치의과학석사 학위논문

Inhibitory effects of surface-grafted polylactide-co-glycolide nanoparticles on the pro-inflammatory polarization of macrophages

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이 논문을 치의과학 석사 학위논문으로 제출함

2022년 6월

서울대학교 대학원

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최상훈의 치의과학석사 학위논문을 인준함

2022년 7월

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Abstract

Inhibitory effects of surface-grafted polylactide-co-glycolide nanoparticles on the pro-inflammatory polarization of macrophages

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(Directed by Professor Hyeong-Cheol Yang, Ph.D)

Macrophages are known to play a key role in the inflammatory response and regeneration process. M1 macrophages, which are pro-inflammatory macrophages, create an inflammatory environment and produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12. Due to these properties, the sustained expression of M1 macrophages can inhibit the wound healing process. Phosphatidylserine (PS) is a type of phospholipid present inside the cell membrane, and acts as an "eat-me" signal in apoptotic cells, resulting in macrophage phagocytosis and anti-inflammatory activity. In previous studies, it has been reported that PS inhibits the expression of M1 macrophages in the form of liposomes. To increase the utility of PS molecules, we considered the other vehicle to

delivery. PLGA (polylactide-co-glycolide) is known to have excellent biocompatibility and biodegradability, and it has been widely used in nanoparticle fabrication. In this study, the surface of PLGA nanoparticle is modified with PS, and their biological properties are investigated.

PLGA nanoparticles (PLGAnPs) containing phosphatidylcholine (PC) and PS were prepared using emulsification-solvent-evaporation (ESE) technique and classified as follows: 1) PC 100% (PCnP); 2) PS:PC = 50:50 (PSPCnP); and 3) PS 100% (PSnP). The size and distribution of PLGA nanoparticles were analyzed by a nanoparticle analyzer, and the surface charge was measured by a zeta potential analyzer. For cell experiments, mouse bone marrow-derived macrophages (BMDM) were used, and cytotoxicity by nanoparticles was measured after treatment with WST-8 for 12 hours. Lipopolysaccharide (LPS) was used for induction into M1 macrophages, and nanoparticles were treated with LPS to determine the degree of inhibition. Changes in cell morphology were observed with an inverted digital microscope after treatment for 12 hours. The markers representing M1 macrophages (TNF-α, IL-1β, IL-6, IL-12p40, CD86 and iNOS) were analyzed after treated with LPS for 6 hours. Whereas the M2 macrophages markers (Arg-1, YM-1, CD206) were analyzed after 12 hours of treatment. All the gene expression markers were assessed by RT-qPCR.

The size of the nanoparticles was assessed about 210 nm in all groups. The zeta potential was close to 0 mV in the negative control group, PLGAnP. Meanwhile, the surface charges were below -12 mV in the PCnP, PSPCnP, and PSnP groups. None of the nanoparticles used in this study showed cytotoxicity. LPS-treated macrophages differentiated into M1 macrophages, and distinct morphological changes could be observed. In contrast, the M1-type morphological change was inhibited by PSPCnP and

PSP cotreatment with LPS. TNF-α, IL-1β, and IL-6 mRNA expressions were decreased in all nanoparticle-treated groups, and in IL-12p40, only PSPCnP and PSnP were decreased. Although there were no statistically significant differences in the results of CD86 and iNOS, the PSPCnP group showed the highest tendency to inhibit the expression. Therefore, it was demonstrated that the PSPCnP group, in which PS and PC were present in the same ratio, maximally inhibits M1 differentiation of macrophages. However, there was no significant difference in the markers of M2 macrophages compared to the negative control group. The reason of these results is considered as the insufficient numbers of PS attached to PLGA during the particle generation process or insufficient numbers of particles which had interacted with cells.

Keyword : Macrophage; Phospholipid; Phosphatidylserine; PLGA; Nanoparticles; Anti-inflammatory

Student Number: 2020-21982

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Chapter 1. Introduction

1.1. Characteristics and Classification of Macrophages

Macrophages are known to play a vital role in the inflammatory response and regeneration process. When inflammation occurs, neutrophils flow into the inflammation site. Then, monocytes arrive to differentiate into macrophages [1, 2]. Macrophages can be classified into the following phenotypes: broadly M1 (classically activated) and M2 (alternatively activated) states [3]. M1 macrophages are known to secrete a prominent level of pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-12], and reactive oxygen and nitrogen species. [4] Given that the M1 macrophages provoke an inflammatory environment, they can aggravate the inflammation impeding the wound healing procedure [5]. The M2 macrophages, countering the M1 phenotype, are well known for their anti-inflammatory and tissue regeneration effects [6].

1.2. Interaction between Macrophages and Engineered Phosphatidylserine

Phosphatidylserine (PS) is a type of phospholipid present inside of the healthy cell membrane bilayer. PS exposed outside of apoptotic cells acts as an "eat-me" signal, resulting in macrophage phagocytosis and anti-inflammatory activity. PS receptors on the macrophages bind with PS, and

their interaction plays a key role in inflammatory modulation [7]. The PS-dependent ingestion of apoptotic cells can induce the secretion of TGF-β1 which is classified as the anti-inflammatory cytokine under the inflammatory environment. TGF-β1 can also suppress the M1 macrophage by inducing to M2 polarization [8]. The encounter between PS and PS receptors on macrophages provokes the inhibitory effect of inflammation [9]. PS is frequently utilized in PS-contained liposomes (PSLs), which could mimic the anti-inflammatory apoptotic cells [10-12]. PSLs inhibit an inflammation by deregulating the expression of M1 macrophages and inducing M2 polarization [13]. The immunomodulatory effect of PSLs was determined to be reinforced with arginine-glycine-aspartate (RGD) peptides on surface and co-treatment with sodium butyrate [14, 15]. Furthermore, other reports have revealed that PS accelerates the phagocytosis of curcumin loaded acetalated dextran nanoparticles [16]. These studies suggest that PS-contained nanosized particles have a potential for upregulating the immunomodulatory effect with other elements.

