Estrogen Metabolism and the Malignant Potential of Human Papillomavirus Immortalized Keratinocytes (44239)

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Abstract. **Increased 16α-hydroxylation of estradiol has been shown to be associated with heightened cancer risk in estrogen responsive tissue. Certain types of human papillomavirus (HPV) are cofactors for cancer in the cervix, an estrogen sensitive tissue. We have demonstrated that estradiol and 16α-hydroxyestrone increased the number of cells positive for proliferating cell nuclear antigen in HPV immortalized keratinocytes, the *in vitro* correlate of the premalignant keratinocyte. These estrogens caused the abnormal proliferation and anchorage independent growth, which correlates with malignant conversion. Indole-3-carbinol, a phytochemical in cruciferous vegetables known to preferentially induce 2-hydroxylation with minimal effect on 16α-hydroxylation, markedly blocked the ability of estradiol to increase anchorage independent growth. The results indicate that 16α-hydroxyestrone increases the malignant phenotype of HPV immortalized keratinocytes. However, indole-3-carbinol will block this response.** [P.S.E.B.M. 1998, Vol 217]

Estrogen promotes cancer in estrogen responsive tissues. Increased 16α-hydroxylation of estrogen is associated with greater risk for cancer in the cervix (1), breast (2), endometrium (3), and larynx (4). The metabolic pattern of estrogen largely controls the availability of biologically active estrogen, and this metabolism is strongly influenced by phytochemicals. Indoles from cruciferous vegetables (5, 6); certain fatty acids (7), or flavones (8) induce 2-hydroxylation thereby increasing inactive estrogens. In addition, phytoestrogens compete for the estrogen receptor and other estrogen binding proteins (reviewed in Ref. 9).

Estradiol is the primary active estrogen and has a high affinity with the estrogen receptor. Metabolism of estradiol to sulfated estrogens results in compounds that are excreted or stored. Alternately, as shown in Figure 1, estradiol is oxidized to estrone, which may be either hydroxylated at C-2 to give 2-hydroxyestrone or at C-16α to give 16α-hydroxyestrone (16α-OHE1). 2-Hydroxyestrone is a catechol estrogen that is inactive and weakly antiestrogenic (10). Conversely, 16α-OHE1 is fully estrogenic (11) and forms covalent bonds with amino groups (12), including the estrogen receptor (13). 16α-OHE1 has a prolonged activity in an estrogen-receptor-dependent response (14). 16α-OHE1 increases anchorage independent growth (AIG) and induces DNA repair in mammary cells (15).

The human papillomaviruses (HPV) are cofactors for cervical cancer, the focus of this study. Greater than 90% of HPV-related lesions and cancers in the genital tract occur in the most estrogen sensitive cells, the transformation zone of the cervix (16). This is in contrast to nontransforming inapparent infections with HPV. The prevalence of HPV DNA in other parts of the female genitalia or male genitalia is equal or greater than the prevalence of HPV DNA in the transformation zone (17, 18, 19) although significant lesions are primarily in this zone. Cervical cancers take months or years to develop. In a prospective study in Finland, 20% of infections with HPV type 16 in the cervix became precancerous lesions, and 21% of the precancerous lesions became malignant (20). HPVs do not immediately induce cancer. Expression of HPV is essential for initiating transformation, and viral proteins are necessary for maintaining the transformed state (reviewed in Ref. 21). For an HPV-transformed cell to become malignant, the viral activity has to be

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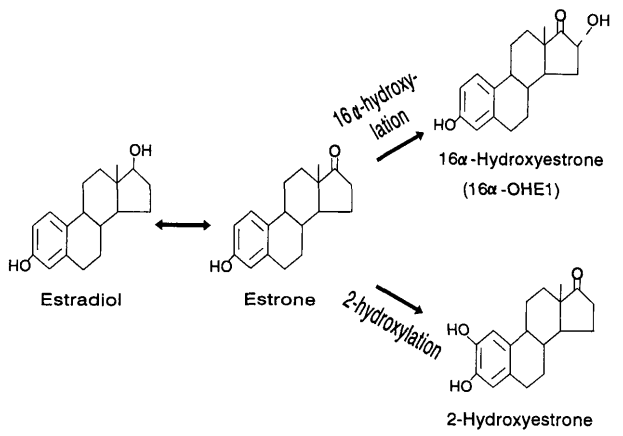


Figure 1. 16α- and 2-Hydroxylation of estradiol.

supported or complemented by additional events, (e.g., DNA damage) (reviewed in Ref. 22).

