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A novel model of HPV infection in meshed human foreskin grafts

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Abstract

The present study describes a novel meshing procedure that provided successful low-risk papillomavirus propagation and reproducible wart induction in human foreskin xenografts. The initial HPV6 and/or 11 inocula were collected from clinically excised human wart tissues and confirmed to be free of HPV16, 18 and 31 by PCR analysis. Human foreskin grafts were collected from a circumcision clinic, and pre-inoculated with HPV virions by scarification. Meshing was carried out with a Zimmer Skin Grafter Mesher. Grafts were cut to appropriate size (1 cm × 1 cm or 5 mm × 5 mm) for cutaneous or subcutaneous grafting to NIH-nu-bg-xid mice under halothane anesthesia. Cutaneous xenografts were dressed with antibiotics and protective band-aids for 3 weeks. In the paralleled experiment using the same viral stock containing both HPV6 and 11, and matched grafts, no visible papillomas were observed in non-meshed cutaneous xenografts ($n = 4$ up to 6 months). In comparison, six of eight cutaneous xenografts treated with the meshing procedure formed visible papillomas within 4 months. This high frequency of distinct papilloma induction over the surface of meshed xenografts were reproduced in subsequent experiments with viral stocks containing both HPV11 and 6 (8 out of 10 grafts), or with a single-type HPV11 inoculum (80–100%). In contrast, an initial viral stock of single-type HPV6 provided lower frequency and more delayed papilloma induction. Serial passage of HPV6 in the meshed xenograft appeared to improve both the induction frequency and growth rate up to the 3rd generation. Histology, in situ hybridization, and immunohistochemical analysis revealed similarity of xenograft warts to those observed in the clinic. The highly reproducible papilloma induction rate and successful viral stock propagation associated with the meshing procedure provide a novel feature in the HPV xenograft model.

Keywords: HPV; Xenograft model; Meshing; Papilloma

1. Introduction

Human papillomavirus (HPV) infection is widespread in the population and associated with a broad spectrum of human diseases ranging from benign condyloma, common skin warts, and laryngeal papillomas to anogenital cancers (Gross, 1997; Howett et al., 1990; Kreider et al., 1990; Pfister, 1984). While HPV-associated anogenital cancers may be life threatening, there is also an important clinical need for more efficacious therapy for benign HPV diseases due to their morbidity and related economic and social costs (Gross, 1997; Phelps et al., 1998; Phelps and Alexander, 1995; Tewari et al., 2000). To assist in the discovery and development of new antivirals against HPV, several in vitro and in vivo models of HPV infections have been developed (Bonnez et al., 1998, 1993; Brandsma et al., 1995; Brown et al., 1998; Bryan et al., 2000; Christensen and Kreider, 1999; Christensen et al., 1997; Culf and Christensen, 2003, 2004; Dollard et al., 1989, 1992; Howett et al., 2000; Iyer et al., 2002; Laimins, 1993; Majewski and Jablonska, 1997; Tewari et al., 2000; Yiu et al., 1990). However, severe limitations exist for all currently described models (Christensen and Kreider, 1999; Stanley et al., 1997).

In vivo modeling of HPV infection faces numerous challenges. Amongst them are the strict host-specific tropism of HPVs and the lack of conventional methods of viral propagation (Gangemi et al., 1994; Kreider et al., 1990; Pfister, 1984). Kreider et al. first described HPV papilloma induction in human xenografts (Kreider et al., 1987a, 1987b, 1986). In this model, human skin tissues were surgically implanted under the renal capsule of the nude mouse. The

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grafts were allowed to remain in the animal (2–3 months) until papillomatosis was observed in recovered grafts. Viral preparations derived from these xenograft warts were used to induce papillomas in subsequent experiments, verifying successful HPV propagation in this model. The choice of renal capsule as the receiving grafting bed was advantageous due to the enriched blood supply at this anatomical location, which might have been critical for survival, growth, and proliferative papillomatosis. However, the choice of this grafting location also created a limitation for the model, specifically a difficulty in accessing the grafts for treatments and observations. Several other laboratories also tried to extend the xenograft model to cutaneous HPV infections, and limited success in HPV16 virus and DNA inoculation have recently been reported (Christensen and Kreider, 1999; Stanley et al., 1997). Nevertheless, all of the previously published models quantify gross papilloma growth by the measurement of whole graft size rather than direct measurement of distinct visible papillomas similar to that observed in the clinic. Therefore, a highly reproducible and convenient model of cutaneous low-risk HPV infection for virus propagation and antiviral drug evaluations has not been established yet.

Following a number of attempts to infect cutaneously grafted human foreskin grafts in NIH-nu-bg-xid mice with low-risk HPV inocula from clinical wart specimens, we have developed a highly reproducible model of low risk HPV infection that features a novel procedure of graft meshing.

2. Materials and methods

2.1. Initial viral extraction from clinically excised human warts

Clinically excised human anogenital wart tissues were obtained from the Jewish General Hospital, Montreal, Que., Canada. The collected warts were kept on dry ice and transported to our laboratories. For viral stock preparations, clinical samples were weighed, minced into small pieces (∼1–2 mm squares) and homogenized with a Polytron™ in cold phosphate-buffered saline (4 °C) to a final volume of 5 ml/g tissue. The homogenate was centrifuged at 3000 × *g* (4 °C) for 30 min. The resulting pellet was optionally subjected to a second extraction using the same procedure. The collected (1st and/or 2nd) supernatant was supplemented with 0.5 mg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Ont., Canada), and stored at −80 °C. The extracted supernatants were the initial HPV stock for infecting xenografted human skin tissue. For single-type HPV stock preparations, warts were swabbed and their DNA was typed using PCR as described below. Samples identified with the same HPV type, but too small to be extracted separately, were pooled for extractions. The final viral extracts were checked for HPV types again by PCR prior to the inoculation of xenografts. All manipulations of infected human tissue were carried out under the Biosafety cabinet.

