**In vitro** infection of normal human keratinocytes by human papillomavirus type 1 followed by amplification of the viral genome in reconstructed epidermis

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Primary cultures of normal human keratinocytes were inoculated in vitro with human papillomavirus type 1 (HPV-1), the agent responsible for deep plantar warts. Upon transfer to dead de-epidermized dermis and growth at the air–liquid interface, keratinocytes reconstituted a pseudoepidermis. Under these highly differentiating conditions, HPV-1 DNA amplification was found to take place in the reconstructed epidermis, being detectable from 7 days after the transfer and persisting for at least 10 days thereafter. The extent of keratinocyte differentiation may be insufficient to allow a complete HPV infectious cycle.

Owing to the lack of an in vitro system for papillomavirus production, experimental in vivo models were developed, allowing limited propagation of these viruses. Thus, papillomas could be serially induced in humans (Oriel, 1971) and in animals (Cook & Olson, 1951) by inoculating filtrates of lesions from homologous donors. Moreover, production of human papillomavirus type 1 (HPV-1), -11 and -16 has been achieved in immuno-deficient animals implanted with experimentally (Kreider et al., 1990) or naturally (Sterling et al., 1990) infected human keratinocytes.

Given that HPV replication is dependent in vivo on the differentiation of target keratinocytes (Orth et al., 1977; Grussendorf & zur Hausen, 1979), the description of epidermis reconstitution upon cultivation of human keratinocytes on dead de-epidermized dermis (DED) (Prunières et al., 1983) revived the hope of achieving well controlled production of HPV in vitro (Taichman et al., 1984). Keratinocytes from a cervical intraepithelial neoplasia grade 1 lesion carrying HPV-31b have recently been found consistently to allow viral DNA amplification when grown in such a highly differentiating environment (Bedell et al., 1991). The results presented extend this finding to HPV-1, which is most commonly associated with deep plantar warts (Fabre et al., 1975; Gissmann et al., 1977). In addition, the system is shown to be usable under purely in vitro conditions, i.e. amplification of the HPV-1 genome could be achieved upon inoculation of isolated virus on to a culture of proliferating normal human keratinocytes, followed by epidermal reconstitution on DED.

Freshly excised plantar warts were individually ground in 3 ml of PBS at 0 °C and passed through a 0.45 µm Millipore filter. The presence of papilloma-like virus was detected by electron microscopy. The virus type was determined by dot-blot hybridization under highly stringent conditions (Tm -10 °C) using 32P-labelled probes prepared from the linearized complete genomes of HPV-1, -2, -3 and -7. Clones of these genomes in pBR322 were a generous gift from H. zur Hausen and E. M. de Villiers (DKFZ, Heidelberg, Germany). Inocula strongly positive for HPV-1 only were selected for further experiments.

The medium used for conventional cultures of keratinocytes was MCDB153 supplemented with insulin (5 mg/ml), epithelial growth factor (10 ng/ml), bovine pituitary extract (70 µg protein/ml) (Tsao et al., 1982), hydrocortisone (0.4 µg/ml) and 0.1 mM-ethanolamine. This medium also contained either 0.1 mM-Ca2+ or 1.8 mM-Sr2+ and was designated complete MCDB153 with calcium or strontium, respectively. DEDs were prepared from human skin specimens obtained from plastic breast surgery. After maceration of the skin in PBS, the epidermis was lifted from the dermis. The dermis layer was cut into pieces of approximately 4 cm² each, subjected to 10 cycles of freezing and thawing, and exposed to gamma rays (2 × 10⁴ Gy) from a 60Co source. Cells do not survive such drastic treatment and no
Fig. 1. Histological analysis of HPV-1 and mock-infected keratinocytes cultivated on DED. (A, B and C) Haematoxylin- and eosin-stained cross-sections of DED prior to (A) and 17 days after (B and C) the transfer of HPV-1-infected keratinocytes. (C) Dermal invagination repopulated by seeded keratinocytes. (D and E) In situ hybridization of HPV-1- (D) and mock- (E) infected keratinocytes grown on DED, probed with 35S-labelled HPV-1 DNA and examined by autoradiography. (D) The same repopulated invagination as in (C) (from serial sections). Bar marker represents 20 μm.

Remnants of the original epidermis remained, as ascertained by histological examination of DED (Fig. 1A). Cultures on DED (differentiating conditions) were grown in Joklik's medium supplemented with 10% foetal calf serum, insulin (5 mg/ml), hydrocortisone (0.4 μg/ml), cholera toxin (100 ng/ml) and 1.2 mM-Ca2+. This medium was designated complete Joklik's medium.

