ELSEVIER

Antiviral Research 24 (1994) 1–15

Antiviral Research

Mini-Review

Replication of human papillomaviruses in cell culture

Margaret A. Stanley

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

(Received 12 October 1993; accepted 31 January 1994)

Key words: Papillomavirus; Wart; Condyloma acuminata; Viral replication

1. Introduction

The papillomaviruses are a large group of pathogens which infect mucocutaneous surfaces where they induce a variety of proliferative lesions ranging from benign warts to malignant neoplasms. The papillomaviruses are not classified serologically but by nucleotide sequence homology, a new genotype being identified if it bears less than 50% homology to an existing isolate by liquid phase hybridisation. At present there are about 70 human papillomavirus (HPV) genotypes which have been cloned from clinical biopsies (Fig. 1) and each shows a predilection for a cutaneous or mucosal surface (De Villiers, 1989). Those genotypes infecting the genital tract have received close attention because of the strong association between infection with specific genital viruses and anogenital cancer, particularly cancer of the uterine cervix (Gissmann, 1992). About 20 genotypes infect the genital tract and they can be divided roughly into two groups. There are those HPVs associated with exophytic condyloma acuminata or true genital warts found predominantly in the lower genital tract and mainly HPV types 6 and 11 and those associated with intra-epithelial lesions, the so-called flat condyloma, caused by HPV types 16, 18, 31, 33, 35 and related types (de Villiers, 1989). These distinctions are not absolute but the association between infection with types 16 and 18 in particular and the development of cervical cancer is very strong to the extent that infection with these viruses is now regarded as the major risk factor for the subsequent development of this malignancy (Schiffmann, 1992). This raises the possibility that intervention in the natural history of HPV infection in the genital tract by either prevention of infection or treatment of

*Corresponding author. Fax: +44 223 333735.

SSDI 0166-3542(94)00004-R

DISEASE	HPV TYPE
Skin	
Common warts, hands and feet etc.	1, 2, 4, (26), (27), 29, 57
Plane warts	3, 10, 28, (49)
Butchers' warts	7
Epidermodysplasia verruciformis, benign	5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 46, 47, 50
Epidermodysplasia verruciformis, SCC	5, 8, 14, 17, 20
Keratocanthoma	37
Malignant melanoma	38
Actinic keratosis	5, 8
SCC	41, (48)
Epidermoid cyst	60
Genitalia and mucous membranes	
Normal cervix	16, 53
Genital warts	6, 11, 44, 54
Buschke Lowenstein tumours	6, 11
Cervical intraepithelial neoplasia	6 ,11, 16, 18, 30, 31, 33, 34, 35, 39, 40, 42, [43], [44], 45, 51, 52, 56, 57, 58, 66*
Cervical carcinoma	16, 18, 31, 33, 35, 39, 45, 51, 52, 56,.66*
Vulvar intraepithelial neoplasia	16, 18, 43, 59
Penile intraepithelial neoplasia	16, 18, 39, 40
Bowen's disease	34
Bowenoid papulosis	16, 39, 55
Laryngeal papillomas	6, 11
Laryngeal carcinoma	30
Focal epithelial hyperplasia (Heck's)	13, 32
Oral papillomas	32

Fig. 1. Papillomavirus genotypes and the diseases with which they are associated (de Villiers 1989). Types in brackets () refer to isolates from immunosuppressed patients. Types in brackets [] refer to isolates from macroscopically normal cervix. HPV 66* reported by Tahweed et al. (1991).

established lesions will be an effective anti-cancer strategy.

Clearly the oncogenic HPVs are important but disease induced by the so-called non-oncogenic genital viruses is not trivial. Genital warts are the most commonly diagnosed viral sexually-transmitted disease in the UK (PHLS 1989) and cause considerable discomfort and disruption to the lives of patients. Current therapies are unsatisfactory with a high frequency of recurrent disease and a proportion of patients with refractory lesions (Krebs et al., 1989). A third and increasingly important group of patients with HPV infections are those who are immunosuppressed either as a consequence of organ transplantation (Benton et al., 1992) or HIV infection (Kiviat et al., 1993). Infections with both cutaneous and mucosal HPV types are one of the most frequent viral complications in those individuals. For example, individuals immunosuppressed as a consequence of renal transplantation exhibit not only a high frequency of cutaneous warts but these are florid growths refractory to most therapeutic strategies (Benton et al., 1992). Importantly, malignant transformation can occur in these lesions which tend to arise on sun-exposed surfaces and it has been estimated that such individuals have a 20 times risk of cutaneous malignancy compared to the general population (Rudlinger et al., 1986). HIV-induced immunosuppression increases the severity and duration of anogenital warts, increases their infectiousness and reduces treatment efficacy (Rudlinger et al., 1988). In advanced immunosuppression, significant increases in rates of HPV-associated anogenital intraepithelial neoplasia including cervical intraepithelial neoplasia (CIN) are found and in areas of high HIV prevalence there is an increased incidence of anal carcinoma in men (Palefsky et al., 1990). The HPVs are not simply the cause of trivial, albeit unsightly, excrescences but induce significant, life-threatening human disease and therapeutic strategies to prevent infection or treat-established disease are imperative. Clearly with an infectious agent, immunological intervention by prophylactic vaccination or immunotherapy is an attractive prospect but the large number of HPV types – each of which may be serologically distinct – raise potential problems and the increasing problem of HPV infection and neoplastic progression in immunosuppressed individuals identifies the need for chemotherapy in the form of specific anti-HPV drugs.

