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Induction of productive human papillomavirus type 11 life cycle in epithelial cells grown in organotypic raft cultures

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Abstract

The study of the human papillomavirus (HPV) life cycle was hampered for more than 50 years by the lack of a conventional cell culture system for propagating HPV. Considerable progress has been made in the production of several HPV types using either organotypic rafts or human epithelial xenografts in immunocompromised mice. In this study, we demonstrated episomal maintenance of HPV-11 DNA in N-Tert cells. HPV-11 episomal DNA containing cell populations grown in raft culture showed induction of the productive viral life cycle. HPV-11 DNA amplification and viral capsid antigen synthesis were detected in differentiated layers of epithelia. The viruses generated were able to infect keratinocytes in vitro, which indicate that viruses generated were infectious. The demonstration of the productive HPV-11 life cycle in raft culture from cloned HPV-11 DNA will facilitate genetic analyses of viral gene functions that was not possible using the human xenograft athymic mouse model.

Keywords: Human papillomavirus; Cervical cancer; Organotypic raft culture

Introduction

Human papillomaviruses (HPV) are small DNA viruses that infect human epithelial cells including those in the anogenital tract (Howley, 1996). These anogenital HPV are divided into two groups: the low-risk types, which associate with benign genital warts and the high-risk types, which associate with cervical cancer (Bosch et al., 1995). The HPV life cycle is tightly linked to the differentiation program of human keratinocytes (Hummel et al., 1992). The viral life cycle can be divided into early and late stages. The early stage of the viral life cycle occurs after infection through skin wounds or microabrasions in the basal layer of the epithelia, and viral DNA is replicated and maintained at a low copy number (~50–100 copies per cell) (Frattini et al., 1996). The late stage of the viral life cycle occurs with epithelial cell differentiation and migration towards the epithelial cell surface, and viral DNA is amplified to a high copy number (500–1000 copies per cell) (Flores and Lambert, 1997; Hummel et al., 1992). Viral DNA amplification may be followed by virus capsid antigen synthesis and assembly of the progeny virions in the terminally differentiated epithelial layers (Stubenrauch and Laimins, 1999). Since the life cycle is tightly linked to the differentiation program of human epithelial cells, HPV cannot be propagated using ordinary, monolayer, cell cultures in general except in one case, where HPV-59 was propagated in monolayer culture after calcium stimulation (Lehr et al., 2003). The high level of tissue specificity of HPV also precludes the direct infection of experimental animals with HPV. The production of HPV and the investigation of the complete HPV life cycle have therefore been hindered by the lack of in vitro cell culture systems and animal models. Fortunately, significant progress has been made in the past 20 years. Human epithelium xenografts/immunocompromised mouse systems and organotypic raft culture systems have been developed and used successfully for production of multiple HPV types and for investigation of the HPV life cycle (Bedell et al., 1991; Kreider and Howett, 1987; Meyers et al., 1992).

The human epithelium xenografts/immunocompromised mouse system was first developed by Kreider et al. (1985) in

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1985. Split-thickness human epithelial chips were incubated with a pooled cell-free virus extract from patients with *condylomata acuminata*. Infected tissue chips were then grafted under the renal capsule of athymic mouse kidneys. After 3 months of incubation, xenografts develop into virus producing epithelial cysts, which closely resemble naturally occurring *condyloma acuminata* with characteristics of koilocytosis. These papillomas stain highly positive for HPV DNA, RNA, and capsid antigens. The grafts as well as the keratin plugs within the centers of the grafts are a rich source of infectious virus (Kreider et al., 1986). Further infections in additional animals can be initiated using cell-free extracts of keratin preparations (Kreider et al., 1987). The xenograft system can serve as a rich source of infectious virus for continued propagation of viruses by reinitiating infection in epithelial targets and in vivo production of a papilloma. This model is therefore outstanding for studying virus production events and the molecular events associated with pathogenesis of papilloma formation. It is also highly utilized for anti-viral, microbicidal and anti-neoplastic testing. The human xenograft model has been limited, however, by the inability to initiate infection via HPV DNA and by the limited ability to extract infections, pools of "high risk" HPV virus stocks, from clinical material, except in rare cases, such as HPV-59 (Lehr et al., 2003).