1.3. PLGA Nanoparticle Synthesis

Polylactide-co-glycolide (PLGA) is a copolymer of lactic and glycolic acid. The ratio of two monomers affects their properties, including the biodegradation rate and hydrophilicity [17, 18]. PLGA has been used widely because of its biocompatibility, biodegradability, and mechanical strength. [19]. Due to these attributes, the PLGAnP is considered as one of the most suitable nanoparticles for drug delivery systems [20]. the PLGAnP could be synthesized by numerous techniques, such as

emulsification-evaporation [or emulsification-solvent-evaporation (ESE)], emulsification-diffusion, interfacial deposition, salting out, dialysis, and nanoprecipitation [21]. The PLGAnP can be dissolved in highly hydrophobic and volatile solvents, such as dichloromethane, and chloroform, using the ESE method [21-23].

Lipid surface-engineered PLGAnPs have been used in numerous studies owing to their biomimetic and biocompatible advantages [24, 25]. The synthesis methods can be categorized into the classical two-step and contemporary single-step processes [26]. In the two-step method, PLGAnPs are synthesized first and mixed with preformed liposomes later [27]. In the single-step method, however, lipids are self-assembled around the PLGAnP core by hydrophobic interactions [28]. The appropriate method for the experiment depends on numerous factors, including size, shape, and characteristics of the lipids (Fig. 1) [29].

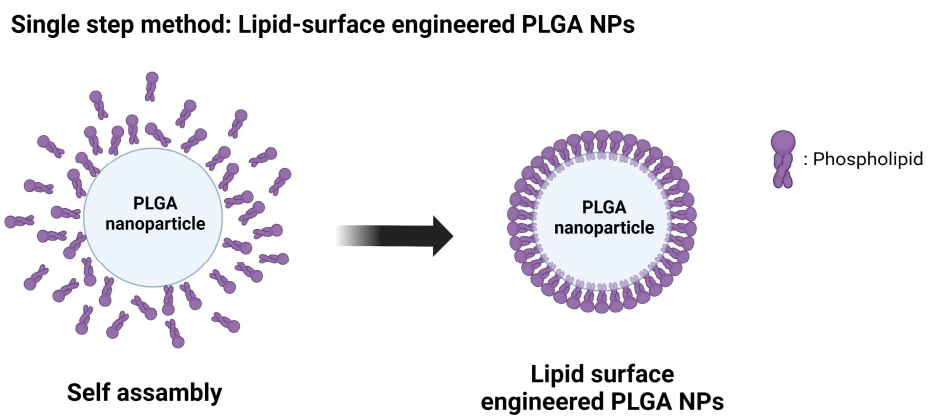

Figure 1. Single step method for lipid-surface engineered PLGA nanoparticle

1.4. Purpose of Research

In the present study, we hypothesized that PS on the surface of PLGAnPs would mimic the apoptotic cells to upregulate the immunomodulatory effects of macrophages. Furthermore, PS-PLGAnPs had been speculated to be able to improve the stability and stockage, as a carrier of PS.

Chapter 2. Materials and methods

2.1. Nanoparticle preparation

L-α-phosphatidylserine (PS, porcine brain) and L-α-phosphatidylcholine (PC, egg yolk) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Poly(D,L-lactide-co-glycolide) (PLGA, lactide:glycolide (50:50), MW: 30,000-60,000) and other reagents were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise specified. The single ESE technique (w/o) was exploited to synthesize the nanoparticles [22, 23]. The brief procedure is as follows: PS and PC were dissolved in chloroform/methanol (9:1 v/v) solvent; each group contains 24 μmol PC (PCnP) / 12 μmol PC, 12 μmol PS (PSPCnP) / 24 μmol PS (PSnP) respectively. The organic solvent was evaporated by nitrogen gas streaming for 30 min in a glass tube and vacuum chamber for 2 h. The remaining phospholipid film was dissolved in polyvinyl alcohol (PVA, Mowiol® 4-88) 2% (in DW, w/v) / dimethyl sulfoxide (DMSO) (9:1 v/v) solution by stirring for 1 h. PVA is emulsifier for synthesize nanoparticles and DMSO is solution for dissolve PS and PC. Then 40 mg PLGA, dissolved in dichloromethane (DCM), is add to the aqueous phase dropwise with vortexing; the PLGA nanoparticles were dispersed and combined with phospholipids by sonication (1/4″ probe, 40% amplification) (VCX 130, Sonics & Materials, Newtown, USA). To demonstrate the effect of phospholipid in nanoparticles, pure PLGA nanoparticle group (PLGAnP) begun to be included from this procedure. The organic phase was removed by a stirring in vacuum chamber for overnight. For an efficient

stirring, 0.1% PVA solution (in DW, w/v) was added to the solutions. The samples were extruded through Minisart® Syringe Filter (Surfactant-free Cellulose Acetate (SFCA), Pore Size 1.2 μm) purchased from Sartorius AG (Göttingen, Germany) for size determination, purification, and facile quantification [30]. After this procedure, nanoparticles were collected by centrifugation (15,000 rpm, 20 min) (Avanti J-E, Beckman Coulter, California, US) and the supernatant was removed. The pallet was completely suspended in DW with sonicator and vortexer. Subsequently, these steps were repeated twice more for completely remove the other impurities. Before transfer to deep freezer, added 15 mg trehalose (D-(+)-Trehalose dihydrate) for a cryoprotectant. After freezing the nanoparticles overnight at -80 °C, freeze-drying in lyophilizer (Alpha 1-4 LSCbasic, Martin Christ, Osterode am Harz, Germany) 48 h, and stock the samples in 4 °C. Whenever the samples were utilized for experiment, the syringe filter was employed to remove the agglomerated nanoparticles.

