Characterization of factors involved in human papillomavirus type 16-mediated immortalization of oral keratinocytes


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We have examined intrinsic and external factors that influence human papillomavirus type 16 (HPV-16)-mediated immortalization of oral keratinocytes. The efficiency with which HPV can immortalize human oral keratinocytes was quantified and a considerable difference in the transfection and immortalization competence of the cells was detected. The ability of HPV-16 to immortalize oral cells appeared to be linked to the age of the culture upon transfection. The addition of dexamethasone to the transfected cultures increased the efficiency of immortalization, possibly indicating a role for a critical level of HPV gene expression in initial outgrowth of immortalized colonies. We also document in detail the changes in the oral keratinocyte induced by HPV-16 immortalization. These include alterations associated with crisis and feeder independence as well as basic changes in keratin expression and differentiation.

Human papillomaviruses (HPVs) are ubiquitous pathogens associated with both benign and malignant lesions of epithelial tissue (reviewed in zur Hausen & Schneider, 1987). This is particularly apparent in the cervix where the oncogenic HPV types 16, 18, 31 and 33 have been isolated from cervical carcinoma tissue (Boshart et al., 1984; Dürst et al., 1983; Lorincz et al., 1987; Syrjanen et al., 1985). In contrast, HPV types 6 and 11 are predominantly associated with benign lesions of the oral and genital mucosa (Gissmann et al., 1983). Immortalization of foreskin and cervical keratinocytes with the oncogenic HPV types has been well documented (Piris et al., 1987; Schlegel et al., 1988; Woodworth et al., 1988, 1989; Pecoraro et al., 1989) and this immortalizing activity appears to be primarily encoded by the viral oncogenes E6 and E7 (Vousden et al., 1988; Hawley-Nelson et al., 1989; Bedell et al., 1989; Hudson et al., 1990). These studies have emphasized the differences between the HPV types associated with benign disease, where no immortalizing activity has been seen, and those virus types associated with malignancy which give a high frequency of cell immortalization.

The role of HPV infection in generating oral cavity malignancy is less clearly understood than in the cervix. Epidemiological studies have demonstrated the presence of HPV DNA in normal, benign and malignant lesions of the oral cavity (Sculley et al., 1988; Syrjanen et al., 1988; Maitland et al., 1989; Yeudall & Campo, 1991). In the anogenital region the most common site for HPV-associated malignancy is the transitional zone epithelium and this transitional region in the oral mucosa is a common site for squamous carcinoma. In both these transitional regions, two epithelial phenotypes co-exist in close proximity requiring greater cellular differentiation which may render these cells more vulnerable to transformation.

The ability of HPV-16 to immortalize oral keratinocytes has been previously reported by Park et al. (1991). The studies presented here support these observations and also document the inability of HPV-11 to immortalize oral cells. We have also extended these observations by comparing immortalization capacity with transfection efficiency in a series of 20 separate transfections in oral keratinocytes. We then followed two of the cell lines through crisis and loss of feeder dependence and report in detail changes of cell morphology and growth characteristics over a defined period of time.

Oral keratinocytes were obtained from biopsies of normal buccal and gingival tissue. The establishment of keratinocyte cultures from oral tissue has been previously described (Langdon et al., 1991). Cultures were maintained in RM+ medium, a mixture of Dulbecco's MEM and Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and mitogens (Rheinwald & Green, 1975), and irradiated 3T3 fibroblasts at 37°C in the presence of 10% CO2.
Table 1. Immortalization frequencies of HPV-16-transfected oral keratinocytes

| Expt.* | Line† | TP/SP‡ | LacZ§ | 16*'/11† || 16*/Z‡ (%)¶ | DEX** |
|--------|-------|--------|-------|----------|----------------|--------|
| 1      | T103C | 3/5    | 323   | 4/0      | 1.2            | -      |
| 2      | T121CN| 1/4    | 37    | 3/0      | 80             | -      |
| 3      | T93C  | 3/6    | 493   | 4/0      | 0.8            | -      |
| 4      | T66C  | 6/9    | 105   | 3/0      | 2.8            | -      |
| 5      | T142CN| 2/4    | 220   | 6/0      | 2.7            | -      |
| 6      | T172CN| 2/5    | 55    | 3/0      | 5.4            | -      |
| 7      | None  | 5/8    | 110   | 0/0      | 0              | -      |
| 8      | None  | 7/9    | 102   | 0/0      | 0              | -      |
| 9      | T133C | 3/5    | 85    | 16/0     | 188            | +      |
| 10     | T192CN| 2/6    | 125   | 21/0     | 160            | +      |

