Optimization of Recombinant Human Platelet-Derived Growth Factor-BB Encapsulated in Poly (Lactic-co-Glycolic Acid) Microspheres for Applications in Wound Healing

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Growth factors play multiple and critical roles in wound repair processes. Platelet-derived growth factor (PDGF) is a potent growth factor that is particularly important in the early inflammatory phase of wound healing. In order to extend the half-life of PDGF, polymeric encapsulation is used. In the current study, Poly (lactic-co-glycolic acid) (PLGA) microspheres containing recombinant human (rh) PDGF-BB were prepared to prolong the effectiveness of this growth factor. PLGA microspheres were optimized using a modified w/o/w-double-emulsion/solvent evaporation method by changing the processing conditions of stirring speed and emulsifier (polyvinyl alcohol) concentration. Microspheres prepared using the optimized method released rhPDGF-BB for up to three weeks. An *in vitro* migration assay showed a significant decrease in the wound area in cells treated with rhPDGF-BB microspheres compared to control cells. These findings demonstrate the potential of rhPDGF-BB encapsulated in microspheres to enhance wound healing. **Tissue Eng Regen Med 2016;13(1):13-20**

Key Words: Recombinant human platelet-derived growth factor-BB; Poly (lactic-co-glycolic acid) microsphere; Wound healing; Human immortalized keratinocyte cell line; Migration

INTRODUCTION

Soft tissue repair is a well-orchestrated, complex process involving a series of cellular and molecular interactions that, in part, are regulated by locally acting polypeptide growth factors [1]. Wound healing can be roughly divided into three overlapping phases: inflammation, proliferation, and remodeling. Wound healing processes are strictly regulated by multiple growth factors and cytokines released at the wound site. In an acute wound, activated neutrophils are virtually nonexistent, whereas in a chronic wound neutrophils are present throughout the healing process. The large number of activated neutrophils found in a chronic wound leads to excessive amounts of

degradative matrix metalloproteinases. As a result, an abnormal ratio of degradative to protective enzymes ensues, inflammatory cytokines predominate, and concentrations of factors that promote proliferation and healing, such as platelet-derived growth factor (PDGF), are reduced [2,3].

Growth factors are defined as agents that promote cell proliferation and metabolism through their interaction with specific membrane-bound receptors. In addition, these factors may induce the migration of cells into the wound space, serving as chemoattractants to recruit important cells such as leukocytes and fibroblasts [1]. During normal wound healing, PDGF is a mitogen for various cell types including fibroblasts and smooth muscle cells [4]. PDGF purified from platelets accelerates wound healing by stimulating the chemotaxis and proliferation of fibroblasts, smooth muscle cells, neutrophils, and macrophages [5]. PDGF is stored in the alpha-granules of circulating platelets and is released at wound sites during blood clotting. Subsequently, PDGF may be synthesized by infiltrat-

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ing macrophages, epithelial cells and fibroblasts at cutaneous wound sites [6,7].

Advances in molecular biology have made possible the production of highly purified recombinant human (rh) proteins. rh growth factors have emerged as potential therapeutic wound healing agents. rhPDGF-BB (the homodimer of the PDGF B subunit) has quickly emerged as a leading candidate for clinical trials. In animal models, rhPDGF-BB demonstrated wound healing activity, predominantly by enhancing the formation of granulation tissue. However, despite its superior functions in enhancing tissue regeneration, intravenously injected PDGF is rapidly cleared by the circulation resulting in a half-life of less than two minutes *in vivo* [8,9]. Thus, high or repeated doses of PDGF must be administered frequently to maintain sufficient therapeutic concentrations to help repair soft tissue defects. Due to these disadvantages, research is needed to develop an improved delivery system for the effective use of growth factors to promote tissue regeneration.

