area of mass chromatography signals in HPLC. *(A)*, The ratio of each glycan structure was expressed by taking the total peak area of each FN as 100%. *(B)*, Ratios of glycans possessing various numbers of *O*-acetyl groups at the *N*-acetyl neuraminic acid residues. The ratios of each glycan with different numbers of *O*-acetyl group are expressed by taking the sum of mass chromatography signals of the each glycan in position as 100%. Of the structures not expressed in (B), BiNA(1)F, TriNA(3), and TriNA(3)F, all without *O*-acetyl group (Ac(0)) were detected.

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Fig. 1

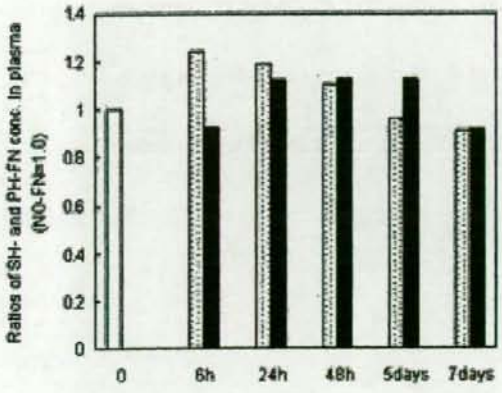

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Fig. 2

