Indole-3-Carbinol Prevents Cervical Cancer in Human Papilloma Virus Type 16 (HPV16) Transgenic Mice

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ABSTRACT

Mice that express transgenes for human papillomavirus type 16 under a keratin 14 promoter (K14-HPV16 mice) develop cervical cancer when they are given 17β-estradiol chronically. We asked whether the antiestrogenic phytochemical indole-3-carbinol (I3C), found in cruciferous vegetables, administered at physiological doses, would prevent the cervical-vaginal cancer that is promoted in these mice by high doses of estrogen. We compared mice that were fed a control diet with those that were fed a diet supplemented with 2000 ppm I3C. In the group fed the control diet, at a dose of estradiol of 0.125 mg per 60-day release, 19 of 25 transgenic mice developed cervical-vaginal cancer within 6 months, and the remainder had dysplasia. Only 2 mice of 24 in the group fed the I3C supplemented diet developed cancer, and the remainder had dysplasia or hyperplasia. I3C reduced dysplasia in the nontransgenic mice. Similar results were obtained at a higher dose of estradiol (0.250 mg per 60-day release), and I3C helped to prevent morbidity associated with retention of fluid in the bladder that frequently occurred with the higher estradiol dose. Additionally, I3C appeared to reduce skin cancer in transgenic mice. These data indicate that I3C is a useful preventive for cervical-vaginal cancer and, possibly, other cancers with a papillomavirus component.

INTRODUCTION

Infection with certain types of HPVs predisposes cells to developing cancer (reviewed in Refs. 1 and 2). Most clearly, HPV infection is a risk for cancer of the cervix, which is the second most common cancer in women and the seventh most common form of cancer worldwide (3). The highly oncogenic HPVs inactivate the cellular proteins Rb (4) and p53 (5). These tumor suppressor proteins exert negative effects on cell growth and increase apoptosis. However, infection with the high-risk HPVs is not sufficient to cause cancer. Of all the women who contract cervical HPV infections, only a fraction exhibit preneoplastic stages (cervical intraepithelial neoplasia usually graded as SIN I, II, or III), and only a small percentage of those women develop invasive cervical cancer (6). Evidence indicates that HPV infection increases the risk of other cancers, such as skin cancer (reviewed in Refs. 7 and 8). This is consistent with the loss of p53 and/or Rb in a variety of other cancers.

In the case of cervical cancer, both circumstantial and direct evidence indicate that estrogen increases the risk of HPV-infected cells becoming precancerous and malignant. The most compelling circumstantial evidence is that the most estrogen-sensitive genital site (the transformation zone of the cervix) is also the site where >90% of HPV-induced lesions and cervical cancers occur (9). This is in contrast to simple infection by HPV (presence of HPV DNA), which is similar in all parts of the genital tract of both men and women (10, 11). The most compelling direct evidence is a mouse model for cervical-vaginal cancer (12). This mouse has transgenes for HPV16 and expresses viral genes E6 and E7, the viral proteins that inactivate p53 and Rb, respectively. These mice develop cervical cancer when they are given estrogen chronically. The reasons why estrogen promotes cervical cancer are likely to be multiple. Estrogen increases expression of HPV16 in the cervical cancer lines SiHa (13) and CaSki (14) cervical cell lines. Estrogen also increases proliferation of estrogen-sensitive cells, including HPV-infected cells (15, 16), and prevents apoptosis (17, 18). Some metabolites of estradiol are carcinogenic (reviewed in Ref. 19). Infection with HPV increases 16α-hydroxylation of estradiol (20), thereby increasing the amount of 16α-hydroxyestrone, a metabolite that is carcinogenic. 16α-Hydroxyestrone dramatically increases anchorage-independent growth of HPV-immortalized genital cells (16).

Cofactors for skin cancers associated with papillomavirus are different. Both the human disease epidermoplasia verruciformis and the disease caused by the cotton tail rabbit papillomavirus provide evidence that papillomavirus and other factors (e.g., sun exposure, trauma, or certain chemical carcinogens) act synergistically with papillomavirus to induce malignant conversion (8, 21).