One way to increase the therapeutic efficacy of growth factors is to encapsulate them in a dosage form that is capable of releasing the macromolecule continuously at a controlled rate [10]. Biocompatible and biodegradable polymers such as poly (L-lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactic-co-glycolic acid) (PLGA), and polycaprolactone have been used as drug carrier materials for many years. Biodegradable microspheres that encapsulate proteins have gained importance as preferred dosage forms in the field of protein delivery [11,12]. PLGA is degraded by hydrolysis into biocompatible byproducts, including lactic and glycolic acid monomers. PLGA microspheres can be readily fabricated using the double-emulsion solvent-extraction technique. This technique allows the control of sphere diameter and degradation kinetics, while maintaining the stability and bioactivity of the encapsulated growth factors [13-15]. However, microencapsulation of drugs by solvent evaporation techniques remains challenging, especially with materials having a low yield and encapsulation efficiency.

The aim of the current study was to prepare microspheres optimized to have a high yield and encapsulation efficiency. Biocompatible and biodegradable PLGA microspheres were prepared via the double emulsion solvent evaporation method as a way to overcome the disadvantages of microspheres made of a single polymer [16,17]. In addition, formulation conditions to maximize the yield and encapsulation efficiency of microspheres were determined by changing the stirring speed and emulsifier [polyvinyl alcohol (PVA)] concentration. Scanning electron microscopy was used to characterize the size and surface morphology of the microspheres. *In vitro* release kinetics of encapsulated rhPDGF-BB were assessed by quantitative analyses of the rhPDGF-BB released from optimized microspheres.

Cytotoxicity and *in vitro* migration induced by free and encapsulated rhPDGF-BB were measured using a human immortalized keratinocyte cell line (HaCaT). The results indicate that encapsulated rhPDGF-BB is non-toxic, exhibits bioactivity, and may become a superior candidate for regenerative therapeutics.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used were analytical or clinical grade unless otherwise stated. PLGA copolymers (50:50; Resomer RG 505, MW 78000 g mol⁻¹) were purchased from Evonik (Krefeld, Germany). PVA (MW 9000–11000 g mol⁻¹) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (Junsei Chemical, Tokyo, Japan) and deionized distilled water were used as solvents. rhPDGF-BB was provided by the Korea Institute of Industrial Technology. The rhPDGF-BB ELISA kit was purchased from Abcam Inc (Cambridge, MA, USA). Media and reagents for cell culture were purchased from Gibco BRL (Gaithersburg, MD, USA).

Preparation of rhPDGF-BB encapsulated in PLGA microspheres

rhPDGF-BB was encapsulated into PLGA microspheres using the water-in-oil-in-water (W₁/O/W₂) microencapsulation method (Fig. 1). Briefly, 100 µL of the rhPDGF-BB buffer solution (rhPDGF-BB in 10 mM sodium acetate buffer with pH=6.3) was emulsified into 1.5 mL of PLGA/dichloromethane solution to form the primary water-in-oil (W₁/O) emulsion. The W₁/O emulsion was then poured into aqueous 0.5% (w/v) PVA solutions with stirring at 500, 600, 700, and 800 rpm, respectively. And the W₁/O emulsion was then poured into aqueous 0.1, 0.3, 0.5, and 0.7% (w/v) PVA solutions respectively with stirring at 600 rpm. This resulted in W₁/O/W₂ double emulsions. The solution was then stirred at room temperature for at least 3 hours to evaporate the dichloromethane. The microspheres were harvested by centrifugation, washed with distilled water to remove PVA remaining on the microsphere surface, and lyophilized to a dry powder. The collected microspheres were stored at -20°C.

Characterization of the microspheres

The morphology of rhPDGF-BB encapsulated in PLGA microspheres prepared with various stirring speeds and PVA concentrations was observed by scanning electron microscopy (SU 8020, Hitachi Co., Tokyo, Japan). One drop of the microsphere suspension was placed on a metallic surface. After drying under vacuum, the sample was coated with a gold layer. Observation of microspheres was carried out at 1 kV.

Determination of rhPDGF-BB encapsulation efficiency

Microspheres (10 mg) were dissolved in 400 μ L of dichloromethane, vortexed for 3 minutes, and added to 400 μ L of Dulbecco's phosphate buffered saline (PBS) to extract encapsulated rhPDGF-BB. The amount of rhPDGF-BB was measured using an ELISA kit according to the manufacturer's instructions. Encapsulation efficiency was calculated as the percentage of encapsulated rhPDGF-BB relative to the total initial amount of rhPDGF-BB used for microsphere formulation.

