Immortalization by Human Papillomavirus Type 16 Alters Retinoid Regulation of Human Ectocervical Epithelial Cell Differentiation

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ABSTRACT

Human cervical cells are a primary site of papillomavirus infection and 90% of all cervical tumors are positive for human papillomavirus (HPV) DNA. Over one-half million cases of HPV-associated cervical, valvar, and penile cancers are reported per year. Yet, in spite of the magnitude of this problem, the effects of HPV infection on cervical cell growth and differentiation are not well characterized. To study these effects we have developed a clonal cell line of HPV-16-immortalized ectocervical epithelial cells, ECE16-1.

In the present study we demonstrate that under normal growth conditions the cytokeratin content of ECE16-1 cells is dramatically altered compared to normal cervical cells; the level of K5, K6, K14, K16, and K17 is reduced and the level of K7, K8, and K19 is increased. We demonstrate that this change is largely due to a difference in the response of the cells to retinoids, as growth in retinoid-free medium produces a complete normalization of cytokeratin levels. Upon addition of natural and synthetic retinoids, the levels of cytokeratins K5, K6, K14, K16, and K17 are reduced, while the levels of cytokeratins K19, K7, and K8 are increased. Cytokeratin K13 levels are only slightly altered.

The level of involucrin, a precursor of the cervical cell envelope (superficial cell), is not changed by immortalization nor is it regulated by retinoids. Transglutaminase activity is also not appreciably altered by immortalization; however, ECE16-1 cells make fewer envelopes than normal ECE cells.

Our results clearly indicate that natural and synthetic retinoids suppress the differentiation of HPV transformed cervical cells. In early, low grade, cervical intraepithelial neoplasia, transcription of the HPV E6/E7 oncoproteins is confined to the suprabasal layers. Our results suggest that retinoids, because they inhibit the differentiation of HPV16 immortalized cervical cells, may reduce the extent of viral oncoprotein transcription and thus be useful in slowing the neoplastic process.

INTRODUCTION

Cervical cancer is a major health problem worldwide (1). The high-risk group of human papillomaviruses (HPV16, HPV18, HPV31, and HPV33) are suspected etiological agents associated with the development of cervical carcinoma (2-5). Integrated forms of HPV\(^2\) DNA are found in high-grade cervical tumors (6, 7). This integration nearly always occurs within the E1-E2 reading frames which disrupts the product of the E2 gene, a trans-acting regulator of the HPV promoter/enhancer responsible for transcription of the viral immortalizing genes, E6 and E7 (8, 9). Transcripts encoded by the E6 and E7 reading frames are nearly always expressed in these tumors (10) and experiments in cultured cells using mutant viruses indicate that the E6 and E7 genes are sufficient and necessary for cellular transformation (11). Moreover, these genes are known to interact with other proteins, p105-RB and p53, that are thought to be involved in regulation of cell proliferation (12, 13).

An important problem that has not been adequately explored is the effect of HPV on the biochemical phenotype of human ectocervical epithelial cells and the role of exogenous agents in modulating the response of these cells to the viral gene products. Of particular interest are agents that regulate epithelial cell differentiation. Vitamin A and its derivatives are known to be important modulators of epithelial cell differentiation (14-16), and recent experiments indicate that they regulate the differentiation of normal human ectocervical epithelial cells (ECE cells) (17, 18). In the present study we immortalize primary cultures of human ECE cells with HPV16 and demonstrate that the cells are relatively undifferentiated compared to normal ECE cells. However, when the cells are grown in medium devoid of vitamin A and other retinoids, the phenotype nearly completely normalizes.

Our results indicate that retinoids dramatically suppress the differentiation of HPV16-immortalized cervical cells. Since HPV viral production and transcription appear to be restricted to more differentiated cells, inhibition of HPV-infected cervical cell differentiation by retinoids may be useful in suppressing the incidence and/or progression of HPV-associated cervical tumors.

MATERIALS AND METHODS

Cell Culture. Normal ectocervical cells were grown by using \(^{60}\)Co-irradiated 3T3 feeder cells by the method of Rheinwald and Green as previously described (19). The growth medium was a mixture of Dulbecco's modified Eagle's medium/F12 supplemented as described previously (19). Trans-Retinoic acid and the synthetic retinoids Ro 13-6298 and Ro 13-7410 were prepared as 1000-fold concentrated stocks in dimethyl sulfoxide. For experiments, near-confluent cultures were shifted to retinoid-free medium where the fetal calf serum was replaced by 5% delpidized fetal calf serum.