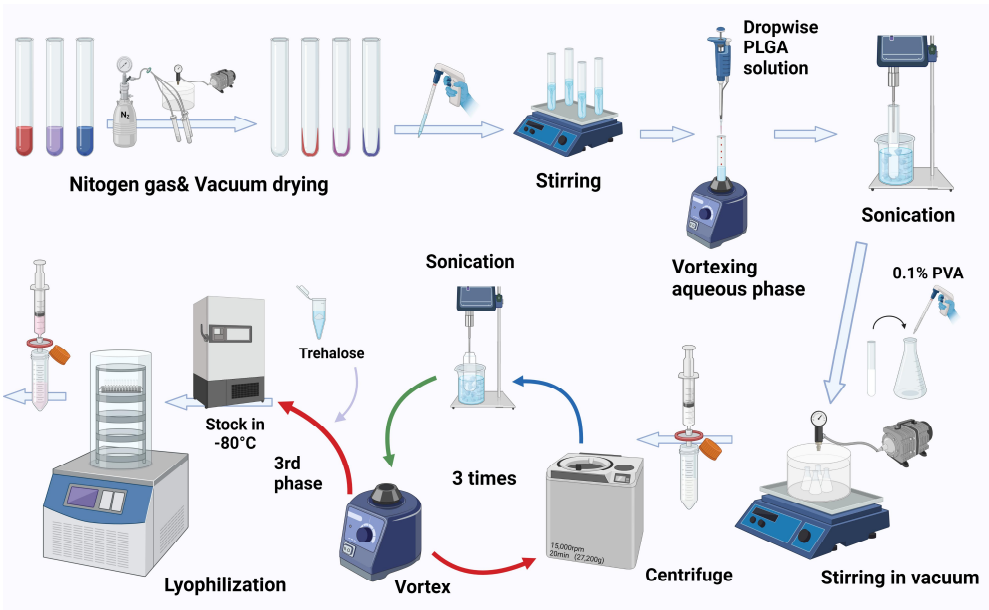

Figure 2. Nanoparticles preparation

2.2. Characterization of nanoparticles

The size of the PLGAnP was measured by using a nanoparticle tracking analyzer (NanoSight, Malvern Panalytical, Malvern, UK). The surface charge of the nanoparticles was surveyed by using a zeta potential analyzer (ELSZ-1000, Otsuka Electronics Co., Ltd, Osaka, Japan). The zeta potential was gauged in distilled water using the following parameters: avg. electric field, -16.50 V/cm; avg. current, 0.00 mA; temperature, 25.0 ℃; refractive index, 1.3328; viscosity, 0.8878 cP; and dielectric constant, 78.3.

2.3. Cell culture of mouse bone marrow-derived macrophages

Mouse bone marrow-derived macrophages (BMDMs) were used to investigate the effects of nanoparticles on the polarization of macrophages. For this study, 5-week-old Institute of Cancer Research (ICR) male mice (OrientBio Inc. Seongnam, Korea) were used with humanely sacrifice. After harvesting femur from the mice, bone marrow was extracted with flushing DPBS (pH 7.4) from the end of the femur by using a syringe under aseptic conditions. The extract was centrifuged at 1500 rpm for 5 min (MF80, Hanil science, Daejeon, Korea) and followed by resuspension of pellet in R10 media (RPMI 1640 supplemented with 10% non-heated FBS, 1% antibiotics, 2 mM l-glutamine, and 20 mM HEPES; pH 7.0) containing macrophage colony-stimulating factor (M-CSF, 20 ng/mL). All constituents of fresh R10 media were purchased from Welgene (Daegu, Korea). The cell suspension was prepared as 1 x 10^6 cells/mL counted by LUNA-II™ automated cell counter (Logos Biosystems, Anyang, Korea). A 1

mL cell suspension was subsequently cultured in each well of a 12-well plate for 24 h and then 1 mL of fresh R10 medium was added to each well during further 24 h culture. Finally, half of the culture media was exchanged by fresh R10 media containing M-CSF for the last 24 h culture before the treatment of macrophages.

2.4. Cell viability and cytotoxicity assay

Cell viability and cytotoxicity were measured using with water soluble tetrazolium salt (WST-8) assay kit (EZ-Cytox, Dogenbio, Seoul, Korea). After 12 h of treatment, washed out with DPBS twice and incubated on R10 media with 10% EZ-Cytox for 4 h. Absorbance was gauged using an enzyme-linked immunosorbent assay reader (Sunrise, TECAN, Salzburg, Austria) at 450 nm.

2.5. Morphological analysis

The mode of M1 macrophage polarization provides a useful system to study the macrophages in vitro. For the polarization of M1 phenotypes in this study, 50 ng/mL lipopolysaccharide (LPS) (Escherichia coli; serotype 0111:B4) was added to the culture medium for 12 h. To demonstrate the anti-inflammatory effect, 200 μg/mL of each nanoparticle was treated together with LPS; 20 ng/mL IL-4 was used for a positive control for LPS co-treatment group. The cellular morphology images were taken by DS-Ri2 / Nikon Ti (Nikon, Tokyo, Japan).

