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High efficiency, long-term clinical expression of cottontail rabbit papillomavirus (CRPV) DNA in rabbit skin following particle-mediated DNA transfer

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ABSTRACT

The ability of skin to support long lasting expression of genes delivered with a particle-mediated system was evaluated in rabbits inoculated with cottontail rabbit papillomavirus (CRPV) DNA. The optimal delivery force for maximal gene expression in rabbit skin was determined in transient β-galactosidase assays. Forty-five sites in four rabbits were then inoculated at 350–400 p.s.i. with CRPV DNA. All sites (100%) formed papillomas with multiple papillomas at most sites. These results support the feasibility of using a particle-mediated delivery system for gene therapy and suggest that some papillomavirus features, such an origin of replication, may be well suited for use in vectors to target long term expression to skin.

Skin is considered an ideal target for gene therapy (1). The ability of particle-mediated gene transfer *in vivo* to induce short term expression in skin has been demonstrated (1,2). Documentation of long lasting expression has been more difficult (3). In the cottontail rabbit papillomavirus (CRPV) DNA-rabbit system, the formation of cutaneous papillomas provides a phenotype to document accurately the number of sites that retain and express a DNA inoculum (4). Domestic rabbits do not replicate infectious CRPV virus. Papillomas persist because the daughters of infected basal cells—that proliferate and replenish the epidermis—continue to express viral genes. Papillomavirus genomes contain an origin of DNA replication and are maintained in keratinocytes as extrachromosomal episomes (5).

In previous studies, we induced rabbit papillomas by inoculating skin with CRPV DNA in an aqueous solution, under hydraulic pressure, through an intradermal nozzle of a jet injector (6). Jet injectors were designed for delivering vaccines into human skin. Jet injection of CRPV DNA was a significant advance over the older methods of scarification and intradermal inoculation and puncture because it allowed rabbits to be inoculated rapidly at ≥100 sites each. We used the method to study the papilloma-inducing activity of several CRPV mutant genomes, including some with only one fourth the activity of wild-type CRPV DNA (6–9). The activity of mutants with much lower activity might be missed in this assay, however, because the rates of papilloma formation in individual rabbits vary widely: from ≤20 to ≥80% of wild-type CRPV DNA-injected sites. The genetic heterogeneity of rabbits probably contributes to the observed variability, but so does the injection device. Furthermore, jet injection requires substantial amounts of purified DNA, e.g. 50 µg per site.

Particle-mediated methods of DNA delivery *in vivo* are simple. Small quantities of DNA are precipitated onto inert particles of gold and accelerated to high velocity, for example, by the instantaneous release of high pressure helium, resulting in penetration of target tissues. This approach has been used to express individual genes in skin (2), and we also used it to show that human papillomavirus type 16 (HPV 16) genomes could induce papillomas in human foreskin xenografts on severe combined immunodeficient (*scid*) mice (10). The current study demonstrates that particle-mediated delivery of CRPV DNA can induce persistent papillomas in rabbit skin with very high frequency. This will facilitate the study of CRPV genetics *in vivo* and also has implications for the use of papillomavirus-based vectors for gene therapy of skin.

Two plasmids were used. pCMV-β (Clonetech, Palo Alto, CA) expressed the β-galactosidase (β-gal) gene from the cytomegalovirus (CMV) promoter. CRPV-pLAII contained a full-length CRPV genome in plasmid pLAII. Supercoiled plasmids were purified (6) and precipitated (12 and Dennis McCabe, personal communication) onto gold particles (average diameter 1–3 µm), at a ratio of 1 µg DNA:0.5 mg gold, in 0.1 M spermidine and 2.5 M $CaCl_2$ during a 10 min incubation at 20°C. The DNA-coated gold particles were pelleted at 12 000 r.p.m. for 30 s, washed three times with 100% ethanol, and resuspended at 2 µg DNA/mg gold/ml ethanol. The DNA–gold–ethanol suspension was introduced into a 22″ section of Tefzel tubing (1/8″ outside diameter, 3/32″ internal diameter) (McMaster–Carr, Elmhurst, IL). Particles were allowed to settle onto the bottom of the tubing and the ethanol was then evacuated using a peristaltic pump. The tubing was rotated at 20 r.p.m. for 30 s in a device (Agracetus, Inc., Middletown, WI) designed to distribute the gold evenly over the inner walls of the tubing. Rotation was continued as the DNA–gold was dried under a continuous stream of nitrogen gas delivered at 250 ml/min. The tubing was sliced into 1/2 ″ lengths

