Human Papillomavirus-immortalized Keratinocytes Are Resistant to the Effects of Retinoic Acid on Terminal Differentiation

Daniel T. Merrick, Allen M. Gown, Christine L. Halbert, Rebecca A. Blanton, and James K. McDougal


Abstract
In order to study how human papillomaviruses (HPVs) can alter normal epithelial cell differentiation, we looked at the response to retinoic acid (RA) of HPV-immortalized keratinocytes grown on organotypic cultures. Ten- to 30-fold higher concentrations of RA were required to block terminal differentiation in these cultures when compared to organotypic cultures of control cells. This resistance to RA was associated with maintained expression of differentiation-specific markers and, for keratin K1, Northern analysis showed that K1 mRNA was also detectable at 30-fold higher concentrations of RA in HPV organotypic cultures when compared to controls. These differences were reproducible and characteristic of all HPV cell lines studied, including very early passage HPV16-containing cell lines, suggesting that expression of HPV genes leads to this phenotype. Expression of epithelia-specific components of the RA response pathway was also studied by Northern analysis. At all RA concentrations, there were no detectable differences in overall levels of retinoic acid receptor γ or cytosolic RA-binding protein II mRNA found. Retinoid X receptor α expression was also evaluated, and, in two of three HPV-immortalized cell lines, it was found to be 2 to 3 times as abundant as in controls. Although this difference in retinoid X receptor α expression could contribute to RA resistance, the mechanism involved in producing this resistance could not be fully elucidated in these studies. However, resistance to the effects of RA on epithelial differentiation is demonstrated in organotypic cultures of HPV-containing cells, and it is shown that this is associated with maintenance of RNA and protein expression of differentiation-associated genes at abnormally high concentrations of RA.

Introduction
HPVs are epitheliotropic viruses which cause a variety of lesions. HPV types 16 and 18 have been associated with a number of cervical lesions and have been shown to play a significant role in the development of nearly all cervical carcinomas (1, 2). The effects of HPV16 and -18 on normal processes in epithelia have been the subject of a number of studies (3–8). Expression of HPV16 or -18 E6 and E7 ORFs is associated with abnormal epithelial morphology when grown in organotypic cultures (3, 4) or similar stratified cultures (5). In addition, abnormal maintenance of cell growth potential in suprabasal layers of epithelia has been described in HPV-containing epithelia (6–8). RA also has significant and diverse effects on a number of cellular processes, including inhibition of cellular differentiation and growth in epithelial and other cell types (9, 10). In addition, preventive and therapeutic effects of RA on squamous cell carcinomas have been described (11–15). Although both RA and HPV exert influence on similar processes in epithelia, the effects of their interaction on these processes has not been fully characterized.

RA in high concentrations blocks keratinization of stratified squamous epithelium in vivo and in organotypic cultures (16, 17), at least in part by negatively regulating transcription of a number of differentiation-specific genes. RA and its metabolites exert transcriptional control through nuclearRARs and RXRs (18–22). There are three subtypes (α, β, γ) of each of these receptor types, and when RA or its metabolites complex with these receptors, they form dimers that act via retinoic acid response elements in the promoter sequences of a number of genes. RARs in the presence of RA can inhibit transcription from reporter constructs that are linked to 5′ sequences from keratin genes involved in the differentiation of keratinocytes (23). These receptors can form either homodimers or heterodimers, and a number of recent studies suggest that some important effects of RA are mediated through RAR heterodimers, especially RAR-RXR heterodimers (24–27). RARs also associate with a number of other transcription factors such as AP-1 and thyroid hormone receptors (27–33), suggesting that a number of other signaling pathways may modify the ultimate effects of RA. In addition to the nuclear receptors, RA also complexes with CRABP and II. Although the function of these binding proteins has not been fully defined, a number of recent studies suggest that they may act to control intracellular RA concentration (34–36). Overexpression of CRABP in F9 embryonal carcinoma cells inhibits RA-induced differentiation of these cells, pointing to such a role for CRABPs (34).

Several defects involving components of the retinoic acid response network have been correlated with disease and abnormal phenotypes. In lung cancers and squamous cell

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2 To whom requests for reprints should be addressed, at Program in Cancer Biology, M616, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.

