The use of modified mRNA encoding platelet-derived growth factor-BB as an innovation in periodontal regeneration

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Abstract

Since mRNA platform has been recently introduced to several fields in medicine and the achievement of periodontal regeneration is currently unpredictable, the use of mRNA technology tends to be a promising approach to pursue the complete periodontal regeneration. This study aims to learn if mRNA encoding platelet-derived growth factor-BB (PDGF-BB) induces PDGF production in human periodontal ligament cells (PDLCs) as well as to investigate the effect of secreted PDGF on PDLCs proliferation. PDLCs were obtained from extracted teeth of healthy periodontal patients. The modified N1-methylpseudouridine mRNA encoding PDGF-BB were transfected into PDLCs. The supernatants were collected from 24-, 48- and 72-h time points and measured the protein production using ELISA. The viability of transfected PDLCs was also assessed. In addition, the supernatants collected at the 24-h time point were used for the proliferation assay using AlamarBlue. The result showed that PDLCs, transfected with mRNA encoding PDGF-BB, produced a higher level of intracellular PDGF-BB than controls at 24 h. PDGF-BB has also detected in the supernatants started from 24 h and constantly secreted up to 72 h. The transfection of mRNA encoding PDGF-BB did not affect PDLCs viability. The supernatants containing PDGF-BB were able to promote PDLCs proliferation. Thus, this mRNA platform technology is possibly applicable for periodontal tissue regeneration.

***Keywords**: Periodontal regeneration, mRNA, periodontitis, Platelet-derived growth factor-BB (PDGF-BB)*

1. Introduction

Periodontal disease is known as an immunity condition that responds to oral pathogens. The disease progression contributes to the tooth loss and subsequently affects an individual's functions including eating, speaking, aesthetics and the quality of life. In general, periodontal disease can be roughly divided into 2 major groups. The first group called "Gingivitis" can be found at any ages. The inflammation is confined within the gingiva. The other called "Periodontitis" commonly affects middle-aged and elderly people. The inflammation spreads down to periodontal structures, deepens periodontal pockets, loosens teeth later led to a loss of the dentition in severe cases. The severe form of periodontitis is considered as a major oral health problem ranked as the sixth among the most common chronic diseases in the world (Kassebaum et al., 2014). Even though periodontitis is not a life-threatening disease, it is considered as a huge public health burden which requires global attention.

The treatment of periodontitis is to eliminate dental plaque, the cause of the disease as well as other contributing factors, such as dental calculus. Current treatments are professional cleaning, along with the instruction of oral health care. In severe cases, deep plaque removal and bony defect correction will be further performed with the surgical approach. To the best of our knowledge, guided tissue regeneration (GTR) with or without bone substitution are acceptable approaches commonly used for periodontal regeneration.

The shortcoming is the difficulty of obtaining complete periodontal regeneration by GTR and bone grafting due to several limitations such as systemic conditions and patient's behavior, types of periodontal defects, previous restorations, and surgical techniques (Bashutski et al., 2011). Furthermore, both treatments were also considered to be high expense procedures (Kao et al., 2015, Avila-Ortiz et al., 2015).

During the past decade, tissue engineering has been introduced as an innovation to regenerate the destructed periodontium. There are three essential constituents: stem cells, scaffolds and signaling molecules. Periodontal ligament cells are a major source of mesenchymal stem cells in the oral cavity which has a regenerative potential (Bartold et al., 2000). To enhance the success in periodontal regeneration, mesenchymal stem cells have been used in the combination with either scaffold such as collagen, fibrin, hydrogel, and gelatin or non-scaffold materials such as cell sheets (Akizuki 2005). However, the uses of mesenchymal stem cells and cell sheets in this field are limited due to the lack of cell source, time-consuming and complicated procedures.

Growth factors are considered as one of the developed therapies for periodontal regeneration by stimulating stem cell proliferation and differentiation. For decades, numerous growth factors were used in regeneration (Kao et al., 2009). Platelet-derived growth factors (PDGFs) are known as a group of growth factors promoting tissue regeneration and wound healing (Dereka et al., 2006). PDGF consists of 3 forms: PDGF-AA, PDGF-BB and PDGF-AB. The use of recombinant human PDGF-BB (rhPDGF-BB) positively impacted on defect bone fill, bone height gain and clinical attachment gain in periodontal defects (Li *et al*, 2017) and provided comparable outcomes to GTR or bone grafts (Darby and Morris 2013). Food and Drug Administration (FDA) also granted for the use of 0.03% PDGF-BB in combination with β-tricalcium phosphate to treat intrabony defects, furcation defects and gingival recession (Suarez-Lopez Del Amo et al., 2015). Even though PDGF-BB has potential in stimulating PDL stem cells to proliferate and differentiate, the leakage of protein from the treated area as well as its high cost is major limitations.

