Characterization of human keratinocytes transformed by high risk human papillomavirus types 16 or 18 and herpes simplex virus type 2

Kavita R. Dhanwada,1 Lenora Garrett,2 Patricia Smith,2 Kenneth D. Thompson,1 Alan Doster3 and Clinton Jones*  

1 Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, Illinois 60153,  
2 Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 and 3 Center for Biotechnology,  
Department of Veterinary Science, East Campus, University of Nebraska, Lincoln, Nebraska 68583-0905, U.S.A.

Recent reports implicate two DNA tumour viruses, herpes simplex virus type 2 (HSV-2) and human papillomavirus types 16 or 18 (HPV-16 and -18), in the pathogenesis of cervical cancer. Previous studies have indicated that primary human fibroblasts transfected with HPV-16 and HSV-2 morphologically transforming region III (mtr III) are more aneuploid than fibroblasts immortalized with HPV-16 and that HSV-2 DNA sequences are retained in transformed cells. Since HSV-2 and HPV typically infect cells of epithelial origin, the interactions of these viruses with respect to morphological transformation were examined in human keratinocytes. HPV-16- or HPV-18-immortalized keratinocytes (FEPL and FEA cells, respectively) were transfected with fragments derived from HSV-2 mtr III. When compared to their normal counterparts, FEPL cells and FEA cells transfected with mtr III fragments grew to higher saturation densities and were morphologically transformed. FEPL cells transformed by HSV-2 were capable of growth in soft agar and, when injected into nu/nu mice, lesions developed at the site of injection. Histological examination of the lesions revealed a benign mass which was composed of squamous epithelial cells that were producing keratin. In contrast, immortalized keratinocytes (FEPL or FEA) or FEA cells transfected with HSV-2 did not produce these lesions. These observations suggest that sequences within mtr III can alter the growth properties of human keratinocytes immortalized by HPV-16 or HPV-18.

Introduction

Recent epidemiological evidence indicates that women infected with herpes simplex virus type 2 (HSV-2) and human papillomavirus types 16 or 18 (HPV-16 or -18) are at greater risk with respect to cervical carcinoma compared to women infected with only one virus (Hildesheim et al., 1991). To assess possible interactions between HSV-2 and high-risk HPVs (HPV-16, -18) in the pathology of cervical carcinoma, Di Luca et al. (1987, 1989) examined cervical tumours for the presence of the respective viral DNAs. Six of eight tumours (Di Luca et al., 1987) and all of eight tumours (Di Luca et al., 1989) contained both HSV-2 and HPV-16 or -18 DNA sequences. Previous studies demonstrated that primary human fibroblasts transfected with HPV-16 and HSV-2 morphological transforming region III (mtr III) are more aneuploid than fibroblasts immortalized with HPV-16 alone and that HSV-2 DNA sequences are retained in these transformed cells (Dhanwada et al., 1992). Furthermore, mtr II of HSV-2 can convert cervical keratinocytes immortalized with HPV-16 to a tumorigenic cell line (DiPaolo et al., 1990). Taken together, these observations suggest that high-risk HPVs and HSV-2 can be cofactors during the development of cervical cancer.

Papillomaviruses are small DNA tumour viruses which induce proliferative lesions of the skin and mucosa (reviewed in zur Hausen, 1977; Pfister, 1984). Although many of the lesions from HPV infections are benign, infection with specific types of HPV are associated with cervical carcinoma. HPV sequences are present in 80 to 85% of cervical carcinoma biopsies, with HPV-16 and -18 being the most prevalent (Broker & Botchan, 1986; Dürst et al., 1983). Malignant lesions frequently contain HPV DNA integrated into the cellular genome with only a portion of the viral DNA present (Dürst et al., 1985). It is well established that HPV-16 and -18 can immortalize primary human cells, such as keratinocytes (Dürst et al., 1987; Kaur & McDougall 1988; Woodworth et al., 1989; Matsukura et al., 1986; Pater & Pater, 1985; Seedorf et al., 1987) and fibroblasts (Pirisi et al., 1987; Watanabe et al., 1989). The E6 and E7 genes are required for this process (Matlashewski et al., 1987; Münger et al., 1989; Watanabe et al., 1989). HSV-2 is an oncogenic DNA virus spread primarily by
sexual contact (Ginsberg, 1980). Within the HSV-2 genome there are two unique morphological transforming regions designated mtr II (map units 0·585 to 0·63) and mtr III (map units 0·42 to 0·58) (Galloway & McDougall, 1981; Jariwalla et al., 1983). The HSV-2 mtr III contains a minimal transforming fragment of 486 bp (486 TF) (Jones et al., 1986). When linked to a reporter gene, 486 TF can function as a complex transcriptional regulatory element (Jones, 1989). However the mechanism by which mtr III accomplishes neoplastic transformation has not been determined.

