Efficient Expression of Naked Plasmid DNA in Mucosal Epithelium: Prospective for the Treatment of Skin Lesions

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Mucocutaneous gene therapy offers exciting new treatment modalities for skin lesions. Transient expression of naked plasmid DNA could be used as a local treatment of various skin lesions where the corresponding gene product (protein) has therapeutic or immunization potential. We analyzed the time course, magnitude, and histologic expression of the indicator plasmid DNA (pCMV:β-Gal) in mucosal epithelium and papilloma lesions. Upon direct injection of naked plasmid DNA (20 μg) into oral mucosa, expression occurred at high local concentrations, up to 35-fold higher than in comparable injections into the epidermis. Due to the accelerated turnover of mucosal epithelium β-galactosidase positive epithelial cells were detected in the basal and suprabasal layers as early as 3 h after injection, whereas only the most superficial mucosal layers demonstrated β-galactosidase staining at 24 h post-injection. These biologic characteristics need to be taken into consideration when clinical applications of expressing naked plasmid DNA in epithelial tissues are considered. ***Key words: gene therapy/mucosa/wart.***
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Gene therapy offers exciting new treatment modalities for skin lesions. The genetic manipulation of epidermis with the expression of biologic response modifiers represents a novel therapeutic approach to treating skin lesions (Vogel, 1993; Hengge, 1997). Recently, we have demonstrated the ability of epidermal keratinocytes to take up and transiently express naked (uncoated) plasmid DNA upon direct intradermal injection, producing high local levels of the corresponding biologically active protein (Hengge *et al*, 1995, 1996). This novel technique could be used as a local treatment of various skin lesions where the transient production of the appropriate gene product (protein) has therapeutic potential, and for genetic immunizations. These entities include Kaposi's sarcoma, basal cell and squamous cell carcinoma, and chronic wounds, as well as papilloma (zur Hausen *et al*, 1991; Phelps, 1995; Stingl *et al*, 1996; Yang and Huang, 1996). Expression of immunomodulators such as interferon-α, granulocyte-macrophage colony-stimulating factor genes, or costimulatory molecules (e.g., B7.1) at high local levels in the actual tissue might be advantageous over current treatments (Stingl *et al*, 1996; Yang and Huang, 1996). For example, the treatment of HPV-induced papilloma or condyloma with expression of the therapeutic interferon-α gene for extended periods of time may be superior to the therapy with recombinant protein, which is rapidly degraded upon intralesional injection.

In this study, we show the expression of the indicator plasmid DNA (pCMV:β-Gal) in mucosal epithelium and papilloma lesions at high local concentrations upon direct injection. Due to the accelerated turnover of mucosal epithelium, time course and quantitation experiments were performed in preparation for the design of a study evaluating the therapeutic effects of canine interferon-α plasmid DNA in the beagle model of oral papillomatosis.

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Abbreviation: β-Gal, β-galactosidase.

MATERIALS AND METHODS

Plasmid DNA injection Plasmid DNA (pCMV:β-Gal, Clontech, Palo Alto, CA, containing the β-galactosidase (β-Gal) indicator gene or pcDNA3 vector control DNA) was injected into the buccal mucosa of 2–4 mo old beagles or minature swine, which were anesthetized with ketamine, xylazine, butorphan, and atropine (1:1:1:1). The plasmid DNA was analyzed for β-Gal enzymatic activity to exclude pseudo-transfection prior to use (Alexander *et al*, 1997). It was diluted in phosphate-buffered saline to the concentrations indicated. Injection was performed into the lamina propria and the papilloma lesion using a tuberculin syringe and a 30 g needle. The total injected volume was 50 μl.

All plasmid DNA preps were subjected to double cesium chloride purification and endotoxin levels were monitored using the limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD), which detects endotoxins chromogenically (Hurley, 1994; Duner, 1995). When necessary, contaminating endotoxins were removed from the plasmid DNA using the triton X-114 detergent phase separation technique (Aida and Pabst, 1990; Cotten *et al*, 1994). Endotoxin levels were typically less than 0.005 ng per μg plasmid DNA.

Detection of β-Gal A histochemical staining method was used to detect β-Gal activity in mucosal specimens as previously described (Hengge *et al*, 1995). Briefly, specimens were fixed with paraformaldehyde, washed in phosphate-buffered saline, and stained with Bluo-Gal staining solution (1 mg per ml in N-N-dimethylformamide) at room temperature for 4–16 h.

