Differentiation-independent constitutive expression of the human papillomavirus type 16 E6 and E7 oncogenes in the CaSki cervical tumour cell line

Chee K. Choo, Ellen A. Rorke and Richard L. Eckert*1,2,3,5

Departments of 1 Physiology and Biophysics, 2 Reproductive Biology, 3 Dermatology, 4 Environmental Health Sciences and 5 Biochemistry, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, Ohio 44106-4970, U.S.A.

CaSki cells are a human papillomavirus type 16 (HPV-16)-positive cell line that serves as a model for the study of advanced cervical carcinoma. Calcium is an important regulator of normal ectocervical epithelial cell differentiation. HPV E6 and E7 gene products are thought to be important in the process of cervical cell immortalization and hence important in the development of cervical cancer. In the present study we examine the relationship between CaSki cell differentiation and expression of the papillomavirus oncogenes. Shifting CaSki cells from medium containing low (0.06 mM) to high (1-4 mM) calcium results in an increase in cell-cell contact and increased differentiation as measured by an increase in the level of mRNA encoding cytokeratin K13, involucrin and type 1 transglutaminase, which are markers of differentiation in the cervical epithelium. In contrast, E6/E7 transcripts are produced in a differentiation-independent constitutive manner. These results and those from our previous experiments with HPV-16-immortalized but non-tumorigenic cell lines suggest that the constitutive, differentiation-independent expression of E6/E7 levels is a property of both tumorigenic and non-tumorigenic HPV-16-positive cancer cells.

Introduction

The high-risk group of human papillomaviruses (HPV-16, -18, -31 and -33, etc.) are strongly implicated in the pathogenesis of cervical cancer (Beaudenon et al., 1986; Bosshart et al., 1984; Broker, 1987; Di Luca et al., 1986; Durst et al., 1983; Pirisi et al., 1987; Woodworth et al., 1989; zur Hausen, 1977). The HPV genome is approximately 8 kb of DNA including two reading frames encoding the E6 and E7 oncogenes (Broker, 1987; Hirochika et al., 1988; Seedorf et al., 1985). Expression of E6 and E7 is sufficient for immortalization of cultured epidermal keratinocytes (Munger et al., 1989) and the activity of the P97 promoter, which regulates E6/E7 production, is regulated by both HPV and cellular proteins (Chan et al., 1989, 1990; Gloss et al., 1987; Swift et al., 1987).

In low-grade cervical intraepithelial neoplasia involving HPV-16 or in benign tumours produced by low-risk viruses, expression of E6/E7 has been reported to be localized to the upper layers of the cervical epithelium (Crum et al., 1988; Durst et al., 1991; Stoler & Broker, 1986; Stoler et al., 1990). In contrast, basal layer expression of E6 and E7 is observed when cervical cells containing integrated HPV DNA are implanted in nude mice (Durst et al., 1991). In other studies, expression of the E6/E7 reading frames was confined to the basal cell layer in anogenital papillomas produced by HPV-6 (Itfner et al., 1992) and unchanged by induction of differentiation in cells containing an HPV-31b episome (Hummel et al., 1992). Hence there is some disagreement as to whether E6 and E7 expression is differentiation-dependent or independent.

