Liposomes as Immunomodulator
—Inhibitory Effect of Liposomes on NO Production from Macrophages—

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Liposomes have been artificially made into membranous vesicles composed essentially of naturally occurring phospholipids and have been found to serve as a carrier of drugs and an immunological adjuvant. After being intravenously injected, they are quickly removed from the blood circulation and trapped by Kupffer cells of the liver and macrophages of spleen. However, the changes liposomes exert in these cells with which liposomes interact remain unresolved. To clarify this point is very important to assure the safe use of liposomes as drug carriers. Macrophages have many unique functions, and nitric oxide (NO) produced by NO synthase (NOS) which is induced in response to some cytokines and bacterial products such as lipopolysaccharide (LPS) is responsible for the bactericidal, tumoricidal and immune regulatory activities. On the other hand, overexpressed NO is implicated in the development of atherosclerosis, DNA injury, and hypotension associated with septic shock. This article focuses on the effects of liposomes on NO production from LPS-stimulated mice peritoneal macrophages in vitro; we found that liposomes composed of phosphatidylyserine inhibit NO production. We also discuss the mechanism of the inhibitory activity of liposomes.

Key words macrophage; liposome; nitric oxide

INTRODUCTION

Liposomes are of considerable interest as carriers for the controlled delivery of drugs because many substances can be encapsulated in aqueous and lipid phases.\(^{1}\) Cationic liposomes are particularly expected to be a candidate as a non-viral vector, because DNA readily forms a complex with cationic liposomes via electrostatic interactions\(^{2-4}\); liposomes labeled with the ligands transferrin\(^{5}\), folate\(^{6}\), epidermal growth factor (EGF)\(^{7}\), and asialoglycoprotein\(^{8}\) are reported to be candidate carriers of DNA to the expected tissues in vivo.

One of the best known observations in the field of liposome research is that intravenously injected naked liposomes are rapidly taken up by macrophages in the reticuloendothelial system such as the liver and spleen.\(^{9,10,11}\) Since this characteristic is advantageous for directing antigens or immunomodulating agents toward macrophages, many investigators have used liposomes as an adjuvant, depots for theslow release of antigens, and the targeting antigens to macrophages, which are one of the antigen-presenting cells. Macrophages have many unique functions, such as enhancing phagocytosis, protease secretion, increasing the production of interleukin-1, and cellular cytotoxicity, when activated by various naturally occurring synthetic agents.\(^{12}\) But, it is not yet clear how liposomes affect these macrophage functions. It is essential that this be clarified to assure that liposomes can be used safely as controlled delivery systems. We previously investigated the effects of liposomes on Fcγ-receptor-mediated phagocytosis, one of the most important functions of macrophages, in vitro\(^{13}\) and in vivo\(^{14,15}\) and found that they activated macrophages to phagocytize the opsonized foreign body through Fcγ-receptor irrespective of liposomal charge; this suggested that liposomes could alter macrophage functions.

Nitric oxide (NO), a reactive free-radical gas, was found to be generated enzymatically from 1-arginine and molecular oxygen by constitutive or inducible NO synthase (NOS) in a variety of cells; neuronal cells, endothelial cells, neutrophiles, Kupffer cells, and activated macrophages (Table 1).\(^{16,17}\) Endothelial and neuronal NOS are expressed constitutively and are Ca\(^{2+}\) and calmodulin dependent, and NO produced by these NOS acts as an intracellular messenger, participating in neurotransmission and vascular homeostasis.\(^{18,19}\) In macrophages, NOS is expressed only by transcriptional induction, and macrophages activated with some cytokines or lipopolysaccharide (LPS) produce large quantities of NO which is generated by inducible NOS (i-NOS). NO produced by i-NOS in macrophages is partially responsible for the bactericidal, tumoricidal, and possibly immune regulatory activities. Once induced, i-NOS synthesizes high levels of NO at a constant rate for long periods, and overex-

| Table 1. Characteristics of Three Isoforms of NOS |
|---|---|---|
| Representative cell | Expression | Regulation of NO production |
| n-NOS (NOS I) | Neuron | Constitutive | Ca\(^{2+}\)/calmodulin dependent |
| e-NOS (NOS III) | epithelial cell | Constitutive | Ca\(^{2+}\)/calmodulin dependent |
| i-NOS (NOS II) | Macrophage | Inducible | Depends on amount of i-NOS protein but not on Ca\(^{2+}\)/calmodulin |

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pressed NO and its metabolite ONOO⁻ have been implicated in the development of atherosclerosis, DNA injury, and hypotension associated with septic shock. Consequently, it is crucial to regulate the excess NO produced by iNOS in macrophages.