Quantitative assay for β-Gal activity A chemiluminescence assay was used to quantitate the β-Gal activity in oral mucosa and epidermis (Jain and Magrath, 1991). Briefly, epithelial, and where appropriate epidermal sheets from 8 mm biopsies were obtained at various times after injection of 20 μg pCMV:β-Gal into canine mucosa using dispase (10 U per ml for 1 h at 37°C, Boehringer, Indianapolis, IA) and lyzed in lysis buffer (0.1 M potassium phosphate pH 7.8, 0.2% triton X-100, and 1 mM 1.4-Dithio-DL-threit; Sigma, St. Louis, MO) using a mortar and pestle. Following centrifugation, a 2 μl aliquot of the cell extract was mixed with reaction buffer (0.1 M potassium phosphate pH 8.0, 1 mM magnesium chloride, and 0.035 mM Galacton chemiluminescent substrate, Tropix, Bedford, MA). After a 1 h incubation at room temperature, 10% Emerald luminescence amplifier in 0.2N NaOH (Tropix, Bedford, MA)

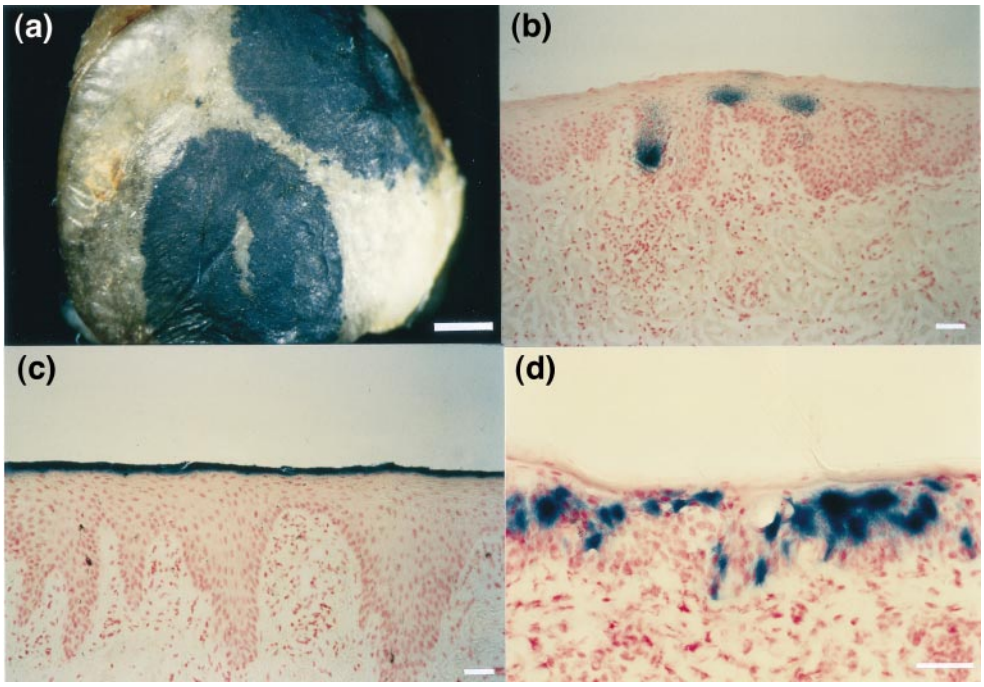

Figure 1. Mucosal epitheliae cells expressing β-Gal move upward with time. (*a*) Gross section of mucosal epithelia (8 mm punch biopsy) 12 h after injection of 20 μg of β-Gal plasmid DNA to two different sites (*scale bar*: 1 mm); (*b*) histologic section of an injected and stained biopsy 3 h after injection of β-Gal plasmid DNA shows blue staining (β-Gal protein) in basal and suprabasal layers of the mucosa with absent staining in the spinous and granular layers (*scale bar*: 100 μm); (*c*) histologic section of injected mucosa 24 h after β-Gal plasmid DNA injection demonstrating β-Gal staining only in the most superficial mucosal layers (*scale bar*: 50 μm); (*d*) histologic section of porcine epidermis 24 h after injection showing abundant β-Gal protein in keratinocytes in all epidermal layers (*scale bar*: 50 μm).

was added immediately before measurement of the chemiluminescence in a chemiluminometer (Monolight 1005, Analytical Luminescence, San Diego, CA). The β-Gal specific activity in epithelial extracts was calculated as the ratio of light units divided by the protein content (Biorad, Hercules, CA). Background β-Gal specific activity in epithelial extracts was determined by injecting non-β-Gal expressing plasmids (e.g., vector control DNA) with the background activity being subtracted from positive samples. Assays were performed in duplicates and compared with a standard curve obtained by spiking β-Gal-negative cell extracts with known amounts of purified β-Gal (Sigma).