In this report, we examined what effects HSV-2 DNA fragments from mtr III have on human keratinocytes immortalized with HPV-16 or HPV-18 (FEPL and FEA cells, respectively). The studies demonstrated HPV-16/HSV-2-transformed cells grew to higher saturation densities when compared to their immortalized counterparts. The data also imply that differences exist with respect to progression of HPV-16-immortalized cells compared with HPV-18-immortalized cells after transfection with HSV-2 mtr III sequences.

### Methods

**Cells and plasmids.** Keratinocytes were maintained in serum-free keratinocyte medium (Gibco) supplemented with epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 μg/ml), penicillin (10 units/ml) and streptomycin (100 μg/ml). Cells were split 1:4 weekly. Primary keratinocytes immortalized with a 3·4 kb tumour clone of HPV-16 were designated FEPL cells (Kaur et al., 1989) and keratinocytes immortalized with a plasmid containing the entire HPV-18 genome (7857 bp) were designated FEA cells (Kaur & McDougall, 1988). Plasmid pEJ6, containing the Ha-ras oncogene was obtained from ATCC (41028). Two c-myc plasmids were used in the analysis. Plasmid p5-UTR containing a 1·6 kb SacI fragment of the 5' untranslated region of the c-myc gene, was obtained from Dr Len Erickson.

**Polybrene transfection of keratinocytes.** Keratinocytes were transfected by the polybrene method (Rhim et al., 1986). FEPL cells at passage 55 were used for all transfections. FEA cells at passage 75 were used for all transfections. Cells were transfected with 10 μg of the designated plasmid DNA using 10 μg polybrene (1 mg/ml) in 1 ml serum-free medium. Cultures were incubated at 37 °C and rocked every hour. A 30% DMSO shock (4 min) was performed after 6 h to facilitate uptake of DNA. All cultures were cotransfected with pSV2neo (2 μg/60 mm dish). Forty-eight to 72 h after transfection, geneticin was added to cultures (50 μg/ml for FEA cells and 500 μg/ml for FEPL cells) to select for cells with plasmids. After 1 week in the presence of geneticin, FEPL cells were split twofold and fresh medium containing 500 μg/ml geneticin was added. This procedure was repeated in the third week of geneticin selection. Selection was carried out for 3 to 5 weeks post-transfection or until the control cells were dead. When densely growing geneticin-resistant cells became macroscopic, cells were trypsinized and cultured in the absence of geneticin. The resulting cell lines were designated as follows: PTF (FEPL/HSV-2 486 TF); PPC (FEPL/HSV-2 PsrI-L); PBE (FEPL/HSV-2 BamHI-E); Pras (FEPL/Ha-ras); Pneo (FEPL/pSV2neo); ATP (FEA/HSV-2 486 TF); APC (FEA/HSV-2 Psrl-C); ABE (FEA/HSV-2 BamHI-E); Aras (FEA/Ha-ras); Aneo (FEA/pSV2neo).

**DNA preparation and Southern blot hybridization.** Protocols for the preparation of high M DNA and Southern blot hybridization were described previously (Dhanwada et al., 1992).

**RNA extraction and Northern blot hybridization.** Total cellular RNA was extracted from cells by the method of Peppel & Baglioni (1990). Cells were washed with PBS and lysed with a solution containing 2% SDS, 200 mm-Tris–HCl pH 7·5 and 1 mm-EDTA. DNA and proteins were precipitated from the lysate by addition of 4·37 m-potassium acetate. The RNA-containing supernatant was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). RNA was precipitated with isopropanol and stored at −70 °C in formamide.

Total cellular RNA (10 μg) was electrophoresed through a 1% agarose gel containing 0·66 m-formaldehyde, 1 × (200 mm) MOPS buffer, 5 mm-sodium acetate pH 7·0 and 1 mm-disodium EDTA. Prior to electrophoresis, 10 μg RNA was precipitated and redissolved in sample buffer containing 0·75 ml denisoned formamide, 0·15 ml 10 × MOPS, 0·24 ml formamidé, 0·1 ml RNase-free water, 0·1 ml glycerol and 0·08 ml 10% (w/v) bromophenol blue. RNA was transfused to nylon filters (Hybond N+, Amersham) by blotting overnight in 20 × SSPE (1 × SSPE contains 0·18 M-NaCl, 0·01 M-sodium phosphate, 1 mm-disodium EDTA, pH 7·7). Probes labelled with [α-32P]dCTP (1 × 106 c.p.m./μg DNA) were prepared by random priming and hybridized to filters in a solution containing 5 × SSPE, 5 × Denhardt's solution, 0·5% SDS, 50% formamide and 50 μg/ml sheared salmon sperm DNA. Hybridization was carried out at 42 °C for 16 h. The filters were washed with 2 × SSPE and 0·1% SDS twice for 10 min at room temperature followed by 1 × SSPE and 0·1% SDS for 15 min at 65 °C. Filters were air-dried and autoradiographed.