This article describes the inhibitory effects of liposomes on the NO production from mouse peritoneal macrophages stimulated with LPS in vitro and the inhibitory mechanism of action.

Effects of Liposomes on NO Production from Macrophages C3H/HeN mice (male, 6–8 weeks old) were injected with 1 ml of 2% thioglycollate intraperitoneally. On day 4, peritoneal macrophages were collected and then incubated with liposomes for 24 h. The macrophages were then further incubated with 10 μg/ml LPS for 48 h in order to elicit NO production. NO production was estimated by measurement of nitrite in culture supernatant using Griess reagent. As shown in Fig. 1(A), NO production induced by LPS was inhibited by the treatment of macrophages with negatively charged liposomes composed of phosphatidylserine (PS-liposomes), phosphatidylcholine (PC : PS : cholesterol = 1 : 2 : 1 (by mol)) and phosphatidic acid (PA-liposomes, PC : PA : cholesterol = 1 : 2 : 1), while no inhibition was observed in neutral liposomes composed of phosphatidylcholine (PC-liposomes, PC : cholesterol = 3 : 1). PS-liposomes inhibited NO production in a dose dependent manner, and complete inhibition was observed at 500 μg/ml of PS (Fig. 1(B)). No differences in the inhibitory activities of PS-liposomes were observed between multilamellar vesicles and small unilamellar vesicles. Furthermore, macrophage viability after liposome treatment was estimated by Trypan blue dye exclusion test, and no change in viability was observed (data not shown). Hence, NO production was inhibited by negatively charged liposomes.

The kinetics of NO inhibitory activity of PS-liposomes on macrophages was examined, and at least 21 h of PS-liposome treatment were needed to decrease NO production to control levels (Fig. 2). Preincubation of macrophages with PS-liposomes for 3, 6, or 9 h resulted in no inhibition of their potential to produce NO; nor did the addition of PS-liposomes to macrophage culture at the time of LPS stimulation interfere with their normal production of NO. Therefore, pretreatment appears to be required in order to observe the inhibitory activity of PS-liposomes in NO production induced by LPS.

Figure 3 shows the results of Western blot analysis for iNOS protein. An immunoreactive band at 130 kDa reacting with anti-iNOS antibody was observed in the extract of the control and PC-liposome-treated macrophages, whereas a faint or no band was observed in PS-liposome-treated ones. The intensity of the immunoreactive band was inversely propor-
Fig. 4. Effects of Liposomes on the Expression of i-NOS mRNA
Macrophages (1×10⁶ cells) were treated with 500 μg phospholipid/ml of PC-liposomes (A) or PS-liposomes (B) for the indicated times. Total RNAs were extracted and then RT-PCR was performed. Lower panel shows the changes in ratio of i-NOS mRNA to β-actin which was evaluated from the band intensity using NIH image.

Table 2. Scavenger Receptor and Ligand Binding

<table>
<thead>
<tr>
<th>SRA</th>
<th>CD36</th>
<th>SR-B1</th>
<th>SR-C1</th>
<th>FcyRIIB2</th>
<th>Macroladin</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxLDL</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AcLDL</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maleylated BSA</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>PS-liposome</td>
<td>-</td>
<td>+</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
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</tbody>
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Liposomes inhibit the i-NOS expression in macrophages following LPS stimulation, but do not inhibit i-NOS activity. The effects of liposomes on i-NOS mRNA levels were also examined by RT-PCR analysis. Figure 4(A) indicates the effects of PC-liposomes on i-NOS mRNA levels following LPS stimulation. The i-NOS mRNA band was observed at 3 h, and reached the maximum level at 6 h after stimulation with LPS. The changes in ratio of i-NOS mRNA to β-actin mRNA were estimated by NIH image, and no changes in the ratios between PC-liposome-treated and LPS treated ones (control) were observed. On the other hand, the i-NOS mRNA band from the extract of macrophages treated with PS-liposomes was faint, and the ratio of i-NOS mRNA to β-actin mRNA showed less than half that of the control (Fig. 4(B)), suggesting that PS-liposomes inhibit NO production at the up-stream of transcription of i-NOS mRNA following stimulation with LPS. These findings correspond well with data of Western blot analysis and of NO production.

