Comparison of Inhibitory Effects of Polyanions on Nitric Oxide Production by Macrophages Stimulated with LPS

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In this paper, we investigated the inhibitory mechanism of the production of nitric oxide (NO) by polyanions and liposomes composed of phosphatidylserine (PS-liposomes) focusing on cytokine production and mitogen activated protein kinase (MAP kinase) activation. NO production by macrophages was inhibited by treatment with oxidized lipoprotein (OxLDL), maleylated bovine serum albumin (mBSA), and heparin. No inhibitory effect was exhibited by poly-cytidilic acid (PolyC). To clarify the mechanism of the inhibitory effect of polyanions on NO production, we evaluated the productions of transforming growth factor-β (TGF-β) and interleukin (IL)-10 which are known to be anti-inflammatory cytokines. TGF-β was produced when macrophages were treated with OxLDL as was the case with PS-liposomes. No increase in TGF-β production was observed for mBSA, heparin, and PolyC. On the other hand, significant production of IL-10 was observed using mBSA. Extracellular signal-regulated kinase (ERK), a member of the MAP kinase superfamily, was activated when macrophages were treated with OxLDL as well as PS-liposomes. In the case of mBSA, the activation of ERK and c-Jun N-terminal kinase (JNK) was observed. No activation of p38 MAP kinase was observed using any of the polyanions. Although heparin had an inhibitory effect on NO production by macrophages, no activation of MAP kinase or production of TGF-β and IL-10 was observed. The inhibitory effect of these ligands on NO production may be regulated *via* different signaling pathways.

Key words nitric oxide (NO); macrophage; polyanion; mitogen activated protein (MAP) kinase; transforming growth factor-β (TGF-β); interleukin (IL)-10

Nitric oxide (NO) is an endogenously synthesized free radical gas that has been demonstrated to modulate a variety of cellular and physiological processes.[1,2] Activated macrophages express inducible NO synthase (i-NOS) in response to lipopolysaccharide (LPS) or a variety of cytokines, and NO derived from i-NOS is an important mediator of acute or chronic inflammation, and also contributes to the killing of virally infected cells, tumor cells and some pathogens.[3—5]

Negatively charged liposomes composed of anionic phospholipids such as phosphatidylserine (PS) are preferentially taken up by phagocytic cells, especially macrophages, through receptors such as class A and B scavenger receptors,[6,7] CD68,[8] vitronectin receptor,[9] and PS-specific receptor.[10] Fadok *et al.* reported extensively on the PS-specific receptor-mediated recognition and endocytosis of PS-expressing apoptotic cells by macrophages.[11] We have already reported that PS-liposomes inhibited the production of NO by macrophages stimulated with LPS, and demonstrated that PS-liposomes interact with macrophages through PS-specific receptors, leading to the production of transforming growth factor-β (TGF-β) *via* the activation of extracellular signal-regulated kinase (ERK) and finally, the TGF-β produced by PS-liposomes inhibited NO production in macrophages.[12,13] We also investigated the effects of other negatively charged materials (polyanions), which are recognized as ligands for scavenger receptors, on NO production, and found that oxidized lipoprotein (OxLDL), maleylated bovine serum albumin (mBSA), and heparin, but not acetyl-LDL or PolyC, inhibited NO production by macrophages stimulated with LPS.[14] However, the receptors for these ligands differ, and it is not clear how the ligands inhibit the production of NO in macrophages stimulated with LPS.

In this paper, we investigated the inhibitory mechanism of NO production induced by these ligands in mouse peritoneal macrophages focusing on cytokine production and mitogen activated protein (MAP) kinase activation.

MATERIALS AND METHODS

Materials PS from bovine brain, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111; B4), Poly-Cytidylic acid (PolyC), human LDL, and recombinant human interleukin (IL)-10 were purchased from Sigma Co., Ltd. Heparin and bovine serum albumin (BSA, fraction V) were purchased from LKT Laboratories, Inc. and Boehringer Mannheim Co., respectively. Anti-mouse TGF-β1 antibody, biotinylated anti-mouse TGF-β1 antibody, anti-mouse IL-10 antibody, biotinylated anti-mouse IL-10 antibody and recombinant human TGF-β were purchased from R&D systems.

