Protein expression after delivery of mRNA encoding *PDGF-BB* into rat gingiva: A Pilot Study

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ABSTRACT

Messenger RNA (mRNA) has been emerged as a novel therapeutic modality in medical fields, including regenerative medicine. The concept of mRNA-based therapy is the use of synthesized mRNA encoding a potentially therapeutic protein delivered into targeted tissue. Thus, utilizing mRNA encoding growth factor for protein replacement therapy could be a promising alternative to recombinant protein. Platelet-derived growth factor (PDGF) is one of most extensively studied growth factors for periodontal regeneration. To date, the potential of mRNA therapy has never been explored in the field of periodontal regeneration. The aim of the study is to examine the effect of nucleoside-modified mRNA encoding PDGF-BB on the level of PDGF-BB protein and the inflammatory response at local tissue upon intragingival injection. Sprague-Dawley rats were injected with pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer at palatal gingiva. Gingiva were collected at days 1, 2, 3, 5 and 7 for protein analysis of PDGF-BB, VEGF-A protein and pro-inflammatory cytokines (IL-6 and TNF-α) in tissue homogenates. Enzyme-linked immunosorbent assay revealed that a single gingival injection of pseudouridine-modified mRNA encoding PDGF-BB significantly promoted transient PDGF-BB protein expression up to 40- to 100-fold as compared to control. PDGF-BB production peaked at 24-hour post-injection and declined to baseline within 3 days. Neither IL-6 nor TNF-α in gingiva were affected. The findings from this study demonstrated that intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer results in transient PDGF protein production with minimal local immune response. mRNA technology might be a potential approach for periodontal regeneration.

Keywords: Periodontal Regeneration, Mrna, Platelet-Derived Growth Factor, Rat Gingiva

1. Introduction

Periodontitis is one of the most common chronic diseases, affecting up to 743 million adults worldwide. In Thailand, more than 25% of middle-aged and 35% in elderly population are diagnosed with periodontitis. The breakdown of tooth-supporting structures, in particular alveolar bone, is the hallmark of the disease. This eventually leads to early tooth loss in severe cases, which significantly impinges on patients' quality of life.

Regeneration of damaged tooth-supporting structures is indeed required to restore full function of the periodontium and become the ultimate goal of periodontal treatment. Several regenerative approaches have been investigated yet the results of current treatments are mostly unpredictable. Despite the fact that guided tissue regeneration (GTR) and/or bone grafting procedures have showed to be effective for treating some types of periodontal defects, they have failed to attain complete periodontal regeneration. New therapeutic approaches to promote regeneration such as the use of growth factors, stem cells or gene-based therapy have been investigated. Nevertheless, the clinical results are limited and inconsistent. Recombinant growth factor delivery has gained more interest and various commercial products are currently available. However, the rapid degradation *in situ* and the cost of treatment probably hinder its use (Mitchell, Briquez, Hubbell, & Cochran, 2016). Recently, gene-therapy has been introduced to enhance the ability to regenerate periodontal tissues.

Gene therapy could be achieved by viral vector or plasmid DNA (pDNA). This approach has been studied in the field of periodontology since early 2000s, for example, adenovirus encoding PDGF-A transduced gingival fibroblast and enhanced cell proliferation and migration *in vitro* (Chen & Giannobile, 2002). In animal model, plasmid DNA encoding BMP-4 with a scaffold delivery system was found to enhance bone formation in rat cranial defect (Huang, Simmons, Kaigler, Rice, & Mooney, 2005). Injection of plasmid DNA (pDNA) encoding Osteoprotegerin (OPG) was found to reduce progressive alveolar bone resorption in experimental-induced periodontitis in rat (Tang et al., 2015). However, integration of DNA into host genome may pose the risk of insertional mutagenesis and often associated with low levels of protein expression. Therefore, mRNA-based approach has recently emerged as a novel alternative in the nonviral gene therapy. In contrast to DNA, mRNA does not integrate into host genome, transiently expresses protein of interest and subsequently be degraded through metabolic pathway (Sahin, Kariko, & Tureci, 2014; Weissman, 2015).