Calcium is an important physiological regulator of normal human epithelial cell differentiation. Mouse or human keratinocytes grown in media containing ≤ 0·1 mM-calcium express biochemical markers of non-differentiated cells and continue to proliferate (Choo et al., 1993; Hennings et al., 1980; Kasturi et al., 1993; Pillai et al., 1990; Rubin & Rice, 1986, 1988; Sharpe et al., 1993; Watt & Green, 1982; Yuspa et al., 1989). Addition of ≥ 0·5 mM-calcium causes the cells to stop dividing, to synthesize differentiation-specific biochemical markers and to form stratified layers and cornified envelopes (Choo et al., 1993; Heenen et al., 1992; Kasturi et al., 1993; Rothnagel et al., 1987; Yuspa et al., 1989). Moreover, a physiological calcium gradient has been observed in stratifying epithelia. In the skin the free...
calcium concentration is significantly higher in the superficial layer than in the basal layer (Forslind et al., 1984; Malmqvist et al., 1984; Menon et al., 1985); the higher concentration appears to be important in inducing cell differentiation, and as the cells differentiate they become increasingly permeable to Ca\(^{2+}\). Patch-clamp experiments (Mauro et al., 1993) indicate the presence of a Ca\(^{2+}\)-activated cation channel and a Cl\(^{-}\) channel in keratinocytes. The cation channel is permeable to Ca\(^{2+}\), and entry of Cl\(^{-}\) into the cells through the Cl\(^{-}\) channel may increase the transmembrane electrical potential allowing Ca\(^{2+}\) to enter. Measurement of intracellular Ca\(^{2+}\) levels indicates that differentiated human keratinocytes have significantly high intracellular Ca\(^{2+}\) levels (Sharpe et al., 1993). Moreover, La\(^{3+}\) (lanthanum), a trivalent competitor of Ca\(^{2+}\), prevents cornified envelope formation and transglutaminase (TG) activity in keratinocytes (Pillai & Bikle, 1992). La\(^{3+}\) displaces Ca\(^{2+}\) from its binding sites on the cell surface and blocks Ca\(^{2+}\) influx and efflux (Lettvin et al., 1964; Van Breeman et al., 1979; Weiss, 1974).

The mechanism by which Ca\(^{2+}\) stimulates epithelial cell differentiation may involve the activation of phospholipase C (PLC) (Jeng et al., 1985). PLC hydrolyses membrane phosphatidylinositol into diacylglycerol (DAG) and inositol-(1,3,5)-triphosphate (IP\(_3\)) (Berridge, 1988). DAG activates the membrane-bound protein kinase C (PKC), and IP\(_3\) triggers the release of intracellular Ca\(^{2+}\) stores. DAG analogues such as phorbol esters (Yuspa et al., 1980, 1982) and agents that induce the release of sequestered Ca\(^{2+}\) such as ionophores A23187 and ionomycin (Jaken & Yuspa, 1988; Moscat et al., 1989; Tang et al., 1988) mimic the effects of PLC suggesting that both agents are important in regulating differentiation.

We have previously shown, in ECE16-L cells, an HPV-16-immortalized but non-tumorigenic cell line (Agarwal et al., 1991), that calcium regulates differentiation marker gene expression, but not the level of the E6/E7 oncogenes (Choo et al., 1993). These results are consistent with differentiation-independent, constitutive expression of E6/E7. However the effects of calcium on papillomavirus transcription in a tumorigenic HPV-16-positive cell line has not been studied. In the present report we characterize the effects of calcium on cell differentiation and E6/E7 expression in CaSki cells, a tumorigenic cell line derived from cervical carcinoma and a model for advanced cervical cancer. Our results are consistent with expression of E6/E7 in a differentiation-independent, constitutive manner in tumorigenic cervical cancer cells.

**Methods**

**Cell culture.** The HPV-16-immortalized cell line ECE16-L was grown as previously described (Agarwal et al., 1991). The growth medium was a mixture of Dulbecco's modified Eagle's medium/F12 supplemented with 50% fetal calf serum (FCS), non-essential amino acids, insulin (5 μg/ml), cholera toxin (1 × 10\(^{-10}\) M), transferrin (5 μg/ml), 1,3,5-triiodothyronine (T\(_3\)) (2 × 10\(^{-9}\) M), hydrocortisone (1 × 10\(^{-8}\) M), epidermal growth factor (10 ng/ml), t-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), gentamicin (50 μg/ml) and adenine (1.8 × 10\(^{-5}\) M) (Gorodeski et al., 1990a, b). CaSki cells were grown under identical conditions. Cell lines were thawed and used within 20 passages. For experiments, near-confluent cultures were shifted to calcium-free medium and the serum was replaced with 5% delipidized and chelated FCS (DLCXCS). Delipidization removes retinoids and chelation treatment removes calcium (Brennan et al., 1975; Rothblat et al., 1976; Choo et al., 1993). The calcium concentration was adjusted by addition of calcium chloride to the culture medium.

