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Topical delivery of low-cost protein drug candidates made in chloroplasts for biofilm disruption and uptake by oral epithelial cells

Yuan Liu [a, 1], Aditya C. Kamesh [b, 1], Yuhong Xiao [b], Victor Sun [a], Michael Hayes [b], Henry Daniell [b, *], Hyun Koo [a, **]

[a] Department of Orthodontics, Divisions of Pediatric Dentistry and Community Oral Health, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104-6030, USA
[b] Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104-6030, USA

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ABSTRACT

Protein drugs (PD) are minimally utilized in dental medicine due to high cost and invasive surgical delivery. There is limited clinical advancement in disrupting virulent oral biofilms, despite their high prevalence in causing dental caries. Poor efficacy of antimicrobials following topical treatments or to penetrate and disrupt formed biofilms is a major challenge. We report an exciting low-cost approach using plant-made antimicrobial peptides (PMAMPs) retrocyclin or protegrin with complex secondary structures (cyclic/hairpin) for topical use to control biofilms. The PMAMPs rapidly killed the pathogen *Streptococcus mutans* and impaired biofilm formation following a single topical application of tooth-mimetic surface. Furthermore, we developed a synergistic approach using PMAMPs combined with matrix-degrading enzymes to facilitate their access into biofilms and kill the embedded bacteria. In addition, we identified a novel role for PMAMPs in delivering drugs to periodontal and gingival cells, 13–48 folds more efficiently than any other tested cell penetrating peptides. Therefore, PDs fused with protegrin expressed in plant cells could potentially play a dual role in delivering therapeutic proteins to gum tissues while killing pathogenic bacteria when delivered as topical oral formulations or in chewing gums. Recent FDA approval of plant-produced PDs augurs well for clinical advancement of this novel concept.

1. Introduction

Biopharmaceuticals produced in current systems are prohibitively expensive for a large majority of the global population. The cost of protein drugs ($140 billion in 2013) exceeds GDP of >75% of countries around the globe [1], making them unaffordable. One third of global population earns <$2 per day or the low socio-economic/underprivileged in the US can't afford protein drugs. Such high costs are associated with their production in prohibitively expensive fermenters, purification, cold transportation/storage, short shelf life and sterile delivery methods [2,3]. In order to address these concerns, low cost PDs can be made in plant cells for their topical [4] or oral delivery [2,3,5].

Many infectious diseases in humans are caused by biofilms, including those occurring in the mouth [6,7]. For example, dental caries continues to be the single most prevalent biofilm-associated oral disease, afflicting mostly underprivileged children and adults in the US and worldwide, resulting in expenditures of >$40 billion annually [8–10]. Caries-causing (cariogenic) biofilms develop when bacteria interact with dietary sugars and accumulate on tooth surface, forming organized clusters that are firmly adherent and enmeshed in an extracellular matrix of polymeric substances such as exopolysaccharides (EPS) [9]. *Streptococcus mutans* is one of the major pathogens causing dental caries, although additional organisms may be involved [6,8–10]. This bacterium expresses multiple exoenzymes (glucosyltransferases) that make it a primary EPS producer in oral cavity, while it is also highly acidogenic and aciduric [9]. Current topical antimicrobial modalities for controlling

* Corresponding author. School of Dental Medicine, University of Pennsylvania, 240 South 40th St, Rm#547, Levy Building, Philadelphia, PA 19104-6030, USA.
** Corresponding author. Department of Orthodontics, Divisions of Pediatric Dentistry and Community Oral Health, School of Dental Medicine, University of Pennsylvania, 240 South 40th St, Rm#417, Levy Building, Philadelphia, PA 19104-6030 USA.
E-mail addresses: hdaniell@upenn.edu (H. Daniell), koohy@upenn.edu (H. Koo).
[1] Both authors made equal contributions.

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cariogenic biofilms are limited. Chlorhexidine (CHX) is considered the 'gold standard' for oral antimicrobial therapy, but has adverse side effects including tooth staining and calculus formation, and is not recommended for daily therapeutic use [11]. As an alternative, several antimicrobial peptides (AMPs) have emerged with potential antibiofilm effects against caries-causing oral pathogens, including *S. mutans* [12,13].

When compared with conventional antibiotics, AMPs provide additional advantages for oral antimicrobial therapy. For example, AMPs not only possess bactericidal activity but also have other biological functions like immunomodulation by activating mast cells and wound healing [14], while playing a critical role in angiogenesis [15]. Furthermore, they are potently active against bacteria (particularly Gram-positive), fungi and viruses and can be tailored to target specific pathogens by fusion with their surface antigens [14,16,17]. AMPs can kill and restrict microbial infection by multiple mechanisms, including altered cell surface charge, disruption of membrane integrity and pore formation while also neutralizing lipopolysaccharides-induced endotoxin shock [14,16–19]. Although development of resistance is less likely with AMPs, previous studies have shown that resistance mechanisms can be developed by pathogens, including up-regulation of proteolytic activity, release of scavenging anionic compounds such as EPS and glycosaminoglycan, as well as amidation and related surface conjugations of membrane lipids and/or peptidoglycan [18,19]. However, AMP structure and bioactivity varies greatly.