Apart from using recombinant growth factors, the use of DNA in gene therapy has been introduced in treating several diseases. The gene therapy can be performed by processing plasmid DNA or viral vector. Foreign DNA is delivered to the nucleus by passaging through the cell and nuclear membranes. Foreign DNA is integrated into the host genome and sustained transgene expression even after host cells replicate (Kim and Eberwine 2010). Nonetheless, gene therapy should be used with caution due to safety issues including mutation and tumorigenesis (Kim and Eberwine 2010).

Recently, a new technique called "mRNA therapy" is introduced. Briefly, this technique is the use of specific mRNA delivered with a carrier into the cytoplasm of the patient's cell to achieve the process of translation for the desired protein. These proteins have functions or properties as signaling molecules or growth factors that induce cell proliferation and differentiation in the periodontal tissue regeneration. The process of mRNA technology is an advanced innovation that will transform the medical treatment base with biotechnology for safe and affordable treatment. There are many advantages that are better than DNA therapy. Due to good delivery efficiency and no risk of integration with the host genome (Kim and Eberwine 2010).

Currently, the use of mRNA has been developed by various methods to prevent mRNA degradation and increasing the effectiveness in protein expression. The encapsulation of mRNA enhances its stability and helps in uptake mRNA into the cell. The mRNA encapsulation or transfection can be performed in several forms such as lipids encapsulation (Mintzer and Simanek 2009), polymers (Pack et al., 2005) and peptides (Martin and Rice 2007). Furthermore, increasing the stability of mRNA by the gene sequence coding modification, such as 5′cap, 5 ′ and 3 ′ UTR and the length of poly-A, especially chemical modification of nucleosides, leads to the improvement of protein production (Zhang et al.,2019). In addition, to avoid the immune activation by modifying the base part of the mRNA was suggested. Particularly, the base adjustment at pseudouridine or N1-methylpseudouridine was able to inhibit the response of the innate immune system via type 1 interferon, and promoting protein production (Karikó et al., 2008, Andries et al., 2015).

As mentioned above, it is possible to introduce the advanced innovation in the development of mRNA technology platform for the medical tissue regeneration. This study will develop an *in vitro* design of the mRNA biometrics by selecting an mRNA which is encoded PDGF and modifying the bases which are the most effective in the expression in human periodontal ligament cells. This study will also explore

appropriate bioactivities such as human periodontal ligament cell proliferation, Vascular endothelial growth factor (VEGF) production and the ability to induce tube formation by endothelial cells.

2. Objectives

To learn if mRNA encoding PDGF-BB induces PDGF-BB production in human periodontal ligament cells (PDLCs) and investigate the effect of PDGF-BB induced by mRNA encoding PDGF-BB transfection on PDLCs proliferation.

3. Materials and Methods

3.1 Construction of mRNA encoding PDGF-BB

Nucleotide sequence of human PDGF-BB was designed by our immunology laboratory. In collaboration, the synthesis of N1-methylpseudouridine mRNA was provided by Dr. Norbert Pardi of University of Pennsylvania (Pardi et al., 2017, Pardi et al., 2018, Pardi et al., 2018).

3.2 Medium and reagents

Minimum Essential Medium with Alpha modification (Alpha MEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM GlutaMax-I, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Life Technologies) was used throughout the study. Opti-MEM I, Lipofectamine 2000 was purchased from Invitrogen. Human recombinant PDGF-BB was obtained from R&D Systems.

3.3 Cells isolation and culture

All participants were provided written informed consent. The study protocol was approved by the medical ethical committee from Chulalongkorn University, Faculty of Dentistry, Bangkok, Thailand.