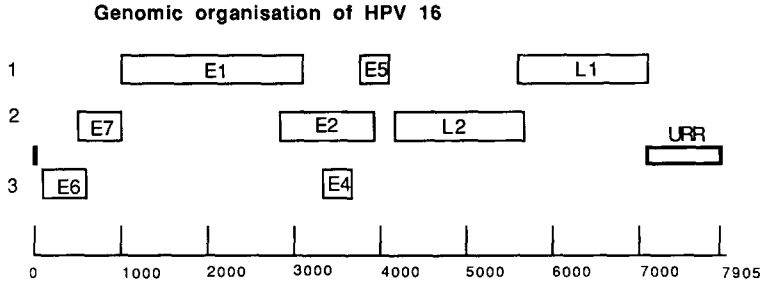
2. Genomic organisation and molecular biology of the HPVs

The implementation of prophylactic or therapeutic strategies is absolutely dependent upon a knowledge of the biology of the virus and its interaction with the target cell for infection, the keratinocyte. Despite some important advances in the past 2–3 years, this information remains fragmentary because of the unique replication strategy of HPV – a strategy in which virus replication and differentiation proceed in parallel and in which vegetative viral growth occurs only in a terminally differentiating epithelium. The difficulties of reproducing this scenario in vitro have contributed significantly to the problems in understanding papillomavirus/keratinocyte interactions and the regulation of viral gene expression during permissive growth. However the application of recombinant DNA technology has permitted the analysis of viral

genomic organisation. The DNA of approximately 45 genotypes have been sequenced and overall show a remarkable similarity of genomic organisation. Typically, (Fig. 2) there are 3 domains; an approximately 1 kb non-coding region, the upstream regulatory region (URR), an early (E) region (open reading frames E6, E7, E1, E2, E4, E5) and a late (L) region (ORFs L1 and L2). The URR contains the origin (*ori*) of DNA replication and binding sites for numerous transcription factors including NF1, AP1, Oct 1, TEF1 and TEF2 and other as yet unidentified factors (Apt et al., 1993 and references therein): the exquisite epithelial specificity of the HPVs is considered to result very largely from the cooperative interaction of these ubiquitous transcription factors. However the activity of the URR has been studied to date only in cell lines in vitro, and since the control of the URR in vivo is differentiation or lineage-specific, the complete elucidation of its functions will require the use of in vitro systems which support a complete infectious cycle.

The E and L regions are all transcribed from the same strand, and despite the apparently simple genetic organisation, the actual protein products are complex because of the use of alternative promoters and multiple RNA splicing events (Smotkin et al., 1989; Doorbar et al., 1990; Palermo-Dilts et al., 1991; Rohlfs et al., 1991; Chiang et al., 1991). The application of recombinant DNA technology to analyse viral genomic organisation and viral gene expression has resulted in an understanding of some of the functions of the proteins encoded by these ORFs.

The E6 and E7 genes encode proteins which induce and regulate the expression of cellular proteins, such as proliferating cell nuclear antigen (PCNA) and DNA polymerases, essential for viral replication (Dollard et al., 1993; Chen, Broker and Chow,



Protein functions of ORF products

E1 Viral DNA replication
E2 Transactivation
E6 Transactivation, binds p53, co-operates with E7 in immortalisation
E7 Immortalisation, binds RB and cyclin A/p33cdk2
E5 Affects growth factor receptors (EGF-R, PDGF-R)
E4 Interacts with keratin intermediate filaments
L2 Minor capsid protein
L1 Major capsid protein

Fig. 2. The genomic organisation of HPV 16 and the functions of the proteins encoded by the ORFs.

unpublished data). In the oncogenic HPVs, expression of these ORFs is essential for transformation of primary human keratinocytes (Barbosa and Schlegel, 1988) and this region of the genome is always retained in cervical malignancies. Both E6 and E7 form complexes with cell proteins which play a role in negatively regulating cell growth (Vousden 1993). Several proteins are known to form complexes with E7 but perhaps the best understood interaction is the association between E7 and the retinoblastoma (*Rb-1*) gene product (Munger and Phelps 1993). The interaction between Rb and E7 disrupts the association of Rb with the transcription factor E2F (Chellappan et al., 1992) and E7 expression one consequence of which could be the inappropriate transcription of E2F responsive genes releasing the normal blocks on progress through the cell cycle. The E6 proteins from the high risk HPVs bind to p53 resulting in the rapid degradation of this protein via the ubiquitin proteolytic system (Scheffner et al., 1990) and human cells expressing E6 show a marked reduction in the half-life of endogenous p53 (Lechner et al., 1992, Hubbert et al., 1992). Like Rb, p53 belongs to the class of proteins which negatively regulate cell growth and since the E6 proteins from the low risk HPVs bind p53 less well and, at least in in vitro assays do not appear to target p53 for degradation, this interaction between the E6 of high risk HPVs and p53 may contribute to neoplastic transformation and progression (Vousden 1993).

Studies on BPV-1 have shown that E1 encodes proteins essential for viral replication and plasmid maintenance (reviewed in Lambert 1991) and in vitro assays have shown that the products of this ORF form protein/protein complexes with an E2 protein and bind to viral DNA initiating viral DNA replication (Ustav and Stenlund 1991; Ustav et al., 1991; Yang et al., 1991). Recent studies (Chiang et al., 1992) have shown that, as in BPV-1, the E1 and E2 proteins of HPV 11 are essential for the replication of the homologous viral *ori*. Interestingly this work also showed that both homologous and heterologous papillomavirus *ori* replication was supported in a range of cell types of both human and rodent origin, an observation which suggests that the tissue specificities of the HPVs are not a reflection of direct restrictions on replication but that transcriptional control must dominate.

