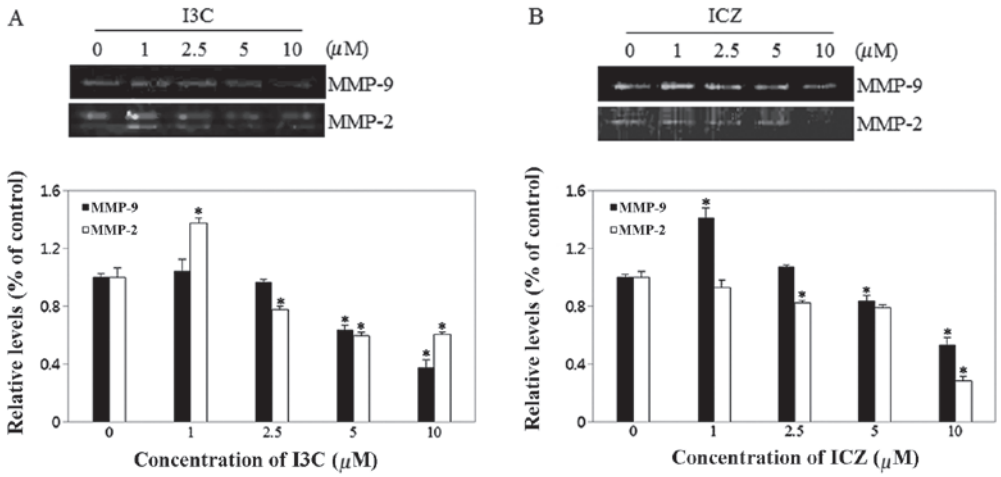

Figure 5. Effects of I3C and ICZ on MMP-2 and -9 activity in MCF-7 cells. (A) I3C; (B) ICZ. Cells were treated with I3C and ICZ and MMP-2 and -9 activity was measured using zymography in conditioned medium, as described in Materials and methods. The results are presented as the means ± standard deviation (SD) of at least three independent experiments, each performed in triplicate. *Statistically significant difference ($P<0.05$), as determined by Tukey's multiple range tests. I3C, indole-3-carbinol; ICZ, indole[3,2-b] carbazole; MMP, matrix metalloproteinase.

tion (17). In this study, to determine whether FAK is a target of I3C and ICZ in the inhibition of breast cancer cell migration, mRNA expression of FAK was measured using RT-PCR. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control, the mRNA expression of FAK was dose-dependently reduced by 44 and 34%, in 10 μM I3C- and ICZ-treated cells, respectively.

Effect of I3C and ICZ on MMP activity. Cell migration may be promoted by overexpression of MMPs (11). Therefore, MMP-2 and -9 activity was measured using zymography following treatment with various concentrations of I3C and ICZ. As shown in Fig. 5, MMP-2 and -9 activity of MCF-7 cells was noticeably downregulated by I3C and ICZ in a dose-dependent manner. In particular, I3C levels of 10 μM inhibited MMP-9 and -2 activity by 63 and 40%, respectively, compared to untreated control cells. ICZ levels of 10 μM significantly suppressed MMP-9 and -2 activity by 47 and 72%, respectively, compared to untreated control cells.

Discussion

Cell migration is a highly integrated multistep process including detachment, translocation and protrusion. It is involved in various biological processes, such as embryogenesis, cancer and chronic inflammatory diseases (21,22). Recently, studies of breast and other types of cancers have suggested that EMT may be a potential mechanism by which epithelial tumor cells acquire a more motile and invasive phenotype and the ability to escape from the primary tumor (23,24). In other words, EMT has been implicated in cancer migration, invasion and metastasis and therefore may also represent a major mechanism of tumor progression (5,24). EMT is characterized by a downregulation of E-cadherin with the concomitant acquisition of vimentin and secretion of MMPs (25). In particular, E-cadherin is regarded as a 'master' controller of the epithelial/mesenchymal phenotype switch due to its repression being sufficient to induce and to complete EMT, and its re-activation potentially resulting in the reverse process, mesenchymal-epithelial transition (MET) (23). In this study, I3C and ICZ significantly increased E-cadherin mRNA expression in MCF-7 cells. In their study, Meng *et al* (17) demonstrated that similar effects of I3C were observed in other breast cancer cell lines, such as T-47D and MDA-MB468. In addition, I3C and ICZ significantly attenuated mesenchymal markers, such as vimentin. Thus, our findings demonstrate that I3C and ICZ inhibit cell migration through suppression of EMT.

MMPs (a family of zinc-dependent endopeptidases) are highly involved in the degradation and reconstruction of ECM (26,27). The misregulation of MMPs is widespread in several pathological settings and especially in cancer, whereby MMP overexpression contributes to tumorigenesis and tumor progression through multiple mechanisms (28). In particular, MMP-1, -2 and -9 are highly expressed in breast carcinoma cell lines, and studies suggest that MMPs play a critical role in breast cancer invasion, metastasis and tumor angiogenesis (14). Moreover, cancer cells that undergo EMT may produce more MMPs, and elevated levels of MMPs may directly induce EMT with enhanced invasion and metastasis (28,29). Therefore, we analyzed MMP-2 and -9 activity and revealed decreases in MMP-2 and -9 activity associated with I3C and ICZ treatment. This finding is consistent with other reports that showed that I3C inhibited MMP-2 expression (27). The present study showed that downregulation of MMP-2 and -9 activity, a reduction of EMT and consequential inhibition of migration in breast cancer cells are associated with I3C and ICZ treatment.

However, increased levels of FAK play a crucial role in various biological processes such as embryonic development, cell adhesion, survival, differentiation, migration, invasion and angiogenesis (2,30,31). FAK-/- fibroblasts derived from FAK knockdown mouse embryos yield a significant decrease in cell migration compared to the cells from wild-type mice (12,32). Moreover, recent studies suggest that FAK may be a critical mediator in TGF-β-induced EMT in hepatocytes and renal tubular epithelial cells (33,34). According to Deng *et al* (34), FAK may induce the loss of E-cadherin and the decrease in MMP-9 secretion (34). In the present study, subsequent to

treatment with ICZ and ICZ, FAK mRNA expression was significantly decreased in a dose-dependent manner. These data demonstrate that I3C and ICZ significantly inhibit the EMT process and cell migration through reduction of mRNA expression of FAK in MCF-7 cells. Previous studies report that I3C and ICZ exhibit anticancer activity in various cancer cell lines (17,27,35,36). However, to the best of our knowledge, this study is the first to indicate that I3C and ICZ inhibit migration through suppression of the EMT process and FAK expression in MCF-7 breast cancer cells.

In conclusion, treatment with I3C and ICZ significantly inhibits the migration of breast cancer cells by suppression of the EMT process and by reduction of MMP-2 and -9 activity through repression of FAK mRNA expression. Our findings suggest that I3C and ICZ are potential target compounds for the inhibition of cancer cell migration.

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