3 The abbreviations used are: HPV, human papillomavirus; ORF, open reading frame; RA, retinoic acid; RAR, RA receptor; RXR, retinoic X receptor; CRABP, cytosolic RA-binding protein; HIF, human foreskin epidermal cells; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; bp, base pairs; DLS, defolpizid serum.
carcinomas of the epidermis and oral cavity, lost or decreased expression of RAR-α, β, or γ has been described for a large portion of tumors studied (37, 38). In addition, loss of RA-mediated induction of RAR-β in tumorigenic HeLa cell hybrids distinguishes these cells from nontumorigenic hybrids (39). Despite the fact that a number of acute promyelocytic leukemias respond to RA treatment with induction of differentiation and inhibition of growth (40), many are resistant to RA. Recent studies of some of these resistant cell lines have shown that they either express abnormally low levels of RAR-α (41) or have abnormal RAR-α with decreased affinity for RA (42).

Abnormalities in CRABP expression have also been correlated with disease. Elevated CRABP transcripts have been identified in affected skin from patients with psoriasis (43). In patients treated with RA to induce remission of some acute promyelocytic leukemias, induction of CRABP expression in bone marrow cells that do not usually express CRABP has been described (44). The possibility that CRABP expression in these cells might contribute to secondary resistance to RA therapy in patients who relapse has been proposed (44).

Since both RA and HPV have effects on epithelial differentiation and tumorigenesis, we have decided to study the effects of RA on the growth and differentiation of HPV-immortalized epithelial cells in organotypic cultures. We have identified an abnormal resistance to the effects of RA on terminal differentiation in organotypic cultures of HPV-immortalized cells. Our results show that 10–30-fold higher concentrations of RA are required to achieve similar reductions in the expression of differentiation-specific markers at both the RNA and protein levels in HPV-containing cultures compared to normal controls. In addition, we have studied the retinoid response pathway in organotypic cultures of HPV-immortalized keratinocytes in an effort to uncover any aberrations that might explain this phenotype. Expression of RAR-γ, RXR-α, and CRABPII was evaluated by Northern analysis. An elevation of RAR-α was seen in most HPV-containing cultures, but no significant differences in the levels of RAR-γ or CRABPII mRNAs were noted between normal and HPV-immortalized organotypic cultures.

**Results**

**Effects of Retinoic Acid on Organotypic Cultures.** Organotypic cultures of normal control and HPV-immortalized keratinocytes were grown in parallel in a range of concentrations of RA. As with epidermis in vivo, increasing concentrations of retinoic acid block terminal differentiation of keratinocyte controls (LXSN) grown in organotypic cultures. Fig. 1 shows that whereas organotypic cultures of LXSN cells grown with medium containing 3 $\times$ 10^{-9} M retinoic acid show K1 protein expression and a well formed cornified layer, at 10^{-8} M RA, these cells show substantially decreased K1 expression. Growth with media containing concentrations of RA above 10^{-8} M RA blocked cornification and K1 expression in organotypic cultures of LXSNs (Fig. 1). Organotypic cultures of the HPV16-expressing LXSN16E6E7 cells also lost characteristic terminal differentiation in increasing concentrations of RA; however, 30-fold higher concentrations of RA were required to block this process. Fig. 2 shows that even at 10^{-7} M RA, organotypic cultures of LXSN16E6E7 cells demonstrate reduced but abundant K1 expression and formation of a cornified layer. Organotypic cultures of LXSN16E6E7 grown at concentrations above 10^{-7} M RA lost their ability to cornify and expressed only trace amounts of K1 protein. The passage level of LXSN16E6E7 cells used in these organotypic cultures differed very little from that of the vector-infected cells, suggesting that immortalization with HPV16 E6 and E7 ORFs is linked to this observed resistance to retinoic acid and that other factors, such as length of time in tissue culture, are not responsible for this phenomenon.

In addition to the LXSN16E6E7 cell line pictured, several other established HPV16 or -18 immortalized cells were studied, and all demonstrated resistance to the effects of retinoic acid on terminal differentiation (data not shown). Organotypic cultures of these cell lines, which ranged in passage level from passage 15 to passage 65, also required 10–30-fold higher concentrations of retinoic acid to block cornification and expression of K1 in comparison to organotypic cultures of normal HFEs grown in parallel.

