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표면 개질된 PLGA 나노입자에 의한 대식세포의 염증유발 분극 억제효과

최상훈, 선경미, 보쾅칸, 손기태, 양형철*

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

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기존 연구에서 포스파티딜세린(PS)은 대식세포의 PS 수용체와 결합하여 사멸세포의 항염증 작용을 모방하였다. 본 연구에서는 PS로 표면 개질된 PLGA (polylactide-co-glycolide) 나노입자가 마크로파지 분극에 미치는 영향을 알아보고자 하였다. 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). PS를 함유한 PLGA 나노입자는 LPS에 의한 M1 마크로파지로의 형태변화 및 M1 마커 (TNF-α, IL-1β, IL-6, IL-12p40, CD86, iNOS)의 mRNA 발현을 저해하는 경향을 보였다. 특히, 양성대조군 및 인지질이 부착되지 않은 PLGAnP군에 비해 PSPCnP군에서 TNF-α, IL-6, IL-12p40 발현이 유의미하게 감소하였다($p<0.05$). 따라서, 이 연구의 결과는 염증을 약화시키고 약물 전달 시스템을 조절함에 있어 PS 이식 PLGA 나노입자의 잠재력을 보여준다.

색인단어 : 대식세포, 포스파티딜세린, PLGA, 나노입자

Introduction

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

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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).

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 encounter between PS and PS receptors on macrophages provokes the inhibitory effect of inflammation (8). PS is frequently utilized in PS-contained liposomes (PSLs), which could mimic the anti-inflammatory apoptotic cells (9). PSLs inhibit an inflammation by deregulating the expression of M1 macrophages and inducing M2 polarization (10). The immunomodulatory effect of PSLs was determined to be reinforced with arginine-glycine-aspartate (RGD) peptides on surface and co-treatment with sodium butyrate (11, 12). These studies suggest that PS-contained nanosized particles have a potential for upregulating the immunomodulatory effect with other elements.

Polylactide-co-glycolide (PLGA) is a copolymer of lactic acid and glycolic acid. The ratio of two monomers affects their properties, including the biodegradation rate and hydrophilicity (13). PLGA has been used widely because of its biocompatibility, biodegradability, and mechanical strength. Due to these attributes, the PLGAnP is considered as one of the most suitable nanoparticles for drug delivery systems (14). 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. The PLGAnP can be dissolved in highly hydrophobic and volatile solvents, such as dichloromethane, and chloroform using the ESE method (15).

Lipid surface-engineered PLGAnPs have been used in numerous studies owing to their biomimetic and biocompatible advantages (16). The synthesis methods can be categorized into the classical two-step and contemporary single-step processes (17). In the two-step method, the PLGAnPs are synthesized first and mixed with preformed liposomes later (18). In the single-step method, however, lipids are self-assembled around the PLGAnP core by hydrophobic interactions (19). The appropriate method for the experiment depends on numerous factors, including size, shape, and characteristics of the lipids (20).

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.

Materials and Methods

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 (15). The composition of nanoparticles was shown in Table 1.

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

Table 1. Composition of nanoparticles

Nanoparticles	PLGA	PC	PS
PLGAnP	40 mg	0	0
PCnP	40 mg	24 μmol	0
PSPCnP	40 mg	12 μmol	12 μmol
PSnP	40 mg	0	24 μmol

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.

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 femur (OrientBio Inc. Seongnam, Korea) were used with humanely sacrifice.

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.

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.

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 sequence of primers for RT-qPCR was shown in Table 2. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was employed as a control housekeeper gene. 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 2. 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

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.

Results

1. Characteristics of nanoparticles

The effect of size of nanoparticle is substantial on their immunomodulatory function (21). The size differences of nanoparticles caused by phospholipids were measured and evaluated. As shown in Fig. 1, 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 (22). The zeta potential of PLGAnP, PCnP, PSPCnP, and PSnP was -2.38, -12.01, -15.47, and -16.28, respectively (Figure 1).

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 showed no difference on the respective concentration of PLGAnP) The cell viability of BMDM was not different with concentration dependency for PLGAnP (Figure 2A.). In Figure 2B, the treatment with different types of nanoparticles showed no significant difference in cytotoxicity. Therefore, none of the nanoparticles used in this study showed cytotoxicity.

