Tetrasomy Is Induced by Human Papillomavirus Type 18 *E7* Gene Expression in Keratinocyte Raft Cultures[1]

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ABSTRACT

We have demonstrated previously that oncogenic human papillomaviruses (HPVs) induce basal cell tetrasomy in low-grade squamous intraepithelial lesions of the cervix. To identify HPV genes and growth conditions involved in this process, we analyzed: (*a*) organotypic raft cultures of primary human keratinocytes transfected with whole HPV-18 genomes; and (*b*) organotypic raft cultures acutely infected with recombinant retroviruses expressing the HPV-18 *E6, E7,* or *E6/E7* genes from the differentiation-dependent HPV-18 enhancer-promoter. Cultures were examined for HPV DNA by *in situ* hybridization and for karyotype by interphase cytogenetics. Tetrasomy occurred in the suprabasal strata of raft cultures expressing *E7* and *E6/E7* but not in those expressing *E6* alone or in a control culture. These data indicate that suprabasal tetrasomy occurs in association with expression of the *E7* gene alone. Basal cell tetrasomy was additionally observed in the raft culture transfected with whole HPV-18 genomes, consistent with observations in low-grade squamous intraepithelial lesions. The distribution of tetrasomic cells in these raft cultures may reflect the involvement of additional viral genes or possibly differences in the pattern of viral oncogene and host gene expression.

INTRODUCTION

Tetraploidy is a natural biological occurrence in plants, some fish, such as the common carp (1), and even the occasional mammal such as the red viscacha desert rat (2). It has also been associated with the process of wound healing (3). However, the development of tetraploidy is also related to the process of tumor formation. *In vitro* studies have suggested that tetraploidy is a transitional state between diploidy and aneuploidy in chemically induced neoplastic transformation (4). In addition, viral proteins from SV40 and oncogenic HPV[3] types can induce a similar change in DNA content in cultured cells. We have demonstrated in a previous study that tetrasomy occurs in naturally occurring condylomas and low-grade SILs of the cervix infected with HPVs. Tetrasomy of suprabasal keratinocytes, without involvement of basal keratinocytes, was identified in lesions infected with oncogenic and non-oncogenic HPV types. By contrast, basal keratinocyte tetrasomy was restricted to lesions infected with oncogenic HPV types (5). Although tetraploidy refers to reduplication of the cellular DNA content (4N) and tetrasomy represents sister chromatid separation after DNA re-replication, both events are likely to involve dysregulation of cell cycle control.

HPVs are small epitheliotropic DNA viruses of which there are >80 different types (6). The cervical epithelium is a particularly receptive target to >30 HPVs (7). These viruses are grouped into non-oncogenic types associated with benign hyper-proliferative "warty" lesions (*i.e.,* papillomas and condylomata) and oncogenic types that, at a low frequency, can additionally cause preinvasive intraepithelial neoplasias and progression to cervical carcinomas (8). Although HPVs are a major contributor to oncogenesis, *in vitro* studies have shown that the viral genes are only capable of inducing immortalization (9), and that mutations in cellular genes are required for full malignant transformation.

Terminal squamous differentiation is necessary for HPV to initiate the productive phase of infection (10, 11). Because of their small genome size (7.9 kb), HPVs rely heavily on cellular replication proteins to support their own DNA replication and therefore have developed ways to subvert key host factors that regulate the expression of these. Consequently, postmitotic, differentiated keratinocytes can be stimulated to reenter S-phase to support viral DNA replication (12, 13). If, however, the viral oncoproteins were up-regulated in proliferating stem or reserve cells, surveillance mechanisms that prevent abnormal replication of cellular DNA could be overcome, and accumulation of genetic abnormalities would then ensue (14).

