Benzyl Isothiocyanate Inhibits Epithelial-Mesenchymal Transition in Cultured and Xenografted Human Breast Cancer Cells

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Abstract

We showed previously that cruciferous vegetable constituent benzyl isothiocyanate (BITC) inhibits growth of cultured and xenografted human breast cancer cells and suppresses mammary cancer development in a transgenic mouse model. We now show, for the first time, that BITC inhibits epithelial-mesenchymal transition (EMT) in human breast cancer cells. Exposure of estrogen-independent MDA-MB-231 and estrogen-responsive MCF-7 human breast cancer cell lines and a pancreatic cancer cell line (PL-45) to BITC resulted in upregulation of epithelial markers (e.g., E-cadherin and/or occludin) with a concomitant decrease in protein levels of mesenchymal markers, including vimentin, fibronectin, snail, and/or c-Met. The BITC-mediated induction of E-cadherin protein was accompanied by an increase in its transcription, whereas BITC-treated MDA-MB-231 cells exhibited suppression of vimentin, snail, and slug mRNA levels. Experimental EMT induced by exposure to TGFβ and TNFα or Rb knockdown in a spontaneously immortalized nontumorigenic human mammary epithelial cell line (MCF-10A) was also partially reversed by BITC treatment. The TGFβ-/TNFα-induced migration of MCF-10A cells was inhibited in the presence of BITC, which was partially attenuated by RNA interference of E-cadherin. Inhibition of MDA-MB-231 xenograft growth in vivo in female athymic mice by BITC administration was associated with an increase in protein level of E-cadherin and suppression of vimentin and fibronectin protein expression. In conclusion, this study reports a novel anticancer effect of BITC involving inhibition of EMT, a process triggered during progression of cancer to invasive state. Cancer Prev Res; 4(7); 1107–17. ©2011 AACR.

Introduction

Breast cancer is a serious public health concern for women worldwide (1, 2). Despite remarkable progress toward screening efforts and unremitting advancement of targeted therapies, breast cancer is still a leading cause of cancer-related deaths in women (1–4). A number of mammary cancer risk factors have been identified, including family history, Li-Fraumeni syndrome, atypical hyperplasia of the breast, late age at first full-term pregnancy, early menarche, and late menopause (5–7). Because many of the risk factors associated with mammary cancer development are not easily adjustable, other strategies for prevention of this dreadful disease are appealing to reduce disease-related cost, mortality, and morbidity for a large segment of women population. Prevention of breast cancer is feasible considering successful clinical applications of selective estrogen-receptor modulators, including tamoxifen and raloxifene (8–10). Unfortunately, this strategy is effective only against estrogen-receptor–positive breast cancers and suffers from adverse side effects, including increased risk of uterine cancer, thromboembolism, and perimenopausal symptoms (8–10). Discovery of agents efficacious for prevention of breast cancer regardless of the estrogen-receptor status is desired. Dietary plants have drawn increasing attention in recent years for the discovery of cancer preventive and therapeutic agents (11–13). Population-based case-control studies have documented an inverse association between intake of cruciferous vegetables and the risk of breast cancer (14, 15). For example, a case-control study involving more than 300 breast cancer cases and matched controls showed an inverse association between urinary levels of isothiocyanates (ITC) as a biological measure of cruciferous vegetable intake and the risk of developing breast cancer (14). Broccoli consumption was also found to be inversely associated with the risk of developing mammary cancer in premenopausal women (15). Anticancer property of cruciferous vegetables is ascribed to organic ITCs, which are produced upon cutting or chewing of these vegetables (16). Benzyl ITC (BITC) is
one such ITC compound that seems promising for prevention of breast cancer on the basis of our own work. We showed previously that administration of BITC in the diet (3 μmol BITC/g diet) inhibited mammary hyperplasia and carcinoma incidence and/or burden in MMTV neu transgenic mice in association with suppression of cell proliferation, apoptosis induction, and tumor infiltration of T cells (17). We also found that the growth of MDA-MB-231 human breast cancer cells implanted in female athymic mice was inhibited significantly by BITC administration (18).

