Retention of low copy number human papillomavirus DNA in cultured cutaneous and mucosal wart keratinocytes

A. T. Williams,1* C. J. Sexton,1,5 A. L. Sinclair,2 K. J. Purdie,1 M. S. Thomas,6 N. A. Hanna,7 B. T. Goh,3 D. M. Williams4 and I. M. Leigh1

Departments of 1 Experimental Dermatology, 2 Virology, 3 Genito-Urinary Medicine and 4 Oral Pathology, The London Hospital Medical College, London E1 2AD, 5 Department of Cell Biology, Wellcome Research Laboratories, Beckenham, Kent, 6 International Center for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy and 7 Department of Genito-Urinary Medicine, St Mary's Hospital, London, U.K.

Cultured wart keratinocytes have previously been described as having a limited proclivity to maintain episomal human papillomavirus (HPV) DNA. To investigate the nature of episode loss, and to determine keratinocyte-specific factors involved in it, we have examined a large series of anogenital and oral wart keratinocyte cultures, tracing episomal copy number with culture passage. We report that a higher proportion of oral wart keratinocytes maintain episomal HPV DNA to first passage (70% compared with 37% of anogenital wart cultures) when screened by slot blot hybridization. Furthermore, oral wart keratinocytes maintain episomal HPV copy through a greater number of passages (60% positive at passage 2 compared with 2% of anogenital wart cultures) with this technique. When anogenital cultures were examined at first passage for HPV infection by PCR with Southern blot hybridization of the product, a further 34% were found to be HPV-positive. To determine the mechanism of loss of episomal DNA from these cultures we examined the relative HPV copy number in cells which adhered to the culture vessel following passage and in those which did not adhere. Those which remained floating contained episomal HPV at high copy number whereas those which adhered were negative by slot blotting. The adherent cells, however, remained positive by PCR at subsequent passages until senescence. We conclude that a subpopulation of HPV-positive keratinocytes may be maintained in culture through serial passage until senescence.

Introduction

The natural host of the human papillomavirus (HPV) is the keratinocyte of stratified squamous epithelia. HPV-infected keratinocytes derived from anogenital lesions have been subject to particularly intense investigation as a result of a strong aetiological association with oncogenesis at these sites (Broker & Botchan, 1986; Howley, 1991). The vegetative reproductive cycle, however, remains relatively uncharacterized. In benign infection the viral genome is presumed to replicate as a stable low copy number episome within cells of the basal stratum. The subsequent stages of the viral life cycle appear to be irrevocably dependent upon keratinocyte differentiation. Amplification of the viral genome occurs in the suprabasal layers and virus maturation proceeds in the upper layers culminating in the shedding of virus particles with sloughed keratinocytes at the epithelial surface (Crum & Nuovo, 1991).

We are interested in determining the interactions of the replicating virus with its native host cell, as the keratinocyte undergoes its differentiation programme. Tissue culture systems such as collagen rafts have been developed and are currently being exploited by groups including ourselves to induce vegetative viral replication (Bedell et al., 1991). In contrast to these more advanced systems, keratinocyte monolayer culture favours cellular proliferation at the expense of differentiation (Rheinwald & Green, 1975), reflecting a basal environment. Monolayer culture of wart keratinocytes therefore permits, and restricts, the examination of the viral life cycle to a simulated hyperplastic basal epithelial compartment. As immortalization by HPV involves deregulated control of normal cellular proliferation and differentiation capability, and frequently loss of episomal DNA, one of the truest model systems for examining viral replication is the cultured primary wart keratinocyte. These cells remain responsive to differentiation signals which may be used to induce and maintain the keratinocyte within varying degrees of differentiation, and therefore represent an excellent substrate for identifying virus-host cell interactions within a differentiating environment.
The extent to which HPV replication may be supported in keratinocytes derived from warty lesions has previously been examined in cultured laryngeal and anogenital tissue. Laryngeal papilloma cultures were shown to contain episomal HPV DNA which was lost with continued passage (Di Lorenzo et al., 1992) apparently in spite of a rate of DNA replication which exceeded that of the cell. In addition, two studies have shown that episomal HPV DNA may persist in anogenital wart keratinocytes grown as explant cultures (Rose et al., 1987) or in primary monolayer culture (Ishiwata et al., 1991), although the effects of passage on viral maintenance were not examined.