Central to the disruption of cell cycle control by HPV is the ability of the viral E7 protein to bind to and inactivate the unphosphorylated pRb protein and other members of the pRb family including p107 and p130 through an LxCxE motif (15, 16). This interaction leads to dissociation of the E2F/DP transcription factors from pRb and transcriptional derepression of genes that are regulated by E2F and are required for S-phase entry and progression, including cyclins E and A. Up-regulation of these cyclins *in vitro* by HPV-16 causes disruption of the G_1-S cell cycle transition and precedes neoplastic transformation in normal human fibroblasts (17). In addition, the HPV E6 oncoprotein targets the p53 tumor suppressor protein, which is a critical regulator of G_1-S and G_2-M cell cycle checkpoints (14). Furthermore, HPV E6 and E7 oncoproteins independently abrogate G_2-M checkpoints, including that which couples completion of S-phase and initiation of mitosis (18). Loss of such a checkpoint can result in cellular DNA re-replication in cycling cells, in part because of S-phase entry without progression into mitosis. Inactivation of both of these tumor suppressor proteins in stem cells compromises the ability of the cells to maintain DNA integrity, accurate chromosome segregation, and cell division, as manifested by chromosome breaks, polyploidy, or aneuploidy.

Numerical chromosome abnormalities have been associated with expression of the *E6* or *E7* gene of oncogenic HPV types in submerged cultures, although these observations were not consistent. For instance, transfection of HPV-16 *E7* expressed from the SV40 promoter induced doubling of chromosome number in primary human epidermal keratinocytes, as identified by metaphase cytogenetics (19). In the same study, the effect of *E6* gene function was analyzed in primary mouse keratinocytes and was not associated with detectable numerical chromosome abnormality (19). However, in another study, aneuploidy was identified in late-passage fibroblasts expressing

HPV-16 *E6* but not in those expressing HPV-16 *E7* alone (17). The explanation for these different observations is not clear.

To investigate the viral gene or genes responsible for the induction of tetrasomy observed in naturally occurring cervical lesions (5), we used epithelial raft cultures derived from human keratinocyte cell lines produced by transfection of primary NHEKs with the whole HPV-18 genome. Such cultures support viral DNA amplification and progeny production in some of the suprabasal cells (11). We also examined raft cultures of primary human keratinocytes at early passages that were acutely infected with retroviruses expressing the *E6, E7,* or *E6/E7* genes of HPV-18. In these transduced cells, HPV gene expression is under the control of the native HPV-18 1.1-kb URR, the activity of which is dependent in turn upon squamous differentiation (20). Differentiation-dependent activation of the URR has been substantiated by using raft cultures transduced with a retrovirus containing URR-lacZ as a reporter (21). These experiments have established that the URRs of the oncogenic HPV-18 and the non-oncogenic HPV-11 are active in submerged proliferating PHKs, a condition possibly resembling wound healing. The promoter is then turned off in basal cells within a few days upon stratification and squamous differentiation in the raft cultures. However, it is reactivated in the differentiated strata. Consequently, in the HPV-18 URR-E7 or URR-E6/E7 raft cultures, there is very little *E7* expression in basal cells in the established squamous epithelium.

These two types of raft culture systems enabled us to analyze the induction of tetrasomy by HPV. Cultures were examined for HPV DNA by *in situ* hybridization and karyotype by interphase cytogenetics. Dual hybridization for HPV DNA and interphase cytogenetics was used to investigate the relationship between viral DNA replication and chromosome abnormalities.

MATERIALS AND METHODS

Raft Cultures of NHEK Cell Lines Transfected with Whole HPV-18 Genomic DNA. Raft cultures were prepared as described previously (11). Briefly, full-length HPV-18 viral DNA (~8 kb) and a plasmid encoding a hygromycin B-selectable marker were transfected into NHEKs by electroporation. After selection with hygromycin, the cells were seeded onto collagen matrices containing J2 3T3 fibroblast feeder cells and when confluent, raised onto steel grids and lifted to the air-liquid interface. Viral gene expression was shown to peak at 12 days after stratification and differentiation of cultures.