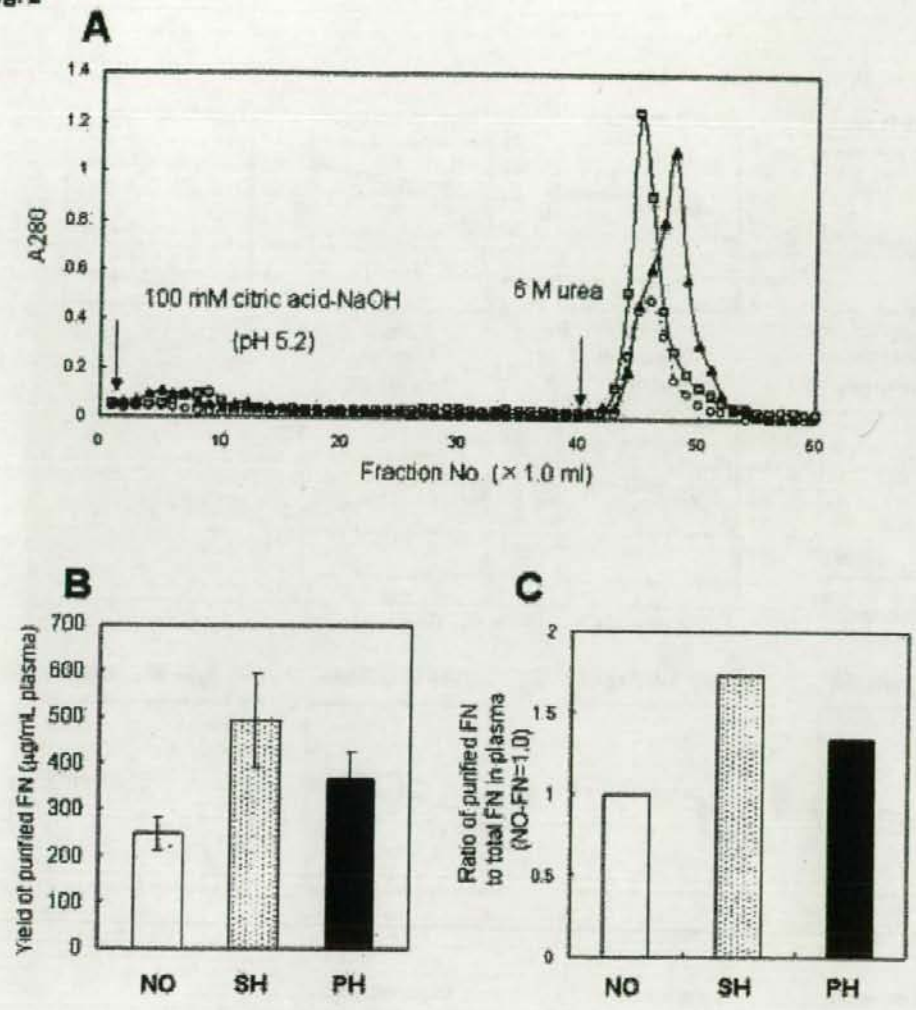

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Fig. 3

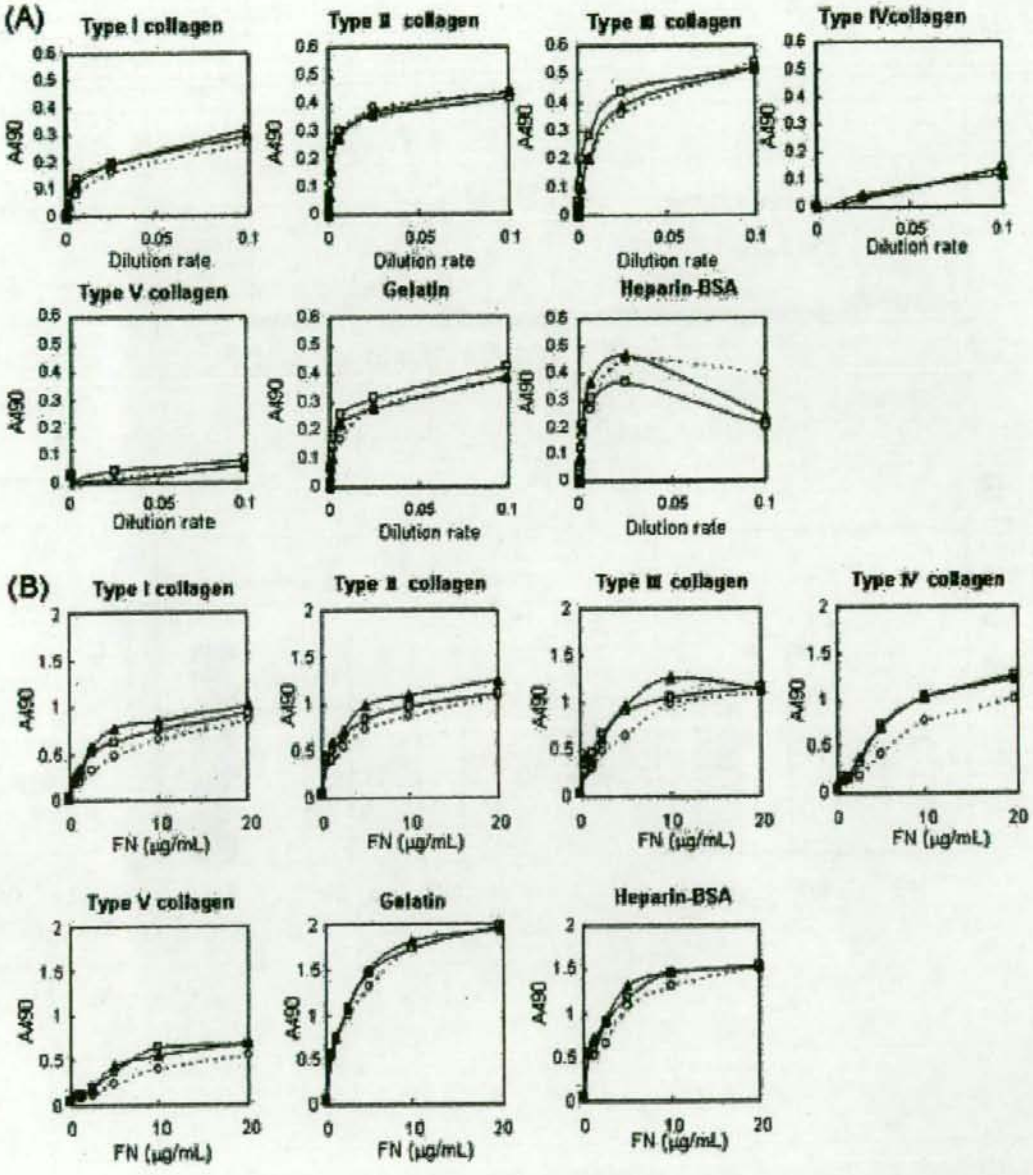

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Fig. 4

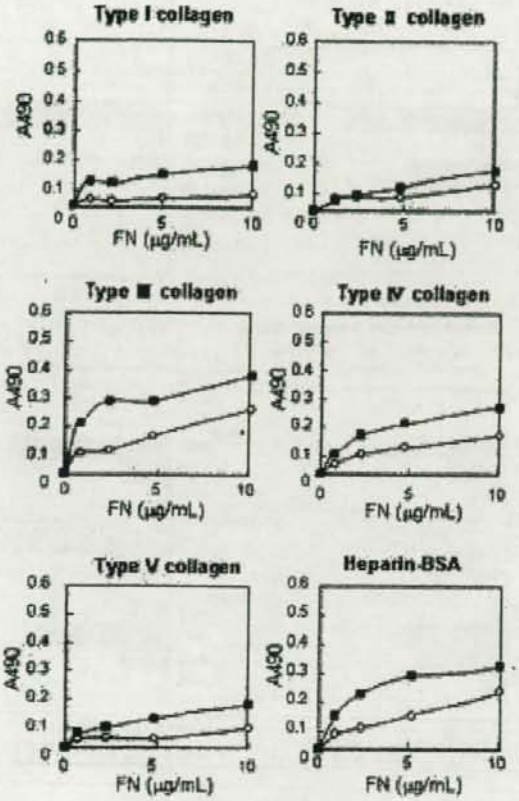

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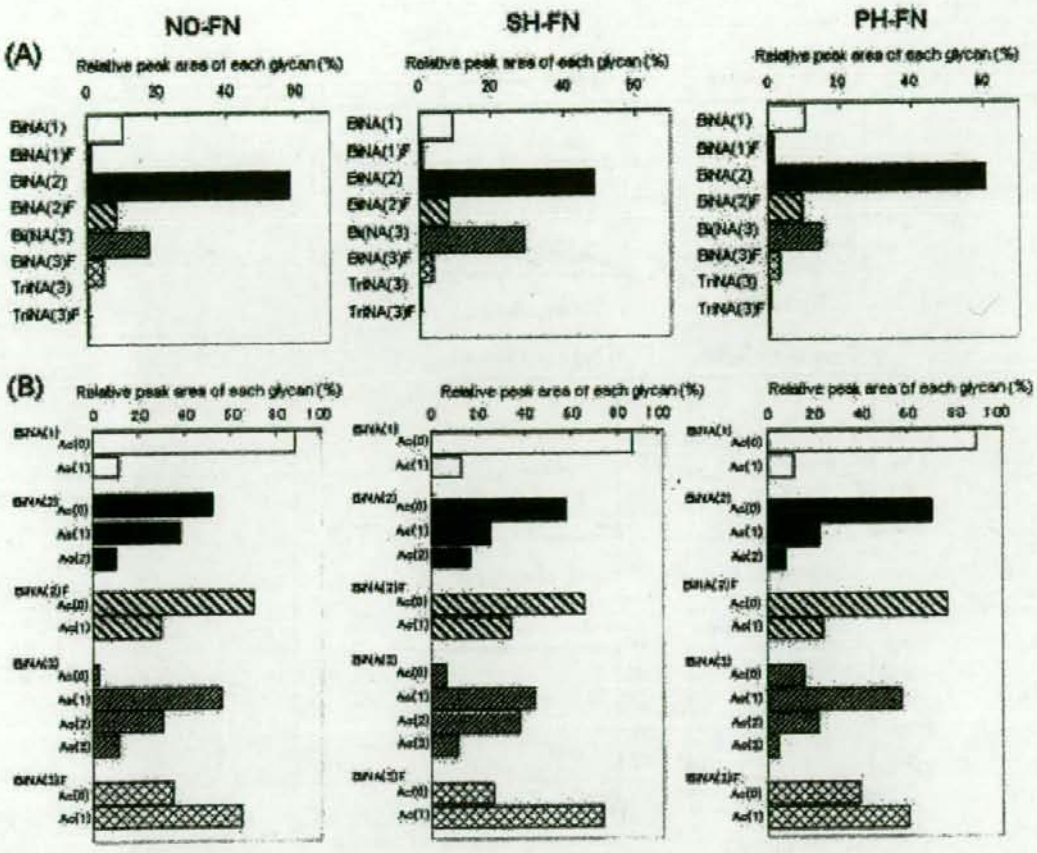
Fig. 5