3. Effect of nanoparticles on cell morphology

The polarization of macrophages accompanies remarkable changes in cell shape. M1 macrophage exhibit a flat and spread like pancake shape, meanwhile M2 exhibit a spindle and elongated shape (23). Therefore, cell morphological changes could be observed under the optical microscope after 12 h treatment. Figure 3 shows

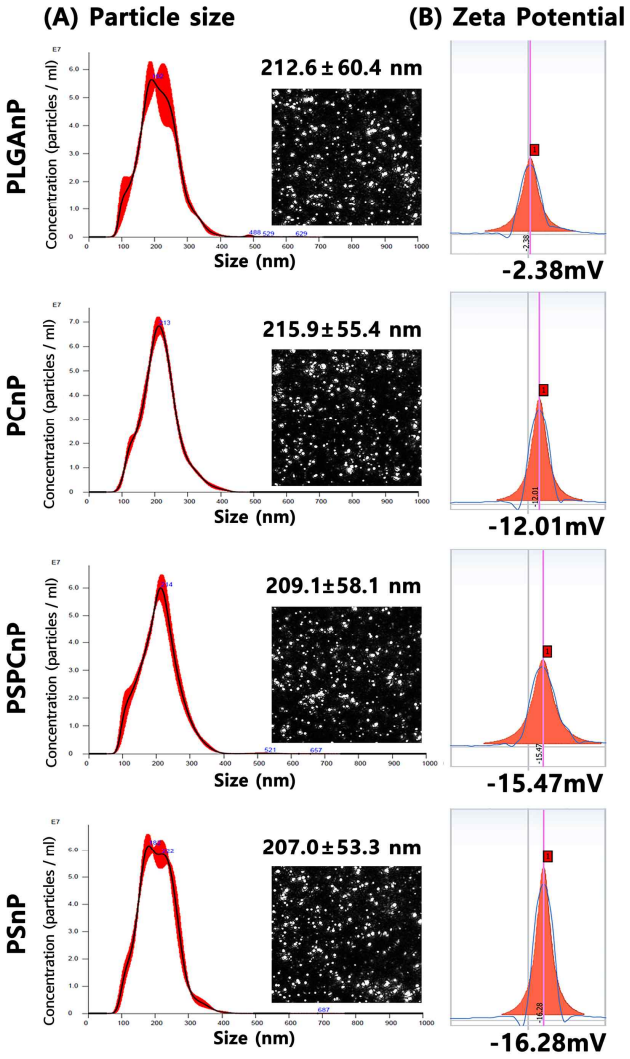

Figure 1. Nanoparticle size and zeta potential analyses. The size and particle distribution were no significant different between the PLGAnP, PCnP, PSPCnP, and PSnP groups. The zeta potentials were -2.38, -12.01, -15.47, and -16.28 for the PLGAnP, PCnP, PSPCnP, and PSnP groups, respectively. PLGAnPs, polylactide-co-glycolide nanoparticles; PCnP, phosphatidylcholine 100%; PSPCnP, phosphatidylserine:phosphatidylcholine = 50:50; PSnP, phosphatidylserine 100%

that bone-marrow-derived macrophages are slightly spindle or round in 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 change to the typical LPS-induced M1 shape. These figures suggest that cell shape is important cue for anti-inflammation effect.

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 (Figure 4A, C). Approximately 50% of IL-1β mRNA expression was declined by nanoparticles. However, there was no statistically significant difference among the experiment groups (Figure 4B). PLGAnP and PCnP did not affect the LPS-stimulated mRNA expression on the IL-12p40 marker, while PSPCnP and PSnP remarkably suppressed the expression (Figure 4D). Although there was no statistically significant difference in mRNA expression of CD86 and iNOS, PSPCnP showed a tendency to inhibit the expression (Figure 4E, F).

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 (24). The mean size of nanoparticles is a substantial factor in biomaterials, which affects various biosystems, such as cytotoxicity,

