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***In Vitro* and *In Vivo* Effects of Phenethyl Isothiocyanate Treatment on Vimentin Protein Expression in Cancer Cells**

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Abstract

We have shown previously that cancer prevention by cruciferous vegetable constituent phenethyl isothiocyanate (PEITC) in a transgenic mouse model of prostate cancer is associated with induction of E-cadherin protein expression. Because suppression of E-cadherin protein concomitant with induction of mesenchymal markers (e.g., vimentin) is a biochemical hallmark of epithelial-mesenchymal transition, a process implicated in cancer metastasis, we hypothesized that PEITC treatment was likely to suppress vimentin protein expression. Contrary to this prediction, exposure of human breast (MDA-MB-231) and prostate cancer cells (PC-3 and DU145) to PEITC resulted in a dose-dependent increase in vimentin protein level, which was observed as early as 6 hours post-treatment and persisted for the duration of the experiment (24 hours). RNA interference of vimentin resulted in a modest augmentation of PEITC-mediated inhibition of MDA-MB-231 and PC-3 cell migration as well as cell viability. Furthermore, the PEITC-induced apoptosis was moderately increased upon siRNA knockdown of vimentin protein in MDA-MB-231 and PC-3 cells. To our surprise, PEITC treatment caused a marked decrease in vimentin protein expression in breast and prostate carcinoma *in vivo* in transgenic mouse models; although the difference was statistically significant only in the breast carcinomas. The present study highlights the importance of *in vivo* correlative studies for validation of the *in vitro* mechanistic observations.

Keywords

Phenethyl isothiocyanate; Vimentin; Cancer chemoprevention

INTRODUCTION

Natural products represent a rich source of potential cancer chemopreventive and therapeutic agents. Constituents of edible fruits and vegetables are particularly attractive for the discovery of novel cancer fighting agents. Phenethyl isothiocyanate (PEITC) is one such small-molecule compound present in substantial amounts in some cruciferous vegetables such as watercress (1). Cancer chemopreventive potential of PEITC was first observed by Wattenberg (2) in a chemically-induced rodent carcinogenesis model. Specifically, the PEITC administration 4 hours before challenge with 7,12-dimethylbenz[a]anthracene resulted in inhibition of mammary carcinogenesis in rats (2). Likewise, rats fed a diet supplemented with 3 and 6 mmol PEITC/kg diet developed significantly fewer esophageal tumors compared with rats fed a control diet (3). Lung tumorigenesis induced by the tobacco-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone was inhibited significantly by dietary administration of 4 and 8 mmol PEITC/kg diet in rats (4). Other

examples of PEITC-mediated inhibition of chemical carcinogenesis include azoxymethane-induced colonic aberrant crypt foci in rats (5) and *N*-nitrosomethylbenzylamine-induced buccal pouch cancer in hamsters (6). More recent studies, including those from our laboratory, have established cancer chemopreventive efficacy of PEITC in transgenic mouse models of prostate and breast cancers (7-9). Interestingly, PEITC treatment sensitized human prostate cancer cells to docetaxel *in vitro* as well as *in vivo* (10).

Research over the past few decades has provided wealth of knowledge concerning the mechanisms underlying cancer chemopreventive response to PEITC. Evidence continues to accumulate to suggest that PEITC can not only inhibit cancer initiation by modulating pathways relevant to carcinogen metabolism (e.g., induction of phase 2 enzymes) but also confer protection against post-initiation cancer development by suppressing cell proliferation and angiogenesis and inducing apoptotic as well as autophagic cell death (11-14). Despite these advances, however, a full appreciation of the molecular mechanisms contributing to cancer prevention by PEITC is necessary for its clinical development. For example, mechanistic understanding using *in vitro* models (e.g., cultured cancer cells) coupled with *in vivo* correlative validation of the molecular alterations could lead to identification of biomarker(s) predictive of PEITC response potentially useful in future clinical trials.

We have shown previously that cancer prevention by PEITC *in vivo* in a transgenic mouse model of prostate cancer (Transgenic Adenocarcinoma of Mouse Prostate; hereafter abbreviated as TRAMP) is associated with a marked increase in expression of E-cadherin (8). Suppression of E-cadherin concomitant with induction of mesenchymal markers (e.g., vimentin) is a biochemical hallmark of epithelial-mesenchymal transition (EMT), a process implicated in cancer metastasis (15,16). The present study was undertaken to determine the effect of PEITC treatment on vimentin protein expression using *in vitro* (cultured cancer cells) and *in vivo* (tissues from transgenic mouse models) models of breast and prostate cancer.