**Primer extension analysis.** This was performed using 50 μg of total RNA from immortalized and transformed keratinocyte cell lines as previously described (Ausbil et al., 1989). Oligonucleotide primers, synthesized with an Applied Biosystems 391 PCR Mate DNA synthesizer, were 30 nucleotides (nt) in length. HPV-16 E6/E7 transcripts were detected using a primer corresponding to nt 191 to 220 (5' TGTGTGTACTGCAAGCAACAGTTACTGCGA 3') of the HPV-18 genome. HPV-18 E6/E7 transcripts were detected using a primer corresponding to nt 175 to 204 (5' CACTGAAAGACCATAGAATAACCTGTTGAT 3') of the HPV-18 genome (Colec & Danos, 1987). Samples were separated on an 8% polyacrylamide–7 m-urea gel. The single-stranded oligonucleotides were end-labelled with [γ-32P]ATP.

**PCR analysis.** High M DNA was prepared from various cell lines as previously described (Dhanwada et al., 1992) and digested with EcoRI overnight at 37 °C. Oligonucleotide primers, 21 bases in length, were chosen to amplify a target sequence of 366 bp located within the HSV-2 mtr III 486 TF sequence. The primers were synthesized according to the published sequence of 486 TF (Jones et al., 1986). Primer 971 contained nt 917 to 937 of the plus strand (5' TCGTGTTACTGCAAACAGTTACTGCGA 3') and primer 1262 contained nt 1262 to 1282 of the minus strand (5' GGCGTTCCGGGTCCTGTGGA 3'). The probe used to detect the amplified sequence was the 486 TF sequence (486 bp). The protocol for PCR analysis was previously described in detail (Dhanwada et al., 1992).

**Tumorigenicity assay in nude mice and histological analysis.** To determine the tumorigenic potential of transformed cell lines, 5 × 104 cells were injected subcutaneously into nu/nu mice, males 3 to 5 weeks old (Harlan Sprague Dawley). Mice were observed weekly for the
presence of lesions. Seven to 8 weeks post-injection, animals were sacrificed and lesions removed.

Tissue sections for histology analysis were fixed in 50% formalin, embedded in paraffin wax and sections were stained with haematoxylin and eosin. Karyotyping analysis of keratinocyte cell lines was performed using the procedure described by Yunis et al. (1981).

Results

Transformation of immortalized human keratinocytes by mtr III fragments

To test whether fragments within HSV-2 mtr III can alter the growth properties of FEPL or FEA cells, plasmids containing various regions of mtr III (Jones et al., 1986; Fig. 1) were transfected into the parental cell lines. A plasmid containing an activated Ha-ras gene, pEJ6.6, was also transfected into immortalized cells to serve as a positive control for cooperation with HPVs (Matlashewski et al., 1987). A comparison of HPV-immortalized and HPV/HSV-2-transformed cell lines was undertaken to determine whether differences existed. Saturation density analysis indicated that FEPL or FEA cells transformed by mtr III DNA fragments contained twice the number of cells after 1 week in culture compared to immortalized cells (Table 1). Several independent FEPL cell lines transfected with the various regions of mtr III also grew more efficiently in soft agar compared to the parental cells (data not shown). All three HPV-16/HSV-2-transformed cell lines grew three times more efficiently than immortalized FEPL cells in soft agar (Table 1). In contrast, none of the FEA cells, normal or those transfected with mtr III, grew in soft agar. For each mtr III fragment (BamHI-E, PstI-C or 486 TF) three additional cell lines were prepared after transfection of FEA cells and none of these grew in soft agar (data not shown). In summary, both HPV-16 and HPV-18 cell lines transfected with mtr III had saturation density values twice that of their immortalized counterpart. Only HPV-16/HSV-2-transformed cells exhibited efficient anchorage-independent growth capabilities.

Tumorigenicity analysis

To determine whether the respective cell lines could induce tumours in animals, cell lines were injected subcutaneously into nu/nu mice (5 x 10⁶ cells/animal). FEPL cells transfected with HSV-2 mtr III sequences formed lesions which grew slowly (Table 2). Histological analysis indicated that the lesions were benign squamous cell masses (data not shown). When immortalized FEPL cells were injected into nu/nu mice, small nodules were evident 1 to 2 weeks after injection. By 8 weeks post-injection, the nodules regressed and no sign of a lesion was evident. FEPL cells transfected with Ha-ras also formed squamous cell lesions; however, they were not invasive, as Matlashewski et al. (1987) had demonstrated with rodent cells.

