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Mouse salivary glands and human β -defensin-2 as a study model for antimicrobial gene therapy: technical considerations*

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Abstract

Transduction of salivary glands with antimicrobial peptide genes has great potential for oral infection control. Our ultimate goal is to introduce antimicrobial peptide genes into salivary glands that secrete these peptides into saliva to control bacterial/fungal infection in the oral cavity. However, an animal study model to test this potential has not been established. Therefore, we determined to test (i) whether the potent antimicrobial peptide human β -defensin-2 (hBD-2) can be overexpressed in saliva after transduction of salivary glands and (ii) whether oral fungal infection can be developed in a NOD/SCID murine model. Lentiviral vector SIN18cPPTRhMLV bearing hBD-2 cDNA was introduced into SCID mouse submandibular glands via cannulation. Reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry or enzyme-linked immunosorbent assay (ELISA) were performed to detect hBD-2 expression in glands or in saliva. *Candida albicans* 613p was inoculated orally into SCID mice to establish oral candidiasis. Whilst expression of hBD-2 was detected in mouse salivary glands by RT-PCR and immunohistochemistry 1 day or 1 week following delivery of lentivirus, hBD-2 was not detected in saliva. There was recoverable *C. albicans* from the oral cavity and gastrointestinal tract 4 days to 4 weeks after infection, but there was no establishment of observable oral candidiasis in SCID mice under a stereomicroscope. Our data indicate that lentiviral vectors transduce mouse salivary glands, but not at a sufficient level to allow hBD-2 detection in saliva. Other vectors for gene transduction and additional treatment of SCID mice to establish oral candidiasis are needed in order to utilise mouse salivary glands to test antimicrobial gene therapy.

Keywords

hBD-2; Lentiviral vectors; Mouse salivary glands; *Candida albicans*; SCID mice

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1. Introduction

Since the discovery of natural antimicrobial peptides/proteins, researchers have tried not just to understand how these peptides/proteins work and applied them in topical use for infection control, but have also explored the possibility of linking their use to gene-based therapeutics, i.e. transducing cells with these peptide genes to augment the innate resistance of tissues/organs against infection [1–5]. Tracheal antimicrobial peptide (TAP), a member of the β -defensin family of antibiotic peptides found in the tracheal mucosa of cows, was used to generate transgenic animals to produce TAP in milk that is antimicrobial [3]. Among a long list of naturally occurring antimicrobial peptides, some well characterised ones are encoded by single genes and their products do not need post-translational processing, making them good candidates for antimicrobial gene therapy. Human β -defensins (hBDs or DEFB) have drawn considerable attention for this purpose as they have potent antimicrobial abilities and some also exert inhibitory effects on viral infections [6–13]. Although only four hBDs (hBD-1–4) have been well characterised, up to 28 hBDs may exist based on genomic surveys using a computational search strategy [14,15]. Fourteen hBD or DEFB gene transcripts (DEFB-1, -4 and -103–114) were investigated using reverse transcription polymerase chain reaction (RT-PCR) to detect their expression in gingival keratinocytes [16]. Transduction of epithelial cells in skin or oral mucosa with hBDs has been tested for its potential to enhance infection control [8,12,17]. Primary fibroblasts have also been proposed as target cells to express hBDs, but the amount of antimicrobial peptide secreted may be insufficient to exert an effect [17,18].

Salivary glands have been considered an excellent target organ for gene-based therapeutics compared with other tissues or organs (e.g. liver and skeletal muscle) both for systemic and upper gastrointestinal tract gene therapeutic applications. Salivary glands are well encapsulated, which limits undesired extraglandular vector dissemination. In addition to being an exocrine secretion organ, physiological existence of endocrine secretory pathways in these tissues has been reported [19–21]. In 1996, O'Connell et al. [2] first utilised salivary glands as a target organ for antimicrobial gene-based therapy to control oral infection. The gene encoding the anticandidal protein histatin 3 was transferred to rat salivary glands using an adenovirus-directed approach, and up to 1 mg/mL of histatin exerting a strong candidal effect was detected in saliva. However, the study did not test whether the secreted histatin 3 in saliva controls oral fungal infection in vivo.

hBDs are expressed in salivary glands and are detected in saliva [22–25]. They are presumably among a plethora of defence mechanisms in the oral cavity to combat infections. hBD-2 is a potent antimicrobial peptide effective against a broad spectrum of bacteria, including Gram-positive bacteria, Gram-negative bacteria and fungi. Among these microbes, *Streptococcus mutans* and *Lactobacillus acidophilus*, which are associated with dental caries, and certain strains of periodontal bacteria such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* have been shown to be sensitive to hBD-2 [18,26]. hBD2 is chemotactic for T-cells, immature dendritic cells, mast cells and tumour necrosis factor- α (TNF- α)-primed neutrophils [10,27,28]. Its expression in saliva may enhance not only the innate but also adaptive immunity of the oral mucosa. Together with its ability to inhibit human immunodeficiency virus (HIV) infectibility [11,13], these characteristics make hBD-2 an ideal candidate for a study model of antimicrobial gene therapy to control oral infection.

