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Human Papillomavirus Types 16 E6 and E7 Contribute Differently to Carcinogenesis

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High-risk human papillomaviruses (HPVs) are etiologically implicated in human cervical cancer. Two viral genes, E6 and E7, are commonly found expressed in these cancer cells. We have previously shown that mice transgenic for the HPV-16 E6 gene or E7 gene, in which the E6 or E7 was expressed in the basal layer of epithelia, developed skin tumors. The spectrum of tumors derived from E6 and E7 mice differed, however; although most tumors derived from the E7-transgenic mice were benign, the majority of the tumors from the E6-transgenic mice were malignant. These findings led us to hypothesize that E6 and E7 play different roles in carcinogenesis. To assess at what stages in carcinogenesis E6 and E7 act, we treated the skin of K14E6- and K14E7-transgenic mice with chemical carcinogens known to contribute to distinct stages in carcinogenesis. Both E6 and E7 were found to synergize with chemical carcinogens in causing tumor formation. E6 was found to act weakly at the promotion stage of carcinogenesis in the formation of benign tumors but strongly at the progression stage which involves the malignant conversion of benign tumors. In contrast, E7 primarily affected the promotion stage of carcinogenesis. These results provide direct evidence that E6 and E7 contribute differently to carcinogenesis; E7 promotes the formation of benign tumors, and E6 acts primarily to accelerate progression of these benign tumors to the malignant stage. Consistent with this model, we found E6 and E7 to cooperate in inducing tumor formation in mice expressing both oncogenes.

INTRODUCTION

Human papillomaviruses (HPVs) are small DNA tumor viruses that cause benign tumors or warts in human skin. A subset of anogenital HPVs, the high-risk HPVs, are also associated with malignant tumors, including the majority of cervical cancers (zur Hausen, 1996). HPV-associated cervical carcinogenesis is a multistep process, in which infected cells develop into squamous intraepithelial lesions (SIL) (Wright *et al.,* 1994). In these lesions, cells become atypical and mitotically active and lose their ability to terminally differentiate. In low-grade SIL stage, these abnormal cells are limited to the lower portion of the epithelia. Low-grade SIL associated with high-risk HPVs can progress to high-grade SIL, in which abnormal cells are evident throughout the entire thickness of the epithelia, and eventually to cervical carcinoma. During this multistep process, high-risk HPV genomes commonly integrate into cellular chromosomes (Baker *et al.,* 1987; Choo *et al.,* 1987; Cooper *et al.,* 1992; Daniel *et al.,* 1997), and two early genes, E6 and E7, become selectively overexpressed. E6 and E7 genes can transform cells in culture, and their transforming ability is due, at least in part, to their ability to interact with cellular tumor suppressor proteins. E6 binds the tumor suppressor p53 through its interaction with another cellular protein, E6 associated protein (E6-AP), leading to the degradation of p53 via the ubiquitin-mediated protein degradation pathway (Huibregtse *et al.,* 1991; Scheffner *et al.,* 1993, 1990; Werness *et al.,* 1990). The inactivation of p53 appears to be required but may not be sufficient for E6 to transform most cell types, because the loss of p53 function does not transform or immortalize cells as efficiently as does E6 (Dalal *et al.,* 1996; Nakagawa *et al.,* 1995). Recently, several other cellular factors have been found to associate with E6 protein; these cellular proteins include E6 binding protein (Chen *et al.,* 1995), paxillin (Tong and Howley, 1997), the human homolog of *Drosophila* discs large tumor suppressor protein (Kiyono *et al.,* 1997; Lee *et al.,* 1997), and the newly identified protein E6 target protein 1, a putative GAP protein (Gao *et al.,* 1999). E6's association with these proteins may contribute to E6's transforming ability. E7 binds and degrades the retinoblastoma tumor susceptibility gene product (pRb) (Dyson *et al.,* 1989; Jones *et al.,* 1997). E7 also binds the other members of the pRb "pocket" protein family: p107 and p130 (Dyson *et al.,* 1992). The binding of E7 to the pocket proteins leads to deregulation of the G_1 to S transition in the cell cycle. Mutations in the conserved region (CR) 2 domain of E7 that mediates its binding to pRb abrogate E7's ability to transform cells, indicating the likely importance of pRb's inactivation in E7's transforming activity (Watanabe *et al.,* 1990). Recently, E7 was also demonstrated to bind to the cyclin/CDk inhibitor p21 (Jones *et al.,* 1997) and p27 (Zerfass-Thome *et al.,* 1996) and to associate directly with cyclins

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TABLE 1

Histological Characterization of Skin Tumors Derived from K14E6 and K14E7 Transgenic Mice

Transgene	No. of tumors examined	No. of benign tumors	Carcinoma grade I	Carcinoma grade II	Carcinoma grade III	Malignancy[c]
K14E6[a]	24	6	5	11	2	75%
K14E7[b]	9	8	1	0	0	11%

[a] Data taken from ref. 31; the tumors were scored between 6 and 15 months of age.