2.6. Gene expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

To evaluate the anti-inflammatory effects of nanoparticles, macrophages were treated with nanoparticles in the presence of 50 ng/mL LPS for 6 h; then, the mRNA expression of pro-inflammatory genes (TNF-α, IL-1β, IL-6, IL-12p40, CD86, and iNOS) was assessed by RT-qPCR. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was employed as a control housekeeper gene. RT-qPCR was performed as follows: total RNA was isolated using an RNA isolation reagent (QIAzol Lysis Reagent, QIAGEN, Hilden, Germany); cDNA was synthesized from total RNA using a 100 mM dNTP set, $Oligo(dT)_{18}$ primer PrimeScript™ Reverse Transcriptase, and RNaseOUT™ Ribonuclease Inhibitor; and PCR was performed using TB Green™ Premix Ex Taq (Tli RNaseH Plus) and ROX Reference Dye II, cDNA, and gene-specific primers (Table 1). 100mM dNTP set and RNaseOUT were purchased from Invitrogen (Massachusetts, USA); $Oligo(dT)_{18}$ was purchased from thermoscientific (Massachusetts, USA); PrimeScript, TB Green and ROX dye II were purchased from Takara Bio, (Otsu, Japan). Thermo-cycling conditions for RT-qPCR were consisting of holding stage (50 °C for 2 min and 95 °C for 10 min), and denaturation & annealing stage (40 cycles of 95 °C for 15 s and 60 °C for 1 min) (ABI PRISM 7500 (Applied Biosystem, Carlsbad, CA, USA). The expression levels of all targeted cytokines were calculated based on their threshold cycle values and were noted as the relative mRNA expression ratios normalized to a reference gene (GAPDH).

Table 1. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primers

Genes	Forward sequence	Reverse sequence
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGCCAGTGAATTCGG
IL-1β	TGGAGAGTGTGGATCCCAAG	GGTGCTGATGTACCA GTTGG
IL-6	ATAGTCCTTCCTACCCCAATTTCC	GATGAATTGGATGGTCTTGGTCC
IL-12p40	AGCAGTAGCAGTTCCCCTGA	AGTCCCTTTGGTCCAGTGTG
CD86	TCTCCACGGAAACAGCATCT	CTTACGGAAGCACCCATGAT
iNOS	ACCATGGAGCATCCCAAGTA	CCATGTACCAACCATTGAAGG
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	CACGGCACCTCCTAAATTGT
YM-1	CAGGGTAATGAGTGGGTTGG	CACGGCACCTCCTAAATTGT
CD206	GTCAGAACAGACTGCGTGGA	GCATTCCAGAGAAGCCTGAC

2.7. Statistical analysis

All data obtained from three independent experiments were presented as the mean ± standard deviation (SD). Differences among the groups were assessed by one-way analysis of variance (one-way ANOVA) followed by Tukey's test. Statistical analyses were performed using IBM SPSS 26 statistics software (IBM, Armonk, NY, USA). P values of <0.05 were considered statistically significant.

Chapter 3. Results

3.1. Characteristics of nanoparticles

The effect of size of nanoparticle is substantial on their immunomodulatory function [31]. The size differences of nanoparticles caused by phospholipids were measured and evaluated. As shown in Fig. 3, the mean diameters of PLGAnP, PCnP, PSPCnP, and PSnP were 212.6 ± 60.4, 215.9 ± 55.4, 209.1 ± 58.1, and 207.0 ± 53.3 nm, respectively. The average size among all the groups was not statistically significantly different. The zeta potential is a significant parameter used to assess the phospholipids attached to the surface of nanoparticles. PS is known as the most negatively charged glycerophospholipid in eukaryotic membranes [32]. The zeta potentials of PLGAnP, PCnP, PSPCnP, and PSnP were -2.38, -12.01, -15.47, and -16.28, respectively (Fig. 4).