**Northern Analysis of K1 Expression.** Total RNA extracted from organotypic cultures of LXSN- and LXSN16E6E7-infected cells was analyzed for K1 transcripts. The LXSN cultures showed abundant K1 transcripts in the absence of RA (Fig. 3, Lanes 1 and 2) and decreased, but detectable, K1 mRNA at 3 $\times$ 10^{-9} M RA (Fig. 3, Lanes 2). No K1 transcripts were not detectable in organotypic cultures grown in the presence of 10^{-8} M or higher concentrations of retinoic acid (Fig. 3, Lanes 4–7). The amount of K1 transcripts found in organotypic cultures of LXSN16E6E7 cells also decreased with increasing concentrations of RA, but transcripts were detectable at concentrations as high as 10^{-7} M RA (Fig. 3, Lanes 8–14). Thus, K1 RNA was expressed in LXSN16E6E7 cultures at 30-fold higher RA concentrations than it was in LXSN cultures. This blot was also stripped and reprobed with an internal standard, α-tubulin, which showed that similar amounts of RNA were loaded in each lane. These results do not distinguish between the possibilities that higher levels of K1 RNA are present in LXSN16E6E7 cultures as a result of either higher levels of K1 gene transcription or increased K1 mRNA stability. They do, however, show that steady-state levels of K1 mRNA are detectable in organotypic cultures of LXSN16E6E7 cells at 30-fold higher concentrations of RA than the highest concentration of RA at which they are detected in LXSN cultures, suggesting that the resistance to retinoic acid observed in LXSN16E6E7 cells may be due to a resistance to the transcriptional effects of RA.

**Northern Analysis of RAR-γ and CRABPII Expression.** RNA from organotypic cultures of uninfected normal keratinocytes (HFEs) derived from the same primary culture of keratinocytes as the LXSN16E6E67 cells was compared to LXSN16E6E7 RNA for RAR-γ expression. In both HFEs and LXSN16E6E7, RAR-γ transcripts were found at approximately equal levels at all concentrations of RA (Fig. 4A). In addition, the transcripts appear to be identical in size, suggesting that the HFE and LXSN16E6E7 cells express the same subtypes of RAR-γ. This transcript is approximately 3.3 kilobases in length, which corresponds to the size of RAR-γ transcript that is normally expressed in epidermis (19). There did not appear to be any significant difference in levels of RAR-γ transcripts between organotypic cultures of HFE and LXSN16E6E7 cells that might explain their different responses to retinoic acid. This blot was also used to compare steady-state levels of CRABPII mRNA in organotypic cultures of uninfected parental HFEs and LXSN16E6E7 cells. Both cell types expressed CRABPII RNA, and, although RNA levels were slightly higher in 16E6E7 cultures, the difference was very small (1.1–1.3-fold by densitometry; data not shown) and probably insignificant (Fig. 4B). For each cell type, induction of CRABPII message was observed at 10^{-7} M and 3 $\times$ 10^{-9} M RA (Fig. 4B, Lanes 5, 6, 11, and 12).
Fig. 1. LXSN RA titration. Organotypic cultures of control LXSN foreskin keratinocytes grown in a range of RA concentrations (molar concentrations indicated at left). These keratinocytes were infected with a retroviral vector encoding G418 resistance in the absence of other test genes. Organotypic cultures were fixed in methocarn solution, embedded in paraffin, sectioned, and then subjected to either hematoxylin and eosin staining (left) or immunohistochemical analysis of keratin K1 expression (right). Note loss of K1 expression at $10^{-9}$ M RA and loss of cornification at concentrations of RA above $10^{-8}$ M.
Fig. 2. LXSN16E6E7 RA titration. Organotypic cultures of LXSN16E6E7 foreskin keratinocytes grown in a range of RA concentrations (molar concentrations indicated at left). These keratinocytes were derived from the same parental culture as the LXSN keratinocytes and were infected with a similar retroviral vector which, in addition to expressing G418 resistance, also expressed HPV16 E6 and E7 ORFs. Organotypic cultures were fixed in methocarn solution, embedded in paraffin, sectioned, and then subjected to either hematoxylin and eosin staining (left) or immunohistochemical analysis of keratin K1 expression (right). Note maintenance of cornification and K1 expression at RA concentrations as high as $10^{-7}$ M.
Fig. 3. Northern analysis of keratin K1. Expression of keratin K1 mRNA in organotypic cultures of LXSN and LXSN16E6E7 cells grown at various concentrations of RA (molar concentrations indicated above each lane; DLS and DMSO samples are the same as described in legend to Fig. 3). Ten µg of total RNA were analyzed in each lane. K1 blot (top) was stripped and reprobed for tubulin expression (bottom), which was used as an internal standard.

