Development and characterization of a novel xenograft model permissive for human papillomavirus DNA amplification and late gene expression

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Human papillomaviruses (HPVs) are important human pathogens associated with a range of epithelial neoplasia. The rising incidence of HPV infection and association of HPV with malignancy has led to increased interest in appropriate management of these infections. Development of new therapies for viral warts has been frustrated by the lack of availability of models permissive for viral replication. Here we describe the development of a HPV–severe combined immunodeficient mouse model which reproduces mature HPV-infected epithelia. Grafting of anogenital and laryngeal papillomas harbouring either HPV-6 or HPV-11 resulted in the formation of a differentiated neo-epithelium exhibiting the hallmark features of HPV infection including basal hyperplasia, acanthosis and koilocytosis. The reformed warty epithelium contained amplified HPV DNA and expressed capsid protein in the differentiated layers. A striking feature is the production of macroscopic papillomata in an anatomically relevant and accessible site, providing a system of particular relevance for the temporal evaluation of developing lesions and selection of antiviral agents.

Human papillomaviruses (HPVs) are strictly epitheliotropic viruses. HPVs associated with the anogenital region are the best characterized and may be subdivided into those predominantly causing benign warts (HPV-6 and -11) and those associated with squamous cell carcinoma (HPV-16, -18, -31 and -33; Androphy, 1994). Little is known about the early infectious cycle as macroscopic wart formation represents a fairly late event. Data from analysis of wart biopsies are limited by the inability to examine the time-course of infection and so provide only a snapshot view of the disease process. Examination of the early events in the infectious cycle requires the development of models permissive for both keratinocyte differentiation and HPV late gene expression. Such models will provide detailed information on the temporal regulation of HPV replication and gene expression, and the association of viral proteins with those of the host.

Considerable progress has been made in this area by culturing infected tissue or cells in organotypic and xenograft systems. Two keratinocyte lines, CIN612 (Bedell et al., 1991) and W12 (Stanley et al., 1989), which maintain episomal HPV-31 and -16 DNA, respectively, have been used with favourable results; both lines can undergo differentiation resulting in limited virion production (Meyers et al., 1992; Sterling et al., 1990). Whilst these cell lines provide useful models for oncogenic HPV infection, they are not the most appropriate models for benign HPV infection. The best characterized model for benign warts involves the xenografting of human foreskin epithelium infected with HPV-11 to mice, as described by Kreider et al. (1987). Although this successfully generates virus particles, a number of problems exist. Extensive use has been confined to a single virus strain, and epithelial morphogenesis is compromised by an inappropriate extracellular matrix substratum and the absence of an air-exposed interface.

To overcome some of these difficulties we have modified existing techniques (Worst et al., 1974) to xenograft endogenously infected tissue. Our initial experiments were performed using nude mice, but subsequent use was precluded by a rejection phenomenon observed at around 4 weeks post-grafting, in which the grafted epithelia became necrotic and the bed was associated with an inflammatory infiltrate. Experiments were subsequently confined to severe combined immunodeficient (SCID) mice, where the success rates were nearly doubled.

Prior to grafting, a granulation bed was formed by inserting a glass coverslip under the skin of the anaesthetized mouse’s flank. The incision was closed with suture clips and swabbed with iodine. After 7–14
Fig. 1. Histopathology of xenografts and expression of cellular markers. (A) Haematoxylin- and eosin-stained section of a normal skin graft showing reformed normal epithelium growing on the surface of the granulation bed (b). The graft is overlaid with a necrotic scab (a). (B) Haematoxylin- and eosin-stained section of anogenital wart graft. The graft in all cases is located on the surface of the granulation bed (b) and again overlaid with a necrotic scab (a). Wart xenografts also show acanthosis and koliocytosis (c). Immunohistochemical analysis was carried out on grafted wart tissue; (C) negative control with secondary antibody only; (D) keratin 10 (LH2) reactivity displayed in the most differentiated layers; (E) involucrin staining in the suprabasal layers; and (F) PCNA (PC10) reactivity in patches of basal cells extending through the stratum granulosum, including some staining in koliocytes. All grafts were taken at 10 weeks. Magnification × 20.

days the coverslip was removed and a silicone grafting chamber (Renner) was secured to the granulation bed. Normal skin was obtained from breast reduction operations; dysplastic tissue was taken from wide excision of the backs of the hands of renal transplant patients; benign wart tissue, including giant condyloma
and laryngeal papilloma, was obtained from otherwise healthy patients and from one patient on long-term steroid therapy. Freshly excised tissue was divided and a portion snap-frozen in liquid nitrogen for further analysis. The remainder was placed in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum, antibiotics and nystatin (250 U/ml). Tissue fragments (1 mm slices) were oriented to ensure that the basal face of the tissue slice was apposed to the granulation tissue bed. Collagen was laid around the graft to secure the tissue chip.