[b] Four were papillomas, three were benign sebaceous adenomas, one was a keratoacanthoma, and one was a papilloma with foci of probable early malignant transformation. The tumors were scored between 6 and 15 months of age.

[c] The percentage of malignancy between the two groups was significantly different ($P < 0.01$).

A and E, or to increase the expression of these cyclins, leading to deregulation of the cell cycle (Dyson *et al.,* 1992; McIntyre *et al.,* 1996; Zerfass *et al.,* 1995). Taken together, it appears that E7 primarily associates with cell cycle-regulating proteins that control cell cycle progression at physiological conditions, whereas E6 primarily associates with proteins important for genome stability or associated with signal transduction pathways that involve cell–cell interactions and contribute to the maintenance of cellular morphology.

The oncogenic activities of E6 and E7 have been studied *in vivo* through the generation and characterization of E6- and E7-transgenic mice. When expressed in various tissues by different transcriptional promoters, E6 and E7 together can induce various types of tumors, depending on the type of tissue expressing these two genes (Arbeit *et al.,* 1993; Greenhalgh *et al.,* 1994; Griep *et al.,* 1993). To define the roles of E6 and E7 individually in carcinogenesis, workers at our laboratory generated E6 (K14E6)- or E7 (K14E7)-transgenic mice in which each gene was directed in its expression to basal epithelial cells of the epidermis using the human keratin 14 (hK14) transcriptional promoter. E6 and E7 each could induce skin hyperplasia and skin tumors (Herber *et al.,* 1996). However, the spectrum of tumors derived from E6- and E7-transgenic mice appeared to differed. E6 primarily induced malignant skin tumors, whereas E7 primarily induced benign skin tumors. Based on these observations, we hypothesized that E6 and E7 play distinct roles in HPV carcinogenesis. To determine how E6 and E7 contribute to HPV-induced carcinogenesis, we studied E6 and E7 in the context of a model for multistage skin carcinogenesis, in which the carcinogenic process is divided into three stages: initiation, promotion, and progression (see review in Boutwell, 1989). Chemical carcinogens and oncogenes preferentially affect specific stages. For instance, the *ras* mutation is considered an initiating event in carcinogenesis (Brown *et al.,* 1986). To assess the stages in the carcinogenesis in which E6 and E7 act, skin tumor induction experiments were carried out on the K14E6 and K14E7 mice with chemical carcinogens known to act at distinct stages in carcinogenesis. E6 was found to increase malignant progression of chemically induced skin papillomas, whereas E7 only promoted the formation of papillomas. This result supports our hypothesis that E6 and E7 act differently at the multiple stages in carcinogenesis. Consistent with these findings, E6 and E7 were found to act cooperatively to induce the development of cancer.

RESULTS

Skin tumors derived from K14E6- and K14E7-transgenic mice differ in grade of malignancy

We reported earlier that E6 and E7 induce skin tumors in K14E6- and K14E7-transgenic mice, respectively, when these genes were expressed individually in the undifferentiated epithelial cells of the epidermis (Herber *et al.,* 1996; Song *et al.,* 1999). E6 and E7 induced different types of tumors. Grossly, the tumors arising in the K14E6 mice were mostly open and invasive, indicative of malignancy, and arose with an average age of onset of 12 months; whereas tumors arising in K14E7 mice were mostly self-contained and indicative of benign papillomas and arose with an average age of onset of 10.4 months. Histopathological analysis was performed to compare the degree of malignancy of tumors from both groups of mice (Table 1). The skin tumors from K14E7 mice were primarily benign papillomas and sebaceous epitheliomas, and only one of the nine skin tumors examined had any signs of malignancy; it was a papilloma with early malignant transformation, which was categorized as a grade I epidermoid carcinomas in Table 1. In contrast, the majority of the tumors from K14E6 mice were malignant epidermoid carcinomas (18 of 24, or 75%) ($P < 0.005$). Of the 18 malignant tumors arising in the K14E6 mice, 5 were grade I, 11 were grade II, and 2 were grade III epidermoid carcinomas. Thus the range in grade of malignancy of tumors derived from K14E6- and K17-transgenic mice also differed significantly. From these results we conclude that E6 primarily induces malignant tumors, whereas E7 primarily induces benign tumors.