Fig. 4. Northern analysis of RAR-γ and CRABPII. Expression of RAR-γ and CRABPII mRNAs in organotypic cultures grown at various concentrations of RA (molar concentrations indicated above each lane; DLS and DMSO samples are the same as described in legend to Fig. 3). HFE samples are from organotypic cultures of uninfected keratinocytes which were part of the same parental culture from which the LXSN and LXSN16E6E7 cells were derived. Twenty-five µg of total RNA were analyzed in each lane. RAR-γ blot (A) was stripped and reprobed for CRABPII expression (B), and then stripped again and reprobed for tubulin expression (C), which was used as an internal standard.

Fig. 5. Northern analysis of RXR-α. Expression of RXR-α mRNA in organotypic cultures grown at various concentrations of RA (molar concentrations indicated above each lane; DLS and DMSO samples are the same as described in legend to Fig. 3). HFE samples are from organotypic cultures of normal foreskin keratinocytes. 18-5 samples are from organotypic cultures of foreskin keratinocytes previously immortalized by transfection of HPV18 sequences (see “Materials and Methods” for complete description). Twenty-five µg of total RNA were analyzed in each lane. RXR-α blot (A) was stripped and reprobed for tubulin expression (B), which was used as an internal standard.

Densitometric analysis of the blot revealed that this induction was approximately 2–2.3-fold, with induction being slightly greater in HFEs (data not shown). CRABPII induction was not quite as dramatic as has been previously reported (45), but it may have increased at higher RA concentration and, nevertheless, does constitute a normal response to RA.

Northern Analysis of RXR-α Expression. A comparison of the steady-state levels of RXR-α mRNA between HFE and HPV18 immortalized cells grown in organotypic culture revealed some differences. The organotypic cultures of HPV-immortalized (18-5) cells expressed RXR-α at higher levels than that seen in the organotypic cultures of HFEs (Fig. 5A; compare Lanes 1–7 to Lanes 8–14). This blot was subjected to densitometric analysis, then stripped and rehybridized with a tubulin probe used as an internal control, and again assessed by densitometry. The expression of tubulin was similar in all samples (Fig. 5B). After correction of RXR-α densitometry readings with tubulin readings, the results showed that RXR-α levels were 1.5–5-fold higher in HPV-immortalized organotypic cultures, with the greatest differences seen at the highest RA concentrations. In addition to expression in organotypic cultures, we also compared the expression of RXR-α in normal HFEs to expression in 18-5s and the HPV16-immortalized F13 and LXSN16E6E7 cell lines grown in monolayer. These cells were grown in the presence of various concentrations of RA for 6 h before RNA was extracted. Northern analysis revealed that in 18-5s and F13s, RXR-α was expressed at 1.5–3-fold higher levels than in normals (data not shown), showing that overexpression of RXR-α can occur in both HPV16- and 18-immortalized cells and suggesting that this overexpression is not dependent on the state of differentiation of the HPV-immortalized keratinocytes. RXR-α expression in the early passage LXSN16E6E7 cell line, however, did not appear to be significantly elevated in comparison to normals, suggesting that overexpression of RXR-α may not be a ubiquitous feature among HPV-immortalized cells. Whether this overexpression of RXR-α might play a role in producing the resistance to RA in some or all of the HPV-immortalized cells in which this phenotype has been observed could not be fully determined in this study; however, the possibility that it might contribute to the phenotype described cannot be overlooked and will be discussed.
Effects of RA on HPV16 E6 and E7 Expression. The expression of the HPV16 E6 and E7 ORFs (E6/E7) was evaluated by Northern analysis in the LXS16E6E7 and F13 cell lines both in organotypic cultures and in monolayer cells treated for 6 h with RA. E6/E7 expression is controlled by the natural HPV16 NCR in F13 cells, and by the Moloney murine leukemia virus long terminal repeat in the LXS16E6E7 cells. In monolayer, RA has little effect on E6/E7 expression in either cell line (Fig. 6A), although slight induction of E6/E7 expression may be present at higher RA concentrations in the LXS16E6E7 cell line. These results are somewhat in contrast to previous reports on the effects of RA on these promoters (46, 47). In a recent study of Moloney murine leukemia virus-driven expression in the same retroviral construct used in the LXS16E6E7 cell line, RA was shown to enhance transcription (46). This study, however, was done in the hematopoietic cell line HL60, and thus these different results may reflect tissue-specific differences. It should also be noted that the greatest induction in the HL60 cells was noted after 24 h of RA treatment; thus, length of RA exposure may be the source of these differences. Length of exposure to RA may also explain the disagreement between our results and those reported by Khan et al. (47) in which RA was shown to inhibit E6/E7 expression from the natural HPV16 NCR promoter. In their study, expression was evaluated after 72 h of RA treatment, and it is possible that this inhibition of NCR-controlled E6/E7 expression is not apparent after 6 h of RA treatment. Interestingly, in organotypic cultures, high levels of RA seemed to inhibit E6/E7 expression in LXS16E6E7 to some degree, while not affecting E6/E7 expression in F13 (Fig. 6B). Thus, although RA treatment has some effect on E6/E7 expression, it probably does not significantly affect the actions of these proteins in these cell lines.