The organotypic raft culture is a cell culture system where epithelial cells are seeded on top of a dermal equivalent (collagen matrix containing fibroblasts) and raised onto a wire grid and fed from below in such a way that the keratinocytes grow and differentiate at the air–liquid interface (Bedell et al., 1991; Meyers, 1996; Meyers et al., 1992). The raft culture has been shown to closely mimic the differentiation and organization of normal epithelial tissues. Several cell lines that stably maintain HPV episomal DNA and derived from low-grade cervical intraepithelial neoplastic lesions have been used to study the viral life cycle in the organotypic raft cultures or in the athymic mouse xenografts (Bedell et al., 1991; Flores et al., 1999; Meyers et al., 1992; Sterling et al., 1990). Subsequently, methods have also been developed to introduce the HPV genome into human foreskin keratinocytes by either electroporation (Meyers et al., 1997, 2002) or transfection (Frattini et al., 1996) to establish HPV episomal containing cell lines and to establish the viral life cycle in organotypic raft cultures or semisolid media (Thomas et al., 2001). In addition, an adenoviral delivery vector that contained a full HPV-16 genome flanked by Lox P homologous recombination sites was used to deliver HPV genome to cervical epithelial cells and differentiated human airway epithelia in the presence of Cre recombinase (Lee et al., 2004). The advantage of introducing a selected HPV genome into human keratinocytes and subsequently inducing differentiation in the raft culture is that it provides a powerful means for the genetic analysis of viral gene functions in the context of the complete HPV viral life cycle. To further overcome the difficulties associated with culturing primary human keratinocytes and selecting stable HPV episomal containing keratinocytes before senescence due to the limited life span of primary human keratinocytes, researchers turned to spontaneously immortalized human foreskin keratinocytes to allow maintenance of episomal HPV DNA (Brune and Durst, 1995) in monolayer cultures and to support the life cycle of certain types of HPV in raft cultures (Flores et al., 1999).

Although HPV types 1, 2, 11, 40, and 83 (LVX82/MM7) have been successfully propagated in the xenograft system (Brown et al., 1998; Christensen et al., 1997; Kreider et al., 1990), none of the viruses have been propagated in tissue xenografts starting from HPV DNA. Using raft cultures, HPV types 31b, 18, 45, 16, and 18/16 chimera (Lee et al., 2004; McLaughlin-Drubin et al., 2003, 2004; Meyers et al., 1992, 1997, 2002) have been propagated starting from HPV DNA. In this study, we report for the first time the establishment of the HPV-11 life cycle in a human keratinocyte cell line immortalized by the catalytic subunit of human telomerase (N-Tert) grown in raft cultures, starting from HPV-11 DNA, the pBT-1 clone of HPV-11-Hershey. N-Tert cells have been shown previously to have normal growth and differentiation characteristics in raft cultures and xenografts (Dickson et al., 2000). HPV-11 episomal DNA containing N-Tert cell populations grown in raft culture showed induction of the productive stages of the viral life cycle. HPV-11 DNA amplification was detected by in situ hybridization and viral capsid antigen synthesis was detected by immunohistochemistry staining for HPV group specific antigen (GSA). Infectious virus stocks could be derived. These data indicated that the N-Tert cell line can support the life cycle of HPV-11 when grown in raft cultures and may serve as an alternative target cell line for the study of HPV life cycle.

Results

Establishment of HPV-11 episomal DNA containing N-Tert cell lines

HPV-11 DNA, the pBT-1 clone of HPV-11-Hershey, was linearized and separated from the vector sequence by digestion with *Bam*HI, which has a cutting site at nucleotide 7072 in the L1 region. N-Tert cells were electroporated with linear HPV-11 DNA and a plasmid that encodes the neomycin resistance gene. Electroporated cells were subsequently subjected to G418 selection for HPV-11 DNA containing cell populations. Southern blot analysis of total DNA extracted from HPV-11 electroporated and neomycin selected N-Tert cell populations revealed the presence of episomal copies of HPV-11 genome and the copy number was estimated in the range of 10 to greater than 100 copies per cell in about 70% of the cell populations examined. Representative Southern blot analysis probed with HPV-11 DNA showed that Form I DNA was evident in the lanes digested with a restriction enzyme that will not cut HPV-11 DNA (Fig. 1).

Temporal differentiation and stratification of HPV-11 episomal DNA containing N-Tert cell populations grown in organotypic raft cultures

N-Tert cells electroporated with linear HPV-11 and neomycin selective marker were selected in the presence of G418. The resulting HPV-11 episomal DNA containing cell population

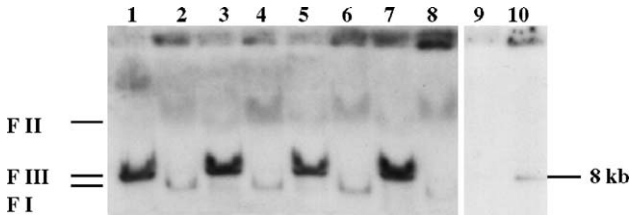

Fig. 1. Southern blot analysis for the state of HPV-11 DNA in electroporated N-Tert cell populations. Four representative and individually derived HPV-11 DNA eletroporated N-Tert cell populations 11A (Lanes 1 and 2), 11B (Lanes 3 and 4), 11C (Lanes 5 and 6), and 11D (Lanes 7 and 8) were analyzed for the state and copy numbers of HPV-11 DNA. The whole gel is shown and bands at the top of the gel are in the wells. Total cellular DNA (5 μg) was digested with restriction enzyme *Bam*HI, which is a single cutter of HPV-11 genome (Lanes 1, 3, 5, and 7), or *Xho*I, which is a non-cutter of HPV-11 genome (Lanes 2, 4, 6, and 8). HPV-11 plasmid of 10 copies per cell and 100 copies per cell are shown as controls (Lanes 9 and 10). Episomal HPV-11 DNA is detected in all four cell populations with an estimated copy number ranged from 10 to greater than 100 copies per cell. Form I, II, and III DNA are shown as indicated.