Nevertheless, delivery of mRNA encoding growth factor remains challenging in the aspect of its immunogenicity and limited stability (Weissman, 2015). Several methods have been applied to overcome these limitations, including base modification and delivery system optimization. Base or nucleoside modification, such as replacing uridine with pseudouridine, evidently offers an advantage of diminishing immunogenicity when deliver into target cells (Kariko et al., 2008). In term of stability, many studies have showed that the stability of mRNA can be improved when the proper vehicles, for example, lipids, polymers and peptides are employed (Martin & Rice, 2007; Mintzer, Merkel, Kissel, & Simanek, 2009; Pack, Hoffman, Pun, & Stayton, 2005). Recently, citrate-buffer has also been proved to be a potential carrier for mRNA (Sultana et al., 2017). This knowledge has advanced the

used of mRNA for a therapeutic purpose. mRNA has now been employed for immunotherapy, inhibition of pathogenic mRNA translation, genetic reprogramming and protein replacement/growth factor therapy. A pioneered work on mRNA-based therapy in the field of tissue regeneration by Zangi *et al.* has demonstrated that a single dose of mRNA encoding vascular endothelial growth factor-A (VEGF-A) could successfully restore the ischemic heart in mice model. Currently, mRNA encoding VEGF-A has now been under clinical trials, aiming to develop regenerative therapies for the treatment of cardiometabolic diseases. Nevertheless, the use of nucleoside modified mRNA has never been investigated in periodontal tissue regeneration.

Given the potential of mRNA-based therapy as a tool for tissue regeneration, we hypothesized that mRNA encoding specific growth factor could be a promising alternative for periodontal therapy. We previously developed nucleoside-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) which its transfection and expression into target cells have been successfully demonstrated *in vitro.* Therefore, in this study, we aimed to examine the effect of nucleoside-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) upon locally delivered *in vivo* via gingival injection. PDGF-BB protein expression in rat gingival tissue at different time points and the inflammation at sites of mRNA delivery were investigated.

2. Objectives of the study

To examine the PDGF-BB protein expression, as well as VEGF-A, IL-6 and TNF-α production in gingival tissue upon local delivery of pseudouridine-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) in sucrose citrate buffer *in vivo*

3. Materials and methods

Construction of modified mRNA encoding PDGF-BB

Open reading frame of human PDGF-BB gene was provided to Biotechnology company (TriLink Biotechnologies Co., Ltd., San Diego, CA, USA) for mRNA transcription service. A specially designed cDNA with the required elements for transcription: proprietary 5'and 3' UTR and its designed to incorporate a 120nt poly-A tail via polymerase chain reaction (PCR) was constructed. Since pseudouridine-modified mRNA has been reported to achieve higher protein expression compared to unmodified mRNA or other formulation (Kariko et al., 2008). The modified mRNA encoding PDGF-BB was synthesized, utilizing pseudouridine modification.

Animals

All rat experimental protocols in this study were reviewed and approved by Institute of Animal Care and Use Committee of Faculty of Tropical Medicine, Mahidol University (FTM-IACUC) and the Ethics committee of the Faculty of Dentistry, Chulalongkorn University. Wild-type Sprague-Dawley male rats, aged 6-week old were purchased from Nomura Siam International Co., Ltd. (Bangkok, Thailand) and adopted in individually ventilated cages with 12-hours light/dark cycle for a week before beginning of the experiment. Rats were randomly divided

into six groups of three mice each (Zangi et al., 2013). One group, served as a control, was injected with sucrose citrate buffer only and then sacrificed 1 day post-injection. The rest were injected with pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer and then sacrificed at five different timepoints post-injection (days 1, 2, 3, 5, 7).