Preparation of OxLDL and mBSA Copper OxLDL was prepared by incubating LDL (250 μg/ml) with $Cu(Ac)_2$ (5 μM) in phosphate-buffered saline at 37 °C for 24 h as described by Yang *et al.*[15] The extent of oxidation was estimated by measuring lipid peroxidation in terms of the amount of thiobarbituric acid reactive substance expressed as nmol of malondialdehyde. mBSA was prepared according to the procedures of Sakai *et al.*[16]

Preparation of Liposomes Multilamellar liposomes were prepared by vortexing and passed through a membrane filter (0.45 μm; Corning Glassworks, Corning, NY, U.S.A.) before use. The lipid composition of liposomes was PS : PC : cholesterol = 2 : 1 : 1 (PS-liposomes).

Preparation of Macrophages Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee on the Care and Use of Laboratory Animals. C3H/HeN mice (male, 6—8 weeks old, Japan SLC Inc., Shizuoka) were injected intraperitoneally with 1.0 ml of 3% thioglycollate (Difco Labo-

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ratory, Detroit, MI, U.S.A.). On day 4, the peritoneal macrophages were prepared according to our previous method.[17]

Nitrite Assay Macrophages (1×10^5 cells/well) were treated with several negatively charged ligands for at least 24 h, and then further incubated for 48 h with LPS (10 μg/ml). NO production was estimated by measuring the amount of nitrite in the culture supernatant using Griess reagent as described by Stuehr and Nathan.[18] In brief, an aliquot of the conditioned medium was mixed with an equal volume of 1% sulfanilamide and 0.1% *N*-1-naphthyletylenediamine dihydrochloride in 5% phosphoric acid. The absorbance at 540 nm was measured to evaluate NO production. Sodium nitrite diluted in HBSS was used to generate a standard curve.

Detection of TGF-β and IL-10 Macrophages (5×10^5 cells/well) were treated with several polyanions and supernatants were collected the appropriate time. TGF-β1 and IL-10 concentrations were determined by ELISA using pairs of purified capture and biotinylated detection antibodies recognizing murine TGF-β1, β2, β3 and IL-10, respectively, according to the manufacturer's instructions (BD Biosciences).

Western Blotting Analysis Macrophages (1×10^6 cells/well) were treated with several negatively charged ligands for specific periods. Cells were then lysed using our previous method. For the determination of phosphorylated ERK, p38, or c-Jun N-terminal kinase (JNK) protein, cell lysates were separated by 12% SDS-PAGE, blotted on Immobilion P membranes (Nihon Millipore, Tokyo, Japan) and analyzed using a Phospho plus p44/p42 MAP Kinase (Thr202/Tyr204) Antibody Kit, Phospho plus p38 MAP Kinase (Thr180/Tyr182) Antibody Kit or Phospho plus SAPK/JNK (Thr183/Tyr185) Antibody Kit (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.), respectively.

Statistical Analysis The statistical significance of mean comparisons was determined by ANOVA with Duncan's test for multiple comparisons. The *p*-values for significance were set at 0.01.

RESULTS AND DISCUSSION

Inhibition of NO Production by Various Negatively Charged Ligands We previously reported that negatively charged liposomes inhibited the production of NO in mouse peritoneal macrophages stimulated with LPS.[17] It is well established that negatively charged liposomes are recognized and taken up *via* scavenger receptors.[19] Several types of scavenger receptors have been reported to be present on the macrophage surface,[20] and not only chemically modified LDL, mBSA, and selected polysaccharides but also negatively charged phospholipids such as PS could serve as ligands. Therefore, we investigated the effects of negatively charged ligands (polyanions) on NO production in thioglycollate-elicited mouse peritoneal macrophages. The production of NO was inhibited by treatment with PS-liposome, mBSA, OxLDL, and heparin in a dose dependent manner. However, in comparison with PS-liposomes (125 μg lipid/ml), the inhibitory effects of these anions were weak (Figs. 1A—D). No inhibitory effect was exhibited by PolyC (Fig. 1E).