**Involucrin (INV) assay.** INV is a precursor of the cornified envelope in cervical cells and is a marker of cervical cell differentiation (Agarwal et al., 1991; Choo et al., 1993; Gorodeski et al., 1989; Kasturi et al., 1993). INV levels were measured by electrophoresing equal cell equivalents (normalized based on cell counts) of total cell extract on an acrylamide gel, transferring to nitrocellulose and incubating with an antibody specific for human INV (Rice & Green, 1979). Binding of the primary antibody was visualized by incubation with \(^{125}\)I-Protein A followed by exposure on X-ray film. Band intensity was compared by densitometry.

**Cytokeratin profile.** Cytokeratins form a 20-member family (designated K1 to K20) of intermediate filament proteins that are expressed...
in epithelial cells in a cell type-specific and differentiation-dependent manner (Eckert, 1989; Sun et al., 1985). They serve as useful markers for diagnosing the differentiation status of ectocervical cells (Agarwal et al., 1991; Choo et al., 1993; Czernobilsky et al., 1984; Dixon & Stanley, 1984; Gorodeski et al., 1990a; Levy et al., 1988; Moll et al., 1983; Smedts et al., 1990; Weikel et al., 1987). To analyse for cytokeratins, CaSki cells were incubated for 20 h in [35S]methionine-containing medium supplemented with the appropriate concentration of calcium chloride exactly as previously described (Choo et al., 1993). The cytokeratins were then isolated (Agarwal & Eckert, 1990), electrophoresed on a two-dimensional gel and visualized by fluorography (O'Farrell et al., 1977).

Nucleic acid methods. Poly(A)+ RNA was isolated from cultured cells by guanidine lysis-CsCl centrifugation and oligo(dT)-cellulose chromatography (Aviv & Leder, 1972). Poly(A)+ RNA (3 to 10 µg/lane) was electrophoresed on RNA denaturing gels (Boedtker, 1971), transferred to a Biodyne A membrane and hybridized with 32P-labelled DNA (Gilfix & Eckert, 1985). The plasmids utilized in this study include pA-1 (actin) (Eckert & Green, 1984), pK13-1 (cytokeratin K13) (Eckert & Green, 1984), pSP642I-3 H6B (INV) (Eckert & Green, 1986) and pTG-7 (type 1 TG, TG1) (Floyd & Jetten, 1989). HPV-16 transcripts encoding the E6/E7 open reading frame were detected using a 0.83 kb PCR product including positions +51 to +883 of the HPV-16 viral DNA sequence (Choo et al., 1993; Smedts et al., 1990). The hybridization probes were labelled by random priming in the presence of [32P]dCTP.

**TGI assay.** TGI is the enzyme responsible for assembly of the cornified envelope in cervical epithelial cells and is a marker of cervical cell differentiation (Agarwal et al., 1991; Choo et al., 1993; Kasturi et al., 1993). Total, soluble and membrane-bound (particulate) TGI activity was assayed as previously described using methylated casein as
Fig. 4. Comparison of INV, K13 and TG1 levels in ECE16-1 and CaSki cells. Near-confluent CaSki or ECE16-1 cells were shifted to experimental medium containing 1.4 mM-calcium and grown until 10 days post-confluence. (a) Equivalent quantities of CaSki (lane C) or ECE16-1 (lane E) cell extract, normalized based on cell number, were electrophoresed on an 8% acrylamide gel and transferred to nitrocellulose. The membrane was then incubated sequentially with anti-INV antibody and 125I-labelled Protein A and exposed on X-ray film (Agarwal et al., 1991). No signal was observed when CaSki cell extract was incubated with secondary antibody alone (lane SA). (b) CaSki (panel C) and ECE16-1 (panel E) cells were labelled for 20 h in the presence of [35S]methionine. The cytokeratin fraction was prepared, electrophoresed in two dimensions and exposed on X-ray film. The cytokeratins are labelled as previously described (Agarwal et al., 1991; Choo et al., 1993; Moll et al., 1982); actin and vimentin are indicated by a and v, respectively. (c) Soluble and membrane-bound (particulate) TG activity was assayed as previously described (Agarwal et al., 1991). The results are expressed as pmoles of [3H]putrescine incorporated per µg of casein (pmol/µg protein) and the activity level is normalized based on protein concentration. Similar results were observed in each of three experiments. (©), low calcium, CaSki; (l-], high calcium, CaSki; (l), high calcium, ECE16-1.

the amine acceptor and [3H]putrescine as the amine donor (Agarwal et al., 1991).

