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Development of porous PLGA/PEI_{1.8k} biodegradable microspheres for the delivery of Mesenchymal Stem Cells (MSCs)

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Abstract

Multipotent mesenchymal stem cells (MSCs) promise a therapeutic alternative for many debilitating and incurable diseases. However, one of the major limitations for the therapeutic application of human MSC (hMSC) is the lengthy *ex vivo* expansion time for preparing a sufficient amount of cells due to the low engraftment rate after transplantation. To solve this conundrum, a porous biodegradable polymeric microsphere was investigated as a potential scaffold for the delivery of MSCs. The modified water/oil/water (W₁/O/W₂) double emulsion solvent evaporation method was used for the construction of porous microspheres. PEI_{1.8k} was blended with Poly(lactic-co-glycolic acid) (PLGA) to enhance electrostatic cellular attachment to the microspheres. The porous PLGA/PEI_{1.8k} (PPP) particles demonstrated an average particle size of 290 μm and an average pore size of 14.3 μm, providing a micro-carrier for the MSC delivery. PPP particles allowed for better attachment of rMSCs than nonporous PLGA/PEI_{1.8k} (NPP) particles and non-porous (NP) and porous PLGA (PP) microspheres. rMSC successfully grew on the PPP particles for 2 weeks *in vitro*. Next, PPP particles loaded with 3 different amounts of hMSC showed increased *in vivo* engraftment rates and maintained the stemness characteristics of hMSC compared with hMSCs-alone group in rats 2 weeks after intramyocardial administration. These customized PPP particles for MSC delivery are a biodegradable and injectable scaffold that can be used for clinical applications.

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Keywords

PLGA; Porous microparticle; Mesenchymal Stem Cell; Human Stem Cell; Cell Therapy; PEI_{1.8k}

1. Introduction

Mesenchymal stem cells (MSCs) have the multipotent potential to differentiate into various phenotypes, especially after trauma, disease, or aging [1–4]. MSCs are hypoimmunogenic, do not express HLA-II (DR) or blood group antigens, and therefore can evade immunologic complications after transplantation [2–5]. MSCs can be easily obtained from a small volume of bone marrow aspiration, can be compatible with different delivery methods and formulations with stable phenotypes, and can be made as a standardized cell product [2–5]. However, MSCs must be expanded on a large scale for clinical applications through lengthy *ex vivo* expansion for 3–4 weeks, which reduces transfectability of MSCs, increases cost, and risks contamination and alteration of cellular properties [2, 5]. Also, the beneficial effects of adult stem cells, particularly MSCs, administered after organ injury are primarily mediated via combined paracrine, endocrine, and homing actions [1–4, 6, 7]. Transplantation of MSCs by intravenous or intra-arterial infusion demonstrated low engraftment rate and homing varies from less than 1% to about 10% of systemically administered MSCs at a week following injection [3, 5, 6]. To overcome the discussed hurdles of MSC transplantation, various cell delivery systems including microsphere carriers, hydrogels, natural and synthetic scaffolds, and cell-sheets have been considered [8–13].

Poly(lactic-co-glycolic acid) (PLGA) copolymers, which have been approved by FDA for diverse applications, has been most widely used as a biodegradable and biocompatible scaffold, in the form of either suspensions for cell cultivation or injections for the cultivated cells [14–16]. The porous PLGA scaffolds can be produced by the porogen leaching method using salts, carbohydrates, and hydrocarbon waxes [12, 15–17]. These PLGA scaffolds with open pores afford a large surface area for cell attachment, which increases the cell seeding density, promotes cell growth by facilitating mass transport of nutrients and oxygen, and results in improved regenerating and reconstructing potentials [15, 16]. Increase in hydrophilicity of PLGA microparticle by the incorporation of biocompatible molecules was suggested to promote cell attachment on the microparticles [18, 19]. Polyethylenimine (PEI) is one of the most potent polymers for the gene delivery. However, at high molecular weight (MW; 25 kDa or above), it is toxic, causing aggregation with erythrocytes [20]. Recently, incorporation of PEI_{25k} in PLGA microparticles was reported to produce large porous particles with a uniform distribution of pores, resulting in higher entrapment efficiency and prolonged release effect of drug [21, 22]. PEI_{1.8k} is less potent but also less toxic than high MW PEI.

