Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice

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ABSTRACT High-risk human papillomaviruses (HPVs), including type 16, have been identified as factors in cervical carcinogenesis. However, the presence and expression of the virus per se appear to be insufficient for carcinogenesis. Rather, cofactors most likely are necessary in addition to viral gene expression to initiate neoplasia. One candidate cofactor is prolonged exposure to sex hormones. To examine the possible effects of estrogen on HPV-associated neoplasia, we treated transgenic mice expressing the oncopgenes of HPV16 under control of the human keratin-14 promoter (K14-HPV16 transgenic mice) and nontransgenic control mice with slow-release pellets of 17β-estradiol. Squamous carcinomas developed in a multistage pathway exclusively in the vagina and cervix of K14-HPV16 transgenic mice. Estrogen-induced carcinogenesis was accompanied by an incremental increase in the incidence and distribution of proliferating cells solely within the cervical and vaginal squamous epithelium of K14-HPV16 mice. Expression of the HPV transgenes in untreated transgenic mice was detectable only during estrus; estrogen treatment resulted in transgene expression that was persistent but not further upregulated, remaining at low levels at all stages of carcinogenesis. The data demonstrate a novel mechanism of synergistic cooperation between chronic estrogen exposure and the oncopgenes of HPV16 that coordinates squamous carcinogenesis in the female reproductive tract of K14-HPV16 transgenic mice.

The "high-risk" human papillomavirus (HPV) types, such as HPV16 and -18, are found in 80–90% of invasive cancers of the uterine cervix (1). High-risk HPV types encode two oncoproteins, E7 and E6, which inactivate the function of the retino-blastoma protein and p53 tumor suppressor proteins (2) and which have been shown to immortalize cells in culture. However, in human cervical squamous epithelium, most lesions containing high-risk HPVVs do not progress to in situ or invasive carcinomas (3, 4), implicating either environmental or genetic cofactors in those rare cases where progression occurs. For example, both cigarette smoking and genetic predisposition have been linked to cervical carcinogenesis associated with high-risk HPV (5, 6). Another cofactor that has been repeatedly associated with HPV neoplasia is exposure to estrogen. Pregnancy appears to be a permissive environment for persistent HPV infection (7), while the prolonged use of oral contraceptives, most of which contain estrogen, has been shown to double the risk of HPV neoplasia and malignancy (8).

The precise mechanism by which estrogen or progesterone might contribute to HPV neoplasia is obscure. The 1-kb enhancer/promoter of both HPV16 and -18 has been shown to contain response elements for progesterone and glucocorticoids (9). Further, estrogen itself has been shown to transactivate the viral genome in HPV containing malignant cell lines (10). Thus, a current hypothesis is that estrogen contributes to HPV persistence and subsequent neoplastic progression by increasing viral gene expression. However, other studies suggest that estrogen and the HPV oncopgenes cooperate on other levels to facilitate neoplastic progression. For example, cervical squamous epithelium has been shown to contain estrogen receptors (11) and to respond to chronic estrogen administration with persistent proliferation (12), resulting in benign hyperplasia. These data suggest that estrogen and the HPV oncopgenes may act in concert to induce neoplastic proliferation within the squamous epithelium of the cervix, setting the stage for secondary genetic events leading to carcinogenesis (13). In addition, estrogen has been shown to be a direct carcinogen, an effect apparently linked to a specific pathway of oxidative hormone metabolism (14). Moreover, HPV infection has been shown to markedly increase the formation of these potentially carcinogenic estrogen metabolites (15).

In this study, we investigated whether estrogen and the HPV oncopgenes could cooperate to induce cervical neoplasia in the absence of the confounding variable of direct hormonal transactivation of the viral genome. We asked whether chronic exposure of female keratin 14 (K14)-HPV16 transgenic mice to estrogen could render the reproductive tract squamous epithelium permissive for HPV neoplasia. K14-HPV16 transgenic mice contain within their genomes the entire HPV16 early region. Instead of the endogenous long control region, the viral genes are regulated by 2 kb of the human K14 enhancer/promoter (16). Mice within several transgenic lines develop a progressive epidermal neoplasia, beginning with diffuse hyperplasia, from which foci of dysplasia appear, that become more frequent and advanced with increasing age (17). When backcrossed into an inbred background (FBV/n) susceptible to epidermal malignancy (18), squamous cancers develop from dysplastic lesions (19). Importantly, none of the K14-HPV16 transgenic mice spontaneously develop gynecological pathology when analyzed up to 1.5 years of age, irrespective of the number of pregnancies. In contrast, the present study demonstrates that chronic estrogen exposure specifically induces a multistage neoplastic progression in the squamous epithelium of the cervix and vagina in 100% of transgenic mice treated with the hormone, culminating in invasive cancers in 60% of the treated mice. Expression of the HPV transgene remained unchanged throughout the reproductive tract during neoplastic progression and in cervical and vaginal carcinomas. These data are evidence for a novel synergism between estrogen and the HPV oncopgenes, distinct from transactivation of the viral gene expression, in the squamous epithelium of the female reproductive tract.