Raft Cultures of PHKs Acutely Transduced with HPV-18 URR-E6, URR-E7, URR-E6/E7, or Vector-only Retrovirus. The HPV retrovirus constructs and the production of virus particles and raft culture systems have been described (20, 21). Briefly, retroviral vectors containing the HPV-18 URR linked to *E6, E7,* or *E6/E7* were electroporated into the ecotropic packaging cell line psi-cre. Media containing virus particles were then used to infect the amphotropic cell line GP+E AM 12. After selection with G418 (Geneticin), media containing infectious particles were used to infect PHKs. After a 2-day selection with G418, the surviving keratinocytes were used immediately to prepare the raft cultures. Negative control rafts were infected with retrovirus vector-only. All cultures were prepared using the same batch of PHKs at the same time, and all cultures were formalin fixed and paraffin embedded after being cultured for 9 days at the air-medium interface.

HPV Localization. ISH using a digoxigenin-labeled genomic probe to HPV-18 was as described previously (5). Briefly, 6-μm sections of formalin-fixed, paraffin-embedded raft tissues were predigested with proteinase K before overnight hybridization at 37°C in buffer containing 2 ng/μl HPV-18 probe, 50% formamide, 2× SSC, and 10% dextran sulfate. After posthybridization washes in 4× SSC at 37°C, the signals were detected after sequential incubations with mouse anti-digoxin (1:5000; Sigma Chemical Co.), biotinylated sheep antimouse (1:200; Amersham, Life Sciences), streptavidin-conjugated AP (1:100; Dako), and visualized with Fast Red AP substrate (Vector Laboratories).

Interphase Cytogenetics. Sections were pretreated with sodium thiocyanate, pepsin/HCl, and hybridized as described previously (5) using a digoxi-

genin-labeled chromosome 1 satellite III (pUC1.77) probe kindly provided by Dr. A. H. N. Hopman (University of Limburg, Maastricht, the Netherlands) and biotinylated chromosome 3 and 17 probes (Oncor, Gaithersburg, MD). Signal detection was with peroxidase-conjugated antibodies, diaminobenzidine and hydrogen peroxide. Signal number was determined at a magnification of ×630 as previously defined and validated (22). To determine the frequency of tetrasomy, 2000 keratinocytes were assessed for each raft.

Dual Hybridization for HPV DNA and Interphase Cytogenetics. Dual hybridization was based mainly on the interphase cytogenetic methodology. Sections were pretreated with sodium thiocyanate and pepsin/HCl and hybridization overnight at 37°C in hybridization buffer containing both digoxigenin-labeled chromosome 1 probe and HPV-18 biotinylated by nick translation. After a 60% formamide, 2× SSC posthybridization wash at 42°C for 20 min, signals were detected with a series of combined antibody steps: mouse anti-digoxin (1:5000; Sigma Chemical Co.) and streptavidin conjugated with AP (1:100; Dako); rabbit antimouse conjugated with HRP (1:80; Dako) and biotinylated goat antiavidin (1:100; Vector Laboratories); swine antirabbit HRP (1:100; Dako), and Streptavidin AP. AP signal development was with Fast Red TR/naphthol AS-MX phosphate and then HRP with diaminobenzidine/hydrogen peroxide.

RESULTS

Histology. H&E-stained, 5-μm sections of the URR-E6, URR-E7, URR-E6/E7, and vector-alone raft cultures revealed a well-organized, differentiated epithelium with distinct basal, spinous, and granular layers and cornified surface. There was no basal atypia or mitotic figures. A number of binucleate cells were present in the suprabasal layers in the E7 and E6/E7 rafts but not in the E6 or vector-alone cultures. The whole genomic HPV-18 raft culture was also differentiated but showed mild basal atypia with scattered mitoses resembling a low-grade SIL. In addition, some binucleation and koilocytosis was observed in the upper suprabasal strata.

