A Role for HPV16 E5 in Cervical Carcinogenesis

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Abstract

A subset of the mucosotropic human papillomaviruses (HPV), including HPV16, are etiologic agents for the vast majority of cervical cancers, other anogenital cancers, and a subset of head and neck squamous cell carcinomas. HPV16 encodes three oncogenes: E5, E6, and E7. Although E6 and E7 have been well-studied and clearly shown to be important contributors to these cancers, less is known about E5. In this study, we used E5 transgenic mice to investigate the role of E5 in cervical cancer. When treated for 6 months with estrogen, a cofactor for cervical carcinogenesis, E5 transgenic mice developed more severe neoplastic cervical disease than similarly treated nontransgenic mice, although no frank cancers were detected. In addition, E5 when combined with either E6 or E7 induced more severe neoplastic disease than seen in mice expressing only one viral oncogene. Prolonged treatment of E5 transgenic mice with exogenous estrogen uncovered an ability of E5 to cause frank cancer. These data indicate that E5 acts as an oncogene in the reproductive tracts of female mice. Cancer Res; 70(7); 2924–31. ©2010 AACR.

Introduction

Human papillomavirus (HPV) 16 E5 is an 83-amino acid hydrophobic membrane–associated protein that localizes to the endoplasmic reticulum (1, 2). Studies with bovine papillomavirus initially identified E5 as a potent oncogene (3). HPV16 E5 was subsequently shown to transform murine fibroblasts and keratinocytes in tissue culture (4–6), enhance the immortalization potential of HPV16 E6 and E7 (7), and cooperate with HPV16 E7 to stimulate the proliferation of human and mouse primary cells (8, 9). HPV16 E5 could activate the epidermal growth factor receptor (EGFR) in a ligand-dependent manner (6, 10, 11) and associates with the 16 kDa subunit of the vacuolar ATPase. This latter interaction could block the acidification of endosomes, which is necessary for the degradation of cell surface receptors including EGFR, leading to increased recycling of these receptors back to the cell surface. This, in turn, could explain the ability of E5 to increase EGFR signaling (6, 12). Other activities that could contribute to the oncogenic abilities of E5 include the inhibition of apoptosis (13–15), inhibition of gap junction–mediated cell-cell communication (16), down-regulation of surface expression of MHC class I and II molecules (17, 18), interaction with EVER1/2 (19), and reduced transcription of p21 (20).

To examine the oncogenic properties of E5 in vivo, K14E5 transgenic mice were created in which expression of E5 was directed to the basal layer of the stratified squamous epithelia. These mice display epidermal hyperplasia, aberrant differentiation of the epithelium, and are susceptible to spontaneous skin tumors (21). E5 was shown to contribute to the promotion and progression stages in skin carcinogenesis similar to what was seen previously in E6 transgenic mice (22, 23). In contrast, E7 was found to only contribute to the promotion stage of skin carcinogenesis (23). Thus, in skin carcinogenesis, E5 and E6 are the more potent oncogenes. In contrast to the skin, we found that E7 is the more potent oncogene in cervical cancers compared with E6, using transgenic mice (24). Specifically, after 6 months of treatment with exogenous estrogen, a cofactor in cervical carcinogenesis, E7 transgenic mice developed cancers throughout the reproductive tract whereas E6 transgenic mice did not. E6E7 double transgenic mice developed larger cancers, demonstrating a subtle role for E6 in cervical carcinogenesis (24). E6 mice do develop cancers when treated with 9 months of exogenous estrogen (25).

