Malignant Transformation of Cutaneous Lesions in Renal Allograft Patients: A Role for Human Papillomavirus


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ABSTRACT

Renal allograft recipients are at greatly increased risk of developing squamous cell carcinomas. As these frequently arise adjacent to areas of multiple viral warts, we have investigated a possible role for human papillomavirus in malignant transformation within this population. We established, as primary cultures, keratinocytes from 24 lesions of varying degrees of squamous atypia from 9 patients. Ten of 14 cultures screened for the presence of episomal human papillomavirus DNA were positive using a mixed probe for cutaneous human papillomaviruses, although episomal copy was universally lost with continued passage. Three cultures, 2 of which were derived from malignant tissue and 1 from a benign lesion, were positive when screened with a probe for potentially oncogenic human papillomavirus DNAs 5 or 8. Both positive cultures of malignant origin exhibited extended lifespan and have been briefly characterized by morphology and growth requirements.

INTRODUCTION

It is well recognized that among renal allograft recipients there is an increased incidence of benign warts, premalignant skin lesions, and NMSC. In particular, the incidence of SCC is approximately 36 times greater than in the normal population (1). Factors associated with the development of SCC include the duration of immunosuppressive therapy, exposure to UV light, and skin type. In recognition of the complications associated with immunosuppression, treatment regimes have been revised to include new types of immunosuppressive drugs such as cyclosporin A, and the use of lower dosages. Although these precautions appear to lower the risk of developing warts and neoplasms (2, 3), the development of cutaneous cancers remains a serious problem within this population.

HPV has been implicated in malignant transformation of cutaneous lesions from allograft patients by clinical observations that viral warts appear to progress through squamous atypia to carcinoma in situ or invasive SCC (4). In addition, similarities have been noted between the lesions of transplant patients and those of individuals with the rare genetic skin disease EV. In this condition, patients suffer from multiple flat, sheeted warts that in sun-exposed sites evolve into carcinoma in situ or invasive squamous cell carcinoma in about 30% of cases. The benign lesions have been shown to be infected by a broad spectrum of unusual HPV types (5, 6), while predominantly HPV 5 and 8 DNA sequences have been detected in malignant EV lesions (7). These latter HPV types, shown to be capable of transforming rat embryonal fibroblasts (8, 9), have been implicated in the malignant conversion of sun-exposed skin in EV patients (7). This incidence of HPV types appears to be mirrored in the renal allograft recipient population, with the discovery of novel HPV types in lesions from renal allograft recipients (10) and, in one study (4), a 60% detection rate of HPV 5 and 8 DNA sequences in invasive and in situ carcinomas. Other studies have disputed this high frequency, however (11), and the role of HPV in cutaneous cancer among immunosuppressed individuals remains unclear.

The progression from normal epidermis through various degrees of dysplasia to cancer can be studied to a limited extent at a histological level, in fixed sections of biopsied tissue. The availability of cultured cells from benign, dysplastic, and malignant lesions provides a better opportunity to study the process of malignant transformation and to investigate the role of various factors in this conversion in an in vitro setting. In this study, we have established in culture lesions of different degrees of squamous atypia and investigated the prevalence of HPVs in these cultures, with a particular focus on EV-associated types. In addition, we have characterized 2 spontaneously immortalized keratinocyte lines that contained potentially oncogenic HPV at early passage.

MATERIALS AND METHODS

Patients. A total of 291 renal transplant recipients were monitored for the development of skin lesions. Over a period of a few months, lesions were removed from patients attending weekly transplant clinics, and from these a selection covering the full spectrum of histological atypia was used in this study. A total of 63 skin lesions from 21 renal allograft recipients were examined. The period since the patients’ first transplant ranged from 2 to 22 years (mean time, 10 years). Patients whose transplant was before 1985 had received immunosuppressive therapy consisting of prednisolone and azathioprine, while those with a transplant date after this had also received cyclosporin A.

Primary Care. Lesions were removed by excisional biopsy or curettage, and a part of all samples was sent for histological diagnosis. The remainder were placed immediately after removal into collection media (DMEM; Gibco) containing 5% v/v FCS (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin.