Recombinant Plasmids. The complete HPV16 genome, kindly provided by Dr. Harold zur Hausen (20), was inserted into the BamHI site of the plasmid pECENE.\(^3\) pECENE contains the neomycin phosphotransferase gene from Tn5 transcribed from the SV40 promoter and terminated by the SV40 terminator.

Transfection. Secondary cultures of normal ectocervical cells (strain ECTO-5) were plated at \(2 \times 10^5\) cells/cm\(^2\) dish. After attachment the cells were incubated with 20 \(\mu\)g of pHPV16-NEO in 3.0 ml of media containing 10 mg/ml Polybrene for 6 h, shocked with 30% dimethyl sulfoxide for 4 min, washed with Hank's balanced salt solution, and refed with fresh growth medium (21).

Protein Methods. ECE16-1 and normal ECE cells were incubated in medium containing one-tenth the normal level of radiocinet methionine and 50 \(\mu\)Ci/ml [\(^{35}\)S]methionine for 20 h. After labelling, cytokeratins were extracted as previously described (22) and characterized by 2-dimensional electrophoresis (23). Relative expression of cytokeratin bands was quantitated by laser densitometric scanning of the gels. Involucrin was detected immunologically by using an involucrin-specific antibody (24) and visualized by using \(\text{\footnotesize}^{125}\text{I}-\text{protein A (18).}\)

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\(\text{\footnotesize}^{2}\) The abbreviations used are: HPV, human papillomavirus; poly(A)\(^+\) RNA, polyadenylated RNA; ECE cells, ectocervical epithelial cells; TG, transglutaminase.

\(\text{\footnotesize}^{3}\) R. L. Eckert, unpublished data.

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HPV-16 IMMORTALIZATION OF ECE CELLS

Fig. 1. HPV16/SV40-NEO transfer vector. Plasmid map of pHPV16-NEO showing the gene encoding resistance to G418 (neo-r) under the control of the SV40 promoter and terminator. HPV16 DNA is delineated by the cross-hatched bar. The complete HPV16 genome was cloned into the pECENE0 vector as a BamHI fragment. The relative orientation of the HPV16 BamHI insert relative to the neo-r gene has not been determined. The HPV16 open reading frames E6, E7, Eia, Elb, E2, E4, E5, L2, and L1 are shown. 1, 2, and 3 represent the three reading frames. The upstream regulatory region of HPV16 is located between the L1 and E6 reading frames.

Envelope Quantitation. Viable cell counts and envelope counts were determined as previously described (18).

RNA Analysis. RNA was isolated from ECE cells and ECE16-1 cell lines by lysis in guanidine (16) followed by centrifugation through cesium chloride. Poly(A)+ RNA was selected by binding total RNA to oligodeoxythymidyate cellulose. RNA gel electrophoresis (25) and transfer blotting were exactly as previously described (16). Keratin and HPV16 transcripts were detected by using random-primed probes labeled with [32P]dCTP.

Transglutaminase Assay. Cells were homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, containing 2 mM EDTA, 2 mM dithiothreitol, and 3.9 µg/ml phenylmethylsulfonyl fluoride, and the supernatant was obtained by centrifugation (soluble TG). The pellet was reconhomogenized in the above buffer containing 0.002% Triton X-100, left on ice for 30 min, and the supernatant was obtained by centrifugation (particulate TG). Both the soluble and particulate fractions were assayed for TG activity by using methylated casein and [3H]putrescine as substrates (26, 27) in assay buffer containing 100 mM Tris-Cl, pH 7.6, 50 µM CaCl2, 2 mM dithiothreitol, and 3.9 µg/ml phenylmethylsulfonyl fluoride. Casein bound [3H]putrescine was recovered by trichloroacetic acid precipitation. After washing, the trichloroacetic acid pellet was dissolved in tissue solubilizer and was scintillation counted. The results were normalized based on protein content of the extracts.

RESULTS

Fig. 1 shows the structure of the plasmid utilized to transfer the complete HPV16 genome to primary cultures of human ectocervical epithelial cells. The HPV16 genome (20) was cloned as a BamHI fragment into the unique BamHI site in pECENE0 to generate pHPV16-NEO. Purified pHPV16-NEO was transferred to human ectocervical epithelial cells by Polybrene transfection (21). Clonal cell lines were selected with G418 and one cell line, ECE16-1, was selected for detailed characterization. This cell line displays a relatively normal epithelial cell morphology and has been in continuous culture for over 8 months.