Estrogen metabolism and the pathology of an HPV infection appear to be interdependent. The transformation zone of the cervix, where HPV lesions usually form, has a high constitutive level of 16α-hydroxylation of estradiol (1), an amount comparable to breast ductal cells (23). On the other hand, virtually no 16α-hydroxylation occurs in male genitalia (1). Active infection with HPV (24) or immortalization with HPV increases 16α-hydroxylation (1). This increase even occurs in immortalized foreskin keratinocytes, cells that do not normally hydroxylate estradiol. However, the level of 16α-hydroxylation in HPV immortalized foreskin cells is still less than in normal cells derived from the transformation zone (1). The implication is that there is a spiraling effect whereby 16α-hydroxylation exacerbates an HPV infection, and HPV increases 16α-hydroxylation.

In this study, we explored the promotional role of 16α-OHE1 on the HPV16 immortalized genital keratinocytes to the malignant phenotype and methods for blocking this response.

Materials and Methods

Cells and Cell Culture. All of the HPV16 cell lines had been previously evaluated for the ability to carry out 2- and 16α-hydroxylation (1). The HPV16 immortalized foreskin cell line used in this study was evaluated for estrogen metabolism before and after the immortalization of cultured foreskin keratinocytes with cloned HPV16 DNA (1). The CX162S are HPV16 immortalized keratinocytes from the cervix (25). While immortalized, neither cell line is malignant. The cell lines were expanded in FAD medium (1:3 mix of Ham's F12 and DME plus 24.3 mg/ml adenine) supplemented with 5% fetal bovine serum, epidermal growth factor, insulin, cholera toxin, and hydrocortisone. Defined medium was keratinocyte basal medium purchased from Clonetics (San Diego, CA) without methyl red indicator and supplemented with epidermal growth factor, insulin, hydrocortisone, transferrin, retinoic acid, and pituitary extract. Charcoal stripped serum was used in selected experiments as indicated. 16α-OHE1 was purchased from Steraloids (Wilton, NH).

Estrogen biotransformation assays. The radiometric procedure to evaluate the conversion of estradiol to 16α-OHE1 has been previously described (26). Briefly, the formation of ^3H_2O from stereospecifically labeled ^3H (C16)-estradiol was determined. Labeled estrogen was added to confluent cell monolayers. After incubation for 24 hr, the sublimed water from the culture medium was counted for radioactivity. Corrections were made for the spontaneous biotransformation in the medium and normalized by protein content of cells using the Pierce (Rockford, IL) modification of the Lowry method. To evaluate water soluble (sulfated) estrogens, 6,7-^3H was used as above. Free and conjugated estrogen were partitioned between chloroform and medium. The percentage of counts remaining in the medium was determined.

Anchorage independent growth. Trypsinized cells were suspended in supplemented FAD medium and 5% charcoal stripped fetal bovine serum with a final concentration of 0.6% agar and plated in 60-mm tissue culture dishes. Cells were fed twice weekly with medium containing 0.3% agar without removing the basal medium. Results were evaluated at 4 and 5 weeks after plating.

Unscheduled DNA synthesis. The method was identical to that used by us for mammary cells (15). Briefly, cells were treated with hydroxyurea and ^3H thymidine for 24 hrs, and incorporation of tritium in a trichloroacetic acid precipitate of cell lysates was determined.

Assays for PCNA. The percentage of cells positive for proliferating cell nuclear antigen (PCNA) was determined as described by us previously (24). Briefly, cells were fixed with 1% paraformaldehyde and treated with antibody to PCNA (Boehringer Mannheim, Indianapolis, IN) followed by fluorescein-labeled anti-rabbit IgG antibody.

Results

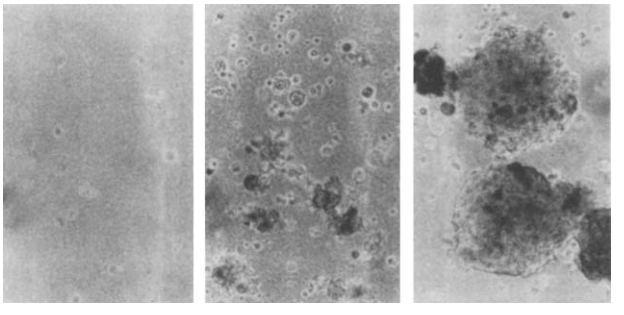
The results show that 16α-OHE1 promotes the continued transformation of HPV immortalized cells to a more malignant phenotype. Hydroxylation of estradiol is increased in cells derived from papilloma lesions (24) and increased in cells immortalized with HPV type 16 (1). High-risk HPV types like 16 are cofactors in cervical cancer. They transform keratinocytes but are not sufficient to fully transform cells into the malignant phenotype (reviewed in Refs. 21, 22). Because 16α-OHE1 is a DNA-damaging agent in mammary cells (15), we hypothesized it would be more pronounced in HPV immortalized cells. The viral oncoprotein E6 expressed by HPV16 degrades p53 protein (27). Therefore, p53 cannot stop cell replication and repair DNA or induce apoptosis in response to DNA damage (reviewed in Ref. 28).