Epidermal keratinocyte cultures were initiated from this tissue as described by Rheinwald and Green (12). Cells were grown as a monolayer in the presence of γ-irradiated Swiss 3T3 feeder cells in DMEM/Ham’s F12 (3:1) medium supplemented with 10% FCS and various mitogens (13). Cultures were grown at 37°C in 10% CO2. Upon reaching 80% confluence, cells were washed with PBS, detached with 0.05% trypsin in 0.25% EDTA, and reseeded into new flasks at a 1:3 ratio. At the first passage, parallel cultures were set up with and without 10-7 M dexamethasone. Cultures were coded RT for renal transplant recipient, with the third letter indicating the histology of the original lesion (S for SCC, B for basal cell carcinoma, D for dysplastic, and W for wart). Patients were numbered at their initial presentation, with each separate lesion biopsied being denoted by a letter.

Growth Requirements. Cultures that had not senesced after 10 passages were examined to determine whether extended lifespan was accompanied by any characteristics of transformation. Growth factor requirements were investigated by comparing the growth rates of cultures in the presence and absence of 3T3 feeder cells and in normal and low serum conditions.

The effects of anchorage deprivation were studied by culturing cells in soft agar. Briefly, cells were mixed with top agar (DMEM with 0.3% Difco Noble

Received 3/1/93; accepted 8/25/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was funded by the Wellcome Foundation Ltd.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma; HPV, human papillomavirus; DMEM, Dulbecco’s modified Eagle’s medium; EV, epidermodysplasia verruciformis; FCS, fetal calf serum; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.


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Agar, 10% FCS) and plated at 10^3, 10^4, or 10^5 cells onto base agar (DMEM with 0.5% agar, 10% FCS) in 35-mm-diameter tissue culture dishes. Fresh top agar was added each week for 3 weeks, after which dishes were examined for colony formation. Caski cells were used as a positive control.

**Tumorigenicity in Nude Mice.** Keratinocytes (5 × 10^5) were suspended in Matrigel, which has been shown to accelerate tumor growth (14), and injected s.c. into each of 5 athymic mice. Animals were examined weekly for the development of enlarged lymph nodes or superficial tumors. The animals were sacrificed and examined for the presence of tumors after 12 weeks (or earlier with the development of large tumors, in compliance with animal licensing regulations).

**Karyotype.** Metaphase spreads were prepared as described by Verma and Babu (15). Briefly, Colcemid was added at a final concentration of 0.3 μg/ml to growing cells for 3 h. Cells were then harvested, washed with PBS, and resuspended in hypotonic solution (10% FCS in distilled water) for 15 min. After pelleting by centrifugation, fixative (methanol:glacial acetic acid, 3:1) was gently added for 30 s. This step was repeated 3 times with subsequent periods of fixation being 10 min. Cells were then spread onto slides by dropping from a height of approximately 1 meter and later stained with 10% Giemsa stain in PBS.

**Slot-Blot Analysis.** Low molecular weight DNA was extracted, as described by Hirt (16), from one-third of the cells from an 80% confluent 25-cm² flask (approximately 1 × 10^6 cells) after each passage up to and including passage 5. Hirt supernatant equivalent to 5 × 10⁵ cells was denatured as described by Clare et al. (17), and then transferred under vacuum to Hybond N* membranes (Amersham) through a Hybri-slot manifold (BRL Life Technologies). Control HPV DNA (equivalent to 100 copies/cell for 10⁶ cells) was included on all slot-blots.

**Southern Blot Analysis.** Total cellular DNA was extracted by proteinase K digestion and phenol extraction (18) from a confluent 175-cm² (1–2 × 10⁷ cells) flask of any culture with an extended lifespan. For Southern blot analysis, 10 μg of high molecular weight DNA or the remainder of the Hirt supernatant (corresponding to approximately 5 × 10⁵ cells) from any sample positive for HPV by slot-blot analysis was digested with PstI restriction endonuclease (Pharmacia LKB Biotechnology) and separated by 1% agarose gel electrophoresis (19). PstI-digested HPV DNAs 2, 3, 5, 8, and 10 were used as positive controls. DNA was denatured and transferred to Hybond N* membranes as described in the manufacturer’s protocol.