In this current study, we investigated whether E5 alone or in combination with the other HPV16 oncogenes, E6 and E7, contributes to the development of cervical cancer. Groups of HPV16 transgenic mice expressing one or more of the three viral oncoproteins in stratified epithelia or control, nontransgenic mice, were treated for 6 or 9 months with exogenous estrogen and the reproductive tracts were analyzed for the presence of disease and cancer formation. Expression of E5 led to more severe neoplastic disease of the cervix compared with that observed in nontransgenic mice, although frank cancers only arose after the longer treatment with estrogen. This finding is similar to what was observed with mice...
Expressing E6, E5, when expressed together with E6 or E7, led to greater tumor burden than seen with any oncogene alone; however, the contributions of E5 were lost when all three oncogenes were expressed. Together, these findings indicate that E5 could contribute to cervical carcinogenesis alone or cooperatively with one other viral oncogene, and that its potency is similar to that of E6. However, it remains unclear whether its oncogenic potency is manifest in the presence of all three oncogenes.

Materials and Methods

**Mouse lines and estrogen treatment.** The K14E5 (line 32; ref. 21), K14E6 (line 5737; ref. 26), and K14E7 (line 2304; ref. 27) transgenic mouse strains carrying the HPV16 E5, E6, or E7 oncogenes, respectively, under the control of the human keratin 14 (K14) promoter were maintained on an inbred FVB/N genetic background. These lines were crossed to each other or to nontransgenic FVB/N mice to generate mice hemizygous for none, one, two, or all three HPV16 transgenes. Mice were genotyped by PCR as previously described (21, 26, 27). To monitor for cervical carcinogenesis, 5-wk-old virgin female transgenic or nontransgenic FVB/N mice were either treated or not treated with 17β-estradiol (0.05 mg, 60-d release pellets) for a period of 6 or 9 mo. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care–approved McArdle Laboratory Animal Care Facility in accordance with an institutionally approved animal protocol.

**Analysis of reproductive tracts.** The reproductive tracts of estrogen-treated or nontreated female mice were harvested after 6 or 9 mo of estrogen treatment and analyzed as described previously (24). Briefly, tissues were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and every 10th 5-μm section stained with H&E and histologically examined for tumors and/or dysplastic disease with the worst grade of lesion scored as the final diagnosis. Frank cancers were measured at the largest cross-sectional area using the Zeiss Axiovision (version 3.1) program (Zeiss). Any cancer with a cross-sectional area of >0.5 mm² was classified as a large invasive cancer (LIC). All other cancers were classified as microinvasive cancers (MIC).

**Statistical analysis.** Fisher’s exact test was used to determine the significance in tumor incidence. Two-sided Wilcoxon rank-sum test was used to determine the significance of all other data. Statistical analysis was carried out using the Mstat program (28).

**Immunohistochemistry.** Histologic sections were deparaffinized in xylene, rehydrated in a series of alcohols, boiled in 10 mmol/L of citrate buffer for 17 min to unmask antigens, blocked in 10% horse serum in PBS for 1 h, then incubated overnight at 4°C with primary antibody specific for either bromodeoxyuridine (BrdUrd; Ab-2; Calbiochem) or p-ERK1/2 (Cell Signaling), each diluted 1:100 in block. A universally biotinylated secondary antibody was applied for 30 min (Vectorstain Universal Secondary), washed in PBS, and incubated in ABC (Vectorstain, Vector Labs) reagent for 30 min. Sections were developed with 3,3′-diaminobenzidine reagent for appropriate times, counterstained with hematoxylin, dehydrated in a series of alcohols, and coverslipped.

Results

**HPV16 E5 acts as an oncogene in the cervix.** HPV16 E7 alone has been shown to induce cervical cancer in mice treated for 6 months with physiologic levels of exogenous estrogen (17β-estradiol) which is sufficient to induce continuous estrus (24). In contrast, in mice treated for 6 months, HPV16 E6 was unable to induce frank cancer, but was able to contribute to the severity of disease by increasing the incidence of LIC in E6E7 double transgenic mice compared with E7 mice alone (24). To determine what role HPV16 E5 may have in cervical carcinogenesis, female K14E5 transgenic mice were treated for 6 months with the same dose of 17β-estradiol used in prior studies of K14E6 and K14E7 mice, and reproductive tracts were harvested, embedded, and sectioned throughout. Every 10th section was stained with H&E and scored for the worst stage of neoplastic disease, ranging from hyperplasia to dysplasia, to frank cancer (either microinvasive (MIC), or large invasive (LIC) cancers, the latter defined as having a cross-sectional area of >0.5 mm²) present within the lower reproductive tract (Table 1; Supplementary Table S1). As was the case for nontransgenic as well as E6 transgenic mice, none of the E5 transgenic mice (n = 15) developed frank cancer of the cervix, cervico-vaginal junction, or the vagina (Table 1). Interestingly, 3 of 19 E5E6 double transgenic mice did develop cancers (Table 1) and those arose within the cervix or cervico-vaginal junction, but this low incidence was not statistically significant when compared with nontransgenic mice (P = 0.24).