Induction of TGF-β and IL-10 by Various Negatively Charged Ligands TGF-β and IL-10 are known to be anti-inflammatory cytokines, and both inhibit the production of NO by macrophages.[12,21] The former contributes to numerous physiological processes, including cell growth, differentiation, and the down-regulation of proinflammatory cytokine production in macrophages.[22,23] TGF-β was produced when macrophages were treated with PS-liposomes, and TGF-β was involved in the inhibitory effect on NO production by

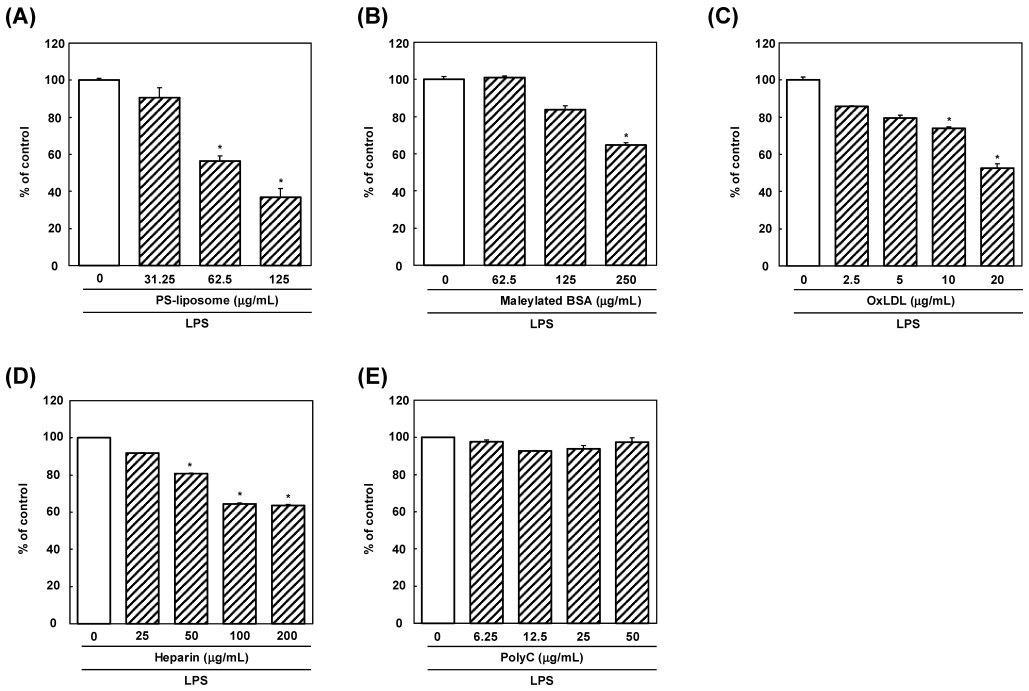

Fig. 1. Effects of Polyanions on NO Production in Macrophages Stimulated with LPS

Macrophages (5×10^5 cells/well) were pretreated with various concentrations of PS-liposomes, mBSA, OxLDL, heparin, and PolyC for 24 h. The macrophages were then stimulated with 10 μg/ml of LPS for 48 h, and NO production was evaluated as described in Materials and Methods. Asterisks (*) indicate *p* values of <0.01 *versus* LPS only stimulated.

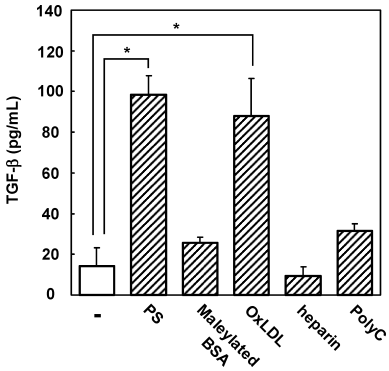

Fig. 2. Production of TGF-β by Macrophages Treated with Indicated Polyanions

Macrophages (5×10^5 cells/well) were treated with PS-liposomes (125 μg lipid/ml), mBSA (250 μg/ml), OxLDL (20 μg/ml), heparin (100 μg/ml), and PolyC (50 μg/ml) for 24 h. The supernatants of macrophage cultures were collected and TGF-β was measured as described in Materials and Methods. The values are means±S.D. of triplicate cultures from three independent experiments. Asterisks (*) indicate p values of <0.01 *versus* unstimulated cells.