The E2 gene products are complex encoding important regulatory proteins. A notable feature of the URR of papillomaviruses is the presence of multiple copies of a sequence motif $ACCGN_4CGGT$ which is the binding site of E2 dimers (Androphy et al., 1987; Knight et al., 1991). The effect of E2 binding at these sites on viral gene expression may be stimulatory or inhibitory depending upon the position of the binding motif and the nature of the E2 gene product. In BPV-1 expression of E2 from different promoters and differential splicing results in the production of at least three E2 proteins, a full length E2 transactivator protein and two shorter proteins which act as transrepressors blocking transactivation by the full length product by competing for its binding site (Choe et al., 1989; McBride et al., 1989; Vaillancourt et al., 1990). A weak transcriptional repressor activity has also been reported for the HPV18 E2 protein (Romanczuk et al., 1990) but recent studies reveal that the full length E2 of HPV 16 is a potent transcriptional activator in cervical keratinocytes (Bouvard et al., 1994). These complex interactions of E2 proteins must be a reflection of the differentiation-dependent and time-dependent repli-

cation cycle, and as with many other aspects of HPV biology, require for experimental analysis an in vitro culture system which supports the complete infectious cycle of the virus.

The E5 ORF encodes a small intensely hydrophobic protein one function of which appears to be to alter the function of membrane-bound protein kinases such as the EGF or PDGF receptors (Pim et al., 1992; Cohen et al., 1992). The dominant cellular locale of this protein is, however, in the membranes of the Golgi and endoplasmic reticulum (Banks and Matlashewski 1993; Sun, Zhou and Frazer, unpublished data) and it is likely that there are unknown but important functions of the E5 protein which are related to this intracellular location. The E4 ORF encodes proteins which appear late in the viral cycle (Doorbar et al., 1986; Breitburd et al., 1987). In simple epithelial cells infected with a vaccinia recombinant virus expressing the HPV 16 E4, the E4 protein binds to keratin intermediate filaments and collapses the keratin cytoskeleton (Doorbar et al., 1991). In lesions E4 is expressed in the upper spinous and granular layers of the epithelium where the simple epithelial keratins (K8 and K18 in the main) are not present: in these sites the dominant keratin polypeptides are K1 and K10 in the skin and K4 and K13 in the cervix. There is no evidence for cytoskeletal collapse in differentiated cervical keratinocytes as a consequence of E4 expression (Sterling et al., 1993) and the interaction of E4 and keratins remains to be elucidated. The L region contains two ORFs, the L1 which encodes the major capsid protein and L2 the minor capsid protein; expression of these genes is absolutely dependent upon keratinocyte terminal differentiation and the regulation of late gene expression is poorly understood although there is evidence that the regulation of expression of HPV 16 L1 is at the level of RNA processing (Kennedy et al., 1991).

3. In vitro culture of HPVs

The HPVs are exclusively epitheliotropic viruses and they have evolved a unique replication strategy which depends absolutely upon the differentiation programme of the keratinocyte. Only keratinocytes – or cells with the potential for squamous maturation – can be infected and only in terminally differentiating keratinocytes are viral capsid proteins synthesised and virions assembled. In vitro culture systems which permit the serial passage of primary epidermal keratinocytes were first described by Rheinwald and Green (1975) in a seminal series of experiments. Infection of such monolayer cultures of keratinocytes with HPV virions derived either from cutaneous (HPV 1) or genital (HPV 11) warts or transfection with HPV DNA has been disappointing with transient replication only of the episome and rapid loss of DNA from the cells (La Porta and Taichmann 1982; Mungal et al., 1992). Transfection or electroporation of cloned DNA from the oncogenic HPVs 16 (Kaur et al., 1989) and 18 (Kaur and McDougall, 1988) together with a selectable marker has resulted consistently in the derivation of immortalised lines in which viral DNA is integrated into the host genome making such cells useless for studies on episomal replication.

An alternative approach has been to derive keratinocyte lines from low grade cervical lesions. One such line, W12, is an established but non-malignant keratinocyte line containing on average 100 copies/cell of HPV 16 predominantly as the episome (Stanley et al., 1989). In conventional submerged cultures these cells undergo limited stratification, express basal cell specific keratins but do not express markers of keratinocyte terminal differentiation such as loricrin or filaggrin (Greenfield and Stanley, unpublished observations). The HPV 16 early region genes are transcribed and the E6 and E7 proteins are detectable by immuneprecipitation albeit at low levels. Late gene transcripts are present in trace amounts and this is attributable to the presence of rare cells with a very high viral copy number (Higgins and Stanley, unpublished observations). The dependence upon a differentiated environment for permissive viral growth is clearly illustrated by transplanting W12 cells to a skin pocket on the flank of a nude mouse using a transplantation technique (Hammond et al., 1987) which permits epithelial reformation. This results in the expression of a completely differentiated phenotype by W12 cells and major changes in HPV 16 gene expression. These changes are time-dependent and parallel those seen in clinical lesions. Ten days after grafting, W12 cells have reformed an epithelium which morphologically appears identical to that of the normal ectocervical epithelium; at this time viral sequences cannot be detected by DNA:DNA in situ hybridisation (ISH). At 28 days post-grafting the epithelium has thickened and has the histological appearance of a low grade cervical intra-epithelial neoplasm. Viral sequences can now be detected by ISH in the superficial cells and immunohistochemistry reveals isolated cells positive for the L1 major capsid protein. At 54 days post-grafting, many cells are positive for the L1 protein, the E4 protein is expressed throughout the stratum granulosum and viral particles can be identified in the degenerating nuclei close to the keratinized superficial layers of the graft (Sterling et al., 1990). Immunoelectron microscopy with L1 and E4 monoclonal antibodies confirm that these viral particles are HPV 16 (Sterling et al., 1993).