was then allowed to differentiate and stratify using raft cultures (Figs. 2A–C) compared with N-Tert rafts without HPV-11 (Figs. 2D–F). Rafts harvested at various time points were stained with hematoxylin and eosin to examine the histology for the temporal differentiation and stratification of the epithelium. Cross sections of N-Tert and HPV-11 N-Tert rafts showed that the epithelia thickened over time (Fig. 2). Progressive stratification and differentiation of HPV-11 N-Tert epithelia took place from days 12 to 18 (Figs. 2A–C). More specifically, the epithelium was 3–4 cell layers thick at day 12 (Fig. 2A), and became 5–6 cell layers thick by day 15 (Fig. 2B). By day 18, the epithelium became 8–9 cell layers thick with koilocytes and extensive keratinization (Fig. 2C). The morphology of day 18 HPV-11 N-Tert rafts was suggestive of low-grade dysplasias with koilocytosis and parakeratosis (Fig. 2C). HPV-11 N-Tert rafts appear to have a more proliferative morphology compared to N-Tert rafts especially at day 18 (Figs. 2C and F), but further experiments are needed to definitively determine whether HPV-11 affects differentiation/proliferation in N-Tert raft cultures.

Detection of HPV-11 DNA amplification and capsid antigen in HPV-11 N-Tert cell populations grown in raft cultures

As shown above, rafts grown from HPV-11 episomal DNA containing N-Tert cell populations were able to stratify and differentiate over time. To examine the late stages of HPV-11 viral life cycle in HPV-11 N-Tert rafts, viral DNA amplification and capsid antigen synthesis indicative of late stages of viral life cycle were analyzed. Viral DNA amplification and capsid antigen synthesis were examined by in situ hybridization and immunohistochemical staining for viral capsid antigen, respectively. HPV-11 positive signals of high intensity were detected in a number of cells in the upper layers of epithelium in day 18 rafts (Figs. 3A and B). Immunohistochemical staining for HPV group specific antigen also detected weak staining of cells in upper layers of epithelium in some area of cells positively stained for HPV-11 DNA in day 18 rafts (Figs. 3C and D). N-Tert rafts alone without HPV-11 DNA or HPV-11 N-Tert rafts at early time points did not show any positive signal (data not shown). These