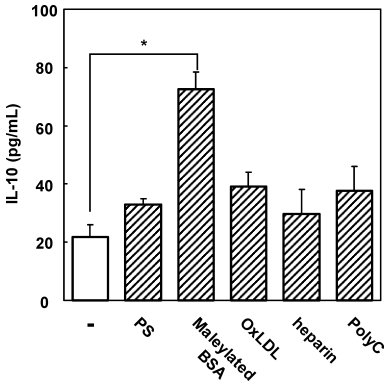

Fig. 3. Production of IL-10 by Macrophages Treated with Indicated Polyanions

Macrophages (5×10^5 cells/well) were treated with PS-liposomes (125 μg lipid/ml), mBSA (250 μg/ml), OxLDL (20 μg/ml), heparin (100 μg/ml), and PolyC (50 μg/ml) for 24 h. The supernatants of macrophage cultures were collected and IL-10 was measured as described in Materials and Methods. The values are means±S.D. of triplicate cultures from three independent experiments. Asterisks (*) indicate p values of <0.01 *versus* unstimulated cells.

PS-liposomes. However, no production of IL-10 was observed.[13] To clarify the mechanism of the inhibitory effect of polyanions on NO production, we evaluated the production of these cytokines by macrophages following treatment with polyanions. As shown in Fig. 2, TGF-β production was observed when macrophages were treated with OxLDL as is the case with PS-liposomes. No increase in TGF-β production was observed using mBSA, heparin, and PolyC.

On the other hand, significant production of IL-10 was observed when macrophages were treated with mBSA (Fig. 3). Although PS-liposomes, OxLDL, mBSA and heparin all have show inhibitory effects on NO production, their effects on cytokine production differ, suggesting the involvement of a different inhibitory mechanism for each ligand.

Effects of Negatively Charged Ligands on MAP Kinase Activation Mitogen-activated protein (MAP) kinases are composed of three principal family members, extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase.[24] These kinases have been shown to be intimately involved in cell proliferation, differentiation, apoptosis, and inflammation.[25,26] We have recently reported that PS-liposomes activated ERK, and the ERK signaling pathway might be one of the mechanisms that lead to TGF-β production following PS-liposome treatment. This is supported by the finding that the addition of an inhibitor for ERK reduced significantly the production of TGF-β. Consequently, we evaluated the effects of polyanions on MAP kinase activation by measuring tyrosine phosphorylation with a Western blotting. As shown in Fig. 4, tyrosine phosphorylation of ERK increased when macrophages were treated with OxLDL for 5 and 15 min as well as PS-liposomes. In the case of mBSA, band intensities of tyrosine phosphorylated ERK were remarkably high at 5 min and returned to the control levels at 15 min after treatment. Furthermore, mBSA activated JNK at 5 min. Additionally, PolyC activated both ERK and JNK at 15 min after treatment. No activation of p38 MAP kinase was observed with any of the polyanions. Although heparin had an inhibitory effect on NO production in macrophages, no activation of the MAP kinase superfamily was observed under our experimental conditions.

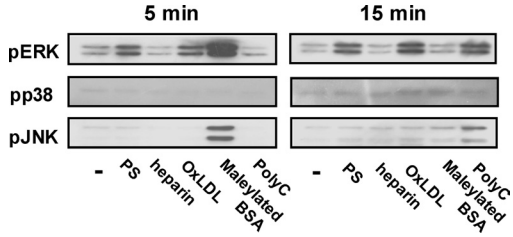

Fig. 4. Effects of Various Polyanions on Phosphorylation of the MAP Kinase Superfamily, ERK, JNK, and p38

Macrophages (1×10^6 cells/dish) were treated with PS-liposomes (125 μg lipid/ml), heparin (100 μg/ml), OxLDL (20 μg/ml), mBSA (250 μg/ml), and PolyC (50 μg/ml) for the periods indicated. Macrophage lysates were analyzed by Western blotting as described in Materials and Methods.