In this study we have compared the maintenance of episomal HPV DNA in cultured keratinocytes derived from anogenital and oral warts using slot blot hybridization, PCR and Southern blot hybridization of the PCR product. The use of more sensitive techniques demonstrates that although high copy number HPV is lost from cultures with passage, residual infection may persist until culture senescence. The rate of episomal loss varies with keratinocyte phenotype, with episomal DNA being maintained for a greater number of passages in cultures derived from oral warts.

**Methods**

**Tissue collection.** Anogenital wart biopsies were obtained from patients presenting at routine genito-urinary medicine outpatient clinics (Dr B. Goh and the Royal London Hospital, Whitechapel and Dr N. Hanna at St Mary's Hospital, Paddington, London, U.K.). Oral warts were obtained from excised lesions sent for histopathological examination (Professor D. M. Williams at the Royal London Hospital). Clinical diagnosis, site and duration of lesion were noted. Specimens were collected in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B and stored overnight at 4°C. Where possible a sample of the original lesion was snap-frozen in liquid nitrogen for later analysis.

**Cell culture.** Immediately before processing, tissue was soaked briefly in 70% ethanol and washed twice in DMEM. Larger samples were chopped and the tissue was incubated in 0.5% trypsin at 37°C for 1 h. The trypsinized sample was disaggregated with needle and forceps and a single-cell suspension obtained by pipetting the sample in trypsin. Keratinocytes were seeded into a 25 cm² flask on a lawn of lethally irradiated Swiss 3T3 fibroblasts (Green et al., 1979) in RM⁻ medium [DMEM: Ham's F12 (3:1) supplemented with 5 μg/ml insulin, 2×10⁻¹¹ M-thiouronium, 1×10⁻⁴ M-adrenaline, 5 μg/ml transferrin, 0.4 μg/ml hydrocortisone and 10⁻¹⁸ M-cholera toxin] containing 10% fetal bovine serum (FBS). After a 24 h incubation at 37°C in the presence of 10% CO₂, the culture medium was replaced with RM⁺ medium (i.e. RM⁺ medium with 10 ng/ml epidermal growth factor) with 10% FBS. Cultures were subsequently maintained in RM⁺, with media changed twice weekly and fresh feeder cells added as required. Cultures were passaged at 80 to 90% confluence and split at 50%. Feeders were removed from confluent cultures, keratinocytes were detached from the substratum and disaggregated in 0.05% trypsin in 0.25% EDTA at 37°C and the cells were counted. One half of the cells were reserved for DNA isolation and the remainder reseeded into a 25 cm² flask containing irradiated feeder cells. Cell cultures were maintained in this way until the keratinocytes senesced.

**Isolation and hybridization of low Mᵦ DNA.** Episomal DNA was extracted from fresh cell pellets using a method adapted from that of Hirt (1967). Briefly, cell suspensions were washed in PBS, pelleted and resuspended at 10⁶ cells per ml TE buffer (10 mM-Tris–HCl, 1 mM-EDTA pH 7.5). A 1/10 volume 10% SDS was added dropwise to the cell suspension and incubated at room temperature for 5 min. A 3/10 volume of 5 M-NaCl was added dropwise to the partially lysed cells, the suspension was gently mixed and incubated overnight at 4°C. The lysed cells were pelleted, the supernatant was collected and the pellet reserved for isolation of cellular genomic DNA. The supernatant was extracted with phenol and chloroform and precipitated in ethanol.