Delivery of mRNA encoding PDGF-BB in sucrose citrate buffer into rat gingiva

The 30 μg of pseudouridine-modified mRNA encoding PDGF-BB (Ψ-modified mRNA) was prepared in sucrose citrate buffer. All rats received gingival injection of the solution of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer at the palatal gingiva, using needle gauge 31 under the loupe magnification. The injection was performed at 6 sites with the volume of 3 μl solution containing 5 μg Ψ-modified mRNA per site (Fig.1). Thus, the total Ψ-modified mRNA of 30 μg in total volume of 18 μl solution was given to each rat.

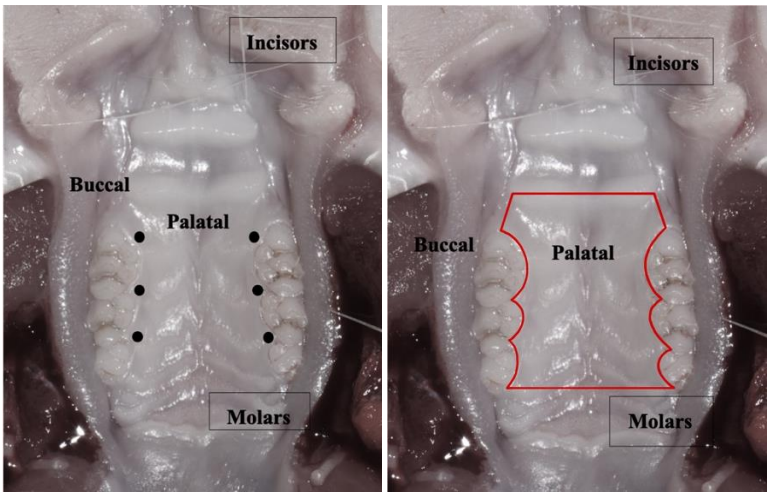

Figure 1 Diagram of rat maxillary teeth: Gingival injection was performed 6 sites per rat. (A) Black dot (●) indicated site for gingival injection. (B) Red line outlines the part of palatal gingiva that was collected.

Preparation of gingival tissue homogenates

Rats were sacrificed at 1, 2, 3, 5 and 7-day post-injection, regarding the experimental groups previously assigned. Palatal gingival tissues were collected as shown in Fig.1. The collected gingival tissues were weighed and homogenized with a micro tissue homogenizer in RIPA Lysis and Extraction Buffer (Pierce™ RIPA Buffer; Thermo scientific, Co., Ltd, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The homogenates were centrifugated at 16,000 rpm for 15 minutes at 4°C, and the supernatant was collected for further protein analysis by enzyme-linked immunosorbent assay (ELISA).

Measurement of protein expression *in vivo*

The total protein in tissue homogenates was measured using BCA protein assay kit (Pierce™ BCA Protein Assay; Thermo scientific, Co., Ltd, Rockford, IL, USA). The production of PDGF-BB, VEGF-A and pro-inflammatory cytokines including TNF-α and IL-6 was determined by commercially available ELISA kits (Quantikine® ELISA; R&D Systems, Inc., Minneapolis, MN, USA). ELISAs were performed according to the manufacturer's protocols. The minimal levels of rat TNF-α and IL-6 detected by ELISA kit were 5 and 21 pg/ml,

respectively. The results were presented as the amount of interested growth factors or proinflammatory cytokines per total protein.

Statistical analysis

The statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Normal distribution of data was tested by Shapiro-Wilk normality test. Non-parametric Kruskal-Wallis and Mann-Whitney U test were performed for within group and between group comparisons, respectively. A *P*-value<0.05 was considered statistically significant.