In vitro systems permissive for HPV must reproduce the environment which supports in vivo differentiation. The regulation of keratinocyte differentiation is extremely complex but depends very substantially on signals from the subepithelial stroma or dermis (Smola et al., 1993). Culture systems which partially reproduce this connective tissue matrix have been developed. In essence all these systems consist of a collagen matrix seeded with fibroblasts of murine (Kopan et al., 1987) or human (Bell et al., 1979) origin. Keratinocytes are seeded thickly onto this matrix in submerged culture and when confluent the collagen keratinocyte sandwich is raised on an inert support so that the keratinocyte layer is at the air/liquid interface (Fig. 3). In such organotypic or "raft" cultures the keratinocytes stratify and differentiate with the expression of differentiation specific keratins (Kopan et al., 1987). Alternatively de-epidermised dermis has been used as a dermal substitute in in vitro skin equivalents with comparable results in terms of epidermal organisation (Regnier et al., 1981).

Using organotypic culture systems immortal keratinocyte cell lines transformed by HPV 16 or 18 DNA have been shown to stratify and undergo abnormal differentiation comparable to those seen in CIN in vivo (McCance et al., 1988; Merrick et

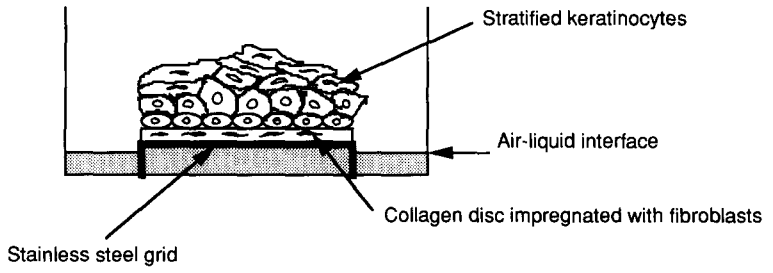
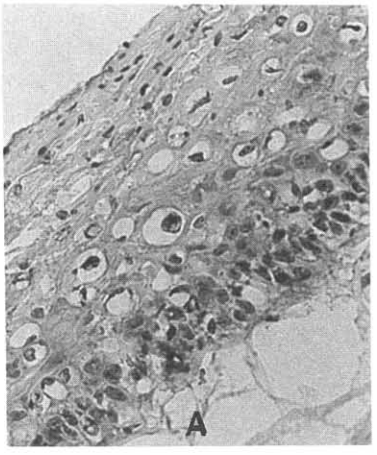
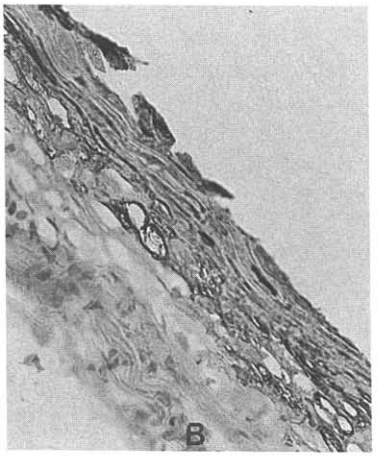


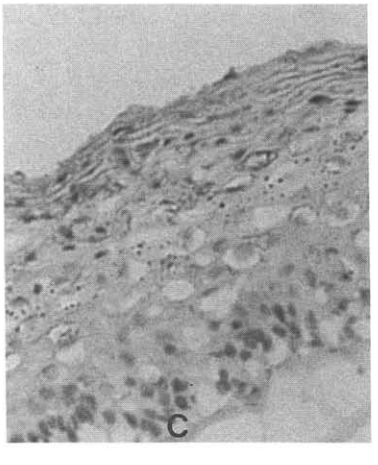
Fig. 3. A schematic representation of organotypic "raft" culture systems for keratinocytes. Keratinocytes are seeded onto collagen matrices under conventional submerged culture conditions and are then raised to the air/liquid interface as shown in the cartoon after which stratification and differentiation occur.

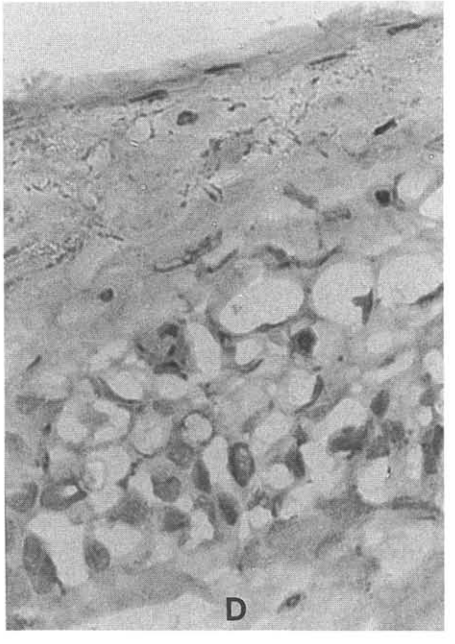
al., 1992). Cell lines containing episomal copies of HPV stratify and differentiate and viral DNA amplification can be shown by in situ hybridisation, to occur in the upper layers of the stratified cells (Greenfield and Stanley, unpublished data; Bedell et al., 1991). Transcriptional analysis of HPV 31b in the CIN 612 line in organotypic culture shows significant differences compared to monolayer cultures with evidence for a novel promoter, p742 in the E7 gene from which abundant E1E4 and E5 transcripts are initiated (Hummel et al., 1992). Recent developments with organotypic cultures have achieved permissive HPV replication and viral assembly in vitro when keratinocytes containing HPV as the episome are used to initiate the cultures. Dollard and colleagues (1992) explanted small fragments of HPV 11 containing condylomas (generated as nude mouse xenografts) onto collagen matrices seeded with the A31 strain of Balb/C 3T3 fibroblasts. Keratinocytes grew out from these explanted fragments, stratified and differentiated and within 3 weeks of culture complete viral transcription and replication leading to viral assembly occurred. Viral gene expression in this system is exquisitely dependent upon the source of the