2.2. HPV typing of clinically excised human warts or xenograft warts by PCR

HPV typing was performed on DNA collected from the swab samples or the extracts of clinical or xenograft warts. Swab samples were obtained from both visible papillomas and non-infected xenografts (for comparison purposes). Briefly, the uppermost layer of warts or grafts was swabbed first with a cotton swab moistened with PBS, followed by gentle rubbing with a dry swab. Both swab samples were extracted by incubating overnight at 55 °C in a volume of 0.5 ml of digestion buffer containing 100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0.5% SDS and proteinase K at a final concentration of 0.2 mg/ml. At the end of the incubation period, the swabs were squeezed to remove excess liquid and discarded. For DNA extractions from viral stocks prepared from clinical and xenograft warts, the same buffer and digestion procedures were used. The digested samples (from either swab or stocks) were mixed with an equal volume of solvent containing phenol:chloroform:isoamyl alcohol (25:24:1), followed by centrifugation at 16,000 × *g* for 1 min to separate the phases. The aqueous phase was passed through a Microcon-50 micro-concentrator (Millipore Canada Ltd.) and the DNA was eluted in 25 μl of 0.25× Tris–EDTA, pH 7.4, buffer. The eluted DNA was digested with the restriction enzyme *Hin*dIII (0.5–1 U/μl, New England Biolabs).

Aliquots of 5 μl of the *Hin*dIII-digested DNA were co-amplified with primer pairs specific to HPV types 6, 11, 16, 18 and 31 as described by Mant et al. (1997). The primer pair S-GH20/S-PCO04, specific for the human β-globin gene, was used as a negative control for non-infected human tissues. The positive controls are standard HPV plasmids pUC19-HPV6, pBR322-HPV11, pBluescript-HPV16, pBR322-HPV18 and pBR322-HPV31 containing HPV6, 11, 16, 18 and 31 DNA, respectively, obtained from American Type Culture Collection (Manassas, VA, USA). Amplification of these plasmids produces amplification products greater than 3 kb for the control plasmids of HPV6, 11, 16 and 31. For HPV18, only the L1 open reading frame of 216 bp is amplified.

The amplification reactions were carried out in a Perkin-Elmer GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT), in a 50 μl volume containing 5 μl of 10× PCR buffer, 6 μl of 25 mM $MgCl_2$, 1 μl of 12.5 mM dNTP mix, 2 μl of each primer at 10 μM and 0.5 U/μl of AmpliTaq Gold™ (Applied Biosystems, Mississauga, Ont.) under the following conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplification products were analyzed by electrophoresis on a 1% agarose gel and visualized with 0.5% ethidium bromide.

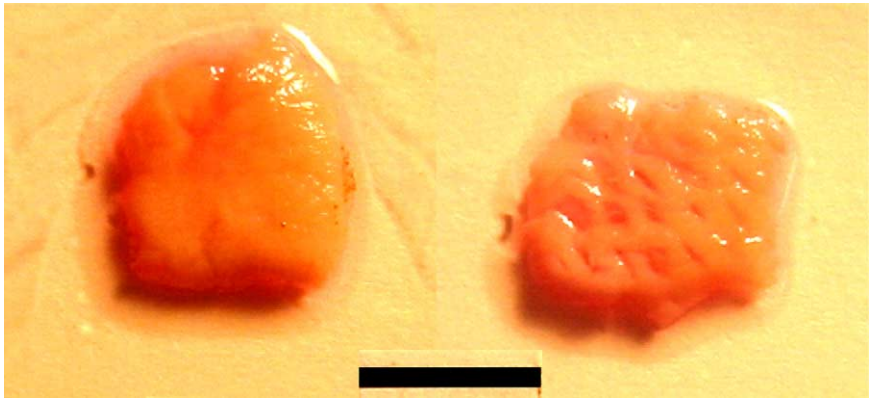

Fig. 1. Gross morphology of control (left panel) and meshed human foreskin graft (right panel). Calibration bar indicates a length of 1 cm.

2.3. Preparation of human foreskin

Neonatal foreskins from routine circumcisions were collected at the Tiny Tots Clinic, Dollard-des-Ormeaux, Que., Canada. Samples were placed in alpha-modified Eagle medium with Earle's salts and L-glutamate (Cellgro, Ont., Canada), supplemented with antibiotics (0.05 mg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin) and transported to our laboratories. All manipulations of human tissues were conducted under a class II Bio-safety cabinet (NuAire^{TM}, Plymouth, MN, USA). The foreskins were processed by removing occluded tissue and part of the underlying dermis. The split-thickness foreskin tissue was cut into pieces measuring 1 cm^2 (for cutaneous grafting) or 5 mm × 5 mm squares (for subcutaneous grafting) with or without pre-scarification with HPV extracts prepared from clinical warts. Scarification was carried out with a no. 10 surgical blade and inoculated with 70 μl of viral stock for each square centimeter. Scarification lines were made as crosses separated at a distance of ∼1 mm, with the depth of scarification controlled to less than 1 mm so that there was no cut through. Another 30 μl of viral stock was added for each square centimeter of tissue prior to 1-h incubation at 37 °C.

For the meshed grafts (Fig. 1), meshing was carried out with the Zimmer Skin Graft Mesher^{TM} (Zimmer Bureau Regional, Montreal, Que., Canada), prior to cutting into the desired graft sizes, but following the scarification. When grafted, these meshed grafts were carefully stretched so that approximately 40% of the grafting bed would be exposed to the meshed holes that would be covered by neo-epithelium initialized from the edges of the original donor skin bridges (Harries et al., 1995), within 2 weeks post grafting.

2.4. Cutaneous and subcutaneous grafting

NIH-nu-bg-xid mice were obtained from Charles River Laboratories (Wilmington, Boston, USA) or Taconic (NY, USA). Animals were housed in microisolator cages inside semi-rigid isolators with sterile food, water and bedding. All experiments were carried out within class II-type safety cabinets (NuAire^{TM}, Plymouth, MN, USA), according to protocols approved by the institutional Animal Care Committee, following the guidelines of the Canadian Council for Animal Care (Ottawa, Ont., Canada).