RESULTS

Buccal mucosa expresses β-Gal gene To evaluate the feasibility of gene therapy in mucosa we determined whether canine buccal mucosa is capable of taking up and expressing plasmid DNA and then characterized that expression in terms of histologic localization, time course, and level of expression. Therefore, 20 μg of β-Gal plasmid DNA were injected superficially into the buccal mucosa of beagles. β-Gal was chosen because it can be easily detected in macroscopic and histologic specimens. Buccal mucosa was found to take up and express naked plasmid DNA as shown grossly by the intense blue staining of an 8 mm punch biopsy 12 h after injection (**Fig 1*a***). Histologically, the level of mucosal keratinocytes expressing β-Gal protein was analyzed at 3, 6, 12, and 24 h after injection of 20 μg of β-Gal plasmid DNA. β-Gal protein could be detected as early as 3 h after injection of β-Gal plasmid DNA in basal and suprabasal layers of the mucosa with absent staining in the spinous and granular layers (**Fig 1*b***). Keratinocytes expressing β-Gal plasmid DNA were found in the spinous and granular layers at 6 and 12 h after injection, whereas β-Gal protein was not detected in the basal layers at this and later time points (data not shown). Twenty-four hours after injection, only the most superficial mucosal layers demonstrated β-Gal staining (**Fig 1*c***) being consistent with the rapid turnover of mucosal epithelial cells (Cutright and Bauer, 1967). For comparison, a histologic section of porcine epidermis is depicted 24 h after injection of β-Gal plasmid DNA showing abundant β-Gal protein in keratinocytes in all epidermal layers (**Fig 1*d***). Synthesized β-Gal protein seems to be carried along within the cells as they migrate upwards and eventually get shed from the mucosal surface. Uptake and expression of plasmid DNA at later time points (e.g., 12 h after injection) did not occur because there was no β-Gal staining in any but the most superficial layers at these later time points. This might be due to complete uptake of plasmid DNA following injection or to the instability/degradation of injected plasmid DNA at the site of injection.

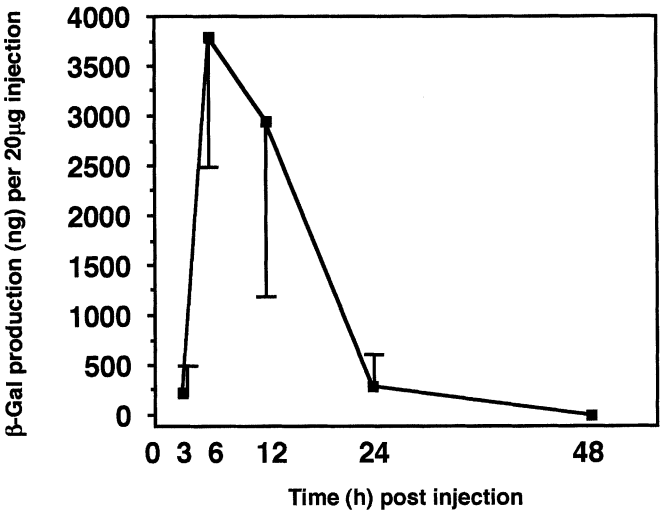

Figure 2. Quantitative time course of β-Gal expression in dog mucosa. The amounts of β-Gal protein were measured over time in 8 mm epithelial sheets using a chemiluminescent assay. Six to eight independent samples were analyzed per time point. *Error bars*: means ± SD.

Time course of β-Gal expression in mucosa Quantitatively, the amounts of β-Gal protein in 8 mm epithelial sheets were measured over time using a chemiluminescence assay. Consistent with the gross impression, maximal amounts of β-Gal (3750 ng per 8 mm epithelial surface) were detected 6 h after the injection of β-Gal with baseline levels detectable at 48 h after injection (**Fig 2**).