* Experiment refers to individual transfection sets.
† Line: HPV-16 lines that were established.
‡ TP/SP: Passage numbers at transfection and senescence.
§ LacZ: Number of β-galactosidase-positive cells 48 h after transfection.
|| 16*/11**: Number of immortalized clones that grew out after each transfection.
¶ 16*/Z: Percentage of cells that became immortalized in comparison to the number that took up and expressed LacZ.
** DEX: Presence of dexamethasone in the medium during selection.

The oral keratinocytes were indistinguishable from epidermal keratinocytes derived from foreskin biopsies in culture morphology and colony formation. However, they grew more rapidly and persisted in culture for longer, up to nine (average of six) passages. We selected 20 keratinocyte strains for this study.

We first compared the immortalization efficiency of HPV-11 and -16 in oral keratinocytes. Twenty oral keratinocyte strains were transfected and scored to detect any differences in immortalization frequency that may be attributed to variation in keratinocyte strains. Prior to transfection, oral cultures were treated with EDTA to remove the feeder layers and then incubated in KGM (Clonetics) overnight. Following transfection the feeders were replaced and the cells maintained in RM medium. Ten micrograms of either pJ4/11 or pJ4/16 (Storey et al., 1988) was transfected either alone or with 10 μg of pSV2Neo using lipofectin (BRL) in OptiMem (Gibco). pSV2Neo transfectants were selected in the presence of 0.1 mg/ml G418. To monitor the efficiency of transfection, 10 μg of SVLacZ was also included and β-galactosidase expression measured after 48 h by staining with a solution containing X-gal. The number of positive blue cells per flask was scored to determine the transfection efficiency. The immortalization frequency for HPV-11 and -16 was determined by counting the colonies that grew out and dividing by the number of LacZ-positive transfectants as scored above. The results from 10 of the 20 transfections are shown in Table 1. Of the other 10 transfections seven were late passage and failed to immortalize and three early passage transfections gave results similar to those shown. Following transfection, colonies of keratinocytes that grew out and could be passaged were scored as being immortalized; many of these lines have now been grown continuously in culture for over 18 months. No lines were obtained using either the pJ4Ω vector or pJ4Ω11, both of which were used in parallel. As HPV-11 did not yield any immortalized colonies in any of the experiments, the rest of the results are for the HPV-16 transfections.

As can be seen from Table 1, HPV-16 transfectants regularly gave rise to immortalized colonies although at a low frequency. In contrast two transfections that failed to produce immortalized lines are shown in experiments 7 and 8; both transfections were done when cells were at passage 5 or greater. Out of a total of 20 transfections only one culture of passage greater than 5 resulted in an immortalized line (experiment 4). Although there were differences in the transfection efficiencies of the different keratinocyte cultures used, transfection efficiency did not appear to change significantly with increasing passage number. Outgrowth of HPV-16 colonies was generally noted 6 to 10 weeks post-transfection. The lifespan of the keratinocyte cultures used was monitored and is recorded in Table 1.

Dexamethasone (10⁻⁷ M) was added to the culture medium during selection to determine whether steroid hormones could modulate immortalization frequencies as previously shown in cervical keratinocytes (Dürst et al., 1989; Storey et al., 1992) and primary rodent cells (Crook et al., 1988; Pater et al., 1988; Lees et al., 1990). In these studies the increase in immortalization was mediated by increased viral gene expression, stimulated by the glucocorticoid response element in the viral regulatory region. Experiments 9 and 10 in Table 1 show that the addition of dexamethasone to the culture medium markedly increased the immortalization frequency for HPV-16. No differences in the control cells or HPV-11-transfected cells were noted following the addition of dexamethasone, suggesting that this effect
requires the presence of HPV-16 DNA sequences. The use of G418 selection did not result in any significant difference in the number of immortalized colonies obtained for HPV-16, HPV-11, or the vector.