All grafting surgeries were carried out in mice anesthetized with halothane. For cutaneous grafting, a 1 cm^2 area of the host skin in the laterodorsal area was removed carefully to preserve the underlying fascia and avoid bleeding. The graft was fitted into the receiving bed and fixed in position with size 6–0 silk suture. The grafted area was dressed with polysporin antibiotic cream and Sofra-tulle antibiotic dressing, and covered with a layer of Vaseline-impregnated gauze. Finally, the graft and inner dressing were fixed in position with a flexible band-aid strip. Animals were maintained dressed for 3 weeks with redressing every 3–4 days or as necessary. For subcutaneous grafting, the foreskin tissues were further cut into squares of 5 mm × 5 mm and introduced into the subcutaneous space via a small opening in the central dorsal area. No dressing was required for these grafts. Starting from day 0 post-grafting, all mice were supplied with Septra antibiotic in the drinking water at a concentration of 1:800 (v/v) dilution.

2.5. Measurements of xenograft sizes and HPV DNA identification by simple swab tests

Graft sites were examined daily for the development of papillomas or other infections. In some experiments, upon the observation that subcutaneously grafted tissues reached a plateau in their growth pattern, a procedure of re-exteriorization was developed at the tenth week post-grafting. The skin covering the apex of the subcutaneous papilloma was cut with an incision; the skin was gently retracted and fixed to the grafted tissue using sutures, allowing the papillomas to grow outward and to protrude through the skin. These exposed subcutaneous papillomas developed a similar morphological and histological appearance to cutaneously growing xenograft papillomas. The host skin over the cyst was removed under halothane anesthesia, and the edges of the host skin were sutured with the cyst. The surgical sites were

dressed the same way as for the cutaneously implanted grafts until the wound was securely rejoined.

The size of distinct papilloma growth over the cutaneous xenografts was measured using digital calipers against the surface of the xenografts (Stoelting, IL, USA). The size of subcutaneous xenografts was measured against the flank surface. Volume of cutaneous warts or subcutaneous xenografts was calculated as the product of the length, width and height or its cubic root (geometric mean diameter, GMD). Following the analysis of experimental data, it was observed that in the cutaneous model, papilloma size did not always reflect the extent of papillomatosis, mostly complicated by the relative size to the surviving graft. For example, a confluent papilloma with dense keratinization on a smaller graft may have a smaller volume than a semi-confluent papilloma on a large graft. Therefore, a scoring system was developed to score cutaneous papillomas with the following criteria: (0) normal; (1) roughness; (2) small warts with 1–2 mm in each dimension; (3) large warts >2 mm in any two dimensions; (4) semi-confluent papillomas covering up to half of the graft surface; (5) confluent papillomas covering >1/2 of the graft surface; (6) confluent papillomas with dense keratinization. Although there is some subjectivity in the scoring system, inter-observer variability tested with four scientists in the blind fashion never exceeded 10% (including a student following the 1st training). This high reproducibility may be contributable to the fact that in this system, only the distinct papilloma growth over the surface of xenografts was scored. For the identification of HPV types involved, a swab test and PCR typing were carried out as already described.

2.6. Collection of the uppermost layers of xenograft warts and preparation of the HPV stock

The xenograft papillomas were surgically excised and HPV type re-confirmed by PCR. Viral stock was collected from these tissues following the same procedure as that used for collection from clinical warts. The harvested stock was stored in phosphate-buffered saline supplemented with antibiotics at 1% (v/v) of gentamycin (50 mg/ml), penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) at −80 °C, until it was used for subsequent serial passages.

2.7. Histology, in situ hybridization, and immunohistochemistry features of xenograft warts

Formalin-fixed tissues (both negative control skin and warts) were sent to Pathology Associates International (Frederick, MD, USA) for histology, in situ hybridization and immunohistochemistry studies. For histology, the tissue was fixed with formalin immediately upon collection. Samples were trimmed across epidermis to subcutis, desiccated, processed through xylene, and perfused with paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin.

For in situ hybridization, biotinylated DNA probes specific for HPV6 or 11 were obtained from DAKO Corporation (Via Real, Carpinteria, USA). Tissue sections were deparaffined in xylene and re-hydrated through graded ethanol and water. Following protease digestion, probe solution was added to the slides. The slides were covered without sealing and incubated for 6 min at 92 °C to denature HPV and probe DNA. Slides were then placed in a humid chamber for 1 h at 37 °C. Following hybridization, slides were subjected to a high stringency wash to reduce non-specific hybridization. Specific hybridization was visualized by catalyzed reporter deposition using a tryamide signal amplification kit (GenPoint, DAKO Corporation), and dark-brown intracellular staining was identified as a positive signal.

For immunohistochemistry, murine IgG1 monoclonal antibody (Novocastra Laboratories Ltd., UK) directed against HPV6 L1 coat fusion protein (amino acids 40–233) common to HPV types 6, 11, and 18, was used to detect HPV6 or 11 L1 expression in wart tissues. Biotinylated goat anti-mouse IgG1 was added to react with the antibody followed by immunoperoxidase staining which labels positive cells in dark-brown.

3. Results

3.1. Initial HPV collection from clinical warts and typing by PCR

An initial viral stock was prepared from clinical warts and shown to contain both HPV6 and 11 DNA by PCR (Fig. 2).