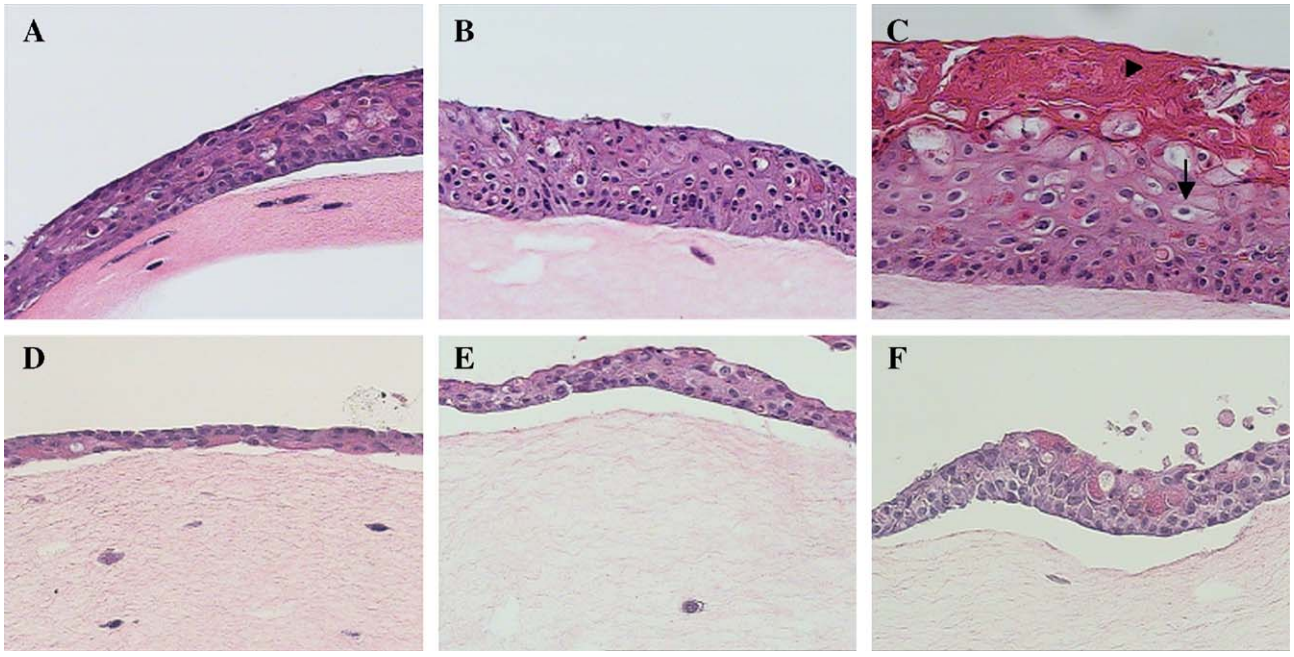

Fig. 2. Temporal differentiation and stratification of HPV-11 episomal DNA containing N-Tert cell population grown in organotypic raft culture. N-Tert cells electroporated with linear HPV-11 and neomycin selective marker were selected in the presence of G418. The resulting HPV-11 episomal DNA containing cell population was then allowed to differentiate and stratify using raft cultures compared with N-Tert rafts without HPV-11. Rafts harvested at various time points were stained with hematoxylin and eosin to examine the histology for the temporal differentiation and stratification of the epithelium. (A) Day 12 HPV-11 N-Tert raft showed 3–4 cell layers thick epithelium. (B) Day 15 HPV-11 N-Tert raft showed 5–6 cell layers thick epithelium. (C) Day 18 HPV-11 N-Tert raft showed 8–9 cell layers thick epithelium with koilocytosis (➡) and extensive keratinization (▶). (D) Day 12 N-Tert raft showed 2–3 cell layers thick epithelium. (E) Day 15 N-Tert raft showed 3–4 cell layers thick epithelium. (F) Day 18 N-Tert raft showed 3–5 cell layers thick epithelium. Magnification was 200× for all panels.

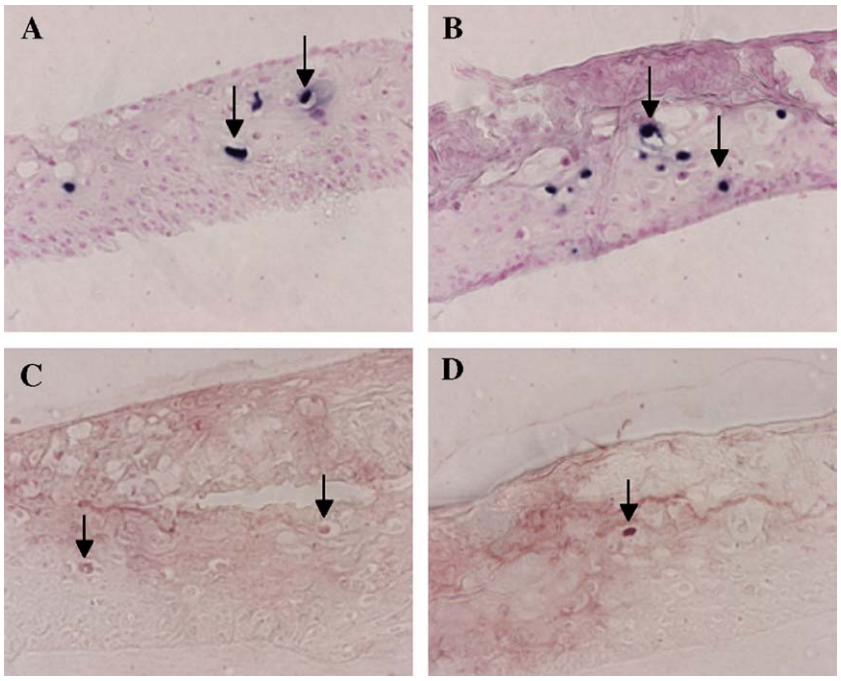

Fig. 3. Detection of HPV-11 late functions of HPV-11 N-Tert cell populations grown in raft cultures. Rafts were grown from HPV-11 episomal DNA containing N-Tert cell populations. (A and B) Two representative views of day 18 rafts analyzed by in situ hybridization stained with biotin-labeled HPV-11 DNA. HPV-11 positive signals were detected in a number of cells in the upper layers of the epithelium as indicated by arrows. (C and D) Immunohistochemical staining for HPV group specific antigen detected weak staining of cells in upper layers of the epithelium in two representative areas of day 18 rafts as indicated by arrows. Magnification was 200× for all panels.

results indicated that HPV-11 late stages of viral life cycle were successfully induced by differentiation of HPV-11 episomal DNA containing N-Tert cells grown in raft cultures.

Demonstration of infectivity of generated HPV-11 viruses

In order to test whether the viral stocks generated from these HPV-11 raft cultures are infectious, virus stocks were prepared from rafts generated from two individually derived HPV-11 episomal DNA containing N-Tert cell populations and cervical carcinoma cell line A431 cells were infected by serial dilutions of viral stocks from the undiluted to the 10^{-5} diluted virus stocks. Total RNA extracted from infected cells was subjected to nested RT-PCR detection of viral transcript E1^E4. Cells infected by a virus stock, which isolated from raft cultures derived from an individual HPV-11 episomal DNA containing cell population (W-2), were positive for E1^E4 transcripts from the undiluted and the 10^{-1} diluted stocks (Fig. 4, Lanes 2 and 3) indicating that virus stocks were infectious and were able to infect keratinocytes in vitro. Same results were obtained for another individually derived HPV-11 episomal DNA containing cell population (W-6) (data not shown). The titer of these virus stocks tested was at least 20 infectious does/ml.