Localization of Viral DNA by *in Situ* Hybridization. Individual punctate signals, which correlate with the presence of integrated HPV DNA (23), were observed in the cell nuclei of both types of raft culture (Fig. 1, *A* and *C*). In cultures transduced with URR-E6, URR-E7, or URR-E6/E7, the signals were small but present in most cells along the length of the epithelium and also throughout the epithelial layers (Fig. 1*C*). In the HPV-18 raft culture, this signal was much larger in size, as would be predicted when detecting the full viral genome, as opposed to individual genes (Fig. 1*A*). Also present in this culture were a small number of cells containing diffuse signals in the suprabasal layers, consistent with viral DNA amplification (Fig. 1*B*). These cells were scattered along the length of raft epithelium overlying cells containing punctate signals. No signals were observed in the nuclei of keratinocytes transduced with vector-only retrovirus, attesting to probe specificity.

Induction of Numerical Chromosome Abnormalities by HPV-18 *E7*. To identify numerical chromosome abnormalities, *i.e.,* tetrasomy, interphase cytogenetics was performed with biotinylated pericentromeric probes specific to chromosomes 1, 3, and 17. Only a limited number of probes were deemed necessary, because previous work on SILs has shown that HPV-induced chromosome reduplication involves at least chromosomes 1, 3, 4, 6, 10, 11, 17, 18, and X (24).

Basal cell tetrasomy involving all three chromosomes was present in the raft cultures transfected with the whole HPV-18 genome (Figs. 1, *D* and *F*, and 2*A*), similar to tetrasomy observed in low-grade SILs (5). No differences were observed between the individual chromosomes. Four signals were identified in isolated and small clusters of basal cells scattered along the length of the raft, but in most cases, the adjacent suprabasal cells were disomic. This spatial relationship suggests that basal tetrasomy may indicate a delay in, rather than an abrogation of, mitosis. Individual and small clusters of tetrasomic
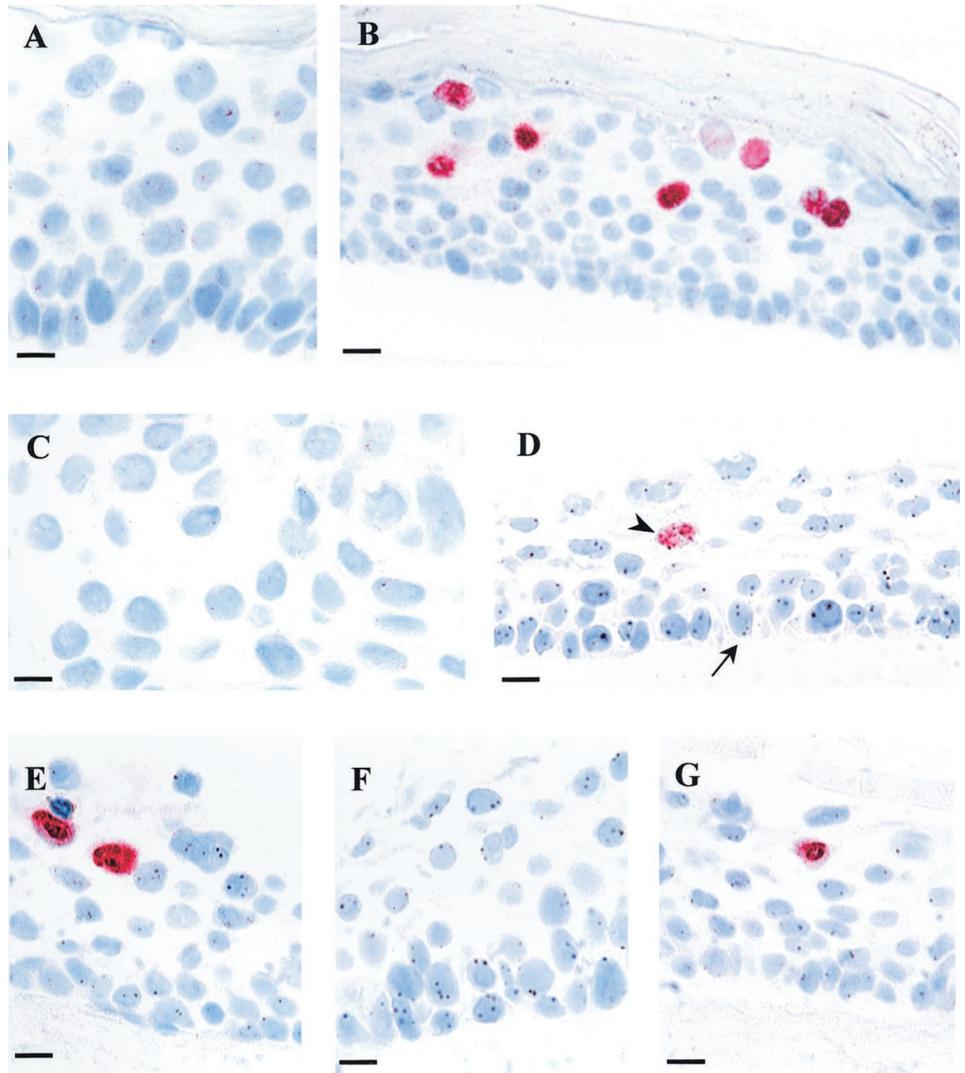
Fig. 1. HPV ISH showing both individual punctate signals (*A*) and diffuse signals (*B*) in rafts containing the whole HPV-18 genome. Only small punctate signals are present in the HPV-18 URR-E6/E7 raft (*C*). Dual hybridization for HPV (*red*) and chromosome 1 (*brown*) on the raft culture containing the whole HPV-18 genome (*D–G*). Tetrasomy is observed in the basal layer (*arrow*) below cells showing a diffuse HPV ISH staining pattern (*arrowhead*), indicative of viral DNA amplification (*D*) and in suprabasal cells adjacent to those showing viral DNA amplification (*E*). Basal tetrasomy is present in regions of the culture where no viral DNA amplification has taken place (*F*). Viral amplification also occurs in areas showing no numerical chromosome abnormalities (*G*). *Bar: A* and *C,* 10 µm; *B* and *D–G,* 15 µm.