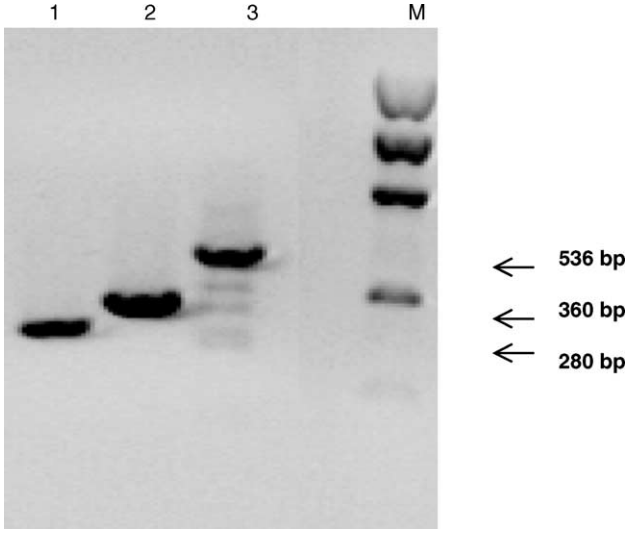

Fig. 2. HPV typing of the initial virus stock prepared from clinical wart extract containing a mixture of HPV6 and 11. In lane 1, primer pair VdB-6-U/D that comprises the HPV6 L2 open reading frame, amplified the expected 280 bp product. In lane 2, primer pair VdB-11-U/D that comprises the HPV11 L1 open reading frame, amplified the expected 360 bp product. In lane 3, a positive control, primer pair KM29/RS42 specific for human β-globin, amplified the expected 536 bp product. Lane M illustrates a DNA molecular weight ladder representing 2000, 1200, 800, and 400 bp, from top to bottom. The arrows on the right indicate the molecular weights of the amplification products.

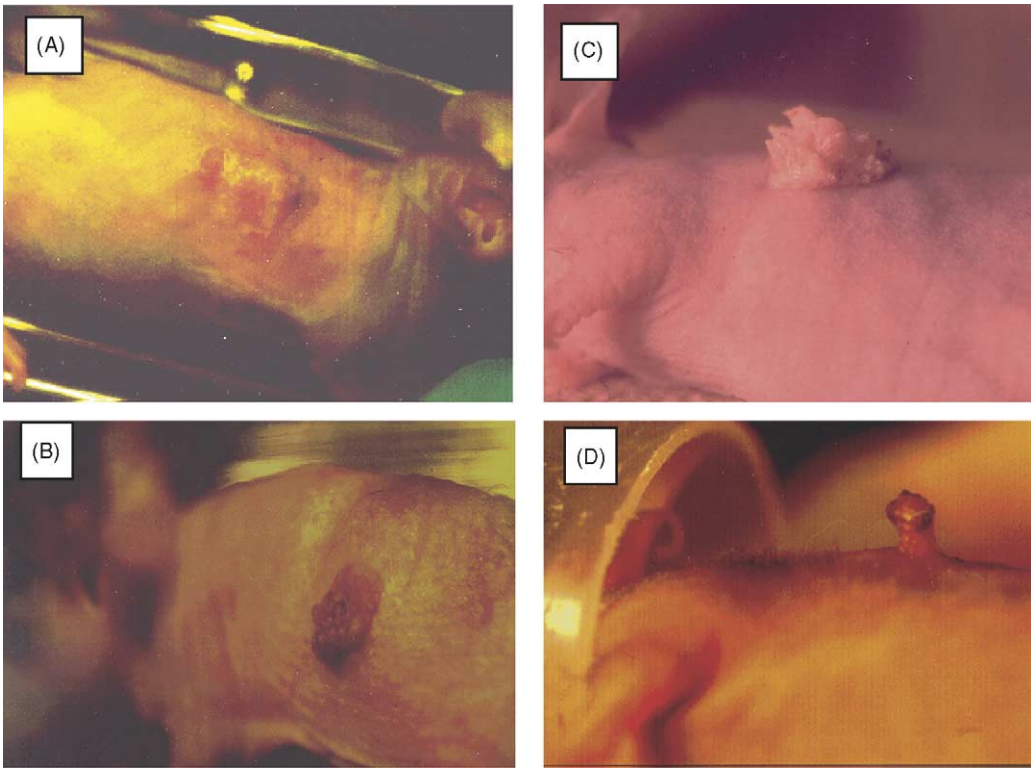

Fig. 3. Gross morphology of cutaneous human foreskin graft and human papillomas. Panel A shows the appearance of a non-infected human foreskin graft (with meshing prior to grafting). Panel B shows a 1st generation papilloma induced by the viral stock containing both HPV6 and 11 extracted from clinical warts in the meshed graft. Panel C shows a typical HPV papilloma induced by the inoculum containing HPV11 single-type virus in the meshed graft. Panel D shows the morphology of an exposed subcutaneous papilloma induced in meshed grafts.

Bands of 280 bp and 360 bp corresponding to the VdB6 and VdB11 primers confirmed the presence of both HPV types 6 and 11 in the clinical wart extract analyzed in Fig. 2. Similar PCR typing technique was used to verify single-type HPV6 or 11 viral extracts which were PCR-negative for the high-risk HPV genotypes HPV16, 18, and 31 (data not shown).

3.2. Papilloma induction in cutaneous grafts

Following several failed attempts to induce visible papillomas in the human foreskin xenografts (0 out of 16 grafts), we introduced a meshing protocol that processes the scarified human foreskin grafts into skin meshes with reproducible pore sizes. In the cutaneous model, the wound healing process with obvious neo-epithelialization covers the pores within about 2 weeks. Using the initial viral preparation containing both HPV11 and HPV6 as inoculum, six of eight cutaneous human grafts treated with the meshing procedure formed visible papillomas within 2–4 months. The appearance of a cutaneous papilloma is shown in Fig. 3B. In comparison, in the same paralleled experiment using the same viral stock and matched cutaneous grafts without meshing, no visible papillomas were induced up to 6 months ($n = 4$), similar to the previous non-paralleled experiments with non-meshed grafts. Visible pailloma induction frequency and rate in meshed xenografts were reproducibly repeated with the viral stock containing both HPV11 and HPV6 in the subsequent experiment (8 out of 10 grafts). Statistically, there was no significant inter-experimental differences of papilloma induction rate in either non-meshed grafts (0 in all experiments with 20 inoculated cutaneous grafts), or in the meshed grafts inoculated with the viral stock containing both HPV11 and 6 (6/8 versus 8/10). Yet, the induction rate of visible papillomas in the meshed grafts was significantly improved over that in the non-meshed grafts ($P < 0.05$ by Fisher Exact Test).