E7 increases the yield of chemically induced papillomas and primarily enhances promotion in carcinogenesis

Carcinogenesis is divided into three stages: initiation, promotion, and progression (Boutwell, 1989; Drinkwater, 1990). At appropriate doses, the mutagen 7,12-dimethylbenz[*a*]anthracene (DMBA) can only initiate. In contrast, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) can only promote (Ordman *et al.,* 1985). To examine the role of E7 in carcinogenesis, we tested how E7 affected carcinogenesis induced by chemical carcinogens. We treated groups of K14E7-transgenic mice with a single dose of initiator DMBA (0.01 or 0.03 μmol) to the skin on the back, followed by twice-weekly treatments with the promoting agent TPA for 20 weeks. Groups of nontransgenic mice were treated with these two agents on the same schedules. Papillomas were counted every other week. A significantly greater number of papillomas ($P < 0.001$) were observed on the K14E7-transgenic mice in both treatment groups compared with that in the nontransgenic mouse groups, and these tumors developed earlier in K14E7-transgenic mice (Figs. 1A and 1B). A high percentage of the DMBA- and TPA-treated K14E7 mice, but no DMBA- or TPA-treated nontransgenic mice, developed benign sebaceous epitheliomas during the 20-week treatment period. This is a tumor type that can arise spontaneously in older K14E7 mice (Herber, *et al.,* 1996); their more rapid development in the treated mice indicates that E7 can synergize with DMBA and/or TPA to induce this benign tumor type as well. Together, these results indicate that E7 acts in the early stages of carcinogenesis, leading to papilloma formation.

To determine whether E7 also affects the later, malignant progression stage of carcinogenesis, the papillomas that had formed on the K14E7 and nontransgenic mice induced by DMBA and TPA were monitored for malignant progression after termination of the TPA treatment. Due to the large number of benign sebaceous epitheliomas and large papillomas on the K14E7 mice, these mice had to be sacrificed for humane reasons by 14 weeks into this monitoring period. Papillomas that became open, ulcerated, and flattened with a wide base were clinically diagnosed to have progressed to a malignant state. Mice with these tumors were sacrificed, and tumors were collected for histopathology. Papillomas from both nontransgenic mice and K14E7-transgenic mice were able to progress to carcinomas. The frequency of malignant progression was low in both groups of mice (Table 2). At 14 weeks after termination of skin treatment with chemical carcinogens, the percentage of papillomas that progressed to malignant carcinomas in the nontransgenic and K14E7-transgenic mice was not statistically different ($P = 0.45$). Thus E7 does not play a role in progression.

Papilloma formation requires the action of two types of carcinogens: an initiator and a promoter. To determine at which of the early stages E7 might act, we treated groups of nontransgenic and K14E7 mice either with a single application of the initiator DMBA or with twice-weekly applications of the promoter TPA for 20 weeks. As expected, no tumors developed in the groups of nontransgenic mice treated with either DMBA only or TPA only, which is consistent with the requirement of both initiator and promoter for papilloma formation. Likewise, the K14E7 mice treated with TPA only did not develop any skin tumors. However, the group of K14E7 mice treated with DMBA only developed papillomas, with a multiplicity of 0.8 per mouse at the end of 20 weeks (Fig. 1C; $P = 0.04$). That an initiator could synergize with E7 to induce papillomas indicates that E7 acts as a promoter in carcinogenesis. The inability of E7 to synergize with TPA to induce tumors indicates that E7 does not act as an initiator.

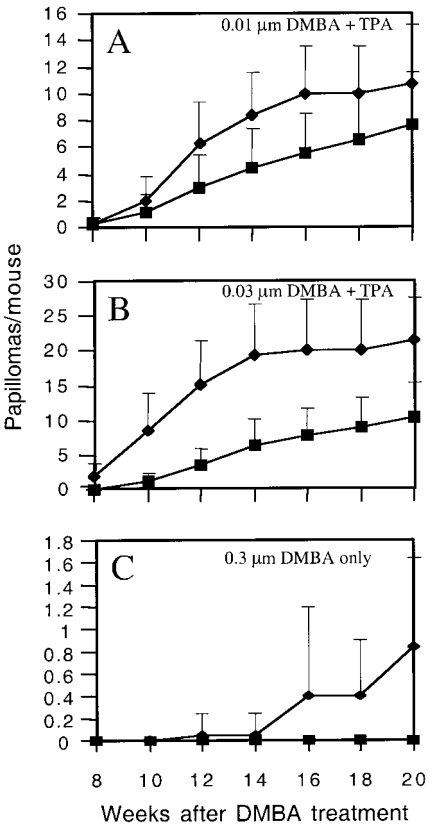

FIG. 1. Tumor induction with chemical carcinogens in K14E7-transgenic mice. Groups of 20–30 K14E7-transgenic mice (◆) and nontransgenic control mice (■) were treated with a single dose of DMBA, 0.01 μmol (A) or 0.03 μmol (B) on day 1, followed by TPA treatment (2.5 μg/treatment) twice a week for 20 weeks. Papillomas were counted every 2 weeks during TPA treatment. To examine the effect of E7 on promotion in carcinogenesis, groups of mice were treated with a single dose of DMBA (0.3 μmol) and monitored for tumor formation for 20 weeks (C). Statistical analysis was performed for each treatment ($P <$ 0.01 for panel A and B, $P = 0.04$ for panel C).