Transduction of salivary glands with antimicrobial peptide genes has great potential for oral infection control. Our ultimate goal is to introduce antimicrobial peptide genes into salivary glands, which secrete these peptides into saliva to control bacterial/fungal/viral infection in the oral cavity. However, an animal study model to test this potential has not yet been

established. In the present study, our objective was to address two questions before establishing this model: (i) whether the potent hBD-2 can be expressed in saliva after transduction of salivary glands using lentiviral vectors; and (ii) whether oral fungal infection can be developed in a NOD/SCID murine model. Our findings provide an insightful understanding on the technical issues and concerns for future investigation regarding salivary gland antimicrobial gene therapy for oral infection control.

2. Materials and methods

2.1. In vitro antimicrobial assays

The antimicrobial activity of recombinant (r)hBD-2 was assayed as previously described [6,18]. The following microorganisms were used for the assays: *Candida albicans* 613p (from Dr C. Haidaris, University of Rochester, Rochester, NY) [29]; *P. gingivalis* ATCC 33277 (from Dr S.K. Haake, UCLA, Los Angeles, CA); and *Escherichia coli* ML-35p (from Dr T. Ganz, UCLA) [6]. *Candida albicans* 613p was grown in yeast extract–peptone–dextrose (YEPD) broth, *P. gingivalis* was grown in mycoplasma broth base (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.5 µg/mL hemin SS (Sigma, St Louis, MO) and 0.5 µg/mL menadione (Sigma) and *E. coli* ML-35p was grown in Lauria–Bertani broth. Microorganisms were cultured at 37 °C in aerobic conditions (except *P. gingivalis*, which used anaerobic conditions), grown to exponential phase, washed with phosphate-buffered saline (PBS) twice and re-suspended in low or high salt buffer (10 mM Na phosphate, pH 7.4 or 100 mM Na phosphate, pH 7.4, respectively, each containing 0.03% trypticase soy broth) or in filter-sterilised (0.2 µm) human saliva from healthy donors. The input concentration was adjusted to 2×10^6 cells/mL and the suspension was incubated with high-performance liquid chromatography (HPLC)-purified rhBD-2 (from Dr T. Ganz) with final hBD-2 concentrations of 0, 0.1, 1 and 10 µM. Mixtures of microbes and hBD-2 were then incubated under aerobic or anaerobic (*P. gingivalis*) conditions for 1 h at 37 °C. Subsequently, the mixtures were serially diluted and plated onto appropriate agar plates to quantitate surviving microbes for colony-forming unit (CFU) analysis.

2.2. Degradation of hBD-2 in saliva

Whole saliva from healthy mouse or human donors was collected. Mouse saliva was collected by administering 0.5 mg/kg pilocarpine via subcutaneous injection to induce saliva secretion. Saliva was filtered (0.2 µm) and stored at –80 °C until use. Forty-five microlitres of saliva was mixed with 5 µL of hBD-2 (16 ng/mL) and incubated at 37 °C for 0, 10, 30 and 60 min. At each time point, samples were snap frozen in liquid N₂ until enzyme-linked immunosorbent assay (ELISA).

2.3. Cell cultures

HT-1080 (human fibrosarcoma cell line, ATCC CCL-121) was grown in Alpha-Minimum Essential Medium (Life Technologies/GIBCO BRL, Gaithersburg, MD) with 10% fetal bovine serum supplemented with 100 units/mL penicillin G, 100 µg/mL streptomycin and 0.25 µg/mL fungizone (Gemini Bio-Products, Inc., Woodland, CA).

2.4. Construction and production of viral vectors

A lentiviral vector SIN18cPPTRhMLV-E (SIN18-E), the packaging plasmid pCMVR8.2DVPR and the vesicular stomatitis virus G protein expression plasmid (pHCMV-G) were obtained from Dr I.S.Y. Chen, UCLA [30]. The SIN18-E vector containing the enhanced green fluorescent protein (EGFP) reporter cDNA was released and inserted with hBD-2 cDNA yielding SIN18-hBD-2 as described previously [17].