Human keratinocytes were obtained from newborn or young boys' foreskins. The epidermis was separated from the dermis by means of a keratotome and trypsinized. Dissociated cells were collected and seeded in complete MCDB153 medium with Ca2+ (10⁶ cells/9 cm dish). When this primary culture reached 80% confluence, keratinocytes were passaged at a 1:3 ratio in the same medium. For subsequent exposure to the virus inoculum and initial post-infection cultivation, cells were maintained under conditions assumed to keep them in a state similar to that of the natural targets of HPV, basal keratinocytes. To this end, cultures were incubated in the presence of SrCl2 at a concentration shown previously to stimulate the growth of keratinocytes while inhibiting their differentiation and the establishment of intercellular contacts (Praeger et al., 1987). Thus, subcultures grown to 30% confluence were exposed to 1.5 ml of either virus inoculum or PBS alone, each supplemented with 0.45 mM-MgCl2 and 1.8 mM-SrCl2, for 1 h at 37 °C with intermittent gentle stirring. After removal of the inoculum, cells were rinsed and cultivated in complete MCDB153 medium with Sr2+ for 24 h and with Ca2+ thereafter.

When mock- and virus-treated cultures became 70 to 80% confluent (1 to 4 days post-infection), keratinocytes were harvested by trypsinization, resuspended in complete Joklik's medium (5 × 10⁶ cells/ml) and seeded onto DED (2 × 10⁵ cells/fragment). Prior to keratinocyte seeding, DED pieces were set on stainless steel grids in Petri dishes at the surface of complete Joklik's medium, so that only the bottom face of the DED came into contact with the medium. As a control, mock- or virus-treated keratinocytes (10⁵ cells) were replated on the bottom of Petri dishes for conventional cultivation in complete MCDB153 medium with Ca2+. These cultures were maintained, if possible, for the same period as their counterparts grown on DED (about 4 weeks), requiring two to seven passages.

In contrast to conventional cultures, which keep proliferating without visible signs of differentiation for four to seven passages (Boyce & Ham, 1983), normal human keratinocytes grown at the liquid–air interface on a DED substrate reconstituted a stratified pseudo-
epidermis (Fig. 1B). However, it should be stated that the in vitro reconstituted epithelium was not fully equivalent to a natural epidermis (Asselineau et al., 1986). Thus, the polarity and the cuboidal shape of basal cells were not always complete. Moreover, although it proceeded to the terminal (squamous) stage, differentiation in the reconstructed epithelium with thinner intermediate layers and an accelerated turnover of about 7 days. In addition, the artificial epidermis had a limited lifespan and degenerated after 2 to 4 weeks in culture. Good epidermal reconstruction was often found in dermal invaginations, presumed remnants of hair follicles and sebaceous glands, which were repopulated by seeded keratinocytes (Fig. 1C). It is noteworthy that the HPV-1 inocula had no detectable effect on the appearance, growth or lifespan of conventional keratinocyte cultures nor those of reconstructed epidermis.

With the object of determining the fate of HPV-1 DNA, keratinocytes were harvested at different times either by trypsinization of conventional cultures or by vigorous rinsing of reconstituted epithelia with conditioned medium to remove differentiated (squamous) cells. Cells were then lysed in 10 mM-Tris–HCl, 50 mM-EDTA (pH 7.2) containing 0.5% SDS and digested with proteinase K (100 µg/ml) for 60 min at 37°C. After phenol and ether extractions, total DNA was ethanol-precipitated and redissolved in 10 mM-Tris–HCl, 1 mM-EDTA (pH 7.2). After photometric determination of the nucleic acids content, 10 ng samples were denatured with 0.5 M-NaOH for 5 min at 20°C, and slot-blotted on nylon membranes. The membranes were processed for DNA hybridization under highly stringent conditions using a 32P-labelled linear full-size HPV-1 probe (10 µg/ml; 10^8 c.p.m./µg), and examined by autoradiography.

As shown in Fig. 2(a), conventional cultures failed to amplify input viral DNA, which rapidly decayed and/or became diluted during cell proliferation. Within 5 to 13 days after infection, the residual HPV-1 DNA content of daughter cells was under the lower sensitivity threshold of the assay, estimated at about five viral DNA copies per human genome. This observation confirms previous reports indicating that undifferentiated keratinocytes are unable to amplify HPV DNA (La Porta & Taichman, 1982). In contrast, positive results were obtained with the cells washed away from five of seven reconstituted epithelia, as illustrated in Fig. 2(b) for two representative experiments. Shed material recovered from an epithelium at a few days of age gave weak HPV-1-specific hybridization signals. These low amounts of DNA may correspond to residual parental HPV-1 genomes that remained after degradation and dilution of the original input virus carried by transferred keratinocytes, as seen with conventional cultures. However, starting from 5 to 14 days after DED seeding (i.e. an interval allowing squama formation), cells released by reconstituted epidermis showed a marked increase (10⁴ to 10^3-fold) in their relative content of HPV-1 DNA (Fig. 2b). The release of HPV-1 DNA-enriched material persisted for up to 18 days, but declined progressively as the cultures aged (Fig. 2c).