4. Results

The delivery of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer attained efficient PDGF-BB protein production *in vivo*. Direct single intragingival injection of this modified mRNA yielded a robust PDGF-BB protein expression, 40- to 100-fold increase compared to control. The amount of PDGF-BB production in gingiva peaked at 24-hour post-injection, then rapidly decreased on the second day and returned to baseline at day-3 (Fig.2). The mean concentration of PDGF-BB protein at 24-hour was 6886.32 ± 1213.81 pg/mg protein. At 48-hour, the mean concentration dropped by 98% to 191.34 ± 68.10 pg/mg protein. The elevated level of PDGF-BB protein significantly decreased to the level comparable to that of control at day-3 (68.21 ± 9.50 pg/mg protein, $p<0.05$). A statistically significant difference was observed between test and control group only at day 1 ($p<0.05$).

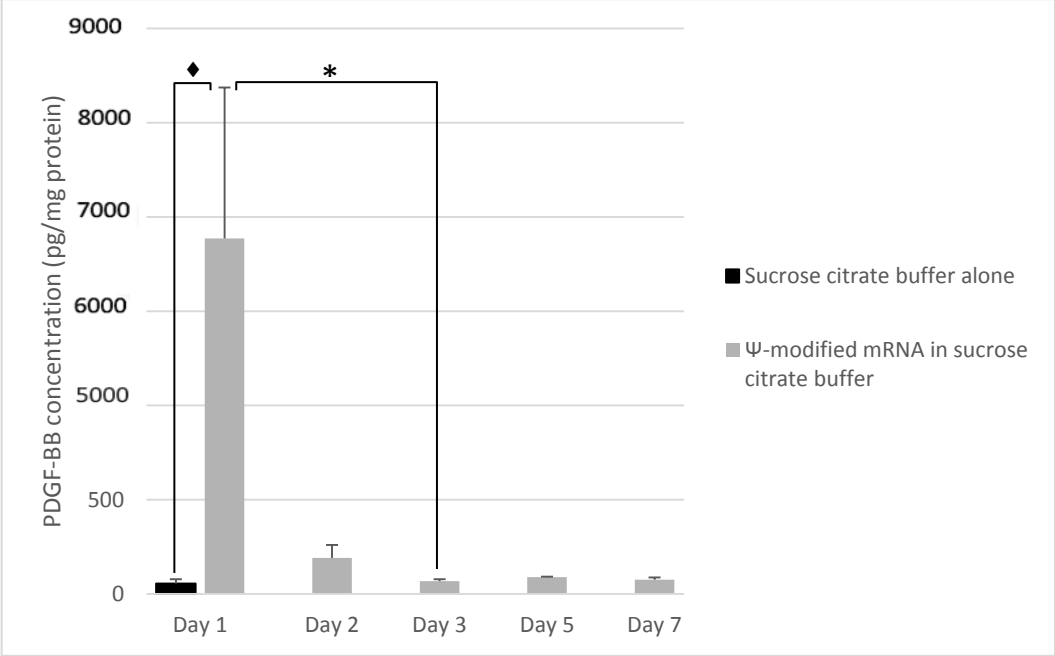

Figure 2 Kinetic expression of PDGF-BB protein in rat gingival tissue after injection with pseudouridine-modified mRNA encoding PDGF-BB (N = 3 for Day 1, 2, 3 and 5; N = 2 for Day 7, Data are presented as mean ± SD).

* *indicates statistically significance, p<0.05; Kruskal-Wallis with pairwise comparison;* ♦ *indicates statistically significance, p<0.05; Mann-Whitney U test*

Previous study demonstrated that PDGF-BB could promote angiogenesis through stimulating vascular endothelial growth factor-A (VEGF-A) production (Affleck et al., 2002). Therefore, the level of VEGF-A protein was assessed. However, the levels of VEGF-A protein in experimental and control group were not statistically different at all time points (Fig.3).