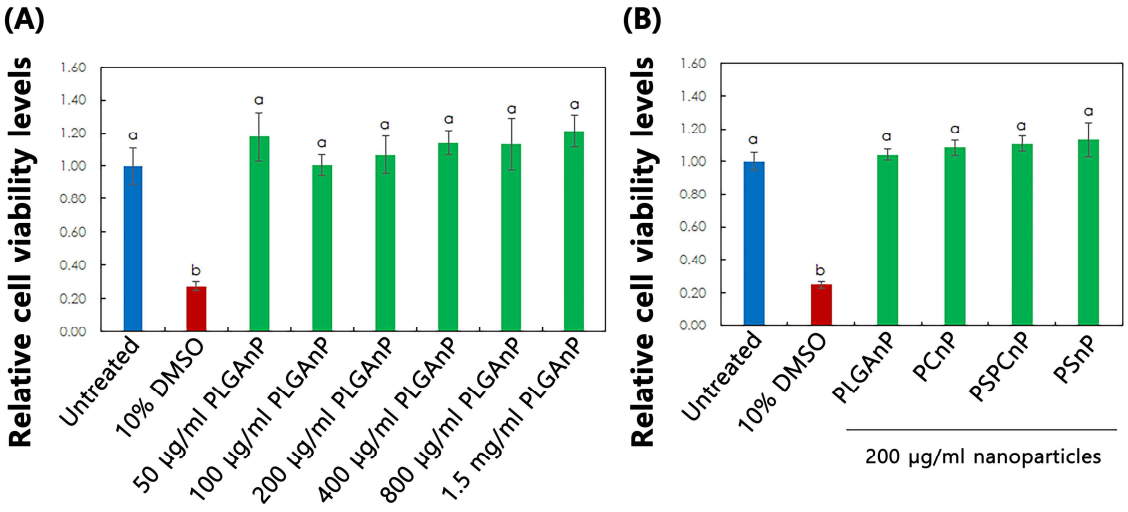

Figure 2. Cell viability assay for nanoparticles. The WST-8 assay was performed with different concentrations of PLGAnP (A), and with various kinds of nanoparticles at 200 μg/mL (B). Relative cell viability indicates the ratio between test group and control group.

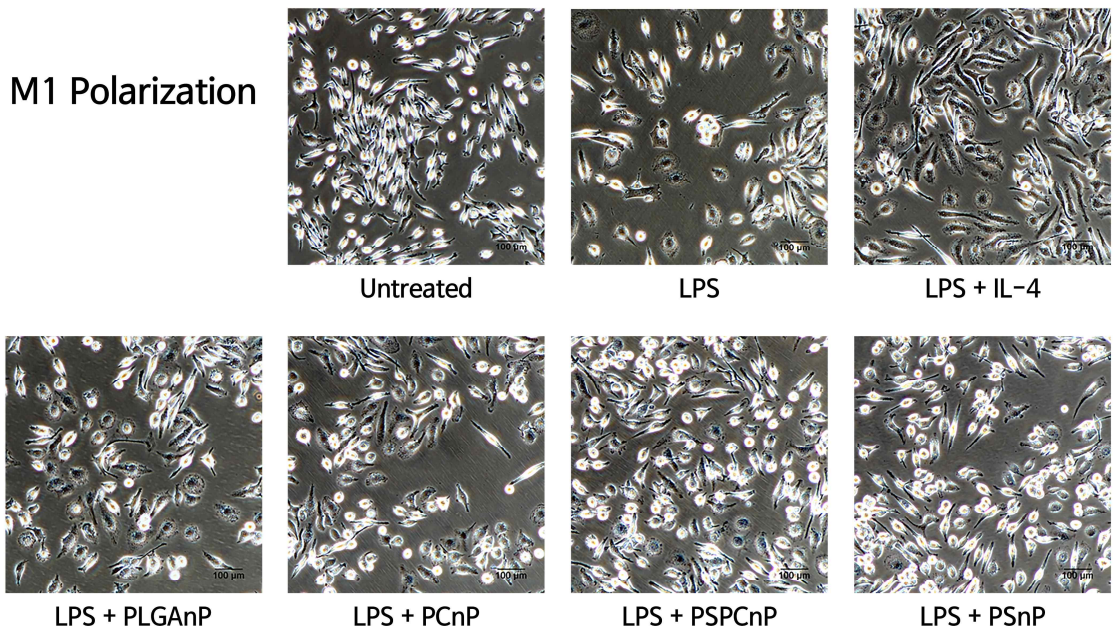

Figure 3. Cellular morphological analysis. Cell shape changes are observed under an optical microscope. LPS stimulation induced the M1 polarization, while co-treatment with PSPCnP and PSP could prevent the cell morphological changes. Scale bar is 100 μm.

macrophage polarization, and mesenchymal stem cell osteogenesis (21, 25). 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) (26). The proper size of PLGAnP ranged from 100 to 300 nm (27). The average size of nanoparticles was approximately 210 nm, which was not significantly different among the groups (Figure 1). The zeta potential also plays a crucial role in cytotoxicity and cellular interactions (22). Cytotoxicity