The ongoing loss of cardiomyocytes, in which the apoptotic and necrotic cardiomyocytes are replaced by fibroblasts that form scar tissue, is one of the early pathological characteristics of myocardial infarction (MI) [2, 23]. This leads to adverse cardiac remodeling that causes contractile dysfunction, heart failure, and mortality [2, 23]. Transplantation of skeletal

myoblasts has been tried as a promising alternative method for the treatment of MI, but it is difficult to obtain donor cells and can be arrhythmogenic [2, 24]. The tissue regenerating properties of MSCs provide a promising new therapeutic approach for many unsolved medical needs including adverse post-MI remodeling [1–4, 6, 8, 25]. In this study, we developed injectable porous PLAG/PEI_{1.8k} (PPP) microspheres for the MSCs delivery. Here, the characteristics of PPP scaffold and its potentials for the MSC delivery were evaluated.

2. Materials and Methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (lactide:glycolide ratio 50:50, Mw 80,000) (PLGA) was purchased from Polysciotech® (West Lafayette, IN). Polyethylenimine (Mw 1,800, PEI_{1.8k}) and polyvinyl alcohol (PVA, 87–89% hydrolyzed, Mw 31,000–50,000) were purchased from Sigma-Aldrich® (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), phosphate buffered saline pH7.4 (PBS, pH7.4), and Fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). All others reagents were of analytical grade.

2.2. Preparation of porous PLGA-PEI_{1.8k} (PPP) particles

Porous microspheres were prepared by the modified water/oil/water (W₁/O/W₂) double emulsion solvent evaporation method (Suppl. Fig. 1). 250 mg of PLGA was dissolved in 10 ml of methylene chloride (DCM). 125 µl of PEI_{1.8k} in the distilled water (100 mg/ml) was diluted in 4.875 ml of acetone, making the 2.5% of working concentration (2.5% v/v). 250 µl of prepared 2.5% PEI_{1.8k} solution was blended with 2 ml of 25 mg/ml PLGA solution and then stirred at 400 rpm for 30 min. To produce porous PLGA (PP) particles and porous PLGA/PEI_{1.8k} (PPP) particles, the 250 µl of 5% sodium chloride solution was added to PLGA/PEI_{1.8k} solution. The non-porous PLGA (NP) particles and non-porous PLGA/PEI_{1.8k} (NPP) particles were produced without adding the salts. The first W₁/O emulsion was prepared using a homogenizer (Ultra Turrax IKA® T18 basic; IKA® Works Inc., Wilmington, NC) at 22,000 rpm for 3 min. This primary emulsion was immediately poured into 400 ml of 0.4% (w/v) PVA solution and then was re-emulsified using stirring at 400 rpm for overnight. After the solvent was evaporated, the microparticles were separated by centrifugation, and washed three times with distilled water. Then, the microparticles were lyophilized using a freeze dryer.

2.3. Characterization of PPP microspheres

PLGA-PEI_{1.8k} porous beads were visualized using optical microscope with digital camera. Then, the gross morphology and pores of particles were detected by scanning electron microscopy (SEM, Hitachi S-3000N). The average diameter of microspheres and size distribution were determined by SEM. To determine the surface pore size of microspheres, five microspheres were analyzed using Image software (Image J, US NIH). The average surface pore size of microspheres was measured in five microspheres (pores of <5 µm were excluded).

2.4. Preparation of mesenchymal stem cell (MSC)

Rat MSC (rMSC) was purchased from American Type Culture Collection (ATCC[®], Manassas, VA). After thawing, rMSCs were cultivated in low-glucose Dulbecco modified eagles' medium (DMEM) containing 10% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator at 37 °C under 5% CO₂.

Human MSCs (hMSCs) transferred from the Pharmicell Co., Ltd. (Sungnam, South Korea) were used in this study. hMSCs were isolated from bone marrow (BM) aspiration in healthy adult male donors with informed consent. Briefly, the BM aspirate is diluted with phosphate buffered saline (PBS) and then layered over Ficoll[®] liquid by density gradient centrifugation. Mononuclear cells were placed into 75cm² flask and were cultivated in lowglucose DMEM containing 10% FBS and 20 µg/ml gentamicin in a humidified incubator at 37°C under 5% CO₂ for 5 to 7days. The medium was changed to remove non-adherent cells. hMSCs displayed a fibroblast-like spindle-shaped appearance, and were characterized by their ability to adhere to plastic in standard culture conditions and to form colony forming units in every passage. When these primary cultures of MSCs reached 80% confluence, the cells were trypsinized and subcultured. The procedure was repeated for continuous maintenance of cells. For experiments, the expanded cells were stored in liquid nitrogen. The hMSC was characterized by flow cytometry (BD Biosciences), using specific positive surface markers CD105, CD73 and while being negative for hematopoietic markers such as CD45, CD34, and CD14. The cryopreserved cells were thawed and used for this study. After thawing, hMSCs were cultivated in low-glucose DMEM containing 10% FBS, 20 µg/ml gentamicin in a humidified incubator at 37 °C under 5% CO₂.