Contribution of Scavenger Receptor Negatively charged liposomes composed of anionic phospholipids such as PS are preferentially taken up by phagocytic cells, especially macrophages. Several studies have demonstrated that scavenger receptors (SRs) which bind chemically modified lipoproteins such as oxidized lipoprotein (OxLDL) and acetylated LDL (Acetyl LDL) may in some cases function as anionic phospholipid receptors. Yang et al., Thai et al., and Hamilton et al. showed the expression of inflammatory cytokine mRNAs and release of NO to be inhibited by Ox-LDL in macrophages. These findings suggest that SR is involved in the inhibition of NO production, but the mechanism of this effect remains to be established. Many types of scavenger receptors have been reported, and the relationships between scavenger receptors and ligand binding specificity are shown in Table 2. Thus, we investigated the effects of several ligands for SRs on NO production from macrophages stimulated by LPS to determine the contribution of SR to the regulation of NO production.

NO production from macrophages treated with Ox-LDL, heparin, and maleylated BSA was inhibited as well as from those treated with PS-liposomes, while no inhibition was observed by native LDL, acetyl-LDL, or dextran sulfate treatment (Fig. 5). Western blot analysis of i-NOS protein revealed that the pretreatment of macrophages with Ox-LDL, heparin, and maleylated BSA also inhibited the induction of i-NOS (Fig. 6). Furthermore, the pretreatment of macrophages with Ox-LDL was required to decrease NO production to control levels as well as PS-liposomes (data not shown).

CD14, a 55 kDa protein on the surface of mononuclear phagocytes, is thought to be a receptor for LPS and is closely related to the signal transduction of LPS for NO production. The inhibitory effect of several ligands for SR(s) on NO production induced by LPS would thus appear to be derived from the inhibition of LPS binding to the receptor.
through ligand–LPS complex formation, or ligands may compete for LPS receptor. Pretreatment appears essential to detect the inhibitory activities of Ox-LDL and PS-liposomes on NO production, and no inhibitory effects were observed following concomitant treatment of macrophages with these ligands and LPS. Thus, the inhibition of NO production by several ligands for SR(s) does not appear to be due to the formation of a ligand–LPS complex or PS-liposomes (or Ox-LDL) competing for the LPS receptor; SR(s), especially CD36 and/or macroscialin, are suggested to contribute this inhibition, because of their ligand binding specificity as indicated in Table 2.

Sambrano and Steinberg\textsuperscript{37} and co-workers\textsuperscript{38} reported that a plasma membrane protein of 94–97 kDa (macrosialin) was one of the SRs of macrophage for Ox-LDL; PS-liposomes showed strong binding to the protein and this binding clearly inhibited Ox-LDL binding to macrophages. To determine whether macroscialin participates in the inhibitory effects of PS-liposomes on NO production, the effects of antimacrosialin monoclonal antibody (FA/11, kindly given by Professor S. Gordon, Oxford University, U.K.) on the inhibition of NO production by PS-liposomes were investigated.\textsuperscript{39} FA/11 itself showed no effect on NO production induced by LPS. Binding of FA/11 to macrophages was inhibited by the addition of PS-liposomes, indicating that the liposomes have an affinity for macrosialin. However, the inhibitory effects of these ligands on NO production were not reduced even in the presence of FA/11 (Fig. 7). These findings suggest that macrosialin does not contribute to the inhibitory effects of PS-liposomes on NO production from macrophages stimulated with LPS, and the contribution of other type of receptors.

Normally, very little, if any, PS is found on the inner leaflet of the plasma membrane of cells, and the maintenance of this asymmetry is an energy-consuming process attributed to an aminophospholipid translocase located within the plasma membrane.\textsuperscript{40,41} Early after the initiation of programmed cell death (apoptosis), the loss of phospholipid asymmetry and the exposure of PS on the outer surface of the plasma membrane occur.\textsuperscript{42} The exposure of PS on the outer surface of apoptotic cells triggers specific recognition and removal by macrophages.\textsuperscript{43,44} In this recognition, the contributions of different receptors such as the vitronectin receptor,\textsuperscript{45} CD36,\textsuperscript{46} CD14,\textsuperscript{47} and class B scavenger receptor\textsuperscript{48} as well as macrosialin have been reported. Furthermore, Fadok et al.\textsuperscript{49} recently cloned a gene that appears to recognize PS on apoptotic cells. Therefore, signal(s) generated following the binding of PS-liposomes to macrophages through these receptors, but not macrosialin, probably interfere with NO production from mouse peritoneal macrophages stimulated with LPS.
Inhibitory Mechanism of PS-Liposomes on NO Production