To test this concept, we measured AIG in HPV immortalized cells. AIG is the transformed phenotype that most closely correlates with the malignancy (29). We used the

HPV16 immortalized foreskin keratinocytes because estrogen metabolism is low in these cells compared to HPV16 immortalized cervical keratinocytes (1). As shown in Figure 2 and Table I, AIG occurred in the cells treated with estradiol or 16α-OHE1, but not in the untreated cells. The most AIG occurred with 16α-OHE1 treatment. Since indole-3-carbinol (I3C), a dietary component of cruciferous vegetables induces alternate metabolism, 2-hydroxylation (30), we measured the response to the addition of I3C to AIG in cells treated with estradiol. As shown in Table I, I3C abrogated the ability of estradiol to enhance the AIG of the HPV immortalized cells. From these results, it is clear that 16α-OHE1 causes a more transformed phenotype of HPV immortalized keratinocytes, and 2-hydroxyestrone inhibits this transformation.

Both estradiol and 16α-OHE1 increase proliferation of estrogen sensitive cells including breast and larynx (10, 24). Errors in DNA can be incorporated in replicating cells. The extent of proliferation was measured by PCNA positive nuclei. Addition of estradiol and 16α-OHE1 resulted in increased numbers of proliferating HPV immortalized foreskin and cervical keratinocytes (Fig. 3). However, the increase for the foreskin cells was not significant (Mann-Whitney, one tailed analysis). At least in HPV transformed cervical keratinocytes, estradiol and 16α-OHE1 would contribute to the continued transformation by increasing proliferation with incorporation of DNA errors.

Estradiol can also be metabolized by conjugation to form water soluble sulfates that can be excreted or stored. We determined that defined medium without serum favors sulfation and blocks hydroxylation. Such medium without serum favors formation of water soluble estrogens. 16α-Hydroxylation was prevented with no ^3H at the C16 position being converted to H_2O. The effect was medium dependent since hydroxylation was restored when serum was added to the medium. However, tritiated estradiol became soluble in the defined medium. Greater than 65% of the estradiol became soluble in 24 hrs whereas less than 4% estradiol in the control medium became soluble. This observation is not unique to these genital cells because endometrial cells no longer hydroxylate estradiol in defined medium without serum (43).



Control Estradiol 16α-Hydroxyestrone

Figure 2. Estradiol and 16α-hydroxyestrone increase anchorage independent growth of HPV16 immortalized keratinocytes. HPV16 immortalized foreskin keratinocytes were plated in soft agar (0.6% in supplemented F12/DME, 10% charcoal stripped fetal calf serum) at a density of 10^4 cells/ml. The medium contained no estrogen (control), 10^{-7} estradiol, or 10^{-7} 16α-hydroxyestrone. Cells were maintained as described in methods. Original photography (5 weeks after plating) was at 62×.

Table I. Estradiol and 16α-Hydroxyestrone Increase Anchorage Independent Growth; Indole-3-Carbinol Abrogates Effect of Estradiol

	Starting density	Number of colonies (> 5 cells)			
		Control	Estradiol	16α-OHE1	Estradiol and I3C
Expt 1	10^4	0	20	28	0
Expt 2	10^5	112	281	tntc	40
Expt 3	10^4	2	13	53	8
Expt 4	10^4	5	15	35	10

Note. tntc = too numerous to count
HPV16 immortalized foreskin keratinocytes were plated in soft agar (0.6% in supplemented F12/DME, 10% charcoal stripped fetal calf serum) at a density of 10^4 or 10^5 cells/ml. The medium contained no estrogen (control), 10^{-7} estradiol, 10^{-7} 16α-OHE1, or both 10^{-7} estradiol and I3C. Cells were maintained as described in methods. After 4 weeks, the number of colonies (> 5 cells in a cluster) on a 60-mm dish was determined.