The phytochemical I3C is anticarcinogenic and antiestrogenic. I3C inhibits growth of benign tumors of laryngeal tissue caused by HPV11 in a mouse model (15) and is effective in the treatment and prevention of laryngeal papillomas caused by HPVs (22–24). I3C, other indoles, brassinins, and isothiocyanates result from the breakdown of glucosinolates, compounds that are found at high levels in cruciferous vegetables (e.g., cabbage, broccoli, Brussels sprouts, and cauliflower). Dietary I3C functions as a potent inducer of 2-hydroxylation of estradiol in rodents (25) and humans (26), thus increasing the anti-proliferative metabolite 2-hydroxyestrone and decreasing 16α-hydroxyestrone. This change in estrogen metabolism may be the reason, at least in part, why I3C inhibits mammary tumorigenesis in various mouse models (25, 27).

Here, we determined that I3C inhibited the papillomavirus-initiated and estrogen-promoted cervical-vaginal cancer in mice with HPV16 transgenes. We further determined that I3C reduced other estrogen-associated pathology in both transgenic and nontransgenic mice. Finally, I3C appeared to reduce dysplasia and cancer of skin in the transgenic mice, pathologies that are not promoted by estrogen.

MATERIALS AND METHODS

Transgenic Mice. The K14-HPV16 transgenic mice were described previously (28). The transgenes consist of the entire early region of the HPV16 genome controlled by the K14 promoter. Established lines expressed E6 and E7 but not the E1 and E2 transgenes. The mice in these studies were extensively back-crossed into the FVB/n background. Nontransgenic littermates were used in comparative experiments. Presence of the viral sequences was determined by PCR using primers consisting of 141–160 nt (5′) and 491–510 nt (3′) or 651–670 nt (5′) and 751–777 nt (3′) of HPV16. After transfer by Southern blot, PCR products were hybridized with DNA fragments (271–290 and 751–770 nt) to verify the presence of DNA coding for HPV16 E6 and E7, respectively.

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Diet Studies. Four to 5-week-old virgin control and transgenic female mice were used in these studies. Mice were implanted s.c. with either 0.125 or 0.250 mg per 60-day release pellets of 17\(\beta\)-estradiol. Implants were repeated every 60 days until the end of study. Mice were fed ab libitum with AIN76a diet or the AIN76a diet enriched with 2000 ppm I3C. I3C was purchased from Sigma Chemical Co. (St. Louis, MO), and diets were prepared by Zeigler Brothers Inc. (Gardner, PA). Mice were euthanized by CO\(_2\) asphyxiation at 24 weeks. Any mouse showing signs of morbidity were euthanized prior to 24 weeks.

Tissue Procurement and Histology. After euthanasia, the vagina, cervix, and both uterine horns were removed and immediately fixed in 10% formalin in PBS overnight. Similarly, ear skin was removed and fixed. Tissues were dehydrated through graded alcohol and xylene and embedded in paraffin. Five-\(\mu\)m serial sections through the full tissue were prepared, mounted, deparaffinized, and stained with H&E.

Proliferation Assay (PCNA Assay). The presence of the PCNA, a component of the \(\delta\) DNA polymerase, was used to determine which cells were proliferating. Five-\(\mu\)m paraffin sections were air-dried overnight and rehydrated through graded alcohol and PBS. After endogenous peroxidase activity was blocked with 0.1% H\(_2\)O\(_2\) for 15 min, sections were treated with proteinase K (Boehringer Mannheim, Indianapolis, IN) using 20 \(\mu\)g/ml in PBS for 20 min to expose antigens. Horse serum (1.5% in PBS) was used to suppress nonspecific binding. Sections were incubated with a 1:200 dilution of PCNA mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C and developed with the avidin-biotin-immunoperoxidase ABC immunostain system from Santa Cruz Biotechnology.

Statistical Analysis. The \(\chi^2\) test was used for data analysis.
RESULTS

Our goal was to determine whether dietary I3C would prevent cervical and epidermal cancer, using a previously described mouse model (12). FVB/n mice develop cervical dysplasia when they are given 17β-estradiol chronically. K14-HPV16 transgenic mice develop cervical cancer when they are given estradiol chronically. Additionally, the transgenic mice develop epidermal dysplasia and tumors, independent of estradiol.