Results
Calcium regulates CaSki cell morphology
As shown in Fig. 1, in the presence of a low extracellular calcium concentration (panel L, 0.06 mM), the CaSki cells remain well separated in the culture dish. Increasing the calcium concentration causes the cells to flatten and form close associations (panel H, 1.4 mM). If maintained in high calcium-containing medium for extended periods of time, these cells form a multilayered system.

Calcium regulation of keratin and INV protein levels
Calcium is known to regulate the levels of markers of differentiation (keratins, INV and TG1) in normal cervical cells (Kasturi et al., 1993). We therefore examined the effects of low (panel L, 0.06 mM) and high (panel H, 1.4 mM) calcium on CaSki cell cytokeratin expression (Fig. 2a). CaSki cells express cytokeratins K5, K6, K7, K8, K14, K16/K17 and K19 as well as a low level of vimentin (Fig. 2a). None of the cytokeratin levels are significantly modulated by the calcium shift and cytokeratin K13, a marker of ectocervical cell differentiation (Gorodeski et al., 1990a), is not detected. However K13 is detected when the CaSki cells are grown on a permeable filter (Gorodeski et al., 1994). In contrast, INV levels are modulated (Fig. 2b). Levels increase by 10-fold when cells are shifted from low (lane L) to high calcium-containing medium (lane H).

Calcium regulation of mRNA levels
The mRNAs encoding various markers of differentiation are regulated by calcium (Fig. 3). K13 and INV mRNA levels increase five- and sixfold when the cells are shifted from low (lanes L, 0.06 mM) to high (lanes H, 1.4 mM) calcium. TG1 mRNA levels also increase in response to calcium (Fig. 3, TG1). Actin (A) is included as a control, as its levels are not modulated by calcium. Time-course studies indicate that the longer the cultures are maintained at confluence, the larger the increase in the level of each respective marker mRNA (not shown).

Comparison of marker expression in ECE16-1 and CaSki cells
Fig. 4 compares INV, K13 and TG1 levels in ECE16-1 and CaSki cells. The immunoblot in (a) shows that ECE16-1 cells express at least 10 times more INV than CaSki cells. A comparison of cytokeratin levels (b) indicates that K13 is reduced in CaSki compared to ECE16-1 cells. In addition, ECE16-1 cells produce high levels of particle-associated TG1 activity, whereas ac-
**HPV gene expression**

Fig. 5. Comparisons of INV, K13 and TG1 mRNA levels in ECE16-1 and CaSki cells. Poly(A)⁺ RNA was prepared from ECE16-1 (lanes E) or CaSki (lanes C) cells, electrophoresed on a 1.4% agarose gel and transferred to a Biodyne A membrane. Parallel sets of lanes were hybridized with ³²P-labelled cDNAs encoding cytokeratin K13, INV, TG1 and actin (A) (Eckert & Green, 1984, 1986; Floyd et al., 1989). The membranes were then washed and exposed on X-ray film. Prolonged exposure of the gels resulted in detection of INV, TG1 and K13 mRNA in the CaSki cells (not shown). Similar results were observed in each of three experiments.

