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Human papillomavirus type 16 alters human epithelial cell differentiation *in vitro*

(keratinocyte/transformation/cervical cancer)

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ABSTRACT **Human papillomavirus (HPV) types 16, 18, 31, and 33 have been implicated as etiologic agents of cervical and penile cancer. Using a cell culture system for keratinocytes which allows stratification and production of differentiation-specific keratins, we have examined the effects of one of these viruses, HPV-16, on the differentiation capabilities of human epithelial cells. A plasmid containing the HPV-16 genome and a neomycin-selectable marker was transfected into primary human epidermal cells and SCC-13 cells, an immortalized squamous cell carcinoma cell line. Cloned neomycin-resistant cell lines were isolated and examined by cell culture on raised collagen rafts. Cell lines containing HPV-16 DNA retained the ability to stratify and express differentiation-specific keratins in the raft system but otherwise failed to differentiate normally. The histological abnormalities induced by HPV-16 closely resembled those seen in genital intraepithelial neoplasia *in vivo*. Hence, our results support the role of HPV-16 as an etiologic agent in the development of genital neoplasias and suggest a specific system for the study of HPV-16-induced epithelial cancers.**

Papillomaviruses are small DNA viruses that are responsible for a wide variety of proliferative lesions (papillomas) in many animals including humans. Over 50 types of human papilloma viruses (HPVs) have been identified. While the majority of the HPVs are associated with benign lesions, several of these viruses are found at a high frequency in cervical and penile cancers. HPV types 16, 18, 31, and 33 are found in >90% of all cervical cancers, thereby implicating them as etiologic agents (1–4). The most common papillomavirus subtype associated with these neoplasias is HPV-16 (1, 5, 6). The lesions produced by papillomaviruses in the urogenital tract (cervix, vagina, vulva, and penis) share common histological features including lack of differentiation, koilocytosis, parakeratosis, and, in the most severe cases, abnormal mitotic figures. Premalignant disease or intraepithelial neoplasia is graded I (mild) through III (severe) depending on the degree to which a lack of differentiation is observed. Intraepithelial neoplasia of the cervix (cervical intraepithelial neoplasia) is the most frequently observed papillomavirus-induced precursor of genital cancer, and cervical cancer is the second most common female-specific malignancy worldwide (7).

The development of a model system for the study of HPV-induced neoplasia has been hampered by the lack of both a permissive tissue culture system for viral propagation as well as an easy method for growing normal stratified epithelium *in vitro*. Kreider and coworkers (8) have implanted papillomavirus-infected tissue under the renal capsule of nude mice and observed, after 50 days, the histological features of HPV infection *in vivo*. However, this system requires surgical dexterity and may not be amenable to genetic manipulation. Moreover, the technique may be limited to a particular strain of HPV-11. Most *in vitro* transformation studies on HPV-16 and -18 have been performed in rodent cells. While these studies have identified a major transforming function of HPV-16 and -18 in the E6/E7 region (9, 10), the culture systems do not accurately mimic the transformation process in differentiating epithelial cells and as such may not allow a complete understanding of HPV-induced neoplasia.

In this study, we use a system originally developed by Asselineau and Prunieras (11) and modified by Kopan *et al.* (12), which allows stratification and production of differentiation-specific keratins. In this system, human epithelial cells containing transfected copies of HPV-16 retain the ability to stratify and express differentiation-specific keratins but lose their ability to differentiate morphologically. The histopathological changes in epithelial morphology seen in HPV-transfected keratinocytes cultured in this fashion are typical of intraepithelial neoplasia, supporting an etiologic role for HPV-16 in premalignant disease of the urogenital tract.

MATERIALS AND METHODS

Culture of Cells. SCC-13 cells are an established cell line derived from a squamous cell carcinoma of the skin (13). Human keratinocytes were cultured from neonatal foreskin and used at 10–15 cell generations. Both cell types were grown on plastic in the presence of mitomycin C-treated 3T3 J2 cells in E medium: 3 parts Dulbecco's modified Eagle's medium/1 part Ham's F-12 medium/10% fetal calf serum (Hyclone, Logan, UT); hydrocortisone (0.4 μg/ml) (Calbiochem); 0.1 nM cholera toxin (Schwarz-Mann)/transferrin (5 μg/ml) (Sigma)/2 nM 3,3′-5-triodo-L-thyronine (Sigma) supplemented with epidermal growth factor (5 ng/ml) and insulin (5 μg/ml; Sigma) (14).