As seen in women, cervical cancers arise in mice as a result of a progressive neoplastic disease characterized by the onset of benign lesions (CIN1–3) within the cervical epithelia that become progressively less differentiated and more dysplastic, ultimately leading to the development of microinvasive, then large invasive carcinomas. To more thoroughly assess the influence of viral oncogenes on the complete range of the progressive disease that arises within the HPV16 transgenic mice, we performed a two-sided Wilcoxon rank sum test in which each mouse was ranked by the worst stage of disease present in their cervix (i.e., hyperplasia was given a score of 1; CIN1, 2; CIN2, 3; CIN3, 4; MIC, 5; and LIC, 6) as reported in Table 1. Using this test, we found that E5 significantly increased the severity of cervical disease over that observed in the nontransgenic mice (P = 0.002). The progressive disease was more severe in E5 mice than in E6 mice (P = 0.05), and E5 contributed strongly to the increased severity of disease in E5E6 double transgenic mice (E6 versus E5E6, P = 0.0006). A similar pattern of increased dysplastic disease in E5 transgenic mice was also observed in the vaginal epithelium (Supplementary Table S1). Based on these observations, we conclude that HPV16 E5 contributes to the development of neoplasia in the lower reproductive tract to a degree that is similar if not greater than that of HPV16 E6.

**E5 cooperates with E7 to cause cervical cancer.** E7 has been previously shown to be a very potent oncogene in the
reproductive tracts of female mice (24). In the present study, the incidence of cervical cancer in E7 transgenic mice treated with estrogen for 6 months was 56%, consistent with prior observations (Table 1). E5E6 double transgenic mice were generated to determine if E5 could cooperate with E7 in inducing cervical cancers. We found a marginal (P = 0.13) increase in the incidence of cervical cancer in E5E7 mice (85%) compared with E7 mice (56%). Tumor multiplicities and tumor sizes were also compared between groups of transgenic mice. The mean number of tumors in E5E7 (4.4) double transgenic mice was significantly higher than in E7 (2.0) mice alone (P = 0.04; Fig. 1; Table 1) and was similar to that of E6E7 double transgenic mice (4.2). There was also a significant increase in the size of tumors between E5E7 (0.17 mm²) double transgenic mice and E7 (0.12 mm²) transgenic mice (P = 0.01; Fig. 1; Table 1). To analyze whether E5 was somehow altering the levels of E7 protein thereby indirectly contributing to increased oncogenesis, we monitored levels of expression of MCM7, a gene induced in its expression via E7’s inactivation of pRB and consequent activation of E2F transcription factors. E7 alone led to the potent induction of MCM7 throughout the cervical epithelium (Supplementary Fig. S1), consistent with our prior studies (29). The levels of induction of MCM7 in the cervical epithelium of E5E6 and E6E7 double transgenic mice and E5E6E7 triple transgenic mice was indistinguishable from that observed in the E7 singly transgenic mice (Supplementary Fig. S1). Thus, E5 does not alter the activity levels of E7 protein in the cervix. In sum, these data show that E5 and E7, when expressed together, lead to increased tumor multiplicity and tumor size. This is similar to what is seen between E6 and E7 (ref. 25; Table 1).