Discussion

We have described the growth of normal or vector-infected and HPV16- or -18-immortalized foreskin epithelial cells on organotypic cultures and have demonstrated that 10–30-fold higher concentrations of retinoic acid are required to block terminal differentiation of the HPV-containing cells compared to the controls. All five HPV-containing cell lines tested showed this resistance, and, for each cell line, between two and four repeats of the study all produced identical results. In addition to showing that this was a reproducible phenomenon, we also studied HPV16-containing cells shortly after the introduction of HPV sequences such that they were closely matched in passage level with the normal or vector-infected control cells. These HPV-immortalized cells also demonstrated resistance to retinoic acid action, strongly supporting the suggestion that immortalization with HPV16 E6 and E7 ORFs leads to this resistance. These observations may, at first look, seem contrary to those reported in some previous studies, in which HPV-containing cells were reported to have increased sensitivity to RA (48, 49). The increased sensitivity to retinoic acid was mainly seen in the response of these cells to the growth-inhibitory properties of RA, which were generally demonstrated only after several days of RA treatment. The kinetics of the growth effects of RA suggest they may be an indirect consequence of RA exposure (48, 50) and thus are probably mediated by a different pathway than that involved in the differentiation response to RA. Nonetheless, the fact that RARs have now been shown to exert their effects via a variety of different homo- and heterodimers (24–33) suggests that, even for genes whose expression is directly controlled by RA, there may be a number of distinct regulatory pathways involved. Indeed, preliminary experiments aimed at determining whether HPV-immortalized cells were resistant to the effects of RA on genes whose transcription is enhanced by RA have shown that this may not be the case. These studies were started with one such keratin gene, K19, but it was found that this gene is aberrantly expressed, at least to some degree, by all HPV-immortalized cell lines studied (51). Studies of the interleukin 1β gene, however, showed that treatment of normal HFES and HPV-immortalized cells grown in the presence of a range of RA concentrations produced similar induction of interleukin 1β transcription. This study was therefore aimed at more fully characterizing the effects of RA on terminal differentiation and the expression of genes involved in this process in HPV-immortalized cells.

The ability to terminally differentiate at higher RA concentrations was evaluated by examining expression of the differentiation-associated keratin K1 protein and RNA. In addition, expression of the differentiation-associated protein filaggrin was studied and, like K1 protein, was found to be
inhibited in organotypic cultures of HPV-immortalized cells only at concentrations of RA 30-fold higher than that needed to block its expression in normal control cultures (data not shown). Northern analysis showed that, like K1 and filaggrin protein expression, K1 mRNA expression could be demonstrated in organotypic cultures of HPV-immortalized cell lines grown with media containing 30-fold higher concentrations of RA than the highest concentration of RA at which K1 mRNA could be found in control cultures (Fig. 3). This suggested that there is a resistance to the transcriptional effects of RA in HPV-containing cells, although it did not rule out other explanations such as increased RNA half-life. Recent reports have implicated 5' promoter sequences in the RA-mediated control of expression of keratin genes, including the RA-inhibited, terminal differentiation-associated K10 gene (23). This report also showed that RARs acting via negative response elements were responsible for inhibition via these 5' promoter sequences. In addition, another report has found that the kinetics of such inhibition suggests that RA has a direct effect on expression of these genes (50).