MATERIALS AND METHODS

Transgenic Mice. Details of the initial characterization of epidermal neoplasia in the K14-HPV16 transgenic mice are as
were sacrificed after 1, 2, and 3 months of treatment. K14-wt-1 mice treated with 0.72-mg 60-day 17β-estradiol pellets with two high-power 5-min microwave pulses. Subsequently, with 20 ml of ice-cold phosphate-buffered saline (PBS). Immunohistochemistry for paraformaldehyde. The vagina, cervix, and both uterine horns were made from this template using T7 polymerase (Promega). Subsequently, microwave treatment for optimal signal. Slides were immersed in a citrate solution (Biogenex, San Ramon, CA) and treated with RNase A (20 μg/ml; Worthington) for 30 min at 37°C. After repeated washes in 2X SSC/10 mM 2-mercaptoethanol/1 mM EDTA, the sections underwent a high-stringency wash in 0.1X SSC/10 mM 2-mercaptoethanol/1 mM EDTA at 55°C for 2 hr and the repeated washes in 0.5X SSC. Finally, the slides were dehydrated in graded alcohols containing 0.3 M ammonium acetate and air-dried. For autoradiography, slides were dipped in NTB-2 emulsion (Eastman Kodak), air-dried, and exposed for 4 weeks at 4°C. Slides were developed in D19 developer (Kodak), fixed, washed, and lightly counterstained with hematoxylin/cosin.

**Transfections.** HeLa cells were maintained for 72 hr in Dulbecco's modified Eagle's medium without phenol red, supplemented with fetal bovine serum treated with dextran-coated charcoal to remove endogenous estrogens. The cells were seeded at 1 x 10⁶ cells per 60-mm dish and, after attachment, were cotransfected using calcium phosphate precipitation with 0.5 μg of pCMV β-galactosidase and various combinations of the following estrogen expression vectors (20): 5 ng of pHoe, a plasmid encoding the full-length human estrogen receptor cDNA; 50 ng of pTTCO-CAT, a plasmid containing the chloramphenicol acetyltransferase (CAT) cDNA linked to a minimal thymidine kinase promoter; 50 ng of pGL45-CAT, a plasmid containing two tandem estrogen response elements of the vitellogenin gene linked to a minimal thymidine kinase promoter and CAT; and 10 or 100 ng of pK14-CAT, a plasmid containing 2 kb of the human K14 enhancer/promoter linked to CAT (21). Transfected cells were maintained for 48 hr in the absence or presence of 10⁻⁶ or 10⁻⁸ M 17β-estradiol. Cells were harvested and extracts were used to assay for CAT (22) by using a Molecular Imager (Bio-Rad). CAT activity data were normalized for transfection efficiency based on β-galactosidase activity.

**RESULTS**

The study investigating the possible contribution of estrogen to the initiation of neoplastic progression in control and K14-HPV16 transgenic mice consisted of two parts. In the first part, virgin 1-month-old transgenic and negative littermate females (n = 22), from a single transgenic line (K14-wt-1) prone to epidermal carcinogenesis, were treated with 17β-estradiol for 1, 2, and 3 months (Table 1). After 1 month of treatment, there were diffuse changes in the squamous epithelium of the vagina

**Table 1. Summary of squamous epithelial histopathology in the cervix and vagina in K14-HPV16 transgenic mice treated with 17β-estradiol for 1–6 months**

<table>
<thead>
<tr>
<th>Estrogen dose, mg/60 days</th>
<th>Genotype</th>
<th>Duration of treatment, months</th>
<th>Cervicovaginal phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>NTG (10)</td>
<td>1–3</td>
<td>Acanthosis/hyperplasia</td>
</tr>
<tr>
<td></td>
<td>TG (12)</td>
<td>1–3</td>
<td>Low to moderate grade dysplasia</td>
</tr>
<tr>
<td>0.25</td>
<td>NTG (10)</td>
<td>6</td>
<td>Acanthosis/hyperplasia</td>
</tr>
<tr>
<td></td>
<td>TG (11)*</td>
<td>6</td>
<td>Six invasive carcinomas eight high-grade dysplasia</td>
</tr>
</tbody>
</table>

Data presented are from the K14-wt-1 line. NTG, nontransgenic control mice; TG, transgenic mice. Acanthosis is an increase in thickness of all layers of the squamous epithelium with normal differentiation.