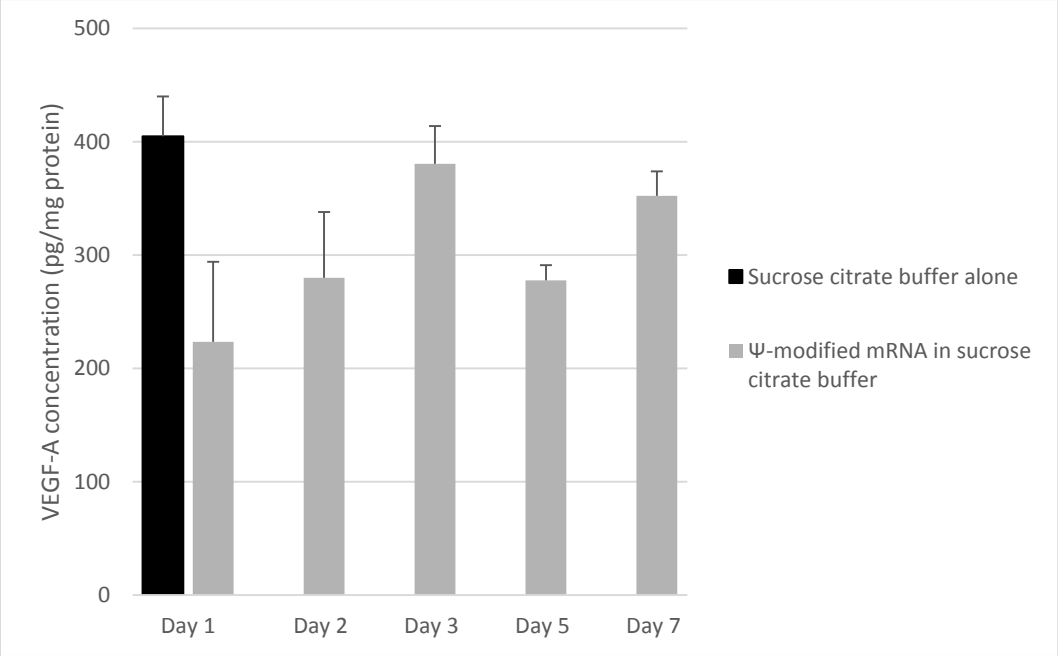

Figure 3 Kinetic expression of VEGF-A protein in rat gingival tissue after injection with pseudouridine-modified mRNA encoding PDGF-BB (N = 3 for Day 1, 2, 3 and 5; N = 2 for Day 7, Data are presented as mean ± SD).

Next, we evaluated the effects of intragingival injection of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer on the gingival levels of IL-6 and TNF-α. TNF-α and IL-6 were not detected at all time points in Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer. This suggested that intragingival injection of Ψ-modified mRNA encoding PDGF-BB did not alter gingival levels of both TNF-α and IL-6, since the levels of both proinflammatory cytokines in control group were also below the detection limit of the ELISA. Data were not shown.

5. Discussion

This study is the first pre-clinical study utilizing mRNA for periodontal regeneration. The keys to delivery of growth factor for therapeutics are to deliver a transient, robust signal at a precise time and target site. Although recombinant PDGF-BB offers a great therapeutic potential, the major drawbacks are the high cost and short half-

life. At the periodontal defect, the half-life of recombinant growth factors are significantly reduced due to the proteolytic breakdown and rapid dilution by gingival crevicular fluid and saliva (Anusaksathien & Giannobile, 2002). In contrast to recombinant protein, the pseudouridine-modified mRNA encoding PDGF-BB in this study provided a transient, strong signal directly to the target site, which resulted in a high production of PDGF-BB protein that could retain around the target tissue.