cells were also identified in the suprabasal layer (Fig. 2*B*). Overall, tetrasomy was present in 1.8% of keratinocytes in this raft. In both the URR-E7 and URR-E6/E7 raft cultures, no basal cell tetrasomy was identified, but occasional suprabasal cells were tetrasomic (Fig. 2, *C* and *D*) with all three chromosome probes. Overall, suprabasal tetrasomy was present in 0.9% of keratinocytes in the URR-E7 raft and 1.0% of keratinocytes in the URR-E6/E7 raft. Tetrasomy was not observed in the basal or suprabasal layers in the URR-E6 or vector-alone control raft cultures, both of which were disomic (Fig. 2, *E* and *F*).

Tetrasomy and Viral DNA Amplification. To determine whether tetrasomy was associated with viral DNA amplification, dual hybridization for HPV and interphase cytogenetics was carried out in the culture containing the whole HPV-18 genome (Fig. 1, *D–G*). Although occasional suprabasal cells with a diffuse HPV ISH staining pattern, consistent with productive viral DNA synthesis, contained four chromosome signals, most tetrasomic cells, particularly those in the basal layer, did not show evidence of viral DNA amplification (Fig. 1, *D* and *F*). Moreover, tetrasomy was present in both the basal and suprabasal strata of the culture in regions where no viral DNA amplification had taken place (Fig. 1*F*). Conversely, viral DNA amplification was also present in areas showing no evidence of tetrasomy (Fig. 1*G*).