Packaging cells 293T (2×10^7) were co-transfected with 12.5 μ g of SIN18-hBD-2 or SIN18-E, 12.5 μ g of pCMVR8.2DVPR and 5 μ g of pHCMV-G plasmids using a calcium phosphate precipitation method [31]. The viral supernatant was collected on Days 2 and 3 after transfection, filtered (0.45 μ m) and concentrated ca. 100-fold by ultracentrifugation as described previously [17]. Viral concentration was measured against known concentrations of viral protein p24 and virus titration was performed using 293T cells with various dilutions of the viral stock and analysed for EGFP expression by fluorescence microscopy and flow cytometry on Day 3 post infection. Vector titres were routinely 10^8 infectious units/mL. Expression of hBD-2 was analysed by ELISA as described below.

2.5. ELISA

The amount of secreted hBD-2 was determined by ELISA as described previously [9,17] using monoclonal anti-human hBD-2 antibodies (1:5000 dilution) as anchoring antibodies, polyclonal rabbit anti-human hBD-2 antibodies (1:2000 dilution) as detecting antibodies and horseradish peroxidase (HRPO)-labelled polyclonal goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL) as a second-step antibody. Bound HRPO was visualised with fresh developing buffer containing a substrate of optimal concentrations of *o*-phenylenediamine dihydrochloride (Sigma), 0.01% H_2O_2 and 20 mM sodium citrate pH 4.0. The developing reaction was stopped with the addition of 2.5 M sulphuric acid. Absorbance was determined at 490 nm with a microplate reader (Bio-Tek Instrument, Inc., Laguna Hills, CA) and the concentrations were determined with Delta Soft III software. rhBD-2 produced in baculovirus-transfected insect cells was used as a standard. All antibodies were kindly provided by Dr T. Ganz (UCLA).

2.6. Viral infection of target cells and subcloning

Virus from the packaging cell line 293T was used to infect HT-1080 cells. Cells (5×10^5) were exposed to 400 ng of virus at 37 °C in the presence of 5 μ g/mL polybrene. After overnight incubation, cell cultures were replaced with fresh medium and incubated for 3 days. Secreted hBD-2 was detected by ELISA. To select clones with the highest hBD-2 expression, transduced cells were subsequently diluted to 1 cell/well in 96-well plates for subcloning. Single cell colonies were selected and hBD-2 expression was detected by ELISA. Colonies expressing high levels of hBD-2 were further subcultured for subsequent use.

2.7. In vivo gene transfer into submandibular glands of NOD/SCID mice

Male 6–8-week-old NOD/SCID mice (SCID, NOD.CB17-*Prkdc-scid/J*; Jackson Laboratory, Bar Harbor, ME) were anaesthetised via intraperitoneal injection with a mixture of 80 mg/kg ketamine and 8 mg/kg xylazine and mounted on a custom-made operating board. Mouse jaws were held open with a custom-made device for cannulation under a stereomicroscope. Extended polyethylene tubes (PE-10; Intramedic, Sparks, MD) were inserted into the openings of glandular ducts and 50 μ L of SIN18-hBD-2 or SIN18-E viral vector was administered into each submandibular gland by retrograde ductal instillation. Virus with an average HIV-1 p24 concentration of 12×10^3 ng/mL was used. NOD/SCID mice were euthanised 24 h or 1 week after gene transfer. Submandibular glands were removed from mice at the time of sacrifice and subjected to hBD-2 detection. Mice were maintained in the animal care centre at the UCLA Division of Laboratory Animal Medicine. All procedures involving experimentation and handling of mice described herein followed the protocols approved by the UCLA Animal Research Committee.

2.8. Formation of subcutaneous tumours in NOD/SCID mice using SIN18-hBD-2-transduced HT-1080 cells

Tumours were generated by transcutaneous injection of 10^7 HT-1080 cells transduced with pBabe-hBD-2 as described previously [9,17] or SIN18-hBD-2 in 200 μ L of Hank's balanced salt solution, using a 26 gauge needle, into the back of NOD/SCID mice (Jackson Laboratory). Tumours were grown until they reached ca. 1.5 cm $\times$ 1.5 cm in size. Mice were then euthanised and tumours were resected for subsequent hBD-2 detection by RT-PCR and immunostaining.