FEA cells (HPV-18-immortalized) were unable to produce any lesions in nu/nu mice. Tumour formation in mice injected with HPV-18/HSV-2 mtr III cell lines was not readily observed at 8 weeks post-injection. Lesions

Fig. 1. Schematic map of HPV-16, HPV-18 and HSV-2 mtr III. (a) Restriction enzyme map of HPV-16 genome and location of ORFs. The prototype HPV-16 genome is depicted. Map indicates the 3.4 kb fragment of HPV-16 DNA retained in a tumour clone (black boxes) used to immortalize primary genital keratinocytes (Kaur et al., 1989). These HPV-16-immortalized keratinocytes were subsequently used to generate HPV-16/HSV-2 cell lines (designated FEPL cells). (b) Restriction map of HPV-18. pHPV-18, containing the HPV-18 genome and pBR322 vector sequences, was used to transfect primary genital keratinocytes (Kaur & McDougall, 1988). The resulting HPV-18-immortalized cell lines were used to generate HPV-18/HSV-2 cell lines (designated FEA cells). (c) Restriction map of HSV-2 mtr III region containing 486 TF. Plasmids containing subregions of mtr III, BamHI-E, PstI-C and 486 TF, were used to generate HPV/HSV-2-transformed cell lines.
Table 1. Growth characteristics of keratinocyte cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>0.3% Agarose colony formation (%)†</th>
<th>Saturation density‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEA-N (HPV-18)</td>
<td>&lt; 1</td>
<td>1.0 ± 0.3 × 10⁶</td>
</tr>
<tr>
<td>ATF-1 (HPV-18/486 TF)</td>
<td>&lt; 1</td>
<td>2.0 ± 0.5 × 10⁵</td>
</tr>
<tr>
<td>APC-1 (HPV-18/PstI-C)</td>
<td>&lt; 1</td>
<td>2.0 ± 0.4 × 10⁵</td>
</tr>
<tr>
<td>ABE-1 (HPV-18/BamHI-E)</td>
<td>&lt; 1</td>
<td>2.0 ± 0.4 × 10⁵</td>
</tr>
<tr>
<td>FEPL-N (HPV-16)</td>
<td>5</td>
<td>3.2 ± 1.0 × 10⁵</td>
</tr>
<tr>
<td>PTF-1 (HPV-16/486 TF)</td>
<td>15</td>
<td>6.3 ± 1.0 × 10⁵</td>
</tr>
<tr>
<td>PPC-1 (HPV-16/PstI-C)</td>
<td>15</td>
<td>5.9 ± 1.0 × 10⁵</td>
</tr>
<tr>
<td>PBE-1 (HPV-16/BamHI-E)</td>
<td>15</td>
<td>6.4 ± 1.0 × 10⁵</td>
</tr>
</tbody>
</table>

† Growth characteristics of keratinocyte cell lines were analysed for anchorage-independent growth and saturation density rates. Keratinocytes transfected with the respective HSV-2 fragments were passaged 10 times prior to the respective assays. Immortalized keratinocytes (FEPL or FEA cells) were passaged an equivalent number of times.

‡ Saturation density was the number of cells/100 mm dish at confluence. Five plates were seeded with 3 × 10⁶ cells and after 1 week in culture, cells were trypsinized and counted using a hemocytometer. The values presented are the mean ± S.E.M. of the number of cells in a 100 mm dish.

Table 2. Tumorigenicity analysis of keratinocyte cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumour formation</th>
<th>Type of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEPL-N (HPV-16)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>PTF-1 (HPV-16/486 TF)</td>
<td>2/5</td>
<td>Epithelial cyst</td>
</tr>
<tr>
<td>PPC-1 (HPV-16/PstI-C)</td>
<td>5/7</td>
<td>Epithelial cyst</td>
</tr>
<tr>
<td>PBE-1 (HPV-18/BamHI-E)</td>
<td>4/5</td>
<td>Epithelial cyst</td>
</tr>
<tr>
<td>Pras (HPV-16/Ha-ras)</td>
<td>4/5</td>
<td>Epithelial cyst</td>
</tr>
<tr>
<td>FE-AX (HPV-18)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>ATF-2 (HPV-18/486 TF)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>APC-2 (HPV-18/PstI-C)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>ABE-2 (HPV-18/BamHI-E)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>Aras (HPV-18/Ha-ras)</td>
<td>0/5</td>
<td>None observed</td>
</tr>
<tr>
<td>HeLa (HPV-18 cervical carcinoma)</td>
<td>2/2</td>
<td>Anaplastic carcinoma</td>
</tr>
</tbody>
</table>