2.5. Cell attachment and proliferation analysis

Dry PLGA microspheres (NP and PP) and PLGA/PEI_{1.8k} microspheres (NPP and PPP) were sterilized by soaking into 70% ethanol at 4 °C for 4 h, and then washed with phosphate buffered saline (PBS, pH7.4). Subsequently in 96-well plates, 2×10⁵ rMSCs suspended in 200 µl DMEM containing 10% FBS were added to 1 mg/ml of microspheres in a 37 °C incubator under 5% CO₂ condition with continuous agitation for 24 hrs. The next day, the floating rMSC-loaded microparticles were moved onto a new 24-well plate to measure the attachment of rMSCs to the microspheres. On 1, 3, and 6 days, microscopic images were taken to evaluate the attachment of rMSCs on the microparticles. After 30 minutes of incubation with blue Hoechst 33342 stain, cell permeable nucleic acid stain, the fluorescence microscopic images were taken. Next, to quantify the time-dependent growth rate of rMSC anchored on the rMSC-loaded particles for 2 weeks, cell proliferation was evaluated using the Cell Counting Kit (CCK-8, Dojindo Molecular Technologies, Inc., Rockville, MD), which determined the number of viable cells in cell proliferation. Cell number was measured by MTT following manufacturer's protocol. To evaluate rMSC growth, rMSC-loaded microparticles were moved to a new plate every 3 to 4 days.

2.6. Estimated in vitro loading capacity of hMSC-loaded PPP particles

1×10⁵ cells/well of hMSCs in 200 µl DMEM containing 10% FBS were mixed with 1 mg/ml of PPP in 96-well plates. The hMSC and PPP were incubated for 24 hrs using continuous agitation at 37 °C under 5% CO₂ to attach the cells. The next day, the floating

hMSC loaded-PPP was collected and moved into a new 24-well plate. After 7 days, the floating cells and particles were removed. The newly expanded hMSCs, attached in the 24-well plate were evaluated to identify hMSCs immunophenotype with CD44+ (Santa Cruz, SC-18849) and CD 34- (Santa Cruz, SC-7324). After the removal of the floating hMSC loaded-PPP microparticles, the remaining amount of CD44+ hMSCs was compared with the amount of CD44+ cells in the hMSC only group without particles to estimate the loading capacity of the PPP particles.

2.7. In vivo Engraftment rate of hMSC in Myocardial infarction (MI) model

MI was induced in 7–8-week-old male Sprague–Dawley (SD) rats (200–250 g) by surgical occlusion of the left anterior descending (LAD) coronary artery as previously described [23]. Briefly, under mechanical ventilation, the LAD coronary artery was ligated for 30-min occlusion. Following successful ischemia–reperfusion (I/R), the animals were assigned to one of six groups: sham thoracotomy, I/R only, injection of hMSC-alone, injection of hMSC-loaded PPP particles. In the hMSC-alone group, 20×10^5 hMSCs were administered. The hMSC-loaded PPP particle group was administered with three different hMSC amounts: 20×10^5 (High group), 10×10^5 (Medium group), and 5×10^5 (Low group). The amount of PPP was fixed at 1mg per rat. Right after reperfusion, the rats received a total injection volume of 200 μ l delivered to four separate intramyocardial sites with three injections to the border zone of the infarct in left ventricle (LVb) and one injection to the fibrotic central zone in left ventricle (LVc).

Immunohistochemical (IHC) staining was performed on the 4 μ m thick sections of formalin-fixed, paraffin-embedded rat hearts tissue. Slides were deparaffinized in xylene and then hydrated by incubation in a graded series of alcohols. Endogenous peroxidase activity in the sections was blocked with 3% hydrogen peroxide, and slides were blocked with Protein Block Serum-Free (DAKO, Glostrup, Denmark) for 20 minutes at room temperature. Slides were also stained with Anti-Human CD105 (Bio-Rad AbD Serotec, Oxford, UK; MCA1557F), Anti-Human CD73 (BD Biosciences, Franklin Lakes, NJ; #550257), Anti-Human CD45 (BD Biosciences, Franklin Lakes, NJ; #555482) and Anti-Human CD14 (BD Biosciences, Franklin Lakes, NJ; #555397) in Ab diluents (DAKO, Glostrup, Denmark). After incubating with primary antibodies at 4 °C overnight, the sections were washed twice in PBS and incubated with goat anti-mouse IgG (H+L)-HRP (Southern Biotech, Birmingham, AL) for 2 hours at room temperature. Diaminobenzidine/hydrogen peroxidase (DAKO, Carpinteria, CA) was used as the chromogen substrate. All slides were counterstained with Mayer's hematoxylin and examined by light microscopy at 400 $\times$ magnification.