2.9. RT-PCR for hBD-2 mRNA detection

Total RNA was extracted from resected submandibular glands or tumours from NOD/SCID mice using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesised using Advantage RT-for-PCR kit (BD Biosciences Clontech, Palo Alto, CA). The appropriate amounts of cDNA and specific primers (final concentration 0.4 μ M each) for hBD-2 in the reaction mixture plus *Taq* DNA polymerase were then used for PCR. Amplification was performed at 95 °C for 1 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 1.5 min and 72 °C for 7 min as the final step. Sequences of the primer set for hBD-2 were 5'-CCAGCCATCAGCCATGAGGGT-3' (sense) and 5'-GGAGCCCTTTCTGAATCCGCA-3' (antisense) with an expected target size of 255 bp. PCR products were analysed by 1% agarose gel electrophoresis with ethidium bromide staining. Human placenta RNA and human glyceraldehyde-3-phosphate dehydrogenase primers provided in the RT kit were used to ensure the accuracy of the RT-PCR procedures and to determine the quality of the sample RNA.

2.10. Immunohistochemistry for hBD-2 in submandibular glands and tumours

Resected submandibular glands and tumours were divided into several fragments and some were snap frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and stored at -80 °C. Cryostatic sections (10 μ m) were fixed in cold acetone and blocked in serum followed by overnight incubation with primary antibodies (polyclonal rabbit anti-human hBD-2 (1:500)) at 4 °C. Sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (Sigma) for 2 h at room temperature, washed, cover-slipped with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope.

2.11. *Candida albicans* infection of the mouse oral cavity

The oral mucosal surfaces of male NOD/SCID mice (8–10 weeks old; Jackson Laboratory) or normal wild-type C57BL/6 mice were swabbed using cotton-tipped applicators soaked with *C. albicans* (10^7 blastoconidia/mL) grown to exponential phase in YEPD broth. Visual observation under a stereomicroscope was performed at different time points to determine the establishment of oral candidiasis (white patches of plaque) on the tongue and cheek, followed by an oral swab with PBS-wetted cotton-tip applicators. The cotton tips were then placed in 5 mL of YEPD broth containing 100 units/mL penicillin G and 100 μ g/mL streptomycin and incubated overnight at 37 °C. Some mice were euthanised and the tongue and intestine were resected. One-half of the resected tissues were homogenised and added to 5 mL of YEPD broth with antibiotics as above for overnight incubation at 37 °C. The other half of the tissues were fixed in 10% formalin for standard haematoxylin and eosin (H&E) staining for histological observation of fungal colonisation of the mucosa.

3. Results

3.1. Antifungal activities of hBD-2 against *C. albicans* and *P. gingivalis*

Candida albicans 613p is known to establish oral candidal infection in rats after surgical hyposalivation [29]. It was therefore chosen as the infectious agent to establish oral fungal infection for the study model. We first tested its sensitivity to recombinant hBD-2 by an in vitro antimicrobial assay. The potency of hBD-2 was verified by performing parallel experiments using *E. coli* and *P. gingivalis* ATCC 33277 as positive controls, which are reported to be sensitive to hBD-2 at various concentrations [6,32].

Microorganisms grown to exponential phase were incubated with HPLC-purified hBD-2 under low or high salt conditions. After 1 h at 37 °C, the mixtures were plated on agar plates to quantitate surviving microbes (CFU analysis). The findings for *E. coli* susceptibility to hBD-2 (Fig. 1A) are in accordance with a report by Liu et al. [6], which showed that *E. coli* is only sensitive to hBD-2 under low salt conditions. *Porphyromonas gingivalis* ATCC 33277 was also sensitive to hBD-2 but at a higher concentration, as reported by Joly et al. [32], although it was more resistant than *E. coli*. Under low salt conditions, growth of *P. gingivalis* at 10 µM hBD-2 was completely inhibited (Fig. 1B), similar to the findings in a report by Mineshiba et al. [26]. Growth of *C. albicans* 613p was inhibited by 1 µM hBD-2 by 87% or 97% under high and low salt conditions, respectively (Fig. 1C), suggesting that this *C. albicans* 613p strain appears to be a suitable candidate for the proposed study model.

3.2. Effect of saliva on hBD-2 integrity

In order for hBD-2 secreted into saliva to exert its function in the oral cavity, it is important that the secreted hBD-2 is not degraded in saliva, which contains many enzymes and proteases. To verify this possibility, whole saliva collected from NOD/SCID mice or a human donor was incubated with rhBD-2 at 37 °C for various time intervals. The samples were then subjected to ELISA to detect hBD-2. The results shown in Fig. 2 indicate no degradation of hBD-2 by mouse saliva after 60 min of incubation, whereas intact hBD-2 was reduced to ca. 60% in human saliva after 30–60 min of incubation, suggesting that hBD-2 is resistant to mouse salivary enzyme but is sensitive to human salivary enzyme degradation to some extent. It should be noted that mouse saliva was collected by stimulation of salivation with pilocarpine.