TABLE 2

Comparison of Malignant Progression in Nontransgenic versus K14E7 Transgenic Mice

Mice[a]	No. of mice	No. of carcinomas/ mouse	Percentage of papillomas progressed[b]
Nontransgenic	31	0.226	2.97
K14E7	24	0.333	3.11

[a] The mouse groups that were monitored for progression were those described in the legend to Fig. 1A [i.e., those treated with single dose of DMBA (0.01 μmol), followed by treatment with TPA (2.5 μg), twice a week for 20 weeks]. Mice were monitored for an additional 14 weeks for tumor progression. Data were obtained at 34 weeks after treatment with DMBA (0.01 μmol). All malignant tumors were confirmed by histopathology.

[b] Calculated based on the number of carcinomas that had developed by week 34 as a fraction of the number of papillomas observed at the termination of TPA treatment (week 20). The difference between the two groups was not statistically significant ($P = 0.45$).

E6 weakly potentiates tumor development after chemical carcinogenesis and primarily enhances malignant progression

Similarly, we asked at what stages E6 acts in carcinogenesis. We treated groups of K14E6 mice with DMBA and TPA and monitored the development of papillomas. K14E6-transgenic mice treated with 0.01 μmol of DMBA produced a statistically ($P < 0.01$) higher number of papillomas than did nontransgenic mice (Fig. 2A). However, the numbers of papillomas induced in the K14E6 mice treated with a higher dose of DMBA (0.03 μmol) did not differ from that seen in nontransgenic mice ($P = 0.4$) (Fig. 2B). This result suggested that E6 might synergize with chemical carcinogens in the induction of papillomas but that its action is weak and therefore easily masked when higher levels of chemical carcinogens are used. This is in contrast to E7, which could synergize even with high levels of chemical carcinogens to induce papilloma formation. Sebaceous epithelioma was not commonly seen in the K14E6 mice with or without treatment with chemical carcinogens.

To determine at which stage, initiation or promotion, E6 weakly acts, we treated K14E6 and nontransgenic mice with DMBA or TPA only. No tumors developed in the groups of K14E6 or nontransgenic mice treated with TPA only (data not shown), indicating the E6 does not act as an initiator. However, tumors did develop in the group of K14E6-transgenic mice treated with DMBA only (Fig. 2C), although the multiplicity of only 0.156 papilloma per mouse was far less than that seen in the K14E7 mice. This result indicates that E6 acts as a promoter in the formation of papillomas but that its action at this stage of carcinogenesis is weak compared with E7.

To study the function of E6 in progression of carcinogenesis, the papillomas induced by treatment with DMBA and TPA were monitored for malignant progression for an additional 20 weeks after termination of skin painting with the promoting agent TPA. The chemically induced papillomas on the E6-transgenic mice converted to a malignant state earlier than papillomas from nontransgenic mice, and the rate of malignant conversion was higher than the papillomas in nontransgenic mice (Table 3). The difference in the percentage of papillomas progressing to malignant tumors between E6-transgenic mice and nontransgenic mice was statistically significant ($P < 0.05$), indicating that E6 increases the malignant progression of chemically induce papillomas. The increase in malignant conversion in the K14E6 mice could also be seen by 14 weeks after the termination of TPA treatment, the end point used in the analysis of progression in the K14E7 mice. Thus E6 contrasts with E7 in its ability to cause malignant progression.

