Laboratory production of infectious stocks of rabbit oral papillomavirus

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Several small, raised lesions from the underside of the tongue of domestic rabbits were isolated, and an extract prepared and tested for the presence of rabbit oral papillomavirus (ROPV). Two weeks after inoculation of this extract into the underside of rabbit tongues, multiple small discrete, grey-white nodules were observed that reached a maximum size of 2 mm in diameter by 5 weeks. These lesions showed typical ROPV pathology, and nuclei stained positive for papillomavirus (PV) group-specific antigen (GSA) by immunocytochemistry. Tissue fragments from rabbit tongues were incubated with a suspension of ROPV and placed subcutaneously into athymic mice. After 60 days, cysts were removed, sections cut for histology, and a virus stock prepared. GSA staining and in situ hybridization demonstrated that the xenografts were morphologically transformed with areas showing strong nuclear staining for viral capsid antigen and ROPV DNA. Extracts prepared from the pooled xenografts contained infectious ROPV as demonstrated by inoculation into the undersurface of tongues of non-immune New Zealand White rabbits. The results demonstrated that stocks of infectious ROPV can be prepared in the athymic mouse xenograft system for use in studies on the experimental transmission of a mucosal-targeting animal papillomavirus.

Introduction

Rabbit oral papillomavirus (ROPV) is a mucosal-targeting papillomavirus (PV) that causes benign lesions on the tongue and in the oral cavity of domestic rabbits (Parsons & Kidd, 1936, 1942; Richter et al., 1964; Rdzok et al., 1966; Weisbroth & Scher, 1970; Dominguez et al., 1981; Sundberg et al., 1985; O'Barion et al., 1988). ROPV, in contrast to cottontail rabbit papillomavirus (CRPV) (a cutaneous PV associated with squamous cell carcinomas), does not infect cutaneous sites, is antigenically unrelated to CRPV, and induces lesions that often regress spontaneously (Parsons & Kidd, 1942; Rdzok et al., 1966). There are currently two animal model systems (bovine PV type 4 and cattle; canine oral PV and dogs) that are available for studying viral infection, pathogenesis and host immunity to a mucosal-targeting PV (Campo et al., 1980; Patel et al., 1987; DeMonbreun & Goodpasture, 1932; Delius et al., 1994; Isegawa et al., 1995). These two animal model systems have been used to determine some key events in PV infection of mucosal surfaces and host immune responses to PV infection (Smith & Campo, 1989; Jarrett et al., 1990; Chandrachud et al., 1995; Chambers & Evans, 1959; Cheville & Olson, 1964; Bregman et al., 1987; Bel et al., 1994), but suffer from some logistical difficulties such as the cost and housing of these large animals. We therefore sought to establish a mucosal PV animal model system in a smaller laboratory animal by using ROPV, which has been reported as an occasional infection of domestic rabbits (Parsons & Kidd, 1942; Weisbroth & Scher, 1970; Sundberg et al., 1985). The addition of a second rabbit PV to the CRPV/rabbit model system could provide additional information with respect to virus tissue specificity and host immunity that more closely identifies with the situation in humans, who are often infected with more than one human PV (HPV) type.

Domestic New Zealand White (NZW) rabbits were examined for the presence of papilloma-like lesions on the undersurface of the tongue, and several small raised lesions were observed. Infectivity with an extract from these lesions demonstrated the presence of infectious ROPV, and a high-titre stock of ROPV was prepared using the athymic mouse xenograft system (Kreider et al., 1985). ROPV was detected indirectly by using PCR amplification with a set of primers designed from previously published partial ROPV sequence...
data (O’Banion et al., 1988). The results demonstrated that stocks of ROPV were developed that can be used to study various aspects of tissue specificity and host mucosal immunity in an animal model system.

Methods

Detection of oral papillomas and isolation of infectious stocks of ROPV. Domestic NZW rabbits that were obtained from local breeders were first anesthetized, then examined for oral papilloma-like lesions on the undersurface of the tongue. Several small lesions were identified on 3/20 animals, and the lesions were removed, pooled and homogenized in a buffer containing 1 m-NaCl, 0.02 m-Tris (pH 7.4) in a Brinkman homogenizer as previously described (Christensen et al., 1994).