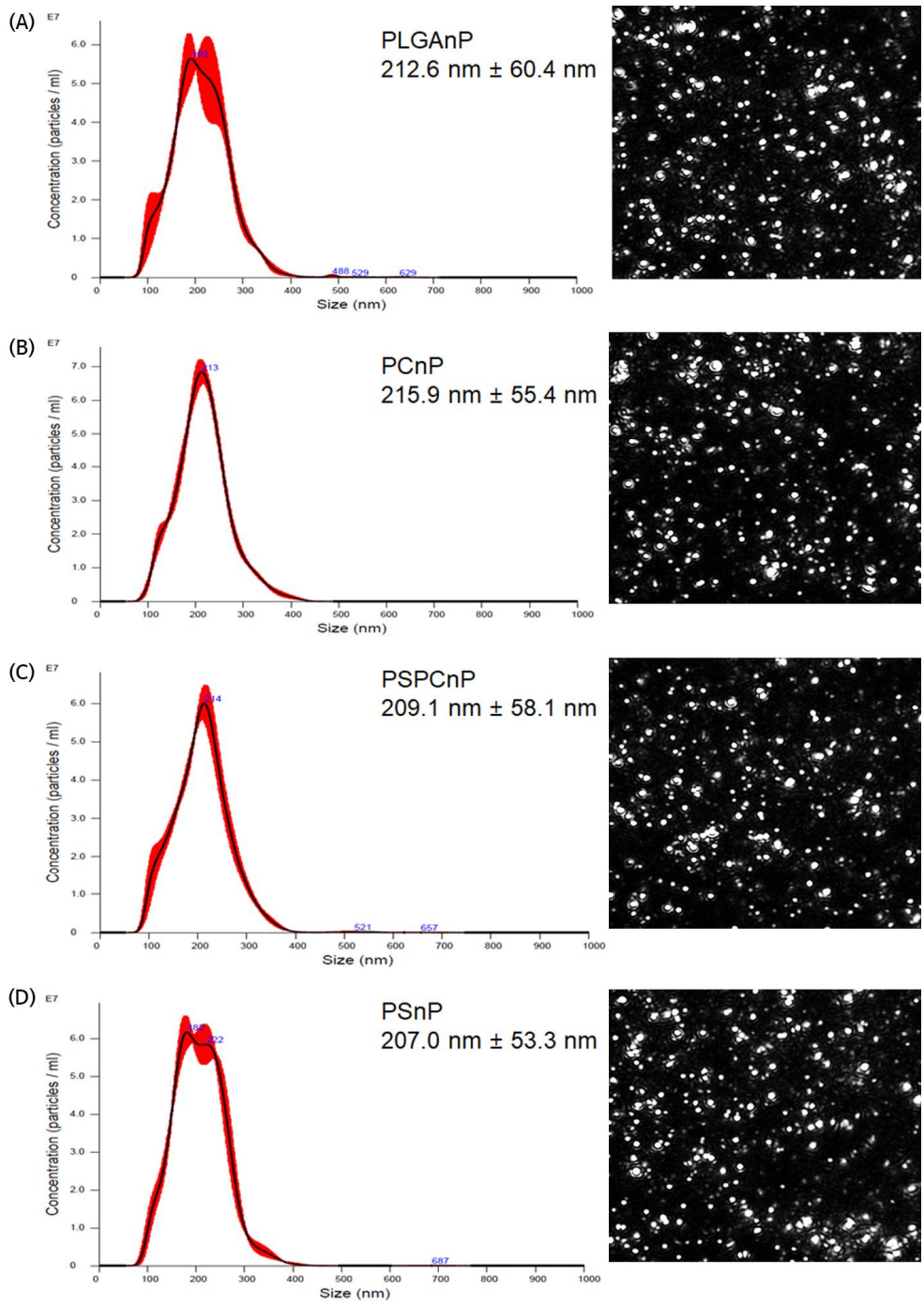

Figure 3. Size and distribution analysis of nanoparticles. Size analysis and particle distribution was no significant difference in (A) PLGAnP, (B) PCnP, (C) PSPCnP, and (D) PSnP.

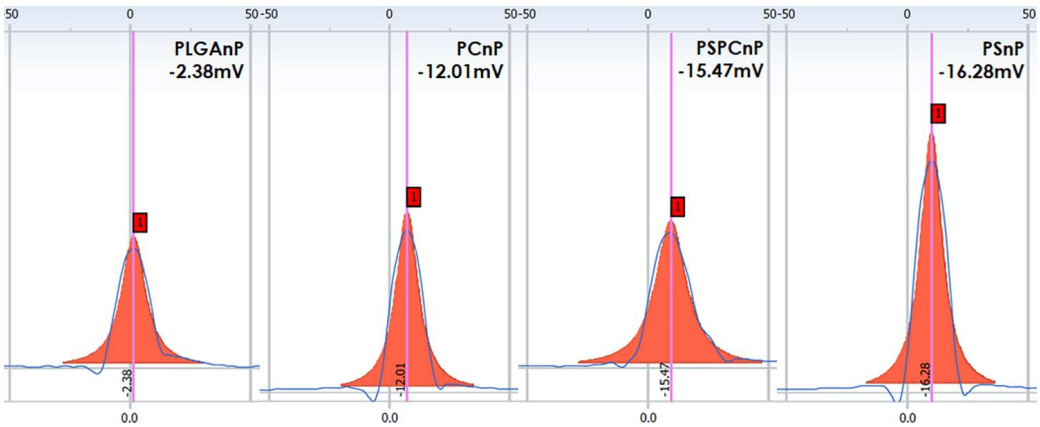

Figure 4. Zeta potential analysis of nanoparticles. The zeta potential was -2.38, -12.01, -15.47, and -16.28 for PLGAnP, PCnP, PSPCnP, and PSnP, respectively.

3.2. Effect of nanoparticles on cell viability and cytotoxicity assay

The effects of nanoparticles on the viability of BMDM were evaluated depending on concentration and type of nanoparticles. The cell viability of BMDM was not different according to the concentration of PLGAnPs (Fig 5A.). In Figure 5B, the treatment with different types of nanoparticles showed no significant difference in cytotoxicity. Therefore, none of the nanoparticles used in this study showed cytotoxicity.

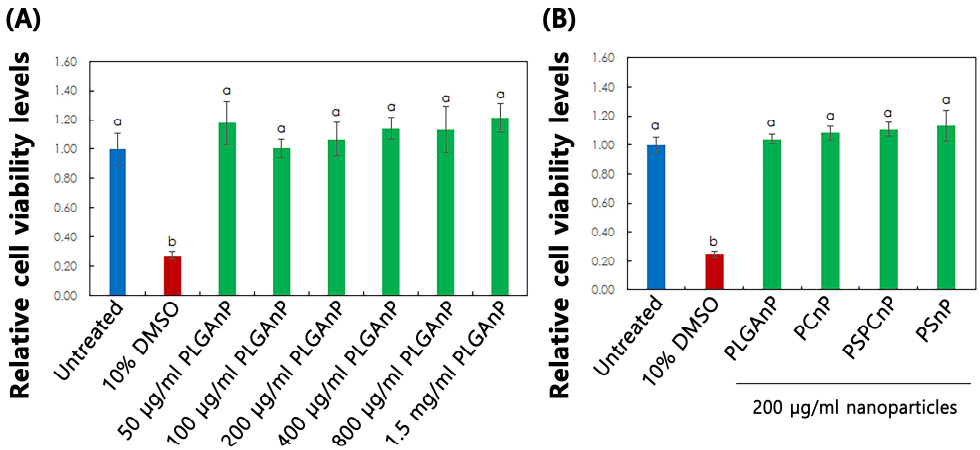

Figure 5. Cell viability assay for nanoparticles. The WST-8 assay depends on (A) concentration of PLGAnP, and (B) types of nanoparticles.