All HPV-16-positive transfectants underwent a phase of rapid growth which lasted for approximately seven to 10 passages. At this stage the transfectants entered a crisis period. During crisis, the cells could not be passaged, but remained intact and viable. The ability to retrieve cells from crisis was linked to their density upon entering crisis. Cultures maintained in flasks larger than T25 size proved difficult to rescue, whereas those which were maintained in T25 flasks at low split ratios were much easier to retrieve. The length of time spent in crisis varied between different cultures and did not appear to reflect levels of E7 protein prior to entering crisis (Fig. 1) because both T103C and T121CN underwent similar crisis periods.

The morphology of the immortalized cells grown on plastic differed from normal oral keratinocyte cultures. Immortalized cells appeared larger and colonies less compact than the non-immortalized keratinocyte cultures. Crisis did not alter the morphology of the lines. HPV-16 lines became feeder-independent approximately 10 passages post-crisis for the two lines studied. Attempts to remove the feeders prior to this stage were unsuccessful regardless of whether the line was clonal or pooled. Following feeder independence, the morphology of T121CN and T103C grown on plastic was altered. T121CN cells appeared larger with wider intercellular spaces whereas the T103C cells appeared smaller and were more irregularly shaped. The cell lines were injected into athymic mice (5 × 10⁶ cells/mouse) before and after feeder independence. To date we have tested lines up to passage 35 and none of the lines have been found to be tumorigenic. This is consistent with previous reports on HPV-16-immortalized cervical and foreskin keratinocytes (Kaur & McDougall, 1988; Pirisi et al., 1988).

Aberrant keratin expression, in particular the overexpression of simple epithelial keratins 8 and 18, is a useful marker of malignancy in human tissue. Such studies must, however, take into account the normal expression of a particular keratin marker in normal tissue and the sensitivity of the antibody used. (Considerable variation exists in the reactivity of certain keratin antibodies to the same keratin.) We examined the effect of HPV-16 immortalization of oral keratinocytes upon keratin expression using a panel of well characterized monoclonal antibodies. Monolayer cultures of the HPV-16 cell lines were grown on glass Sera-Tek slides, then fixed and processed for immunocytochemistry as previously described (Leigh et al., 1987). The results are summarized in Table 2 and are representative of results from the other HPV-16 lines. In our own analysis, basal cell markers were lost or greatly diminished which is consistent with results obtained in epidermal keratinocytes (Kaur & McDougall, 1988) and may reflect the more primitive nature of the immortalized cell. Keratins 1 and 10, markers of terminal differentiation in epidermis, were absent from both the parental and derived cell lines. Keratin 19 expression varied between cultures. Keratins 4 and 13, markers of orogenital mucosal differentiation, were equally expressed in keratinocytes and lines. No change in the levels of the hyperproliferative markers, keratins 6 and 16, or keratin 14 (as detected by antibody LL001) were observed. These findings are in contrast to results from foreskin keratinocyte lines which showed a variation in keratin 13 or 16 protein levels following immortalization (McCance et al., 1988; Woodworth et al., 1988). Detection of the simple epithelial keratins 8 and 18 was highly antibody-dependent. Keratins 8 and 18 were
Table 2. Keratin expression in parental and immortalized keratinocytes

<table>
<thead>
<tr>
<th>Keratin Antibody</th>
<th>Type</th>
<th>T103C</th>
<th>T121CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 LL001</td>
<td>Stratified squamous</td>
<td>P</td>
<td>L</td>
</tr>
<tr>
<td>14 LH8</td>
<td>Basal</td>
<td>+/-</td>
<td>+/ -</td>
</tr>
<tr>
<td>10 LH1</td>
<td>Differentiation-specific</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 LL017</td>
<td>(cornified)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 6B10(3)</td>
<td>Differentiation-specific</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13 IC7</td>
<td>(mucosal)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16 LL023</td>
<td>Hyperproliferative</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 LL020(4)</td>
<td>Simple/Switch</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8 M20(5)</td>
<td>Simple epithelial</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>8 LE41(6)</td>
<td>Simple epithelial</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18 LE61</td>
<td>Simple epithelial</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18 RCK106(7)</td>
<td>Simple epithelial</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*P and L, Non-immortalized parental cells and the HPV-16 line, respectively.
† +/-, Some positive and some negative cells.

found in normal oral keratinocyte cultures and this expression increased with passage of these cells as did expression of keratins 16 and 19 (C. J. Sexton et al., unpublished). Thus although the HPV-16 lines did express simple keratins, no significant difference from normal oral keratinocytes could be detected by immunofluorescence.