The crude extract was then placed onto the wounded undersurface (multiple pin-pricks with a 26-gauge needle) of the tongue of fresh rabbits and the sites monitored weekly. Three weeks after inoculation, many tiny spherical grey-white lesions at the sites of the needle punctures were observed which grew to a maximum size of 1 x 1 x 1 mm by 35 days, and then began to regress. Tongue tissue was prepared from these infections and subsequently examined by haematoxylin and eosin, papillomavirus group specific antigen (GSA) antibody staining and in situ hybridization with an ROPV DNA subgenomic probe for detection of ROPV (see below).

Production of infectious stocks of ROPV in the athymic mouse xenograft system. Infectious stocks of ROPV were prepared using the athymic mouse xenograft system (Kreider et al., 1985). Fetal rabbit tongue fragments were incubated for 1 h at 37 °C with the above described extract diluted 1:4 with water to reduce the salt concentration to near physiological conditions, and 10% normal rabbit serum (NRS). Additional tissue fragments from the gums, cheek and external genital tissue of fetal rabbits were also prepared. For each tissue source, split-thickness epithelial fragments (2 x 2 x 1 mm) were prepared, and punctured several times with a scalp el point prior to incubation with ROPV virus suspension. The ROPV-infected tissue fragments were implanted subepithelially in athymic mice and grafts harvested after 60 days at which time the grafts had reached an average size of between 4 and 6 mm in diameter. Sections of the grafts were prepared for histological analysis, and the remainder used to prepare stocks of infectious ROPV. The cysts were ground in a Brinkman homogenizer in PBS to make a 10% crude extract to be used in infectivity studies. Titration studies using non-immune rabbits demonstrated that the infectious titre of the xenograft-derived extract was greater than 10⁵, with no papillomas developing at sites injected with this extract diluted to 10⁻⁴ (data not shown).

Detection of ROPV in xenografts and infected rabbit tongues. Several techniques were used to detect ROPV in the experimentally induced lesions. For cloning purposes, an extensive search using 33 restriction enzymes revealed only three enzymes (BstEI, Ndel and SpeI) that linearized the ROPV genome. An SpeI cloning site was established in the multiple cloning site of the plasmid pUC19 using linkers obtained from New England Biobs, as per the manufacturer’s instructions, and the ROPV genome was cloned into this site. Enzymes that did not cut the ROPV genome included AatII, Apal, BalI, BglII, BglIII, Csp45I, EcoRI, NotI, SacII, SalI, XbaI and XhoI. Enzymes with two sites included Bsm36I, ClaI, EcoRV, Ndel, Poul, SacI, Smal and SphI; and those that cut three or more times included BamHII, BanII, DdeI, HaeIII, Hinfl, HpaI, HpyII, NcoI and SspI. For in situ studies, a subgenomic fragment of ROPV (a 3.8 kb EcoRV fragment) was cloned into the plasmid pcDNA3 (Invitrogen). The viral insert was purified from the plasmid, biotinylated (Amersham) and used as an in situ probe for the detection of ROPV DNA. A control probe was generated from CRPV DNA and control tissues were xenografts derived from cottontail rabbit skin chips infected with CRPV. Antisera generated against the papillomavirus GSA (Biogenex, San Ramon, Calif., USA) was used to detect ROPV capsid antigen by immunocytochemical staining. Sections of infected tongue and xenografts were analysed.

A set of primers was designed from the partial sequence of ROPV (O’Banion et al., 1988) and tested by PCR with ROPV and CRPV template DNA as an indirect test for specific ROPV DNA sequences. Purified viral DNA was extracted from CsCl-banded ROPV and CRPV that was prepared from xenograft-derived material and used as template DNA. The primer sequences were as follows: sense, 5' GGGGTACCTAGCACTGTGATGAGC 3' (#41) and 5' ATAGGTACCTGAAGTGGGTGC 3' (#43); antisense, 5' CGGCATCCGTTGCGAGCTCTAG 3' (#42), 5' TATGATAGGGTTCCTTGTA 3' (#43) and 5' GCATACTGTCCTGGATCGGT 3' (#44). The sizes of the PCR products were predicted to be 2102 bp (primers 41 and 42), 1519 bp (primers 41 and 43), 1031 bp (primers 45 and 42), 770 bp (primers 41 and 44) and 498 bp (primers 45 and 43).