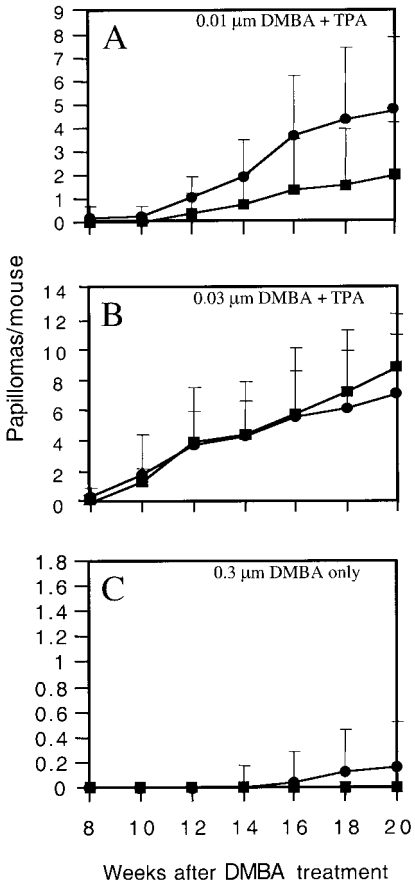

FIG. 2. Tumor induction with chemical carcinogens in K14E6-transgenic mice. Groups of 20–30 K14E6-transgenic mice (●) and nontransgenic control mice (■) were treated with a single dose of DMBA, 0.01 μmol (A) or 0.03 μmol (B) on day 1, followed by TPA treatment (2.5 μg/treatment) twice a week for 20 weeks. Papillomas were counted every 2 weeks during TPA treatment. To examine the effect of E6 on promotion in carcinogenesis, groups of mice of each strain were treated with a single dose of DMBA (0.3 μmol) and monitored for tumor formation for 20 weeks (C). Statistical analysis was performed for each treatment ($P < 0.01$ for panel A, $P = 0.4$ for panel B, and 0.08 for panel C).

TABLE 3

Comparison of Malignant Progression in Nontransgenic versus K14E6 Transgenic Mice

Mice[a]	No. of mice	No. of carcinomas/ mouse	Percentage of papillomas progressed[b]
Nontransgenic	18	0.11 (0)[c]	5.56 (0)
K14E6	19	0.894 (0.37)	19.78 (7.78)

[a] The mouse groups that were monitored for progression were those described in the legend to Fig. 2A [i.e., those treated with single dose of DMBA (0.01 μmol), followed by treatment with TPA (2.5 μg), twice a week for 20 weeks]. Mice were monitored for an additional 20 weeks for tumor progression. Data were obtained at 40 weeks after treatment with DMBA (0.01 μmol). All malignant tumors were confirmed by histopathology.

[b] Calculated based on the number of carcinomas that had developed by week 40 as a fraction of the number of papillomas observed at the termination of TPA treatment (week 20). The difference between the two groups was statistically significant ($P < 0.05$).

[c] Numbers in parentheses indicate data obtained at week 34. Note that differences in number of carcinomas per mouse in nontransgenic controls at this time point compared with that reported in Table 2 reflect differences in specific activity of DMBA used in these two experimental studies, as evidenced by the 4-fold difference in frequency of papillomas (compare data in Fig. 2A with that in Fig. 1A).

E6 cooperates with E7 in induction of carcinogenesis

Taken together, our skin-painting experiments indicate that E7 acts at the promotion stage and that E6 acts primarily at the progression stage in carcinogenesis. To further test this hypothesis, we looked at whether E6 and E7 cooperate in carcinogenesis. A synergistic effect between these genes would be consistent with the hypothesis that these two genes act at different stages in carcinogenesis. We generated K14E6xK14E7 double-transgenic mice by crossing our E6-transgenic mice (line 5737) and E7-transgenic mice (line 2304). By doing so, the gene doses of E6 or E7 in these double-transgenic mice are equivalent to that in K14E6- or K14E7-transgenic mice, respectively. Tumor incidence in K14E6 × K14E7 double-transgenic mice was monitored and compared with that of K14E6- or K14E7-transgenic mice. Skin tumors developed earlier in the K14E6 × K14E7 mice, and the tumor incidence was statistically significantly higher ($P < 0.001$) than that seen in either K14E6 or K14E7 mice (Fig. 3). The tumors that arose in these mice were a combination of sebaceous epitheliomas (~30%) and squamous tumors (~70%), with the vast majority of the latter being malignant carcinomas. Both tumor types arose earlier and with higher frequency in the doubly transgenic mice than in the singly transgenic mice. The sebaceous epitheliomas were seen only in the singly transgenic K14E7 mice, not in the K14E6 mice; their earlier onset and higher frequency in the doubly transgenic mice compared with the K14E7 mice suggest that E6 can contribute to the development of this tumor type when coexpressed with E7. These results clearly indicate that E6 and E7 cooperate in carcinogenesis.

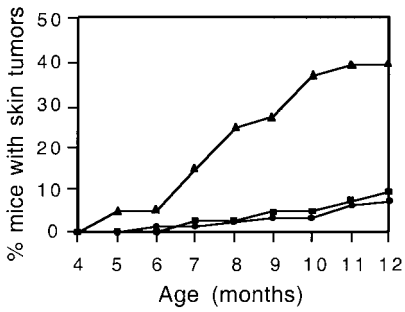

FIG. 3. Cooperation between E6 and E7 genes in tumorigenesis. The K14E6 × K14E7 double transgenics (▲) were generated by crossing K14E6 (line 5737, ●) and K14E7 (line 2304, ■) mice that had been used in this study for analysis of spontaneous tumors and for tumor induction assays.

