Indole-3-Carbinol Prevents PTEN Loss in Cervical Cancer In Vivo

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Indole-3-carbinol (I3C) is a phytochemical (derived from broccoli, cabbage, and other cruciferous vegetables) with proven anticancer efficacy including the reduction of cervical intraepithelial neoplasia (CIN) and its progression to cervical cancer. In a breast cancer cell line, I3C inhibited cell adhesion, spreading, and invasion associated with an upregulation of the tumor suppressor gene *PTEN*, suggesting that PTEN is important in inhibition of late stages in the development of cancer. The goal of this study was to determine the expression of PTEN during the development of cervical cancer and whether I3C affected expression of PTEN in vivo. We show diminished *PTEN* expression during the progression from low-grade to high-grade cervical dysplasia in humans and in a mouse model for cervical cancer, the K14HPV16 transgenic mice promoted with estrogen. The implication is that loss of *PTEN* function is required for this transition. Additionally, dietary I3C increased PTEN expression in the cervical epithelium of the transgenic mouse, an observation that suggests *PTEN* upregulation by I3C is one mechanism by which I3C inhibits development of cervical cancer.

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INTRODUCTION

Indole-3-carbinol (I3C) is a promising cancer-preventive phytochemical found abundantly in cruciferous vegetables, such as broccoli and Brussels sprouts. In animal models, I3C prevents the development of a plethora of malignancies, including cervical cancer (1), breast cancer (2), prostate cancer (3), endometrial cancer (4), and skin cancer (5). Diindolylmethane (DIM), the congener of I3C formed in the stomach, alters the expression of many genes (6). As a result, I3C alters estrogen signaling (7-9), inactivates many carcinogens (10,11), causes growth arrest (12-14), induces the endoplasmic reticulum response (15), and induces apoptosis (16,17). Relevant to this study with *PTEN*, it is clear that I3C inactivates Akt in tumor cells (18,19).

The ability of I3C to upregulate the tumor suppressor PTEN is controversial, however. Meng et al. (20) reported that I3C upregulated *PTEN* in T47-D human breast cancer cells, whereas Howells et al. (21) observed no change of *PTEN* levels in response to I3C in a different breast cancer cell line. We therefore sought to determine the effect of I3C on PTEN in relation to I3C's ability to prevent the development of cervical cancer in vivo.

Cervical cancer and precancerous lesions are well characterized (for example, hyperplasia, low-grade dysplasia, high-grade dysplasia [with intermediate gradations], and cancer). Additionally, a mouse model with HPV transgenes (cofactor for cervical cancer) develops cervical cancer when given estrogen chronically (22). The mouse's development of cervical cancer resembles the development of cervical abnormalities and progression to cancer as seen in humans (1,22). I3C prevents cervical cancer in this mouse model (1) and has efficacy in the treatment of cervical dysplasia in both mice and humans (1,23).

Mutations in *PTEN* are common in many human cancers (24-26) but not in cervical cancer (27-29); however, inactivation by methylation of the promoter does occur (30-33). PTEN is a dual-specificity phosphatase capable of dephosphorylating phospholipids as well as phosphoproteins (34). The in vivo lipid substrate of PTEN is believed to be phosphatidylinositol-3,4,5-triphosphate (PIP_3) (35), a PI3 kinase product that recruits Akt to the plasma membrane (36,37) where it is phosphorylated by the activating kinases PDK1 (38) and PDK2/ILK (39,40). The C3-specific lipid phosphatase activity of PTEN generates PIP_2. *PTEN*, therefore, is a negative regulator of the Akt branch of PI3 kinase pathway (41). Negative regulation of Akt signaling by *PTEN* decreases both the level and the nuclear localization of cyclin D1, thereby markedly reducing Rb phosphorylation and increasing cell-cycle arrest at G_1 (42). Reducing Akt signaling by PTEN also activates the Forkhead family of transcription factors (43) as well as Bad (44); both are pro-death regulators. As a protein phosphatase, PTEN mediates, either directly or indirectly, the dephosphorylation, and hence the inactivation, of phosphoSTAT3 (45). Other phosphoprotein substrates for PTEN include focal adhesion kinase (FAK) and adaptor protein Shc (46). As PTEN functions to tilt survival/death toward the pro-death side, the consequence of inactivating *PTEN* is tumor promotion, and the consequence of increasing functional *PTEN* expression is tumor suppression.