Fig. 3C shows the typical human papillomas induced by a single-type low risk HPV11 in a meshed graft. The frequency of papilloma induction in meshed grafts by HPV11 or HPV6 single-type collected from clinical warts in the primary infection experiments is summarized in Table 1. In comparison to the high frequency of papilloma induction following infection with HPV11 in the meshed grafts, HPV6 infection generally resulted in a lower frequency of papilloma induction and the extent of papillomatosis was much milder and

Table 1
The frequency of papilloma induction by HPV11 and 6 single-type collected from clinical warts in the primary infection experiment

Type	Mouse survival	Graft survival	Induction frequency
HPV11	5/7	10	10/10 (7 weeks)
HPV6 Expt. 1	2/12	4	1/4 (14 weeks)
HPV6 Expt. 2	13/16	13	8/13 (14 weeks)

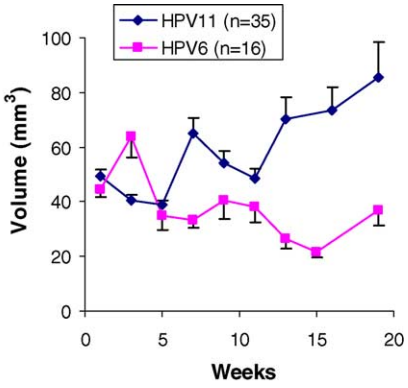

Fig. 4. Growth rates of papillomas in subcutaneously grafted human foreskin induced by single-type HPV11 or 6 in the primary infection experiments. The volume was measured as that detailed in the text as the product of length × width × height. HPV11-induced warts were collected at 20 weeks post-grafting for virus collection. Only a few HPV6 infected grafts had moderate growth after 7 months. Data were presented as mean ± S.E.M.

delayed (Table 1). In the first experiment with HPV6 infection, premature deaths of immunodeficient mice were caused by contamination from the clinical wart extracts not related to HPV infection. In subsequent experiments, introduction of a 30 min centrifugation at 15,000 × *g*, minimizing bacterial contamination, reduced premature deaths of the nude mice to less than 30%.

3.3. Papilloma induction in sub-cutaneous xenografts

In primary infections with the mixed type HPV6 and 11 inoculum, 4 of the 16 subcutaneously implanted meshed grafts showed significant growth within 10 weeks, followed by a plateau period of 2 weeks. Upon re-exteriorization, 5 of the 16 grafts grew further into cutaneous papillomas within the next 7 weeks. One of the re-exteriorized warts is shown in Fig. 3D. In the paralleled experiments using the same viral stock and matched donor grafts without meshing, no apparent growth was observed in all 10 non-meshed subcutaneous grafts over the same period of 17 weeks. Limited growth was observed in three of them following repeated exteriorizations at 20 weeks post-grafting.

In experiments with single-type HPV inocula, 1st generation HPV11 infected meshed sub-cutaneous xenografts grew significantly. In comparison, 1st generation single-type HPV6 infected tissues did not have obvious growth in most of the grafts, despite meshing (Fig. 4).

3.4. Histology, in situ hybridization, and immunohistochemistry of xenograft warts

In addition to their gross morphological similarity to clinical papillomas, these cutaneous and subcutaneous warts induced by either mixed or single-type HPV shared similar histological, cellular, in situ hybridization, and immunohistochemical characteristics to those in clinical warts (Fig. 5). In comparison to the non-infected xenograft (Fig. 5A), all tested papilloma tissues (10/10) exhibited extensive acanthosis, koilocytosis, parakeratosis and hyperkeratosis (Fig. 5B). As exemplified in panels C and D, papillomas induced by HPV11 were only specifically hybridized with HPV11 probes, but not with HPV6 probes (panel C versus panel D), and vice versa. Immunohistochemistry data also confirmed the positive HPV6 or 11 L1 expressions in xenograft wart tissues (Fig. 5E).

3.5. HPV DNA typing

The results in Fig. 6 confirm the presence of HPV types 6 and 11, and the absence of HPV types 16, 18 and 31 in the DNA isolated from the 1st generation cutaneous xenograft

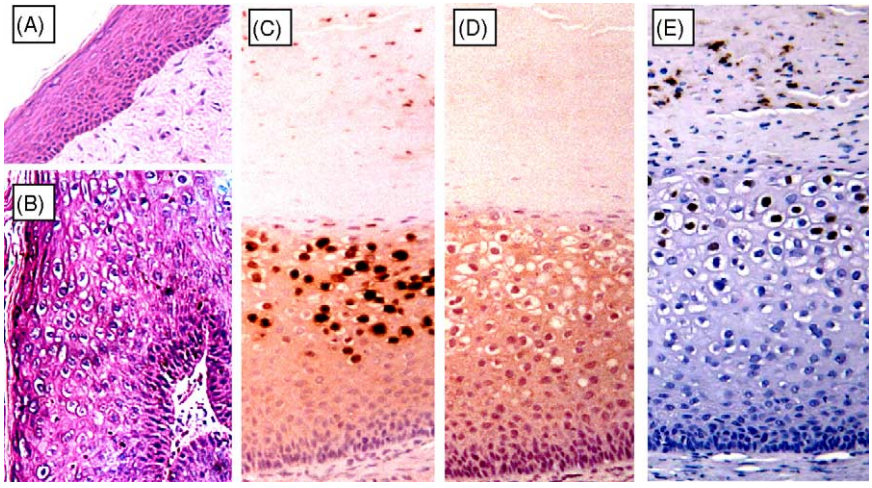

Fig. 5. Typical histology, in situ hybridization, and immunohistochemistry of HPV-induced xenograft warts. Panel A: typical histology of non-infected xenograft skin; Panel B: typical histology of xenograft papilloma induced by HPV11 in the cutaneously grafted human foreskin. Panels C and D: a typical subcutaneous xenograft papilloma induced by HPV11 was examined by in situ hybridization. It was clear that this HPV11-induced wart was positive for HPV11 probes (panel C), but not for HPV6 probes (panel D). Panel E: a typical subcutaneous xenograft papilloma examined by immunohistochemistry.