A total of 146 SCID mice, excluding animals which showed contamination, were grafted with human tissue. Our initial experiments were hampered by a high incidence of *Staphylococcus* infection. The use of mice from a *Staphylococcus*-free colony, in addition to treatment with tetracycline (200 mg/l), virtually eliminated this problem. The animals were inspected weekly and sacrificed after 2–10 weeks. To preserve the orientation, grafts were removed with the chamber in situ and fixed in formal saline. Fifty-four mice were grafted with normal human skin, 39 with benign anogenital and laryngeal wart tissue, and 53 with dysplastic skin. Successful grafts were observed in 27 (50%), 13 (33%), and 36 (68%) mice, respectively. Take rates in each group were in general related to the individual sample and ranged from 25–85%. The success rate of grafting of benign wart tissue was notably less than that of both normal and dysplastic skin. As inflammatory cells are frequently found around the base of the lesion at excision, the warts may already have been subject to processes of immunological rejection. In addition, anogenital lesions are frequently colonized by secondary fungal infections and these factors may combine to reduce the viability of wart tissue. The enhanced proliferation of dysplastic tissue reflects the natural history of these lesions *in vivo*, where growth is often relentless.

**Normal and dysplastic human skin**

Normal human skin was grafted as a control to determine whether the epithelial phenotype would be preserved in this system. Early time-points demonstrated basal cell streaming across the graft bed from the proliferating compartment of the tissue chip. Colonization of the graft bed was accompanied by necrosis of the original tissue and neovascularization of the bed in successful grafts. In failed grafts, the granulation bed became fibrous and avascular, and features of keratinocyte proliferation were absent. Later time-points were histologically similar to the original biopsy, although some depigmentation was noted in melanotic skin. Overall, this provides good evidence that a proliferating and differentiating epithelium developed by outgrowth from the grafted tissue rather than the tissue being statically maintained.

Interestingly, growth of the neo-epithelium was confined to regions which contained what appeared morphologically to be human dermis. No keratinocyte growth was noted on the granulation tissue bed, which lacked...
this dermal component. This parallels observations in which cultured keratinocyte grafts were significantly enhanced by prior grafting of de-epidermalized dermis (Kangesu et al., 1993). As vascular growth and dilation appears to be an essential factor for graft take, the proliferating fibroblasts within the human dermis may enhance neo-vascularization by secretion of basic fibroblast growth factor, a potent angiogenic cytokine (Folkman & Klagsburn, 1987). Keratinocyte proliferation may also be enhanced by the elaboration of extracellular matrix proteins and cytokines from the dermal fibroblastic cells. In addition, the presence of the dermal components may be a factor in the supposed stem cell niche which is likely to be important in re-epithelialization. For both normal and dysplastic tissue, the reformed epithelium was histologically indistinguishable from the native lesions.

Benign wart tissue

We next examined the extent to which benign wart tissue could be phenotypically reproduced in this system. The histology of biopsies and graft was remarkably similar, showing basal hyperplasia, acanthosis and koilocytosis consistent with the pathology of viral warts (Fig. 1A, B). Examination of early grafts again revealed basal cell streaming and cell nest development. The graft bed itself demonstrated neovascularization with the development of a well-dilated stromal vasculature. The wart epithelium was overlaid with a necrotic scab, probably representing the remnants of the original grafted tissue. The developing graft was histologically identifiable at all time-points and in over 80% of the grafts remaining after 12 weeks, macroscopic warts developed. These reached a size of 4-8 mm in diameter and 2-4 mm in height at 12 weeks (Fig. 2). Clearly the success of this system is not confined to reproducing a number of histopathological features of HPV infection but rather enables the formation of wholly identical tissue structures.