The present study demonstrated that direct intragingival injection of modified mRNA encoding PDGF-BB in sucrose citrate buffer successfully resulted in PDGF-BB protein expression at the injection size. The highest PDGF-BB expression was at 24 hours, which was significantly higher compared to control. The superior protein level was observed until day-3 post-injection. Our findings are in line with the previous studies examining the kinetics of mRNA *in vivo*. Zangi *et al.* showed that cardiac injection of 100 μg modified mRNA encoding Luciferase (Luc) in lipid nanoparticle (RNAiMAX) resulted in an immediate Luc expression that reached high level after 3 hours, peaked at 18 hours and returned to baseline at 144-150 hours (Zangi et al., 2013). Sultana *et al.* studied the kinetic expression of modified mRNA encoding Luc in mice model and found that cardiac injection of the modified mRNA in sucrose citrate buffer yielded an increased Luc expression, significantly above baseline within 10 minutes. The highest expression was at 24 hours and returned to the basal level at 96 hours (Sultana et al., 2017). Carlsson *et al.* revealed that injection of 15 μg modified mRNA encoding Luc in sucrose citrate saline into cardiac muscle could provide rapid Luc expression within 30 minutes. The expression peaked at 24 hours, persisted throughout 4 days and became negative by day-7 post-injection. However, intradermal injection of this mRNA could prolong the skin expression to more than 216 hours. In skeletal muscle, protein expression lasted more than 30 days (Carlsson et al., 2018). Thus, delivery of mRNA into different tissues might need further optimization in order to obtain the most effective protein production. Nevertheless, the transient controlled expression may be more important, as prolonged exposure of PDGF could lead to adverse effect on osteoblastic cell collagen production, differentiation and bone formation (Yu, Hsieh, Bao, & Graves, 1997).

To minimize the number of animals used in the study, control group was conducted only at one time point. The amount of PDGF-BB in group receiving sucrose citrate buffer alone at day-1 was used as a control or baseline level of PDGF-BB in rat gingiva to eliminate the bias from cross-reactivity of human PDGF-BB ELISA kit with rat PDGF-BB. Single intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer significantly increased the level of PDGF-BB protein production from baseline. This difference indicated the newly synthesized protein in gingival tissues after the mRNA administration.

Although VEGF production could be induced by PDGF, the patterns of expression between these two proteins were different. VEGF level peaks at a later timepoint after PDGF delivery. In periodontal lesion after stimulating with recombinant PDGF-BB, the level of VEGF was found to immediately increase, but peaked at day-12 to 15 (Cooke et al., 2006). In the present study, the levels of VEGF-A protein were assessed until day-7 after PDGF-BB delivery. Thus, the significant increase of VEGF-A expression might not be observed.

The result in this study demonstrated low toxicity and immunogenicity of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer with intragingival injection. Although the gingival levels of IL-6 and TNF-α were below the detection limit of the ELISA, the results were similar to the basal levels of both proinflammatory cytokines. The larger amount of protein sample could possibly increase the sensitivity of ELISA. The gingival tissues harvested from rat were, however, limited. Other assays, such as a PCR test, might be performed in further study to examine the difference in proinflammatory cytokines gene expression. Nevertheless, this preliminary data suggested that mRNA formulation used in this study did not provoke the local immune response. The results were in accordance with previous data showing that pseudouridine-modified mRNA could limit TLRs recognition due to the changes in its secondary structures (Kariko et al., 2008). Moreover, using sucrose citrate buffer as a delivery vehicle may benefit in reducing immunogenicity. Sucrose increases the viscosity of modified mRNA solution, thus prevents the accumulation of single-stranded modified mRNAs in the mixture. This accumulation might inhibit double-stranded modified mRNA translation, which could result in an immune activation via TLR-3 (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Together, these data support that modified mRNA in sucrose citrate buffer could be an effective approach for a transient localized mRNA delivery to gingival tissue *in vivo*. This preliminary study provides a set of data encouraging the potential of mRNA encoding PDGF-BB as therapeutic approaches in the field of tissue regeneration.

6. Conclusion

The present study provides *in vivo* evidence that intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer transiently yields rapid and robust PDGF-BB protein production and does not stimulate local tissue inflammation. These preliminary results suggest the potential of mRNA-based therapy application in periodontal regeneration. The larger sample size should be employed in further study.

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