Cellular studies have provided novel insights into the mechanisms underlying anticancer effects of BITC (19–23). For example, our own work has revealed that BITC inhibits growth of cultured human breast cancer cells (MDA-MB-231 and MCF-7) by causing G2-M phase cell-cycle arrest and p53-independent apoptotic cell death (19, 22, 23). Notably, a spontaneously immortalized non-tumorigenic human mammary epithelial cell line (MCF-10A) is significantly more resistant to growth inhibition and apoptosis induction by BITC than breast cancer cells (19, 22). Molecular circuitry of BITC-induced apoptosis involves the production of reactive oxygen species due to inhibition of complex III of the mitochondrial respiratory chain (22). This study builds on these observations and shows, for the first time, that BITC inhibits epithelial-mesenchymal transition (EMT), which is implicated in progression of cancers to invasive state (24–26).

Materials and Methods

Reagents

BITC (purity >98%) was purchased from LKT Laboratories. Reagents for cell culture including medium, fetal bovine serum, and antibiotics were purchased from Invitrogen-Life Technologies. TGFβ was from Calbiochem, and TNFα was obtained from Sigma-Aldrich. Antibodies against E-cadherin and β-catenin were purchased from BD Transduction Laboratories; anti-snail antibody was from Abgent; antibodies against vimentin and actin were from Sigma-Aldrich; anti-fibronectin and anti-c-Met antibodies were from Santa Cruz Biotechnology; anti-Rb antibody was from Cell Signaling; anti-occludin antibody was from Invitrogen-Life Technologies; and anti-p53 antibody was from Calbiochem. The vimentin antibody used for immunofluorescence microscopy was from Santa Cruz Biotechnology. Alexa Fluor 488–conjugated goat anti-mouse and Alexa Fluor 568–conjugated goat anti-mouse antibodies were from Invitrogen-Life Technologies. E-cadherin promoter reporter construct was purchased from Addgene. E-cadherin–targeted siRNA was from Ambion, Rb-targeted siRNA was from Thermo Fisher Scientific-Dharmacon, and a control nonspecific siRNA was purchased from Qiagen.

Cell lines

The MDA-MB-231, MCF-7, MCF-10A, and PL-45 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to the supplier’s instructions. Authentication of MDA-MB-231, MCF-7, and MCF-10A cell lines was done by Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO). The cells were last tested in February 2011. The MDA-MB-231, MCF-7, and MCF-10A cells were found to be of human origin and no mammalian interspecies contamination was detected. Moreover, the genetic profile for each cell line was consistent with the corresponding genetic profile in the ATCC database. The PL-45 cell line was not authenticated.

Immunoblotting

Stock solution of BITC was prepared in dimethyl sulfoxide (DMSO) and an equal volume of DMSO (final concentration <0.05%) was added to the controls. Control and BITC-treated cells and tumor tissues from control and BITC-treated mice were processed for immunoblotting, as described by us previously (18, 19, 27). Lysate proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with the desired primary antibody for 1 hour at room temperature or overnight at 4°C. Immunoreactive bands were visualized by enhanced chemiluminescence method. Densitometric quantitation was done by UN-SCAN-IT software version 5.1 (Silk Scientific Corporation).

Immunocytochemical analysis for E-cadherin and vimentin

Cells (2.5 × 10⁴ cells/mL) were cultured on coverslips, allowed to attach, and then exposed to DMSO (control), 2.5 μmol/L BITC, and/or a combination of 10 ng/mL each of TGFβ and TNFα for specified time period. The cells were fixed with 2% paraformaldehyde for 1 hour, permeabilized with PBS containing 0.05% Triton X-100 for 10 minutes, and blocked with 0.5% bovine serum albumin in PBS for 1 hour at room temperature. For single-antibody staining (E-cadherin and vimentin), the cells were incubated overnight with anti-E-cadherin or anti-vimentin antibody at 4°C, washed with PBS, and incubated with Alexa Fluor 568–conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature. For E-cadherin/actin double staining, the cells were incubated with E-cadherin antibody overnight at 4°C, followed by extensive washing with PBS and incubation with Alexa Fluor 568–conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature. A monoclonal anti-actin antibody was then added followed by 2 hours of incubation at room temperature. Cells were then washed 3 times with PBS and stained with Alexa Fluor 488–conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature. Cell nuclei were stained with 4′, 6 diamidino 2 phenylindole (DAPI; 10 ng/mL; 5 minutes at room temperature). Cells were washed twice with PBS, mounted, and examined under a Leica DC300F microscope.
Reverse-transcription PCR