Amounts of β-Gal protein were compared between trunk skin (pig) and mucosa (dog) at 12 h after injection of 20 μg of β-Gal plasmid DNA. Whereas 8 mm epidermal sheets (n = 10) from (porcine) trunk skin produced ≈100 ng of β-Gal, the same amount of plasmid DNA led to the synthesis of 3650 ng of β-Gal protein in a 8 mm epithelial sheet from (canine) buccal mucosa. When this experiment was performed with β-Gal plasmid injections into porcine mucosa, comparable high levels of gene product have been obtained (data not shown). Thus, on a quantitative level it seems that mucosa is able to produce about 30–35-fold more protein following the injection of β-Gal plasmid DNA. A potential explanation lies in the different

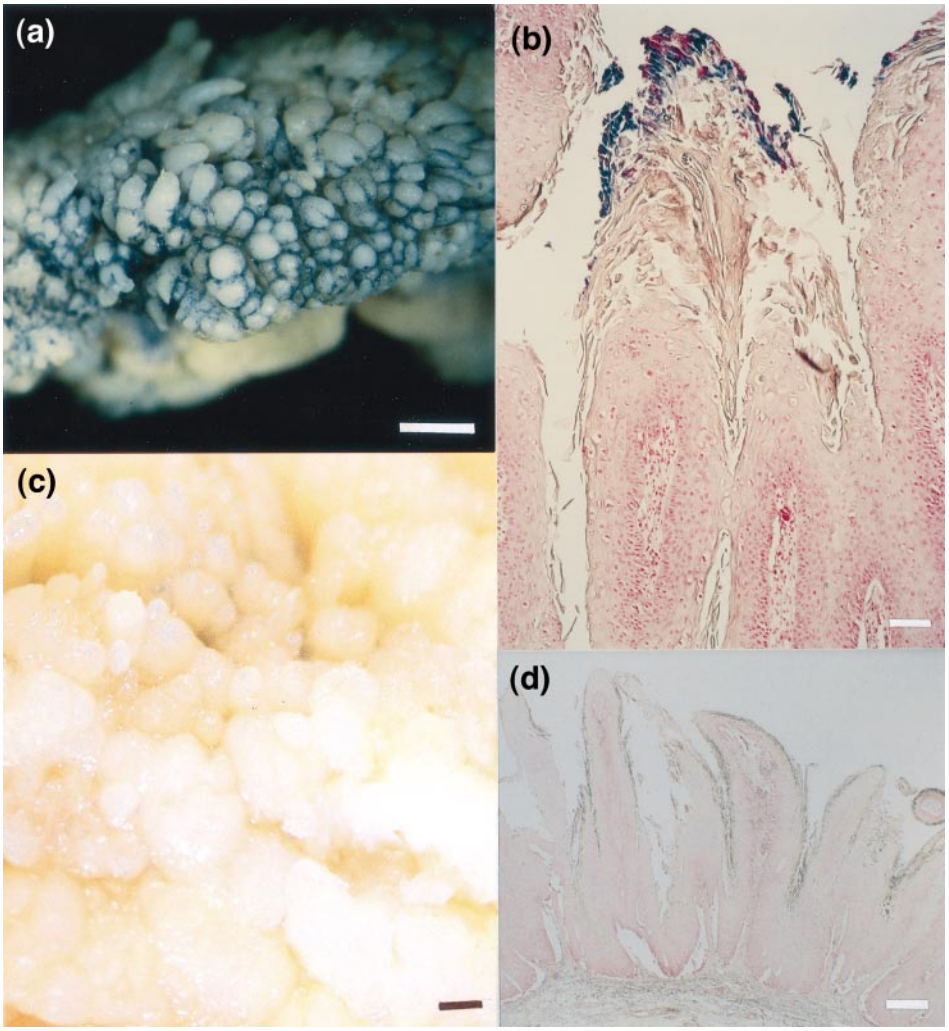

Figure 3. Oral papillomas take up and express intralesionally injected β-Gal indicator plasmid DNA. Two to four centimeter papilloma lesions on the gums of 2–4 mo old beagles were injected with 20 μg β-Gal plasmid DNA. (*a*) β-Gal activity could be detected grossly 12 h after injection (*scale bar*: 1 mm); (*b*) on histologic examination, β-Gal protein was detected 12 h after injection in the most superficial layers, consistent with the increased turnover in a hyperproliferative epithelial lesion (*scale bar*: 50 μm); (*c*) gross impression of pcDNA3 control DNA-injected papilloma upon analysis for β-Gal activity (*scale bar*: 1 mm); (*d*) histologic evaluation did not reveal any blue staining (β-Gal activity) (*scale bar*: 100 μm).

morphology of buccal mucosa, which has a higher number of keratinocyte layers (10–15) than epidermis, accounting for about 5–8 layers. It is important to remember, however, that β-Gal protein expression in mucosa has a different time course. Because the turnover for buccal mucosa is extremely rapid (about 2 d), protein produced following the uptake of naked plasmid DNA can only be detected for about 24–48 h, in contrast to epidermally produced protein that was detectable over a 3 wk period (Hengge *et al*, 1995).