We compared the nontransgenic mice that were fed the control diet to those that were fed the I3C-supplemented diet to evaluate whether I3C would reduce cervical dysplasia. Similarly, we compared the K14-HPV16 mice that were fed the control diet to those that were fed...
the supplemented diet to determine whether I3C would prevent cervical-vaginal or epidermal cancer and dysplasia. We evaluated two doses of estradiol: 0.125 and 0.250 mg per 60-day release. After euthanasia, histological exams were performed. Precursor lesions were divided by grade, as follows: hyperplasia (grade I), moderate dysplasia (grade II), severe dysplasia (grade III), and cancer (grade IV). Examples of a normal epithelium and stages II, III, and IV are shown in Fig. 1.

Dietary I3C Prevented Cervical Cancer. At the lower dose of estradiol, which is estimated to be ~15 times the normal dose, based on estrogen replacement therapy (29), cervical-vaginal epithelium was evaluated 24 weeks after estradiol and special diet treatments were started. As shown in Fig. 2, 76% of the transgenic mice fed the control diet developed cancer. The remainder had moderate or severe dysplasia. Only two cancers (8%) were detected in the mice fed the diet supplemented with I3C. The remainder of the mice had hyperplasia (12.5%), moderate dysplasia (41%), or severe dysplasia (37.5%). The reduction in numbers of cancers was significant (P < 0.001). Fig. 3 shows the prevalent cervical-vaginal histologies of transgenic (Fig. 3, A, C, and E) and nontransgenic mice (Fig. 3, B, D, and F) that were untreated (no estrogen/control diet; Fig. 3, A and B) and treated with estrogen (control diet, Fig. 3, C and D; diet with I3C, Fig. 3, E and F). Notably, hyperplasia was present in the transgenic mice that were not given estrogen. Similar results were obtained with the mice at the higher dose of estradiol. At 24 weeks, all transgenic mice had cervical cancer or high-grade dysplasia in the group fed the control diet. No cancer was detected in the group given diet supplemented with I3C. However, because morbidity (see below) occurred at this higher dose of estradiol, only six and eight mice (control and I3C-supplemented diets) were evaluated at 6 months.

Only one cancer occurred in the nontransgenic mice at either dose of estradiol (Fig. 2). I3C significantly reduced dysplasia at the lower dose of estradiol (P < 0.05) and reduced the severity of dysplasia in the group given the higher dose of estradiol.

I3C Reduced Morbidity Associated with High-Dose Estrogen. As shown in Fig. 4, these highly inbred mice, given estradiol at 0.250 mg per 60-day release, had significant morbidity. The morbidity was caused by retention of fluid in the bladder, as determined by autopsy after euthanasia. No morbidity occurred in mice that were not given estradiol (results not shown), and very little occurred at the lower dose of estradiol. In mice given the high-dose estradiol, more morbidity was seen in the K14-HPV16 mice (74%) than in their nontransgenic litter mates (40%). In both groups, the number of mice that became sick was reduced when mice were fed the diet with I3C. Sixty four % of transgenic and 37% of nontransgenic mice developed morbidity.

Cervical Dysplasia and Cervical-Vaginal Cancer Increased with Time. Mice on this higher dose of estradiol were euthanized when morbidity was apparent, as detected by difficulty in walking. The cervical-vaginal preparations were examined histologically. If no morbidity was apparent, mice were sacrificed at 24 weeks after start of diet. A summary of this analysis is shown in Table 1. For both the transgenic mice and the nontransgenic mice, increasing pathology occurred with time of estrogen treatment when mice were fed the normal diet. Higher grades of dysplasia were seen in the K14-HPV16 mice compared to the nontransgenic mice.