Total RNA was prepared from DMSO-treated control and BITC-treated MDA-MB-231 or MCF-7 cells by using RNAasy Kit (Invitrogen) and reverse transcribed with reverse transcriptase and oligo(dT)$_{20}$ to synthesize complementary cDNA. Reverse-transcription PCR (RT-PCR) reaction was carried out using high-fidelity Taq polymerase (Invitrogen-Life Technologies), gene-specific primers, and 2 μL of cDNA. The E-cadherin primers were: forward, 5'-TGGGTTATTTCTTCTTCCAG-3' and reverse, 5'-TTTGCAGGGACGCCAGAT-3'. The amplification conditions were: 94°C for 5 minutes, 25 (MCF-7 cells) or 55 (MDA-MB-231 cells) cycles 94°C for 60 seconds, 60°C for 60 seconds, 68°C for 60 seconds, and 68°C for 10 minutes. The primers used for vimentin were: forward, 5'-CTCTTCCAAACTTTCTCTCCC-3' and reverse, 5'-AGTTTGATGATAACGGTCC-3'; the primers used for snail were: forward, 5'-CGAAAGGCTTCAAAGTCAAT-3' and reverse, 5'-ACTGTACCTGTCGAT-3'; and the primers used for Slug were: forward, 5'-CGCCCTCCAAAGGCAAAC-3' and reverse, 5'-CGGTAGTCACCACAGTGATG-3'. The amplifications' conditions used were: 94°C for 5 minutes, 30 cycles 94°C for 30 seconds, 58°C for 50 seconds, 68°C for 50 seconds, and 68°C for 7 minutes. The housekeeping gene β-actin or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a control. The β-actin was amplified by the primers: forward, 5'-CAAAGACCTGATAGCCACAG-3' and reverse, 5'-CATACTCTGTTGATGATTCC-3'; and the following amplification cycles: 95°C for 3 minutes, 18 cycles 95°C for 60 seconds, 56°C for 60 seconds, 68°C for 60 seconds, and 68°C for 10 minutes. The GAPDH was amplified by the primers: forward, 5'-TGAATCAGCAGAAGGTGGAAG-3' and reverse, 5'-TCAGTTGGGACCGACATGG-3'; and the following amplification cycles: 94°C for 2 minutes, 25 cycles 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds, and 68°C for 5 minutes. PCR products were resolved by 1% to 2% agarose gel prestained with ethidium bromide and visualized under an UV illuminator.

E-cadherin luciferase assay

MDA-MB-231 cells (2 × 10$^4$ cells/well) were plated in 12-well plates and allowed to attach by overnight incubation at 37°C. The cells were then cotransfected with 8 μg of pGL2Basic-EcadK1-Luc plasmid with human E-cadherin promoter sequence from −108 to +125 and 0.8 μg of pRL-cytomegalovirus plasmid using Fugene6. Twenty-four hours after transfection, the cells were treated with DMSO or BITC for 8 or 16 hours. Luciferase activity was determined by Dual-Luciferase Reporter Assay Kit (Promega).

Migration assay

Effect of BITC administration on MCF-10A cell migration was determined by Transwell Boyden chamber (Corning) containing a polycarbonate filter (8 μm). MCF-10A cell suspension (1 × 10$^5$ cells/mL) in complete medium was mixed with DMSO (control) or BITC in the absence or presence of TGFB and TNFα (10 ng/mL each), and the suspension was added to the upper compartment of the chamber. Lower compartment of the chamber contained 0.6 mL of complete medium containing the same concentrations of DMSO, BITC, and/or TGFB/TNFα. By following incubation at 37°C for 24 hours, nonmigrant cells from the upper face of the membrane were removed using a cotton swab. The membrane was washed with PBS and the migrated cells on the bottom face of the membrane were fixed with 90% ethanol followed by staining with hematoxylin and eosin.