In vitro release studies

The *in vitro* rhPDGF-BB release profile was determined by incubating 50 mg of microspheres in 1 mL of PBS (pH 7.4) at 37°C with orbital rotation at 100 rpm. The medium was removed by centrifugation and replaced with the same quantity of PBS at predetermined time intervals [18]. Samples were taken at specified times for 21 days, and the quantitative analysis of rhPDGF-BB in the medium was measured by an ELISA.

Cell culture

HaCaT cells were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 500 U/mL penicillin, and 500 μ g/mL streptomycin at 37°C under 5% CO₂. Confluent cells were treated with 0.25% trypsin/EDTA (Invitrogen, Karlsruhe, Germany) and centrifuged at 1000 $\times$ g for 5 min to harvest the cells.

Cytotoxicity of PLGA and rhPDGF-BB

The toxicity of PLGA microspheres with and without encapsulated rhPDGF-BB was tested with the MTS cell viability

assay (Promega, Madison, WI, USA). HaCaT cells (2×10^5) were seeded in a 24-well plate and cultured as described above. After 24 hours, microspheres (10, 30, or 50 mg per well) were added. The MTS assay was performed after 24 hours of sample exposure with absorbance being measured at 490 nm.

In vitro wound migration assay

Six-well plates were seeded with HaCaT cells (1×10^6 cells/well) in complete medium and cultured at 37°C under 5% CO₂ for 48 hours to form a confluent monolayer. Gaps, approximately 500 μ m in width, were created by scratching the plates with a sterile pipette tip [19]. After washing with PBS, 50 mg of microspheres were added to the cells in fresh serum-free media. The average width of the gaps at 0, 8, and 24 hours after wound creation was calculated from the photographic microscopic images at the same sites in each well using NIS Element software.

Statistical analyses

All data are expressed as means $\pm$ standard deviation (n=3). One-way analysis of variance was used to evaluate differences among means. Specific group differences were determined by Duncan's multiple range tests at the 95% confidence level. Statistical analyses were performed using SAS software (Window version 8, SAS Institute Inc., Cary, NC, USA).

RESULTS

Optimization of the PLGA microsphere formulation

Choosing a suitable polymer for microsphere formulation via the double emulsion solvent evaporation method is a cru-

Figure 1. Schematic diagram for the formulation of rhPDGF-BB-loaded PLGA microspheres. rh: recombinant human, PDGF: platelet-derived growth factor, PLGA: poly (lactic-co-glycolic acid), PVA: poly (vinyl alcohol), DCM: dichloromethane, W₁/O: water-in-oil, W₁/O/W₂: water-in-oil-in-water.

cial factor for success. In this study, PLGA was used as the main encapsulation matrix as it is safe and approved by the USA Food and Drug Administration (FDA) for use in humans [20].

Table 1. Yield and encapsulation efficiency of rhPDGF-BB PLGA microspheres

	Microspheres yield (%)	Encapsulation efficiency (%)
rpm (PVA 0.5%)		
500	46.9±6.7*	8.33±0.84†
600	51.5±5.5*	14.3±0.4†
700	50±1.5*	12.8±2.2*
800	46.8±2.8*	10.7±0.3‡
PVA concentration (%) (600 rpm)		
0.1	36.3±4.6†	14.1±4.5*
0.3	51±6.5*	12.6±4.7*
0.5	60±11*	18.7±1.4*
0.7	59±3.5*	11.7±4.8*

rhPDGF-BB encapsulation efficiency was highest at 600 rpm and 0.5% (w/v) PVA (18.7±1.4%). The maximal microsphere yield was 120±22 mg under these same conditions. All data are presented as mean ± standard deviation (n=3). Means with *, †, ‡ in the columns are significantly different by Duncan's multiple range tests ($p<0.05$). rh: Recombinant human, PDGF: platelet-derived growth factor, PLGA: poly (lactic-co-glycolic acid), PVA: poly (vinyl alcohol)

We investigated the optimum conditions to encapsulate rhPDGF-BB into microspheres by varying conditions of stirring speed and PVA concentration. The formulation yields using 0.5% PVA concentration ranged from 93.5±5.6 to 103±11 mg depending upon the stirring speed (Table 1). The highest microsphere yield using the double emulsion solvent evaporation method was obtained at 600 rpm. Encapsulation efficiency, measured using the rhPDGF-BB ELISA kit, was also maximal at 600 rpm (14.3±0.4%) compared to the lowest value at 500 rpm (8.3±0.8%).