Canine papilloma express β-Gal Because buccal mucosa did express the β-Gal marker gene upon injection of β-Gal plasmid DNA, we wanted to determine whether papilloma lesions also take up and express naked plasmid DNA. This characteristic would provide the basis for attempts to treat hyperproliferative epithelial tumors with plasmid DNA injections encoding cytokines or biologic response modifiers. To determine this, we injected 20 μg of β-Gal plasmid DNA into 2–4 cm large oral papillomas on the gums of 2–4 mo old beagles. β-Gal activity could be detected 12 h after injection (**Fig 3*a***). On histologic examination, β-Gal activity was detected in the most superficial layers, consistent with the increased turnover in a hyperproliferative epithelial lesion (**Fig 3*b***). At 48 h no β-Gal activity could be detected (data not shown). For comparison, pcDNA3 control DNA has been injected and specimens analyzed for β-Gal activity. No blue staining was seen on macroscopic and histologic examination (**Fig 3*c*–*d***).

DISCUSSION

The ability of mucosal epithelium to take up and express naked plasmid DNA at high local concentrations makes it an attractive target for skin gene therapy. Unlike epidermis, mucosal epithelia have an accelerated transit time from basal to superficial layers (2–4 d, Cutright and Bauer, 1967), resulting in the early loss of epithelial cells expressing the introduced DNA as evidenced by the β-Gal gene. Consequently, mucosa expressed introduced plasmid DNA at all epithelial layers only very early (3 h) after injection, in contrast to epidermis where keratinocytes in all layers expressed the introduced gene 24 h after injection (Hengge *et al*, 1995). Histologically, β-Gal protein could only be detected within 48 h after injection, by then the epithelial cells expressing β-Gal were lost from the mucosal surface. Accordingly, oral papilloma lesions were shown to take up and express the β-Gal indicator plasmid with similar time characteristics as mucosa. In order to detect the produced protein, the following processes need to be accomplished: transport of DNA from the lamina propria/submucosa to the basement membrane zone, crossing of the basement membrane, uptake of DNA by epithelial cells, transport to the nucleus, transcription, and translation. Therefore, the process of uptake and expression in epithelial cells seems very rapid and efficient. Given the thickness of epidermis (5–8 layers) and mucosal epithelium (10–15 layers) and the fact that a constant amount of plasmid DNA (20 μg) was injected, we suspect that uptake of plasmid DNA per surface area is substantially higher in mucosal epithelia than in epidermis. In this regard, projects are ongoing in order to identify DNA binding proteins in epidermal and mucosal tissues. On a quantitative level, the efficiency of protein production in mucosal epithelial cells was found to be significantly (about 30–35-fold) higher in the first 12 h after injection than in epidermal keratinocytes. β-Gal protein could not be detected in epithelial cells later than 48 h after injection due to the loss of the very cells that have expressed the introduced gene at earlier time points. Taken together, the quantitative time course of naked plasmid

DNA expressed in mucosa correlated very well with the histologic time course. These biologic characteristics need to be taken into consideration when clinical applications of expressing naked plasmid DNA in epithelial tissues are considered. In particular, the abundance of protein products for extended periods of time cannot be achieved using mucosal tissues; however, the transient expression of certain biologically relevant proteins at high local quantities may be sufficient for treatment purposes or the elicitation of an immune response (DNA vaccination).

This study lays the groundwork for gene therapy approaches using expression of naked plasmid DNA in epithelium for the treatment of skin and lesions. Because standard therapies for a variety of skin diseases such as the treatment of HPV-associated lesions are unsatisfactory, the injection of naked plasmid DNA encoding biologic response modifiers like interferon-α might offer a valuable alternative. It remains to be shown whether plasmid DNA technology is as effective or even superior to the treatment with recombinant protein.

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