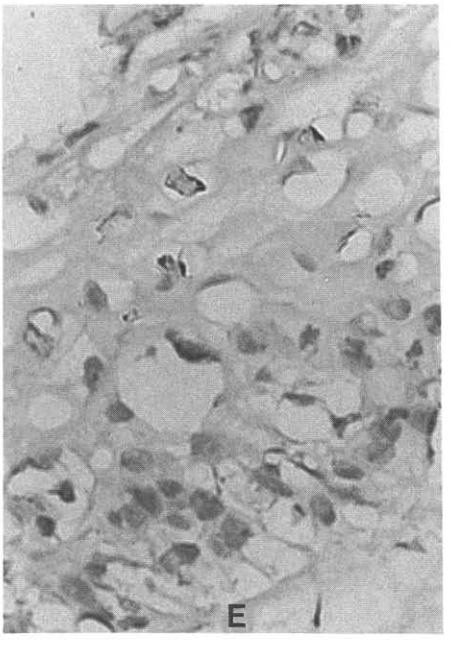
Fig. 4. Expression of keratinocyte differentiation markers and HPV 16 late proteins by the W12 cell line in organotypic culture. W12 cells at passage 9 were seeded out on collagen matrices impregnated with A31 fibroblasts, grown for 9 days in raised culture, fixed in buffered formol saline and paraffine wax embedded and 5 μ sections taken and immunostained using an immunoperoxidase technique (Vectastain, Vector Laboratories, UK). A: Haematoxylin and eosin stain, note the stratification and presence of cells with a koilocytic morphology in the upper layers. Original magnification × 100. B: Immunoperoxidase staining of W12 cells with anti-keratin 13 mAb 7003 (Dako A/S Denmark). Positively-stained cells are confined to the upper third of the stratified epithelium. Original magnification × 100. C: Immunoperoxidase staining of W122 cells with an anti-filaggrin mAb (Biogenesis UK Ltd). Positively-stained cells are confined to the extreme superficial cell layers. Originl magnification × 100. D: Immunoperoxidase staining of W12 cells with an anti-HPV 16 E4 antibody (Doorbar et al., 1991). Positively-stained cells appear in the superficial stratified layers. Original magnification × 200. E: Immunoperoxidase staining of W12 cells with an anti-HPV 16 L1 antibody CamVir 1 (McLean et al., 1990). Occasional positively-stained nuclei are evident in the superficial layers.


A


B


C


D


E

fibroblasts in the collagen matrix. Thus, if the matrices are seeded with human dermal fibroblasts no viral DNA amplification, late gene expression or virion assembly can be demonstrated and seeding with the A31 3T3 strain is essential for permissive viral growth. Viral gene expression in these permissive organotypic cultures was virtually identical to that seen in the clinical biopsies and in the experimental condyloma in the nude mouse with high copy viral DNA and abundant E6/E7 mRNA's in the upper spinous layers. W12 cells grown on matrices seeded with A31 fibroblasts express the differentiation markers keratin 13 and filaggrin in the superficial layers. Low level expression of the the HPV 16 E4 protein is seen in these cultures and occasional cells express the L1 capsid protein (Fig. 4).

A different approach for the induction of the completely differentiated phenotype in raft culture has been taken by Meyers and colleagues (1992). In these studies the CIN 612 cell line which contains episomal copies of HPV 31b were grown in organotypic cultures pulsed with the phorbol ester TPA (Tetradecanoylphorbol-13-Acetate), a potent activator of protein kinase C. Such treatment resulted in an abrupt spinous to granular transition in the upper layers of the cultures, the induction of capsid protein synthesis and the production of HPV 31b virions which could be purified by density gradient separation. Similarly TPA treatment of organotypic cultures of the W12 line results in a massive induction of L1 and L2 messages in these cells and the synthesis of capsid proteins (Higgins and Stanley, unpublished data). Organotypic cultures in which permissive viral growth is occurring represent powerful tools for the screening of novel anti-viral compounds and the exploitation of these systems can be anticipated.

4. Infection in vitro

These recent developments in in vitro culture of HPVs are encouraging but the fact remains that at the present time there is no published report showing that virions generated in vitro can infect keratinocytes and initiate another round or replication in vitro. This inability to infect with virus and initiate episomal replication in vitro parallels the many unsuccessful attempts to introduce cloned or episomal DNA preparations into keratinocytes and elicit viral replication. One should contrast this with the nude mouse xenograft model of Kreider and colleagues (Kreider et al., 1985) in which human foreskin or cervical tissue fragments are infected in vitro with the Hershey strain of HPV 11 then transplanted under the renal capsule of the nude mouse with the subsequent expression of a complete infectious cycle and production of infectious virus (Kreider et al., 1987). Clearly the cell permissive for infection has exquisite growth requirements apparently provided only by the in vivo environment and this raises the issue of the identity of this cell. Intuitively one suspects that a virus so dependent upon keratinocyte differentiation for permissive replication extends this dependence to the complete life cycle, from "the cradle to the grave" so to speak, and that the target cell for infection is the stem cell.