DISCUSSION

Basal cell tetrasomy observed in low-grade SILs is associated with oncogenic HPV infection. *In vitro*, numerical chromosome abnormalities have been associated previously with constitutive expression of HPV-16 *E6* in fibroblasts and HPV-16 *E7* in epidermal keratinocytes (17, 19). The use of raft cultures has clarified that when expressed in a differentiation-dependent manner, the HPV *E7* gene alone is capable of inducing tetrasomy in differentiated squamous epithelium, the natural target tissue of HPV infections. Suprabasal tetrasomy was observed in the *E7* and *E6/E7*-transduced raft cultures but not in *E6* or vector-only raft cultures. These observations are in agreement with the fact that only the E7 protein, but not E6, can induce unscheduled cellular DNA replication in postmitotic, differentiated keratinocytes (12) and additionally demonstrate that E6 is not necessary for the induction of suprabasal tetrasomy. Although the frequency of tetrasomy was relatively low (~1% of keratinocytes), the acquisition of chromosome abnormalities by even a small number of cells may be biologically significant. Moreover, the biological relevance of this observation is supported by the morphological distribution of tetrasomy in these rafts, which is similar to that identified in some naturally occurring condylomata and SILs, in which suprabasal tetrasomy was present without basal tetrasomy in association with either oncogenic or non-oncogenic HPV infection (5). By contrast, both
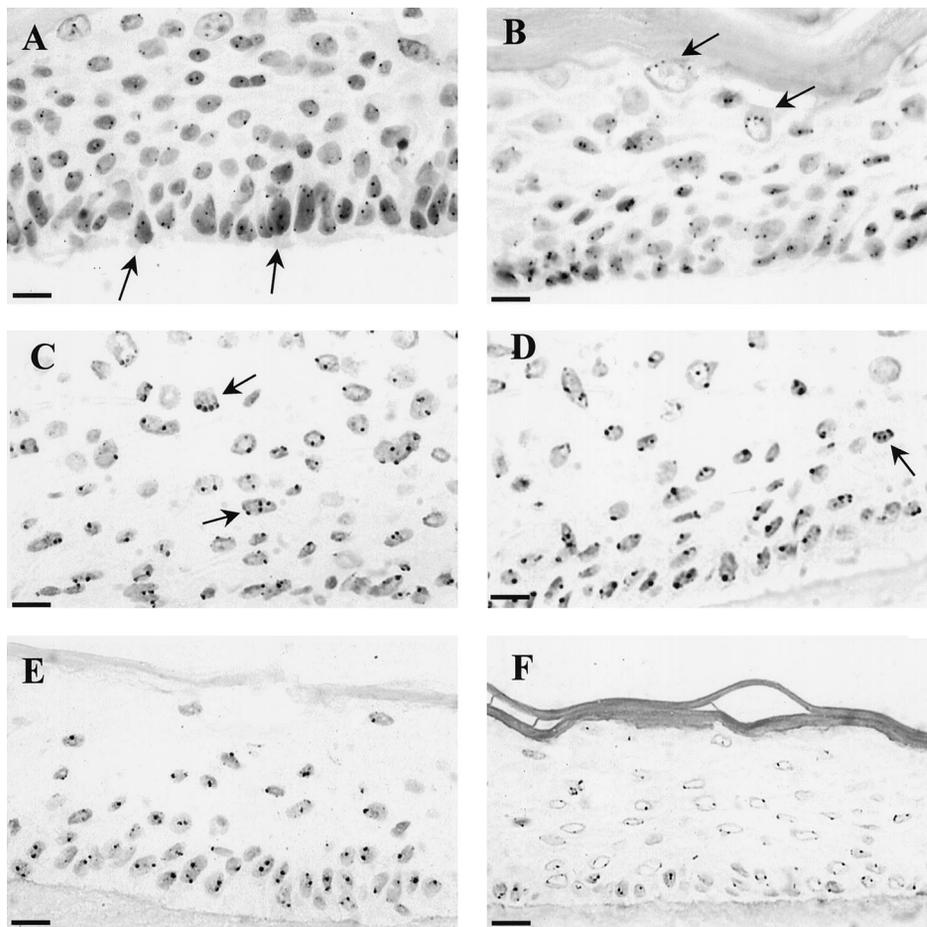
Fig. 2. Tetrasomy in raft cultures expressing HPV genes. Tetrasomy of chromosome 17 (*arrows*) is present in basal cells (*A*) and suprabasal cells (*B*) in the raft containing the whole HPV-18 genome. Suprabasal tetrasomy of chromosome 1 is observed in raft cultures containing HPV-18 URR-E6/E7 (*C*) and HPV-18 URR-E7 (*arrows*; *D*). No numerical chromosome abnormalities are observed in vector-only (*E*) or HPV-18 URR-E6 rafts (*F*). *Bar*: *A–F*, 15 μm.