E6 and E7 Reading Frames Are Important in Viral Immortalization of Ectocervical Epithelial Cells. RNA transcripts corresponding to the HPV16 E6 and E7 open reading frames (see Fig. 1) are frequently present in cervical tumors, suggesting that these reading frames encode genes involved in immortalization and/or transformation (6, 10, 11). Using the complete HPV16 genome as a probe, we identified four distinct transcripts of 1.7, 1.9, 2.4, and approximately 6 kilobases (Fig. 2). To determine whether any of these bands include RNA encoding the E6/E7 open reading frames, we screened with a probe specific for the E6/E7 region of HPV16 generated by using standard polymerase chain reaction procedures. All of the RNA

Fig. 2. Characterization of HPV16 transcripts in ECE16-1 cells. Poly(A)+ RNA (6 µg) isolated from ECE16-1 cells (Lane A) or normal ECE cells (Lane B) was fractionated on a formaldehyde-containing agarose gel (25), transfer blotted to nitrocellulose as previously described (16), and hybridized with the complete HPV16 genome labeled by random priming in the presence of [32P]dCTP. Four RNA species, labeled 1 through 4, were detected with the HPV16 probe. The size of each mRNA species is listed in the text and was determined by comparison with known keratin mRNA transcripts (30).
bands were detected by the E6/E7 probe (Fig. 3). These results indicate that all of the transcribed bands detected with the complete HPV-16 probe include the E6/E7 reading frame. The most abundant transcript is at 1.9 kilobases (Fig. 3). In addition, it is possible that transcripts could result from read-through into the E6/E7 region from the SV40 promoter present in our plasmid which promotes transcription of the neo-r gene (Fig. 1). This is not the case, however, since when the neo-r coding region was utilized as a probe, no radioactive bands corresponding to E6/E7 transcripts were detected (not shown).

HPV16 Immortalization Results in Apparent Reduction in ECE Cell Differentiation. Transformed cell lines derived from human tumors or immortalized in vitro are frequently less differentiated than the corresponding normal cells (15, 22, 28, 29). This is manifest at the biochemical level by a predictable change in the profile of keratin expression. Fig. 4 compares the pattern of keratin expression in normal ECE cells and in ECE16-1 cells. Normal ECE cells, as previously reported (19), express K5, K6, K13, K14, K16, K17, and K19 (Fig. 4A). As shown in Fig. 4B, in the immortalized cells the levels of K6, K14, K17, and K16 are dramatically reduced. Although only slightly reduced in this experiment, K5 is usually dramatically reduced. K13 levels remain unchanged and the levels of K7, K8, and K19 increase.

Keratin mRNA Levels Regulate Level of Keratin Protein. We had previously observed that keratin protein level directly reflects the level of the corresponding mRNA transcript in human tracheal and epidermal keratinocytes (16). We therefore investigated whether these changes in keratin content observed in ECE16-1 cells result from changes in mRNA content. Poly(A)* RNA isolated from ECE (Fig. 5, Lane 2) and ECE16-1 cells (Fig. 5, Lane 1) was gel fractionated and probed with complementary DNAs specific for each keratin transcript (30). In each case the change in keratin protein level reflects the mRNA
Retinoid Sensitivity Appears to Be Basis for Apparent Difference in Differentiation of HPV-16-immortalized Cells Compared to Normal Cells. Retinoids are known to modulate the differentiation of epithelial cell types. It therefore occurred to us that the basis for the apparent change in differentiation, as measured by the changes in keratin content, could be due to a difference in retinoid sensitivity of HPV immortalized cells. To test this hypothesis we grew ECE16-1 cells in retinoid-free medium or in medium containing natural or synthetic retinoids (Fig. 6).

DISCUSSION

Highly transforming forms of human papilloma virus are frequently associated with human cervical tumors (2-4) and are thought to be important etiological agents in the genesis of cervical cancer (3, 4). In spite of the importance of HPV as a disease agent, very few studies have examined its effects on...
cervical cell differentiation under controlled in vitro conditions. In the present study we describe a line of cervical cells developed by transfection with molecularly cloned HPV16.