3.3. Effect of nanoparticles on cell morphology

The polarization of macrophages is accompanied by remarkable changes in cell shape. The M1 macrophages exhibit a flat and spread-like pancake shape, meanwhile the M2 macrophages exhibit a spindle and elongated shape [33]. Therefore, the morphological changes of cells could be observed under an optical microscope after 12 h of treatment. Figure 6 shows bone-marrow-derived macrophages that are slightly spindle or round in the untreated group. LPS stimulation induces the cells to become large, flat, and pancake-like, and co-treatment with PLGAnP and PCnP showed no significant difference. However, PSPCnP and PSnP prevent cell morphological changes to the typical LPS-induced M1 shape. Figure 6 suggests that cell shape is an important cue for anti-inflammatory effects.

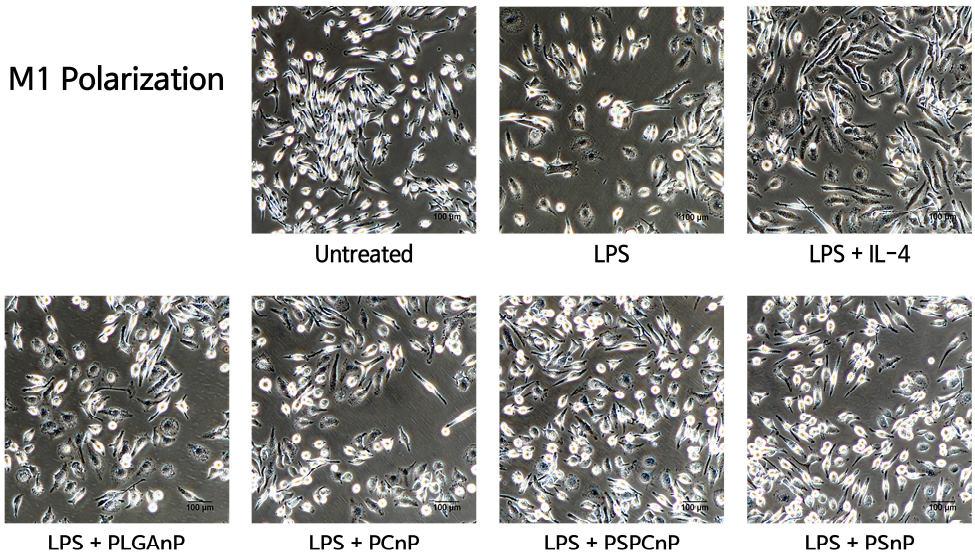

Figure 6. Cellular morphological analysis. Cell shape changes are observed under an optical microscope. LPS stimulation induced the M1 polarization, while co-treatment with PSPCnP and PSP could prevent the cell morphological changes.

3.4. Effects of nanoparticles on macrophage mRNA expression of inflammation and polarization genes

To evaluate the anti-inflammatory effect of nanoparticles, mouse bone-marrow-derived macrophages were treated with nanoparticles in the presence of LPS. Then, the mRNA expression of inflammatory cytokines (TNF-a, IL-1β, IL-6, and IL-12p40), immunoglobulins (CD86), and enzyme release (iNOS) was assessed after 6 h of treatment by RT-qPCR. Figure 4A–F shows that LPS (50 ng/mL) induced higher levels of these mRNAs, as compared to expression in the untreated cells. The mRNA expression of TNF-α and IL-6 was downregulated by nanoparticles, especially in the PSPCnP group (Fig. 7A, C). Approximately 50% of IL-1β mRNA expression was declined by nanoparticles. However, there was no statistically significant difference among the experiment groups (Fig. 7B). PLGAnP and PCnP did not affect the LPS-stimulated mRNA expression on the IL-12p40 marker, while PSPCnP and PSnP remarkably suppressed the expression (Fig. 7D). Although there was no statistically significant difference in mRNA expression of CD86 and iNOS, PSPCnP showed a tendency to inhibit the expression. (Fig. 7E, F). However, M2 marker genes including Arg-1, YM-1, and CD206, did not showed statistically difference in RT-qPCR assay (Fig. 7G-I).