The characteristics of several types of scavenger receptor have been reported. Scavenger receptor class-A (type I/II) has affinity for OxLDL, acetyl LDL, mBSA, and dextran sulfate, but not for PS-liposomes. Scavenger receptor class-B (SR/BI and CD36) has affinity for OxLDL, acetyl-LDL, and PS-liposomes.[14,27,28] Fadok *et al.* reported extensively on the PS-specific receptor-mediated recognition and endocytosis of PS-expressing apoptotic cells by macrophages.[10] PS-liposomes interact with macrophages through PS-specific receptors, leading to the production of TGF-β *via* the activation of ERK, and finally, the TGF-β produced by PS-liposomes inhibited NO production by macrophages.[12] OxLDL affected cytokine production and MAP kinase activation similar to PS-liposomes, suggesting the contribution of a PS-specific receptor to the inhibitory effect of OxLDL on NO production. Furthermore, the contribution of scavenger receptor class-B to inhibitory effect of OxLDL was also suggested. With mBSA, cytokine production and MAP kinase activation were different from those with PS-liposomes. Scavenger receptor class-A has affinity for mBSA, suggesting that ligation scavenger receptor class-A causes the activation of MAP kinases (ERK and JNK) and production of IL-10, and this pathway contributes to the inhibition of NO production. Macrophage scavenger receptors exhibit an unusually broad specificity, and polynucleotide binding to macrophages

depends on the formation of base-quartet-stabilized four-strand helices, and PolyC is not a ligand for scavenger receptors.[29] On the other hand, complement receptor Mac-1 (CD11b/CD18) functioned as a receptor for the binding of an oligodeoxynucleotide,[30] suggesting that signaling *via* complement receptors has no effect on the production of NO by macrophages stimulated with LPS. Nakamura *et al.* reported that heparin has an affinity for scavenger receptor class-A in Kupffer cells.[31] However, heparin did not affect MAP kinase activation and cytokine production, suggesting the contribution of some receptor(s) other than scavenger receptors or the PS-receptor.

The inhibition of NO production by several ligands for scavenger receptors does not appear to be due to the formation of a ligand-LPS or ligands competing for the LPS receptor, and scavenger receptors and or some other receptor(s) are suggested to contribute to this inhibition.[14,32,33] Studies are currently underway to clarify the difference between these ligands in the inhibitory effect on NO production by macrophages stimulated with LPS.