Does TGF-β Act as a Factor That Inhibits the NO Production?: Pretreatment of macrophages with PS-liposomes appeared to be required in order to observe the inhibitory activity in NO production induced by LPS as mentioned above; no effect was observed when PS-liposomes were added to macrophage culture at the same time as LPS stimulation. We thus speculated that a factor interfering with NO production could be secreted from macrophages following the PS-liposome treatment. Macrophages were treated with liposomes for 24 h, and culture supernatant was collected by ultracentrifugation. The supernatant was added to the naive macrophages and incubated with LPS for 48 h. NO production induced by LPS was only inhibited by the culture supernatant prepared by incubating the macrophages with PS-liposomes (data not shown). The intensity of inhibition was almost the same as that of the positive control, macrophages that were treated with PS-liposomes directly. These results suggest that macrophages secrete an inhibitory factor and this factor inhibits NO production from macrophages stimulated with LPS.

Apoptotic cells express cell surface changes that allow recognition and removal by macrophages. Removal occurs before lysis, which prevents the release of potentially toxic and immunogenic intracellular contents from the apoptotic cells into the surrounding tissue, and inflammation is avoided. For the removal of apoptotic cells, macrophages recognize PS on these cells and phagocytose them through CD36 as a predominant scavenger receptor.50,51 In consequence, macrophages produce transforming growth factor (TGF)-β, prostaglandin E2 (PGE2) and platelet-activating factor (PAF), and these products inhibit the production of inflammatory cytokines such as interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF)-α and granulocyte macrophage-colony stimulating factor (GM-CSF) from macrophages stimulated with LPS. TGF-β is a multifunctional polypeptide growth factor that has been increasingly recognized as an important immunoregulatory molecule, although its reported effects on immunological responses are often contradictory. TGF-β was shown previously to downregulate the production of proinflammatory mediators in macrophages.52–54 Therefore, we turned our attention to TGF-β as a factor that would modulate NO production from macrophages following PS-liposome treatment, and its production was evaluated by RT-PCR analysis. Figure 8 shows the expression of TGF-β mRNA following the treatment of macrophages with liposomes. TGF-β mRNA was constitutively expressed in macrophages, its level began to increase 1 h after the addition of PS-liposomes, and reached maximum at 3 h. No change in TGF-β levels was observed in macrophages treated with PC-liposomes, however, and the same levels as control were shown throughout the experimental period. Furthermore, NO production from macrophages stimulated with LPS was inhibited in a dose dependent manner by the addition of TGF-β (Fig. 9), but this inhibitory effect on NO production was not complete, suggesting the contribution of other factor(s).

There are several reports52,53 describing that TGF-β could inhibit NO production from macrophages. Vedovozv et al.54 also reported the inhibitory activity of TGF-β on NO production from macrophages, and suggested that the potency of TGF-β reflects the suppression of i-NOS expression by three distinct mechanisms, decreased stability and translation of i-NOS mRNA, and increased degradation of i-NOS protein. Our findings obtained in Fig. 4, in contrast, clearly showed that the expression of i-NOS mRNA was inhibited by the treatment of macrophages with PS-liposomes. Studies are now underway to clarify how PS-liposomes affect macrophages to induce TGF-β and how TGF-β inhibits NO production.
PS-Liposomes Inhibit p38 MAP Kinase, but Not NF-κB Activation: The details of the signal transduction cascade involved in the induction of i-NOS in response to LPS are an active area of investigation. Although LPS-induced i-NOS induction in macrophages, the molecular mechanism involved in this process is not fully understood.

Nuclear transcriptional factor (NF)-κB appears to play a primary role in the transcriptional regulation of the i-NOS gene in macrophages. In unstimulated macrophages, NF-κB is present in the cytoplasm in an inactive form, complexed with NF-κB inhibitor protein I-κB. Upon stimulation, I-κB is phosphorylated on specific serine residues, and degraded in an ubiquitin-dependent process. Then the activated NF-κB is translocated into the nucleus, binds to the κB-site on DNA, and induces the expression of i-NOS mRNA. Therefore, we first investigated the effects of PS-liposomes on the degradation of I-κBα using Western blot analysis. Immediately after the addition of LPS, I-κBα protein disappeared and then reappeared during incubation (Fig. 10). In the case of macrophages pre-treated with PC- or PS-liposomes, the band intensity of I-κB exhibits the same profile as that of control pre-treated with buffer solution. These results suggest that PS-liposomes do not interfere with the degradation of I-κB in macrophages stimulated with LPS.