HPV16 Immortalized Cells Produce Transcripts Encoding E6 and E7. It has been noted that HeLa and C4-1 cells produce HPV18 poly(A)+ RNA transcripts ranging in size from 1.5 to 6.5 kilobases that encode the early E6/E7/E1 region (10). These are fusion transcripts where genomic sequences are fused to the 3' end of the mRNA (10). Early gene transcription has also been reported in cervical cancer-derived CaSki cells (34). Based on these findings, we examined whether the ECE16-1 cell, which we immortalized with molecularly cloned HPV16, expressed similar products. Four transcripts at 1.7, 1.9, 2.4, and 6.0 kilobases were detected when probed with the complete HPV16 genome. All four transcripts were detected with a DNA probe specific for the E6/E7 coding region. The most abundant band was at 1.9 kilobases. Thus, all of the detectable mRNAs produced from the integrated HPV16 genome include the E6/E7 region. A simple explanation of these results is that each mRNA is initiated at a start site within the HPV promoter (2) but terminated at different poly(A) addition sites. This pattern of transcription of E6 and E7 fits with existing literature and is consistent with the idea that E6 and E7 expression is required for the immortalization of epithelial cells (11, 35, 36).

Retinoids Are Responsible for Altered ECE16-1 Cell Cytokeratin Profile. A previous report indicated that HPV-immortalized ECE cells display an altered pattern of keratin gene expression compared to normal cervical cells (36). We verified this effect by growing ECE16-1 cells and normal ECE cells under identical conditions and then analyzing cytokeratin peptide expression by 2-dimensional gel electrophoresis. The results indicate that cytokeratins K5, K6, K14, K16, and K17 are reduced, while K19, K7, and K8 are increased. The level of K13 is unchanged. We hypothesized that this effect could be explained by an altered sensitivity of the cells to a hormonal agent that might be present in the growth medium. Retinoids are potential candidates since they are important regulators of epithelial cell differentiation that reduce squamous epithelial cell differentiation (15, 37, 38). In particular, retinoic acid and various synthetic retinoids modulate keratin gene expression in normal human foreskin and conjunctival keratinocytes (16, 30), normal human ectocervical cells (17, 18), and SV40 large T-antigen-transformed human foreskin keratinocytes (22). When ECE16-1 cells were shifted to retinoid-free culture medium, the cytokeratin profile normalized and was indistinguishable from that of normal ECE cells. This suggests that HPV16-immortalized cervical cells are differentially more sensitive to retinoids than normal ECE cells. In normal ECE cells the keratins are regulated by retinoids, but the changes are less dramatic. Moreover, some keratins that are regulated by retinoids in ECE16-1 cells are not retinoid regulated in normal ECE cells. Thus, the response of the retinoid regulation of keratin gene expression in ECE16-1 cells is significantly different from the normal ECE cell pattern of regulation, an effect that must in some way be linked to the expression of the E6 and E7 gene products.

Retinoids Alter Morphology of HPV16-immortalized Cervical Cells. Since altered retinoid sensitivity appears to be responsible for the changed cytokeratin profile in ECE16-1 cells, we next examined whether other indices of ECE cell differentiation are also altered. Retinoids produced striking changes in ECE16-1 cell morphology by creating blank areas on the plastic surface, an effect that was completely absent in normal ECE cell cultures. The cells appear to be detached and bundled along the lines of least stress. We do not know the mechanism responsible for this change. It could result from retinoid-dependent changes in cell-cell or cell-substratum adhesion (39–41), changes in protease secretion (42–44), or changes in cytoskeletal structure.

* E. A. Rorke et al., manuscript in preparation.
different tumors, complicating the design of retinoids for cancer therapy.

Involucrin Cross-Linking and Transglutaminase Activity Are Elevated in ECE16-1 Cells in Absence of Retinoids. A major structure formed during terminal differentiation of ECE cells is the superficial cell which is utilized by clinicians to determine the phase of the menstrual cycle in women (33). This is the terminal product of ECE cell differentiation and consists of an envelope of cross-linked protein (18, 32). An important precursor of this structure is involucrin, a $M_r$ 60,000 protein (18, 32) which acts as an amine receptor in the cross-linking reaction (24). Normal ECE cells differentiate into superficial cells (envelopes) in culture, a process that is inhibited by retinoids (17, 18). As an index of the extent to which ECE16-1 cell differentiation is regulated by retinoids, we monitored the ability of the ECE16-1 cells to cross-link involucrin and produce transglutaminase in normal (retinoid-containing) medium and retinoid-free medium. ECE16-1 cells were found to express comparable levels of involucrin, in the presence or absence of retinoids.