MATERIALS AND METHODS

Materials

PEITC (purity 98%) was purchased from LKT Laboratories (St. Paul, MN). Stock solution of PEITC was prepared in dimethyl sulfoxide (DMSO), and diluted with complete media before use. Same concentration of DMSO (final concentration <0.1%) was added to the controls. Cell culture reagents were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Vimentin-targeted small interfering RNA (siRNA) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas a nonspecific control siRNA was from Qiagen (Valencia, CA). Vimentin antibody used for western blotting was purchased from Sigma-Aldrich (St. Louis, MO), an antibody against vimentin used for immunohistochemistry was purchased from Santa Cruz Biotechnology, and anti-actin antibody was from Sigma-Aldrich. Transwell Permeable Support (8 μ m polycarbonate membrane) chambers used for cell migration assay were purchased from Corning (Corning, NY). Annexin V-FITC/Propidium Iodide Apoptosis Detection kit was purchased from BD Biosciences (San Diego, CA).

Cell Lines

The MDA-MB-231 human breast cancer cell line and prostate cancer cells (PC-3 and DU145) were purchased from American Type Culture Collection (Manassas, VA) and maintained as described by us previously (10,12,17). Authentication of these cells was done by Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO).

Western Blotting Assay

The MDA-MB-231, PC-3 and DU145 cells were treated with DMSO (control) or PEITC (2.5 or 5 μ M) for 6 hours, 12 hours or 24 hours. Cells were collected and lysed as described by us previously (18). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Perkin Elmer, Boston, MA). Immunoblotting was done as described by us previously (12,13,18).

RNA Interference and Cell Migration Assay

The MDA-MB-231 or PC-3 cells were seeded in six-well plates and transfected at 60-80% confluency with control nonspecific siRNA (100 nM) or vimentin-targeted siRNA (100 nM) using Oligofectamine. Twenty-four hours after transfection, the cells were treated with DMSO or PEITC for 24 hours. The cells were collected and processed for western blotting or cell migration, cell viability and apoptosis assays. For cell migration assay, control siRNA-transfected and vimentin siRNA-transfected MDA-MB-231 or PC-3 cells (2×10^5) were suspended in serum-free medium containing DMSO or the indicated concentrations of PEITC and placed in the upper compartment of the Transwell chamber. After 24 hours of incubation, non-motile cells from the upper surface of the filter were removed using a cotton swab. The motile cells from the bottom face of the filter were fixed with methanol and stained with hematoxylin and eosin. At least four randomly selected areas were scored for cell migration count.

Cell Viability Assay

The MDA-MB-231 or PC-3 cells were seeded in 12-well plates and transfected at 60-80% confluency with control nonspecific siRNA (100 nM) or vimentin-targeted siRNA (100 nM) using Oligofectamine. The control siRNA-transfected and vimentin siRNA-transfected MDA-MB-231 or PC-3 cells were treated with DMSO (control) or specified concentrations of PEITC for desired time period. Subsequently, the cells were collected, washed with phosphate-buffered saline, and processed for trypan blue dye exclusion assay as described by us previously (19).

Determination of Apoptosis

Apoptosis induction was assessed using Annexin V-FITC/Propidium Iodide Apoptosis Detection kit from BD Biosciences according to the manufacturer's instructions with some modifications. Briefly, MDA-MB-231 and PC-3 cells transfected with control siRNA or vimentin-targeted siRNA were incubated in the absence or presence of indicated concentrations of PEITC for 24 h and then trypsinized and washed with phosphate-buffered saline. About 1×10^5 cells were suspended in 100 μ L of binding buffer and stained with 3 μ L of Annexin V-FITC and 2 μ L of PI solution for 15 minutes at room temperature in the dark. The stained cells were analyzed using a flow cytometer.