*Three deaths prior to autopsy.
and cervix of both transgenic (Fig. 1B) and nontransgenic mice (Fig. 1A), consisting of cornification of the vaginal and cervical squamous epithelium, as well as squamous metaplasia of the columnar epithelium lining glands of the lower uterus (data not shown) (13). The squamous epithelium of the vagina and cervix in transgenic mice was distinctive in that the basal and suprabasal layers contain individual enlarged cells with a marked degree of nuclear atypia and pleiomorphism (Fig. 1B). These abnormalities are hallmarks of cervical dysplasia, which is classified as low to high grade depending both on the degree of the atypia and the extent to which the normally differentiating epithelium is replaced by the less-differentiated basal-like cells. The dysplasia was of intermediate grade in the transgenic mice implanted with estrogen for 2 months (Fig. 1C). At 3 months, moderate to severe dysplasia of the cervix and the vagina was apparent, with some degree of papillomatosis (Fig. 1D). In contrast, the squamous epithelium of nontransgenic control mice increased in thickness, without evidence of dysplasia (data not shown). Unexpectedly, urinary obstruction at the base of the bladder developed late in the third month of treatment, necessitating termination of the study.

In the second part of the study, 22 K14-wt-1 transgenic mice were implanted with pellets releasing a lower dose of 17β-estradiol, 0.25 mg over 60 days, and the treatment was continued for 6 months (Table 1). Additionally, a second K14-HPV16 transgenic line, K14-Eltt1-1, was also treated with this lower dose of 17β-estradiol for 6 months. All of the K14-wt-1 transgenic mice developed high-grade dysplasia and carcinoma in situ (Table 1 and Fig. 1E). Six of 8 K14-wt-1 transgenic mice evidenced invasive well-differentiated squamous carcinomas of either the vagina or cervix (Table 1 and Fig. 1G and H); three cancers were microinvasive (Fig. 1G) and three were frankly invasive (Fig. 1H). In contrast, nontransgenic mice

![Fig. 1. Multistep cervical and vaginal carcinogenesis in K14-HPV16 transgenic mice treated with chronic 17β-estradiol. The squamous epithelium of the cervix and vagina of a nontransgenic mouse after 1 month of 17β-estradiol treatment (0.72-mg 60-day pellet) is hyperplastic but retains a normal pattern of differentiation (4). Similarly treated transgenic mice evidence low grade dysplasia (B). After 2 (C) and 3 (D) months of estrogen treatment, transgenic mice demonstrate a progressive increase in the grade of the dysplasia. Transgenic mice treated for 6 months with 17β-estradiol (0.25-mg 60-day pellet) evidence high-grade dysplasia and carcinoma in situ (E). Microinvasive (G) or frankly invasive (H) squamous cancers developed in six of eight transgenic mice. Nontransgenic mice treated for 6 months evidence a further increase in thickness of the squamous epithelium (F), with a persistently normal differentiation pattern. (Bars: A-F, 25 µm; G, 20 µm; H, 40 µm.)](image-url)
treated with the same estrogen dose for 6 months developed a further increase in the thickness of cervical and vaginal squamous mucosa, but none evidenced dysplasia (Fig. 1F). Treatment of the K14-Eltet1-1 line with 0.25-mg 60-day 17β-estradiol for 6 months also produced similar well differentiated squamous cancers of the cervix and vagina albeit at a lower frequency, two of eight transgenic mice.

Estrogen treatment did not accelerate neoplastic progression or malignant conversion in the epidermis of transgenic mice. Similarly estrogen did not induce proliferation or hyperplasia in the epidermis of nontransgenic mice. The divergent neoplastic effect of estrogen on the reproductive tract compared to the skin was particularly notable in the K14-E1tet1 transgenic mice, in which the epidermis remained hyperplastic, and resistant to progression to either dysplasia or malignancy in the presence of chronic estrogen (ref. 17, L. Coussens, D.H., and J.M.A., unpublished data), while concurrently developing invasive cancers of the cervix. The specific neoplastic effect of estrogen on the cervical and vaginal squamous epithelium of the HPV16 transgenic mice implicates a synergism between the viral oncoproteins and estrogen and suggests that cervical and vaginal carcinogenesis involves a pathway distinct from spontaneous epidermal carcinogenesis.