3.3. In vivo detection of hBD-2 in mouse submandibular glands and tumours

3.3.1. RT-PCR detection of hBD-2 mRNA—One day after hBD-2 gene transfer into submandibular glands of SCID mice, glands were removed and RNA was isolated to detect hBD-2 mRNA expression using RT-PCR. A positive control experiment was performed in parallel to ensure that in vivo hBD-2 expression can be detected. Selected clones of HT-1080 transduced with either pBabe-hBD-2 or SIN18-hBD-2 that secrete hBD-2 in vitro were injected into SCID mice subcutaneously to form tumours. RNA was isolated from the resected tumours and underwent the same procedures as for mouse submandibular glands. hBD-2 was detected via RT-PCR in tumours (Fig. 3A) and in submandibular glands (Fig. 3B).

3.3.2. Immunostaining of hBD-2—To verify the presence of hBD-2 peptides in submandibular glands 1 week after gene transfer, immunohistochemistry staining was performed. hBD-2 was observed in submandibular glands as well as in HT-1080 tumours (Fig. 4). The data confirmed successful hBD-2 gene integration into salivary gland acinar cells following salivary duct cannulation. In addition, salivary gland staining (Fig. 5) verified the positive expression of hBD-2 in gland cells. However, hBD-2 was not detected by ELISA in saliva collected (after pilocarpine stimulation) from mice transduced with

hBD-2 in the submandibular glands after 1 day or 1 week, nor was it detected from mice transduced after 2–3 months.

3.4. Minimal fungal infection in the oral cavity of NOD/SCID mice

On Day 0 (before candidal infection), no fungus was detected either in NOD/SCID or normal C57BL/6 mice. On Days 4 and 12 after oral inoculation, no *C. albicans* was detected from the oral cavities of C57BL/6 mice, whereas a large number of *C. albicans* was recovered from the oral cavity swab samples and from homogenised tissues (tongue and intestine) of NOD/SCID mice after overnight incubation in YEPD broth. However, no oral manifestation of oral candidiasis was observed in any of the tested mice under stereomicroscopic examination, nor was there detection of any fungal colonisation or invasion into the epithelial or subjacent tissues of oral and intestinal mucosa using H&E analysis. Similar findings were noted 4 weeks after fungal infection of NOD/SCID mice, although some mice appeared ill and died after several weeks of infection.

4. Discussion

The ultimate goal of this study was to utilise salivary glands as a target organ for antimicrobial gene therapy against oral infections, especially for immunocompromised individuals. Although there are available animal models and a potent hBD-2 ready to test this potentially applicable clinical treatment modality, several questions need to be addressed before a workable animal study model may be established. We stepwise tested the antimicrobial activity against *C. albicans* 613p that was to be used as part of the study model and verified its sensitivity to hBD-2, which has been shown to be a potent antimicrobial agent against some strains of *C. albicans* [6,32,33]. The effective concentrations of hBD-2 against *C. albicans* are 1–14 μM based on published reports, which coincides with our finding using *C. albicans* 613p.

The presence of hBD-2 in human saliva was detected by Mathews et al. [24] with an estimated concentration of 150 ng/mL, which is below the level required for effective antimicrobial function (1 μM hBD-2 = 4.3 $\mu\text{g/mL}$). The exact source of hBD-2 in saliva remains elusive. It was speculated to be from oral keratinocytes (an abundant source of this peptide) and from submandibular glands and minor salivary glands in which hBD-2 mRNA, not its peptide, was detected in a few human samples [25,34]. hBD-2 was not detected in human parotid glands [24,34]. In contrast, hBD-1 mRNA is expressed in all human salivary glands and its peptides in duct cells of minor salivary glands as well as in saliva [34,35]. Since hBD-2 is more potent than hBD-1, overexpressing hBD-2 in human saliva appears to be a logical approach for oral infection control. A study has shown that individuals who develop oral candidiasis have lower hBD expression in saliva, including hBD-2 [36]. Together with our finding of some loss of hBD-2 integrity (down to 60%) in human saliva, these facts further support the approach of overexpressing hBD-2 in saliva via gene transfer of salivary glands to compensate for this loss.