* Tumorigenic potential of keratinocyte cell lines was analysed for production of palpable tumours. Cells (5 × 10⁶) were injected subcutaneously into nu/nu mice. FEA or FEA cells transfected with fragments derived from mtr III were passaged 10 times after geneticin selection prior to injection. Immortalized keratinocytes were passaged an equivalent number of times prior to injection. Eight weeks post-injection, mice were anaesthetized and observed for the presence of lesions. Lesions were classified based on histological analysis.

were not observed even in FEA cells transfected with Ha-ras. When HeLa cells, an HPV-18-containing cervical carcinoma cell line, were injected into nude mice, rapidly growing tumours were detected 2 weeks post-injection. In summary, HPV-16/HSV-2 cell lines were able to form benign epithelial lesions when injected into nude mice in contrast to HPV-18/HSV-2 cell lines.

Chromosome analysis was performed to determine what effect transfection with mtr III had on FEA and FEPL cells. Analysis of HPV/HSV-2-transformed cell lines indicated abnormalities in some of the cell lines. Cell line PTF-1 had a mean chromosome number of 75 and cells consistently contained abnormal chromosomes. Cell line PPC-1 contained a mean chromosome number of 43. However, PPC-1 cells were consistently missing chromosomes 1, 5, 7, 20 and Y. Cell line APC-2 contained a mean chromosome number of 62 with numerous rearranged chromosomes. Other cell lines (ATF-2, ABE-2 and PBE-1) did not exhibit significant aberrations from the parental cells. In summary, three of six immortalized cell lines that were transfected with mtr III contained abnormal karyotypes.

Genomic organization of HPV DNA in keratinocyte cell lines

To examine the organization of HPV DNA after transfection by HSV-2 mtr III or Ha-ras, Southern blot analysis was performed. FEA and FEPL cell lines have one to two copies of integrated viral genomes/cell (Kaur & McDougall, 1988; Kaur et al., 1989). Genomic DNA from FEPL cell lines was hybridized to a large 6-6 kb probe spanning the entire HPV-16 early genome region. Two HPV-16-specific fragments were detected when genomic DNA was probed with the HPV-16 probe (Fig. 2a). A similar pattern of hybridization was also observed in cells transfected with HSV-2 or Ha-ras.

High Mr DNA prepared from immortalized or transfected FEA cells was hybridized to the entire genome of HPV-18. Rearrangements of HPV-18 DNA were detected in HPV-18/HSV-2-transformed cells (Fig. 2b, lanes 2 to 6) when compared to immortalized (lane 1), pSV2neo (lane 8) or HPV/Ha-ras (lane 7) cell lines. Deletions were detected in transfected cells with the loss of a 2-3 kb band in APC-1 (lane 4) and ABE-2 (lane 6). In contrast, cell line ABE-1 contained an additional 2-6 kb hybridizing fragment (lane 5). In the cell line ATF-1 (lane 2), it appears that amplification of HPV-18 DNA sequences has occurred. When genomic DNA of FEA cell lines was hybridized to an HPV-18 probe spanning E6, E7 and the long control region (LCR) no changes were detected, suggesting the rearrangements were outside this region (data not shown). In summary, rearrangements were frequently detected in HPV-18 DNA after FEA cells were transfected with HSV-2 mtr III sequences.

Analysis of cellular genes, c-myc and p53, in transformed keratinocyte cell lines

To determine whether the c-myc locus was disrupted after FEA or FEPL cells were transfected by HSV-2 mtr III, organization of the c-myc gene was examined.
Earlier studies demonstrated that integration of high-risk HPVs occurs near the c-myc proto-oncogene or led to disruption of the c-myc gene (Riou et al., 1987; Dürst et al., 1987; Crook et al., 1990). Furthermore, it was of importance to determine whether rearrangement of cellular genes commonly occurred after transfection with HSV-2 fragments from mtr III, specifically in FEA-derived cell lines. A 420 bp PstI fragment of exon 2 of c-myc was used to examine the gene organization of c-myc DNA. No detectable rearrangements were observed in