Since estrogen increases uterine epithelial cell proliferation in vivo (12), we examined expression of PCNA, a marker of cells in late G1 and S phases (23), in the cervix and vagina of transgenic and control mice (Fig. 2). There was an incremental increase in the labeling index within the squamous epithelium of the transgenic mice, correlating with the duration of estrogen treatment (Fig. 2 A and B). By 6 months, proliferating keratinocytes occupied the entire thickness of high-grade dysplastic lesions (Fig. 2C). In contrast, in similarly treated nontransgenic control mice proliferating squamous epithelial cells remained localized to the basal and inner suprabasal layers (Fig. 2D). Thus estrogen induced a striking increase in proliferation solely within the squamous epithelium of transgenic mice.

One mechanism whereby estrogen could induce proliferation of HPV16 oncogene expression. Therefore, we analyzed the mRNA levels of the two principle HPV oncoprobes, E6 and E7, by in situ hybridization (Fig. 3). Biopsies of ear skin from hormone-treated mice were embedded along with the reproductive tracts to serve as internal positive controls. In addition, epidermal squamous cancers from untreated transgenic mice, embedded in separate blocks, were also run concurrently as parallel positive controls. An initial analysis of cervix and vagina of untreated transgenic mice revealed detectable E6/E7 mRNA only during estrus (Fig. 3B), which was not present on day 1 of diestrus (Fig. 3A). In transgenic mice treated with estrogen for 1, 2 and 3 months, transgene expression was consistently detected at levels similar to estrus of untreated mice (Fig. 3 C–E). This level of E6/E7 mRNA was also comparable to transgene expression in hyperplastic epidermal lesions (data not shown). After 6 months of estrogen exposure, the levels of E6/E7 mRNA within both high-grade dysplasias (Fig. 3F) and well-differentiated cervical and vaginal squamous carcinomas (Fig. 3G) remained constant and comparable to expression levels seen transiently during estrus. In contrast, transgene expression during spontaneous epidermal carcinogenesis in untreated K14-HPV16 transgenic mice incrementally increased (data not shown), such that E6/E7 mRNA was abundant in invasive skin cancers (Fig. 3I). Additional controls in this analysis included a section of reproductive tract from a nontransgenic mouse treated with estrogen for 6 months and hybridized with the HPV16 E6/E7 RNA probe, and section of an invasive vaginal cancer in a K14-HPV16 transgenic mouse hybridized with the murine K14 RNA probe. There was a very low nonspecific background signal in the estrogen-treated metaplastic vaginal and cervical squamous epithelium of the nontransgenic negative control (Fig. 3H). In marked contrast to E6/E7 transgene expression, endogenous murine K14 expression was easily detectable within the invasive squamous cancers of the vagina and cervix (Fig. 3J), indicating that the weak signal for E6/E7 expression in the adjacent sections (Fig. 3G) was not due to degraded mRNA.

To further examine the apparent lack of estrogen responsiveness of the human K14 promoter fragment contained within the transgene, a K14-CAT construct was cotransfected along with an estrogen receptor expression vector in HeLa cells. CAT activity did not change when estrogen was added to the medium. In contrast, when an estrogen response element-CAT reporter plasmid containing an estrogen response element was substituted for K14-CAT, there was a 7- to 20-fold increase in CAT activity after hormone addition (data not shown). Finally, in situ hybridization analysis revealed that endogenous murine K14 expression was similar in both control and transgenic mice, regardless of hormone treatment (data not shown and Fig. 3J). These data are further evidence that neither the human enhancer/promoter fragment nor the murine K14 genes are upregulated by estrogen. Collectively, it is apparent that the induction of cervical and vaginal carcino-

![Fig. 2. Proliferation within the squamous epithelium of the cervix and the vagina in estrogen-treated K14-HPV16 transgenic and nontransgenic control mice. Transgenic mice treated with 17β-estradiol for 1 (A), 3 (B), and 6 (C) months evidence an incremental increase in the proportion of nuclei expressing PCNA (19). The hyperplastic cervical and vaginal epithelium of nontransgenic mice treated with estrogen for 6 months (D) demonstrate PCNA expression restricted to the basal and innermost suprabasal layers. (Bar = 20 µm.)](image-url)
with estrogenic stimulation at estrus, is not sufficient to initiate mice (26). However, it is important to recognize that the carcinogenesis in the cervix and vagina of the K14-HPV16 amount and frequency of administered estrogen in these mediated by direct upregulation of transgene expression. Thus chronic estrogen exposure is an essential cofactor required for elaboration of squamous epithelial reproductive tract carcinogenesis by the HPV16 oncogenes. The data suggest that the benign hyperplasia induced by the combination of chronic estrogen and persistent HPV oncogene expression is sufficient to initiate neoplastic development in the cervix and vagina of K14-HPV16 transgenic mice, which frequently progresses to invasive squamous cancers.

