Supraboral expression of the human papillomavirus type 16 oncoproteins in mouse epidermis alters expression of cell cycle regulatory proteins

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Introduction

Human papillomaviruses (HPVs) are double-stranded DNA tumor viruses that induce hyperproliferative lesions in cutaneous and mucosal epithelia. Various HPV types have been implicated as causative agents in the genesis of epithelial diseases in laryngeal, oral, epidermal and cervical epithelia (1, 2). HPVs encode two proteins, E6 and E7, that are important for cell immortalization (3). E6 and E7 interact with the tumor suppressor proteins p53 and pRb, respectively. E6 facilitates degradation of p53 through association with an accessory protein, E6-AP, a component of the ubiquitin proteolytic degradation pathway (4–6). p53 normally acts to increase the levels of p21 (7), a cyclin kinase inhibitor (8, 9). A reduced p53 level results in less p21 expression with an accompanying loss of cell cycle checkpoint regulation. E7 binds to pRb, and the related pocket proteins p107 and p130, leading to functional inactivation of these proliferation regulators (10, 11). In quiescent cells, pRb is complexed to the transcription factor E2F (12, 13). Approaching S phase of the cell cycle, pRb becomes hyperphosphorylated, causing release of E2F, which then activates expression of growth-associated genes. E7 bypasses this pRb-dependent control by binding to pRb and causing the non-physiological release of active E2F (12).

Recent studies have significantly advanced our understanding of the effects of E6 and E7 on the keratinocyte phenotype and on the level of cell cycle regulatory proteins. In normal stratifying surface epithelia, keratinocytes migrate out from the basal layer towards the body surface. The suprabasal cells exit the cell cycle and undergo a process of biochemical and structural remodeling that results in specific changes in gene expression and alteration of cell morphology (14). The morphological changes include loss of cell organelles, including the nucleus, and assembly of the cornified envelope (14, 15). The ultimate fate of these cells is desquamation from the body surface. Biochemical changes include the differentiation-dependent expression of markers of keratinocyte differentiation. For example, the involucrin and transglutaminase genes, and the keratin K1 and K10 genes are turned on when cells differentiate, while the α4/β6 integrin, K5 and K14 genes are turned off (reviewed in refs 16, 17). In HPV-infected tissue, this differentiation process is altered and the keratinocytes do not completely differentiate.

In cultured keratinocytes, E6 (18) and E7 (19–23) have been reported to regulate differentially the level and/or activity of cell cycle regulatory proteins. These studies implicate the E7 protein as being primarily responsible for these phenotypic changes and the loss of ability to exit the cell cycle (24–26). Moreover, these studies suggest that the HPV promoter activates transcription in the differentiated epithelial layers (26). Some of these changes have been confirmed in studies of human tumor tissues (27); however, the effects of E6/E7 on cell cycle regulatory protein levels have not been examined using an animal model of HPV-dependent disease. We have targeted HPV16 E6/E7 expression to the epidermal suprabasal layers using a promoter that mimics the pattern of HPV transcription. We use these animals to study the effect of E6/E7 reading frame expression on cell cycle regulatory protein expression.

Materials and methods

Antibodies

Mouse monoclonal antibodies specific for cyclin D1 (sc-246), p27/Kip1 (sc-1641), p53 (sc-099), pRb (sc-102) and p21/Waf1 (sc-6246), rabbit polyclonal antibodies specific for cyclin E (sc-481), cyclin-dependent kinase (cdk) 2 (sc-163) and cdk 6 (sc-7181), and a goat polyclonal antibody for cdk 4 (sc-601) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at dilutions specified by the manufacturer.

Preparation of buccal, tongue and epidermal cell lysates

Mice were killed by cervical dislocation, the dorsal skin was removed, and the epidermis was separated from the dermis by scraping. The epidermis was homogenized in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, pH 7.4) and placed on ice for 30 min. The lysate was centrifuged at 15 000 g for 15 min at 4°C and the supernatant (total cell lysate) was used immediately or

Abbreviations:

BrdU, 5-bromo-2’-deoxyuridine; HPVs, human papillomaviruses.

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stored at −80°C. Buccal epithelium (25 mg) and tongue epithelium (100 mg) were harvested by scraping (buccal) or slicing (tongue) and homogenized in 50 or 200 μl of lysis buffer, respectively. The samples were held on ice for 30 min, clarified by centrifugation for 10 min at 15 000 g and stored at −80°C.