Collagen Raft Cultures. Collagen rafts were made with type 1 collagen (Seikagaku America, St. Petersburg, FL)/10× E medium/buffer (8:1:1) at 4°C as described by the manufacturer. After addition of 1.5×10^5 3T3 J2 cells per ml, 2 ml of solution was added to each 35-mm plastic Petri dish, and the collagen was allowed to gel at 37°C. Keratinocytes were then seeded onto the collagen rafts at 3.0×10^5 cells per raft, medium was added, and the cells were grown to confluence. At confluence, the collagen rafts were raised onto stainless steel grids such that subsequent feeding occurred from below (see Fig. 1).

Morphological Studies. After 14–18 days of culture on the metal grids the collagen rafts were harvested. A part of each raft was fixed in 4% paraformaldehyde or 1% glutaraldehyde, embedded in paraffin wax, sectioned, and stained with

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Abbreviation: HPV, human papillomavirus.

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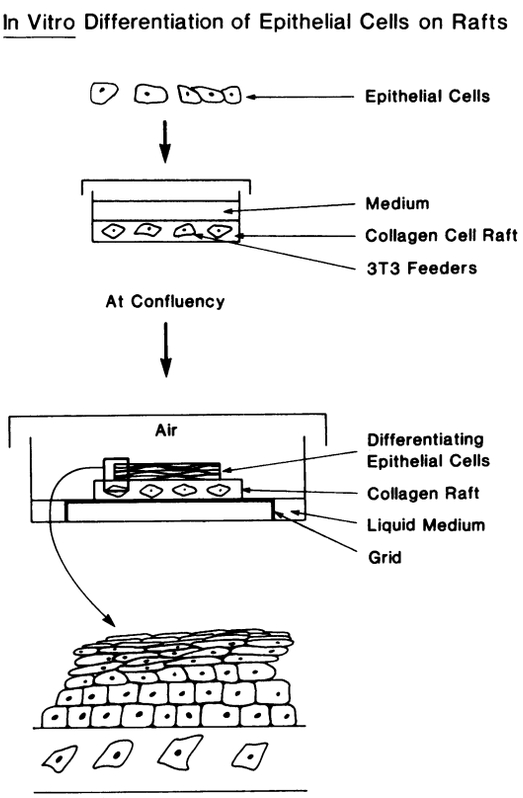

FIG. 1. Diagram of collagen raft system. Epithelial cells are plated into a submerged collagen gel containing 3T3 feeders in the presence of medium. When the cells reach confluence, the gel is raised onto a metal grid and subsequent feeding occurs from underneath by passage through the gel. Cells stratify and differentiate typically over a 2-week period.

hematoxylin and eosin. Chromosome analysis was performed on cells grown on plastic by standard methods.

Plasmids. pSV2neo/16 was cloned from an episomal copy of HPV-16 isolated from a premalignant cervical neoplasia (P. M. Chesters and D.J.M., unpublished data). This plasmid contains the complete HPV-16 genome cloned at the *Bam*HI (15) site of pSV2neo, thereby interrupting the HPV-16 genome in the late region (Fig. 2). The plasmid pSV2neo has been described (16).

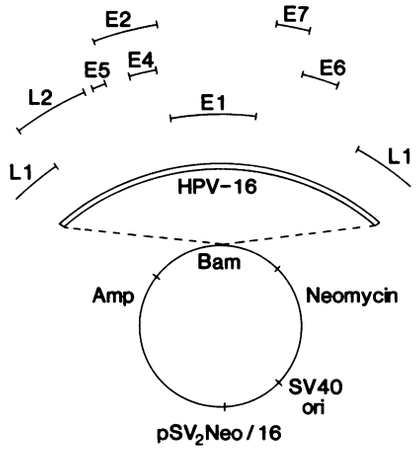

FIG. 2. Diagram of the plasmid pSV2neo/16. HPV-16 DNA was linearized at a *Bam*HI site, leaving an intact early region but a disrupted late region, and cloned into the *Bam*HI site of pSV2neo. The open reading frames of HPV-16 are shown with early genes (E) and late genes (L) indicated. Amp, ampicillin; SV40, simian virus 40; ori, origin.