Estradiol Increases Proliferation; I3C Reduces Proliferation. Because estradiol increases proliferation of precancerous cervical cells in culture (16), we evaluated tissue sections for cells that were positive for PCNA in representative cervical epithelium at 24 weeks (Fig. 5). Such cells would be in late G1 or the S phase of the cell cycle. In transgenic mice without estrogen (Fig. 5A), many cells in the basal layer and suprabasal cells were positive for PCNA, whereas PCNA was only barely detectable in the basal layer in tissue from nontransgenic mice (Fig. 5B). In the estrogen-treated mice, the majority of cells were PCNA positive in the transgenic mice (Fig. 5C). More basal cells and a few suprabasal cells were PCNA positive in the nontransgenic animals receiving the estrogen (Fig. 5D). Fewer cells were PCNA positive in mice given estrogen together with a diet supplemented with I3C (Fig. 5, E and F). However, PCNA-positive cells were not totally confined to the basal layer in the transgenic mice (Fig. 5E).

I3C Reduces Epidermal Dysplasia in Transgenic Mice. The K14-HPV16 transgenic mice develop skin lesions that are mostly hyperplastic but sometimes papillomatous. Some lesions progress to cancers (28). The ear is the most common site. As shown in Fig. 6, skin from the ear of the transgenic mouse was hyperplastic (Fig. 6B) but not in the nontransgenic mice (Fig. 6A). Dysplasia (Fig. 6C) and

![Morbidity of mice given high dose estrogen](image)

Fig. 4. I3C reduces morbidity associated with high-dose estrogen. K14-HPV16 and normal mice were implanted every 60 days with 17β-estradiol (0.250 mg per 60-day release) and fed control diet or diet supplemented with 2000 ppm I3C. Mice were euthanized if morbidity developed (as detected by difficulty walking) or 24 weeks later. Data points, percentages of euthanized mice, determined every 2 weeks. Each of the four groups had 23–25 mice.

### Table 1 Numbers of mice with hyperplasia, dysplasia, and cancerous cervical epithelium

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*K14-HPV16 and nontransgenic mice were implanted every 60 days with 17β-estradiol (0.250 mg per 60-day release) and fed control diet. Mice were euthanized if morbidity developed (as detected by difficulty walking) or 24 weeks later. Cervical-vaginal tissue was fixed, processed, stained (H&E), and evaluated as normal, hyperplastic (grade I), moderate dysplastic (grade II), severely dysplastic (grade III), or cancerous (grade IV) cervical epithelium.
malignant lesions (Fig. 6D) developed in some transgenic mice. These lesions were not exacerbated by estrogen, as in the case of the cervix (12). We, therefore, asked whether dietary I3C would have some efficacy in the prevention of skin lesions in the transgenic mouse. Tissue from the ear was examined by histopathology and evaluated as described for cervical epithelium in mice treated with the lower dose of estradiol (Table 2). Hyperplasia is characteristic in the transgenic mouse. Evaluation of ear epithelium in transgenic mice ranged from hyperplasia (21%), moderate dysplasia (50%), severe dysplasia (21%), and cancer (4%). The evaluation of ear epithelium of mice on the diet with I3C showed rates of 44, 48, and 8% for hyperplasia, moderate dysplasia, and severe dysplasia, respectively. None had cancer. Although significance was not achieved, I3C appeared to reduce both dysplasia and cancer in these skin lesions.
DISCUSSION

In this study, we confirmed the previous observations (12) that chronically administered estrogen promoted papillomavirus-initiated cervical cancer in the K14-HPV16 transgenic mouse model. Using this model, we determined that dietary I3C prevented cervical cancer. Additionally, I3C reduced other pathology caused by estrogen in both transgenic and nontransgenic mice. Additionally, I3C appeared to reduce dysplasia and cancers in skin.