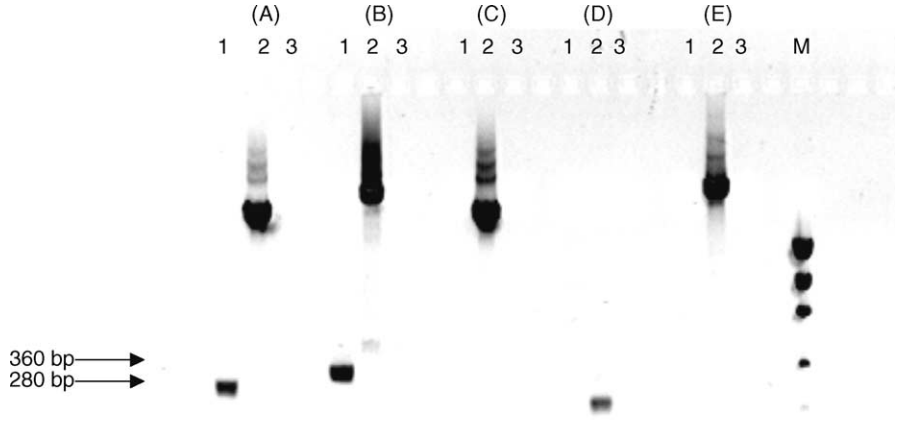

Fig. 6. Verification of low-risk HPV DNA in the cutaneous xenograft papillomas induced with a mixture of HPV6 and 11. The lanes in group A–E correspond to the amplification products of primers specific for HPV6, 11, 16, 18, and 31, respectively. In each group, lane 1 corresponds to the amplification product of DNA extracted from 1st generation cutaneous wart tissue, lane 2 corresponds to a positive control and lane 3 to a negative control (no DNA in the amplification reaction). Lane M illustrates a DNA molecular weight ladder representing 2000, 1200, 800, and 400 bp, from top to bottom. The amplification products demonstrate the presence of HPV6 (lane A1) and 11 (lane B1) in the 1st generation cutaneous papillomas, but not the high-risk types HPV16, 18, and 31.

wart tissue. The presence of low risk HPV6 and 11 was further confirmed with the swab test. DNA isolated from swab samples obtained from the surface of four distinct 1st generation exposed subcutaneous papillomas induced with the same viral stock containing both HPV6 and 11 was analyzed by PCR using HPV types 6 and 11 specific primers. The results confirmed the presence of both HPV types 6 and 11, and the absence of high-risk HPV16, 18 and 31 at all four sites (data not shown). In comparison, subcutaneous and cutaneous papillomas induced by viral stocks containing single-type low risk HPVs only contained single-type HPV6 or 11, respectively (Fig. 7). The selectivity and proportions of PCR positivity of xenograft warts are summarized in Table 2.

3.6. Passaging to 2nd and 3rd generation xenografted animals

Serial single-type HPV11 passages in the 2nd and 3rd generations with inocula collected from previous generation xenograft warts resulted in high frequency and reproducible cutaneous papilloma induction (Fig. 8). The reproducibility of subcutaneous passage of HPV11 is shown in Fig. 9. Although HPV6 single-type induced cutaneous wart formation at a lower frequency (8/13) than HPV11, the 2nd and 3rd passage demonstrated improvements in both induction frequency (3/4, and 7/7, respectively) and growth rate (Fig. 10).

Table 2
Proportions of PCR positivity of HPV xenograft warts

HPV type in the inoculum	No. of xenograft warts tested	Frequency of PCR positivity	
		HPV11	HPV6
	Cutaneous		
HPV11 and 6	12	10	12
HPV11	10	10	0
HPV6	15	0	9
	Subcutaneous		
HPV11 and 6	23	23	22
HPV11	9	9	0
HPV6	5	0	5

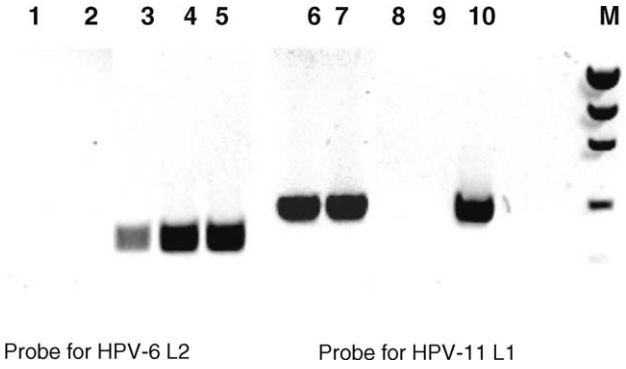

Fig. 7. PCR analysis of single-type HPV6- or 11-induced xenograft warts. Primer pair VdB-6U/D that comprises HPV6 L2 open reading frame amplified the expected 280 bp product from both HPV6-induced subcutaneous warts (lanes 3 and 4), similar to that obtained with control plasmid pUC19-HPV6 (lane 5). In contrast, HPV11 warts did not have positive signals when probed for HPV6 (lanes 1 and 2). In lanes 6 and 7, primer pair VdB-11U/D which comprises HPV11 L1 open reading frame amplified the expected 360 bp products from both HPV11-induced cutaneous and subcutaneous warts, similar to that observed with control plasmid pUC19-HPV11 (lane 10). HPV6 warts gave a negative signal when probed for HPV11 (lanes 8 and 9). Lane M illustrates a DNA molecular weight ladder representing 2000, 1200, 800, 400, and 200 bp, from top to bottom.