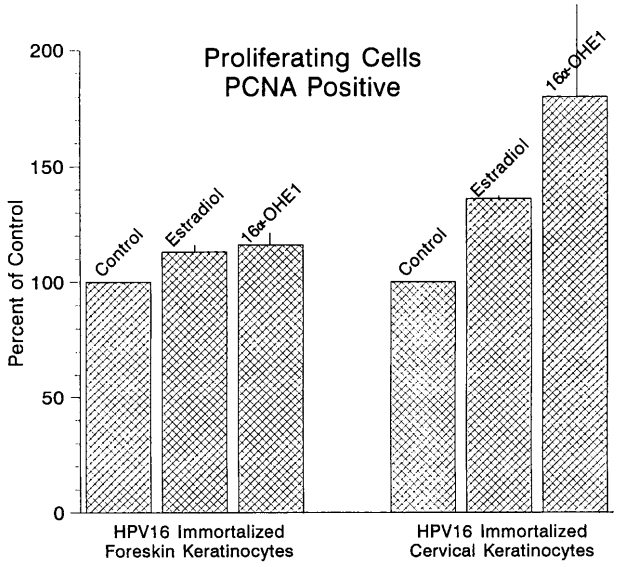


Figure 3. Estradiol and 16α-hydroxyestrone increase proliferation of HPV16 immortalized keratinocytes. HPV16 immortalized foreskin and HPV16 immortalized cervical cells (CX162S) were cultured in defined medium containing no estrogen (control), 10^{-7} *M* estradiol or 10^{-7} *M* 16α-OHE1. Seventy-two hours after the addition of estrogens, the percentage of PCNA positive cells was determined. The percentage positive cells was determined on approximately 1000 cells/assay, and results are from at least three assays (cervical cells) and five assays (foreskin cells). The percentage of positive cells was normalized to control and standard deviations determined.

Discussion

We have shown that 16α-OHE1, a product of estrogen metabolism, enabled keratinocytes immortalized with HPV16 to grow in soft agar, a property of transformed cells that best correlates with the tumorigenic phenotype. A reason for this change in phenotype could be genotoxic damage

induced by 16α-OHE1. Clearly, estradiol and 16α-OHE1 destabilize DNA and cause DNA damage. Estradiol causes aneuploidy (31). 16α-OHE1 forms covalent bonds, nonenzymatically with amino groups (12, 13), a property that should enable this compound to make adducts on DNA. Although genotoxic damage caused by 16α-OHE1 has been validated in breast cells (15), we were unable to show unscheduled DNA synthesis either with estradiol, 16α-OHE1 or the mutagen dimethylbenz(a) anthacene (results not shown). These results are not surprising since p53 is degraded in HPV16 immortalized cells and unable to induce repair of DNA or apoptosis of damaged cells.

Immortalization of keratinocytes by HPV is not sufficient to give cells the property of tumorigenicity. Both *in vivo* and *in vitro,* transformation of keratinocytes occurs in steps. A big range of premalignant and malignant lesions from very mild dysplasias to invasive carcinomas is ascribed to HPV infections in the cervix. Most premalignant lesions do not become malignant (20). *In vitro,* HPVs can immortalize cells. Such cells are not tumorigenic, but several immortalized lines subsequently became tumorigenic after transfection with Harvey *ras* gene (32), infection with Herpes viruses (33, 34) or treatment with mutagens (35, 36). Clinically, DNA damaging agents such as x-rays (37), and ultraviolet light (38) promote the malignant conversion of HPV infections. Typically, HPV sequences from cervical cancers are integrated or have rearrangements (39). Estrogen *via* 16α-hydroxylation enhances DNA damage in estrogen responsive cells, and effects are exacerbated because the virus, in turn, increases 16α-hydroxylation of estradiol.

We have previously determined that although the extent of 16α-OHE1 formation is constitutive and not readily altered, 2-hydroxylation is readily modulated (40). I3C is potent at inducing 2-hydroxylation of estradiol (41). Addition of I3C to the soft agar cultures showed that increasing 2-hydroxylation blocks the ability of estradiol to increase AIG of HPV immortalized cells. In the absence of serum, which is totally nonphysiologic, hydroxylation at 2- or 16α- is diminished, and the major pathway is sulfation. Available estrogen is determined by many factors that are modulated by diet, drugs, and exercise. Aromatation of androgens to yield estrogens and the availability of sex hormone binding globulin are regulated by the degree of obesity. The metabolic pattern is also altered in subjects on vegetarian diets. Phytoestrogens and environmental estrogens have positive or negative effects on available estrogens (reviewed in Ref. 9). HPV disease in estrogen sensitive tissue can be altered by any of these effects.

Increasing 2-hydroxyestrone and decreasing the availability of 16α-OHE1 should not only be beneficial in the prevention of both papillomas (24) but also the malignant conversion. In support of this hypothesis, we recently determined that the ratio of 2- to 16α-metabolites are decreased significantly in women with cervical intraepithelial neoplasia (CIN) and further decreased as the CIN staging increases (42). Additionally, studies in patients with the HPV disease laryngeal papillomatosis have demonstrated that feeding I3C and increasing 2-hydroxylation of estradiol does result in inhibition of the growth of these tumors.

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