In this study, we demonstrated the loss of PTEN during the development of cervical cancer in humans and in a mouse model of cervical cancer. We show that dietary I3C upregulated PTEN in the mouse model.

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MATERIALS AND METHODS

Human Tissues

Archived human specimens from patients with condyloma, varying degrees of cervical intraepithelial neoplasia (CIN), and cervical cancer were from the pathology archive of Long Island Jewish Medical Center. Use of these surgical discards—paraffin-embedded fixed specimens from patients diagnosed with condyloma, low-grade dysplasia, high-grade dysplasia, and invasive squamous carcinoma (more than 20 in each category)—was approved by the Institutional Review Board of the North Shore Long Island Jewish Health System.

Mouse Tissues

Archived mouse specimens were from a study that compared the K14HPV16 transgenic mice and its nontransgenic littermates given control diet or diet supplemented with 2000 ppm I3C (1). Briefly, 5-month-old female mice were given slow release of 17β-estradiol at a dosage of 0.125 mg subcutaneously every 60 days to promote cervical dysplasia. Groups received AIN76a diet with or without I3C from 5 weeks to 7 months of age. Additional paraffin-embedded fixed specimens were from mice at each stage of CIN. The study had the approval of the Institutional Animal Care and Use Committee at Long Island Jewish Medical Center.

Evaluation of Specimens for PTEN

Serial sections (5 μm) were prepared, and pathology was evaluated using H&E-stained sections. Immunohistochemistry was used to visualize PTEN. Briefly, sections were air-dried overnight, rehydrated with graded alcohol and PBS, and treated with 0.1% H_2O_2 and 20 μg/mL proteinase K to block endogenous peroxidase activity and to expose antigens. Horse serum (1.5% in PBS) was used to block nonspecific antibody binding. PTEN monoclonal antibody A2B1 (Santa Cruz) was used at a dilution of 1:100. Immunoreactivity was detected using the Vectastain ABC system (Vector Laboratories). These slides were evaluated by each investigator, including 2 pathologists and 2 others.

Quantitative Evaluation for PTEN

The relative intensity of staining of PTEN in a measured section representing the full width of the epithelium (equivalent to that of the images shown) was performed using Image-Pro Plus 5.0 software (Media Cybernetics, Silver Springs, MD, USA). Briefly, a defined measured area from a ×400 magnification image was displayed in Adobe Photoshop, and the intensity of the staining was evaluated. Imaging was performed on a subset of tissues (minimum of 3) picked at random based on pathology. Immunohistochemistry processing was performed at the same time in these subsets to ensure comparable staining between tissues. Statistical analysis used Student *t* test.

RESULTS

PTEN Expression Decreases as Cervical Dysplasia Increases and Is Absent in Cervical Cancer

To gain insight into the role of PTEN in the development of cervical cancer, we followed its expression in the development of cervical cancer. We evaluated PTEN in various cervical abnormalities including cervical cancer samples obtained from humans. As shown in Figure 1A, cells from condylomata (genital warts or benign tumors caused by infection with an HPV, having very low potential of becoming cancer) expressed *PTEN*. This observation is consistent with previous findings in similar lesions caused by low-risk HPVs in the larynx (45). Similarly, cells in low-grade cervical dysplasia were PTEN-positive. In contrast, little or no PTEN was detected in cells in high-grade cervical dysplasia or in invasive squamous cell carcinoma. This observation indicated that elimination of PTEN expression was associated with progression from low-grade to high-grade cervical dysplasia, a transition when increased angiogenesis is required (47-50).