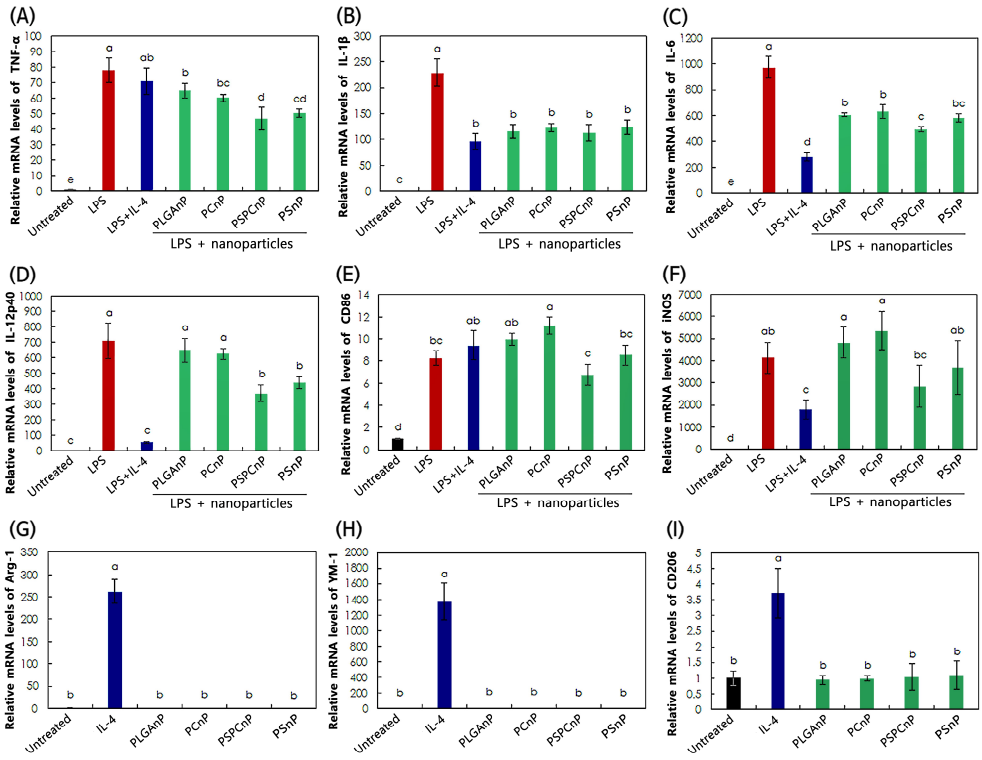

Figure 7. Effects of nanoparticle on mRNA expression of inflammation and polarization genes. (A–F) To assess the anti-inflammatory effect of nanoparticles, macrophages were cotreated with nanoparticles and LPS for 6 h. The mRNA expression of TNF-α, IL-1β, IL-6, IL-12p40, CD86, and iNOS genes were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (G-I) After macrophages were exposed to nanoparticles without LPS for 12 h, the mRNA expression of Arg-1, YM-1, and CD206 were evaluated. The data from four independent experiments are presented as means ± standard deviation (SD). Data bars with distinct letters represent statistically significant differences among the groups ($p < 0.05$), and data bars with the same letters represent no significant differences ($p > 0.05$).

Chapter 4. Discussion

Due to its biodegradability and biocompatibility, PLGA has widely attracted attention as a biomaterial. PLGAnP has been used as a drug delivery system for inflammation, vaccination, cancer, and other diseases [34]. The mean size of nanoparticles is a substantial factor in biomaterials, which affects various biosystems, such as cytotoxicity, macrophage polarization, and mesenchymal stem cell osteogenesis [31, 35]. The mean size of nanoparticles is controlled by 1) PVA concentration in the aqueous phase, 2) surface lipid concentration in the aqueous phase, 3) PLGA concentration in the organic phase, 4) volume ratio of the aqueous solution, and 5) duration of ultrasonic dispersing treatment (sonication) [36, 37]. The proper size of PLGAnP ranged from 100 to 300 nm [38]. The average size of nanoparticles was approximately 210 nm, which was not significantly different among the groups (Fig. 3). The zeta potential also plays a crucial role in cytotoxicity and cellular interactions [32]. Cytotoxicity derived from the PLGAnP was present when the zeta potential ranged from –13.2 to –19.3 mV [38]. In Figure 4, phospholipid grafting has induced a surface charge difference, and the zeta potential of PSPCnP and PSnP was in the proper range. Thus, we could estimate that phospholipids had been attached to PLGAnP, and they had no effect on the size of the particles. The fact that PLGAnP had no cytotoxicity up to 1.5 mg/ml was confirmed in Figure 5A, which support the findings of a previous study [39]. PLGAnP grafted on the surface could stunt the cell viability [40]. We demonstrated that 200 μg/ml of nanoparticles had no cytotoxicity to BMDM (Fig 5B).

In the past, lipid-based surface-engineered PLGAnPs were focused on development of drug and gene delivery platforms. Thus, lipids were mainly used for characterizing nanocarriers [20, 41]. In other previous studies, macrophage polarization was regulated by mimicking the interaction between apoptotic cells and macrophages, induced with PS on the surface of liposomes or titanium [13-15, 42]. Meanwhile, the present study focused on PS as the lipid on the surface of lipid-PLGAnPs and expected that PS-PLGAnPs had some similar effects to PS-liposomes in terms of immunomodulatory effects and macrophage polarization.

As shown in Figure 7A–F, we observed anti-inflammatory effects of the nanoparticles, which was compared with those of IL-4. The LPS-induced gene expressions of IL-1β, IL-6, IL-12p40, and iNOS were repressed by IL-4. However, the mRNA levels of TNF-α and CD86 were not affected by IL-4. These incoherent effects of IL-4 can be described as its pleiotropic properties. The pleiotropic properties wrought the distinctive responsiveness of macrophages to IL-4, which was detected by the characteristic morphological change (Fig 6). Indeed, the co-treatment with LPS and IL-4 showed contrary results on TNF-α for 6 h in the present study and for 12 h in a previous study [14It has been reported that pure PLGAnPs showed a tendency to downregulate the pro-inflammatory cytokines on murine bone-marrow-derived macrophages, and a similar inclination was observed in this study [43, 44]. Among the phospholipid-engineered PLGAnPs, PSPCnP showed the strongest anti-inflammatory effect, which could be assessed by morphological changes and RT-qPCR results (Figs 6 and 7A–F). The M2 macrophage markers, however, showed statistically no significant difference among untreated and nanoparticle groups (Fig 7G-I). Because the hypothesis is supposed that anti-

inflammatory M2 macrophages end inflammation and promote tissue regeneration, M2 polarization of macrophages is recommended for the improvement of biocompatibility.