There have been other murine models of cervical carcinogenesis. In these earlier studies, cervical cancer was induced by applications of high local concentrations of estrogen or coal tar derivatives directly to the cervix, either alone or in combination (24). Notably, when estrogen alone was administered systemically, invasive malignancies developed in only 12–15% of mice after a minimum of 12–18 months of treatment (25). Thus the short period of carcinogenesis in our model and the 60% incidence of squamous carcinogenesis further attest to the neoplastic synergism of estrogen and the HPV oncoproteins in the reproductive tracts of the K14-HPV16 transgenic mice.

It is notable that the estrogen dose used in the second part of our study, which was associated with cervical carcinogenesis, is comparable to early work documenting induction of cervical carcinogenesis by estrogen (25) and is 30% of the dose used to support the growth of xenografts of breast carcinoma in nude mice (26). However, it is important to recognize that the amount and frequency of administered estrogen in these studies differs significantly from that used clinically. For example, estrogen exposure in women taking birth control pills is intermittent with short withdrawal periods, and the proliferative effects may be modulated by concomitant progesterone formulated within these preparations. Women on hormone replacement therapy receive an estrogen dose that is 30 times less per kg than that administered in these murine models (27).

However, drug doses and their resultant effects vary greatly between species due to different rates of metabolism that may be modulated by concomitant progesterone in combination with estrogen. Drug dose equivalency for accurate determination of equivalent drug doses between species is not straightforward. Notwithstanding these difficulties in comparative pharmacology, this mouse model will permit molecular and genetic analyses to identify and test the functional contributions of genes that may regulate HPV associated carcinogenesis in the female genital epithelium.

Presently, our study suggests a model wherein chronic estrogen exposure and HPV16 cooperate to elicit cervical carcinogenesis. Estrogen treatment induces benign proliferation of squamous epithelial cells in the cervix and vagina, presumably by signaling through the estrogen receptor (29). While estrogen is thought to directly upregulate viral transcription in humans infected by HPV 16 via response elements in the viral long control region (9, 10), our study suggests that proliferating squamous cells in the reproductive tract can modestly increase (or maintain) the levels of HPV oncogene expression in other indirect ways, since the viral control region is missing in the transgenic mice, and we have shown that the endogenous K14 gene is not upregulated by estrogen. The mechanism underlying this indirect enhancement of HPV16 oncogene expression is currently unknown.

Thus, the simplest model consistent with the data is that the HPV oncoproteins transform proliferating cells in estrogen-induced hyperplastic lesions in the genital epithelium through...
their ability to both deregulate the cell cycle and generate genomic instability, evoking carcinogenesis. Sequestration of pRB and its family members by the HPV E7 oncoprotein contributes to loss of cell cycle control (2). Epidermal growth factor receptor signaling can be enhanced by the HPV E5 protein, which increases epidermal growth factor receptor recycling (30). Progression to higher grades of neoplasia and malignancy, presumably secondary to an accumulation of additional somatic mutations, would be facilitated by activation of p53 by the HPV E6 oncoprotein, allowing cells with DNA damage to escape repair or apoptosis (31).

Ongoing cellular proliferation initiated by persistent or repetitive inflammation appears to be a common element of papillomaviral transformation in intact tissues. This hypothesis could explain the association of HPV neoplasia with concurrent genital tract infections (32), and the correlation of recurrent trauma and inflammation with the formation of cancers in transgenic mice expressing BPV (33). In this context, continual estrogen-induced proliferation can be viewed as one mechanism by which the squamous epithelial cells of the cervix and vagina become permissive for neoplastic progression in K14-HPV16 transgenic mice.

In sum, cervical and vaginal squamous epithelial carcinogenesis in these estrogen-treated K14-HPV16 transgenic mice document a heretofore unknown synergism between chronic estrogen exposure and the HPV16 oncogenes. This model can now be used to test the contributions of factors such as estrogen receptor signaling, progesterone/estrogen combinations, polycyclic hydrocarbons, genetic background, and each individual HPV oncogene, to cervical carcinogenesis. Moreover, the ability to reproducibly initiate the development of cervical carcinomas through a series of distinctive histological stages will allow preclinical testing of compounds (and combinations thereof) designed to interfere with the actions of the HPV oncogenes or other critical aspects of the cancer phenotype.

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