Low Mᵦ DNA was resuspended at a concentration equivalent to 1.25×10⁸ cells per μl TE buffer. Forty μl of isolated DNA (equivalent to 5×10⁶ cells) was immobilized on a nylon membrane (Hybond N⁺) by slot blotting (Clare et al., 1991) with 100 pg each of full-length HPV-6, -11 and -16 DNA as positive controls, and 100 pg of pJ40 as a vector control. Blots were hybridized (Tₘ - 23°C) with mixed full-length genomic fragments of HPV-6, -11 and -16 labelled with ³²P using a random primer DNA labelling kit according to the manufacturer’s instructions (Gibco-BRL). Blots were washed at high stringency and visualized by autoradiography. Subsequently, blots were stripped according to the manufacturer’s instructions and rehybridized with probes to individual HPV types.

**Isolation and hybridization of cellular DNA.** Total cellular DNA was isolated from the pellets reserved from Hirt extraction (Sambrook et al., 1989) after washing to remove excess salt. Cellular DNA from pellets reserved following Hirt extraction of the cervical keratinocyte cell line CaSki, which contains integrated HPV-16, served as an internal control. Between 3 and 10 μg of isolated DNA was cut with BamHI and electrophoresed through 0.85% agarose gels with control samples of HPV-6, -11 and -16 full-length fragments and 7 μg of total cellular DNA from the CaSki cell line. DNA was transferred by Southern blotting onto nylon membranes (Hybond N⁺) and probed as above.

**Demonstration of HPV DNA by PCR amplification with Southern blotting.** Low Mᵦ DNA was examined for HPV sequences using HPV general primers MY09 and MY11 and as positive controls, and 100 pg of pJ40 as a vector control. Between 3 and 10 μg of isolated DNA was cut with BamHI and electrophoresed through 0.85% agarose gels with control samples of HPV-6, -11 and -16 full-length fragments and 7 μg of total cellular DNA from the CaSki cell line. DNA was transferred by Southern blotting onto nylon membranes (Hybond N⁺) and probed as above.

**Results**

**Morphology and growth characteristics of human wart keratinocytes**

Samples of warts taken from anogenital sites were classified as cutaneous or mucosal to determine whether differences between keratinocyte phenotypes would influence establishment, cell growth and morphology or viral persistence. A small difference between the establishment
rate of anogenital mucosal and cutaneous lesions was noted (47% compared to 56%). Of the 59 established anogenital wart cultures (76%) of the cultures survived until first passage (p1, etc.) with only 10 (17%) still proliferating after p3 (Table 1). The life spans of cultures from mucosal and cutaneous lesions were comparable, with the majority of cell cultures senescing at p1, probably owing to the small size of biopsies obtained.

In contrast to the results from the anogenital samples, successful cultures were established from all of 11 oral wart biopsies, perhaps indicating differences in cell plating efficiencies. The oral wart cultures also survived for a greater period in culture than anogenital wart cultures and the mean time between passages was slightly less suggesting that oral wart keratinocytes proliferate at a greater rate (data not shown). Cultured keratinocytes from both oral and anogenital lesions were morphologically indistinguishable both from each other, and from normal human skin keratinocytes. It is interesting to note that although oral and anogenital sites examined contained keratinizing and non-keratinizing regions, differences in growth kinetics were greatest when considering site rather than epithelial phenotype. Site-specific variations such as growth fraction and cell transit time may have affected cell adhesion, growth, differentiation and life span in vitro.