For IHC of CD44 and CD34, tissue sections were air-dried at room temperature and then placed in a 60 °C oven for 30 min to melt the paraffin. All of the staining steps were performed at 37 °C using an automated immunostainer (BenchMark[®] XT, Ventana Medical Systems) with CD44 (mouse anti-pan CD44 monoclonal antibody; Millipore, Billerica, MA; #MAB4065; 1:6,000) and CD34 (CONFIRM[™] anti-CD34 (QBEnd/10) primary Antibody; #790–2927; Ventana Medical Systems Inc., Tucson, AZ; optimally pre-dilute antibody). The sections were detected using the ULTRAVIEW DAB detection kit (Ventana Medical

Systems). The sections were counterstained with hematoxylin for 8 min. Analysis of all images was carried out with ImageScope (Aperio technologies Inc. Vista, CA) and randomly chosen within the LVb by an investigator blinded to the treatment groups in a high-power field ($\times 20$ magnification) per whole heart specimen.

2.8. Statistical analysis

Statistical calculations were carried out using SPSS 19.0 software (SPSS Inc., Chicago, IL). Data were expressed as the mean $\pm$ SD. Comparisons between multiple groups were performed by analysis of variance (ANOVA) followed by Tukey *post-hoc* testing. Groups with *P* values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Synthesis and characterization of PLGA and PLGA/PEI_{1.8k} microparticles

The PLGA and PLGA/PEI_{1.8k} microspheres were produced by the W₁/O/W₂ double emulsion solvent evaporation method using sodium chloride as a salting-out agent, which formed large, isolated, and scattered pores in the inner region due to the immediate coalescence of the aqueous droplets during the solvent removal [15]. The oil phase was methylene chloride containing PLGA and acetone with PEI_{1.8k}. Both non-porous (NP) and porous PLGA (PP) microspheres did not significantly improve the attachment of rMSCs on microspheres. Then, to improve the attachment of rMSC to microparticles, PEI_{1.8k} blended PLGA (PLGA/PEI_{1.8k}) was introduced. We hypothesized that the positive charge of PEI_{1.8k} could increase the attachment of rMSCs and PLGA/PEI_{1.8k} microparticles. PEI_{25k} has a higher positive charge in aqueous solutions and is one of the most notable vectors. PEI_{1.8k} retains a positive charge but has a higher biocompatibility than PEI_{25k}. The formation of open pores in the interior region in the PPP can be seen in the optical microscope as light passing through the particle (Fig. 1). The previous research group suggested that the PLGA microspheres smaller than 100 μm in diameter did not have an open porous structure and would not be suitable for cell delivery [15]. The PPP particles demonstrated an average particle size of 290 μm ($293 \pm 38 \mu\text{m}$ without lyophilization vs $286 \pm 30 \mu\text{m}$ in Fig. 2). Using 5% sodium chloride, the pores ($14.3 \pm 0.4 \mu\text{m}$) in PPP were produced (Fig. 2).

3.2. In vitro attachment, proliferation, and loading capacity of MSCs on microparticles

The characteristics of PPP microspheres, as a suspension micro-carrier for rMSCs and hMSCs were evaluated. After the inoculation of PPP particles mixed with rMSC for 24 hrs, PPP particles showed higher attachment of rMSC compared with other particle groups ($P = 0.001$; Fig. 3). There was no significant attachment in NP and PP microspheres (Fig. 3). The PPP scaffold showed 4 times and 2 times higher attachment of rMSCs than PLGA particles ($P < 0.01$; NP and PP) and NPP ($P < 0.05$) respectively (Fig. 3). Both structural entrapment in the surface pores of the microspheres and physical conjugation with the positive charge of PEI_{1.8k} in the PPP microspheres were assumed as mechanisms for the enhanced attachment (Graphical abstract). This result suggests that microparticles with open pores provide a favorable spatial environment for the attachment and delivery of MSCs.