Transfections and Southern and RNA Blot Analysis. SCC-13 cells or human foreskin epidermal cells were seeded at a density of 5×10^5 cells per 10-cm dish in the presence of 5×10^5 mitomycin C-treated fibroblast feeder cells. SCC-13 cells were transfected with DNA (30 μg/ml), while primary foreskin cells were transfected with 60 μg/ml as described (17). One-half of each keratinocyte collagen raft was snap-frozen in liquid nitrogen at the time of harvesting. Frozen rafts were subjected to either DNA or RNA extraction (10, 18) and followed by electrophoresis and blotting as described (10, 19).

RESULTS

The culture method used for differentiation of keratinocytes *in vitro* is shown in Fig. 1. Epithelial cells were cultured on submerged collagen gels impregnated with 3T3 J2 fibroblasts. When the keratinocytes reached confluence, the rafts were placed on a suspended metal grid, such that subsequent feeding occurred by diffusion of medium through the collagen gels. Under these conditions, keratinocytes stratified and differentiated at the air–liquid interface over a period of 2 weeks. As noted previously (12, 20), expression of the differentiation-specific keratins K1 and K10 was observed only when the rafts were exposed to the air–liquid interface and not when submerged.

HPV-16 Alteration of SCC-13 Cell Line Differentiation. Initially, we used this cell culture system to examine a cell line, SCC-13, from a squamous cell carcinoma of human skin (13). This cell line showed some degree of differentiation on collagen rafts (Fig. 3) and produced two differentiation-specific keratins, K1 (20, 21) and K10 (unpublished data). SCC-13 cells were transfected with either pSV2neo alone or pSV2neo/16, which contains the entire HPV-16 genome. Three neomycin-resistant clones from each transfection were isolated and expanded by coculturing with mitomycin C-treated 3T3 J2 feeder layers on a plastic substratum (conventional method of culture). The three neomycin-resistant pSV2neo/16 cell lines (SCC-13/16-1, -2, and -3) were examined for the presence of HPV-16 DNA by Southern analysis. In two of the three clones, HPV-16 was found as a full-length sequence, suggesting that integration had occurred in plasmid sequences (Fig. 4). In the other clone, the integration event resulted in a disruption of the HPV-16 genome. Two clones were found to contain over 20 copies of HPV-16, while one contained less than 3 copies (Fig. 4). No episomal copies of HPV-16 were observed.

The HPV-16-containing SCC-13 cell lines were cultured for 2–3 weeks on collagen rafts as described. Stratified cells were fixed in gluteraldehyde and examined histologically (Fig. 3). In all clones containing HPV-16, a loss of morphological differentiation was observed (Fig. 3*B*). While differentiated cell types were visible in stratified SCC-13 cells (Fig. 3*A*), HPV-16-containing cells on rafts appeared to consist mainly of a single cell type with a basal-like appearance (Fig. 3*B*). Furthermore, an increase in mitotic figures was sometimes seen in the upper region of the epithelium. Similar altered morphology was observed with another squamous cell carcinoma cell line (SCC-12) (13) transfected with pSV2neo/16 (data not shown). In contrast, all SCC-13 and SCC-12 control cell lines containing the pSV2neo gene alone showed morphology indistinguishable from that of untransfected cells (data not shown). To date, these experiments have been reproduced five times with identical results.