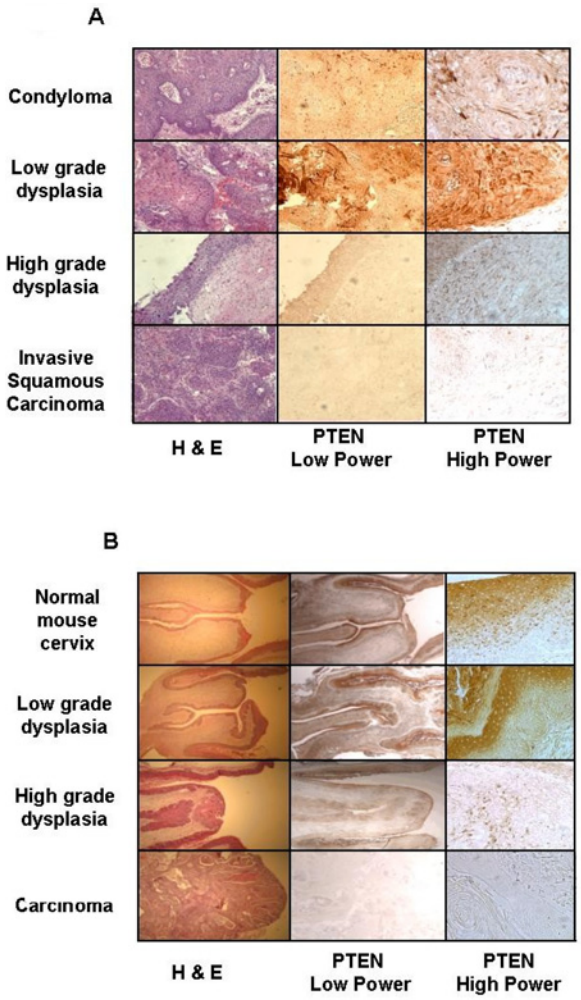

Figure 1. Inhibition of PTEN expression during the development of cervical cancer in humans and in HPV16 transgenic mice. (A) Human condyloma, low-grade cervical dysplasia, high-grade cervical dysplasia, and cervical carcinoma specimens were stained with H&E or immunostained by PTEN monoclonal antibody A2B1 (PTEN) and visualized under low-power (×100) or high-power (×400) magnification. (B) Tissue sections of normal, low-grade dysplasia, high-grade dysplasia, and carcinoma from HPV16 transgenic mice were stained and visualized as in panel A. Images are representative of more than 20 specimens per group.

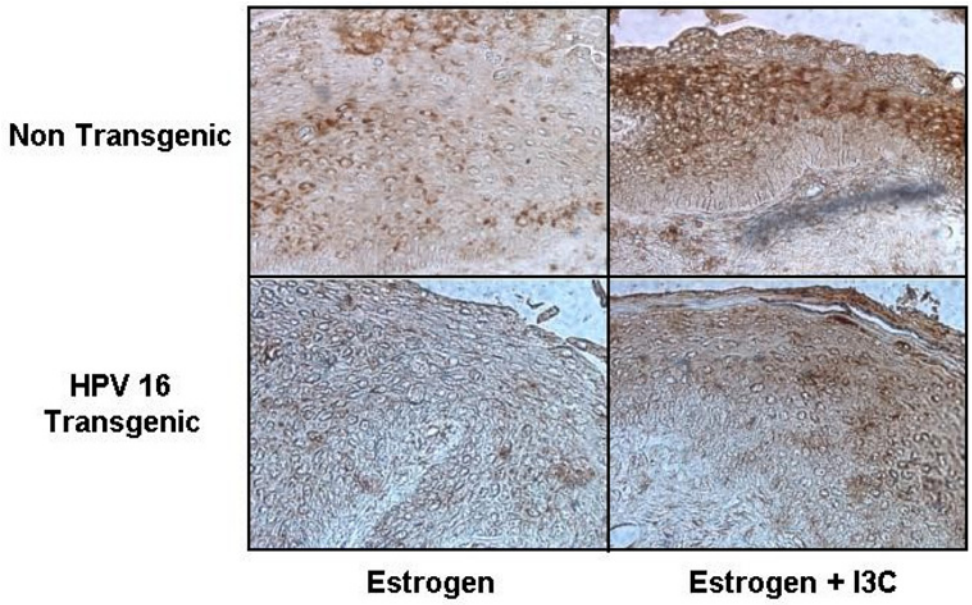

Figure 2. I3C promotes PTEN expression in HPV16 transgenic mice. HPV-16 transgenic mice and their nontransgenic littermates were given slow-release estrogen to promote dysplasia and fed diets with or without I3C. Cervix sections were prepared and immunostained with PTEN monoclonal antibody A2B1. Controls (no I3C treatment) are high-grade dysplasia. Specimens from the I3C-treated groups are age-matched. Images are representative of 20 per category. Magnification ×100 and ×400.

More exact conclusions can be made with lesions found in the K14HPV16 transgenic mouse, the murine model for human cervical cancer, because of its genetic homogeneity. As shown in Figure 1B, cells in normal cervical epithelium or in low-grade dysplasia expressed PTEN, whereas expression of PTEN was mostly undetectable when the dysplasia progressed to high grade or to carcinoma. These observations validated results seen in human lesions and supported our hypothesis that reduction in *PTEN* expression was required for transition from low-grade to high-grade dysplasia.