Human periodontal ligament tissues from healthy periodontal patients (age 15-35 years) undergoing wisdom tooth extraction or tooth extraction due to orthodontic reason were obtained at Chulalongkorn Dental School. Human periodontal ligament cells (PDLCs) were separated from the tooth by enzyme-digestion method (Tanaka et al. 2011). Briefly, the tooth was extensively washed twice with Dulbecco's phosphate-buffered saline (DPBS) and the PDL tissues were scraped out from the middle third of the root under a sterile condition to avoid the contamination from gingival or periapical granulation tissues. Then, PDL tissues were minced into a fragment of 1-2 mm^2 and digested with a solution of 2 mg/ml collagenase and 2 mg/ml dispase for 60 minutes at 37^oC and then filtered through a 70-µm cell strainer. The pass-through was washed twice with culture medium. The PDLCs were cultured with the medium at 37^oC in a humidified atmosphere of 5% CO_2. After a confluent monolayer of cells was reached, PDLCs were trypsinized, washed and then sub-cultured. The cells from 3rd to 8th passages from 3 different donors were used in this study (Iwata et al., 2010).

3.4 *In vitro* cell transfection and expression/secretion of PDGF-BB protein

For *in vitro* transfection of cells, non-modified and modified mRNA encoding PDGF-BB was complexed with Lipofectamine® 2000 (Invitrogen) and transfected into PDLCs -according to manufacturer's instructions. To analyze the PDGF-BB protein production and secretion levels, the transfected cells were cultured for 24-72 h. Supernatants and cells were collected at 24, 48 and 72 h. The cells were lysed using RIPA buffer (Pierce® RIPA buffer, ThermoFisher Scientific) and the lysates were stored for further analysis. PDGF-BB production and secretion were analyzed using ELISA (Quantikine®, R&D System, Minnesota, USA). This experiment was performed in triplicates.

3.5 Cell toxicity

To analyze cell toxicity, PDLCs with either mRNA complexed with the transfecting agent or transfecting agent alone were incubated with 10% AlamarBlue solution (AlamarBlue®, BIO-RAD, California, USA) then incubated at 37^oC in a humidified atmosphere of 5% CO_2. After 4 h., cell culture supernatants were collected and measured the optical density at an absorbance of 570 nm using a

microplate reader (Epoch™, Biotek™, Vermont, USA) according to the manufacturer instruction. This experiment was also performed in triplicates.

3.6 The stimulation of PDLCs proliferation

To determine the effect of produced PDGF on PDLCs proliferation, PDLCs from three different donors were plated in 96-well plate ($3x10^3$ cells per well) and either control medium, supernatants of transfected PDLCs or recombinant PDGF-BB were added. After a 24h. incubation, 10% Alamar Blue solution was added. The cell proliferation ability was measured the optical density at an absorbance of 570 nm.

3.7 Statistical Analysis

Values were presented in mean with standard error. The data were analyzed using a computer program JMP 15.0 (SAS Institute Inc. NC, USA). Student's *t* test was used to determine the difference between the modified mRNA encoded PDGF-BB and the control groups. One-way ANOVA analysis was performed to determine the differences among time-points. *P* values less than 0.05 was regarded as statistically significant.

4. Results

4.1 Intracellular and extracellular PDGF-BB protein production

The amount of intracellular and extracellular PDGF-BB protein secretion was determined from periodontal ligament cell culture which was transfected with either N1-methylpseudouridine mRNA encoding PDG-BB with Lipofectamine 2000 or Lipofectamine 2000 alone. After transfection for 24 h., periodontal ligament cells were collected and digested to measure the amount of intracellular protein production. The result showed that the mRNA PDGF-BB transfected periodontal ligament cells were able to produce significantly higher intracellular proteins than the control at 24 h., with the mean of 155540.3 picograms per milliliter ($P < 0.05$) (Fig. 1A).

The supernatants from the cultures were collected at 24, 48 and 72 h. and taken to measure the amount of extracellular PDGF-BB protein secretion. the protein secretion was detected at 24 h. and continuously secretion up to 72 h., and the protein levels were higher than the control group. The mean concentrations of PDGF-BB were 50,533.33, 73,716.67 and 76,450 picograms per milliliter at 24, 48 and 72 h., respectively (Figure 1B). At each time-points, the protein levels detected in PDGF-BB mRNA group were significantly higher than the control ($P < 0.05$). Despite the fact that, the differences in PDGF-BB production between the time-point was not significant.