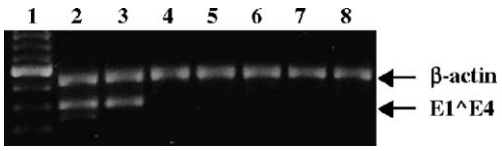

Fig. 4. Demonstration of infectivity of generated HPV-11 viruses. RT-PCR detection of HPV-11 E1^E4 transcripts in virus infected A431 cells. Lane 1: 100 bp marker. Lane 2: RNA from cells infected with undiluted virus isolated from HPV-11 N-Tert rafts. Lanes 3–7: RNA from cells infected with 10^{-1} to 10^{-5} diluted virus isolated from HPV-11 N-Tert rafts. Lane 8: RNA isolated from uninfected A431 cells. The expected size of HPV-11 E1^E4 and β-actin amplicons was 273 bp and 429 bp, respectively, as indicated by arrows.

Discussion

Although several HPVs have been successfully propagated in the human epithelium xenograft system, none of the viruses have been propagated starting from HPV DNA and this system does not currently allow the study of the HPV life cycle by genetic manipulation of HPV genomes. Using raft cultures, several high-risk types of HPV have been propagated starting from HPV DNA and the use of raft culture also allows the study of the HPV life cycle by genetic manipulation of HPV genomes. However, propagation of HPV-11 virus using raft culture starting from HPV-11 DNA has not been previously reported. We therefore attempted to grow HPV-11 starting from HPV-11 DNA using raft cultures.

Our previous attempts of using HPV-11 infected and transfected primary keratinocytes without selection failed to induce the productive HPV-11 viral life cycle in the raft culture systems possibly due to limited life span of primary keratinocytes and low transfection efficiency of primary keratinocytes (Fang, 2003). In order to enrich the number of HPV-11 positively transfected cells and to extend the life span of human foreskin keratinocytes, we decided to use a human keratinocyte cell line immortalized with the catalytic subunit of human telomerase (hTert) with cotransfection of a neomycin resistance

gene as a selective marker. Resulting cell populations that were positive for HPV-11 DNA, demonstrated by Southern blot analysis, were used for raft cultures. Form I HPV-11 DNA was evident in samples that were cut by restriction enzymes that do not cut HPV DNA. We think that they are replicated episomal HPV-11 DNA, since these cells were grown out from isolated single cells to colonies over a month. They are very unlikely to represent the input HPV-11 DNA.

The HPV-11 containing N-Tert cells were able to stratify and differentiate in raft culture with morphology closely resembling low-grade dysplasias with koilocytes and parakeratosis. In situ hybridization showed numerous HPV-11 positive cells in the upper layers of the epithelium. The intensity of the positive signals was comparable to those observed in experimental papillomas generated using the athymic xenograft model, although the number of positively stained cells was fewer. This indicated that HPV-11 DNA amplification was induced in these cells. Immunohistochemical staining for HPV group specific antigen (GSA) detected a few number of cells that were weakly positive in some areas that showed HPV DNA amplification, indicating that viral capsid proteins were synthesized. These results are consistent with a previous report (Thomas et al., 2001), which demonstrated episomal maintenance of HPV-11 with prolonged life span of primary human keratinocytes transfected with recircularized HPV-11 and neomycin selective marker and HPV-11 episomal DNA containing cells grown in raft culture demonstrated mild alteration in differentiation with retention of nuclei in superbasal layers of the epithelium. Weak HPV-11 DNA amplification was demonstrated by Southern blot analysis and induction of several viral late transcripts was detected by Northern blot analysis when HPV-11 episomal DNA containing primary human keratinocytes was induced for differentiation in semisolid media (Thomas et al., 2001). However, the generation of infectious viruses was not demonstrated. In our study, we were able to demonstrate the vegetative viral DNA amplification by in situ hybridization, capsid antigen synthesis by immunohistochemistry staining, and generation of infectious virus stocks from HPV-11 episomal DNA containing N-Tert raft cultures. The infectivity and the titer of the virus stocks were demonstrated in A431 cells with serial dilution of the virus stocks and an in vitro infectivity assay (Ludmerer et al., 2000; Smith et al., 1995). We did not make any attempt to infect normal keratinocytes or N-Tert cells to determine if these cells were infectable, since using A431 cells to check the infectivity of HPV is an established assay. We do know that N-Tert cells can substitute for A431 cells in this assay and are infectable with HPV-11 (Fang et al., 2003). These improvements may be due to the electroporation of HPV-11 viral DNA method used instead of the lipofectamin-mediated transfection of recircularized HPV DNA. Previous studies with electroporation have worked well in generating HPV virus particles in raft cultures for other types (McLaughlin-Drubin et al., 2004; Meyers et al., 1997, 2002).