DNA binding activity of NF-κB in the nucleus of macrophages was examined by electrophoretic mobility shift assay (EMSA) using 32P-labeled NF-κB specific oligonucleotides. PS-liposome treatment did not alter NF-κB translocation into the nucleus or DNA binding in macrophages stimulated with LPS, and gel shift mobility was the same as that of those treated with LPS (Fig. 11). Consequently, the inhibition of NO production from macrophages treated with PS-liposomes did not result from the inhibition of DNA binding of transcription factor NF-κB.

In macrophages, LPS stimulates the simultaneous activation of three classes of mitogen activated protein (MAP) kinases: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase, and p38 with different activation kinetics. Recently, Carter et al. and others reported that the p38 MAP kinase regulates NF-κB-dependent gene transcription by modulating the binding activity of TATA-binding protein (TBP), a basal transcriptional factor, to the TATA box, and the phosphorylation of TBP by the p38 MAP kinase is required for the binding of TBP to the TATA box. Since the p38 MAP kinase activation is regulated by dual phosphorylation on tyrosine and threonine, the effects of liposomes on the phosphorylation of tyrosine was examined by Western blotting. The phosphorylation of tyrosine induced by LPS stimulation was clearly inhibited by the treatment of macrophages with PS-liposomes, while no inhibition was observed in those which were PC-liposome-treated (Fig. 12).

To investigate whether p38 MAP kinase is involved in the LPS-stimulated induction of i-NOS in macrophages, we examined the effects of SB203580, a specific inhibitor of this kinase, on NO production from macrophages stimulated with LPS. SB203580 inhibited LPS-stimulated NO induction in a dose dependent manner, and the complete inhibition was observed at 50 μM (Fig. 13). At the concentrations used, no changes in cell viability were observed (data not shown). Furthermore, the effects of SB203580 on DNA binding of NF-κB were examined by EMSA, and was found to alter...
DNA binding of NF-κB by the evaluating gel shift mobility (Fig. 11), indicating that the p38 MAP kinase may not be involved in the process of NF-κB activation or DNA binding of NF-κB. From these results, one possible mechanism for the inhibitory effects of PS-liposomes on NO production from LPS-stimulated macrophages might be the inhibitory effects of the liposomes on p38 MAP kinase activation. Studies are currently underway to learn how PS-liposome treatment interferes with the p38 MAP kinase activation in macrophages stimulated with LPS.

CONCLUSION

The effects of liposomes on NO production from LPS-stimulated macrophages were examined, and negatively charged liposomes composed of phosphatidylinerine were found to inhibit the production. The proposed inhibitory mechanism of PS-liposomes on NO production is illustrated in Fig. 14: following treatment with these liposomes, macrophages secrete a factor which interferes with i-NOS expression induced by LPS, and TGF-β appears to be a candidate for this factor. PS-liposome treatment did not inhibit transcriptional factor NF-κB activation which appears to play a primary role in the transcriptional regulation of the i-NOS gene in macrophages. The p38 MAP kinase is essential to the LPS-induced i-NOS gene expression, and understood regulates NF-κB-dependent gene transcription by modulating activation of the TATA box binding protein.58 Thus, a factor secreted from macrophages by treatment with PS-liposomes might inhibit the p38 MAP kinase activation induced by LPS treatment.

The exposure of PS on the outer surface of apoptotic cells triggers the removal by macrophages. Removal occurs before lysis, and inflammation is avoided. The mechanism is not yet fully understood, but secretions of TGF-β, PGE_{2}, and PAF which inhibit the production of inflammatory cytokines from macrophages are indicated.50,51 Consequently, PS-liposomes might be a suitable model for understanding the changes in macrophage functions induced by the phagocytosis of apoptotic cells. Furthermore, since macrophages preferentially take up liposomes and macrophages migrate where inflammation occurs, PS-liposomes could be a candidate as DDS carrier for anti-inflammatory drugs, expecting the anti-inflammatory effects of PS-liposomes themselves and the encapsulating drugs.

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