Results

Isolation of infectious ROPV

Several small lesions from the underside of the tongue of NZW rabbits were homogenized to make a crude extract that was tested for the presence of ROPV by inoculation onto the undersurface of tongues of fresh rabbits following wounding by multiple needle pricks as described in Methods. The extract was subsequently demonstrated to contain infectious ROPV and this preparation was used as the starting material for production of high-titre stocks of infectious ROPV using the xenograft system (Kreider et al., 1985).

Production of ROPV in xenografts and tissue susceptibility analysis

Fetal rabbits were used to analyse the tissue susceptibility of ROPV infection in a non-immune environment. Fragments of tissue from the tongue, gums, cheek and external genital area were incubated for 1 h with separate aliquots from a mixture of the initial crude extract containing ROPV (200 µl), water (800 µl) and non-immune rabbit serum (100 µl) to reduce salt to approximately physiological concentrations. The skin chips were transplanted subepithelially as previously described (Kreider et al., 1985), and grafts harvested 60 days later for histological analysis and virus extraction. A summary of these experiments is shown in Table 1. Xenografts derived from tongue and gum tissue showed extensive areas of morphological transformation, with many group-specific antigen (GSA) and in situ positive nuclei. Xenografts derived from genital tissue contained substantial amounts of hair and hair follicles, but no evidence of ROPV infection with the exception of one graft (out of four examined) that contained a small area of 10–20 nuclei that were positive for ROPV DNA by in situ hybridization. This region was confined to one edge of the central keratin core of the xenograft and represented com-
completely differentiated epithelial tissue. Xenografts from cheek tissue did not show morphological transformation and there was no ROPV DNA as determined by

**Table 1. Tissue susceptibility of fetal rabbit tissue to ROPV in the xenograft system**

<table>
<thead>
<tr>
<th>Fetal rabbit tissue</th>
<th>No. of xenografts*</th>
<th>Cyst size (mm)†</th>
<th>GSA staining‡</th>
<th>In situ hybridization§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue</td>
<td>6</td>
<td>5.9 ± 1.2</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Cheek</td>
<td>5</td>
<td>4.7 ± 1.1</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Gums</td>
<td>8</td>
<td>5.0 ± 1.3</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Genital region</td>
<td>8</td>
<td>6.3 ± 1.3</td>
<td>0/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

* No. of viable xenografts 64 days after implanting ROPV-infected skin chips subrenal.
† Mean (± SD) of geometric mean diameters (GMDs) of viable xenograft cysts.
‡ No. of GSA positive xenografts/no. of xenografts examined.
§ No. of ROPV in situ positive xenografts/no. of xenografts examined.

**Discussion**

In this study we describe the laboratory production of infectious stocks of ROPV using the athymic mouse xenograft system. Papillomas derived from ROPV infection were first described by Parsons & Kidd (1936) as a sporadic infection of the oral cavity of domestic rabbits, and extracts of these lesions contained infectious virus as determined by infectivity studies (Parsons & Kidd, 1936, 1942). Since this time, there have been very few published reports describing this PV system (Rich and Scher, 1970; Dominguez et al., 1981; Sundberg et al., 1985; O'Banion et al., 1988), and only limited stocks of infectious virus have been reported. A more recent study has described the cloning and partial sequencing of the ROPV genome (O'Banion et al., 1988), and the published sequence data were used to help confirm indirectly that the PV isolate described in this study was ROPV. In an earlier study, cloning of the ROPV genome was achieved as three separate subgenomic fragments (O'Banion et al., 1988) because a restriction enzyme that cut the ROPV genome once was not found. We have done digestion experiments with an extensive list of restriction enzymes, and found only three enzymes (out of 33 tested) that cut the ROPV genome once. The entire ROPV genome was subsequently cloned at the SpeI site after insertion of a SpeI linker into the multiple cloning site of plasmid pUC19. We also decided initially to clone a subfragment of the ROPV genome (a 3.8 kb EcoRV fragment) in order to make a DNA probe for in situ hybridization studies.