Fig. 2. Genomic organization of HPV DNA in transformed keratinocyte cell lines. Genomic DNA was prepared from HSV-2/HPV-16-transformed cell lines (a; FEPL cells) or HSV-2/HPV-18-transformed cell lines (b; FEA cells), digested with restriction enzymes, and subjected to Southern blotting. Size markers, from HindIII-digested λ phage, are designated by lines (23, 9, 6.6, 4.4, 2.3 and 2 kb). (a) High Mr DNA from FEPL-derived cell lines was digested with PstI and probed with a 6.6 kb BamHI fragment spanning the early region of HPV-16. Lanes: 1, FEPL-N; 2, PTF-2; 3, PPC-1; 4, PPC-2; 5, PBE-2; 6, Pneo; 7, 6.6 kb fragment of HPV-16. (b) Genomic DNA from FEA-derived cell lines was digested with PstI and probed with a 7.9 kb fragment of HPV-18 spanning the entire genome. Lanes: 1, FEA-N; 2, ATF-1; 3, ATF-2; 4, APC-1; 5, ABE-1; 6, ABE-2; 7, Aras; 8, Aneo; 9, 7.9 kb fragment of HPV-18.

Fig. 3. Organization of cellular genes in FEA-derived cell lines. C-myc, hp53 and β-actin gene organization was examined in HPV-18/HSV-2-transformed cell lines to determine whether any disruptions were present in transformed cells compared to HPV-18-immortalized keratinocytes. High Mr DNA from FEA-derived cell lines was prepared and digested with restriction enzymes followed by Southern blotting analysis. Size markers, HindIII-cut λ phage, are denoted by lines (23, 9, 6.6, 4.4, 2.3 and 2.0 kb). (a) Genomic DNA was digested with HincII and probed with the 420 bp PstI fragment of exon 2 of c-myc. Lanes: 1, normal human keratinocytes (NHK); 2, FEA-N; 3, ATF-1; 4, ATF-2; 5, APC-2; 6, ABE-1; 7, ABE-2; 8, Aras. (b) Genomic DNA prepared from FEA-derived cell lines was digested with PstI. The probe used for hybridization was the 1.5 kb XbaI−SmaI fragment of hp53. Lanes: 1, FEA-N; 2, ATF-1; 3, ATF-2; 4, APC-1; 5, APC-2; 6, ABE-1; 7, ABE-2; 8, Aras; 9, Aneo. (c) Genomic DNA digested with HincII was probed with a 2.1 kb BamHI fragment of β-actin. Lanes: 1, FEA-N; 2, ATF-1; 3, ATF-2; 4, APC-1; 5, APC-2; 6, ABE-1; 7, ABE-2; 8, Aras.
RNA analysis of transformed cell lines

Northern blot analysis was performed to determine whether the steady-state levels of HPV-16/-18 E6/E7 differed in HPV-immortalized and HPV/HSV-2-transformed cell lines. Total cellular RNA was hybridized to probes spanning the HPV-16 or HPV-18 E6 and E7 ORFs. Three transcripts were detected ranging in size from 4.5 to 1.8 kb (Fig. 4). However, no obvious differences were observed between immortalized and transformed cell lines.

Primer extension analysis was also performed to measure steady-state RNA levels of HPV-16/-18 E6/E7 transcripts. Total cellular RNA from HPV-immortalized or HPV/HSV-2-transfected cell lines was hybridized to E6/E7-specific primers. There were no significant differences in the levels of HPV-16 E6/E7 transcripts among immortalized and transfected cell lines (Fig. 5). Additionally, no unique transcripts were detected among FEPL transformed cell lines using E6/E7-specific primers. However, in FEA-transfected cells, the level of E6/E7 hybridizing transcripts varied among the cell lines. Compared to FEA cells, higher levels of novel extended products were detected in cells transfected with HSV-2 or Ha-ras, suggesting novel start sites for E6/E7 are utilized. In addition, similar novel start sites were observed in HeLa cells compared to FEA cells. The significance of these differences is yet to be understood.

As judged by Northern blot analysis, the steady-state RNA levels of HPV-16/-18 E6/E7 transcripts in transfected keratinocyte cell lines were similar to that in the immortalized cells. Subtle differences in E6/E7 expression were detected in FEA cells compared to the any cell lines, either HPV-16- (data not shown) or HPV-18-derived (Fig. 3a). Changes in c-myc organization were also not detected in FEPL or FEA cell lines compared to DNA prepared from normal human keratinocytes. As another control, the organization of the human β-actin gene was examined in the various FEA cell lines. No changes were detected in the β-actin gene and its pseudogenes after FEA cells were transfected with mtr III (Fig. 3c).