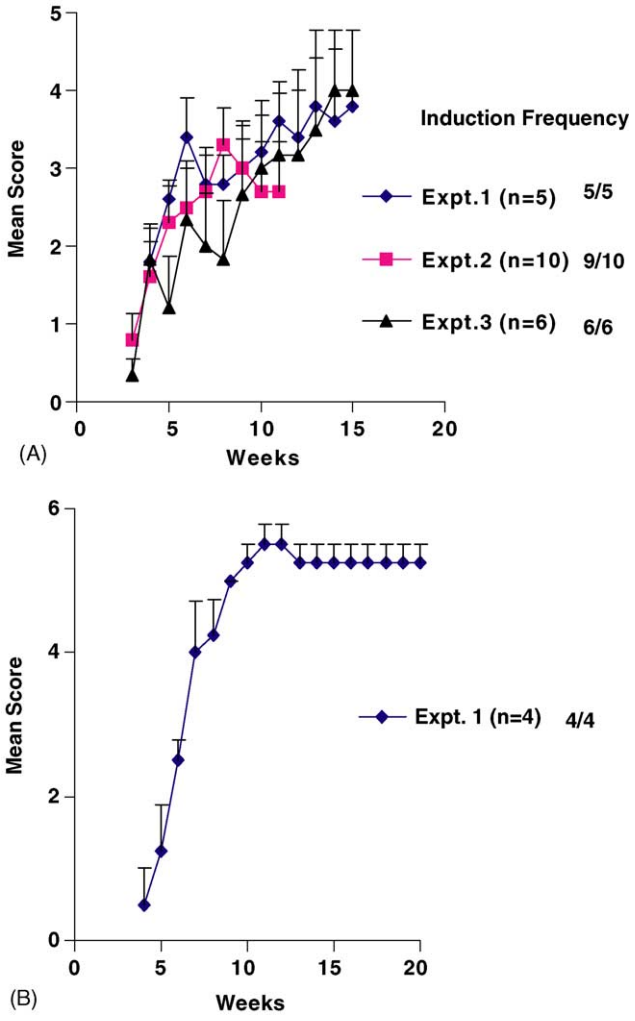

Fig. 8. Single-type HPV11 papilloma induction in the cutaneous model by viral stock originally generated from subcutaneous xenograft warts. Panel A shows 3 individual experiments with highly reproducible papilloma induction frequency and growth rate in this 2nd passage. Panel B shows that the third passage papilloma induction is also highly reproducible. Data were presented as mean ± S.E.M.

4. Discussion

The present study reports a novel meshing procedure that leads to a highly reproducible model that allows us to quantify reproducible papilloma growth over the surface of cutaneously grafted human foreskin. While the subcutaneous model described here is efficient and convenient for viral propagation, the highly reproducible cutaneous wart induction model presented in the current study may be more useful for screening and selecting candidate agents for the treatment or prevention of human papillomavirus infections.

The challenge of HPV wart induction may be related to the unique life cycle of HPV that is closely coupled to keratinocyte differentiation. Infection is believed to initiate at the basal cell layer. Unlike other cells, epithelial cell division continues as the cell undergoes vertical differentiation.

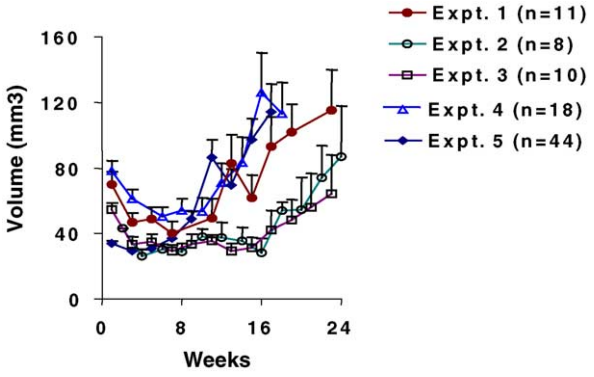

Fig. 9. Subcutaneous growth of single-type HPV11 infected xenograft in the 2nd and 3rd serial passages. Experiments 1–4 show four individual experiments in the 2nd passage, xenografts infected with inoculum collected from 1st generation (or passage) xenograft warts. Experiment 5 shows a third passage in the subcutaneous model (infected with inoculum prepared from 2nd generation xenograft warts). Papilloma induction, passage and volume measurement are as described in the text. Data were presented as mean ± S.E.M.

As infected cells undergo progressive differentiation, viral genome amplification and an increase in viral gene expression occurs. Subsequently, late gene expression and virion assembly follows in terminally differentiated keratinocytes from which viral particles release (Kreider et al., 1990; Stanley et al., 1997). Therefore, sufficient amounts of infectious virus can be collected only at the upper most layer of the warts at the most proper time. The first successfully reported HPV-xenograft model may be attributed to Kreider et al. (Kreider et al., 1987a, 1987b, 1986). This model has been used as an

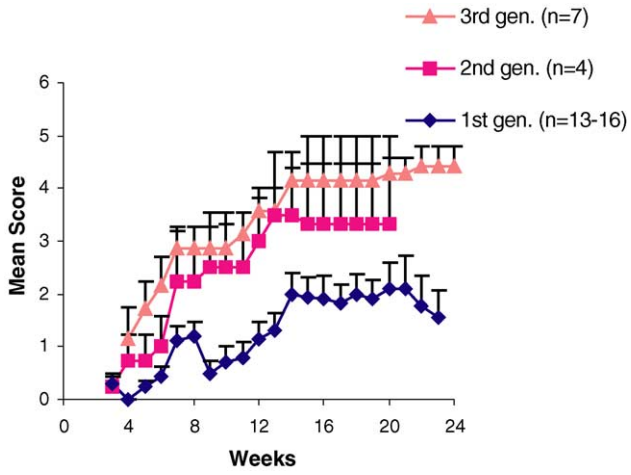

Fig. 10. Comparative growth rates of three generations (gen.) of xenograft papillomas induced by single-type HPV6 in the cutaneous model. Number of animals for the 1st generation (inoculum from clinical warts) was reduced to 13 after day 15 since 3/16 animals developed other infections and were sacrificed. Second generation papillomas were induced by a small viral stock prepared from 24 small subcutaneous and 1 small cutaneous 1st generation warts collected as described in Fig. 4. Third generation papillomas were induced by an inoculum prepared from 2nd generation subcutaneous xenograft warts. Data were presented as mean ± S.E.M.