The dramatic changes in the morphology of HPV-16-containing SCC-13 cells were not accompanied by major changes in keratin expression (data not shown). Antisera specific for K1 (differentiation-specific keratin) and K16 (suprabasal marker) showed reduced and patchy staining, while anti-K14 (basal marker) showed staining throughout all

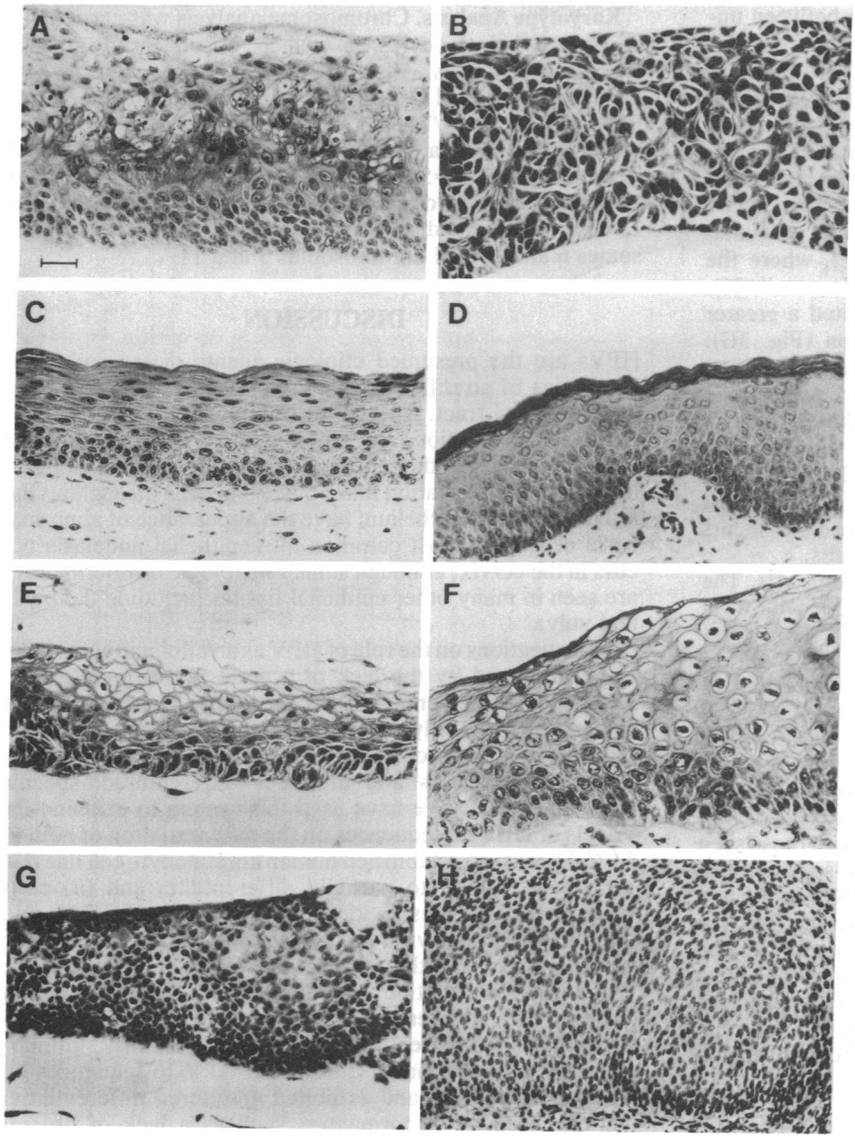

FIG. 3. Hematoxylin and eosin stain of SCC-13 and primary human keratinocytes with and without transfected HPV-16 sequences. (*A*, *B*, *C*, *E*, and *G*) Cells grown *in vitro* on the collagen raft system. (*D*, *F*, and *H*) Cross-sections of tissue samples. (*A*) Stratified and partially differentiated untransfected SCC-13 cells on collagen rafts. Basal epithelium cells, spinous-like cells, and cells containing keratohylin granules are visible. (*B*) Stratified SCC-13 cells containing pSV2neo/16 on collagen rafts. A disruption of differentiation is seen with a majority of basal-like cells present. (*C*) Normal foreskin keratinocytes on collagen rafts at third passage. Normal basal epithelium and differentiation patterns are seen. (*D*) Cross-section of normal stratified squamous epithelium from the foreskin. (*E*) Human foreskin keratinocytes containing pSV2neo/16 (K1/16 cell line) at third passage after transfection as seen in the raft system. Parabasal crowding is present and vacuolated cells (koilocytes) are seen in the upper half of the epithelium. (*F*) A biopsy from penile intraepithelial neoplasia grade I showing koilocytes in upper half of epithelium. (*G*) Human foreskin keratinocytes containing pSV2neo/16 (K1/16) at the 17th subculture after transfection with pSV2neo/16. A lack of differentiation is seen with abnormal cells throughout the full thickness of the epithelium. Mitotic figures are present in the upper portion of the stratified epithelium. (*H*) Cervical intraepithelial neoplasia grade III showing similarities to *in vitro* K1/16 cell cultures. (×165.)