H-ras mutation does not contribute to the increased malignant progression of the chemically induced tumors in E6-transgenic mice

Cells transformed by E6 and E7 could not form tumors when transplanted into nude mice, but cells transformed by these genes in combination with other oncogenes, such as activated *ras*, can form tumors in animals (Crook *et al.,* 1988; Lambert *et al.,* 1993; Liu *et al.,* 1995). To test whether E6's ability to increase progression of chemically induced tumors is associated with increased H-*ras* activation, we looked at the frequency of H-*ras* mutations in the chemically induced tumors. Because DMBA-induced mutations in H-*ras* are almost exclusively A → T transversions at codon 61 (Quintanilla *et al.,* 1986), we analyzed the mutations at this codon 61 by PCR–restrict fragment length polymorphism (RFLP). All the mutations that occurred were A → T transversions at the second position of codon 61. There was no difference in the frequency of H-*ras* mutations at this codon between tumors from nontransgenic and K14E6-transgenic mice (Table 4). Mutations at codon 61 were detected more frequently in carcinomas than papillomas from both groups of mice. We concluded that the increased malignant progression of tumors in E6-transgenic mice was not associated with increases in H-*ras* mutations.

TABLE 4

H-*ras* Mutations at Codon 61 in Tumors Induced with Carcinogens in Nontransgenic versus K14E6-Transgenic Mice

Codon 61	Tumors from nontransgenic	Tumors from K14E6 mice
CAA to GAA	0/25	0/30
CAA to CTA	21/25 (84%)	25/30 (83.3%)
CAA to CAT	0/25	0/30

Note. Values in the table the represent the number of tumors with *ras* mutations as a fraction of the total number of tumors examined.

DISCUSSION

HPV-induced carcinogenesis is a multistep process in which the normal cervical epithelia initially develops cytological changes referred to histologically as SIL (Wright *et al.,* 1994). If the high-risk HPV infection persists, the SIL can progress from low grade to high grade and, after a long latency period, to invasive carcinoma. In this process, the E6 and E7 genes are expressed in the cells and considered to contribute to this carcinogenic process. In this study, we identified the stages in carcinogenesis at which E6 and E7 act. E7 and, to a lesser degree, E6 were found together with the initiator DMBA to induce formation of benign tumors. These data indicate that E7 acts strongly and E6 acts weakly at the promotion stage of carcinogenesis. E6 also contributes to the progression stage of carcinogenesis as evidenced by the increased percentage of papillomas that became malignant. E7 did not affect progression. Thus E6 and E7 play different roles in carcinogenesis; E7 strongly contributes to benign tumor formation, whereas E6 strongly contributes to malignant transformation. Correspondingly, the two genes together were found to act synergistically in the spontaneous induction of tumors.

The role of E7

One common property of carcinogenic promoting agents, including TPA, is their ability to induce cellular hyperplasia (Imamoto *et al.,* 1993; Pence and Reiners, 1987). E7's tumor-promoting activity may reflect its ability to interact with cell cycle-regulating proteins and induce cellular proliferation in cell culture and in animals. In the K14E7 mice, E7 induces epidermal hyperplasia (Herber *et al.,* 1996). The proliferation index in the basal layer of the epithelia of these mice was increased, and cells in the suprabasal compartment of the epidermis, where cells normally cease to proliferate, were still able to synthesize DNA and divide (Gulliver *et al.,* 1997). This activity was dependent on its ability to inactivate pRb and pRb-like proteins (Gulliver *et al.,* 1997). Sebaceous epitheliomas, which can spontaneously develop in K14E7 mice, were also frequently induced by DMBA only in this transgenic strain, indicating that E7 acts to promote two types of benign skin tumor.

In our analysis of spontaneously arising tumors and carcinogen-induced tumors, we concluded that E7 does not contribute significantly to malignant progression in carcinogenesis. The conclusion is consistent with studies that demonstrated E7 is only weakly capable of inducing gross chromosomal alterations, such as aneuploidy, that are indicative of malignancy (Reznikoff *et al.,* 1994; White *et al.,* 1994). This result is also consistent with an early finding that the inactivation of the p16/cdk/cyclin-pRb cascade does not occur during malignant transformation but rather during an early step in the immortalization of cells by HPV-16 or -18 (Nakao *et al.,* 1997). The p16-pRb cascade is disrupted by E7.