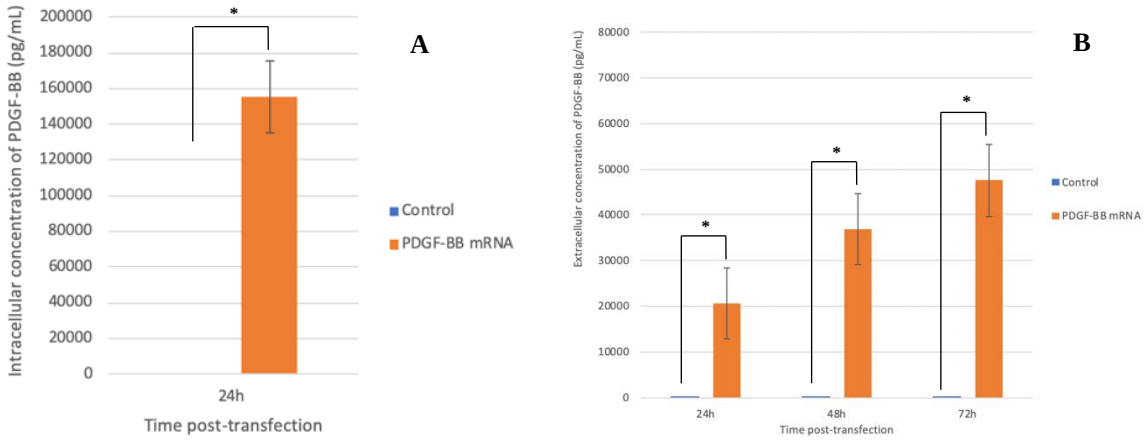

Figure 1 The production of PDFG-BB protein. Human PDLCs at $1x10^5$ cells/well were transfected with 2 µg of PDGF-BB mRNA in Lipofectamine 2000. Culture supernatants were analyzed by ELISA.

A; The mean concentration of intracellular PDGF-BB at 24 h.

B; The mean concentration of extracellular PDF-BB at 24, 48, and 72 h. Data shown are mean ± SE (n=3). *, = significant difference between the mRNA and the control groups (p<0.05)

4.2 Cell viability after transfection with N1-methylpseudouridine modified mRNA encoding PDG-BB with Lipofectamine 2000

The PDCLs were transfected with modified mRNA encoded PDGF-BB, and the cells were then harvested at 24, 48 and 72 h. for analyzing viability using Alamar Blue assay. The results showed that the mRNA encoded PDF-BB did not affect the viability of periodontal cells. At each time-point, the percentages of cell viability were comparable between these two groups. In addition, it showed that cell viability was still greater than 90 percent after 72 h. and similar to the controls (Figure 2)

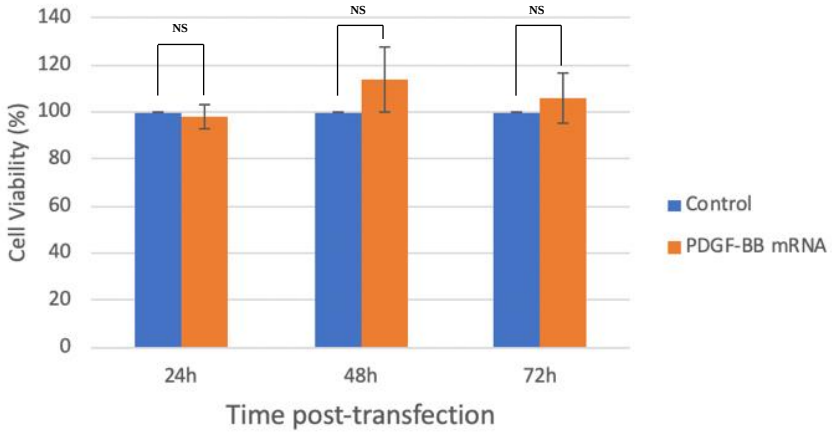

Figure 2 The mean percentage of periodontal cell viability at 24, 48 and 72 h.. Human PDLCs (1x10^5 cells/well) were transfected with 2 µg of PDGF-BB mRNA in Lipofectamine 2000. At each time-point, the cell viability of PDLCs was assessed by AlamarBlue assay. Data shown were mean ± SE (n=3). NS = not significant.

4.3 Biological activity of translated PDGF-BB protein

The periodontal cells were incubated with the clear part of the periodontal cells transfected with a modified mRNA encoded PDGF-BB at 48 h. After 48 h., AlamarBlue was added and cultured for another 4 h. At 48 h. of incubation, the supernatants from periodontal cells transfected with a modified compound of mRNA encoded PDGF-BB stimulated the periodontal ligament cell proliferation. The percentage of cell proliferation in the transfected cell group was greater than the control at 48 h. (P< 0.05) (figure 3).