epithelial cells of the raft. Stratified parental SCC-13 cells exhibited a similar but more intense pattern of K1 and K16 staining and K14 staining was seen only in the basal layer (21). Staining of sections from dysplasias (cervical intraepithelial neoplasia) also shows a lack of K1 and low levels of K16 (22).

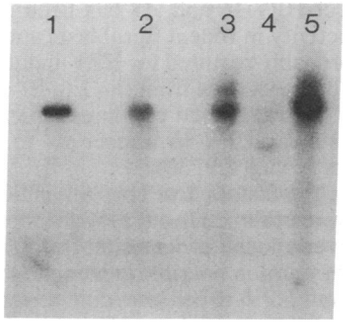

FIG. 4. Southern blot of three SCC-13 cell lines containing pSV2neo/16 and the K1/16 cell line. Lanes: 1, pSV2neo/16 plasmid digested with *Bam*HI; 2, K1/16 cell DNA digested with *Bam*HI; 3–5, three SCC-13 cell line DNAs digested with *Bam*HI. Hybridization was with full-length HPV-16 viral genome.

HPV-16 Alteration of Primary Keratinocyte Differentiation. To verify that the apparent effects of HPV-16 gene products on epidermal differentiation were not limited to carcinoma-derived lines, we examined the effect of HPV-16 sequences on the differentiation program of normal primary keratinocytes. We used gene transfection to introduce pSV2neo/16 into an early passage (second passage, 10–15 cell generations) culture of human epidermal keratinocytes and selected for G418-resistant colonies. One cell line, K1/16, was isolated after pSV2neo/16 transfection and G418 selection. Southern blot hybridizations of DNA isolated from this line showed ≈10 copies of an intact HPV-16 genome had integrated into the chromosomal DNA (Fig. 4). Continued passage of the K1/16 cell line indicates that it is probably a permanent line, since the line has survived more than 1500 cell generations (37 passages; split ratio, 1:40) without any evidence of slowed growth. This is in striking contrast to either primary human keratinocytes (23, 24) or pSV2neo-transfected keratinocytes, which have a limited lifespan of 40–100 cell generations.

The ability of HPV-16 to immortalize primary keratinocytes has been observed by others (23–25); however, subculturing transfected keratinocytes on plastic dishes has not revealed any significant changes in cellular morphology. We examined whether subculturing K1/16 cells on collagen

rafts might uncover morphological differences between untransfected and HPV-16-containing cells derived from primary keratinocytes. While normal foreskin keratinocytes cultured in the raft system (Fig. 3*C*) showed morphology similar to foreskin tissue (Fig. 3*D*), early passage cultures of K1/16 showed histological abnormalities. Parabasal crowding was evident in the lower layers and the upper layers had either enlarged nuclei or small pyknotic nuclei surrounded by vacuolated cytoplasm (Fig. 3*E*). This morphology was analogous to that seen in mild dysplasia (Fig. 3*F*), where the vacuolated cells have been referred to as "koilocytes" (26). At later passage levels, the K1/16 line exhibited a greater degree of altered morphological differentiation (Fig. 3*G*). Cells in the uppermost layers of the stratified epithelium exhibited a small and nucleated morphology typical of basal cells instead of the large and anucleated morphology typical of suprabasal cells. In addition, mitotic figures were found in the uppermost layers of the stratified K1/16 cells. The morphology of these later passage cultures was similar to that seen in severe dysplasia (Fig. 3*H*; ref. 27). These experiments were also repeated five times with similar results.