**Probe Preparation and Hybridization.** Cloned HPV 2, 3, 5, 6, 8, 10, 11, and 16 DNAs were excised from plasmid vectors and gel purified before labeling with [32P]dCTP using the DNA Random Primers Labeling System (BRL Life Technologies, Inc.). Hybridization was performed at stringency Tm = 23°C as described previously (20), modified by the addition of SDS to a final concentration of 0.1%. Blots were initially screened using a mixed probe of cutaneous HPV DNAs (HPVs 2, 3, 5, 8, and 10). After a low stringency wash (2 × standard saline-citrate, 0.1% SDS at 42°C for 15 min), membranes were exposed to Fuji RX100 Medical X-ray film at −70°C for 8–24 h. Samples were classified as negative if no HPV-positive signal could be detected after 7 days of exposure. Hybridization with a mixed probe of mucosal HPV DNAs (HPVs 6, 11, and 16) was similarly performed. Where necessary, membranes were stripped and rehybridized, after checking by autoradiography that the first probe had been completely removed. Membranes were used for a maximum of 3 hybridizations. Samples that hybridized with the mixed cutaneous HPV probe were rescreened with separate combined probes of HPV 2, HPVs 5 and 8, and HPVs 3 and 10. Membranes were washed under stringent conditions (0.1% standard saline-citrate, 0.1% SDS at 65°C for 15 min) and exposed to X-ray film for 24 h.

**RESULTS**

**Establishment and Morphology of Keratinocyte Cultures.** Primary cultures were initiated from 24 of the 63 lesions. Success of establishment correlated closely with histological diagnosis, and was far greater from malignant lesions than from dysplastic or benign tissues (Table 1). Seven of 11 cultures (64%) initiated from the latter group of lesions senesced before first passage, compared with none of 13 malignant cultures. In addition, 37% of malignant lesions gave rise to cultures with extended lifespan (greater than passage 10), compared to only 3% for benign lesions and 0% for dysplastic lesions. It must, however, be noted that 4 of the 6 malignant cultures with prolonged lifespan were established from sequential biopsies of a recurrent lesion from one patient.

Morphological differences were detected from the earliest passage between cultures derived from benign and malignant lesions, with cells from the latter appearing smaller, more rounded, and with a more granular cytoplasm. Cells in these cultures appeared to be only loosely adherent to each other, in contrast to the tightly packed, stratified colonies typical of normal cultures. In at least 2 of the 6 NMSC cultures, a mixed population of keratinocytes of both phenotypes was present at early passage, most likely due to contamination of the SCC biopsy with benign marginal tissue (Fig. 1). Normal keratinocytes

Fig. 1. Different colony morphologies observed in a culture established from SCC tissue. A, cells displaying a loosely adherent arrangement typical of cultured SCC cells. B, tightly packed cells resembling a colony of normal cutaneous keratinocytes.
Keratinocyte cultures were attempted from A3 lesions of different histological origins. Cells were grown as monolayer cultures with 3T3 feeder cells under standard keratinocyte culture conditions, passaged at 80% confluence by detachment with 0.05% trypsin in 0.25% EDTA and reseeded at a ratio of 1:3 into new flasks.

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>% Established&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Senesced&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Extended life&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>Malignant&lt;sup&gt;d&lt;/sup&gt; (n = 16)</td>
<td>81</td>
<td>44</td>
<td>37</td>
</tr>
<tr>
<td>Dysplastic (n = 9)</td>
<td>22</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Benign (n = 38)</td>
<td>24</td>
<td>21</td>
<td>3</td>
</tr>
</tbody>
</table>

* Lesions that were successfully established in culture.
* Cultures that showed finite lifespan similar to that of normal keratinocytes.
* Cultures with lifespan beyond that of normal keratinocytes (>passage 10).
* This group consisted of 1 basal cell carcinoma, 1 poorly differentiated SCC, 6 moderately differentiated SCCs, and 6 well differentiated SCCs.
* Number of lesions cultured of each histological type.

Lesions that were successfully established in culture. Cultures that showed finite lifespan similar to that of normal keratinocytes. Cultures with lifespan beyond that of normal keratinocytes (>passage 10).