Fig. 6. Expression of HPV-16 transcripts in CaSki cells. (a) CaSki cells were grown in the presence of low (lanes L; 0.06 mM) or high (lanes H; 1.4 mM) calcium until 10 days post-confluence. Messenger RNA was then prepared, electrophoresed on a 1.4% agarose gel, transferred to Biodyne A membrane and hybridized with [³²P]dCTP-labelled cDNA encoding actin (A) (Eckert & Green, 1984) or a [³²P]dCTP-labelled PCR product encoding the HPV-16 E6/E7 region (E6/E7) (Choo et al., 1993). The migration of the 2.1 kb actin mRNA is indicated. (b) CaSki (lanes C) or ECE16-1 (lanes E) cells were grown until 10 days post-confluence in medium containing 1.4 mM-calcium chloride. Poly(A)⁺ RNA was prepared, electrophoresed on a 1.4% agarose gel, blotted to a Biodyne A membrane and hybridized with actin cDNA (A) or the HPV-16 PCR product encoding E6/E7 as outlined in (a). The arrowheads indicate the migration of the two major HPV-16 transcripts present in ECE16-1 cells. The actin blot is included in each panel as a control to ensure that equivalent quantities of mRNA had been layered on each lane. Similar results were obtained in each of three experiments.

**Calcium regulation of HPV-16 transcript levels**

The expression of the HPV E6/E7 reading frames has been shown to be necessary and sufficient for epithelial cell immortalization (Munger et al., 1989). Moreover, E6 and E7 have been reported to be expressed in suprabasal (differentiated) layers of HPV-infected cervical samples by some investigators (Durst et al., 1991; Stoler & Broker, 1986), but in the basal layers by others (Iftner et al., 1992). We therefore determined whether E6/E7 transcript levels are regulated when cell differentiation is stimulated by calcium. As shown in Fig. 6(a) CaSki cell E6/E7 transcript levels are not changed by shifting the cells from low (0.06 mM; lanes L) to high (1.4 mM; lanes H) calcium-containing medium. Moreover, a comparison of the level of E6/E7 expression in CaSki (lanes C) and ECE16-1 (lanes E) cells (Fig. 6b) indicates that CaSki cells produce much higher levels of E6/E7 mRNA than ECE16-1 cells.

**Discussion**

Since expression of specific papillomavirus transcripts appears to be linked to the differentiation programme in cervical epithelial cells, it is important to understand the effects of agents that regulate cell differentiation on HPV transcription. Calcium is a known regulator of differentiation in a variety of stratifying epithelial cell types, including epithelial cells derived from epidermis (Hennings et al., 1980; Rubin & Rice, 1986, 1988; Watt & Green, 1982; Yuspa et al., 1989) and ectocervix (Choo et al., 1993; Kasturi et al., 1993). Moreover, it appears to have importance in vivo, as calcium gradients have been described in stratifying squamous epithelial tissues (Malmqvist et al., 1984; Menon et al., 1985). In vitro,
calcium promotes increased differentiation of normal and HPV-16-immortalized ectocervical cells as measured by increased cornified envelope formation and increased K13, INV and TG1 levels (Kasturi et al., 1993; Choo et al., 1993).

In the present study, we have examined calcium regulation of marker expression in a tumorigenic cell line, CaSki, derived from a malignant carcinoma of the uterine cervix (Pattillo et al., 1977). CaSki cells contain between 270 and 500 copies of the HPV-16 genome integrated into the host cell genome at several locations (Herrington et al., 1989; Yee et al., 1985; Baker et al., 1987) and will form tumors in nude mice (C. K. Choo & R. L. Eckert, unpublished). Our results indicate that the cells are relatively undifferentiated compared to normal cervical cells or ECE16-1 cells, as measured by reduced INV, K13 and TG1 levels. This is consistent with a more advanced stage of malignancy and the higher level of E6/E7 transcript expression. However, despite the reduced levels of these markers, the cells differentiate in response to increased levels of extracellular calcium as shown by an increase in the level of mRNA encoding K13, TG1 and INV. These results strongly suggest that although these cells have a basal cell-like, transformed phenotype and were derived from an advanced cancer, they are still able to respond to differentiation stimuli. The change in marker expression is absolutely dependent upon the presence of calcium in the medium and does not occur in medium containing reduced (0.06 mM) calcium.