The role of E6

In this study, we conclude that E6's primary oncogenic contribution to carcinogenesis is to cause tumors to progress to the malignant state. E6 may enhance malignant progression through several mechanisms. First, E6 has been demonstrated in cell culture to induce gross chromosomal alterations that are indicative of malignancy, including chromosomal translocation and aneuploidy (Reznikoff *et al.,* 1994; White *et al.,* 1994). These gross chromosomal changes are also common in malignant cells that have lost p53 function, indicating the E6 may induce the genomic instability through its inactivation of p53. Consistent with this argument, a reduction in p53 gene dose can accelerate malignant progression in carcinogenesis but does not affect tumor promotion in carcinogenesis (Kemp *et al.,* 1993). E6's inactivation of p53 is functionally equivalent to a reduction in p53 gene dose. Second, E6 can inhibit cellular differentiation. In the lens of the mice that express the E6 gene, terminal differentiation was disrupted and the lens fiber cell denucleation process was inhibited (Pan and Griep, 1994). In cell culture, the E6 was found to increase the resistance to calcium-induced differentiation of human keratinocytes through a process that requires, at least in part, p53-independent activities of E6 (Sherman *et al.,* 1997; Sherman and Schlegel, 1996). In addition, we have found E6 to cause an expansion in the undifferentiated compartment in the epithelia of the K14E6 mice; this, too, required p53-independent activities of E6 (Song *et al.,* 1999). Because one of the distinct features between benign and malignant tumors is their differentiation properties, inhibition of cellular differentiation by E6 may contribute to malignant progression. Third, E6 can induce activation of telomerase (Klingelhutz *et al.,* 1996), which can abrogate a late step in senescence of human mammary epithelial cells (Kiyono *et al.,* 1998). Increased activity of telomerase is seen selectively in malignant cervical carcinomas and high-grade CIN III lesions (Klingelhutz *et al.,* 1996; Snijders *et al.,* 1998). Thus E6 may contribute to malignant progression via several of its biological activities. Further studies are necessary to ascertain the contribution of each of these activities of E6 to its role in carcinogenesis.

Activated *ras* can cooperate with E6 or E7 to cause malignant transformation of cells (Liu *et al.,* 1994; Phelps *et al.,* 1988; Storey and Banks, 1993). Cells transformed by E6 or E7 alone usually cannot form tumors in nude mice, but cells transformed by E6 and activated *ras* can (Liu *et al.,* 1994), indicating the cells transformed by cooperation between E6 and activated *ras* were more aggressive. Also, H-*ras* mutation is considered a marker for malignant progression in some skin cancers, such as

melanoma (Ball *et al.,* 1994). We wanted to test whether mutation of the *ras* gene was contributing to the increased malignant progression of the chemically induced papillomas in the K14E6-transgenic mice. Consistent with our earlier result that H-*ras* gene mutations were not involved in the spontaneous tumor development in the K14E6 mice (Song *et al.,* 1999), the frequency of H-*ras* mutations was similar in tumors induced by chemical carcinogens in both the K14E6-transgenic and their control mice, indicating that H-*ras* mutations were not contributing to the increased frequency of malignant progression in the K14E6 mice.

We found that E6 also affected the promotion stage in carcinogenesis. This action was weak compared with that of E7 and probably relates to E6's ability to induce cellular proliferation (Song *et al.,* 1999). Correlative to its weak promotion activity compared with that of E7, the proliferative index of the K14E6 epidermis is significantly less than that of the K14E7 epidermis (Song *et al.,* 1998).

The cooperation between E6 and E7

Supporting the hypothesis that E6 and E7 play different roles in carcinogenesis, these two genes can cooperate in the induction of carcinogenesis. The cooperation between E6 and E7 is consistent with earlier studies demonstrating that both E6 and E7 were required for efficient transformation of human keratinocytes (Munger *et al.,* 1989a). It also fits with the model that E6 and E7 act at different steps in the immortalization of primary human mammary epithelial cells; E7 acts at an early step through inactivation of the p16-pRb cascades leading to an extended life span of primary cells, and E6 acts at a late step through its activation of telomerase.

We speculate that cooperation between E6 and E7 also occurs in cervical carcinogenesis induced by HPVs. First, both transcripts for the E6 and E7 genes are detected in cervical cancer cells. Second, E6 and E7 can complement each other in causing unlimited growth of cells and malignant progression. For example, E7 can prolong the life span of cells and therefore supposedly induces overgrowth of cells. However, E7 can also induce apoptosis (Iglesias *et al.,* 1998; Pan and Griep, 1994); thus E7-induced lesions may not change in size as rapidly as otherwise possible. E6 inhibits this apoptosis induced by E7 (Pan and Griep, 1995; Stoppler *et al.,* 1998), therefore accentuating the effects of E7 on carcinogenesis. Moreover, by contributing to the progression stage of carcinogenesis, E6 likely causes E7-induced benign tumors to convert to a malignant state. Although E7 does not overtly accelerate the malignant conversion (i.e., contribute itself to progression), through its cell proliferation property, E7 may still contribute to the continued growth of malignant tumors. Thus the expression of both E6 and E7 oncogenes in human cervix likely contributes to the formation and maintenance of malignant cervical cancer.