RNA Analysis of Transfected Cells on Collagen Rafts. The K1/16 cell line and HPV-16-containing SCC-13 cells were examined for expression of viral-specific RNAs. Representative RNA blots are shown in Fig. 5. HPV-16-containing SCC-13 lines expressed two viral-specific transcripts of approximately 3.0 and 1.5 kilobases (Fig. 5*a*, lane 2), while no HPV-16 transcripts were detected in parental SCC-13 cells (Fig. 5*a*, lane 1). When probed with the entire viral genome, K1/16 lines at the 22nd passage were observed to express three HPV-specific RNA bands of approximately 4.5, 3.0, and 1.5 kilobases (Fig. 5*b*, lane 1) similar in size to those seen in HPV-16-transfected SCC-13 lines. No HPV-specific transcripts were observed in primary keratinocytes (Fig. 5 *b* and *c*, lanes 2). RNA extracted from an earlier passage (14th) of K1/16 and hybridized with the entire viral genome showed three bands of similar size to that seen at higher passage (data not shown). The same three bands were detected when the blot was rehybridized with an E6/E7-specific RNA probe, indicating that all three contained transcripts from this region (Fig. 5*c*, lane 1).

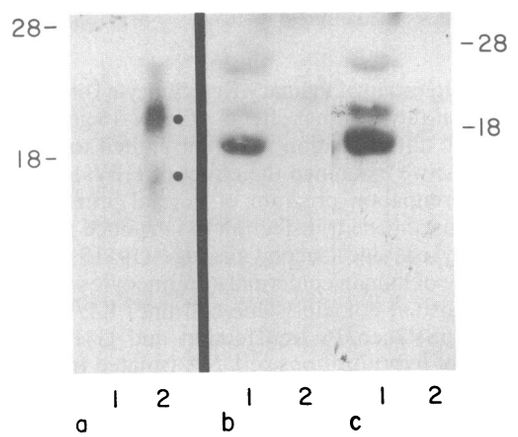

FIG. 5. (*a*) RNA blots from cells after culture on collagen rafts hybridized with ^{32}P-labeled linear HPV-16. Lanes: 1, untransfected SCC-13 cells; 2, SCC-13 cell line transfected with pSV2neo/16 (SCC-13/16-1). Dots indicate the different sizes of HPV-16-specific transcripts; on the left of the figure are the positions of the 28S and 18S ribosomal RNAs. (*b*) RNA blots from 22nd passage K1/16 cells after culture on collagen rafts hybridized with ^{32}P-labeled linear HPV-16. Lanes: 1, 22nd passage keratinocytes (K1/16); 2, normal untransfected keratinocytes. (*c*) Same filter as in *b* but hybridized with T7 polymerase transcripts of the E6/E7 region of HPV-16 DNA labeled with [^{32}P]UTP. In *a*, *b*, and *c*, 30 μg of total RNA was loaded into each well and the exposure time after hybridization was 18 hr.

Karyotype Analysis. Chromosome analysis was carried out on normal keratinocytes as well as on an early and late passage K1/16 cells. All of the normal keratinocytes examined (n = 15) had a normal male chromosomal pattern. Eleven of the 13 K1/16 cells examined at ≈50 cell generations showed trisomy of a number of chromosomes. All of the 22 late passage cells (≈550 cell generations) examined exhibited aneuploidy similar to that seen in early passage cells and 9 of 22 cells showed additional translocations between chromosomes 6 and 10 and chromosomes 3 and 11.

DISCUSSION

HPVs are the presumed etiologic agents of intraepithelial neoplasias of stratified squamous epithelium, especially of the urogenital tract. The target cells are the keratinocytes, which, after infection, are inhibited from differentiating in a normal manner. In the most severe intraepithelial neoplasia, little or no differentation takes place and cells throughout the full depth of the epithelium have the appearance of abnormal basal cells. The most common intraepithelial neoplasia occurs in the cervix, although similar histological abnormalities are seen in many other epithelial tissues including the penis and vulva.