In vitro, keratinocyte differentiation can be increased by provision of a suitable dermal substrate. Primary oral keratinocytes and HPV-16-containing oral cell lines were subcultured on complex substrates consisting of de-epidermalized human dermis (DED) to compare the differentiation capacity of immortalized and normal oral cells. DED is an organ-like culture which until now has not been used to show the differentiation capacity of HPV-immortalized cells. DED was prepared as previously described (Regnier et al., 1981). Keratinocytes and cell lines were plated inside metal rings on top of the substrate and allowed to attach for 24 h at 37 °C in the presence of 10% CO₂, then the rings were removed and the cultures maintained in RM + medium. The substrates were then raised to the air-liquid interface and fed with RM + medium for 14 days. The complex cultures were fixed in formal saline, paraffin-embedded, and sections were prepared and stained with haematoxylin and eosin for histological analysis. Difficulties were encountered when non-immortalized oral cells were raised to the air-liquid interface as this does not constitute a ‘natural’ environment for mucosal keratinocytes. The normal and immortalized oral cells were therefore grown both submerged in a minimal amount of medium and at the air-liquid interface on the substrate. The non-immortalized cells grew and differentiated best when submerged, whereas no difference in the ability of the HPV-16-immortalized cells to differentiate was detected between the two methods. As can be seen from Fig. 2, primary oral keratinocytes formed a differentiated ordered epithelium. In contrast, the HPV-16-immortalized cell lines gave rise to disorganized layers of undifferentiated cells with no orderly stratification.

In summary, we have shown that primary oral human keratinocytes can be immortalized following transfection with HPV-16 DNA as was previously shown by Park et al. (1991). We have extended this observation by showing the failure of HPV-11 to immortalize this cell type. We have documented a striking difference between the transfection efficiency and the immortalization frequency. Assuming the transfection efficiency of the LacZ reported and the HPV-16 construct DNAs to be similar, then a discrepancy exists between the potentially immortalized clones that had taken up the DNA at the time of transfection and the actual number that grew out as scorable colonies. Immortalization efficiency was affected both by the age of the culture as well as the addition of dexamethasone to the cultures during selection. Analysis of the immortalized oral keratinocytes demonstrated continued expression of the HPV-16 E7 protein. This is consistent with this being one of the major transforming genes of the virus whose continued presence is required for maintenance of the transformed phenotype (von Knebel Doeberitz et al., 1988; Crook et al., 1989). In addition, differentiation of the HPV-16-containing cells was greatly impaired and changes in keratin expression, such as loss of the basal keratin 14 marker, were noted.

None of our HPV-16-immortalized cell lines formed tumours in nude mice, and this appears to hold true for most early and medium term passaged HPV-immortalized keratinocytes. In contrast to these findings, late passage HPV-immortalized genital keratinocytes have been found to be capable of forming tumours in
Fig. 2. Growth of HPV-16-immortalized keratinocyte line on DED. Normal oral keratinocytes re-formed a basal layer with differentiated cells in the suprabasal layers. (a, b) The degree of stratification and the absence of a granular layer correlate with the mucosal phenotype of the cells. (c, d) HPV-16-immortalized oral keratinocytes grew readily on the substrate; however, the epithelium that re-formed was composed of relatively undifferentiated cells with no stratification. Bar markers represent 1 mm.

nude mice, an observation which is consistent with the requirement for additional changes within the cell before full transformation takes place (DiPaolo et al., 1989). One line in our study has however grown in soft agar at an early passage and its ability to form tumours in nude mice and corresponding changes in keratin expression are currently being studied. All of the cell lines underwent crisis for a variable period of time and this occurred after an initial rapid outgrowth of the cells. This is an interesting feature in the establishment of many cell lines, and may represent a period during which secondary events necessary for complete immortalization occur.

Current studies are now aimed at determining what factors are involved in the entry and release of cells from crisis. Preliminary studies indicate that the addition of steroid hormone after the initiation of crisis may shorten the crisis period. In contrast, the levels of E7 protein and HPV-induced cytokine expression (D. Bryan et al., unpublished) in the absence of steroid hormone do not appear to change in pre- and post-crisis cells in the initial lines tested.

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