I3C Promotes PTEN Expression in K14HPV16 Transgenic Mice

I3C suppresses the formation of cervical cancer in K14 HPV16 mice (1). Additionally, I3C greatly reduced the incidence of progression to high-grade dysplasia in the background mouse given estradiol (1). More specifically, downstream products of I3C, such as diindolylmethane, formed in the stomach or tissue culture would be responsible for its effects. This observation led to the belief that I3C may exert its antitumor effects, at least in part, by increasing the expression of PTEN, an observation in breast cancer cells in vitro (20). Our evaluations used tissue from 7-month-old mice that had been fed diets with and without I3C from age 5 weeks. Figure 2 demonstrated that expression of *PTEN* was upregulated in cervical tissue from both the background mouse and the HPV16 transgenic mouse.

Quantitative determinations more precisely confirmed our observations. Imaging of the human sections, using immunohistochemistry for staining, indicated that very little PTEN was in the high-grade dysplasias or carcinomas in both human and mouse tissues (Figure 3A and B). In the mouse tissue, diminishing PTEN occurred as the cervical disease stages progressed from normal to low-grade dysplasia to high-grade dysplasia to carcinoma. In the mouse, the amount of PTEN was more variable in the normal cervical tissue than in any of the abnormal tissues. The *P* values comparing the reduced amount of PTEN to normal was significant for the carcinoma ($P = 0.04$) but not for low-grade dysplasia ($P = 0.08$) or high-grade dysplasia ($P = 0.09$), although the trend is clear. Because tissues without pathology (normal) were not available for human samples, the amount of PTEN in high-grade dysplasia and invasive carcinomas was compared with the benign tumors (condylomas). *P* values were highly significant: 0.02 and 0.01 for high-grade lesions and cancers, respectively.

Evaluations (Figure 3C) of mice treated long term with estradiol and diets with or without I3C indicated that mice treated with I3C had significantly more PTEN. The *P* values comparing PTEN in the mice treated with I3C were 0.009 and 0.011 for the background mouse and the HPV16 transgenic mouse, respectively.

The timing of the upregulation of PTEN—i.e., the transition from low-grade dysplasia to high-grade dysplasia (most evident in the human specimens)—coincides with the timing when angiogenesis occurs in the K14HPV16 mouse not treated with I3C (50). Consistently, the I3C congener diindolylmethane (formed from I3C in the stomach) inhibited angiogenesis in a transplantable human breast carcinoma (51).

DISCUSSION

In our in vivo study, PTEN is diminished during the transition from a low-grade to a high-grade dysplasia. I3C prevents this loss of *PTEN* and appears to increase its expression.

As a tumor suppressor, *PTEN* is often inactivated during tumorigenesis, thereby giving progeny tumor cells growth and