O'Connell et al. [2] used pilocarpine to induce rat saliva secretion after adenoviral vector-mediated histatin 3 gene transduction of submandibular glands. In the collected rat saliva, they found >1 mg/mL of histatin 3 expressed. Although adenoviral vectors offer a sufficient level of peptide expression in salivary glands and saliva, its inherited transient effect and elicitation of strong immune reactions preclude its usability for this purpose. Our attempt to use the lentiviral vector to transduce mouse salivary glands indicated that although hBD-2 was detected both at mRNA and protein levels in glands, it was not detectable in saliva. This finding answers our first question regarding whether hBD-2 can be expressed in mouse saliva after transduction by a lentiviral vector. Although the lentiviral vector is powerful in transducing non-dividing cells in vitro and in vivo, we found that it does not appear to be

potent in producing a high level of transgene expression in mouse cells [17]. This is in agreement with the findings of Shai et al. [37] who compared a number of vector systems for transducing mouse salivary glands. Lentiviral vector transduction was found among others to have lower transgene expression levels. None the less, the research group found that feline immunodeficiency virus-based lentiviral vectors appear to provide satisfactory long-term expression of the transgene in mouse salivary glands [38], suggesting a potential future use of this vector for gene transduction of mouse salivary glands.

Rodents, especially mice, have been used to establish oropharyngeal candidiasis or more extensive fungal infections as a study model to test antifungal agents for treatment or for clinical monitoring of infection [29,39–44]. SCID mice have been used by many investigators as a model to study mucosal candidiasis [40,45]. However, a clear establishment of oral candidal infection was only reported by Takakura et al. [39,42] using prednisolone to immunosuppress the mice and they were able to observe macroscopically typical lesions consisting of white patches on the tongues. *Candida albicans* 613p, although showing establishment of oral candidiasis in the rat after surgical hyposalivation by ligation of the parotid salivary ducts plus surgical removal of the submandibular and sublingual salivary glands, based on our finding it did not establish oral candidiasis in NOD/SCID mice. Hyposalivation to induce formation of candidiasis after fungal infection is a contraindication for our purpose. Whilst oral candidiasis was not established in NOD/SCID mice, recovery of live fungus after incubation in broth indicates that there was some degree of fungal colonisation in the mouse oral cavity and gastrointestinal tract. This may be sufficient to serve as a study model for antifungal gene therapy. Nevertheless, the main issue is the level of hBD-2 expression in saliva. In conclusion, our studies addressed a technical barrier that needs to be overcome before an optimal study model can be established to test salivary gland antimicrobial gene therapy for oral infection.

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References

1. Bals R, Weiner DJ, Moscioni AD, Meegalla RL, Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun*. 1999; 67:6084–9. [PubMed: 10531270]
2. O'Connell BC, Xu T, Walsh TJ, et al. Transfer of a gene encoding the anticandidal protein histatin 3 to salivary glands. *Hum Gene Ther*. 1996; 7:2255–61. [PubMed: 8953316]
3. Yarus S, Rosen JM, Cole AM, Diamond G. Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. *Proc Natl Acad Sci USA*. 1996; 93:14118–21. [PubMed: 8943070]
4. Bals R, Weiner DJ, Meegalla RL, Wilson JM. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. *J Clin Invest*. 1999; 103:1113–7. [PubMed: 10207162]
5. Bals R, Xiao W, Sang N, Weiner DJ, Meegalla RL, Wilson JM. Transduction of well-differentiated airway epithelium by recombinant adeno-associated virus is limited by vector entry. *J Virol*. 1999; 73:6085–8. [PubMed: 10364362]
6. Liu AY, Destoumieux D, Wong AV, et al. Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol*. 2002; 118:275–81. [PubMed: 11841544]