We were also interested in learning if there was a further augmentation of carcinogenesis in E5E6E7 triply transgenic mice compared with double transgenic mice. We saw no difference in the cancer incidence between E6E7 mice and E5E6E7 mice. Interestingly, in the context of analyses of the E5E6E7 triple transgenic mice, it seemed that E5 was inhibitory to cancer growth because E5E6E7 tumors were on average smaller than E6E7 tumors (Table 1). However, this difference in average tumor size reflected the fact that there were several very large outlier tumors among those arising in the E6E7 mice (Fig. 1). Indeed the tumor size between the two groups (E6E7 versus E5E6E7) of mice was not statistically significant (P = 0.64). The fact that there was little effect of E5 on the frequency or growth rate of cancers in the presence of both E6 and E7 might explain why, in spite of its capacity in mice to cause cervical cancer when expressed alone or in combination with E6 or E7 (above data), it seems to be dispensable in a significant fraction of human cervical cancers in which both E6 and E7 are always found to be coexpressed.

**E5 alone is sufficient to induce cervical cancer with longer estrogen treatment.** As indicated in Table 1, neither E5 nor E6 alone was sufficient to induce frank cancer when these mice were treated for 6 months with exogenous estrogen, although both contributed to more severe overall disease, and both synergized with E7 to cause cancers in this time period. With longer estrogen treatment (9 months),
E6 alone has been shown to be sufficient to induce frank cancer (25). We therefore investigated whether E5 transgenic mice, when treated for 9 months with exogenous estrogen, developed frank cancer. There were statistically significant increases in cervical cancer incidence ($P = 0.04$) and tumor multiplicities ($P = 0.03$) in E5 compared with nontransgenic mice treated for 9 months with $17\beta$-estradiol (Table 2). Indeed, the incidence of cancer and cancer multiplicities were similar for the E5 and E6 mice (Table 2; Fig. 2). There was no difference in tumor size between E5 and nontransgenic mice whereas E6 transgenic mice developed larger cancers than either E5 or nontransgenic mice (Table 2, Fig. 2). Prior analyses indicated that the ability of E6 alone to induce cancers largely correlates with its capacity to bind to a subset of cellular targets with leucine-rich motifs that includes the ubiquitin ligase E6AP, required for E6's destabilization of p53 (25). Not surprisingly, both the E5 and E6 mice treated for 9 months showed significant increases ($P < 10^{-6}$ and $P < 10^{-6}$, respectively) in the overall severity of cervical disease compared with like-treated nontransgenic mice. These results in the 9-month–treated mice again show a similarity between E5 and E6 in terms of their oncogenic potency in the cervix.

**Effects of E5 on cell cycle progression in the cervical epithelium.** The papillomavirus life cycle is intricately tied to the differentiation program of the host's stratified epithelium.

**Table 2.** Cervical histopathology of mice treated with exogenous estrogen for 9 mo

<table>
<thead>
<tr>
<th>Genotype (total no. of mice)</th>
<th>Grade of disease*</th>
<th>LRT cancer (%)</th>
<th>Average no. of LRT cancers/mouse</th>
<th>Average LRT cancer size (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>CIN1</td>
<td>CIN2</td>
<td>CIN3</td>
</tr>
<tr>
<td>NTG ($n = 23$)$^\dagger$</td>
<td>17</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>E6 ($n = 39$)$^\ddagger$</td>
<td>3</td>
<td>9</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>E5 ($n = 20$)$^\ddagger$</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

*Indicated in each column labeled “H” to “LIC” is the number of mice for which the indicated stage of disease was the worst disease state found throughout the cervix.

$^\dagger$Severity of disease: E5 vs. NTG ($P < 10^{-5}$), two-sided Wilcoxon rank sum test.

$^\ddagger$Severity of disease: E6 vs. NTG ($P < 10^{-5}$), two-sided Wilcoxon rank sum test.

$^5$Percentage of cancers: E5 vs. NTG ($P = 0.04$), one-sided Fisher’s exact test.