The extremely effective chemoprevention elicited by I3C for estrogen-induced cervical cancer likely relates, at least in part, to evidence that I3C is an antiestrogen. I3C reduced estrogen toxicity, thereby substantiating that I3C had antiestrogen effects in this study. Acid condensation products of I3C are ligands for the aryl hydrocarbon receptor (30). This interaction is the reason that I3C alters expression of some CYP450 enzymes that regulate estrogen metabolism (31, 32). I3C increases CYP1A1, thereby increasing 2-hydroxyestrone (an antiestrogen) in breast cells (32). In cervical cells, I3C increases expression of CYP1A1 and CYP1A2, resulting in increased 2-hydroxyestrone (14). Additionally, both I3C and 2-hydroxyestrone can compete with estradiol for the estrogen receptor (14). The antiproliferative effects of I3C, as evidenced by our PCNA studies, may reflect the abrogation of the estrogen effect of increasing proliferation. Other possible anticancer effects of I3C are suggested by the observation that I3C inhibits expression of cyclin-dependent kinase 6 and induces a G1 cell cycle arrest (33) or apoptosis (34, 35). The reason I3C appeared to show efficacy as a chemopreventive for skin papillomas and cancers that spontaneously occur in the K14-HPV16 mouse is likely due to activities of I3C not related to its antiestrogen activities, e.g., G1 cell cycle arrest.

Proliferative cells in the cervical epithelium of the K14-HPV16 mouse were numerous compared to the normal mouse. The viral protein E7, expressed in the transgenic mouse, would most likely induce this excessive proliferation. That many suprabasal and spinous cells were also proliferative implies that the K14 promoter is expressed in these more differentiated cells in the transgenic mouse rather than being limited to the basal layer, again possibly mediated by

Table 2  Percentage of transgenic mice with hyperplasia, dysplasia, and cancerous skin epithelium

<table>
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<td>48</td>
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* K14-HPV16 mice were implanted every 60 days with 17β-estradiol (0.125 mg per 60-day release) and fed control diet or diet supplemented with 2000 ppm I3C. Mice were euthanized 24 weeks later. Skin from ear was fixed, processed for histology, stained (H&E) and evaluated for hyperplasia (grade I), moderate dysplasia (grade II), severe dysplasia (grade III), and cancer (grade IV). The number of mice in control diet group was 24, and the number of mice that were fed the I3C-supplemented diet was 23.
the E7 protein. Although I3C dramatically reduced the number of proliferative cells, PCNA-positive cells could still be detected in cells other than those in the basal layer. We consider this study to be a worst-case scenario because the dose of estrogen was abnormally high, whereas the amount of I3C was not. We wanted to determine the effect of I3C under conditions in which most mice developed cancers. We believe I3C would be even more effective (e.g., prevent more dysplasia) if lower doses of estrogen were used. The dosage of dietary I3C used in this study is an amount that could be achieved with a diet heavily enriched with cruciferous vegetables. A person can usually achieve this level by eating one-half of a head of cabbage per day (36, 37). In some parts of the world, e.g., Northern China, people generally consume large amounts of cruciferous vegetables. One caveat is that these vegetables are extremely variable as to their content of indoles. Another caveat is that the breakdown of glucosinolates and condensation of I3C into its active derivatives depends on acids in the stomach (38, 39) that can be neutralized by antacids. However, I3C and its acid condensation product diindolylmethane are available as supplements. The dosage of estrogen differs from what is used clinically. Women on estrogen replacement therapy receive an estrogen dose 15 times less per kg than that of the lower dose of estradiol used in this study (29). Moreover, species and individual differences occur regarding response to estrogen. The transgenic mice used in this study were extensively inbred into the FVB/n background. They were more sensitive to estrogen than mice in a previous study that had only been back-crossed nine times (12). In women, the amount of estrogen changes dramatically during the menstrual cycle. Additionally, levels of estrogen vary between women. Estrogen levels depend on genetics, diet, exercise, and age.

This study indicates that I3C acts as a chemopreventive for cervical-vaginal cancer and other papillomavirus disease. Our results also indicate that I3C would not only prevent cervical cancer but also reduce cervical hyperplasia and dysplasia. In particular, I3C would be useful for women known to be infected with the highly oncogenic HPVs. I3C already shows promise as a therapy and chemopreventive agent for laryngeal papillomatosis, a disease of benign tumors with a papillomavirus etiology (22–24). This study suggests that I3C could also be a useful preventive for skin warts and possibly some skin cancers. The attractiveness of I3C would not only prevent cervical cancer but also reduce cervical vaginal cancer and other papillomavirus disease. Our results also indicate that I3C could be used as a survival factor. Circulation, 95: 1505–1514, 1997.


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