Cultures were grown in the presence and absence of dexamethasone to evaluate the effects of corticosteroid hormones on cell proliferation and immortalization. No significant differences in cell growth or propensity to immortalize were noted, although, surprisingly, in one instance the addition of dexamethasone to a culture resulted in morphological differences. Cells from the culture RTS7c grown in the presence of dexamethasone assumed a spindle cell morphology after 4 passages. Those grown in the absence of dexamethasone maintained their initial rounded morphology (Fig. 2), suggesting that the presence of dexamethasone selects for a morphologically different subpopulation (although without clonal cultures of RTS7c the possibility that dexamethasone may alter cell morphology cannot be excluded). When dexamethasone was removed, a mixed culture of both subpopulations developed, although, after several passages, cells of spindle morphology were no longer detectable.

**Virological Studies.** The presence of episomal HPV DNA was investigated in cultures from 17 of the 63 lesions examined. Failure to establish in culture or senescence of cultures before reaching first passage prevented the screening of cultures from the remaining 46 lesions, which were of mostly dysplastic and benign origin. Under conditions of low stringency, episomal HPV DNA was detected in 71% of the cultures (6 of 10 malignant and 4 of 4 warts) using the mixed cutaneous HPV probe (Fig. 3). It was estimated that in a Hirt supernatant from 10<sup>5</sup> cells, a copy number as low as 1–2 was detectable with this method, although this decreased with subsequent re-probing of the blot. Under stringent conditions using separate combined probes (Fig. 4), hybridization with HPV DNAs 5 and 8 alone was detected in samples from one culture of benign origin and 2 of malignant origin (21%), and hybridization with HPV 2 DNA alone was detected in samples from 4 cultures, 2 of benign, and 2 of malignant origin. Southern blot analysis of the samples RTS3b and RTS7c confirmed the positive slot-blot results obtained with the combined HPV 5,8 probe, but there was insufficient Hirt supernatant from culture RTW46a to perform this analysis. It was observed that in the sample RTS7c, only one band of about 4.9 kilobases was detectable, correlating with the top band of digested HPV 5 DNA (Fig. 5). There was insufficient Hirt supernatant from samples to confirm the positive slot-blot results with the HPV 2 probe by Southern blot analysis. None of the samples was positive using either HPV 3,10 or HPV 6,11,16 probes. The presence of dexamethasone in the culture media had no consistent effect on episomal copy number or persistence. These results are presented in Table 2. Only the first of the 4 sequential malignant cultures is included, as results were negative for all 4 samples.

Total cellular DNA was extracted from passage 20 cultures of the 4 spontaneously generated cell lines described in Table 2 and screened by Southern blot using mixed probes for cutaneous and mucosal HPVs. HPV DNA was not found in any of the lines in either integrated or episomal form at this passage.

**Characterization of Cultures Positive for HPV 5/8-Homologous DNA.** We next examined the morphology of the 3 cultures that had initially been positive for episomal HPV 5,8 or related types. Cells from the benign culture RTW46a were indistinguishable from those of
showing results obtained with mixed cutaneous probe under low stringency conditions. HPV DMA controls were used at amounts equivalent to $10^4$ copies/cell for If)6 cells.

Interestingly, in both cases crisis occurred within one passage of loss (here as crisis, when the cells stopped dividing but remained viable. However, crisis does not, however, affect episomal copy number or persistence in either of these lines). Both lines were also found to have an absolute requirement for anchorage and showed reduced growth rates in media with less than 5% PCS, although only RTS7c required the presence of dexamethasone. The presence of dexamethasone did not, however, affect episomal copy number or persistence in either cell culture. Once established, however, the cultures provide an ample source of material and may give rise to immortalized lines for further analysis.

At early passage, episomal HPV DNA could be detected in 60% (6 of 10) of NMSC cultures, with loss occurring universally by passage 5. Our method, like other methods that depend upon DNA extraction, does not permit any distinction between HPV in the perilesional tissue and local HPV DNA controls were used at amounts equivalent to $10^4$ copies/cell for If)6 cells.

normal primary keratinocyte cultures and senesced at passage 5. Episomal HPV DNA was present in early passage at a level corresponding to approximately 10 copies/cell, but could not be detected after passage 2.