**Maintenance of episomal HPV DNA**

We next examined the persistence of HPV DNA in the cultured cells as a function of increasing culture age (Table 2 and Fig. 1). Initial experiments using gel electrophoresis and Southern blot hybridization failed to detect any viral DNA integrated within the host genome and was less sensitive than slot blot hybridization for the detection of low Mr viral DNA. Hybridization experiments were consequently confined to slot blotted DNA. Viral DNA was detected by slot blotting with mixed probes for HPV-6, -11 and -16, the most common types found at these sites, in 15/41 (37%) of the anogenital cultures screened at p0. No significant differences were detected between mucosal and cutaneous cultures at this time point. Episomal HPV DNA was rapidly lost with passage, and in all cases HPV was no longer detectable by the third passage. In contrast, episomal HPV DNA was detected in 70% of the oral cultures at p0 and was retained in a higher proportion of cultures with passage (60% still positive at p2). In all cases the episomal HPV DNA was lost within a single passage, although the final HPV passage number ranged from p0 to p2. No hybridization was seen with Hirt supernatants from CaSkI cells, indicating minimal levels of contamination with cellular genomic DNA (data not shown). As with establishment and growth rate, described earlier, no major differences were noted between cutaneous and mucosal anogenital keratinocytes; however, a higher retention rate of episomal HPV DNA was noted with cells derived from the oral cavity.

**Detection of HPV DNA from anogenital wart cultures by PCR**

Where sufficient DNA remained following slot blotting, low Mr DNA samples from cultures at p0 were also examined for HPV sequences by PCR using consensus primers MY09 and MY11. This was possible for nine of the 15 cultures positive by slot blot, and all were positive
Fig. 1. Maintenance of episomal HPV at levels detectable by slot blotting of Hirt supernatants. The blot was hybridized with mixed full-length HPV-6 and -11 probes. Low M, HPV DNA was detected in an anogenital wart culture at p0 (slot 1) but not at p1 (slot 2); episomal HPV in an oral wart culture, through p0 (slot 8), p1 (slot 9) and p2 (slot 10); Hirt supernatants from other anogenital wart cultures at p0 (slots 3, 4 and 11) which senesced before p1. Hirt extraction from CaSki cells (slot 12). Vector control, 100 pg pJ4f~ (slot 5); HPV-6 full-length fragments 200 pg and 100 pg (slots 6 and 13); HPV-II full-length fragments 200 pg and 100 pg (slots 7 and 14).

Table 3. Comparison of HPV maintenance in cultured anogenital wart keratinocytes as detected by slot blot hybridization, PCR and PCR/Southern blot hybridization

<table>
<thead>
<tr>
<th>Technique</th>
<th>Total</th>
<th>HPV-6/-11</th>
<th>HPV-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive by slot blot at p0</td>
<td>15/41</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Positive by slot blot or PCR (gel) at p0*</td>
<td>26/40</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Positive by slot blot, PCR (gel) or PCR/Southern blot at p0*</td>
<td>29/40</td>
<td>25</td>
<td>4</td>
</tr>
</tbody>
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* Results are collated as it was not possible to screen all slot blot-positive samples by PCR. All of the nine positive samples that were screened were also positive by PCR, with no difference in typing selectivity.

Table 4. Effects of HPV type on the life span of anogenital wart keratinocyte cultures

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Passage at senescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p1</td>
</tr>
<tr>
<td>HPV-6/-11</td>
<td>20</td>
</tr>
<tr>
<td>HPV-16</td>
<td>0</td>
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using general primers. Twenty-five of the 26 samples negative by slot blot were subject to analysis by PCR (Table 3). Of these, 11 of 25 examined gave a band when PCR samples were run out on a gel. These primers enable the detection of a range of HPV types, but hybridization analysis was confined to HPV-6, -11 and -16 to permit comparison with detection by slot blotting. Southern blot hybridization revealed that four of these samples were positive for HPV-16 and the remaining seven were positive for HPV-6 and/or HPV-11 (Fig. 2). In addition, Southern blot hybridization revealed three further cultures positive for HPV-6 or -11 which did not demonstrate bands on the original gel. The remaining 11 samples were negative with each technique. Most interestingly, those cultures which were positive for HPV-16 by PCR exhibited an in vitro life span three times longer than the average for HPV-6- or HPV-11-positive cultures (Table 4).