**Immunoblot analysis**

For immunoblot analysis, cell lysates (25 μg protein) were electrophoresed on denaturing 6–10% polyacrylamide gels (28), and the separated proteins were transferred to nitrocellulose membranes. To block non-specific binding, membranes were incubated in 10 mM Tris–HCl pH 7.2 containing 100 mM NaCl, 0.1% Tween-20 and 5% non-fat dry milk. The blot was incubated with the appropriate primary antibody for 1 h at 25°C or for 12 h at 40°C. Antibody binding was detected by incubation with the appropriate horseradish peroxidase-conjugated anti-mouse IgG (Calbiochem, La Jolla, CA) or anti-rabbit IgG (Santa Cruz Biotechnology) at 1:5000 dilution on 2 h at 25°C. The blots were washed and antibody binding was visualized by chemiluminescent detection methods. Relative band intensity was compared by densitometry.

**Construction of hINV-E6/E7-SV40 transgene and production of transgenic mice**

The complete HPV16 genome cloned at the BamHI site in pUC8, kindly provided by Dr Harold zur Hausen (29), was digested with KpnI/EcoRI to release a 1330 bp fragment containing the E6/E7 coding region. This fragment was then digested with KpnI/EcoRI-restricted pUC19 to yield pUC19HPV16 (E7453/K880). This plasmid was digested with EcoRI and treated with Pfu polymerase to release the HPV16 upstream regulatory region, which was replaced with a double-stranded DNA linker design to reconstruct the E6 transcription start site beginning at HPV16 nucleotide 79 (29). The resulting plasmid, pUC19HPV16 (79/K880), was digested with BamHI and the E6/E7 insert was transferred to BamHI/KpnI-restricted pKSM13(-). The pKSM13(-) HPV16 E6/E7 plasmid was digested with XbaI/KpnI and the E6/E7 reading frame fragment was ligated with BamHI/XbaI-restricted pUC19 and a BamHI/KpnI fragment from pCE3 (30) containing the SV40 transcription termination. The resulting plasmid, pUC19HPV16 E6/E7-SV401, contains an E6/E7-SV40 cassette as a HindIII/BamHI fragment. pINV-E6/E7-SV40 contains the involucrin promoter linked to the E6/E7 reading frames and an SV40 terminator. (B) Nucleotide sequence surrounding the fusion point. The upstream box contains sequence derived from the involucrin promoter, unboxed sequences are derived from linker and the downstream box shows sequences derived from HPV16. The arrow indicates the transcription start position of the viral p79 promoter.

**Phenotypic analysis**

The E6/E7 expression produces acanthosis in stratifying epithelia

**Histological methods**

For histological analysis, tissue was collected and fixed immediately in 10% formalin in phosphate-buffered saline. The samples were dehydrated in ethanol and transferred to xylene and embedded in paraffin. Four-micrometer sections were prepared and stained with hematoxylin and eosin prior to examination.

**BrdU labeling**

5-Bromo-2′-deoxyuridine (BrdU) labeling was performed by injecting animals intraperitoneally (100 μg/g body weight) with 5 mg/ml of BrdU (Sigma) in 10 mM Tris–0.9% saline–1 mM EDTA pH 8. After 60 min, the animals were killed. Tissues obtained by biopsy were fixed immediately in 10% buffered formalin and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin prior to examination.

**Detection of HPV RNA expression**

HPV16 transcript production was detected by RT–PCR using the primers and PCR conditions described by Falcinelli et al. (34). Mouse epidermis was shaved and the excised skin was placed in Hank’s balanced salt solution containing 10 mg/ml dispase (35). After 4 h at 4°C, the epidermis was removed by scraping and frozen in liquid nitrogen. After epidermal pulverization, the total RNA was isolated using the single-step method (36). Total RNA (1 μg) was reverse transcribed in a 20 μl reaction containing 50 mM Tris–HCl pH 7.6, 60 mM KCl, 1 mM dithiothreitol, 0.5 μg oligo-dT primer, 8 mM dNTPs, 40 U RNasin, 10 mM MgCl2 and 25 U AMV reverse transcriptase (Boehringer Mannheim, Germany). After 1 h at 42°C, the reaction was boiled for 2 min and diluted in 100 μl of sterile H2O. A 5 μl aliquot was then used as a PCR template using HPV16-specific primers (34). These primers produce bands of 395, 213 and 95 bp for the full-length, E6* and E6** products. The PCR reactions contained 1 μM each primer, 0.8 mM dNTPs, 60 mM Tris–HCl pH 8.5, 3.5 mM MgCl2 and 1.25 U Taq polymerase (Boehringer Mannheim) in a 50 μl reaction. The reaction was pre-incubated with 275 ng Taq Start antibody for 15 min at 25°C (Clontech). The PCR cycling was 2 min at 94°C, 2.5 min at 55°C and 2.5 min at 72°C for 35 cycles. The first and last cycles, respectively, included a 5 min denaturation at 94°C and a 4.5 min extension at 72°C. PCR products were detected by electrophoresing a 10 μl aliquot on a 2% agarose gel for Southern blot analysis.