Morphologically, cells from the 2 positive cultures of malignant origin resembled those HPV-negative cultures derived from lesions of similar squamous atypia, with the exception of the previously described morphological changes observed in RTS7c cultures grown in the presence of dexamethasone. The presence of dexamethasone did not, however, affect episomal copy number or persistence in either culture. Both cultures underwent at early passage a period referred to here as crisis, when the cells stopped dividing but remained viable. Interestingly, in both cases crisis occurred within one passage of loss of the episomal HPV DNA (no evidence of viral integration was detected in either of these lines). Both lines were also found to have an absolute requirement for anchorage and showed reduced growth rates in media with less than 5% FCS, although only RTS7c required the presence of 3T3 feeder cells. Neither culture proved to be tumorigenic in nude mice. Karyotype analysis revealed RTS7c to be diploid, while RTS3b was shown to be hyperploid. Both lines are currently between passages 40 and 50.

DISCUSSION

We and others have established that immunosuppressed patients have a greatly increased incidence of viral warts and squamous cell carcinomas (4), which are frequently observed at adjacent sites in sun-exposed skin. Whether HPV is involved in malignant progression in these individuals remains unclear. The transforming HPVs 5 and 8, which have been implicated in the development of SCCs in EV patients (21), have, however, also been demonstrated in both warts and skin tumors of the immunosuppressed, although the rate of detection varies dramatically between different studies (4, 11, 22).

We have investigated this phenomenon further by culturing keratinocytes from 63 lesions of various degrees of squamous atypia from renal allograft recipients. Success of establishment was found to correlate with histological diagnosis, with keratinocyte isolation seeming to be less difficult from malignant lesions than from benign or dysplastic tissue, probably owing to the comparatively hyperkeratotic nature of the latter. We also observed that the majority of cultures established from benign or dysplastic tissue senesced before reaching first passage, which may indicate that these cells do not grow as well in vitro as keratinocytes from normal skin. In some cases, however, the extremely small size of the original tissue biopsy resulted in the plating of relatively few keratinocytes, hence cultures with a finite number of cell divisions senesced before reaching confluence. The main disadvantage of our approach for studying the incidence and role of HPV in these lesions thus appears to be that only a limited percentage of the samples (in our study 38% overall) can be established in culture. Once established, however, the cultures provide an ample source of material and may give rise to immortalized lines for further analysis.

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and HPV within the SCC. It seems likely, therefore, that some of these positives may be due to contamination with keratinocytes from marginal wart tissue, as in 2 cultures derived from malignant lesions we were able to detect 2 morphologically distinct populations of keratinocytes. This hypothesis is further supported by the observation that the pattern of episomal loss with passage corresponds well with that seen in cultured cutaneous and mucosal anogenital warts from non-immunosuppressed patients. Although we are unable to determine whether the site of infection lies within the lesion or in the surrounding tissue, it seems unlikely that infection by nontransforming HPV types, such as HPV 2-homologous types that we detected in 2 malignant cultures, plays a direct role in immortalizing keratinocytes in these patients. Benign HPV infection may, however, act indirectly by initiating the release of growth factors or further suppressing the local immune system, thus contributing to a more conducive environment for malignant cells.

Although the number of cultures is too small to permit firm conclusions, it is interesting that 2 of the 3 malignant cultures that developed into immortalized lines were found to contain at early passages DNA homologous to that of oncogenic HPV types 5,8, compared with none of the 7 malignant cultures that senesced. As we were only able to detect potentially oncogenic HPV at early passage in these 2 cultures, it would seem that while these HPVs may be involved in immortalization, they do not appear to be needed for maintenance of this phenotype among the cases we studied. This bears similarity to the finding (23) that HPV 6b may initiate transformation of C127 cells yet be unnecessary for persistence of the transformed phenotype. It must, however, be noted that due to the limitations in analysis. The failure of the culture of benign origin positive for HPV 5,8-related DNA to exhibit extended life may indicate that the presence of transforming HPV types alone is insufficient for immortalization. Another possibility is that HPV 5,8 and related viruses have an increased specificity for neoplastic and malignant tissue through alterations in the cell that make it more permissive for infection or viral replication.

It has been suggested (24) that HPV 3 or related types 10 and 28 can be codetected in lesions containing EV-associated HPVs in immunosuppressed patients. In our limited analysis, we could find no evidence for this, although it is feasible that within the lesion there were HPV 3- or 10-positive cells that failed to become established in culture. We also investigated whether HPV 16 DNA or related sequences might be present since these sequences have been found to be associated with NMSCs at sun-exposed sites such as face, neck, and hands in non-immunosuppressed patients (25). As the upstream regulatory regions of these, and possibly other, HPV types contain a glucocorticoid responsive element, cultures were grown in the presence and absence of dexamethasone. In no cultures, however, could HPV 6, 11, or 16 be demonstrated, and dexamethasone did not appear to consistently alter episomal copy number or persistence of the other HPV types detectable.