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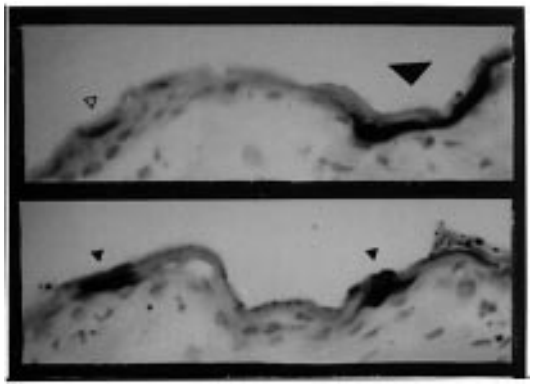

Figure 1. Transient β-galactosidase expression in rabbit skin. Skin sites were inoculated with pCMV-β and collected 24 h later. Frozen sections were stained with X-gal and hematoxylin and eosin. The number of β-gal expressing cells in representative sections were estimated as one (open arrowhead, top panel), eight (large arrowhead, top panel) and two (small closed arrowheads, bottom panel). (10× magnification.)

to generate 'shots' containing 1 µg DNA/0.5 mg gold. The shots were loaded into a 12-shot barrels of a helium-driven Accell™ Gene Delivery Device (Agracetus, Inc., Middleton, WI) (7).

Two kilogram, *Pasteurella*-free female New Zealand white rabbits were anesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg). Hair was clipped from a 100–200 cm^2 area on the rabbit backs, and residual hair and superficial keratin were treated with a commercial depilatory (Nair, Division of Carter-Wallace, Inc., New York, NY 10105). The Accell™ device was held against the skin, and up to 15 sites per rabbit were inoculated.

Transient assays of pCMV-β gal expression were used to evaluate the effectiveness of delivery pressures from 300 to 700 p.s.i. A minimum of two skin sites per rabbit in at least two experiments were evaluated for each pressure. Sites were collected 24 h after inoculation and frozen in cryoembedding medium. Transverse frozen sections (5 or 10 µm thick, ~15 mm long) were fixed in acetone–methanol (1:1) for 10 min at room temperature and stained in standard X-gal buffer (44 mM HEPES–Na salt, 150 mM NaCl, 1.3 mM $MgCl_2$, 3 mM K ferrocyanide, 3 mM ferricyanide, pH 7.4) containing 0.5 mg/ml X-gal at 37°C overnight. Sections were counterstained in hematoxylin and eosin and examined by light microscopy. Sites that had been inoculated with pCMV-β and previously shown to express β-gal served as positive controls. Sites that had been inoculated with gold particles and no DNA or not bombarded served as negative controls. Positivity was quantified as the number of X-gal-expressing cells per field, examined with a 10× objective lens, as illustrated in Figure 1.

Penetration of the gold particles was directly proportional to the inoculation force. Sites inoculated at the lowest pressure (300 p.s.i.) contained the fewest gold particles, located mostly at the *stratum corneum*. At mid-range (350–450 p.s.i.), most particles were within the epidermis. At the highest pressures (≥500 p.s.i.), many particles had penetrated the basement membrane and lodged in the dermis. β-gal-expressing cells were restricted to the epidermis regardless of the inoculation pressure and presumably included keratinocytes, Langerhans's cells and other dendritic cells (Fig. 1). Positive cells generally expressed high levels of β-gal, as determined by visual intensity, and were

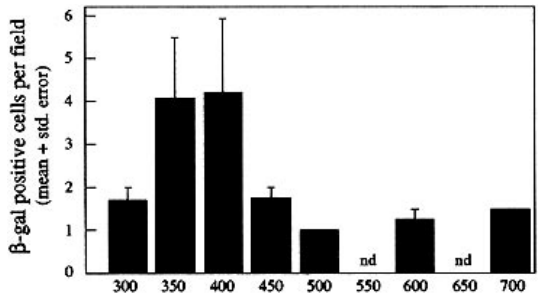

Figure 2. β-galactosidase expression in rabbit skin as a function of delivery force.

frequently found in clusters (Fig. 1). All sites contained some β-gal-positive cells, but their number varied directly with inoculation pressure. β-gal-expression was induced in twice as many cells at sites inoculated at 350 or 400 p.s.i. compared with sites inoculated at 300 or >400 p.s.i. (Fig. 2).