**Results**

**E6/E7 expression produces acanthosis in stratifying epithelia**

HPV16 E6/E7 expression was targeted to stratified epithelia in mice using the vector shown in Figure 1. Eleven independent HPV16-positive mouse lines were developed. These animals displayed visible epidermal phenotypes including hair loss, and epidermal scaling. The severity of these phenotypes varies among the lines from most to least severe as follows: 22, 54 > 12, 15 > 62, 64, 71 > 56, 49, 33, 30. Virtually 100% of the animals display acanthosis, parakeratosis and hyperproliferation in epidermis and in other stratifying surface epithelia. The E6/E7 animal epidermis is much thicker (10–15 cell layers) compared with the epidermis of non-transgenic littermates (Figure 2). Figure 3 shows that numerous suprabasal cells incorporate BrdU in the transgenic animals, indicating cell division in the suprabasal layers. Although not shown in these sections, BrdU-positive cells were also detected in the basal layer. Basal BrdU incorporation was also observed in normal littermates (not shown).

**Epidermal expression of HPV16 E6/E7 reading frames**

The E6/E7 coding region of HPV16 is known to be alternately spliced within the E6 reading frame (37–41). We assayed for HPV transcript production by RT–PCR. Figure 4 compares HPV transcript production in the epidermis of several E6/E7 mouse lines. Most lines produce the E6* transcript and lower levels of transcripts encoding full-length E6 and E6** (i.e. 71, 15, 22, 30 and 64). However, the predominant transcript in lines 12 and 56 is full-length E6. HPV16 transcripts from CaSki cells, shown as a control, encode mostly E6* (41).

**Expression of tumor suppressor genes**

E6 and E7 are thought to interfere with the function of p53 and pRb, respectively (11,42). We therefore measured the level of p53 and pRb in epidermal extracts. As shown in Figure 5, p53 levels are markedly increased in E6/E7 mouse epidermis. Repeated attempts, using multiple antibodies, were made to measure pRb levels, but pRb was not detected in epidermis from normal or E6/E7 mice.
HPV oncoprotein expression in mice

**Fig. 2.** Histological phenotype of the E6/E7 mouse epidermis. Epidermis from line 22 E6/E7 and control mice was sectioned, fixed and stained with hematoxylin–eosin. The extent of the epidermis is indicated (Epi) and the arrows point to the cells of the epidermal basal layer.

**Fig. 3.** Suprabasal BrdU uptake in E6/E7 mice. Twelve-week-old (line 22) E6/E7 mice were injected with BrdU and after 60 min the animals were killed. Biopsy samples were fixed, embedded and sectioned. The sections were then immunostained using a BrdU-specific antibody. The BrdU-positive cell nuclei are stained brown. The arrows indicate the epidermal basal layer.

**Cell cycle regulatory proteins**

Cyclin-dependent kinase activity is regulated by cyclins and cyclin-dependent kinase inhibitors. To assess changes in cell cycle proteins, we measured the level of cyclin-dependent kinases. cdk2, cdk4 and cdk6 levels were slightly increased in E6/E7 mouse epidermis (Figure 6). As shown in Figure 6, cyclin D1, which activates cdk2 and cdk4 (12), is markedly increased in E6/E7 mouse epidermis. In addition to increased cyclin D1 levels, p21 and p27, cyclin-dependent kinase inhibitors, are also elevated (Figure 6).

**E6/E7 effects on cervical and oral cavity epithelium**

We also examined the effects of E6/E7 reading frame expression in other epithelia. Acanthosis and hyperproliferation were evident in the tracheal, buccal mucosa, tongue and the ectocervical epithelium. In the cervix, this was associated with the formation of fingers that project into the underlying stroma (not shown). In the buccal epithelium (Figure 7), HPV16 E6/E7 reading frame expression resulted in marked hyperproliferation. We were able to collect quantities of oral buccal and tongue mucosa to permit monitoring of p53 and p21 levels. As shown in Figure 8, p53 levels are markedly increased in E6/E7 mouse buccal and tongue mucosa. p21 was not detected in either buccal or tongue epithelial extracts.