Linear AMPs have poor stability or antimicrobial activity when compared to AMPs with complex secondary structures. For example, retrocyclin (RC101) and protegrin-1 (PG1) have high antimicrobial activity or stability when cyclized [20] or form hairpin structure [21] with formation of disulfide bonds. RC101 is highly stable at pH 3, 4, 7 and temperature 25 °C–37 °C as well as in human vaginal fluid for 48 h [22], while the antimicrobial activity was maintained for up to six months [23]. Likewise, PG1 is highly stable in salt or human fluids [24,25] but potency is lost when linearized. Furthermore, AMPs displaying cyclic or secondary structures have increased penetrability through the microbial membranes compared to linear peptides [26]. These intriguing characteristics of antimicrobial peptides with complex secondary structures may facilitate development of novel therapeutics. However, the high cost of producing sufficient amounts of antimicrobial peptides is a major barrier for their clinical development and commercialization. Therefore, we have produced several low cost antimicrobial peptides (magainin, retrocyclin, protegrin) in plant chloroplasts [14,16,17].

Clinical therapy of biofilm-associated infections faces yet another challenging problem. Antimicrobial drugs often fail to kill the clusters of microbes that are protected by their extracellular matrix in formed biofilms [27–29]. Therefore, EPS-matrix degrading enzymes from fungi (like dextranase or mutanase) have been explored to disrupt biofilm and prevent dental caries [30–33] but with limited success [34,35]. However, a synergistic approach of combining antimicrobial agents with EPS-matrix degrading enzymes has not yet been developed. In order to address the cost of enzymes, we have developed a low cost strategy by producing them in plant chloroplasts [36,37]. Most importantly, plant cells expressing high levels of therapeutic proteins can be lyophilized and stored at room temperature for several years [2,3,38,39].

Apart from treating oral biofilm, protein therapy is minimally utilized in dental medicine because of invasive surgical delivery. However, there is a great need for delivery of growth hormones or other bioactives to enhance cell adhesion, stimulate osteogenesis, bone regeneration, differentiation of osteoblasts or endothelial cells. In addition to minimal patient compliance, injectable protein drugs often do not contain essential information to reach their target cells or cell penetrating capabilities. Therefore, localized targeting and delivery to cells including osteoblasts, periodontal ligament cells, gingival epithelial cells or fibroblasts is essential to advance oral health. When delivered orally, protein drugs synthesized in plant cells can be released by mechanical grinding (chewing). Therefore, in this study, we investigate the specificity or capability of cyclic or acyclic plant-made AMPs (PMAMPs) fused with green fluorescent protein to target various human periodontal or gingival cells and evaluate their efficacy in protein drug delivery. In parallel, we evaluated the potency of PMAMPs to prevent biofilm formation following a topical treatment and their synergistic activities with matrix degrading enzymes for disruption of formed biofilms. Thus, this study reports a new cost-effective approach for production of protein drugs to prevent or treat biofilm-associated oral diseases and deliver PDs to human oral tissues for enhancing oral health.

2. Materials and methods

2.1. Microorganisms and EPS degrading enzymes

Streptococcus mutans UA159 serotype c (ATCC 700610), *Streptococcus gordonii* DL1 and *Actinomyces naeslundii* ATCC 12104 were used in present study. The strains tested in this research were selected because *S. mutans* is a well-established virulent cariogenic bacteria [40]. *S. gordonii* is an early colonizer and considered an accessory pathogen (that could enhance virulence of periodontopathogens) [41]. *A. naeslundii* is also detected during the early stages of biofilm formation and may be associated with development of dental root caries [42]. All these strains were grown in ultra-filtered (10 kDa molecular-weight cut-off membrane; Prep/Scale, Millipore, MA) buffered tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose to mid-exponential phase (37 °C, 5% CO_2) prior to use. The EPS-degrading enzymes dextranase and mutanase are capable of hydrolyzing α-1,6 and α-1,3 glucosidic linkages present in the EPS glucans derived from *S. mutans* [43]. Dextranase produced from *Penicillium* sp. was commercially purchased from Sigma (St. Louis, MO) and mutanase produced from *Trichoderma harzianum* was kindly provided by Dr. William H. Bowen (Center for Oral Biology, University of Rochester Medical Center).

2.2. Purification of tag-fused GFP proteins

The transplastomic plants expressing green fluorescence protein (GFP) fused with Cholera Toxin B subunit (CTB), Protein Transduction Domain (PTD), retrocyclin and protegrin were created as described in previous studies [16,38,44,45]. Purification of GFP-fused PG1 or RC101 from transplastomic tobacco was done from 0.2 to 1 gm of lyophilized plant material. Subsequent downstream processing was done based on protocols established previously [14,16,44] (also see Supplementary Fig. S1). The lyophilized material was reconstituted in 10–20 ml of plant extraction buffer (0.2 M Tris HCl pH 8.0, 0.1 M NaCl, 0.01 M EDTA, 0.4 M sucrose, 0.2% Triton X supplemented with 2% phenylmethylsulfonylfluoride (PMSF) and a protease inhibitor cocktail (Pierce). The resuspension was incubated in ice for 1 h with vortex homogenization every 15 min. The homogenates were then sonicated (Misonix sonicator 3000) and spun down at 75,000 g at 4 °C for 1 h (Beckman LE-80K optima ultracentrifuge) to obtain the clarified lysate. The lysate was subjected to pretreatment with 70% saturated ammonium sulfate and 1/4th volume of 100% ethanol, followed by vigorous shaking for 2 min. The treated solution was spun down at 2100 g for 3 min. The upper ethanol phase was collected and the process was repeated with 1/16th volume of 100% ethanol. The pooled ethanol phases