Immunohistochemical Analysis for Vimentin Protein Expression

Immunohistochemistry for vimentin was performed essentially as described by us previously for other proteins (8,9). Briefly, dorsolateral prostate sections from TRAMP mice or mammary tumor sections from MMTV-*neu* mice (3-4 μ m) from our previously completed studies (8,9) were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then probed with the anti-vimentin antibody, washed with Tris-buffered saline Wash Buffer (Dako, Carpinteria, CA) and incubated with an appropriate secondary antibody. Color was developed by incubation with 3,3'-diaminobenzidine or vulcan fast red (Biocare Medical, Concord, CA). The sections were counterstained with Harris Hematoxylin (Anatech, Battle Creek, MI), and examined under a Leica microscope (Leica Microsystems, Bannockburn, IL). The images were captured with Image ProPlus 5.0

software (Media Cybernetics, Bethesda, MD) as previously described [8,9]. At least three non-overlapping and non-necrotic images were captured from each section of MMTV-*neu* mice and at least twenty-two non-overlapping and non-necrotic images were captured from each section of TRAMP mice and analyzed with the use of Positive Pixel Count V9 algorithm of Aperio ImageScope software (Aperio Technologies, Vista, CA).

Statistical Analysis

Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test or by two-sided Student's *t*-test. Data are shown as mean $\pm$ SD and $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism v.4.03 software (GraphPad Software, La Jolla, CA).

RESULTS

PEITC Treatment Caused Induction of Vimentin Protein Expression in Cancer Cells

Previous studies from our laboratory have revealed that the PEITC-mediated cancer prevention in TRAMP mice (8) as well as MMTV-*neu* mice (Singh SV, unpublished results) is associated with *in vivo* induction of E-cadherin protein expression. We have also reported previously that benzyl isothiocyanate (BITC), a naturally-occurring analog of PEITC with only subtle-structural difference of varying alkyl chain length, inhibits EMT in cultured breast cancer cells and MDA-MB-231 breast cancer xenografts *in vivo* characterized by induction of E-cadherin protein expression and suppression of vimentin protein level (20). Therefore, it was of interest to determine whether PEITC is capable of inhibiting EMT by suppressing vimentin protein level. Because PEITC is an effective inhibitor of both breast and prostate cancer in transgenic mouse models (8,9), we used cell lines representing these cancers (MDA-MB-231 and PC-3 cells) to test this possibility. To our surprise, PEITC treatment caused a marked and dose-dependent increase in protein level of vimentin that was evident as early as 6 hours after treatment and this effect was sustainable for at least 24 hours (duration of the experiment) (Fig. 1). We used an additional prostate cancer cell line (DU145) to confirm PEITC-mediated induction of vimentin protein *in vitro* (Fig. 1).

Effect of Vimentin Knockdown on PEITC-Mediated Inhibition of Cell Migration and Cell Viability

Because EMT is believed to promote cancer cell migration (15,16), we designed experiments using siRNA to determine functional significance of vimentin induction by PEITC. Level of vimentin protein was decreased markedly upon transient transfection of MDA-MB-231 and PC-3 cells with the vimentin-targeted siRNA when compared with cells transfected with a control nonspecific siRNA (Fig. 2A). Migration in DMSO-treated control and PEITC-treated MDA-MB-231 and PC-3 cells can be visualized in Figure 2B. Knockdown of vimentin alone had no effect on migration ability of either cell line (Fig. 2C). On the other hand, the PEITC treatment dose-dependently inhibited cell migration that was modestly augmented by RNA interference of vimentin at least at the 5 μ M concentration (Fig. 2C).

We also determined the effect of vimentin knockdown on PEITC-mediated inhibition of MDA-MB-231 and PC-3 cell viability, and the results are shown in Figure 3. Consistent with previous observations (21), survival of MDA-MB-231 and PC-3 cells was dose-dependently reduced in the presence of PEITC (Fig. 3). Similar to cell migration, the PEITC-mediated reduction in MDA-MB-231 and PC-3 cell viability was modestly but statistically significantly augmented by knockdown of vimentin protein at least at the 24 hour time point (Fig. 3). Collectively, these results indicated that vimentin induction

resulting from PEITC exposure conferred moderate protection against inhibition of cell migration/cell viability by this agent.