However, this change is not observed when normal ECE cells are treated with similar retinoid concentrations.

Relative Retinoid Potency. An interesting observation is the relative potency of the natural and synthetic retinoids in different cell types immortalized with different oncogenes. trans-Retinoic acid, Ro 13-6298, and Ro 13-7410 regulate morphology and keratin gene expression in normal ECE cells (17, 18) and normal tracheal and conjunctival keratinocytes (16, 45). Ro 13-6298 and Ro 13-7410 are synthetic analogues of trans-retinoic acid (46). Ro 13-6298 is an ethyl ester that is most likely converted to the free acid, Ro 13-7410, by removal of the ethyl group. These types of reactions have been reported for other structurally similar retinoids (47). When we treated the KER-1 cell line, an SV40 large T-antigen immortalized line of human foreskin keratinocytes (22) with these compounds, Ro 13-6298 was completely inactive in modulating differentiation (22). This is in sharp contrast to the potent effects of this compound in ECE16-1 cells. This suggests that (a) the metabolism of the synthetic retinoid may differ in ECE cells versus foreskin keratinocytes, and/or (b) that the effect of SV40 large T-antigen immortalization on this metabolism is different from the effects of HPV16 E6/E7 protein immortalization. It further suggests that retinoid metabolism may differ markedly in different cell types immortalized with different oncogenes.

Fig. 7. Morphology of retinoid-treated ECE16-1 cells. ECE16-1 cells were grown for 8 days in retinoid-free medium containing no added retinoid (A) or supplemented with 100 nm trans-retinoic acid (B), 100 nm Ro 13-6298 (C), or 100 nm Ro 13-7410 (D). Each field is a phase contrast image taken at × 100.

Fig. 8. Involucrin level and cross-linking in normal ECE cells and ECE16-1 cells. Normal ECE cells (Lanes A and B) or ECE16-1 cells (Lanes C and D) were grown 20 days beyond confluence in normal growth medium (Lanes B and D) or in retinoid-free growth medium (Lanes A and C). Total cell extracts were prepared and equal cell equivalents were fractionated on adjacent lanes of an 8% polyacrylamide gel (53). The extract was transferred to nitrocellulose and the blot was incubated with involucrin-specific antiserum followed by visualization with $^{125}$I-protein A.

Fig. 9. Transglutaminase activity in normal ECE cells and ECE16-1 cells. Cells were grown in medium containing normal fetal calf serum (ECE16-1) or in medium containing retinoid-free serum (ECE16-1) (27). Transglutaminase activity is normalized per $\mu$g of total protein.
Moreover, both normal ECE cells and ECE16-1 cells displayed a comparable enhancement of involucrin cross-linking when grown in retinoid-free medium.

A particular form of TG is thought to be responsible for cross-link formation in the cornified envelope (26, 48). ECE16-1 cells and ECE cells displayed low levels of particulate TG activity in normal growth medium and 4- to 5-fold higher activity in retinoid-free medium. These results demonstrate that ECE16-1 cells are competent to produce transglutaminase-mediated cross-linking of involucrin and are stimulated to differentiate in the absence of retinoids.

Retinoids May Be Useful for Clinical Treatment of Cervical Dysplasia and Cancer. Cervical epithelial cells undergo profound changes in morphology and function during differentiation (17, 18, 32, 33). Although the mechanism is not understood, papillomavirus replication and transcription are closely linked to the differentiation process (49). In general vegetative viral replication, and presumably transcription, occur largely in the more differentiated cells. Thus, the quantity of virus within a lesion and the rate of transcript production may be decreased when differentiation is suppressed. Retinoids clearly suppress the differentiation of normal ECE cells (17, 18), and in the present manuscript we demonstrate that they suppress the differentiation of HPV16-immortalized ECE16-1 cells. In low-grade cervical intraepithelial neoplasia, the HPV virus is not integrated into the host DNA and E6/E7 transcription is observed in the upper, more differentiated layers of the epithemis (50, 51). Our results suggest that possibility that retinoids may be useful in the treatment of low-grade cervical cancer by virtue of the fact that they inhibit differentiation of the tumor cells. This could potentially lead to production of fewer viral-immortalizing transcripts (E6/E7) and a reduction in the rate of progression to malignancy. Further studies will be necessary to test this hypothesis.

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