Mechanism of episomal loss

A decline in episomal content is frequently observed in cervical tumour tissue where an integrated HPV copy is commonly detected within the host genome (Dürst et al., 1985). We monitored the wart cultures to determine whether integration was occurring with concurrent
DNA was no longer detectable by slot blot hybridization revealed residual infection which was detectable by PCR, and persisted in cultured cells until senescence at p3 (Fig. 3b). This phenomenon was noted in all of three sequential cultures examined and includes cultures positive both for HPV-6 and -11. Thus it is apparent that HPV DNA may be retained in cultured keratinocytes until senescence.

**Discussion**

The inability of keratinocytes to support protracted HPV replication is a phenomenon which has been described in a variety of culture systems including endogenously infected laryngeal papilloma keratinocytes (La Porta & Taichman, 1982) and cells infected with purified HPV particles (Christian et al., 1987; Reilly & Taichman, 1987). These observations appear to reflect a loss of HPV-positive cells from culture, rather than a failure of episomal replication *per se* (Di Lorenzo et al., 1992). Nonetheless, immortal cell lines which maintain episomal HPV DNA have been derived, in each case from dysplastic cervical epithelium (Stanley et al., 1989; Choo et al., 1989; Bedell et al., 1991). To determine whether such ability to maintain episomal HPV DNA is dependent on the epithelial type from which cell cultures were isolated and to investigate the mechanisms of episomal loss further, we examined keratinocyte cultures derived from oral and anogenital warts to determine the extent of HPV maintenance.

In our analysis, we found striking differences between wart keratinocytes derived from oral and anogenital sites, which appeared to reflect differences in anatomical site rather than keratinocyte phenotype. Oral wart keratinocytes were more efficiently established in culture and survived for a greater number of passages in comparison with anogenital keratinocytes. Similar observations have been made on cultured normal oral keratinocytes and appear to reflect both plating efficiency and the proliferating fraction of cultured cells (H. Navsaria, personal communication). Episomal HPV DNA was also detectable in a notably higher proportion of oral wart cultures at first passage, and subsequently persisted for a greater number of passages at a copy number detectable by slot blotting. These differences between anogenital and oral wart keratinocytes may reflect transcriptional variation, as tissue-specific enhancer elements which have been identified in the HPV-11 upstream regulatory region (Cripe et al., 1987; Steinberg et al., 1989) are more active in oral than in cutaneous keratinocytes. However, the consequences of this specificity for viral replication remain poorly understood. Alternatively, differences in maintenance may stem from demonstrable differences in keratinocyte

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Fig. 3. (a) Hirt supernatants from cultured vulval wart keratinocytes demonstrating episomal loss with continued passage. Slot 1, 100 pg full-length HPV-11 fragment; slot 2, 100 pg full-length HPV-16 fragment; slot 3, 100 pg vector control pJ4Ω. The slot blot was probed with full-length HPV-11 fragment (Tm > 23 °C) and washed at high stringency. Cells from processed tissue were seeded onto a lawn of gamma-irradiated 3T3 feeder cells. Cells which had failed to adhere after 24 h following seeding were removed (slot 4). The remaining keratinocytes were grown to confluence and split at a ratio of 1:2. At 24 h, adherent cells from one culture (slot 5) were harvested separately from floating cells (slot 6). The second culture was grown to confluence and DNA was taken from 50% of cells at each subsequent passage (slots 7 and 8). (b) Persistence of low copy number HPV in anogenital wart keratinocytes until senescence. Hirt supernatants from the culture in Fig. 3(a) were examined for HPV sequences by PCR with primers MY09 and MY11. Samples in lanes 1, 2 and 3 correspond with samples in slots 5, 7 and 8 of (a). The PCR products were separated on a gel (panel i, with 1 kb ladder in lane 5), blotted and probed with full-length HPV-11 fragment (panel ii). The HPV-6-positive control in lanes 4 does not cross-hybridize with the HPV-11 probe.
differentiation or proliferation kinetics in vitro and we are currently investigating these parameters.