Modest Intensification of PEITC-Induced Apoptosis by Vimentin Knockdown

Vimentin has been implicated in apoptosis regulation by some stimuli (22,23). For example, vimentin was shown to confer resistance to nuclear apoptosis after photodynamic treatment with a silicon phthalocyanine in Jurkat cells (22). On the other hand, apoptosis induction by a naturally-occurring small molecule (withaferin A) was mediated by caspase-dependent cleavage of vimentin in soft tissue sarcoma cells (23). Apoptotic response to withaferin A was abrogated after vimentin knockdown or by blockade of caspase-dependent vimentin cleavage (23). We therefore determined the effect of vimentin knockdown on apoptosis induction by PEITC. Figure 4A depicts flow cytometric quantitation of early (Annexin V-positive) and late (Annexin V-positive and PI-positive) apoptotic cells following 24 hour treatment of MDA-MB-231 and PC-3 cells with DMSO (control) or 5 μ M PEITC. Number of Annexin V-positive and PI-positive cells was increased significantly after treatment with PEITC in both cell types and the difference compared with control was significant with 5 μ M dose (Fig. 4B). The PEITC-induced apoptosis was modestly intensified by RNA interference of vimentin. Trend was the same in both cells but the difference compared with control siRNA transfected cells was significant at 5 μ M PEITC only in the PC-3 cell line. These results indicated modest impact of vimentin knockdown on PEITC-induced apoptosis.

PEITC Administration Suppressed Vimentin Protein Level in Breast and Prostate Tumors *In Vivo*

We used archived tissues from our previously published prevention study using MMTV-*neu* mice (9) to determine whether PEITC administration caused *in vivo* induction of vimentin protein. Immunohistochemical analyses for vimentin protein expression in representative tumors from MMTV-*neu* mice are shown in Figure 5A. To our surprise, dietary administration of 3 μ mol PEITC/g diet for 29 weeks not only suppressed mammary carcinogenesis (9) but also resulted in a significant decrease in vimentin protein level (Fig. 5B). Dorsolateral prostates from PEITC-treated TRAMP mice (3 μ mol PEITC/g diet for 19 weeks) also exhibited a decrease in vimentin protein level (Fig. 5C), although the difference did not reach statistical significance probably due to insufficient power (Fig. 5D). Nevertheless these results indicated that, unlike cultured cancer cells, PEITC administration resulted in suppression of vimentin protein expression *in vivo*.

DISCUSSION

The present study shows that PEITC treatment increases vimentin protein level in cultured cancer cells. The PEITC-mediated induction of vimentin protein expression is not a cell line-specific response. Presently it is unclear if PEITC-mediated induction of vimentin protein expression involves transcriptional or post-transcriptional mechanism(s), but vimentin induction confers modest protection against inhibition of cell migration and cell viability resulting from PEITC exposure in cultured cells. Consistent with cell viability data, the PEITC-induced apoptosis is modestly intensified after knockdown of vimentin protein. However, we are puzzled to observe a marked decrease in vimentin protein level *in vivo* after PEITC administration. Reasons for the discrepancy in results between *in vivo* and *in vitro* systems are unclear, but inhibition of metastasis in PEITC-treated TRAMP (8) and MMTV-*neu* mice (Singh SV, unpublished observations) may, at least in part, be mediated by vimentin suppression. Observed mechanistic differences in cultured cells and tumor tissues with regards to the effect of PEITC on vimentin protein expression also highlight importance of *in vivo* correlative studies to confirm *in vitro* cellular observations.

The present study coupled with our previous observations provides yet another example of profound mechanistic differences resulting from even subtle change in the isothiocyanate structure. For example, we have shown previously that B-cell lymphoma 2 interacting mediator of cell death (Bim) is critically involved in regulation of PEITC-induced apoptosis in breast cancer cells including MDA-MB-231 and MCF-7 (24). On the other hand, Bim is totally dispensable for proapoptotic response to BITC in same cell lines (25). Likewise, we have shown previously that BITC treatment suppresses vimentin protein expression in cultured MDA-MB-231 cells as well as in MDA-MB-231 xenografts *in vivo* (20). In contrast, PEITC treatment causes induction of vimentin in cultured MDA-MB-231 cells but suppression of its level in a transgenic mouse model of breast cancer. Marked differences in chemopreventive efficacy of PEITC *versus* BITC *in vivo* have also been observed in some models of chemically-induced cancer in rodents (26,27). These observations raise caution in extrapolation of results between structurally different isothiocyanates even if they have subtle structural differences as exemplified by PEITC and BITC.