Given these observations, we examined the various components involved in the response to RA, to see whether there were differences in the expression of these factors in the organotypic cultures that could be correlated with the resistance to RA seen in HPV-immortalized cells. Of these factors, the most striking results were obtained in the Northern analysis of RXR-α which was expressed at approximately 2–3-fold higher levels in organotypic cultures of the HPV-immortalized 18-5 cell line compared to normals. RNA from monolayer cultures of 18-5 and other HPV-containing cell lines also showed this overexpression of RXR-α, except in the early passage LXSN16E6E7 cell line. These results with the LXSN16E6E7 cell line suggest that overexpression of RXR-α may not be a ubiquitous characteristic of HPV-containing cell lines.

Further studies of early passage HPV16- and -18-expressing cell lines both in monolayer and organotypic cultures should help determine how RXR-α expression is controlled in HPV-containing cells and help show whether or not the LXSN16E6E7 cell line is unique in this respect. Nonetheless, the observed overexpression of RXR-α in these RA-resistant cells is intriguing. It is difficult to say whether it could be contributing to the resistant phenotype, but a number of recent studies have shed light on how RXR-RAR interactions affect RA responses. Several of these papers have shown that RXR-RAR heterodimers as well as other RXR heterodimers have increased affinity for RA response elements over RAR homodimers (24–26, 33, 52–54), making it seem paradoxical that overexpression of RXR-α is correlated with resistance to RA. It should be noted, however, that the response elements for which these observations were made are all involved in the activation of transcription by RA, and that for most of the differentiation-related genes, RA inhibits expression. In fact, it has been shown that, although RARs can lead to repression of collagenase expression, RARs cannot (32), suggesting the possibility that overexpression of RXR might engage RARs in relatively more activating functions than inhibitory ones. The sequences involved in mediating the negative effects of RA on keratin K1 and other differentiation-associated genes have not been precisely identified, and their relation to the response elements just discussed is unknown. A recent study, however, has implicated RARs as being important in mediating the differentiation-inhibiting effects of RA, especially at high (10^{-6} M) RA concentrations (55). Thus, it is difficult to imagine what role, if any, this observed difference in RXR-α expression might play in creating resistance to RA action in HPV-containing cells.

In addition to RXR-α, RAR-γ and CRABPII expression was also studied. No significant differences in the levels of RNA present in HPV or control organotypic cultures were found for either of these products. Overexpression of CRABPII might be associated with a phenotype of resistance to retinoic acid, but lack of increased CRABPII RNA in organotypic culture of HPV-containing cells strongly suggests that this is not a contributing factor. The possibility that differing levels of CRABPII protein are present, however, has not been ruled out. Expression of abnormal levels or alternate isotypes of RAR-γ mRNAs could also have suggested a potential source of resistance to RA. However, the RAR-γ RNA was expressed at similar levels in both normal and HPV organotypic cultures, and the transcripts appeared to be identical in size, strongly suggesting that they are the same isotypes. It is possible that the protein is present at different levels or that they are functionally altered in the HPV cell lines, and previous reports showing that resistance to RA action in some leukemic cell lines can result from functionally altered RAR-α (42) suggest that this may be worth evaluating. Another possibility is that the level of some other transcription factor that might interact with RAR-γ, or in some other way affect its actions, is altered in the HPV cell lines. A number of studies have shown that interactions between RARs and thyroid hormone receptor, COUP-TF protein, and AP-1 complexes can change the transcriptional effects of RA or other ligands (27–33, 56). Thus, it will probably be valuable to assess the status of these factors also in future experiments.