In this study, the lipid-grafted nanoparticles, especially PSPCnP, inhibited the polarization of M1 phenotype in inflammatory environment, whereas M2 polarization was not affected by nanoparticle treatment. These results are considered to be caused by the insufficient number of PS attached to PLGA during the particle generation process or the insufficient number of particles interacted with cells. In the pharmaceutical field, the research on inhibition of macrophage activation has been conducted mainly in the treatment of inflammatory diseases [45]. Furthermore, numerous studies have generally used the single type of phospholipid for lipid surface-engineered PLGAnPs, synthesized using the single-step method [22, 46, 47]. Meanwhile, the anti-inflammatory effect of nanoparticles was optimized at 50% mol of PS in phospholipids. These results correspond to those of the liposome experiments, reporting that the suggested mol% of PS was 30%–50% [14, 48-51]. Based on these results, we anticipate that the PS/PC-grafted PLGAnPs are a promising nanocarrier for inflammation. Although the in vivo effect of PS-nanoparticles is still elusive, a reducing effect in inflammation is expected on the basis of our in vitro results.

Chapter 5. Conclusion

In this study, the therapeutic potential of phospholipid PS-engineered PLGAnPs was evaluated. The surface grafting of PLGAnPs with PS upregulated the anti-inflammatory activity of PLGAnP. The morphological change and gene expression of TNF-α, IL-6, IL-12p40, CD86, and iNOS in LPS-treated macrophages were more substantially suppressed by PSPCnP than by PLGAnP. Overall, the results of this study reveal that PS grafting, particularly PS:PC = 50:50 mol%, indicates the therapeutic potential of PLGAnPs, attenuating inflammation and modulating the drug delivery system.

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Abstract in Korean

대식세포는 염증반응과 재생과정에서 중요한 역할을 하는 것으로 알려져 있다. 전염증성 대식세포인 M1형 세포는 염증성 환경을 조성하고, TNF-α, IL-1β, IL-6, IL-12 등과 같은 전염증성 사이토카인을 생성한다. 이러한 특성으로 인해 M1 대식세포의 지속적 발현은 상처의 치유 과정을 저해할 수 있다. 포스파티딜세린(PS)은 세포막 내부에 존재하는 인지질의 일종으로, 사멸세포에서 “eat-me”신호로 작용하여 대식세포의 탐식작용과 항염증 활성을 초래한다. 기존 연구에서 PS는 리포좀의 형태로 M1 대식세포의 발현을 억제하는 것으로 보고된 바 있다. 본 연구에서는 PS 분자의 활용성을 높이기 위하여 뛰어난 생체적합성과 생분해성을 지닌 것으로 알려져 있는 PLGA (polylactide-co-glycolide)의 표면을 PS로 개질하여 항염증 활성을 지닌 나노입자를 제조하고 이의 생물학적 특성을 조사하였다.

Single emulsification-solvent-evaporation(ESE) 기법을 활용하여 포스파티딜콜린(PC)과 PS를 함유한 PLGA 나노입자를 다음과 같이 제조하였다: 1) PC 100% (PCnP) 2) PS:PC = 50:50 (PSPCnP) 3) PS 100% (PSnP). PLGA 나노입자의 크기와 분포 정도는 나노입자 분석기로 분석하였으며, 표면 전하는 제타 전위 분석기를 통해 측정하였다. 세포실험에는 마우스 BMDM (Bone marrow-drived macrophage)을 이용하였고, 이들에 대한 나노입자의 세포독성은 12시간 처리 후 WST-8을 이용하여 측정하였다. M1 대식세포로 유도를 위해 lipopolysaccharide (LPS)를 사용하였고, 이를 억제하는 정도를 알아보기 위하여 나노입자를 LPS와

함께 처리하였다. 세포 형태의 변화는 12시간 처리 후 도립 디지털 현미경으로 관찰하였다. M1 대식세포를 나타내는 마커들 (TNF-α, IL-1β, IL-6, IL-12p40, CD86 및 iNOS)은 LPS와 함께 6시간 처리 후, M2 대식세포를 나타내는 마커들 (Arg-1, YM-1, CD206)은 12시간 처리 후 RT-qPCR을 통해 분석하였다.

PLGA 나노입자의 크기는 모든 그룹에서 210 nm 정도로 균일하게 관찰되었다. 제타 전위는 음성대조군 PLGAnP에서 0 mV에 근접한 결과를 보였으며, PCnP, PSPCnP, 및 PSnP 군에서는 -12 mV 이하의 표면전하를 나타내었다. 본 연구에서 사용된 모든 나노입자에서는 세포독성이 나타나지 않았다. LPS를 처리한 대식세포는 M1 대식세포로 분화하며 뚜렷한 형태변화를 관찰할 수 있었다. 한편, LPS와 함께 PSPCnP 또는 PSnP를 처리한 군에서는 M1형 형태변화가 저해되는 것으로 나타났다. TNF-α, IL-1β, IL-6 mRNA 발현은 모든 나노입자 처리군에서 감소하였으며, IL-12p40에서는 PSPCnP, PSnP에서만 감소하였다. CD86 및 iNOS의 결과에서는 통계적으로 유의미한 차이는 보이지 않았지만, PSPCnP군이 가장 발현을 저해되는 경향이 나타났다. 따라서, PS와 PC가 같은 비율로 존재하는 PSCP 군이 대식세포의 M1 분화를 가장 잘 억제하는 것으로 판단된다. 다만 M2 대식세포의 마커에서는 음성대조군과 비교해 유의미한 차이를 나타내지 않았다. 이는 입자생성 과정에서 충분한 양의 PS가 PLGA에 부착되지 않았거나 세포와 반응한 입자의 양이 부족한 등의 문제가 있는 것으로 판단된다.

주요어 : 대식세포, 인지질, 포스파티딜세린, PLGA , 나노입자, 항염증
학 번 : 2020-21982