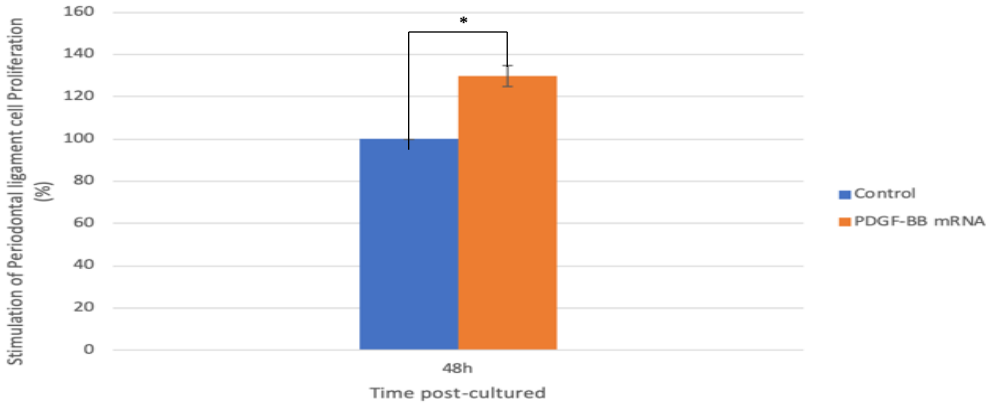

Figure 3 Biological function of PDGF-BB protein translated from mRNA *in vitro*. PDGF-BB produced from mRNA transfection were assessed for cell proliferation. Data shown are mean ± SE (n=3). *, *Significant difference between the mRNA and the control groups ($p<0.05$).*

5. Discussion

This study is the first study using mRNA technology for periodontal regeneration. The cells used in the study are periodontal ligament cells since they have high stem cell potentials. Periodontal ligament cells differentiate into a variety of cell lineages that resembles periodontal ligament and cementum, which is an important target organ for periodontal regeneration. However, the limitation of the study is that the use of periodontal ligament requires tooth extraction (Seo et al., 2004, Chen et al., 2006, Yang et al., 2013).

The success of cell transfection with mRNA implied from our previous experiments that used the same transfecting agent (Lipofectamine 2000). In the previous experiment, periodontal ligament cells were transfected with mRNA encoding green fluorescence protein (GFP) and the results revealed that transfection efficacy of mRNA encoding GFP complexed with Lipofectamine 2000 was over 90% (unpublished data).

According to this *in vitro* experiment, it indicated that mRNA specific to PDGB-BB stimulated target cells (periodontal ligament cells) and effectively induced PDGF-BB in large amount (50,000 pg/ml). The PDGF-BB was released extracellularly at 24 h. and continuously released until 72 h.. Similar to the used of rh PDGF-BB, the amount of protein was released at 24 h. and continuously releasing until day 7 after stimulation (K.U. Zaman et al., 2006). Although there was no previous study in using mRNA specific to PDGF-BB, there was a study using modified mRNA encoding BMP-2 in muscle-derived mesenchymal stem cells. (Zhang et al., 2019) This previous study showed that BMP-2 production was released at first 24 h. and continuously declined. However, cells remained producing and releasing BMP-2 up to Day 6. While our study showed that mRNA specific to PDGF-BB stimulated PDLCs to produce PDGF-BB in relatively large amount; moreover, the production lasted for a longer period.

This *in vitro* study revealed secreted PDGF-BB after stimulating with mRNA had greater ability in inducing PDLCs proliferation than the control group at 48 h.. Like using the recombinant, studies showed that PDGF-BB induced PDL cells proliferation (Mailhot et al., 1996) (K.U Zaman et al., 2006).

Since this *in vitro* study using mRNA platform as a delivery system, not only it stimulated high amount of protein and also a long period of time, but the platform did not affect cell viability of PDL cells. While using plasmid DNA specific to the PDGF-B gene imported into PDL cells showed significantly lower cell viability compare to the controls (Plonka et al.,2017).

6. Conclusion

This study shows that the use of mRNA encoding PDGF-BB can deliver to the periodontal ligament cells and stimulate the cells to produce PDGF-BB without affecting the cell viability. Moreover, produced PDGF-BB stimulates the periodontal ligament cell proliferation. The result of this study will be useful for further *in vivo* studies and clinical trials to acquire the foundation of mRNA technology to restore affected periodontal organs.

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8. References

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