RNA interference

MCF-10A cells (1 × 10$^5$ cells/well) were seeded in 6-well plates 1 day before transfection. The cells were washed thrice with serum/growth factor-free OptiMEM (Invitrogen-Life Technologies) and then transfected with 150 nmol/L E-cadherin–targeted siRNA or Rb-targeted siRNA or control siRNA by using OligofectAMINE. Forty-eight hours after transfection, the cells were treated with DMSO, BITC, and/or TGFB and TNFα (10 ng/mL of each) for 24 hours. The cells were then collected and processed for immunoblotting and migration assay.

Immunohistochemistry for E-cadherin and vimentin in MDA-MB-231 xenografts

We have shown previously that BITC administration significantly retards growth of MDA-MB-231 cells implanted in female athymic mice (18). We used tumor tissues from the same study to determine the effect of BITC administration on expression of E-cadherin and vimentin by immunohistochemistry. Representative tumor sections from control and BITC-treated mice were processed for immunohistochemical analysis of E-cadherin and vimentin essentially, as described by us previously for other proteins (17, 18, 23).

Results

BITC upregulated E-cadherin protein expression in cancer cells

Loss of expression of epithelial adherens junction proteins (e.g., E-cadherin and occludin) with a concomitant gain of mesenchymal marker expression (e.g., vimentin and fibronectin) is a biochemical hallmark of EMT (24–26, 28–30). Loss of E-cadherin expression is associated with increased invasion in breast cancer cells (25, 26). Expression of E-cadherin is very low in the MDA-MB-231 cell line, which is a highly invasive “basal” type and estrogen-independent fibroblastic human breast cancer cell line with stellate morphology (31). Initially, we used this cell line to test whether BITC administration affected EMT. As can be seen in Figure 1A, exposure of MDA-MB-231 cells to pharmacologic concentrations of BITC (1 and 2.5 μmol/L) for 16 and 24 hours resulted in upregulation of E-cadherin expression. The BITC-mediated induction of E-cadherin expression in this cell line was accompanied by a marked decrease in mesenchymal marker vimentin especially at the 2.5 μmol/L concentration (Fig. 1A).
c-Met along with E-cadherin constitutes a marker signature associated with angiogenic and lymphangiogenic factors in breast ductal carcinoma in situ (32). The c-Met expression inversely correlates with E-cadherin expression (32). We observed a decrease in the level of c-Met protein in MDA-MB-231 cells after treatment with 2.5 μmol/L BITC for 16 hours, which was somewhat reversible at the 24 hour time point (Fig. 1A). In addition to regulating canonical Wnt signaling pathway, β-catenin also serves as a component of adherens junctions and links E-cadherin to the cytoskeleton (33). Expression of β-catenin was not affected by BITC administration (results not shown).
shown). We also determined the effect of BITC administration on protein level of snail, a transcription factor that functions as a repressor of *E-cadherin* gene in epithelial tumor cells (34). Expression of snail was also reduced in MDA-MB-231 cells after treatment with 2.5 μmol/L BITC at both 16 and 24 hour time points (Fig. 1A).

Next, we questioned whether the BITC-mediated induction of E-cadherin protein was unique to the “basal” type breast cancer cells. We addressed this question by using MCF-7 and PL-45 cells. The MCF-7 cell line is a well-accepted representative of estrogen-receptor–positive “luminal” type breast cancer (31). Unlike MDA-MB-231 cells, the MCF-7 cell line exhibits epithelial phenotype with high expression of E-cadherin and occludin (31). Expression of mesenchymal marker vimentin is not detectable in MCF-7 cells (31). In contrast to the MDA-MB-231 cell line, expression of E-cadherin and occludin proteins was only modestly increased by BITC administration in the MCF-7 cell line (Fig. 1B). Exposure of a highly invasive pancreatic cancer cell line (PL-45) to BITC (2.5 μmol/L for 24 hours) resulted in a robust induction of E-cadherin protein (5.5-fold increase over DMSO-treated control), which was accompanied by a 70% decrease in fibronectin protein expression (Fig. 1C). We were unable to detect vimentin protein expression in this cell line.

Figure 1D shows the effect of BITC treatment on mRNA levels of *vimentin*, *snail*, and *slug* in MDA-MB-231 cells. The levels of *vimentin*, *snail*, and *slug* mRNA were decreased after treatment of MDA-MB-231 cells with BITC, especially at the 2.5 μmol/L concentration compared with DMSO-treated control at both 16 and 24 hour time points (Fig. 1D).