Keratinocyte proliferation and differentiation

To determine the extent of epithelial maturation, grafted anogenital wart tissue was examined for keratin 10 (Leigh et al., 1993) and involucrin (Simon & Green, 1984) expression. Immunocytochemical analysis was carried out using antibodies LH2 (anti-keratin 10; Leigh et al., 1993) and anti-involucrin (Dako), and a secondary peroxidase-conjugated antibody (Dako). Peroxidase activity was demonstrated with 3-amino 9-ethyl carbazole as chromagen, and counterstained with haematoxylin. Both markers were correctly expressed in grafted tissue, in contrast to organotypic culture systems where expression of involucrin is precocious. Involucrin expression was demonstrated in the epibasal layers, whereas expression of keratin 10 was delayed (Fig. 1E, D), consistent with the features of abnormal wart differentiation (Proby et al., 1993).

The grafts were also investigated for the presence of proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase δ. In grafted normal skin, PCNA staining was confined to the basal layer. This corresponds to the pattern identified in normal skin biopsies where commitment of suprabasal keratinocytes to differentiation is accompanied by a block in cell division. In contrast, tissue reactivity with anti-PCNA antibodies has been demonstrated in cells of the upper layers of benign warts (Penneys et al., 1992). In both benign anogenital warts and the grafts of these tissues, PCNA reactivity was observed in basal cells and extended through approximately two-thirds of the epithelium (Fig. 1F) and may represent activation of host polymerase concomitant with episomal replication.

HPV DNA and capsid expression

Biopsies and grafted tissue were subjected to DNA in situ hybridization using a commercially available kit (Kreatek) with biotinylated HPV-6/11 probes. Amplified viral DNA was observed in the granular layer and koilocytes of biopsies and grafts of laryngeal papilloma (HPV-11), giant condyloma (HPV-11) and anogenital warts (HPV-6, -11). The localization and intensity of the signal was remarkably similar between the original biopsy and grafted tissue (Fig. 3A, B). Grafted normal skin was negative.

Using antibodies to disrupted bovine papillomavirus (BPV) particles (Dako), which cross-react with the L1 capsid antigen of various HPV types, specific staining was demonstrated in the stratum granulosum and cornified layer of both the original biopsy and the grafted wart tissue (Fig. 3C, D). Transmission electron microscopy of the biopsy revealed a small number of scattered nuclei in the stratum corneum which contained viral particles, but a significantly smaller number of virion-positive cells were detected than cells showing L1 reactivity. Virions were not detected in grafted tissue, possibly reflecting the reduced extent of L1 reactivity in the grafted samples. We are currently investigating later time points to determine whether virion synthesis is related to regrowth time.

Passage of xenografted tissue

A portion of fresh tissue from some animals with macroscopic warts was grafted onto further animals. The graft morphology suggested a hyperproliferative human epithelium, devoid of viral infection. Sections were negative for HPV DNA and capsid protein. We are unable to determine at this stage whether this represents
loss of HPV-positive basal cells upon regrafting or a subclinical infection in which the correct signals for viral activation are lacking. This loss of the HPV signal is reminiscent of observations in cultured wart keratinocytes in which high copy-number HPV-positive basal cells are lost with passage (Williams et al., 1994; Di Lorenzo et al., 1992). However, PCR analysis of the cultured cells revealed the persistence of low levels of HPV DNA within these cells, which may represent low copy-number HPV episomes within a subset of the keratinocyte population. An analogous situation may exist here in which smaller numbers of HPV-positive cells are passaged than upon initial grafting, and this, combined with a reduction in the keratinocyte-mesenchymal interactions important in wart proliferation, is insufficient to trigger activation of the viral life cycle within the infected cells.

In summary, the infectious cycle of HPVs appears to involve a complex series of virus-host interactions. Cellular differentiation exerts profound effects on the virus and conversely viral protein expression modifies keratinocyte differentiation. To date, the delineation of early events in HPV infection has been restricted to analysis of wart biopsies, allowing the formulation of a model which predicts the direct infection of basal keratinocytes. Here we have described the development of a novel animal model for HPV infection which supports this hypothesis. In contrast to results from wart biopsies which are limited to a single end-point, our model allows inspection and analysis of the early stages in the formation of wart lesions over an adaptable time-course. The HPV–SCID mouse model reproduces a neo-epithelium which is virtually indistinguishable from the original wart biopsy and which most strikingly results in the formation of macroscopic viral warts. The site of the graft, in addition to being a pharmacologically relevant site and accessible for application of topical agents, permits regular inspection of the graft without sacrifice of the animal. Other advantages of this system include the ability to graft tissue infected with a range of HPV types taken directly from the clinic. The HPV–SCID mouse model, in addition to serving as a model for HPV
antiviral therapies, should prove a useful tool for dissecting the early events in the infectious cycle.

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References


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