**Detection of ROPV by immunocytochemistry, in situ hybridization and PCR**

ROPV was detected in the tongue and xenograft lesions by immunocytochemical staining with antibody reactive to the papillomavirus GSA, and by in situ hybridization using a biotinylated ROPV subgenomic probe as described in Methods. Experimentally infected tongue lesions showed typical ROPV histology (Fig. 1a, b), positive staining with the GSA antibody (Fig. 1c) and were positive by in situ hybridization using the ROPV DNA probe (Fig. 1d). ROPV-induced tongue lesions screened with a CRPV DNA probe were negative (Fig. 1e), and the ROPV DNA probe did not label a CRPV-infected xenograft that was positive for CRPV DNA using the CRPV DNA probe (data not shown). ROPV-infected xenografts derived from tongue skin chips (Fig. 1f) were also strongly positive for ROPV capsid antigen following immunocytochemical staining with antibody to the papillomavirus GSA (data not shown) and positive by in situ hybridization using the ROPV DNA probe (Fig. 1g).

ROPV-specific DNA was indirectly confirmed by PCR using a panel of primers designed from published partial ROPV DNA sequence information (O'Banion et al., 1988). The primers were designed to amplify five ROPV DNA fragments of the following expected sizes: 2102, 1519, 1031, 770 and 498 bp. The results (Fig. 2) demonstrated that bands of the predicted sizes were obtained, and no amplification of CRPV DNA was observed using the ROPV primers (one set shown).
Fig. 1. Immunocytochemical and in situ hybridization analysis of ROPV-infected rabbit tongue (a–e), and ROPV-infected xenograft-derived cyst (f, g). Haematoxylin and eosin staining of sections of ROPV-infected tongue and xenograft (a, b, f); GSA staining of a section of ROPV-infected tongue (c); in situ hybridization using a subgenomic ROPV DNA probe (d, g); in situ hybridization using a CRPV DNA probe (e). Magnification × 250 (a, c–g) and × 800 (b).
whether this small area of positively stained nuclei contained amplification. Lane 1, 1 kb markers; lanes 2–6, ROPV DNA template; lane 43 (lane 2); 41 and 44 (lanes 3 and 8); 45 and 42 (lane 4); 41 and 43 (lane 5); 41 and 42 (lane 6); 45 and 43 (lane 7).

Fig. 2. Ethidium-agarose gel electrophoresis of PCR products using various ROPV-specific primers, purified ROPV DNA and 30 rounds of PCR amplification. Lane 1, 1 kb markers; lanes 2–6, ROPV DNA template; lane 7, no DNA; lane 8, CRPV DNA template. PCR primers used were: 45 and 43 (lane 2); 41 and 44 (lanes 3 and 8); 45 and 42 (lane 4); 41 and 43 (lane 5); 41 and 42 (lane 6); 45 and 43 (lane 7).

showed any ROPV infection, and one small area of one xenograft derived from genital tissue contained ROPV DNA. Whether this small area of positively stained nuclei contained infectious ROPV was unclear based on immunocytochemical staining with anti-GSA antisera. These results, however, suggest that ROPV may have a limited potential for infection of genital tissue, and the above studies indicate that further investigation is required. Early reports demonstrated that ROPV infection of immune competent rabbits was confined to investigations of genital tissue, and the above studies indicate that further studies are required. Earlier reports demonstrating that ROPV infection of immune competent rabbits was confined to the oral cavity, and attempts to generate papillomas on genital tissue, and the above studies indicate that further studies are required.

In summary, infectious stocks of ROPV were prepared using the athymic mouse xenograft system. The production of infectious virus was confirmed by successful re-infection of the tongues of non-immune rabbits with xenograft-derived virus extract, and high-titre stocks of ROPV were established. ROPV together with CRPV may be used to determine some aspects of tissue susceptibility and host immunity to different PVs in individual rabbits using a small laboratory animal model system.

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References


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