REFERENCES

1) Nathan C., *FASEB. J.*, **6**, 3051—3064 (1992).
2) Yook Y. H., Kang K. H., Maeng O., Kim T. R., Lee J. O., Kang K. I., Kim Y. S., Paik S. G., Lee H., *Biochem. Biophys. Res. Commun.*, **321**, 298—305 (2004).
3) Stuehr D. J., Marletta M. A., *Cancer Res.*, **47**, 5590—5594 (1987).
4) Flesch I. E., Kaufmann S. H., *Infect. Immun.*, **59**, 3213—3218 (1991).
5) Fast D. J., Shannon B. J., Herriott M. J., Kennedy M. J., Rummage J. A., Leu R. W., *Infect. Immun.*, **59**, 2987—2993 (1991).
6) Moodley Y., Rigby P., Bundell C., Bunt S., Hayashi H., Misso N., McAnulty R., Laurent G., Scaffidi A., Thompson P., Knight D., *Am. J. Pathol.*, **162**, 771—779 (2003).
7) Kawasaki Y., Nakagawa A., Nagaosa K., Shiratsuchi A., Nakanishi Y., *J. Biol. Chem.*, **277**, 27559—27566. Epub 22002 May 27516 (2002).
8) Yamada Y., Doi T., Hamakubo T., Kodama T., *Cell. Mol. Life Sci.*, **54**, 628—640 (1998).
9) Schwartz B. R., Karsan A., Bombeli T., Harlan J. M., *J. Immunol.*, **162**, 4842—4848 (1999).
10) Fadok V. A., Bratton D. L., Rose D. M., Pearson A., Ezekewitz R. A., Henson P. M., *Nature* (London), **405**, 85—90 (2000).
11) Fadok V. A., Bratton D. L., Henson P. M., *J. Clin. Invest.*, **108**, 957—962 (2001).
12) Otsuka M., Tsuchiya S., Aramaki Y., *Biochem. Biophys. Res. Commun.*, **324**, 1400—1405 (2004).
13) Matsuno R., Aramaki Y., Tsuchiya S., *Biochem. Biophys. Res. Commun.*, **281**, 614—620 (2001).
14) Matsuno R., Aramaki Y., Arima H., Tsuchiya S., *Biochem. Biophys. Res. Commun.*, **237**, 601—605 (1997).
15) Yang X., Cai B., Sciacca R. R., Cannon P. J., *Circ. Res.*, **74**, 318—328 (1994).
16) Sakai M., Miyazaki A., Hakamata H., Kodama T., Suzuki H., Kobori S., Shichiri M., Horiuchi S., *J. Biol. Chem.*, **271**, 27346—27352 (1996).
17) Aramaki Y., Nitta F., Matsuno R., Morimura Y., Tsuchiya S., *Biochem. Biophys. Res. Commun.*, **220**, 1—6 (1996).
18) Stuehr D. J., Nathan C. F., *J. Exp. Med.*, **169**, 1543—1555 (1989).
19) Fukasawa M., Adachi H., Hirota K., Tsujimoto M., Arai H., Inoue K., *Exp. Cell. Res.*, **222**, 246—250 (1996).
20) Krieger M., Herz J., *Annu. Rev. Biochem.*, **63**, 601—637 (1994).
21) Seyler I., Appel M., Devissaguet J. P., Legrand P., Barratt G., *J. Leukoc. Biol.*, **62**, 374—380 (1997).
22) Chesrown S. E., Monnier J., Visner G., Nick H. S., *Biochem. Biophys. Res. Commun.*, **200**, 126—134 (1994).
23) Bogdan C., Paik J., Vodovotz Y., Nathan C., *J. Biol. Chem.*, **267**, 23301—23308 (1992).
24) Feng G. J., Goodridge H. S., Harnett M. M., Wei X. Q., Nikolaev A. V., Higson A. P., Liew F. Y., *J. Immunol.*, **163**, 6403—6412 (1999).
25) Ryang Y. S., Chang J. H., Park J. Y., *Yonsei Med. J.*, **45**, 751—754 (2004).
26) Alexander M., Daniel T., Chaudry I. H., Schwacha M. G., *J. Cell. Physiol.*, **201**, 35—44 (2004).
27) Rhainds D., Falstrault L., Tremblay C., Brissette L., *Eur. J. Biochem.*, **261**, 227—235 (1999).
28) Fukasawa M., Hirota K., Adachi H., Mimura K., Murakami-Murofushi K., Tsujimoto M., Arai H., Inoue K., *J. Biol. Chem.*, **270**, 1921—1927 (1995).
29) Pearson A. M., Rich A., Krieger M., *J. Biol. Chem.*, **268**, 3546—3554 (1993).
30) Benimetskaya L., Loike J. D., Khaled Z., Loike G., Silverstein S. C., Cao L., el Khoury J., Cai T. Q., Stein C. A., *Nat. Med.*, **3**, 414—420 (1997).
31) Nakamura T., Yuasa H., Watanabe J., *Biol. Pharm. Bull.*, **23**, 743—747 (2000).
32) Aramaki Y., Matsuno R., Arima H., Tsuchiya S., *Res. Commun. Mol. Pathol. Pharmacol.*, **104**, 22—30 (1999).
33) Matsuno R., Aramaki Y., Arima H., Adachi Y., Ohno N., Yadomae T., Tsuchiya S., *Biochem. Biophys. Res. Commun.*, **244**, 115—119 (1998).