Synergy between HPV oncogenes and chemical carcinogens

In this study, we demonstrate that the HPV oncogenes can synergize with chemical carcinogens to induce tumors. This result provides direct evidence that environmental carcinogenic factors may affect HPV-associated carcinogenesis. Cell culture studies demonstrated that keratinocytes transformed by HPV-18 could not form tumors in nude mice but could do so after these cells were exposed to the chemical carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, indicating a synergy between high-risk HPVs and chemical carcinogens in the malignant transformation of cells (Shin *et al.,* 1994). Epidemiological and clinical studies have linked smoking to cervical cancer development (Daling *et al.,* 1992; Kalogeraki *et al.,* 1996), and several compounds from smoking can be detected in cervical mucus from smokers, providing direct evidence that smoking may affect the cervix (Prokopczyk *et al.,* 1997). Taken together, these studies suggest that environmental factors may play a role in cervical carcinogenesis together with HPVs. We speculate that the long latency period for HPV-associated cervical carcinogenesis may not be only precipitated by other genetic changes but may also be accelerated by environmental carcinogens.

MATERIALS AND METHODS

Analysis of malignancy of the tumors derived from E6- and E7-transgenic mice

The generation of the K14E6- and K14E7-transgenic mice, in which either HPV-16 E6 or E7 was expressed in the basal layer of the epidermis (Herber *et al.,* 1996; Song *et al.,* 1999), was described previously. Tumors that developed on the K14E6 and K17 were excised, fixed in buffered Formalin, and paraffin embedded. Sections of these tumors were stained with hematoxylin and eosin. Histopathological analysis and evaluation of the degree of the malignancy of the skin tumors were performed by the same pathologist. Tumors were categorized as benign or malignant. Malignant tumors were graded as I through IV based on the degree of differentiation of tumor cells, which is represented by the extent of keratinization, the atypicality, and the architectural pattern of tumor cells.

Treatment of mouse skin with DMBA and TPA

Six- to 8-week-old female mice were shaved on the back. Mice were divided into three treatment groups (20–30 mice per group) that were treated with (1) the carcinogenic initiator DMBA and promoter TPA, (2) DMBA only, or (3) TPA only. For the DMBA plus TPA

treatment groups, a dose of 0.01 or 0.03 μmol of DMBA dissolved in 0.1 ml of acetone was applied to the back skin. Two weeks after the initial treatment with DMBA, treatment with TPA began. TPA was also dissolved in acetone; 0.1 ml of TPA solution containing 2.5 μg of TPA was applied to the backs of mice twice a week for 20 weeks. For the DMBA-only treatment groups, mice were treated similarly as described above but with a single higher dose of DMBA (0.3 μmol) that was not followed by TPA treatment. For the TPA-only treatment groups, mice were not treated with DMBA but were treated with TPA at a dose of 2.5 μg/treatment twice a week for 20 weeks. All mice were examined every 2 weeks to monitor the development of papillomas.

Monitoring malignant progression of chemically induced papillomas in mice

Groups of mice treated with both DMBA and TPA were kept after the termination of TPA treatment. Papillomas were monitored for their progression to the malignant state. Papillomas that became flat, open, and invasive were clinically diagnosed as malignant. These clinically diagnosed tumors were later confirmed to be malignant by histopathology. Mice with open lesions larger than 0.5 cm in diameter were sacrificed for humane reasons. Tumor samples were collected, parts of the tumors were frozen for genomic DNA preparation, and the remaining tumor tissue was fixed in buffered Formalin and processed for histopathological analysis.

Analysis of H-*ras* mutations with PCR and RFLP

H-*ras* mutations in the chemically induced skin tumors were analyzed by PCR and RFLP. Activated H-*ras* mutations induced by DMBA commonly are A → T transversion mutations at the second position of codon 61 in H-*ras* gene (Chakravarti *et al.,* 1995). These mutations create new restriction sites. To determine the frequency and spectrum of H-*ras* mutations in the tumors induced by DMBA and TPA, three possible nucleotide changes, CAA to GAA, CAA to CTA, and CAA to CAT, were analyzed by PCR and RFLP. DNA fragments encompassing codon 61 (CAA) were amplified via PCR from tumor-derived DNA using a pair of primers. The primers were CTCCTACCGGAAACAGGTGGTC and 5′-GCTAGCCATAGGTGGCTCACC. The PCR product was digested with restriction enzymes *Xba*I, *Bsmp*HI, or *Tag*I to detect CAA to CTA, CAA to CAT, or CAA to GAA mutations. PCR products digested with these enzymes were run on 3% Metaphor agarose gels and examined visually by ethidium bromide staining.

Statistical analysis of tumor data

The statistical significance of the difference in the percentage of malignant tumors derived spontaneously from K14E6- and K14E7-transgenic mice and in the frequency of the chemically induced papillomas that progressed to malignant tumors between K14E6 mice and their control or K14E7 mice and their control were analyzed using the χ^2 test. For tumor induction assays, the average multiplicity of tumors per mouse for different experimental groups at a given time point were analyzed for statistical significance using the Student's *t* test.

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