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Dual delivery of PDGF and simvastatin to accelerate periodontal regeneration *in vivo*

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ABSTRACT

The emphasis on periodontal regeneration has been shifted towards the harmonization of bioactive molecules and physiological phases during regeneration. This study investigated whether the combination and sequential-release of platelet-derived growth factor (PDGF, mitogen) and simvastatin (differentiation factor) facilitated periodontal regeneration. PDGF and simvastatin were encapsulated in double-walled poly-(D,L-lactide) and poly-(D,L-lactide-*co*-glycolide) (PDLLA-PLGA) microspheres using the co-axial electrohydrodynamic atomization technique. Critical-sized periodontal defects on rat maxillae were filled with microspheres encapsulating BSA-in-core-shell (BB), PDGF-in-shell (XP), simvastatin-in-core and BSA-in-shell (SB), simvastatin-in-core and PDGF-in-shell, or unfilled with microspheres (XX), and examined at 14 and 28 days post-operatively. The resultant microspheres were around 15 μm diameter with distinct core–shell structure, and the fast-release of PDGF followed by slow-release of simvastatin was noted in the SP group. The SP group demonstrated significantly greater bone volume fraction and decreased trabecular separation compared to the XX group at day 14, and milder inflammatory cells infiltration and elevated tartrate-resistant acid phosphatase level were noted at day 28. Fibers were also well-aligned and obliquely inserted onto the root surface similar to native periodontal ligament with signs of cementogenesis in the SP group. In conclusion, the combination and sequential-release of PDGF-simvastatin accelerates the regeneration of the periodontal apparatus.

1. Introduction

It is still a substantial challenge for the dental profession to achieve predictable and long-term stable results in terms of the recovery of lost periodontal apparatus, regardless of the techniques and surgical approaches employed [1]. Periodontal regeneration requires proper integration of appropriate signals, cells, blood supply and scaffold, in parallel with physiological phases [2]. As such, the utilization of bioactive molecules, such as enamel matrix derivatives, platelet-derived growth factor (PDGF), and bone morphogenetic protein (BMP), with a suitable carrier has gained popular attentions since the last few decades [2,3].

Regeneration can be divided into the early phase of proliferation and the late phase of differentiation, with specific growth factors involved in each phase [4,5]. It is unlikely that a single bioactive molecule will be able to activate the entire regeneration process. A combination of two or more bioactive molecules has been suggested to “mimic the critical aspects” of key events and consequently accelerate the regeneration process [6]. In this sense, combinational approaches, such as PDGF plus insulin-like growth factor (IGF) [7], PDGF plus vasculo-endothelial growth factor (VEGF) [8], and BMP plus IGF [9], has been investigated to promote regeneration. However, regeneration was not always promoted under the combinational approaches, suggesting that harmonization of the dose and time frame of molecule release may play a crucial role in the success of regeneration [10].

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By using the co-axial electrohydrodynamic atomization (CEHDA) technique, we have developed a double-walled poly-(D,L-lactide-co-glycolide)-poly-(D,L-lactide) (PLGA-PDLLA) hybrid microsphere encapsulating PDGF, a potent mitogen, and simvastatin, an anti-hyperlipidemia drug as well as an osteogenic differentiation factor, with parallel or sequential release properties [11]. Our recent investigations demonstrated that sequential PDGF-simvastatin release reduced heat-induced osteonecrosis caused by the implant site preparation [12] and promoted osteogenesis of alveolar bone defects [13]. However, the regeneration of cementum and functionally oriented periodontal ligament (PDL) was only noted under PDGF treatment [5,14]. Thus, in the present study, we aimed at investigating whether periodontal regeneration could be facilitated by the combination of PDGF and simvastatin with the releasing time frame parallel to the physiological phases of regeneration.

2. Materials and methods

2.1. PLGA-PDLLA microspheres

Poly-(D,L-lactide-co-glycolide) (PLGA, Mw: 31,300–43,500) and poly-(D,L-lactide) (PDLLA, Mw: 24,300–75,000) were chosen to fabricate the main components of microspheres according to their good biocompatibility and biodegradable nature, and distinctly different degradation rate, and the double-walled PLGA-PDLLA microsphere microspheres were manufactured using the co-axial electrohydrodynamic atomization (CEHDA) technique as previously described [11]. Briefly, 10% PDLLA and 10% PLGA were dissolved in dichloromethane to formulate the respective matrix solutions respectively. As for loading of the biomolecules, 1 mg simvastatin was dissolved in corresponding matrix solution. On the other hand, PDGF or BSA was first dissolved in DI water and added to the matrix solution.

The mixture was sonicated at 20–30% amplitude for about 60 s with Sonics Vibra cell to form emulsion and then transferred to a co-axial double-channeled needle. The inner channel was occupied with PDLLA to constitute the core compartment of the microsphere, and the outer channel was occupied with PLGA to constitute the shell compartment. Two programmable syringe pumps were used to inject the solutions in the inner and outer channel at a specific rate respectively, and the process was stabilized using a fixed nozzle voltage at 6.5 V and ring voltage at 3.5 kV. The resultant microspheres collected on the aluminum foil were then kept in a freeze-dryer for 3 days.

A total of four types of microspheres were investigated in this study: (i) BSA-in-core-shell (BB), (ii) simvastatin-in-core and BSA-in-shell (SB, simvastatin alone), (iii) PDGF-in-shell (XP, PDGF alone), and (iv) simvastatin-in-core and PDGF-in-shell (SP, Sequential PDGF-simvastatin release).