important tool in understanding HPV biology and drug evaluations; yet, limitations including access to the infection site for treatment and observations have motivated many laboratories to seek improvements. Bonnez et al. (1993) described HPV type 11 infection in human foreskin tissues implanted under the renal capsule, peritoneum and subcutaneously in SCID mice. A total of 58% of the grafts showed signs of HPV infection. In the subcutaneously implanted grafts, 25% were positive for HPV by immunocytochemistry and RT-PCR. No serial passage experiments were published to verify infectious viral propagation from these models. The same group (1998) also reported the isolation and propagation of HPV16. The virus was isolated from clinical samples and used to infect human foreskin prior to subcutaneous implantation into SCID mice. The sites were prepared by inserting glass cover slips at the graft sites 2 weeks prior to grafting. The lesions at the graft sites were exposed 4 weeks after grafting and the animals sacrificed 24 weeks after grafting. Three of the five grafts showed small papillomas. The virions from these papillomas were harvested and used in a subsequent passage containing 60 grafts. Animals were sacrificed 16 weeks after grafting. A total of 34 out of 60 grafts were positive for the presence of HPV DNA and only one was positive for HPV capsid by immunochemistry, indicating a very low frequency of infectious viral particle assembly in this passage.

Brandsma et al. (1995) described HPV papilloma induction with genomic DNA in the SCID xenograft model. In a total of 16 grafts, eight were inoculated with HPV16 DNA pre-grafting and eight post-grafting. Two grafts inoculated post-grafting appeared to develop signs of HPV infection. Again, there were no further passages of the collected virus to verify HPV propagation in this model.

Sexton et al. (1995) described a grafting method whereby a glass cover slip was first inserted into the graft site of a SCID mouse for 1–2 weeks. This was replaced with a silicone-grafting chamber in which benign wart tissue was placed. After 5 weeks, macroscopic warts developed. Attempts to graft the wart tissue resulted in hyperproliferative human epithelium devoid of viral infection.

The meshing procedure described in our present study allowed us to induce papilloma and propagate infectious HPV with high reproducibility in both cutaneous and subcutaneous xenografts. Meshing may increase the success rate of papilloma induction in the xenografts by several different mechanisms. First, meshing may stimulate neoepithelization (Harries et al., 1995), and thus increase the population of basal cells that are the only target cells for HPV infection. Second, meshed grafts have a more pronounced wound healing process. It is known that during the wound healing process, an integrin, $\alpha 6\beta 4$, becomes widely expressed. A recent study (Evander et al., 1997) suggests that this integrin may be a receptor for papillomavirus-binding and entry into the host cells and responsible for the initiation of HPV infection. Therefore, wound healing may be an important factor to consider and mimic for the development of animal models of PV infection. Third, meshed grafts facilitate the transudation of exudates, and thus improve the survival and health of the grafts (Harries et al., 1995).

The selection of the NIH-nu-bg-xid mice has certain advantages over both nu/nu mice and SCID mice. As suggested by Stanley et al. (1997), nu/nu mice may be less immunodeficient than scid and NIH-nu-bg-xid mice, and thus, not easily allow xenograft tissue to survive and grow. The SCID mice are covered with fur, thus necessitating removal of the hair before surgery and before evaluating and measuring experimental endpoints. The NIH-nu-bg-xid mouse is essentially hairless and lacks functional T-cells, B-cells and NK-cells, thus making it the ideal recipient in this animal model.

Although HPV6 has been reported as one of the most common low-risk genotypes associated with genital warts, the reproducibility and frequency of visible papilloma inductions in the xenograft model have not been documented. Bonnez et al. (abstracts from the 15th International Papillomavirus Workshop, Queensland, Australia, 1996, and the 18th International Papillomavirus Workshop, Barcelona, Spain, 2000) reported histological and molecular evidence of HPV6 infection in the subcutaneous xenografts. The same group also reported HPV6-induced warty growth by the measured graft size (Iyer et al., 2002). Yet, neither frequency of distinct wart induction, nor reproducibility was mentioned. In our present study with meshed grafts, HPV11-induced papillomatosis at high frequency and reproducible growth rate, with inocula collected either from initial clinical warts, or from subsequent xenograft passages. In contrast, papilloma induction with initial HPV6 inoculum from clinical warts showed lower induction frequency and milder papillomatosis. With serial passages, both induction frequency and papilloma growth rate improved. An exact explanation for the observed genotype differences is not clear at the present time, but may be attributable to several possibilities. It has been reported that HPV11-associated recurrent respiratory papillomatosis was more aggressive than HPV6-associated disease, and genetic predisposition might explain why African Americans are at a higher risk of acquiring HPV11, while Caucasians may be more susceptible when exposed to HPV6 (Rabah et al., 2001). In a recent study, Brown et al. (1999) reported that the predominant type in lesions from control patients was HPV6, while lesions from immunosuppressed patients (HIV infected or organ transplant recipients) most often contained HPV11. Therefore, the less successful xenograft infection with single-type HPV6 in other laboratories, as well as in our primary HPV6 infection in the present model may be related to either a lower number of infectious particles of HPV6 in the initial inoculum, or the less proliferative (pathogenic) nature of this viral genotype, and/or the lower genetic liability of the donor skin. The observed improvements of serial passages of single-type HPV6 infection, and the productive propagation of HPV6 in the mixed-type infection in our present study seem to favor the first hypothesis. Fang et al. (2003) suggested co-infection with Her-

pes Simplex Virus (HSV) type 2 could suppress HPV gene expression and papillomatosis. It may be possible that the initial viral stock of HPV6 also contains very low titers of HSV, which may be a factor inhibiting papilloma induction. The titers of HSV might have been reduced with serial passages. Nevertheless, further investigation is required to confirm the hypothesis as well as to improve papilloma induction with HPV6.

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