Squamous epithelia consist of self renewing populations of keratinocytes with basal cells forming the proliferative compartment from which cells migrate upward,

differentiating as they progress, to be exfoliated or desquamated from the surface and replaced by new cells from below. Such a self renewing population by definition contains a sub-set of cells with stem cell properties and there is a keratinocyte lineage extending from primitive stem cell to differentiated enucleate squame. In hair bearing skin in rodents, cells with stem cell properties are found in two sites, the bulge zone of the hair follicle (Miller et al., 1993) and in the interfollicular epidermis in the centre of the epidermal proliferative unit (EPU) (Morris et al., 1985). The EPU are hexagonally arranged stacks or columns of cells which comprise the kinetic organisational units of the skin, each EPU has a basal layer of about 10 keratinocytes, all mitotically active but only the central cell has a stem cell phenotype. In relation to the present discussion there is very persuasive evidence from the rabbit model (reviewed by Kreider and Bartlett 1981) that only keratinocytes migrating from the hair follicle after wounding are infectable with the cotton tail rabbit papilloma virus (CRPV). Furthermore a functional mesenchyme or connective tissue is essential for CRPV infection (Breedis and Kreider 1970) and self renewal in epidermis is exquisitely dependent upon the connective tissue matrix (Leary et al., 1992). Relating to this is the recent and fascinating data from Bossens et al. (1992); these workers infected foreskin keratinocytes in vitro with caesium chloride purified HPV-1 virions obtained from plantar warts and the infected keratinocytes were then grown in organotypic culture using as the matrix de-epidermised human dermis. In this skin equivalent system amplification of viral DNA could be shown in the reconstituted epidermis and in squames shed from it. In the context of the present discussion it is of interest that in this system good epidermal reconstruction and evidence of viral DNA amplification were found only in the dermal invaginations which are presumptive remnants of hair follicles and exactly the sites where the connective tissue matrix or niche for stem cells might be retained. If this hypothesis that the cell permissive for infection and immediate early viral functions is the stem cell is substantiated it may well turn out that the observed predilection of the HPVs for cutaneous or mucosal surfaces reflects differences in the transcriptional milieu of the stem cell of hair bearing surfaces compared to mucosal surfaces.

5. Future prospects

The developments of the past 5 years in in vitro culture systems for HPV are impressive. The most powerful aspect of the current sytems is the ability to switch from non-permissive to permissive viral growth either by changing the fibroblast population in the matrix (Dollard et al., 1992) or by TPA treatment (Meyers et al., 1992). This permits the molecular analysis of the regulation both by the cell and virus of this switch and of the control of late gene expression. When these organotypic culture systems are combined with technologies for sorting keratinocyte populations by surface phenotype, methods currently under development in several laboratories, major advances in understanding virus cell interaction can be expected.

Currently the major hole in our knowledge of HPV biology concerns the events which accompany infection and the nature of immediate early viral gene expression.

Investigating these phenomena has been impeded by inadequate culture systems and the difficulties of obtaining virions particularly for the genital HPVs. However the recent demonstration that infection of cells containing episomal BPV with a recombinant vaccinia virus expressing the L1 and L2 capsid proteins of BPV resulted in the assembly of infectious virus (Zhou et al., 1993) provides an avenue for the generation of high titres of virus using episome containing lines and the appropriate vaccinia recombinant. Elucidation of the early events of the infectious cycle is now a real prospect.

Organotypic culture systems will be crucial reagents in the development of drugs effective in the treatment of HPV-induced lesions. The current systems are still not optimal since the complete expression of the keratinocyte lineage does not occur. However these aspects are under intense investigation by several groups and the development of in vitro systems which completely and faithfully reproduce epithelial differentiation is probably quite close. These in vitrosystems combined with animal xenograft systems such as the nude mouse model of Kreider and the more recently described SCID mouse models (Bonnez et al., 1993; Stables and Sexton unpublished observations) provide test systems essential for the evaluation of toxicity and efficacy of anti-HPV drugs.

References

Androphy, E.J., Lowy, D.R. and Schiller, J.T. (1987) Bovine papillomavirus E2 transacting gene product binds to specific sites in papillomavirus DNA. Nature 325, 70–3.

Apt, D., Chong, T., Liu, Y. and Bernard, H.U. (1993) Nuclear factor 1 and epithelial-cell specific transcription of human papillomavirus type 16. J. Virol. 67, 4455–63.

Banks, L. and Matlashewski, G. (1993) Cell transformation and the HPV E5 gene. Papillomavirus Reports 4, 1–4.

Barbosa, M.S. and Schlegel, R. (1989) The E6 and E7 genes of HPV 18 are sufficient for inducing two stage in vitro transformation of human keratinocytes. Oncogene 4, 1529–32.

Bedell, M.A., Hudson, J.B., Golub, T.R., Turyk, M.E., Hosken, M., Wilbanks, G.D. and Laimins, L.A. (1991) Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. J. Virol. 65, 2254–60.

Bell, E., Ivarsson, B. and Merrill, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Nat. Acad. Sci. USA 76, 1274–8.

Benton, C., Shahidullah, H. and Hunter, J.A.A. (1992) Human papillomaviruses in the immunosuppressed. Papillomavirus Reports 3, 23–6.

Bonnez, W., Rose, R.C., DaRin, C., Borkhuis, C., de Mesy Jensen, K.L. and Reichman, R.C. (1993) Propagation of human papillomavirus type 11 in human xenografts using the severe combined immunodeficiency (SCID) mouse and comparison to the nude mouse model. Virology 197, 455–458.

Bossens, M., Van Pachterbeke, C., Tuynder, M., Parent, D., Heenen, M. and Rommelaere, J. (1992) In vitro infection of normal human keratinocytes by human papillomavirus type 1 followed by amplification of the viral genome in reconstructed epidermis. J. Gen. Virol. 73, 3269–73.

Bouvard, V., Storey, A., Pim, D., Baraggino, E., Wisenfeld, U. and Banks, L. (1994) Detection of HPV-16 E2 protein in cervical keratinocytes. In: Stanley, M.A. (Ed.), Immunology of Human Papillomaviruses (HPVs), Proceedings of the Second International Workshop on HPV Immunology. Cambridge 1993, Plenum Press New York, 33–38.

Breedis, C. and Kreider, J.W. (1970) The role of the dermis in the induction of neoplasia by Shope papilloma virus. Cancer Res. 30, 974–80.