We performed immunocytochemistry to confirm results of the immunoblotting about the effect of BITC administration on protein levels of E-cadherin and vimentin by using MDA-MB-231 cells. Constitutive expression of E-cadherin (red fluorescence) was very weak in DMSO-treated MDA-MB-231 cells following 24-hour exposure to 2.5 μmol/L BITC (Fig. 2A). Notably, the BITC-mediated induction of E-cadherin protein expression was evident in both apoptotic (marked by an arrow in Figure 2A) and nonapoptotic cells (marked by an arrowhead in Figure 2A). Constitutive expression of vimentin protein, characterized by filamentous microtentacle–like protrusions, was clearly visible in DMSO-treated control MDA-MB-231 cells (Fig. 2B). Expression of vimentin protein was markedly suppressed after 24 hour treatment of MDA-MB-231 cells with 2.5 μmol/L BITC (Fig. 2B). The BITC administration also resulted in disruption of the vimentin network (Fig. 2B).

**BITC treatment increased *E-cadherin* transcription in breast cancer cells**

The BITC treatment caused an increase in the levels of *E-cadherin* mRNA in MDA-MB-231 and MCF-7 cells at both 8 and 16 hour time points (Fig. 2C). Consistent with the immunoblotting results (Fig. 1), the BITC-mediated increase in *E-cadherin* mRNA expression was relatively more pronounced in the MDA-MB-231 cell line in comparison with MCF-7 cells (Fig. 2C). These results indicate cell line–specific differences in BITC-mediated induction of *E-cadherin* mRNA at least in breast cancer cells.

Luciferase activity in MDA-MB-231 cells transiently transfected with an *E-cadherin* promoter reporter construct (E-cadherin promoter sequence from −108 to +125) was also significantly increased upon treatment with 2.5 μmol/L BITC in comparison with DMSO-treated control at both 8 and 16 hour time points (Fig. 2D). These results indicated that BITC administration increased transcription of *E-cadherin* in both MDA-MB-231 and MCF-7 cells.

**Reversal of TGFβ/-TNFα-induced EMT by BITC in MCF-10A cells**

We used an experimental system involving TGFβ/TNFα (10 ng/mL of each) and MCF-10A cells to confirm BITC-mediated inhibition of EMT. The MCF-10A cell line, which was isolated from fibrocystic breast disease and spontaneously immortalized, is nontumorigenic and widely used as a representative normal mammary epithelial cell line. The DMSO-treated control MCF-10A cells exhibited round and well-packed cobblestone appearance, a morphological feature of epithelial cells (Fig. 3A). Morphology of the MCF-10A cells was altered by a 24-hour exposure to known EMT inducers TGFβ/TNFα with a large fraction of cells exhibiting mesenchymal phenotype characterized by spindle-shaped morphology with cell scattering and loss of cell–cell contact (Fig. 3A). The BITC administration alone did not have an appreciable effect on MCF-10A morphology. The TGFβ-/TNFα-induced EMT was partially reversible in the presence of BITC with restoration of cell–cell contact (Fig. 3A).

Next, immunoblotting was done by using lysates from TGFβ/TNFα and/or BITC-treated cells to confirm reversal of EMT by BITC. As shown in Figure 3B, exposure of MCF-10A cells to TGFβ/TNFα resulted in downregulation of E-cadherin protein (80% decrease compared with DMSO-treated control) and induction of vimentin (1.8-fold induction compared with DMSO-treated control) and fibronectin (3.5-fold induction compared with DMSO-treated control) proteins. The TGFβ-/TNFα-mediated suppression of E-cadherin and induction of fibronectin protein expression were partially reversed in the presence of BITC. The level of β-catenin was not markedly altered by TGFβ/TNFα and/or BITC treatments. On the other hand, the TGFβ-/TNFα-treated MCF-10A cells exhibited a decrease in protein levels of p53 and Rb tumor suppressors (Fig. 3C), which are implicated in EMT (35, 36). The TGFβ-/TNFα-mediated suppression of p53 and Rb protein expression was also fully (p53) or partially reversible (Rb) in the presence of BITC (Fig. 3C).