After the attachment of rMSCs on the PPP microparticles, the rMSCs-loaded PPP particles were moved into a new plate with fresh DMEM containing 10% FBS. Then, the growth of the rMSCs from the PPP particle was imaged up to 6 days (Fig. 4). Next, the time-dependent growth of rMSC anchored on the rMSCs-loaded microparticles was quantified using a CCK-8 kit for 2 weeks (Fig. 5). The proliferation of rMSC on PPP microspheres was significantly higher than PLGA groups (NP and PP) more than 8 days after cultivation (Fig. 5). 14 days after cultivation, the proliferation of rMSC on PPP particles was greater than PLGA groups (NP and PP) and NPP group ($P < 0.01$; Fig. 5). These time-dependent results suggest that the PPP scaffold provides a more biocompatible and favorable microenvironment for the proliferation of MSCs.

The positivity of CD44+ in hMSC was over 95% in flow cytometry analysis and was independent of hMSC cell number (data not shown). The newly grown hMSC attached to the plate surface (excluding floating hMSCs-loaded PPP particles) was compared with the hMSC only group and demonstrated a relative CD44+ positivity of 61.5%. Therefore, the hMSC loading capacity of floating hMSC loaded-PPP was estimated at around a maximum of 38.5%.

3. 3. In vivo Engraftment rate of hMSC-loaded PPP particles

The International Society for Cellular Therapy (ISCT) suggested consensus criteria for the characterization of multipotent human MSC [26]. Two weeks after intramyocardial administration, the *in vivo* engraftment rate and stemness characteristics of hMSC was evaluated in hMSCs alone and three different amounts of hMSC-loaded PPP particles groups in post-infarct hearts of rats. With CD105+, CD73+, and CD44+, the three different amounts of hMSC-loaded PPP particles groups showed increased *in vivo* engraftment rates of hMSCs compared with hMSCs-alone group in rats (Fig. 6). Also, the stemness characteristics of hMSC were supported by the lack of expression of the lymphocytic marker CD45, the lineage marker for hematopoietic stem cell marker CD34, and the monocyte and macrophage marker CD14 (Suppl. Fig. 2). The engraftment rate of hMSC between the three different amounts of hMSC-loaded PPP groups was comparable. In this result, when we fixed 1 mg of the PPP amount per rat, even the 5×10^5 hMSC-loaded PPP (Low) group was appeared to saturate loading capacity of hMSC. The significance of *in vivo* engraftment rates of the three different hMSC-loaded PPP groups must be further elucidated using *in vivo* functional and histo-pathologic analysis.

4. Conclusions

Although the BM-MSCs can be easily harvested from BM, the prolonged *ex vivo* culture time limits their therapeutic efficacy following transplantation in humans because it reduces their potential to differentiate. The PPP group loaded with 5×10^5 hMSCs (Low PPP group) as well as Medium and High PPP group demonstrated significant enhanced *in vivo* engraftment rate compared with the hMSCs alone (20×10^5 hMSCs) at 2 weeks after MI. These results suggest that using only 25% of the current amount of hMSCs could result in greater regenerative potential. This PPP delivery system provides superior survival and engraftment after hMSCs transplantation, shortened *ex vivo* time, lowered costs, and

maintains the characteristics of hMSCs. After transplantation, MSCs act in both endocrine and paracrine pathways, and MSCs themselves are considered an attractive vehicle in cell therapy [3, 27]. Therefore, the MSC-loaded PPP delivery could work as a dual-scaffold system, which results in pathophysiologic and functional improvements. The PPP is a promising injectable scaffold to deliver the hMSC to repair tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Microscopic images of microparticles. Scale bar = 200 μm .

Fig. 2.
SEM images of microparticle. (a) 250×, (b) 2,000× magnification.

Fig. 3.

Attachment of rMSC on PLGA (NP and PP) and PLGA/PEI_{1.8k} (NPP and PPP) particles after 24 hrs. The relative cell number (%) was measured by CCK-8 kit. $*P < 0.01$ vs NP, $\#P < 0.01$ vs PP, $\S P < 0.05$ vs NPP.

Fig. 4. Microscopic images of rMSC proliferation on PPP particles over 6 days. Fluorescence Hoechst 33342 staining. 10× magnification with 400 μ m scale bar.

Fig. 5. Quantification of timedependent rMSC growth on the rMSCs-loaded particles. The cell growth rate was measured by CCK-8 kit for 2 weeks. # $P < 0.05$ vs NP, ## $P < 0.01$ vs NP, † $P < 0.05$ vs PP, †† $P < 0.01$ vs PP, § $P < 0.05$ vs NPP.

Fig. 6.

In vivo engraftment rate of hMSC alone and hMSC-loaded PPP particle at 2 weeks after injections. Representative IHC staining images for CD105+, CD73+, and CD44+ in the LVb from each group.