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FIG. 1.

Immunoblotting for vimentin protein using lysates from MDA-MB-231, PC-3, and DU145 cells treated with DMSO (control) or the indicated concentrations of PEITC for specified time periods. Blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Numbers on top of bands are fold changes in protein expression level relative to corresponding DMSO-treated control. Each experiment was repeated at least twice.

FIG. 2.

A: Immunoblotting for vimentin using lysates from MDA-MB-231 and PC-3 cells transiently transfected with a control siRNA or a vimentin-targeted siRNA and treated for 24 h with DMSO or the indicated concentrations of PEITC. Numbers on top of the immunoreactive bands represent densitometric quantitation of changes in protein levels relative to control siRNA-transfected cells treated with DMSO. Blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. B: Representative images from Transwell chamber assay depicting migration by MDA-MB-231 and PC-3 cells transiently transfected with a control siRNA or a vimentin-targeted siRNA and treated for 24 h with DMSO or the indicated concentrations of PEITC. C: Quantitation of migrated MDA-MB-231 and PC-3 cells from experiment shown in panel B. Experiment was repeated three times and combined results are shown as mean $\pm$ SD ($n = 7$ in MDA-MB-231 cells and $n = 9$ in PC-3 cells). Significantly different ($P < 0.05$) compared with ^acorresponding DMSO-treated control and ^bbetween a control siRNA and a vimentin-targeted siRNA cells at each dose (0, 2.5, and 5 μ M PEITC) by one-way ANOVA followed by Bonferroni's multiple comparison test.

FIG. 3.

Effect of PEITC treatment on cell viability of MDA-MB-231 and PC-3 cells transiently transfected with a control siRNA or vimentin-targeted siRNA and treated for 6 h, 12 h, and 24 h with DMSO or the indicated concentrations of PEITC. The number of viable cells was determined by trypan blue dye exclusion assay. Results shown are mean $\pm$ SD ($n = 3$). Significantly different ($P < 0.05$) compared with ^acorresponding DMSO-treated control and ^bbetween a control siRNA and a vimentin-targeted siRNA cells at each dose (0, 2.5, and 5 μ M PEITC) by one-way ANOVA followed by Bonferroni's multiple comparison test. Each experiment was repeated at least twice and representative data from one such experiment are shown.

FIG. 4.

A: Representative flow histograms depicting apoptotic fraction in MDA-MB-231 and PC-3 cells transiently transfected with a control siRNA or vimentin-targeted siRNA and treated for 24 h with DMSO or 5 μ M of PEITC. B: Quantitation of % apoptotic fraction (early + late apoptotic cells) in MDA-MB-231 and PC-3 cells transiently transfected with a control siRNA or vimentin-targeted siRNA and treated for 24 h with DMSO or the indicated concentrations of PEITC. Experiment was repeated three times and combined results are shown as mean $\pm$ SD (n = 9). Significantly different ($P < 0.05$) compared with ^acorresponding DMSO-treated control and ^bbetween a control siRNA and a vimentin-targeted siRNA cells at each dose (0, 2.5, and 5 μ M PEITC) by one-way ANOVA followed by Bonferroni's multiple comparison test.

FIG. 5.

A: Immunohistochemistry depicting vimentin protein expression in the carcinoma lesions of control and PEITC-treated MMTV-*neu* mice (magnification $\times 200$; scale bar = 100 μm). B: Quantitative analysis of vimentin protein expression in the carcinoma lesions of control and PEITC-treated MMTV-*neu* mice. At least three randomly selected fields from each section were analyzed. Results shown are mean $\pm$ SD ($n = 6$ for both control and PEITC treatment groups). Statistical significance was determined by two-sided Student's *t*-test. C: Immunohistochemical images depicting vimentin protein expression in the poorly-differentiated carcinoma lesions of control and PEITC-treated TRAMP mice (magnification $\times 200$; scale bar = 100 μm). D: Quantitative analysis of vimentin protein expression in the poorly-differentiated carcinoma lesions of control and PEITC-treated TRAMP mice. Results shown are mean $\pm$ SD ($n = 5$ for both control and PEITC treatment groups). Statistical significance was determined by two-sided Student's *t*-test. Vimentin expression in the carcinoma lesions from MMTV-*neu* and TRAMP mice are in brown and pink color, respectively.