From our studies, we can conclude that malignant tissue biopsies are associated with HPV, although with our approach it was not possible to distinguish between HPV contained within the SCC cells and HPV from the marginal tissue. We had hoped to overcome this difficulty by selecting immortal SCC lines through cell culture that could then be screened for HPV DNA, but this was prevented by the apparently universal loss of episomal HPV from tissue culture systems, as a result of either the loss of HPV containing cells from the culture or the loss of episome from cells. It is interesting to note that

<table>
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<th>Histology</th>
<th>Patient</th>
<th>Culture</th>
<th>Site</th>
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<th>MM*</th>
<th>5,8*</th>
<th>3,10*</th>
<th>2*</th>
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* Mixed cutaneous probe (HPV 2,3,5,8,10).
+ Mixed mucosal probe (HPV 6,11,16).
* Cultures were screened with a probe for HPV 5,8 alone.
+ Cultures were screened with a probe for HPV 3,10 alone.
* Cultures were screened with a probe for HPV 2 alone.
* Cultures that grew beyond the finite lifespan of normal keratinocytes (>passage 10).
* Histologically benign.
* Histologically malignant.
* Cultures negative for the mixed probes were not examined with the specific probes.

with commercial primers that in anogenital cultures episomal HPV DNA is often present in low concentrations at late passages, where it is no longer detectable by Southern or slot-blot analysis. In this study, we were unable to perform polymerase chain reaction analysis on late passage DNA samples from initially positive cultures, as due to low cell numbers all DNA obtained was used in slot-blot and Southern blot analysis. The failure of the culture of benign origin positive for HPV 5,8-related DNA to exhibit extended life may indicate that the presence of transforming HPV types alone is insufficient for immortalization. Another possibility is that HPV 5,8 and related viruses have an increased specificity for neoplastic and malignant tissue through alterations in the cell that make it more permissive for infection or viral replication.

It has been suggested (24) that HPV 3 or related types 10 and 28 can be codetected in lesions containing EV-associated HPVs in immunosuppressed patients. In our limited analysis, we could find no evidence for this, although it is feasible that within the lesion there were HPV 3- or 10-positive cells that failed to become established in culture. We also investigated whether HPV 16 DNA or related sequences might be present since these sequences have been found to be associated with NMSCs at sun-exposed sites such as face, neck, and hands in non-immunosuppressed patients (25). As the upstream regulatory regions of these, and possibly other, HPV types contain a glucocorticoid responsive element, cultures were grown in the presence and absence of dexamethasone. In no cultures, however, could HPV 6, 11, or 16 be demonstrated, and dexamethasone did not appear to consistently alter episomal copy number or persistence of the other HPV types detectable.

From our studies, we can conclude that malignant tissue biopsies are associated with HPV, although with our approach it was not possible to distinguish between HPV contained within the SCC cells and HPV from the marginal tissue. We had hoped to overcome this difficulty by selecting immortal SCC lines through cell culture that could then be screened for HPV DNA, but this was prevented by the apparently universal loss of episomal HPV from tissue culture systems, as a result of either the loss of HPV containing cells from the culture or the loss of episome from cells. It is interesting to note that
the only 2 NMSC cultures in which we demonstrated HPV DNA homologous with that of oncogenic types were among the 3 NMSC cultures to demonstrate extended lifespan. To address questions such as site specificity and the involvement of HPV5/8 and related viruses in cutaneous cancer in these patients, we are currently using in situ hybridization of biopsy tissue in conjunction with the outlined approach.

ACKNOWLEDGMENTS

We thank Dr. E. M. de Villiers, Dr. L. Banks, Dr. A. Storey, and Dr. S. Campo for kindly providing the cloned HPV DNAs used in this study. We also thank Dr. A. Lati for her assistance with the karyotyping and Dr. K. Powell, Dr. L. Banks, and Dr. H. Pfister for their helpful critical suggestions.

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