The HPV-1 probe was washed off and the membranes were rehybridized under stringent conditions to a 32P-labelled human DNA probe. A sequence from the

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Fig. 2. Fate of HPV-1 DNA in conventional keratinocyte cultures (a) and reconstructed epidermis (b and c). At the indicated times after virus inoculation (a) or transfer of infected keratinocytes onto DED (b, c), cells were collected by trypsinization of the culture (a) or rinsing of the reconstituted epidermis with conditioned medium (b, c), and total DNA was extracted. (a, b) HPV-1 DNA from total DNA-matched samples was measured by slot-blot hybridization to a 32P-labelled specific probe, followed by autoradiography. Fluctuations in total DNA load per slot were assessed by removal of the HPV-1 probe, rehybridization to a 32P-labelled human DNA probe and densitometric scanning of the autoradiograms. (c) The amount of HPV-1 DNA produced by reconstructed epidermis (experiment 1, b) is expressed relative to the value corresponding to the earliest harvest, considered as 1.0, and is given as a function of epidermal reconstitution time.
Alu family, cloned in plasmid pBLUR-8 (Rubin et al., 1980), was used because it is present in human DNA but not in possible rRNA contaminants (Jelinek & Schmid, 1982). Densitometric scanning of the resultant autoradiograms (Fig. 2b) indicated that variations between total DNA loads of individual slots were less than twofold and did not correlate with the observed differences in the intensities of the signals generated by the HPV-1 probe. Therefore, epithelial reconstruction may actually trigger amplification of HPV-1 DNA, assuming that it did not cause preferential shedding of infected rather than virus-free cells. Consistent with this, in two of five experiments, the absolute amount of HPV-1 DNA collected in successive rinses proved to be greater (up to fivefold) than the original input from seeded keratinocytes. It should be stated that this absolute increase in HPV-1 DNA was low compared with the enhancement of the concentration of HPV-1 relative to that of cellular DNA. This discrepancy may be ascribed to (i) the experimental procedure, revealing only the fraction of HPV-1 DNA associated with cellular material washed away from the epithelium or (ii) the degradation of parental genomes during epidermal reconstitution, before the onset of viral DNA amplification, resulting in an actual input that was lower than the value measured prior to the transfer of infected keratinocytes onto DEDs.

Two to three weeks after keratinocyte seeding, i.e. at a time when HPV-1 DNA was detected in conditioned medium (Fig. 2b, c), reconstructed epithelia on DED substrates were fixed in 10% formalin for 18 to 24 h, embedded in paraffin and cut into 5 μm sections which were set down on poly-L-lysine-coated slides. After removal of paraffin and rehydration, preparations were digested with proteinase K (100 μg/ml) for 15 min at 42 °C and denatured with 0.5 M-NaOH at 20 °C for 5 min. Samples were then processed for in situ hybridization using a genomic 32P-labelled HPV-1 DNA probe (35 ng/ml; 2 × 106 c.p.m./μg). Slides were autoradiographed at 4 °C and stained.

A positive, predominantly nuclear signal was observed in discrete clusters of cells from infected epithelia, especially in repopulated dermal invaginations showing the best epidermal reconstitution (Fig. 1D). Such figures were not found in pseudoeipidermis reconstructed with uninfected keratinocytes (Fig. 1E). The sensitivity of the method used does not allow the detection of a specific DNA sequence unless it is amplified (Schneider, 1987). This fact, together with our failure to observe in situ hybridization signals in infected epithelia analysed before the formation of squames and HPV DNA amplification (data not shown), points to HPV-1 genomes synthesized de novo rather than input viral DNA as being responsible for the signals obtained with infected, differentiated cultures.

Together, these data suggest that the present system fulfils the requirements of HPV-1 DNA replication for keratinocyte differentiation. However, it should be stated that only a minor fraction of incoming HPV-1 genomes were amplified and released by the epithelium because the absolute amount of recovered viral DNA was only slightly greater than that of the original input from seeded keratinocytes. On the other hand, the production of HPV-1 DNA decreased during prolonged cultivation of keratinocytes on DEDs. This may result from the depletion of input virus from the pool of proliferating basal cells in the absence of new infection, and/or from the senescence of reconstituted epidermis, as apparent from histological examination.

In contrast with their capacity for HPV DNA synthesis, foreskin keratinocytes grown on DED did not appear to produce detectable amounts of viral capsid proteins. Indeed, our attempts at immunohistochemical staining of reconstituted epidermis for the 52K capsid protein of HPV-1 have failed (data not shown). A similar defect has recently been reported for epithelia reconstructed from naturally infected cervical keratinocytes (Bedell et al., 1991). In vivo, the completion of late steps of the HPV life cycle has been shown to be restricted to the uppermost layers of the epidermis (zur Hausen, 1985). The apparent abortion of HPV replication in reconstituted epithelia may possibly be due to prevention of maturation in vitro, reducing the pool of cells capable of supporting a productive HPV infection. The failure of these cultures to accomplish late steps of HPV replication may be overcome by producing epidermal reconstructions that mimic more closely the in vivo situation. It would be interesting in this respect to expose the system to agents that are known to modulate keratinocyte differentiation, such as retinoic acid or various growth factors.

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References


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