We have demonstrated that HPV-11 genomes can be established in N-Tert cells. This is consistent with previous observations using a spontaneously immortalized human keratinocyte cell line, HaCaT cell line (Brune and Durst, 1995). Although the establishment of HPV-11 episomal DNA has been reported using primary foreskin keratinocytes transfected with recircularized HPV-11 DNA with a selective marker (Mungal et al., 1992), the limited life span of these cells may not allow sufficient time for the induction of productive viral life cycles using the raft culture system. This was demonstrated by our results showing extensive keratinization and thinning of epithelium from day 15 to day 18 rafts of either HPV-11 virus infected or HPV-11 viral DNA transfected primary foreskin keratinocytes (data not shown). The use of immortalized human keratinocytes extended the life span of primary foreskin keratinocytes to allow sufficient time for the induction of viral DNA amplification and capsid antigen synthesis. This may be especially important for the low risk types of HPVs that cannot efficiently immortalize human keratinocytes and the immortalized cell line may therefore have facilitated the virus propagation in this in vitro system. In addition to an extended life span of the N-Tert cell line, this cell line also showed normal growth characteristics and differentiation and continued to express the terminal differentiation-related proteins involucrin and keratin K10 in suprabasal cells of stratified colonies (Dickson et al., 2000). N-Tert cells grown in raft culture stratify and differentiate and formed a normally differentiating epidermis with formation of a granular layer and stratum corneum (Dickson et al., 2000), unlike HaCaT cells, which are aneuploid and show abnormalities in differentiation when grown in raft cultures (Boukamp et al., 1988).

The normal immortal keratinocyte S (NIKS), an immortalized human foreskin keratinocyte cell line, was used previously to establish the HPV-16 life cycle (Flores et al., 1999). Our data showed that N-Tert cells can be used for the establishment of the HPV-11 life cycle. Although N-Tert cells are immortalized by the subunit of human telomerase, it has been shown to retain normal growth and differentiation characteristics (Dickson et al., 2000). Compared to NIKS cells, N-Tert cells are much easier to grow and differentiate. It provides a suitable host as an additional alternative for studying the HPV life cycle. Further experiments are needed to determine whether this cell line could support the life cycle of high-risk types of HPVs.

Materials and methods

N-Tert cell line

N-Tert cells are human foreskin keratinocytes immortalized with the catalytic subunit of human telomerase (Dickson et al., 2000). N-Tert cell lines were from Dr. J. Rheinwald and Dr. M. Dickson at Department of Medicine and Harvard Skin Disease Research Center. Cells were cultured in E-media with 5% fetal calf serum and 5 ng/ml of epidermal growth factor in the presence of mitomycin C-treated J2 3T3 mouse fibroblasts feeder cells (Meyers et al., 1992). J2 3T3 mouse fibroblasts were kindly provided by Dr. Craig Meyers (Penn State University, College of Medicine, Hershey) and were treated with 8 μg/ml of mitomycin C (Roche Applied Science, Indianapolis, IN) for 2–4 h at 37 °C. Treated cells were then

washed three times with PBS before trypsinization and added to plates containing epithelial cells.

Establishment of HPV-11 episomal DNA containing N-Tert cell lines

As previously described (McLaughlin-Drubin et al., 2003; Meyers et al., 1992, 2002) with minor modifications, for each electroporation, HPV-11 DNA, the pBT-1 clone of HPV-11-Hershey, was linearized and separated from the vector sequence by digestion with *Bam*HI, which has a cutting site at nucleotide 7072 in the L1 region. Linearized HPV-11 DNA (7.5μg), 1 μg pSV_2-neo, which encodes the neomycin resistance gene, and 42.5 μg of salmon sperm DNA, in a total volume of 24.25 μl, was added to N-Tert cells (5×10^6) in a volume of 250 μl of E-media containing 10% fetal bovine serum (FBS) and 5 mM *N*,*N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid and electroporated at 210 V and 960 μF by using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). The electroporated cell suspension was then added to 10 ml E-media containing 10% FBS and centrifuged at 25 × *g* for 10 min. The resulting cell pellet was resuspended in E-media containing 10% FBS and added to a 100 mm tissue culture plate containing mitomycin-treated J2 3T3 feeder cells. Cells were then selected twice with 500 μg/ml of G418 for 24 h each time, beginning 72 h after electroporation. Mitomycin-treated J2 3T3 feeder cells were added and culture was maintained for 24 h without G418 before a second round of G418 selection was carried out. G418 resistant cell populations grown out from a few colonies were maintained in culture for about 1 month without further selection until they were 70–80% confluent. The first week after electroporation, cells were cultured in E-media with 10% fetal calf serum and 5 ng/ml of epidermal growth factor (Collaborative Biomedical Products, Bedford, MA). Cells were then cultured in E-media with 5% fetal calf serum and 5 ng/ml of epidermal growth factor until time for them to be subcultured and analyzed by Southern blotting (70–80% confluent). After splitting, cells were cultured in E-media with 5% fetal calf serum. Mitomycin-treated J2 3T3 feeder cells were added as needed to cover the plate. Cell populations containing HPV-11 DNA were identified by Southern blot analysis.