2.2. Characterization of the microspheres

The surface morphology of the microspheres was examined by a scanning electron microscopy (SEM) (JSM 5600LV, JEOL Technics, Tokyo, Japan), and the particle dimension was analyzed by the SMILEVIEW software (Bioprecision Diagnostics, Somerset, UK). The core–shell structure of the microspheres was assessed by the incorporation of coumarin 6 as the loading molecules in the matrix solution of the outer compartment under a confocal laser scanning microscope (Zeiss LSM 510).

The encapsulation efficiency of the loaded biomolecules was examined by dissolving the microspheres in dichloromethane and PBS. Phase separation occurred after 20 min of centrifuge under 9000 rpm, and PDGF or BSA in the aqueous phase was quantified by ELISA, whereas simvastatin in the organic phase was quantified by HPLC. The *in vitro* release profiles of loaded molecules was evaluated by placing microspheres in an orbital shaker bath under 37 °C and 120 rpm, and the incubated medium was withdrawn and replaced at days 1, 3, 5, 7, 10, and 14. The concentration of PDGF and simvastatin in the resultant medium was quantified by ELISA and HPLC respectively.

2.3. Animal Model Design

All of the procedures performed on the animals followed protocols approved by the Institutional Animal Care and Use Committee of National Taiwan University (20130072). Sixty 4-week-old male Sprague–Dawley rats were utilized (n = 6/treatment/time point based on the Power Analysis); the study design is illustrated in Fig. 1A. Maxillary first molar (M1) from one randomly selected side of the maxillae was removed. After 4 weeks of socket healing, a critical-sized periodontal defect was created on the mesial aspect of the second molar (M2) as previously described (Fig. 1B, C) [5]. Briefly, the defect was 2.0 mm diameter and 1.0 mm depth created by a customized drill with 0.6 mm distance to M2, and the residual bone and cementum on mesial aspect of M2 was manually removed. The defects were filled with BB, XP, SB, or SP microspheres or unfilled with microspheres (XX), and finally closed with Histoacryl® (TissueSeal LLC., Ann Arbor, MI, USA). Animals were covered by ampicillin for 7 days and euthanized at days 14 or 28. The maxillae containing the defects were harvested and fixed in 10% formaldehyde for micro-computed tomography (micro-CT) and histological examinations.

2.4. Micro-CT assessments

The specimens were examined using Siemens Inveon CT System (Siemens Healthcare, Erlangen, Germany) with an effective pixel size of 19 * 19 * 19 μm^3. After the image reconstruction, the image was then re-oriented based on the following criteria: 1) in the transverse plane, the crowns of the second molar (M2) to the third molar (M3) were centrally and vertically positioned; 2) in the sagittal plane, the occlusal surfaces of M2 and M3 were aligned horizontally; and 3) in the frontal plane, the occlusal surface of M2 was aligned horizontally. The entire osteotomy site was selected semi-automatically as the region of interest (ROI) using a customized software (courtesy of Mr. Jason Lim), and the fragments of the root in the ROI were manually excluded by a single-blinded assessor (LYC).

The micro-morphometric bone parameters, including bone volume fraction (BVF), bone mineral density (BMD), tissue mineral density (TMD), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N), were analyzed respectively by using the CTAn software (Skyscan, Kontich, Belgium).

2.5. Histological examinations

After fixation, the maxillae were decalcified with 12.5% ethylenediaminetereacetic acid, cut mesiodistally through the mid-points of M2 and M3, embedded in paraffin, and stained with hematoxylin and eosin. The fiber insertion and collagen matrix deposition on the root surface were evaluated by Masson's Trichome stain, and bone resorptive activity was determined by tartrate-resistant acid phosphatase (TRAP). All of the histological images were acquired utilizing Leica DMD108 system (Leica Microsystems GmbH, Wetzlar, Germany).

Quantitative measurements of cells were performed under 400×. Inflammatory cells were counted from 5 randomly selected areas within the defect. Osteoblasts and osteoclasts (TRAP-positive cells) were counted from 5 randomly selected areas within 30 μm from bone surface in the center of the defect (Fig. 1B). The results were presented as the fraction of inflammatory cells, osteoblasts, or TRAP-positive cells relative to the total amount of cells of the investigated area.

On the instrumented root surface of M2 (Fig. 1C), cementogenesis was measured as the total length of new mineral-like tissue under 400×, and the angulation of the periodontal ligament-like fibers to the instrumented root surface was evaluated by ImageJ software (NIH, USA) under 200× as previously described [15]. The angulation of native periodontal ligament fibers to the un-instrumented root surface in the XX groups at day 28 was measured as the reference.

2.6. Statistical analysis

One-way ANOVA followed by Tukey's *post hoc* test were used to compare the differences between the controls and microsphere-delivery groups at each time point. The data are presented as the mean ± standard deviation (SD) of measurements, with a p value less than 0.05 considered statistically significant.

3. Results

3.1. Characterization of the microspheres

The particle diameter of resultant microspheres was around 15 μm, regardless of the carried molecules, and the encapsulation efficiency was about 60–70% for the shell compartment and 80–90% for the core compartment (Table 1). The distinct core–shell structure was clearly demonstrated under the confocal microscope (Fig. 2A). The *in vitro* release profile varied according to the mode of biomolecules encapsulation. In the SB design, 17.68 ± 2.31% simvastatin was released in the first day, and the release profile was apparently slower until day 14 (<3%/day, Fig. 2B). However, In the XP design, a relatively fast-release of PDGF was noted in the XP design, whereas approximately 82% PDGF was released at day 10 (Fig. 2C). The SP design demonstrated a sequential release profile, which is characterized by a fast release of PDGF and a slow release of simvastatin (Fig. 2D).