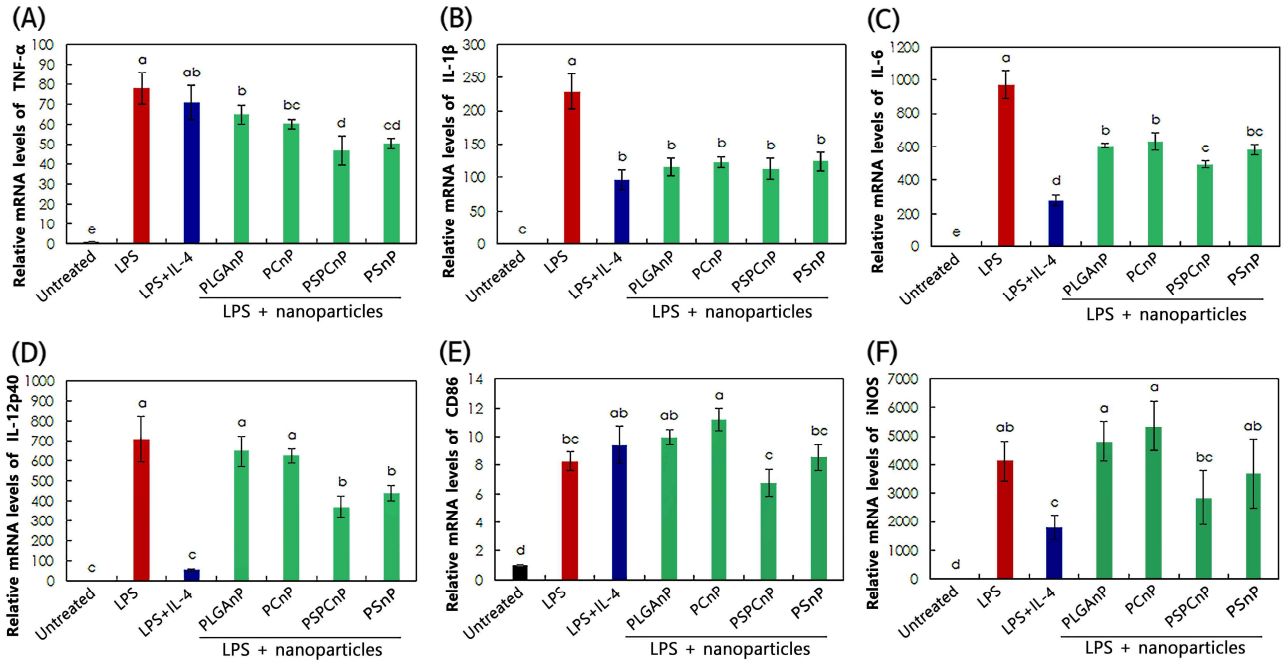

Figure 4. Effects of nanoparticle on mRNA expression of inflammation and polarization genes. (A–F) To assess the anti-inflammatory effect of nanoparticles, macrophages were cotreated with nanoparticles and LPS for 6 h. The mRNA expression of TNF-α, IL-1β, IL-6, IL-12p40, CD86, and iNOS genes were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). 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 ($p>0.05$).

derived from the PLGAnP was present when the zeta potential ranged from -13.2 to -19.3 mV (27). In Figure 1, 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 2A, which support the findings of a previous study (28). PLGAnP grafted on the surface could stunt the cell viability (29). We demonstrated that 200 μg/ml of nanoparticles had no cytotoxicity to BMDM (Figure 2B).

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 (14). 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 (10-12, 30). 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 4A–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 (Figure 3). 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 (11). It 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 (31). Among the phospholipid-engineered PLGAnPs, PSPCnP showed the strongest anti-inflammatory effect, which could be assessed by morphological changes and RT-qPCR results (Figures 3 and 4A–F).

In this study, the lipid-grafted nanoparticles, especially PSPCnP, inhibited the polarization of M1 phenotype in inflammatory environment. In the pharmaceutical field, the research on inhibition of macrophage activation has been conducted mainly in the treatment of inflammatory diseases (32). Furthermore, numerous studies have generally used the single type of phospholipid for lipid surface-engineered PLGAnPs, synthesized using the single-step method (15, 33, 34). 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% (11, 35). Based on these results, we anticipate that the PS/PC-grafted PLGAnPs are a promising nanocarrier for inflammation. For clinical applications, the injections of nanoparticles directly to the inflammatory site is thought to be appropriate.

Conclusions

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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Original Article

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

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Phosphatidylserine (PS) mimics the anti-inflammatory effect of apoptotic cells by binding to the PS receptor of macrophages. In this study, the effect of PS-modified polylactide-co-glycolide (PLGA) nanoparticles on macrophage polarization was investigated. PLGA nanoparticles (PLGAnPs) containing phosphatidylcholine (PC) and PS were prepared using the emulsification-solvent-evaporation (ESE) technique and classified as follows: 1) PC 100% (PCnP); 2) PS:PC = 50:50 (PSPCnP); and 3) PS 100% (PSnP). PS-grafted PLGAnPs tended to inhibit LPS-induced morphological change into M1 macrophages and mRNA expression of the M1 markers (TNF-α, IL-1β, IL-6, IL-12p40, CD86, and iNOS). In particular, the expressions of TNF-α, IL-6, and IL-12p40 were significantly decreased in the PSPCnP group, as compared to those of the positive control and and PLGAnP groups ($p<0.05$). Therefore, the study results demonstrate the potential of PS-grafted PLGAnPs in attenuating inflammation and modulating the drug delivery system.

Keywords : Macrophage, Phosphatidylserine, PLGA, Nanoparticles