Long-term assays of CRPV gene expression were performed at 350 and 400 p.s.i. Four rabbits were inoculated at each pressure at five or 10 sites each with CRPV-pLAII. CRPV DNA-inoculated sites were inspected weekly for 8 weeks, and the number of papillomas at individual inoculation sites was recorded. There was no difference in the rates of papilloma formation between sites inoculated at 350 and 400 p.s.i. Forty-five of 45 sites (100%) inoculated at either pressure in four of four rabbits formed papillomas, with an average of 3.3 per site (149 papillomas/45 sites) (Table 1 and Fig. 3). Histologic evaluation of lesions collected at euthanasia revealed the typical features of viral papillomas, including hyperplasia, acanthosis, parakeratosis and koilocytosis (data not shown). DNAs were extracted from papillomas and amplified by polymerase chain reaction (PCR) (7) using CRPV primers CR986C (5′-GCT ATC CTG TGC GCA GGG C-3′) and CR1440N (5′-GGT TGT CAC AGT CTA AAC AGT CC-3′) that flank a 455 bp region of the CRPV E7-E1 genes. The expected product was amplified from each papilloma, verifying that the inoculum had been maintained (data not shown).

Table 1. Papilloma formation in rabbits inoculated with CRPV DNA

Expt	Rabbit	Rate[a]		Number[b]	
		350 p.s.i.[c]	400 p.s.i.	350 p.s.i.	400 p.s.i.
1	1	5/5	5/5	29/5	27/5
	2	5/5	5/5	25/5	19/5
2	3	5/5	5/5	14/5	16/5
	4	10/10	5/5	11/10	8/5
Total		25/25	20/20	79/25	70/20
Average		100%	100%	3.2	3.5

[a]No. of papilloma-forming sites per no. of inoculation sites.
[b]No. of papillomas per no. of papilloma-forming sites.
[c]Delivery force applied to the DNA inoculum.

In summary, this study reports that particle-mediated DNA delivery can induce long-lasting expression of CRPV genes in rabbit skin with high efficiency. Since papilloma formation requires, at a minimum, the CRPV E1, E2, E6 and E7 genes

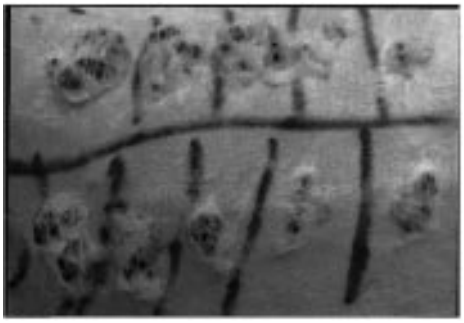

Figure 3. High efficiency CRPV DNA-induced papilloma formation in rabbit skin. CRPV DNA was inoculated at five sites each using a delivery force of 350 p.s.i. (top row) or 400 p.s.i. (bottom row). The sites were marked and the rabbit was photographed 8 weeks after inoculation.

(6–9,13), the results imply that multiple genes were expressed. Furthermore, the persistence of the papillomas and the CRPV inoculum indicates that basal keratinocytes were effectively targeted. Papillomaviruses persist in benign lesions in an episomal state, mediated by the viral origin of replication and functions of the E1 and E2 genes (5). The viral origin and early genes of bovine papillomavirus (BPV) have been used previously to develop an autonomously replicating mammalian expression vector (14,15). Our results suggest that genetic elements of papillomaviruses may also be well suited to the development of vectors for inducing long-lived expression in epidermis. For human gene therapy, the use of low risk human papillomaviruses (HPVs) elements may be appropriate.

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