$^\ddagger$Average number of cancers: E5 vs. NTG ($P = 0.03$), two-sided Wilcoxon rank sum test.
Progeny virus is produced in the suprabasal compartment wherein the virus reprograms terminally differentiated cells to support DNA synthesis (30). E5 and E7 have been shown previously to synergize in driving unscheduled DNA synthesis in the suprabasal compartment of human keratinocytes in organotypic tissue cultures (31, 32). To determine whether E5 and E7 act together to induce suprabasal DNA synthesis in vivo, we monitored the presence of cells supporting DNA synthesis within the cervical epithelium of the different groups of mice used in this study. Mice were injected with BrdUrd 1 hour prior to sacrifice and histologic sections subjected to BrdUrd-specific immunohistochemistry. In the basal compartment, E7 suppresses DNA synthesis in the cervical epithelium (33). This suppression was observed again in our hands (Fig. 3). E5 also had a similar effect (Fig. 3). In the suprabasal compartment, however, E5 in combination with E7 increased the percentage of suprabasal cells supporting DNA synthesis over that seen in E7 alone mice, and this increase was statistically significant \((P = 0.02, \text{E7 versus E5E7}; \text{Fig. 3})\). The rather subtle, yet statistically significant effect of E5 in augmenting the level of suprabasal DNA synthesis in the mouse cervical epithelium is similar to what we observed in our prior studies on the role of E5 in the viral life cycle (31). These data corroborate the prior studies in tissue culture demonstrating a synergy between E5 and E7 in driving unscheduled DNA synthesis.

**E5 activates the mitogen-activated protein kinase pathway in mice treated for 9 months with estrogen.** HPV16 E5 has been shown to enhance ligand-dependent phosphorylation and activation of the EGFR, suggesting that E5 stimulates mitogenic stimulation through the EGFR. To determine if mitogenic signaling is activated in the cervical epithelium of mice, reproductive tracts of transgenic mice treated with estrogen for 9 months were stained for the presence of phosphorylated ERK1/2. Phosphorylated ERK1/2 was not detected in cervical epithelium from nontransgenic and E6 transgenic mice (Fig. 4). In contrast, nuclear phosphorylated ERK1/2 staining was easily detected in the cervical epithelium of E5 transgenic mice treated for 9 months (Fig. 4). This same pattern of positive staining was observed in tumors from E5 transgenic mice treated for 9 months but not in tumors from the nontransgenic or E6 mice (Fig. 4). These data indicate that E5 causes an increased steady state level of activated ERK1/2 and is consistent with E5 being able to activate EGFR signaling. However, this observation was limited to the mice treated for 9 months; E5 did not cause a detectable increase in phosphorylated ERK1/2 in mice treated for only 6 months (data not shown). This finding is consistent with our prior studies in the skin of young E5 transgenic mice wherein we could not detect increased EGFR activity (21). The later age at which phosphorylated ERK1/2 could be selectively detected in the E5 transgenic tissue could point either to increased levels/activity of E5 in these older mice, or the loss of some negative regulator of mitogen-activated protein kinase activity in the cervix of older female mice.

**Discussion**

In this study, we provide evidence that HPV16 E5 could contribute to cervical carcinogenesis. In estrogen-treated mice, E5 was able to induce cervical cancers on its own, and synergize with E6 or E7 to induce more severe cervical
disease. The capacity to cooperate with E6 or E7 in cervical carcinogenesis correlates with its previously demonstrated cooperation with E6 or E7 in the immortalization of human keratinocytes (7), and is consistent with each of these HPV oncogenes contributing to cervical carcinogenesis.

Like E6, E5 was able to induce cancer in mouse reproductive tracts but only after 9 months of treatment with estrogen; whereas, E7 could induce tumor formation at a high frequency with just 6 months of treatment. E5 transgenic mice did develop more severe disease compared with E6 transgenic mice. Nevertheless, E7 remains the most potent HPV oncogene in the context of cervical carcinogenesis. This potency contrasts with what was previously observed in the context of skin carcinogenesis, in which E5 and E6 are the more potent oncogenes (22, 23). In the skin, E5 and E6 both contribute to the promotion and malignant progression stages of carcinogenesis, whereas E7 only contributes to promotion. The underlying reason for the differences in the potency of these oncogenes in these two stratified epithelial tissues remains elusive.