Organotypic raft culture

Epithelial raft cultures were established and maintained as previously described (McCance et al., 1988; Meyers, 1996; Meyers et al., 1992). Briefly, mouse fibroblasts 3T3 J-2 were trypsinized and resuspended in 10% reconstitution buffer (Meyers, 1996), 10% 10× DMEM (Life Technologies, Gaithersburg, MD) (Meyers, 1996), 2.4 μl/ml of 10 M NaOH, and 80% collagen to a concentration of 2.1×10^5 cells/ml on ice. The mixture was then aliquoted into 6 well plates at 3 ml per well and incubated at 37 °C for 2–4 h to allow solidification of the collagen matrix. E-media (2 ml) was then added to each well to allow the matrix to equilibrate. Human epithelial cells were trypsinized and resuspended at 1×10^6 cells/ml in E-media and 1 ml of cell suspension was added to each well of the 6-well plate on top of the dermal equivalent from above. Epithelial cells were allowed to attach to the dermal equivalent for 2–4 h. After removal of E-media, collagen epithelia were transferred onto the grids in 100 mm tissue culture plates using a spatula. E-media were added to the culture plates until the media were just touching the under-side of the collagen. Epithelial tissues were allowed to stratify and differentiate at the air–liquid interphase over 18 days. Rafts were harvested at various time points fixed in 10% buffered formalin and embedded in paraffin for sectioning.

Cell and virus DNA extraction

Cell pellets were resuspended in 3 ml of Hirt extraction buffer containing 400 mM NaCl, 10 mM Tris–Cl, pH 7.4, and 10 mM EDTA with a final concentration of 50 μg/ml RNase A and 0.2% SDS. The mixtures were then incubated at 37 °C overnight with rocking. Proteinase K was then added to a final concentration of 50 μg/ml and the mixtures were rocked at 37 °C for an additional 3 h. DNA was sheared by passing through an 18-gauge needle 10 times. DNA was then extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and extracted once with an equal volume of chloroform. DNA was precipitated overnight at 4 °C with 2 volumes of ethanol and 0.1 volume of 3M NaOAc. Precipitated DNA was centrifuged at 12,000 × *g* for 30 min. Pellets were washed with 70% ethanol and dried. Pellets were then resuspended in 100 μl TE buffer (10 mM Tris, 1 mM EDTA, pH = 7.5).

Southern blot analysis for HPV genome

Total DNA (5 μg) was digested with *Bam*HI to linearize HPV-11 genome. *Xho*I, which does not cut the HPV-11 genomes, was used as a non-cut control. Enzyme digested DNA was separated on a 0.8% agarose gel and transferred to nitrocellulose membrane with an LKB2016-100 VacuGene vacuum blot (Amersham Pharmacia, Piscataway, NJ) as described by the manufacturer. The resulting membrane was pre-hybridized in 35.6%(v/v) deionized formamide, 1× Denhardt's solution, 0.02 M NaH_2PO_4, 5× SSC buffer (0.75 M NaCl and 75 mM sodium citrate, pH 7), and 0.1 mg/ml denatured and sheared salmon DNA. The HPV DNA probes were prepared by gel purification of the 8 kb HPV cloned insert from recombinant vectors and labeled with the "Ready to Go" DNA labeling kit (Amersham Pharmacia, Piscataway, NJ). Labeled probe was purified with a ProbeQuant G50 micro-column (Amersham Pharmacia, Piscataway, NJ). Purified probe was denatured and added to hybridization solution containing 10% (w/v) dextran sulfate, 35.6%(v/v) deionized formamide, 1× Denhardt's solution, 0.02 M NaH_2PO_4, 5× SSC buffer (0.75 M NaCl and 75 mM sodium citrate, pH 7), and incubated with the membrane at 46 °C overnight. Membranes were washed 4 times with 2× SSC/0.1% SDS

for 15 min each at room temperature. The membrane was then washed twice with 0.1× SSC/0.1% SDS for 30 min each at 50 °C and subjected to autoradiography to detect HPV DNA.

In situ hybridization

To produce an in situ DNA probe, a biotin label was incorporated into HPV-11 DNA using the random priming method (Amersham, Cleveland, OH). Deparaffinized, rehydrated sections of formalin fixed paraffin embedded tissues were digested with pepsin (4 mg/ml in 0.1 N HCl) and washed in Tris–HCl, pH 7.5, dehydrated, and covered with a hybridization cocktail containing 0.6 M NaCl, 10 mM Tris pH 7.4, 0.5 mM EDTA, 1 mg/ml bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone (PVP) (w/v), 0.02% Ficoll (w/v), 0.15 mg/ml yeast tRNA, 5 mM DTT, 10% Dextran sulfate (w/v), 50% formamide (v/v), and 1 ng/μl-labeled HPV-11 DNA. The probe and tissue were denatured together by heating the slides at 100 °C for 6 min. After heat denaturation, slides were incubated in a moist chamber at 37 °C for 2 h, washed 3 times for 10 min each in 2× SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7.0), and incubated with avidin conjugated alkaline phosphatase for 30 min. After washing in Tris–HCl buffer (pH 9.5) for 3 × 10 min, the McGady reagent was used to develop signal color and sections were counterstained with Nuclear Fast Red.