basal tetrasomy and suprabasal tetrasomy were present in the HPV-18 raft culture, which resembles low-grade SIL histologically. This is consistent with our previous observations that basal tetrasomy is associated not only with oncogenic HPV infection but also with basal epithelial dysplasia (5).

The E6, E7, and E6/E7 clones used to infect the raft cultures were designed in such a way that transcripts derived from the murine leukemia virus long-terminal repeat would not be translated because of out-of-frame start and stop codons in the URR, the activity of which was dependent in turn upon host cellular differentiation. By using a URR-lacZ construct, it has been established previously that the URR is turned off in basal cells within a few days after the stratified epithelium is formed (21). Therefore, it is likely that little or no E7 protein is present within basal cells. The differences between the morphological distribution of tetrasomic cells in the two raft culture systems may correlate with the localization/distribution or level of E7 expression.

What other differences between the two raft culture systems may account for the distribution of tetrasomic cells? We propose that the mechanism of induction of tetrasomy by HPV may be different in postmitotic, differentiated cells from that in cycling basal cells. To elaborate, in normal differentiated keratinocytes, p21cip1 protein is constitutively expressed but rapidly degraded. However, upon expression of E7 or E6/E7 in these cells, S-phase genes required for viral replication are reactivated in postmitotic cells. In a subset of these cells, p21cip1 is costabilized in a complex with cyclin E and cdk2. These cells are distinct from those that successfully reenter S-phase in which neither cyclin E nor p21cip1 protein is present at detectable levels. In papillomas caused by HPV-6 or HPV-11, postmitotic cells that accumulate high levels of cyclin E and p21cip1 protein, and those

in S-phase or containing abundant viral DNA or mRNA, were also largely found in separate populations (25, 26).[4] It would seem that the high level of endogenous p21cip1 prevents the cells from reentering S-phase. We speculate that it might contribute to blocking cell division upon completion of S-phase, leading to suprabasal tetrasomy. The p21cip1 protein is known to be functionally active in these raft cultures and inhibits cyclin E/cdk2. All raft cultures contain abundant cdk2 but, in the E7-expressing rafts, although there is a considerably higher level of cyclin E and cyclin E/cdk2 complex (as demonstrated by Western blot), the associated H1 kinase activity is not significantly higher than the control raft cultures because the complex also contains p21cip1.[4] Although other studies have reported that p21cip1 is elevated and functionally inactive in high-risk papillomavirus-expressing cells (27, 28), these studies were performed using monolayer cultures of human lung fibroblasts and human keratinocytes and may not necessarily reflect the situation in differentiating epithelium, because the levels of p21cip1 RNA were low relative to that in the differentiated keratinocytes (25, 29). The mechanism by which suprabasal tetrasomy occurs is not restricted to oncogenic HPVs, as it has also been observed in low-grade SILs and in condylomas infected by non-oncogenic HPV types (5). Moreover, a small number of suprabasal cells with a diffuse HPV ISH signal in the HPV-18 raft culture contained four chromosome signals, indicating that tetrasomy can occur in cells replicating viral DNA. This is consistent with the natural function of HPV E7, *i.e.,* the induction of S-phase reentry in postmitotic cells to facilitate viral DNA amplification. However, suprabasal tetrasomy was present in cells that did not show evidence of viral

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[4] F. Noya, W-M. Chien, T. R. Broker, and L. T. Chow, unpublished results.