Investigations on the role of HPV as an etiologic agent have been hampered by the lack of a good *in vitro* system for keratinocyte differentiation. In this study, we have used a system that faithfully reproduces *in vitro* the differentiation patterns of keratinocytes *in vivo*. In the raft system, normal epithelium stratifies and produces differentiation-specific keratins (12, 20). We have used this system to examine the effects of HPV-16 sequences on the differentiation of both (*i*) SCC-13 cells, an established human keratinocyte cell line that retains the ability to partially differentiate, and (*ii*) early passage human foreskin keratinocytes. When SCC-13 cells containing HPV-16 viral sequences were examined in the raft system, cells had the appearance of basal cells with no epithelial differentiation. In contrast, SCC-13 cells containing only the pSV2neo gene exhibited no change in differentiation relative to the parental SCC-13 line. Similarly, primary keratinocytes containing transfected HPV-16 sequences became immortalized and exhibited an altered differentiation pattern. Cellular abnormalities such as a lack of ordered differentiation, koilocytosis, abnormal mitotic figures, abnormal chromosome staining, and reduced cytoplasmic/nuclear ratio in the suprabasal cells of the epithelium were seen with the collagen raft system.

Our studies suggest that HPV-16 gene products are capable of altering the differentiation pathway of epithelial cells in a manner similar to that seen in genital intraepithelial neoplasia. In cervical tumors, the E6/E7 region is the primary portion of the viral genome that is transcribed (28–30). The E6/E7 region of HPV-16 and -18 has been shown to have transforming activity in rodent fibroblasts and expression of this region is probably required for HPV-induced neoplasias. The presence of transcripts from the E6/E7 region in RNA from our HPV-16-transfected cell lines indicates that these cells express similar HPV-16 transcripts to those seen in cervical tumors *in vivo* (29, 30).

Remarkably, the histological changes indicative of grade III neoplasia were observed in our system after a few hundred generations, even though progression from grade I to III *in vivo* often takes years. A possible explanation for this may lie in the fact that our HPV-16 cell lines contain integrated copies of HPV DNA. Progression from stage I to III *in vivo* may involve integration of the viral genome, a process that we have previously suggested may be an activation event for E6/E7 expression (10). In premalignant lesions *in vivo*, the viral DNA is often found as an episome, while in malignant lesions the viral DNA is usually integrated (1, 2, 31). In our

HPV-containing cells, HPV DNA has already integrated in the genome by the time of selection and hence the process of transformation may be accelerated. Other events such as chromosomal duplication or translocations may also be required for full malignant conversion. In our studies, we observed translocations between chromosomes 6 and 10 and chromosomes 3 and 11 in higher passages of K1/16, which were not observed at earlier passages. We do not know whether this is a significant event in progression *in vitro*.

The processes of immortalization and complete blockage of differentiation are not necessarily linked in transformed keratinocytes. This is perhaps best illustrated by the behavior of SCC-13 cells on collagen rafts. While these cells are immortalized as a result of malignant transformation, they still retain some ability to differentiate. Interestingly, the introduction of HPV-16 sequences into these cells seemed to result in the loss of this limited capacity to differentiate. Similarly, primary keratinocytes transfected with HPV-16 DNA simultaneously appeared to be immortalized and blocked in their program of epidermal differentiation. Whether the HPV transforming proteins E6 and E7 might possess the ability to immortalize primary keratinocytes and inhibit their differentiation is not known. We have examined two other pSV2neo/16 mass-transfected cultures in the raft system (unpublished observations) and observed similar alterations in differentiation as seen in the K1/16 line at late passage; however, we cannot completely exclude the possibility that a spontaneous event contributes to the alteration of differentiation.

HPV-16 and -18 are associated with a wide range of genital intraepithelial neoplasias. HPV-induced lesions from the penis, vulva, and vagina closely resemble those from the cervix. In our study, we have used epithelium from facial skin and foreskin and reproduced the morphological changes seen in HPV-induced lesions of the penis and cervix. These observations suggest that HPV may be able to induce similar changes in keratinocytes derived from many types of stratified squamous epithelia.

Genital epithelial cancers may be viewed as lesions in which both immortalization and inhibition of terminal differentiation have occurred. In fact, histological grading of the disease is based on the proportion of the epithelial thickness occupied by undifferentiated basal-like cells (27). Thus, the results presented in this study are consistent with the proposed role of HPV-16 as the etiologic agent of at least the early stages of cervical cancer. The use of the collagen raft system should enable an *in vitro* study of HPV infection in its normal host cell.

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