Mutations in the tumour suppressor gene, human p53 (hp53) have been associated with hyperproliferation and chromosomal instability (Hudson et al., 1990; Woodworth et al., 1990). The E6 early protein of HPV-16/-18 binds the p53 gene product and promotes degradation of this cellular protein (Scheffner et al., 1990). Therefore, it was of interest to determine whether the p53 gene was rearranged after FEA or FEPL cells were transfected with mtr III. Southern blot analysis indicated no apparent rearrangements in hp53 gene organization of either FEPL-derived (data not shown) or FEA-derived cell lines when a 1.5 kb XbaI-SmaI fragment from the hp53 cDNA clone was used as a probe (Fig. 3b). These studies indicated that transfection of FEA cells by mtr III did not lead to rearrangements of the c-myc, hp53, or β-actin genes.
 HPV-16/-18 and HSV-transformed cells

Fig. 5. Primer extension analysis of keratinocyte cell lines. Primer extension analysis was performed to determine the steady-state RNA levels of HPV-16 (lanes 1 to 8) and HPV-18 (lanes 9 to 16) E6/E7-specific transcripts. Fifty μg of total RNA was hybridized with E6/E7-specific primers. Lanes: 1, NHK; 2, FEPL; 3, PTF-2; 4, PPC-1; 5, PPC-2; 6, PBE-2; 7, Pras; 8, Pneo; 9, NHK; 10, FEA; 11, ATF-2; 12, APC-2; 13, ABE-2; 14, Aras; 15, Aneo; 16, HeLa. The expected size of the HPV-16 E6/E7-extended product is 123 nt and is denoted by an arrow. The expected size of the HPV-18 E6/E7-extended product is 100 nt and is also marked.

(a) Map of HSV-2 mtr III-derived primers, 917 and 1262. (b) Southern blot analysis of PCR reaction using 486 TF as the probe. Lane 1 contains 0.1 μg pCPS-1 plasmid (486 TF cloned into the PstI and SalI sites of pUC19). Lanes 2 to 17 contain DNA prepared from FEPL- and FEA-derived cell lines. Lanes: 2, PTF-1; 3, PPC-1; 4, PPC-2; 5, PBE-1; 6, PBE-2; 7, Pras; 8, Pneo; 9, ATF-2; 10, APC-1; 11, APC-2; 12, ABE-1; 13, ABE-2; 14, Aras; 15, Aneo; 16, normal keratinocyte DNA; 17, 'no-template' control.

Fig. 6. PCR analysis of transformed keratinocyte cell lines. High Mr DNA was prepared from transformed keratinocyte cell lines, digested with PstI and 1 μg of digested DNA was used in the PCR reaction. Primers were derived from the HSV-2 mtr III PstI-C fragment. Size markers are φX174 DNA/HaeIII and positions of these fragments are denoted by lines (1353, 1078, 872, 603, 310, 281, 234, 174 and 118 bp). (a) Map of HSV-2 mtr III-derived primers, 917 and 1262. (b) Southern blot analysis of PCR reaction using 486 TF as the probe. Lane 1 contains 0.1 μg pCPS-1 plasmid (486 TF cloned into the PstI and SalI sites of pUC19). Lanes 2 to 17 contain DNA prepared from FEPL- and FEA-derived cell lines. Lanes: 2, PTF-1; 3, PPC-1; 4, PPC-2; 5, PBE-1; 6, PBE-2; 7, Pras; 8, Pneo; 9, ATF-2; 10, APC-1; 11, APC-2; 12, ABE-1; 13, ABE-2; 14, Aras; 15, Aneo; 16, normal keratinocyte DNA; 17, 'no-template' control.

Retention of HSV-2 DNA sequences in transformed cell lines

HSV-2 DNA sequences have not been consistently detected in transformed cells; however, evidence has demonstrated a correlation between HSV-2 infection and malignant transformation (Di Luca et al., 1989; Hildesheim et al., 1991). The inconsistent nature of HSV-2 DNA retention has led to a 'hit and run' hypothesis for HSV-2-mediated transformation (reviewed by Macnab, 1987). High Mr DNA digested with EcoRI was subjected to PCR analysis using primers derived from HSV-2 486 TF. Analysis of FEPL- and FEA-derived cell lines indicated HSV-2 retention in three FEA cell lines (Fig. 6). None of the five HPV-16/HSV-2 cell lines retained HSV-2 DNA, whereas three of five HPV-18/HSV-2 cell lines retained HSV-2 DNA sequences: ATF-2, APC-2 and ABE-2. The amplified band migrated as a 366 bp fragment. Neither the immortalized keratinocyte cell lines, normal keratinocyte DNA nor the HPV/Ha-ras had 486 TF hybridizing fragments. These results demonstrated HSV-2 DNA sequences were present in 30% of the transformed cell lines.