HPV16 E5 has previously been implicated in the productive stage of the viral life cycle, wherein it contributes to inducing unscheduled DNA synthesis in the normally quiescent suprabasal compartment of stratified epithelia (31). This induction of unscheduled DNA synthesis is thought to allow the vegetative amplification of the viral genome, synthesis of which relies on the cellular DNA replication machinery. In organotypic cultures of human keratinocytes harboring the HPV16 genome, E5 and E7, when expressed together, act synergistically to induce unscheduled DNA synthesis in the suprabasal compartment. It was therefore not surprising to find the same to be true in the context of the cervical epithelia of E5E7 double transgenic mice. It remains unclear, however, whether this capacity of E5 to alter suprabasal cells in combination with E7 relates to its carcinogenic properties.

Our studies also provide new insights into the possible mechanism(s) of action by which E5 contributes to cervical carcinogenesis. That we could see increased levels of phosphorylated ERK in the epithelia and cancers arising in the E5 transgenic mice treated for 9 months with estrogen is consistent with E5 being able to increase the activity of EGFR. However, the absence of such an effect at earlier time points raises the question of whether EGFR activation is sufficient or necessary for E5-mediated oncogenesis, at least at earlier time points. One study suggests that E5 is able to activate ERK1/2 in an EGFR-independent manner, leaving open the possibility that E5 may be able to activate ERK1/2 independent of EGFR activation (34). The ability of E5 to activate

**Figure 3.** Characterization of the proliferative index in cervical epithelia. A, representative pictures of BrdUrd-specific immunohistochemical staining of cervical epithelium from nontransgenic (1), E5 (2), E7 (3), and E5E7 (4) mice. B, quantification of BrdUrd-labeling index of cells within distinct layers of cervical epithelia. The average percentage of basal (yellow) and suprabasal (red) BrdUrd-positive cells was obtained from 10 microscope fields (×40) per mouse. An average of at least three mice per genotype was used to calculate the percentage. The difference in percentages of suprabasal DNA synthesis between E5E7 and E7 cervical epithelium was statistically significant (P = 0.02, two-sided Wilcoxon rank sum test).
ERK1/2 at 9 months, but not at 6 months, might also be due to physiologic differences reflective of the age of the mice. We are currently pursuing efforts to investigate whether EGFR is required for E5-induced phenotypes in our mice and if it is required for E5-induced carcinogenesis.

Mice transgenic for the HPV16 oncogene E5 have increased dysplastic disease in the cervical epithelium and, when treated with estrogen for 9 months, develop frank cancer. In addition, E5 cooperated with E7 to increase tumor multiplicity and size. This data shows that E5 may be important in human cancers and should be looked at more closely. Previous work showing that E5 is involved in the productive stage of the viral life cycle (31), increased suprabasal DNA synthesis in E5 transgenic mice (21), and the ability to stimulate EGF-dependent proliferation in human keratinocytes (6, 8, 9) suggests that E5 might play a role in expanding infected keratinocytes. Although we are not able to show whether EGFR is required for these activities, we did show that E5 activates the mitogen-activated protein kinase pathway consistent with E5 enhancing ligand-dependent EGFR activation. Forty percent of human cancers do not express E5 protein, which correlates with the integration of the HPV genome into the host genome (35). In our studies, there was no appreciable effect of E5 on overall disease severity in mice that expressed both E6 and E7. This raises the possibility that E5 plays a minimal role in cervical carcinogenesis in the context of a natural infection in which both E6 and E7 are expressed. Alternatively, it could indicate that the role of E5 is limited to a subset of human cervical cancers, perhaps a reflection of genetic differences among the human population. Regardless, the data presented in this article shows that E5 could increase the dysplastic environment and aid in inducing DNA synthesis, both of which could expand the population of infected cells within a patient.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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