The pattern of cytokeratin expression in ECE16-1 and CaSki cells is generally very similar (Fig. 4). Both cell lines express cytokeratins K5, K6, K7, K8, K14, K16/17 and K19. However, one striking difference is the very low level expression of K13 mRNA in CaSki cells and the apparent absence of cytokeratin K13 protein (Fig. 2 and 3). K13 is a marker of cervical cell differentiation both in vivo (Czernobilsky et al., 1984; Levy et al., 1988) and in vitro (Agarwal et al., 1991; Choo et al., 1993; Gorodeski et al., 1990a; Sizemore et al., 1993) and is specifically expressed in the suprabasal layers of the cervix. In ECE16-1 cells, K13 levels increase when cells are shifted from low to high calcium-containing medium (Choo et al., 1993). The reason for the absence of K13 protein in CaSki cells is not clear; however, K13 is expressed when CaSki cells are grown on semi-permeable membranes (Gorodeski et al., 1994). The present study indicates that the calcium responsiveness of the K13 gene in CaSki cells is intact; however, the overall level of expression is much reduced, consistent with a loss of differentiated function.

TG1 is the key enzyme responsible for formation of the cornified envelope in ectocervical epithelial cells and is regulated by both calcium and retinoids in normal and HPV-16-immortalized cervical cells (Choo et al., 1993; Kasturi et al., 1993). Our present studies indicate that the calcium regulation of TG1 mRNA expression is intact in CaSki cells; however, the mRNA and protein are produced at such low levels that no TG1 activity can be detected in cell extracts. In this respect, CaSki cells are poorly differentiated, having lost the potential to form cornified envelopes. The absence of TG1 activity is likely to contribute to the transformed phenotype of CaSki cells, as some forms of TG have been implicated as positive agents in promoting the process of programmed cell death (Gentile et al., 1992).

For genital precancers containing HPV-16, E6 and E7 appear to be expressed in both basal and differentiated cells (Crum et al., 1988), and when HPV-16-immortalized cells having various potentials for tumour formation are injected in mice, the HPV-16 E6 reading frame is predominantly detected in the basal cell layer regardless of tumorigenic potential (Durst et al., 1991). Moreover, in a cell line containing episomal HPV-31b, expression of the E6/E7 reading frames is encoded by a polycistronic RNA that is not regulated in a differentiation-dependent manner (Hummel et al., 1992).

In the present experiments we have examined the relationship between cell differentiation and E6/E7 expression in a malignant cervical cancer cell line, CaSki, as a model for advanced cervical cancer. Our studies demonstrate that although CaSki cells differentiate in response to elevated extracellular calcium levels, E6/E7 transcript levels are constitutively produced. Our previous study indicated a similar absence of regulation in ECE16-1 cells (Choo et al., 1993). This suggests that the lack of differentiation-dependent expression of E6/E7 is not always correlated with tumorigenicity of the cells, since E6/E7 expression is differentiation-independent in both ECE16-1 and CaSki cells, but only the CaSki cells form actively growing tumours in nude mice.

Durst et al. (1991) suggested that the physical state of the DNA may influence the pattern of E6/E7 expression (i.e. basal expression in cells containing integrated HPV-16 DNA versus suprabasal expression in cells containing extrachromosomal HPV-16). Our results are consistent with this hypothesis, as both ECE16-1 and CaSki cells contain integrated HPV DNA and constitutively express E6/E7. However, it should be noted that expression of E6/E7 is not regulated with changes in differentiation in a cell line containing HPV-31b as an episome (Hummel et al., 1992). Should this result be verified in other systems, it would suggest that expression of E6/E7 is differentiation-independent regardless of whether the HPV DNA is episomal or integrated.

In summary, our results indicate (i) that elevated levels of E6/E7 expression are correlated with a more poorly differentiated phenotype, (ii) that poorly differentiated cervical cancer cells, such as CaSki, retain the ability to
differentiate in vitro, and (iii) that E6/E7 transcripts are constitutively produced in a differentiation-independent manner.

The authors wish to thank Dr Chapla Agarwal and Joan Hembree for kindly reviewing this manuscript and Dr Anton Jetten for providing the transglutaminase cDNA clone. This work was supported by a grant from the American Institute for Cancer Research (R.L.E.) and the National Institutes of Health (E.A.R.) and utilized the facilities of the Skin Diseases Research Center of Northeast Ohio (NIH, AR39750).

References


(Received 20 August 1993; Accepted 9 December 1993)