Breitburd, F., Croissant, O. and Orth, G. (1987) Expression of human papillomavirus type-1 E4 gene products in warts. In: Steinberg, B.M., Brandsma, J.L. and Taichmann, L.B. (Eds.), Papillomaviruses Cancer Cells 5, pp. 115–22. Cold Spring Laboratory, New York.

Chellapan, S., Kraus, V., Kroger, B., Munger, K., Howley, P.M. and Phelps, W.C. (1992) Adenovirus E1A, simian virus 40 tumor antigen and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA 89, 4549–53.

Chiang, C.M., Broker, T.R. and Chow, L.T. (1991) An E1M E2C fusion protein encoded by human papillomavirus type 11 is a sequence specific transcription repressor. J. Virol. 65, 3317–29.

Chiang, C.M., Ustav, M., Stenlund, A., Ho, T.F., Broker, T.R. and Chow, L.T. (1992) Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Natl. Acad. Sci. USA 89, 5799–803.

Choe, J., Vaiilancourt, P., Stenlund, A. and Botchan, M. (1989) Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNA's. J. Virol. 63, 1743–55.

De Villiers, E.M. (1989) Heterogeneity of the human papillomavirus group. J. Virol. 63, 4898–903.

Dollard, S.C., Broker, T.R. and Chow, L.T. (1993) Regulation of the human papillomavirus type 11 E6 promoter by viral and host transcription factors in primary human keratinocytes. J. Virol. 67, 1721–6.

Dollard, S.C., Wilson, J.L., Demeter, L.M., Bonnez, W., Reichman, R.C., Broker, T.R. and Chow, L.T. (1992) Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. Genes Dev. 6, 1131–42.

Doorbar, J., Campbell, D., Grand, R.J.A. and Gallimore, P.H. (1986) Identification of the HPV-1a E4 gene products. EMBO J. 5, 355–62.

Doorbar, J., Parton, A., Hartley, K., Banks, L., Crook, T., Stanley, M.A. and Crawford, L.V. (1990) Detection of novel splicing patterns in a HPV 16 containing keratinocyte cell line. Virology 178, 254–62.

Doorbar, J., Ely, S., Sterling, J., McLean, C. and Crawford, L. (1991) Specific interaction between HPV 16 E1 E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature 352, 824–7.

Gissmann, L. (1992) Human papillomaviruses and genital cancer. Semin. Cancer Biol. 3, 253–61.

Hammond, E.J., Ng, R.L., Stanley, M.A. and Munro, A.J. (1987) Prolonged survival of cultured keratinocyte allografts in the nonimmunosuppressed mouse. Transplantation 44, 106–12.

Hubbert, N.L., Sedman, S.A. and Schiller, J.T. (1992) Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. J. Virol. 66, 6237–41.

Hummel, M., Hudson, J.B. and Laimins, L.A. (1992) Differentiation induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. 66, 6070–80.

Kaur, P. and McDougall, J.K. (1988) Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J. Virol. 62, 1917–24.

Kaur, P., McDougall, J.K. and Cone, R. (1989) Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. J. Gen. Virol. 70, 1261–6.

Kennedy, I.M., Haddow, J.K. and Clements, J.B. (1991) A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. J. Virol. 65, 2093–7.

Kiviat, N.B., Critchlow, C.W., Holmes, K.K., Kuypers, J., Sayer, J., Dunphy, C., Surawicz, C., Kirby, P., Wood, R. and Daling, J.R. (1993) Association of anal dysplasia and human papillomavirus with immunosuppression and HIV infection among homosexual men. AIDS 7, 43–9.

Knight, J.D., Li, R. and Botchan, M. (1991) The activation domain of the bovine papillomavirus E2 protein mediates association of DNA bound dimers to form DNA loops. Proc. Natl. Acad. Sci. USA 88, 3204–8.

Kopan, R., Traska, G. and Fuchs, E. (1987) Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. J. Cell. Biol. 105, 427–39.

Krebs, H.B. (1989) Genital human papillomavirus infection. Management strategies. Clin. Obstet.