Next, we proceeded to determine the role of Rb in BITC-mediated inhibition of EMT by using MCF-10A cells. Rb depletion results in deregulation of E-cadherin and EMT induction (35). Knockdown of Rb protein level by transient transfection of MCF-10A cells with an Rb-targeted siRNA (Supplementary Fig. S1A) was accompanied by suppression...
of E-cadherin (60% decrease) and occludin protein expression (90% decrease) with a concomitant upregulation of fibronectin protein expression (1.5-fold increase; Supplementary Fig. S1B). These effects mediated by Rb protein knockdown were partially reversible in the presence of BITC (Supplementary Fig. S1B). Another phenotype associated with Rb protein knockdown was increased migratory potential of MCF-10A cells (Supplementary Fig. S1C). The MCF-10A cell migration was markedly reduced in the presence of BITC regardless of Rb expression status (Supplementary Fig. S1C). Together, these results indicated that BITC cooperated with Rb to inhibit EMT in our model.

BITC treatment inhibited TGFβ-/TNFα-induced cell migration

We designed experiments to determine antimigratory effect of BITC in TGFβ-/TNFα-stimulated MCF-10A cells. As shown in Figure 4A, migratory potential of MCF-10A cell was increased markedly in the presence of TGFβ/TNFα. Basal and TGFβ-/TNFα-inducible migration of MCF-10A cells was markedly abolished in the presence of 2.5 μmol/L BITC (Fig. 4A). These results led us to conclude that BITC-mediated inhibition of experimental EMT translates into suppression of cell migration. We utilized RNA interference to further test role of E-cadherin in BITC-mediated
inhibition of EMT and cell migration. The BITC administration (2.5 μmol/L for 24 hours) increased level of E-cadherin protein by about 4.6-fold over DMSO-treated control in MCF-10A cells transiently transfected with a control siRNA (Fig. 4B). Protein level of E-cadherin was reduced by more than 98% in MCF-10A cells transfected with an E-cadherin–specific siRNA (Fig. 4B). Similar to untransfected cells (Fig. 4A), migratory potential of control siRNA-transfected MCF-10A cells was increased markedly in the presence of 10 ng/mL each of TGFβ and TNFα (Fig. 4C). Quantitation of migrated cells was not possible in this experiment due to cell clumping. Knockdown of E-cadherin protein also caused a marked increase in migration capability of MCF-10A cells even in the absence of TGFβ and TNFα (Fig. 4C). Formation of adherens junctions is important for maintenance of the epithelial phenotype (24–26). Expression of E-cadherin protein was decreased in TGFβ- and TNFα-treated MCF-10A cells leading to loss of cell–cell contact and appearance of lamellipodia in some cells (identified by arrows). Moreover, when the cells were cotreated with 2.5 μmol/L BITC and 10 ng/mL each of TGFβ and TNFα, a reversion to epithelial phenotype was discernible as evidenced by an increase in E-cadherin staining and emergence of cell–cell contact (Fig. 5).

**In vivo effect of BITC administration on EMT markers in MDA-MB-231 xenografts**

We have shown previously that growth of MDA-MB-231 xenografts in female athymic mice is inhibited significantly by intraperitoneal BITC administration (2.5 and 7.5 μmol BITC/mouse, 5 times per week; ref. 18). For example,
50 days after tumor cell injection, the average tumor volume in control mice (1581 ± 240 mm³) was about 2.5- to 3-fold higher than in BITC-treated mice (18). We used tumor tissues from the same experiment to determine the effect of BITC administration on expression of EMT markers. E-cadherin protein expression was very low in the tumors from control mice (Fig. 6A). The BITC administration dose dependently caused upregulation of E-cadherin protein expression in MDA-MB-231 xenografts (Fig. 6A). Expression of vimentin protein was markedly decreased in MDA-MB-231 tumors from BITC-treated mice compared with those of control mice (Fig. 6B). Results of immunohistochemical analyses were confirmed by western blotting by using tumor supernatants from control and 7.5 μmol BITC-treated mice (Fig. 6C). Tumors from the BITC-treated mice exhibited increased expression of E-cadherin protein compared with control. In agreement with cellular data, MDA-MB-231 tumors from BITC-treated mice revealed a marked decrease in protein levels of fibronectin and vimentin in comparison with those of control mice (Fig. 6C). Together, these results provided in vivo evidence for BITC-mediated suppression of EMT.