Discussion

In this report, the effect that HSV-2 mtr III sequences have on HPV-16- or HPV-18-immortalized human keratinocytes was examined. HPV-16- or HPV-18-immortalized cells (FEPL and FEA cells, respectively) (Kaur et al., 1989; Kaur & McDougall, 1988) were transfected with fragments derived from HSV-2 mtr III. Saturation density analysis indicated that FEPL or FEA cells transformed by mtr III DNA sequences contained...
twice the number of cells after 1 week in culture compared to their immortalized counterpart (Table 1). Neither FEA cells nor the mtr III-transfected derivatives grew more efficiently in soft agar (Table 1) compared to immortalized FEPL cells. FEPL cells transformed by HSV-2 consistently formed benign lesions in nu/nu mice whereas transformed FEA cells did not (Table 2). These studies suggested that HSV-2 can play a role in the progression of the transformed phenotype in human keratinocytes.

Analysis of HPV DNA organization was performed to determine whether rearrangements of HPV DNA occurred after keratinocytes were transfected with mtr III. No rearrangements of HPV DNA were detected by Southern blot analysis in HPV-16/HSV-2-transformed cell lines (Fig. 2). Integration of HPV-16 DNA sequences into the cellular genome seldom disrupts the LCR, E6 and E7 ORFs (Matsukura et al., 1986; Pater & Pater, 1985; Seedorf et al., 1987) since expression of E6 and E7 are necessary for immortalization. FEPL cells contain only the HPV-16 sequences that are required for immortalization (Kaur et al., 1989; Fig. 1) and thus it was not surprising that rearrangement of HPV DNA did not occur. FEA cells transfected with mtr III were also analysed with respect to HPV-18 DNA organization. FEA cells contain the entire genome of HPV-18, a 7.9 kb DNA (Kaur & McDougall, 1988). When high Mr DNA was hybridized with a probe spanning the entire 7.9 kb, rearrangements of HPV-18 DNA sequences in cells transfected with HSV-2 were frequently detected (Fig. 2). However, no changes were detected when DNA was probed with an HPV-18 probe spanning the LCR, E6 and E7 regions (data not shown). In contrast, the organization of the c-myc, hp53 and ß-actin genes were not changed (Fig. 3) suggesting the rearrangement of HPV-18 DNA sequences was not entirely random.

The overall levels of E6/E7 expression in FEA or FEPL cells was not altered dramatically after transfection by HSV-2 mtr III (Fig. 4). Primer extension analysis confirmed the conclusions drawn from Northern blotting in FEPL cells. Subtle differences in the 5' termini of E6/E7 mRNAs were detected in FEA cells and their mtr III-transfected derivatives. Since these changes were also detected in FEA cells transfected with Ha-ras or pSV2neo alone, it is not clear what significance the novel RNAs have with respect to maintaining the immortalized phenotype.

The events that lead to progression of the malignant phenotype after high-risk HPV infection in vivo are not known. The presence of HPV-16 or HPV-18 alone is generally not sufficient for tumorigenesis (reviewed in Broker & Botchan, 1986). Additional cofactors, environmental or viral, may play a crucial role in tumour progression events. Since HSV-2 and HPV-16/-18 are transmitted sexually, infect the same cell type, and can induce morphological transformation, progression has been suggested to be mediated by both viruses (Hildesheim et al., 1991; zur Hausen, 1983). Human cell lines immortalized by HPV-16/-18 have a distinct phenotype after they were transfected with mtr III. The development of benign lesions by HPV-16/HSV-2-transformed keratinocytes and not by the parental FEPL cells indicated that transfection of HSV-2 sequences led to altered growth properties of these cells when injected into nu/nu mice. HSV-2 is known to induce stable heritable changes in the cell without the need for viral replication or gene expression (Clarke & Clements, 1991). The observation that HSV-2 mtr III frequently induces rearrangements of HPV-18 DNA sequences in FEA cells supports these findings and implies that mtr III may have a role in these processes. Furthermore, it is clear from this study and from a previous study (Dhanwada et al., 1992) that sequences within mtr III frequently induce chromosomal changes in human cells immortalized by HPV-16. Since HSV-2 mtr III frequently induces genetic instability of HPV DNA sequences as well as cellular chromosomes, this could play an important role during cervical pathogenesis. Taken together, these observations suggest that HSV-2 and high-risk HPV-16 and -18 can be cofactors in the development of genital cancer.

This work was supported by Public Health Service grant R29CA47872 to C.J. We thank Lee Johnson for assistance in preparing the manuscript and D. Hamernik and R. Barletta for critically reading the manuscript. This work, in large part, was performed by Kavita Dhanwada in the laboratory of Clinton Jones in partial fulfilment of her Ph.D. requirements.

References


(Received 17 November 1992; Accepted 25 January 1993)