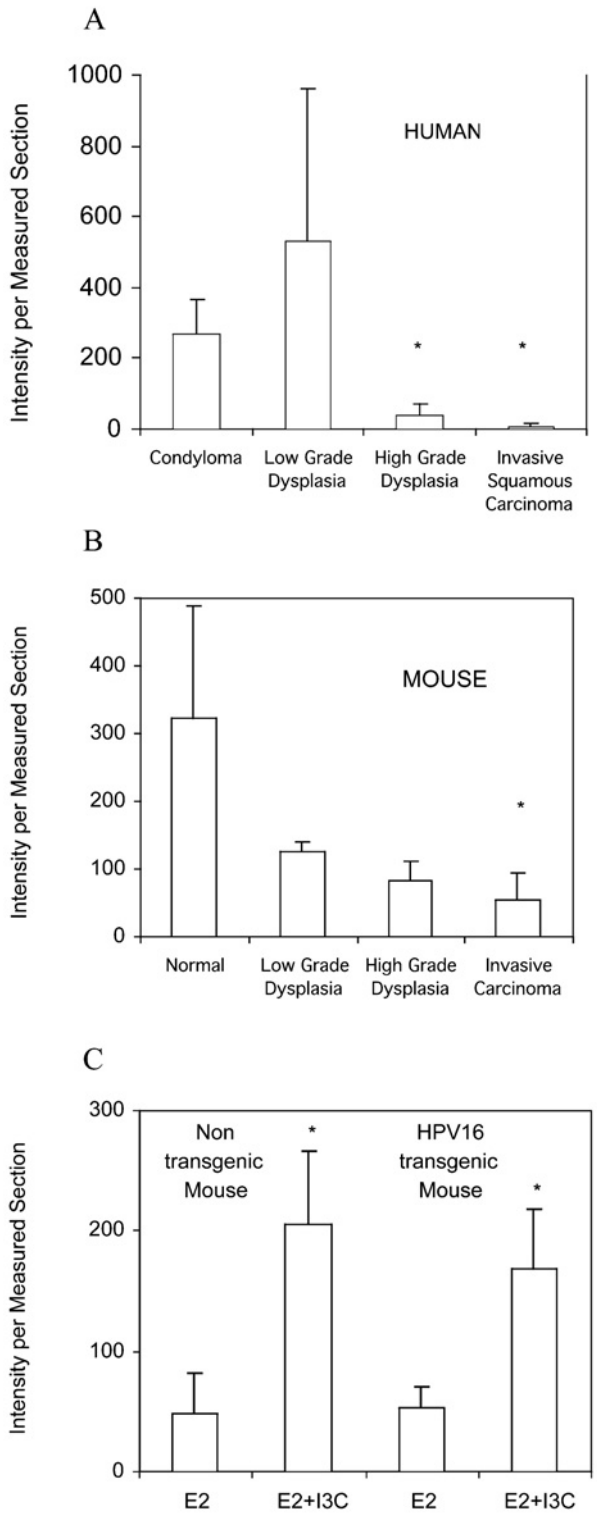

Figure 3. Quantitation confirms inhibition of PTEN expression during development of cervical cancer, and I3C increases PTEN expression. Imaging and quantitation of PTEN immunohistochemistry were done on a minimum of 3 tissues per group for cervix specimens from humans (A) and mice (B) with varying degrees of pathology. Quantitation of PTEN (C) was performed on specimens from nontransgenic and K14HPV16 transgenic mice treated with estradiol (E2) and diets with and without I3C from 5 weeks to 7 months of age. *$P \leq 0.05$.

survival advantages. In endometrial cancer, *PTEN* is mutated with high probability (30% to 50%) (26,27). In cervical cancer, however, genetic alteration that obliterates *PTEN*'s activity appears to be a rare event (28,29). Instead, elimination of *PTEN* expression is achieved in epigenetic ways, such as promoter methylation (32,33). I3C has been shown to inhibit tumor development from low-grade to high-grade dysplasia (1), a process that accompanies the downregulation of PTEN, in the K14HPV16 transgenic mouse model of cervical cancer. This is consistent with the present finding that I3C upregulates PTEN. Our results also suggest that the ability of I3C to upregulate PTEN contributes to its ability to prevent cervical carcinoma.

PTEN could retard tumor progression by inhibiting proliferation and by increasing tumor cell apoptosis. This is supported by our previous findings that I3C decreased proliferating cell nuclear antigen (PCNA)-positive cells (1) and increased TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells (16) in abnormal cervical epithelium of HPV16 mice. PTEN is a known negative regulator of angiogenesis (52). Inhibition of angiogenesis by PTEN can be mediated by reducing the level of activated Akt or STAT3, which leads to diminished transactivation of the VEGF promoter (53). Vascularization has been shown in many in vivo systems to be crucial for tumor progression. Reducing neovasculature restrains tumor growth, and increasing microvasculature is associated with progression from low-grade to high-grade CIN and to cervical carcinoma in humans (47,48,54) as well as in estrogen-induced dysplasia in mice (1). This is also consistent with our result in that I3C induces *PTEN* and retards progression to high-grade dysplasia.

The mechanism by which I3C upregulates *PTEN* is unknown. I3C is a phytoestrogen that can bind to the estrogen receptor and in some cases be an agonist (55), albeit it functions as an antiestrogen in the presence of estrogen by both competing with estrogen for the estrogen receptor (9) and altering estrogen metabolism (7). Other phytoestrogens, for example, genistein, have been shown to increase *PTEN* message (56). Genistein reverses hypermethylation, resulting in reactivation of methylation-silenced genes (57). Thus promoter demethylation of *PTEN* by I3C, which would be very relevant for cervical cancer, might be proposed as a mechanism. Another possibility is the upregulation of Egr-1, a transcription factor known to positively regulate *PTEN* expression (58).

As inactivation of *PTEN* has been implicated in a variety of cancers, upregulation of *PTEN* by I3C should be beneficial in the prevention and adjunctive therapy of a significant number of cancers. Mutations in *PTEN* resulting in a dysfunctional protein would negate this benefit. Nonetheless, I3C should be helpful in increasing *PTEN* levels that result from its underexpression due to a hemizygous deletion or hypermethylation.

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