Using slot blot hybridization we have found that HPV DNA is maintained at early passage in both anogenital and oral wart keratinocytes, but cells containing high copy number viral DNA are lost with passage. This appears to reflect a failure of these cells to re-adhere to the flask upon plating. The average viral copy number per cell appears to be very similar in cells either taken immediately following passage, or following 24 h in suspension, suggesting that this observation does not reflect amplification of viral copy number concomitant with keratinocyte differentiation in suspension. It is not apparent whether the failure of cells to re-adhere upon passage represents a direct effect of HPV on keratinocyte differentiation, or whether cells which contain high copy number HPV are at a more advanced state of early differentiation. No evidence of HPV integration into the host genome was found by Southern blot hybridization either prior to, or coinciding with, episomal loss. We cannot exclude the possibility of low frequency integration events occurring during cell culture although this seems unlikely to have a major effect on the loss of episomal copy number within the entire culture population.

To address further the question of episomal loss, we increased the sensitivity of the screening process by using PCR coupled with Southern blot analysis. In contrast to other studies, we have established, using this technique, that HPV DNA may be retained in keratinocytes in monolayer culture at low copy number until senescence. This finding may be indicative of variation in HPV copy number among different keratinocyte populations. Primary keratinocyte cultures do not constitute a homogeneous cell population, rather they represent cells in a variety of differentiated states with corresponding differences in proliferation capability. Three clonal populations have been identified in primary epidermal keratinocyte cultures, which differ in the number of population doublings observed before senescence (Barrandon & Green, 1987). These clonal subtypes have been suggested to represent basal keratinocytes at different stages of early differentiation, although there is little evidence for such differences in human epithelia in vivo, and cellular markers are currently not available (Albers et al., 1986).

However, recent evidence suggests that keratinocyte clonal populations differ with respect to their expression of cell adhesion molecules, particularly in their expression of the integrins α2 and its partner β1 (Jones & Watt, 1993). More primitive cells with a higher capacity for cell proliferation express these molecules at a higher level. Such cells plate more efficiently and proliferate with their differentiating progeny forming the predominant culture population in serial culture. Maintenance of low copy number HPV in the more primitive cells would thus permit retention of HPV-positive cells until culture senescence. Similarly, if HPV DNA is maintained at a higher copy number in the more differentiated (but still proliferating) keratinocyte subpopulations, a higher copy number would be observed until the eventual senescence of the progeny of these clonal types. A reduction in cell adhesion molecules in the more differentiated cells which may contain higher levels of HPV would therefore result in a failure of these cells to re-adhere upon passage. To characterize more fully the subpopulations of HPV-infected keratinocytes in vitro, we are currently using single cell cloning and isopyknic separation to determine the extent of infection in cells according to their clonogenic capacity and cell density. These studies may provide further insight into the relationship between keratinocyte differentiation and HPV replication in stratifying epithelia.

Episomal amplification is undoubtedly associated with the transition of cells from the basal to the spinous compartments of the stratifying epithelium, whereas little evidence exists for differences in HPV copy number among cells of the basal layer of a warty lesion. In situ hybridization studies have proven to be too insensitive to detect episomal HPV DNA at the levels detected in basal keratinocytes in vitro, and the prior amplification of such levels of copy by in situ PCR does not permit accurate quantification of viral DNA. Our results appear to reflect differences in HPV copy number among populations of basal cells and to demonstrate that HPV may be maintained in culture at low copy number until senescence. HPV has the capacity to infect epithelia latently, and for such latent infection to persist, and subsequently reactivate to form a productive infection, a more primitive population of basal cells must remain infected. These more primitive cells may represent the reservoir for HPV latency and the source of virus reactivation in vivo. Such cells represent targets for further analysis into the mechanism of primary HPV infection and reactivation.

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