We have shown resistance of HPV cell lines to the normal effects of retinoic acid on terminal differentiation of keratinocytes. This may be most interesting in the broader sense that it shows that HPV can alter normal differentiation responses in keratinocytes. Control of expression of a number of HPV genes is linked to the state of differentiation of the keratinocytes with a few HPV ORFs, whose functions are critical to the ultimate formation of a productive lesion, only being expressed in the upper layers of epithelia (57, 58). Given these observations, it would seem important for HPVs to have developed some mechanism by which they could maintain infected epithelial cells on the course to terminal differentiation, even in the presence of such deterrents as RA. In addition, HPV-infected cells maintain the ability to divide even after leaving the basal layer (6). Terminal differentiation is generally considered to proceed in normal epithelial cells only when further cell division has been blocked; thus, there may have evolved in HPVs a mechanism by which terminal differentiation and continued cell division can be simultaneously maintained. We have demonstrated, at a molecular level, that HPV alters the normal differentiation responses of keratinocytes, and further elucidation of the causes of resistance to retinoic acid in HPV-immortalized cells should provide good insight into the mechanisms by which HPV can alter this process.

Materials and Methods

Keratinocyte Cell Lines. Control and HPV-infected cell lines were previously produced by infection of primary foreskin epithelial cells with retroviral vectors pLXSN and pLXS N16E6E7, respectively, by C. Halbert (7, 59). pLXS N16E6E7 contains HPV type 16 E6 and E7 ORFs, and the expression of these proteins in this line (referred to as LXSN16E6E7) has been previously verified (7, 59). Vector
alone (referred to as LXSN) and vector plus HPV16 E6E7 sequences were introduced into foreskin keratinocytes from the same primary cultures, and LXSN and LXSN16E6E7 cells used in organotypic cultures were of approximately the same early passage level (i.e., passages 3–8). The LXSN16E6E7 cell line has also been grown in culture to late passages (i.e., greater than passage 30), demonstrating its immortalized phenotype (59). Included in these experiments were uninfected parental foreskin keratinocytes (referred to as HFEs) whose RNAs were sometimes used as controls. In addition to these cells, other HPV16- or -18-immortalized cells were also used. Although early passage (passages 15–25) FPE1L8 and 18-11 and later passage (passages 50–65) FPE1L13 and 18-5 cell lines all showed very similar phenotypes in organotypic cultures produced in RA titration experiments, only results from experiments using the 18-5 cell line are shown. All of these cell lines had been produced previously by calcium phosphate transfection of HPV16 or -18 sequences derived from cervical carcinomas (51, 60–62). The morphology and marker expression of these cell lines grown in organotypic cultures has been previously characterized (3, 6, 7). Primary foreskin keratinocytes were used as controls in these experiments, and were established and maintained as described previously (3, 6). All cell lines were grown and maintained in keratinocyte-serum-free medium ( Gibco), which was changed every 2 days. Cells were split at a ratio of 1:3 when confluent until enough cells were produced for use in organotypic cultures.

Organotypic Cultures. Construction of dermal equivalents and seeding of epithelial cells have been previously described (3). Organotypic growth medium consists of Dulbecco’s modified Eagle’s medium plus 10% delipidated fetal bovine serum supplemented with hydrocortisone, triiodothyronine, epidermal growth factor, cholera toxin, and insulin as described by Wu et al. (63). The fetal bovine serum was delipidized using a silica-based reagent (International Enzyme, Falbrook, CA) as previously described (6). All-trans-retinoic acid (Sigma, St. Louis, MO) was dissolved in DMSO, and a variety of dilutions were made such that several aliquots of 10,000X solutions could be frozen for each concentration of RA tested. For each experiment, there were two negative control groups in which either nothing or a 1:10,000 dilution of DMSO was added to the organotypic culture medium. In the other groups, RA was added back to the medium at a concentration ranging from 10−10 M to 10−6 M. Since retinoic acid loses its activity rather quickly, new media were made up for each group every 3–4 days. Media were also stored in the dark. The organotypic cultures were grown submerged 4–5 days and raised, at the air-liquid interface, for an additional 11–13 days. At the end of the experiment, the cultures were subjected to one of two procedures. Three or four organotypic cultures were grown for each cell line at each concentration of retinoic acid, and one of these was fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid) for subsequent hematoxylin and eosin staining and immunohistochemistry analysis. The remaining rafts in each group were snap frozen in liquid N2 and stored at −70°C for ultimate RNA extraction.

RNA Preparation. The procedure of Chomczynski et al. (64) was followed. Briefly, snap-frozen rafts were dropped directly into solution D (guanidinium thiocyanate, sodium citrate, sarcosyl, and 2-mercaptoethanol), and sodium acetate, phenol, and chloroform-isomyl alcohol mixture were added sequentially. Following centrifugation, the samples were taken through the various precipitations and extrac-

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References