7. Garcia JR, Krause A, Schulz S, et al. Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB J.* 2001; 15:1819–21. [PubMed: 11481241]
8. Carretero M, Del Rio M, Garcia M, et al. A cutaneous gene therapy approach to treat infection through keratinocyte-targeted overexpression of antimicrobial peptides. *FASEB J.* 2004; 18:1931–3. [PubMed: 15456737]
9. Huang GT, Zhang HB, Kim D, Liu L, Ganz T. A model for antimicrobial gene therapy: demonstration of human beta-defensin 2 antimicrobial activities in vivo. *Hum Gene Ther.* 2002; 13:2017–25. [PubMed: 12489997]
10. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol.* 2004; 22:181–215. [PubMed: 15032578]
11. Quinones-Mateu ME, Lederman MM, Feng Z, et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *AIDS.* 2003; 17:F39–48. [PubMed: 14571200]
12. Sawamura D, Goto M, Shibaki A, et al. Beta defensin-3 engineered epidermis shows highly protective effect for bacterial infection. *Gene Ther.* 2005; 12:857–61. [PubMed: 15729370]
13. Sun L, Finnegan CM, Kish-Catalone T, et al. Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection. *J Virol.* 2005; 79:14318–29. [PubMed: 16254366]
14. Jia HP, Schutte BC, Schudy A, et al. Discovery of new human beta-defensins using a genomics-based approach. *Gene.* 2001; 263:211–8. [PubMed: 11223260]
15. Schutte BC, Mitros JP, Bartlett JA, et al. Discovery of five conserved beta-defensin gene clusters using a computational search strategy. *Proc Natl Acad Sci USA.* 2002; 99:2129–33. [PubMed: 11854508]
16. Premratanachai P, Joly S, Johnson GK, McCray PB Jr, Jia HP, Guthmiller JM. Expression and regulation of novel human beta-defensins in gingival keratinocytes. *Oral Microbiol Immunol.* 2004; 19:111–7. [PubMed: 14871351]
17. Yin C, Dang HN, Zhang HB, et al. Capacity of human beta-defensin expression in gene-transduced and cytokine-induced cells. *Biochem Biophys Res Commun.* 2006; 339:344–54. [PubMed: 16298338]
18. Huang GT, Zhang HB, Yin C, Park SH. Human beta-defensin-2 gene transduction of dental pulp cells: a model for pulp antimicrobial gene therapy. *Int J Oral Biol.* 2004; 29:7–12.
19. Baum BJ, Voutetakis A, Wang J. Salivary glands: novel target sites for gene therapeutics. *Trends Mol Med.* 2004; 10:585–90. [PubMed: 15567328]
20. Voutetakis A, Bossis I, Kok MR, et al. Salivary glands as a potential gene transfer target for gene therapeutics of some monogenetic endocrine disorders. *J Endocrinol.* 2005; 185:363–72. [PubMed: 15930162]
21. Voutetakis A, Kok MR, Zheng C, et al. Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. *Proc Natl Acad Sci USA.* 2004; 101:3053–8. [PubMed: 14978265]
22. Dale BA, Krisanaprakornkit S. Defensin antimicrobial peptides in the oral cavity. *J Oral Pathol Med.* 2001; 30:321–7. [PubMed: 11459317]
23. Dunsche A, Acil Y, Siebert R, Harder J, Schroder JM, Jepsen S. Expression profile of human defensins and antimicrobial proteins in oral tissues. *J Oral Pathol Med.* 2001; 30:154–8. [PubMed: 11271630]
24. Mathews M, Jia HP, Guthmiller JM, et al. Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect Immun.* 1999; 67:2740–5. [PubMed: 10338476]
25. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. *Med Electron Microsc.* 2003; 36:247–52. [PubMed: 16228657]
26. Mineshiba F, Takashiba S, Mineshiba J, Matsuura K, Koikeguchi S, Murayama Y. Antibacterial activity of synthetic human B defensin-2 against periodontal bacteria. *J Int Acad Periodontol.* 2003; 5:35–40. [PubMed: 12760504]
27. Klotman ME, Chang TL. Defensins in innate antiviral immunity. *Nat Rev Immunol.* 2006; 6:447–56. [PubMed: 16724099]