Gynecol. 32, 200–13.
Kreider, J.W. and Bartlett, G.L. (1981) The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression. Adv. Cancer Res. 35, 81–110.
Kreider, J.W., Howett, M.K., Leure Dupree, A.E., Zaino, R.J. and Weber, J.A. (1987) Laboratory production in vivo of infectious human papillomavirus type 11. J. Virol. 61, 590–3.
Kreider, J.W., Howett, M.K., Wolfe, S.A., Bartlett, G.L., Zaino, R.J., Sedlacek, T. and Mortel, R. (1985) Morphological transformation in vivo of human uterine cervix with papillomavirus from condylomata acuminata. Nature 317, 639–41.
Lambert, P.F. (1991) Papillomavirus DNA replication. J. Virol. 65, 3417–20.
LaPorta, R.F. and Taichman, L.B. (1982) Human papilloma viral DNA replicates as a stable episome in cultured epidermal keratinocytes. Proc. Natl. Acad. Sci. USA 79, 3393–7.
Leary, T., Jones, P.L., Appleby, M.W., Blight, A., Parkinson, E.K. and Stanley, M.A. (1992) Epidermal keratinocyte self renewal is dependent upon dermal integrity. J. Invest. Dermatol. 99, 422–30.
Lechner, M.S., Mack, D.H., Finicle, A.B., Crook, T., Vousden, K. and Laimins, L.A. (1992) Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53 mediated repression of transcription. EMBO J. 11, 3045–52.
McBride, A.A., Byrne, J.C. and Howley, P.M. (1989) E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxy-terminal domain transactivation is mediated by the conserved amino-terminal domain. Proc. Natl. Acad. Sci. USA 86, 510–4.
McCance, D.J., Kopan, R., Fuchs, E. and Laimins, L.A. (1988) Human papillomavirus type 16 alters human epithelial cell differentiation in vitro. Proc. Natl. Acad. Sci. USA 85, 7169–73.
McLean, C., Churcher, M., Meinke, J., Smith, G., Higgins, G., Stanley, M. and Minson, A.C. (1990) Production and characterisation of a monoclonal antibody to human papillomavirus type 16 using recombinant vaccinia virus. J. Clin. Pathol. 43, 488–92.
Merrick, D.T., Blanton, R.A., Gown, A.M. and McDougall, J.K. (1992) Altered expression of proliferation and differentiation markers in human papillomavirus 16 and 18 immortalized epithelial cells grown in organotypic culture. Am. J. Pathol. 140, 167–77.
Meyers, C., Frattini, M.G., Hudson, J.B. and Laimins, L.A. (1992) Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science 257, 971–3.
Miller, S.J., Sun, T. and Lavker, R.M. (1993) Hair follicles, stem cells and skin cancer. J. Invest. Dermatol. 100, 289s–94s.
Morris, R.J., Fischer, S.M. and Slaga, T.J. (1985) Evidence that the centrally and peripherally located cells in the murine epidermal proliferative unit are two distinct populations. J. Invest. Dermatol. 84, 277–81.
Mungal, S., Steinberg, B.M. and Taichman, L.B. (1992) Replication of plasmid derived human papillomavirus type 11 DNA in cultured keratinocytes. J. Virol. 66, 3220–4.
Munger, K. and Phelps, W.C. (1993) The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim. Biophys. Acta 1155, 111–23.
Palefsky, J.M., Gonzales, J., Greenblatt, R.M., Ahn, D.K. and Hollander, H. (1990) Anal intraepithelial neoplasia and anal papillomavirus infection among homosexual males with group IV HIV disease. JAMA 263, 2911–6.
Palermo-Dilts, D.A., Broker, T.R. and Chow, L.T. (1991) Human papillomavirus type 1 produces redundant as well as polycistronic messenger RNAs in plantar warts. J. Virol. 64, 3144–49.
PHLS Communicable Disease Centre (1989) Sexually transmitted disease in Britain 1985–86. Genitourinary Med. 65, 117–21.
Pim, D., Collins, M. and Banks, L. (1992) Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene 7, 27–32.
Regnier, M., Prunieras, M. and Woodley, D. (1981) Growth and differentiation of adult human epidermal cells on different dermal substrates. Front Matrix Biol 9, 4–35.
Rheinwald, J.G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinising colonies from single cells. Cell 6, 331–44.
Rohlfs, M.S., Winkenbach, S., Meyer, S., Rupp, T. and Durst, M. (1991) Viral transcription in human keratinocyte cell lines immortalised by human papillomavirus type-16. Virology 183, 331–42.
Romanczuk, H., Thierry, F and Howley, P.M. (1990) Mutational analysis of *cis* elements involved in E2 modulation of human papilloma virus type 16 p97 and type 18 p105 promoters. J. Virol. 64, 2849–59.

Rudlinger, R., Grob, R., Buchmann, P., Christen, D. and Steiner, R. (1988) Anogenital warts of the condyloma acuminatum type in HIV positive patients. Dermatologica 176, 277–81.

Rudlinger, R., Smith, I.W., Bunney, M.H. and Hunter, J.A. (1986) Human papillomavirus infections in a group of renal transplant recipients. Br. J. Dermatol. 115, 681–92.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. and Howley, P.M. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63, 1129–36.

Schiffman, M.H. (1992) Recent progress in defining the epidemiology of human papillomavirus infection and cervical neoplasia. J. Natl. Cancer Inst. 84, 394–8.

Smola, H., Thiekotter, G. and Fusenig, N.E. (1993) Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. J. Cell Biol. 122, 417–29.

Smotkin, D., Prokop, H. and Wettstein, F.O. (1989) Oncogenic and non-oncogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. 63, 1441–7.

Stanley, M.A., Browne, H.M., Appleby, M. and Minson, A.C. (1989) Properties of a non tumorigenic human cervical keratinocyte cell line. Int. J. Cancer 43, 672–6.

Sterling, J.C., Skepper, J. and Stanley, M.A. (1993) Immunoelectronmicroscopic localisation of HPV 16 L1 and E4 proteins in cervical keratinocytes cultured in vivo. J. Invest. Dermatol. 100, 154–58.

Sterling, J., Stanley, M., Gatward, G. and Minson, T. (1990) Production of human papillomavirus type 16 virions in a keratinocyte cell line. J. Virol. 64, 6305–7.

Tawheed, A.R., Beaudenon, S., Favre, M. and Orth, G. (1991) Characterisation of human papillomavirus type 66 from an invasive carcinoma of the uterine cervix. J. Clin. Microbiol. 29, 2656–60.

Ustav, M. and Stenlund, A. (1991) Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. 10, 449–48.

Ustav, M., Ustav, E., Szymanski, P. and Stenlund A. (1991) Identification of the origin of viral replication of bovine papillomavirus and characterisation of the viral origin recognition factor. EMBO J. 10, 4321–29.

Vaillancourt. P., Nottoli, T., Choe, J. and Botchan, M. (1990) The E2 transactivator of bovine papillomavirus type 1 is expressed from multiple promoters. J. Virol. 64, 3927–37.

Vousden, K. (1993) Interactions of human papillomavirus transforming proteins with the products of tumour suppressor genes. FASEB J. 7, 872–9.

Yang, L., Li, R., Mohr, I., Clark, R. and Botchan, M. (1991) Activation of BPV-1 repagel replication in vitro by the transcription factor E2. Nature 353, 628–32.

Zhou, J., Stenzel, D.J., Sun, X.Y. and Frazer, I.H. (1993) Synthesis and assembly of infectious bovine papillomavirus particles in vitro. J. Gen. Virol. 74, 763–8.