4861

amplification, demonstrating that tetrasomy occurs independently of viral DNA replication, perhaps because of a deficiency in viral E1 or E2 proteins necessary to initiate viral DNA replication (12).

We suggest that a different circumstance leads to basal cell tetrasomy. The relatively long-term culture required to enrich the HPV-18 DNA-transfected NHEKs selects for cells with elevated expression of *E6* and *E7* genes, conferring these cells with an extended life span, relative to the normal six or seven passages before senescence. Some of these cells may have integrated viral genomes or have incurred alterations in their transcription properties. Expression of viral oncogenes in proliferating cells may then disrupt cell cycle checkpoints, resulting in basal tetrasomy. Indeed, elevated viral oncogene expression in basal cells has been observed previously in raft cultures of keratinocytes newly immortalized by oncogenic HPVs that have a histology resembling SILs (30). The absence of abundant cyclin E or p21cip1 protein in the basal cells (data not shown) also supports our contention that these cell cycle regulatory proteins are regulated differently in basal proliferating cells *versus* suprabasal differentiated strata that do accumulate these proteins. Indeed, dual hybridization in this culture demonstrated that basal tetrasomy did not coincide with viral DNA amplification. We suggest that, among the HPV-18 transfected NHEKs, a subset of cells maintains the differentiation-dependent expression of viral oncogenes. Unscheduled cellular DNA replication or viral DNA amplification would then take place in some, but not all, postmitotic, differentiated descendants (11, 12, 26).

Factors additional to *E7* gene expression could also be important in the development of basal cell tetrasomy. Expression of the HPV-18 E6 oncoprotein, with consequent degradation of the p53 tumor suppressor protein, which regulates G_1-S and G_2-M cell cycle checkpoints (14), may be particularly critical. This interpretation is in agreement with the observation that constitutive expression of HPV-16 E6 in fibroblasts that have a long life span is associated with numerical chromosome abnormalities (17). The HPV-18 URR-E6 raft, in which expression of E6 was confirmed previously by complete elimination of p53 (25), was prepared with primary human keratinocytes acutely infected with retroviruses expressing E6 at passage 1; therefore, we cannot rule out the possibility that tetrasomy or aneusomy may occur at subsequent passages.

Another mechanism may also contribute to basal cell tetrasomy observed in the HPV-18 raft culture. Both viral gene expression and host responses may additionally be influenced by other early viral gene products, such as E1, E2, E4, and E5. In particular, the E1 protein, the replication initiator, binds both cyclin E and cyclin A with high affinity and is a substrate for associated cdks *in vitro* (31). E2 is a transcription factor and is also the primary viral origin recognition protein (12). In the presence of E1, E2 is also a substrate of these cdks *in vitro*. Thus, both E1 and E2 protein could, in principle, compete with cellular substrates for cdks leading to dysregulated cell-cycle control. Overexpression of E2 increases viral genome replication 5-fold and also induces cellular DNA re-replication in proliferating cells (32). However, the expression of additional viral genes cannot be solely responsible for basal cell tetrasomy because basal tetrasomy was not observed in lesions induced by non-oncogenic HPVs (5). Moreover, the mechanisms of viral DNA replication *in vitro* and differentiation-dependent viral DNA amplification *in vivo* are identical among oncogenic and non-oncogenic HPVs (13).

In conclusion, we have shown that expression of the HPV-18 *E7* gene alone is sufficient to induce suprabasal tetrasomy in keratinocyte raft cultures, and suprabasal tetrasomy does not depend on viral DNA synthesis. By contrast, basal tetrasomy occurs under conditions of cell growth and differentiation and viral gene expression that are different from those that lead to suprabasal tetrasomy. Tetrasomy in cycling basal cells implies loss of cell cycle control and may represent an important early event in neoplastic transformation.

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