Immunohistochemistry

Tissues from organotypic raft cultures were processed by standard formalin fixation and paraffin embedding methods used by the Clinical Pathology Laboratory at the Penn State Milton S. Hershey Medical Center. Paraffin-embedded xenografts were cut to 4-μm thickness and placed on siliconized slides (Fisher Scientific, Atlanta, GA). Slides were baked at 55 °C in a vacuum oven for 1 h before immunohistochemical staining. Tissue sections were dehydrated in xylene and rehydrated in alcohol gradients. Deparaffinized and dehydrated tissue sections were treated to destroy endogenous peroxidases by immersion in 3% hydrogen peroxide in methanol for 10 min. The rehydrated sections were blocked for non-specific binding with 10% normal horse serum in 1% bovine serum albumin (BSA) for 30 min and then blotted. Polyclonal anti-HPV antibody (Signet, Dedham, MA) was applied at 1:100 dilutions and incubated at room temperature for 60 min. After thorough washing in PBS, a biotin-labeled anti-rabbit secondary antibody was applied for 30 min, and then rinsed three times in PBS. A streptavidin/peroxidase complex was used to bind the biotin tag and color visualization of the complex was achieved with 3-amino-9-ethylcarbazol.

Preparation of virus stock and testing for infectivity

Virus stocks were prepared by peeling off the epithelial layers away from the collagen of three organotypic rafts from each individual cell population of HPV-11 episomal DNA containing N-Tert culture. The peeled epithelial layers from three rafts of each individual clone were homogenized in 0.6 ml of PBS on ice and sonicated for 30 s on ice. Virus stocks were kept at −70 °C. The infectivity of the stocks was tested using an in vitro infectivity assay as described previously (Ludmerer et al., 2000; Smith et al., 1995). Briefly, A431 cells established from human epidermoid carcinoma (Giard et al., 1973) were seeded in 6-well plates at 5×10^5 cell/well the day before infection and infected with 0.5 ml of serial dilutions of virus stocks ranging from undiluted to 10^{-5} diluted virus stocks for 50 min at 37 °C with rocking every 10 min. Virus inoculum was removed at the end of the incubation, and 2.5 ml of media was added. Total RNA was extracted 72 h post-infection using a QIAshredder and RNeasy mini kit (Qiagen, Valencia, CA). RT-PCR analysis to detect transient expression of HPV-11 E1^E4 transcript was used to determine whether viruses generated by raft culture were infectious. Total RNA (200 ng) extracted from infected A431 cells was reverse transcribed in the presence of RT buffer, 5 mM $MgCl_2$, 1 mM of dNTPs, 5 μM 11-DO (^{3684}GCCCAATGCCACGTTGAAGA^{3665}) and A-DO (^{1036}GGAGCAATGATCTTGA TCTTC^{1016}) primers, and 2.5 U of reverse transcriptase in a total volume of 20 μl at 42 °C for 1 h. The product was amplified using nested primer sets and two 40-cycle PCR rounds to detect the HPV-11 E1^E4 spliced transcript. Detection of the spliced transcript eliminated possible detection of a signal caused by DNA contamination. β-actin primer sets were included in PCR reactions to serve as an internal control for RNA integrity. Following cDNA synthesis, primers 11-UO (^{778}GCTGGGCACACTAAA TATTGT^{798}) and A-UO (^{395}GATGACCC AGATCATGTTTG^{414}) were used for 40 cycles of PCR (temperature file: 94 °C/30 s, 60 °C/30 s, 72 °C/55 s with a final extension of 72 °C/10 min). A portion (5 μl of 100 μl) of the PCR reaction mixture was then utilized for a second 40 cycles of PCR primed with primers 11-UI (^{808}CTGCGCA CCAAAACCATAACA^{828}), 11-DI (^{3578}TAGGCGTAGCTGCACTGT GA^{3559}), A-DI (^{852}ACTCCATGCCCAGG AAGGAAGG^{831}), and A-UI (^{423}AACACCCCAGCCAT GTACGTTG^{444}) using the same temperature profile. PCRs were conducted according to the manufacturer's instruction (GeneAmp RNA PCR kit from Perkin-Elmer, Branchburg, NJ). The final concentration of deoxynucleotide triphosphates during PCR was 0.2 mM and final concentrations of primers were 0.2 μM in all cases. The expected size of amplified product was 273 bp for the E1^E4 product and 429 bp for the β-actin product.

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