28. Niyonsaba F, Ogawa H, Nagaoka I. Human beta-defensin-2 functions as a chemotactic agent for tumour necrosis factor-alpha-treated human neutrophils. *Immunology*. 2004; 111:273–81. [PubMed: 15009427]
29. Meitner SW, Bowen WH, Haidaris CG. Oral and esophageal *Candida albicans* infection in hyposalivatory rats. *Infect Immun*. 1990; 58:2228–36. [PubMed: 2194965]
30. An DS, Wersto RP, Agricola BA, et al. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. *J Virol*. 2000; 74:1286–95. [PubMed: 10627539]
31. Kung SK, An DS, Chen IS. A murine leukemia virus (MuLV) long terminal repeat derived from rhesus macaques in the context of a lentivirus vector and MuLV gag sequence results in high-level gene expression in human T lymphocytes. *J Virol*. 2000; 74:3668–81. [PubMed: 10729143]
32. Joly S, Maze C, McCray PB Jr, Guthmiller JM. Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. *J Clin Microbiol*. 2004; 42:1024–9. [PubMed: 15004048]
33. Schroder JM, Harder J. Human beta-defensin-2. *Int J Biochem Cell Biol*. 1999; 31:645–51. [PubMed: 10404637]
34. Bonass WA, High AS, Owen PJ, Devine DA. Expression of beta-defensin genes by human salivary glands. *Oral Microbiol Immunol*. 1999; 14:371–4. [PubMed: 10895693]
35. Sahasrabudhe KS, Kimball JR, Morton TH, Weinberg A, Dale BA. Expression of the antimicrobial peptide, human beta-defensin 1, in duct cells of minor salivary glands and detection in saliva. *J Dent Res*. 2000; 79:1669–74. [PubMed: 11023262]
36. Tanida T, Okamoto T, Okamoto A, et al. Decreased excretion of antimicrobial proteins and peptides in saliva of patients with oral candidiasis. *J Oral Pathol Med*. 2003; 32:586–94. [PubMed: 14632933]
37. Shai E, Falk H, Honigman A, Panet A, Palmon A. Gene transfer mediated by different viral vectors following direct cannulation of mouse submandibular salivary glands. *Eur J Oral Sci*. 2002; 110:254–60. [PubMed: 12120712]
38. Shai E, Palmon A, Panet A, et al. Prolonged transgene expression in murine salivary glands following non-primate lentiviral vector transduction. *Mol Ther*. 2005; 12:137–43. [PubMed: 15963929]
39. Takakura N, Sato Y, Ishibashi H, et al. A novel murine model of oral candidiasis with local symptoms characteristic of oral thrush. *Microbiol Immunol*. 2003; 47:321–6. [PubMed: 12825893]
40. Clemons KV, Stevens DA. Efficacy of ravuconazole in treatment of mucosal candidosis in SCID mice. *Antimicrob Agents Chemother*. 2001; 45:3433–6. [PubMed: 11709320]
41. Farah CS, Hong S, Wanasaengsakul S, et al. Irradiation-induced oral candidiasis in an experimental murine model. *Oral Microbiol Immunol*. 2001; 16:358–63. [PubMed: 11737659]
42. Takakura N, Wakabayashi H, Ishibashi H, et al. Oral lactoferrin treatment of experimental oral candidiasis in mice. *Antimicrob Agents Chemother*. 2003; 47:2619–23. [PubMed: 12878528]
43. Sundstrom P, Balish E, Allen CM. Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. *J Infect Dis*. 2002; 185:521–30. [PubMed: 11865405]
44. de Repentigny L, Aumont F, Ripeau JS, et al. Mucosal candidiasis in transgenic mice expressing human immunodeficiency virus type 1. *J Infect Dis*. 2002; 185:1103–14. [PubMed: 11930320]
45. Teichert MC, Jones JW, Usacheva MN, Biel MA. Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2002; 93:155–60. [PubMed: 11862203]

Fig. 1.

Antimicrobial activity of recombinant human β -defensin-2 (rhBD-2) against (A) *Escherichia coli*, (B) *Porphyromonas gingivalis* and (C) *Candida albicans*. The number of colony-forming units was determined after 1 h incubation with rhBD-2. Experiments were made in low or high salt solution or in filter-sterilised human saliva. *Escherichia coli* was determined by a single assay, *C. albicans* in triplicate assays and *P. gingivalis* in duplicate assays. Error bars indicate standard error of the mean.

Fig. 2. Degradation of human β -defensin-2 (hBD-2) in saliva. Whole saliva from a healthy mouse or human donor was mixed with recombinant hBD-2 (16 ng/mL) and incubated at 37 °C. Samples were collected at different time points for enzyme-linked immunosorbent assay (ELISA).

Fig. 3.

In vivo human β -defensin-2 (hBD-2) expression detected by reverse transcription polymerase chain reaction in (A) mouse tumour formed by HT-1080 cells and (B) mouse submandibular glands. M, 1 kb plus DNA ladder; non-trans, non-transduced.

Fig. 4.

Immunohistochemistry of human β -defensin-2 (hBD-2) staining in HT-1080 tumours and in submandibular glands in SCID mice. (A) HT-1080 cells transduced in vitro with hBD-2 carried by either pBabe or SIN18 vector were injected into NOD/SCID mice to form tumours. Resected tumours were then sectioned and stained with anti-hBD-2. Non-trans, tumour cells not transduced with hBD-2. (B) One week after NOD/SCID mice had received SIN18-hBD-2 via intraoral cannulation of salivary ducts, submandibular glands were dissected and processed for hBD-2 detection. Brown deposits indicate immunoreactivity to hBD-2 (arrows). Non-trans, submandibular gland not receiving SIN18-hBD-2 cannulation. Original magnification, 200 $\times$.

Fig. 5. Immunostaining of human β -defensin-2 (hBD-2) in submandibular gland infected with SIN18-hBD-2 in SCID mice. SCID mice received SIN18-hBD-2 by intraoral cannulation of salivary ducts. After 24 h, mice were sacrificed and submandibular glands were dissected for immunofluorescence staining. Blue indicates nucleus staining by 4',6-diamidino-2-phenylindole (DAPI); green indicates hBD-2 by fluorescein isothiocyanate (FITC). (A) Glands not receiving SIN18-hBD-2 cannulation and (B) glands receiving SIN18-hBD-2. Positive staining is indicated by several circled areas demonstrating an overlap of green and blue staining (very bright green). Photographed under fluorescence microscope with a 20 $\times$ objective.