When making microspheres, surfactant is a crucial factor to maintain microsphere size and emulsifying capacity when forming the secondary emulsion. PVA was used as the surfactant emulsifier in the current study. We investigated encapsulation yield and efficiency when microspheres were prepared in 600 rpm using different PVA concentrations (0.1%, 0.3%, 0.5%, and 0.7%). The highest yield of microspheres (120±22 mg) was achieved when using 0.5% (w/v) PVA. Encapsulation efficiency was also maximal (18.7±1.4%) when using 0.5% PVA. Encapsulation efficiency was lowest with 0.7% PVA (11.7±4.8%). An encapsulation efficiency of 9.5±0.3% was reported previously for rhPDGF-BB encapsulated in microspheres using a PLGA 50:50 ratio [21]. In the current study, the encapsulation efficiency of microspheres prepared at 600 rpm with 0.5% (w/v) PVA was more than double this value. Based on these results,

Figure 2. Shape and surface morphology of rhPDGF-BB-loaded microspheres under various formulation conditions. (A and B) Microspheres produced at 500 rpm, 600 rpm, 700 rpm, and 800 rpm in 0.5% PVA concentration. (C and D) Microspheres produced with 0.1, 0.3, 0.5, and 0.7% (w/v) PVA in 600 rpm. The mean size of fabricated microspheres decreased with increasing rpm and increased with increasing PVA concentration. The diameter of the microspheres was affected by both rpm and PVA concentration. All data are presented as mean±standard deviation (n=3). Means with *, †, ‡, § in the columns are significantly different by Duncan's multiple range tests ($p<0.05$). rh: recombinant human, PDGF: platelet-derived growth factor, PVA: poly (vinyl alcohol).

we conclude that the stirring speed and PVA concentration are important factors for the microsphere formulation process.

Characterization of microspheres

The morphological characteristics of PLGA microspheres with encapsulated rhPDGF-BB were observed by scanning electron microscopy (Fig. 2). At 500 rpm, the mean microsphere particle size was $45.7 \pm 10.8 \mu\text{m}$, which was the smallest size obtained. The largest mean particle size was obtained at 700 rpm ($62.0 \pm 14.7 \mu\text{m}$). Microsphere size was maximal at 0.7% (w/v) PVA and was over four times the smallest size seen with 0.1% (w/v) PVA. Based on these results, microsphere formulations, including yield and particle size, can be controlled ac-

cording to the stirring speed and PVA concentration. rhPDGF-BB containing microspheres prepared under various conditions exhibited a smooth surface and completely spherical shape. Further studies were performed using optimized microspheres prepared at 600 rpm with 0.5% (w/v) PVA, which maximized the yield and encapsulation efficiency.

In vitro release of rhPDGF-BB from microspheres

The release kinetics of rhPDGF-BB encapsulated within microspheres are illustrated in Figure 3. In many cases, release profiles are complicated and often contain two main expulsion

Figure 3. Release kinetics of encapsulated rhPDGF-BB from PLGA microspheres. The amount of rhPDGF-BB released from PLGA microspheres during the initial burst was 66.9% of the total encapsulated, increasing to 74.0% after 21 days. Microspheres are capable of releasing encapsulated bioactive rhPDGF-BB in a sustained fashion for prolonged periods of time ($n=3$, $p<0.05$). rh: recombinant human, PDGF: platelet-derived growth factor, PLGA: poly (lactic-co-glycolic acid).