**Discussion**

The EMT is essential for normal physiological processes such as embryonic development, tissue remodeling, and wound healing (37). During EMT, epithelial phenotype characterized by tight cell–cell junctions and polarity changes to a mesenchymal phenotype typified by disruption of the cell–cell contact with conversion to fibroblastic morphology and increased motility (37). Moreover, the
EMT assumes a central place in pathogenesis of aggressive cancers (33). For example, in biopsies of human breast carcinoma detection of overexpressed EMT indicators are associated with tumor aggressiveness, disease recurrence, unfavorable clinicopathologic variables, and shorter survival (25, 29). Recent studies have identified many diet-derived natural products as inhibitors of EMT, including garlic-derived chemicals (38, 39), soy constituent genistein (40, 41), and green tea polyphenols (42). This study shows that cruciferous vegetable constituent BITC inhibits EMT in cancer cells and this effect is not a cell line–specific phenomenon. The BITC-mediated inhibition of EMT is discernible in breast cancer cells and in a pancreatic cancer cell line. More importantly, BITC administration elicits E-cadherin induction and suppression of mesenchymal markers in vivo in MDA-MB-231 xenografts.

Adherens junctions are important for maintenance of epithelial integrity (33, 37, 43). The E-cadherin is regarded as a tumor suppressor because of its role in maintenance of epithelial phenotype (44–46). The E-cadherin is frequently downregulated during cancer progression and correlates with poor prognosis (45). In mesenchymal cells, E-box elements located within 108 bp of the 5′-region of the E-cadherin promoter are responsible for its transcriptional repression by snail and other transcriptional factors (34, 47). Because BITC administration suppresses expression of snail and slug mRNA and increases transcription of E-cadherin, it is reasonable to conclude that BITC-mediated induction of E-cadherin involves E-box elements. Further studies are needed to test this hypothesis.

Vimentin, a mesenchymal filament protein, increases motility of cells undergoing mesenchymal conversion in coordination with detyrosinated microtubules to provide support to microtentacle extension of detached tumor cells (48). The BITC administration clearly decreases vimentin protein level in MDA-MB-231 cells and during experimental EMT in MCF-10A cells. Rb, a classical tumor suppressor, in association with the transcription factor activator protein-2α (AP-2α) binds to E-cadherin promoter sequence and regulates expression of E-cadherin in epithelial cells. During EMT, Rb is degraded and its depletion results in deregulation of E-cadherin and induction of an EMT-like phenotype with increased cell motility (35). We found that the TGFβ- and TNFα-treated MCF-10A cells display morphological alterations from epithelial to mesenchymal phenotype in association with downregulation of Rb, p53, and E-cadherin concomitant with gain of vimentin and fibronectin protein levels. Whereas BITC-mediated reversal of morphological...

Figure 5. Immunocytochemical analysis for E-cadherin and actin in MCF-10A cells treated for 24 hours with DMSO (control) or 10 ng/mL each of TGFβ and TNFα in the absence or presence of 2.5 μmol/L BITC (>100 magnifications). Experiment was repeated twice and representative data from one such experiment are shown.
and molecular changes in TGFβ/TNFα-stimulated MCF-10A cells prompts us to speculate that E-cadherin upregulation in BITC-treated cells may be mediated by AP-2α. Additional work is needed to substantiate this possibility. Nonetheless, this study indicates that E-cadherin is necessary, at least in part, for BITC-mediated inhibition of EMT because E-cadherin knockdown confers partial protection against anti-cell migration effect of BITC.

During EMT, cells undergo cytoskeletal reorganization into actin stress fibers to acquire spindle shape morphology and increased cellular motility (29, 33). The TGFβ/TNFα-treated MCF-10A cells display higher propensity for migration and cytoskeleton remodeling with actin reorganization. The BITC administration is able to inhibit cytoskeletal remodeling and formation of actin stress fibers leading to suppression of TGFβ/TNFα-stimulated cell migration.

In conclusion, this study is the first published report to document inhibition of EMT by BITC that is characterized by upregulation of adherens junction proteins (E-cadherin and occludin), downregulation of mesenchymal markers such as vimentin and fibronectin, and suppression of transcriptional repressors of E-cadherin (snail and slug).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Brightly yellow-orange skin cancer cells.


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