**Discussion**

*Suprabasal expression of HPV16 E6/E7 in stratifying epithelia*

HPV consists of an 8 kb circular double-stranded DNA genome that encodes a small number of genes expressed early in the viral life cycle, and at least two genes expressed late in the life cycle (1,2). After infection, the virus exists as an episome,
but, in the case of the cancer-causing viral forms, the DNA subsequently becomes integrated into the host cell genome. The majority of cervical tumors contain integrated HPV DNA (1,2) and express RNA encoding the E6 and E7 reading frames. Because of the absence of HPV-dependent disease models in animals, an effort has been made to produce HPV-positive mice using transgenic methods. Several gene regulatory units have been used to target HPV reading frame expression to epidermis and cervix, the major HPV-susceptible tissues, including promoters for keratin 14 (43–45), keratin 1 (46) and keratin 6 (47). None of these promoters closely mimic the pattern of HPV expression in human tissues. Each of these promoters, including the modified K1 promoter (46), drive expression in the basal proliferative layer.

Previous studies show that HPV DNA replication and RNA expression are differentiation dependent (i.e. restricted to the upper spinous and granular layers in epidermis) (48,49). This suggests that viral survival depends upon the ability of the virus to reactivate DNA replication in cells that have previously ceased dividing. Based on this evidence, we have developed a mouse model in which HPV16 E6/E7 expression is targeted to the epidermal upper spinous and granular layers (48,49). This was achieved by regulating HPV gene expression using the human involucrin promoter (33,50). Involucrin gene expression is confined to the upper spinous and granular layers in stratifying epithelia (33,50–52), a pattern essentially identical to that of HPV.
**HPV effects on keratinocyte differentiation/proliferation and tumor formation**

Eleven separate animal lines were derived, each displaying a similar phenotype. All animals display extensive epidermal hyperproliferation, acanthosis and parakeratosis. Hyperproliferation is also observed in epithelia, including the esophageal, vaginal, oral and cervical epithelia, and the increased proliferation is associated with dramatically increased desquamation. BrdU incorporation is observed in the suprabasal epidermal layers and basal layers, and visual inspection reveals mitosis in many suprabasal cells. This pattern is reminiscent of that observed in human HPV-positive epithelia (48,49). DNA synthesis is also present in the suprabasal layers when HPV18 E7-expressing keratinocytes are grown in in vitro raft cultures (26).

Tumor formation is the ultimate manifestation of HPV-dependent disease. In humans, this process can have a very long latency period and tumor development is thought to require alterations in additional genes (53,54). We did not observe epithelial papilloma formation in any of these animals, regardless of age (maximum 1.5 years). This is in contrast to some mouse HPV models where papillomas are observed in older animals (46). It is possible that the absence of tumor formation is related to the fact that the E6/E7 reading frames are expressed in the suprabasal location. The absence of tumor formation is reminiscent of the disease process in human cervix, where hyperproliferation is present in HPV-positive lesions, but tumors are not present early in the disease process (53). It is also consistent with observations indicating that HPV can immortalize cells in culture, but that the cells do not grow in soft agar or form tumors in nude mice (55,56). Tumor formation of cells in vitro is enhanced by secondary oncogene activation (56,57), and in studies in progress we have shown that carcinogen treatment can induce papilloma formation.

**HPV16 transcript synthesis**

In human tumors and in HPV-positive cultured human epidermal or cervical keratinocytes, E6/E7 mRNA is produced as a full-length transcript, and as two spliced forms, E6* and E6** (58). The predominant transcript is generally E6* (59–61), and although all three forms are able to produce E7 product (62), it has been suggested that E6** is most important for efficient E7 expression (63). Each of the seven hINV-E6/E7 mouse lines displays similar epidermal hyperproliferation. In most lines E6* is the major transcript, but in several lines the full-length form is most abundant. In addition, the transcript level varies. Based on an analysis of E6/E7 transcript level and splice variant and phenotype, we conclude that neither the specific splice variant present nor the level of RNA produced appears to influence phenotype severity. In human disease, the association of particular E6/E7 spliced products with certain stages of disease remains controversial (59,64). Our results suggest that an equally severe phenotype can be observed whether E6* or the full-length transcript is the major product.

**Alteration of cell cycle control**

Epidermal histology indicates that cell proliferation is not appropriately regulated in hINV-E6/E7 mice. To investigate the molecular basis for this dysfunction, we measured the level of several cell cycle regulatory proteins. p53 regulates progression through the cell cycle via effects on cell cycle regulators, including p21 (7), p53 increases p21 levels via a transcriptional mechanism (7). p21, in turn, inhibits cyclin-dependent kinase activity. In E6-positive cells, the E6 oncoprot-


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