Figure 4. Viability of HaCat cells. (A) HaCat cells were treated with different concentrations of rhPDGF-BB. (B) HaCat cells were treated with different quantities of PLGA microspheres encapsulated with rhPDGF-BB or PBS. Cell viability was determined by the MTS assay. Viability is expressed as a percentage of the controls. Data are means $\pm$ SD ($n=3$). *, † on the bar graphs indicate a significant difference by Duncan's multiple range test ($p<0.05$). rh: recombinant human, PDGF: platelet-derived growth factor, PLGA: poly (lactic-co-glycolic acid), PBS: phosphate-buffered saline, MS: microspheres. MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

processes; the first being an initial burst of expelled medication from the sphere surface and the second a more constant release rate dependent on diffusion and degradation [22-24]. The release of rhPDGF-BB during the first hour was $23.7 \pm 1.3\%$ of the total drug encapsulated; we attribute this to pore-dependent diffusion. After 24 hours, $66.8 \pm 3.2\%$ of the total rhPDGF-BB was released. The release of encapsulated rhPDGF-BB continued for 21 days totaling $75.4 \pm 3.5\%$. These data confirm that the release behavior is sustainable.

Cytotoxicity of PLGA and rhPDGF-BB

Both PLGA, the main encapsulation matrix, and rhPDGF-BB, the drug used for microsphere formulation, are approved by the FDA [25]. However, toxicity testing was done because of

the use of the organic solvent dichloromethane in the microsphere formulation process. MTS assays with the HaCaT cell line were performed on control cells, cells treated with PBS encapsulated in microspheres, and cells treated with rhPDGF-BB encapsulated in microspheres. The toxicity of rhPDGF-BB alone was also assessed. None of the treatments produced any toxicity relative to control cells (Fig. 4).

In vitro migration assay for wound healing

An HaCaT cell monolayer, scratched to simulate a wound, closed more rapidly in the presence of rhPDGF-BB microspheres than the control cells or cells treated with PBS encapsulated in microspheres (Fig. 5). At 24 hours, the extent of wound closure was 67.9% for the control cells and 61.5% for

Figure 5. *In vitro* migration assay. Images were obtained at 0, 8, and 24 h after wound creation. Control: fresh serum-free medium. PBS MS: 50 mg of microspheres encapsulated with PBS in serum-free DMEM medium. rhPDGF-BB MS: 50 mg of microspheres encapsulated with rhPDGF-BB in serum-free DMEM medium. Cells treated with rhPDGF-BB exhibited more rapid wound closure than the control groups (magnification $\times 100$). rh: recombinant human, PDGF: platelet-derived growth factor, PBS: phosphate-buffered saline, MS: microspheres, DMEM: Dulbecco's modified Eagle's medium.

cells treated with PBS microspheres. Complete closure of the scratches was achieved by 24 hours in the cells treated with rhPDGF-BB microspheres. There was no change in cell morphology in this latter group. In contrast, the polygonal morphology began to disappear in control cells treated with serum-free medium. These results are similar to those of Park et al. [26] who found that a high degree of cell migration was observed in response to PDGF-BB solutions compared to α -MEM control media. Overall, these results indicate that the use of rhPDGF-BB encapsulated in microspheres is effective in enhancing cell migration and wound healing.

DISCUSSION

In this study, microspheres using PLGA were prepared for the sustained release of rhPDGF-BB for use in wound healing applications. The hydrophobicity of the polymer is increased at a higher ratio, while the solubility of the polymer is decreased at a lower ratio. Thus, it is important to select an appropriate ratio of polymers [27]. Based on previous studies, a 50:50 PLA/PGA ratio in PLGA was chosen for its intermediate physical properties.

Microspheres prepared by double emulsion solvent evaporation methods have a smooth surface and completely spherical shape. To determine optimal formulation conditions, this study investigated the yield and encapsulation efficiency of microspheres by changing both the stirring speed and PVA concentration. The highest microsphere yield was achieved at a 600 rpm and with 0.5% (w/v) PVA. In many cases, growth factors encapsulated within PLGA microspheres with high hydrophobicity are released initially more than 80%. But quantitative measurements of rhPDGF-BB showed that the release behavior of the microspheres was sustainable for 21 days and that the release kinetics could be controlled by adjusting the stirring speed and PVA concentration [28,29]. The *in vitro* migration assay showed that the migration of HaCaT cells treated with rhPDGF-BB encapsulated in microspheres was enhanced compared with other treatment groups.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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