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Note

Synthesis of Lipid Derivatives of Pyrrole Polyamide and Their Biological Activity

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Novel fatty acyl and phospholipid derivatives of pyrrole polyamide were synthesized. Their cytotoxicity against a cancer cell line of MT-4 cells and those infected by human immunodeficiency virus (HIV) was examined. Although no anti-HIV activity was found, their cytotoxicitty against the cancer cells was significantly enhanced by introducing a lipophilic group into the pyrrole polyamide.

Key words: pyrrole polyamide; lipid; phospholipid; cancer cell; human immunodeficiency virus (HIV)-II

Since the naturally occurring pyrrole polyamide, netropsin, as an antibiotic was reported by Kopka *et al.*,[1,2] a number of pyrrole polyamide analogues has been synthesized so far. Subsequently, their highly sequence-specific binding to DNA[3] has intensively triggered the design of novel functional pyrrole polyamides,[4] and the search for new biological functions including anti-cancer[5] and anti-HIV[6] activities.

As the major components of polyunsaturated fatty acids (PUFA) in marine fish oil, docosahexaenoic acid (DHA) and icosapentaenoic acid (EPA) have a non-conjugated all-*cis*-polyunsaturated olefinic structure. They are known to exhibit a variety of biochemical and physiological functions including enhanced cell membrane permeability,[7] growth regulation and apoptosis-inducing capability to cancer cells,[8–10] cytotoxicity enhancement for some anti-cancer drugs against cancer cells[11,12] and potential anti-malarial activity.[13] Regarding the effect of the lipid modification of bioactive compounds, Zerouga *et al.* have reported that methotrexate, a cytotoxic drug, conjugated to phosphatidylcholine (PC) having a docosahexaenoyl group showed higher anti-proliferative activity against murine leukemia cells than one having a stearoyl group.[14] In our previous study, conjugates of qunine with fatty acid were found to show higher cytotoxicity against tumor cell line FM3A[15] than qunine itself. Parang *et al.* have extensively reviewed the relationship between the lipid modifications of 3′-azido-2′,3′-dideoxythymidine (AZT) and anti-HIV activity.[16] In the present study, novel fatty acyl derivatives of pyrrole polyamide were synthesized by using stearic acid (**3**, a typical saturated fatty acid rich in mammal fats), linoleic acid (**4**, a typical n-6 type of dienoic acid rich in plant lipids) and icosapentaenoic acid (**5**, a typical n-3 type of pentaenoic acid rich in fish oil), and the cytotoxicity of the conjugates was examined by using MT-4 cells.

Pyrrole polyamide has so far been synthesized by a reaction sequence involving the nitration of pyrrole, reduction of the nitro group to an amino group and condensation of the amine with nitro-pyrrole carboxylic acid.[17] This route, however, involves some intermediates having a nitro group that is known to cause allergic symptoms. To minimize the number of these intermediates, a different approach was investigated to obtain a key intermediate (**2**). Briefly, the route involves trichloroacetylation at the 2-position of *N*-methylpyrrole, nitration at the 4-position of the pyrrole nucleus, conversion of the trichloroacetyl group to a methoxycarbonyl group, reduction of the nitro group to an amino group, condensation of the amine with *N*-methylpyrrole

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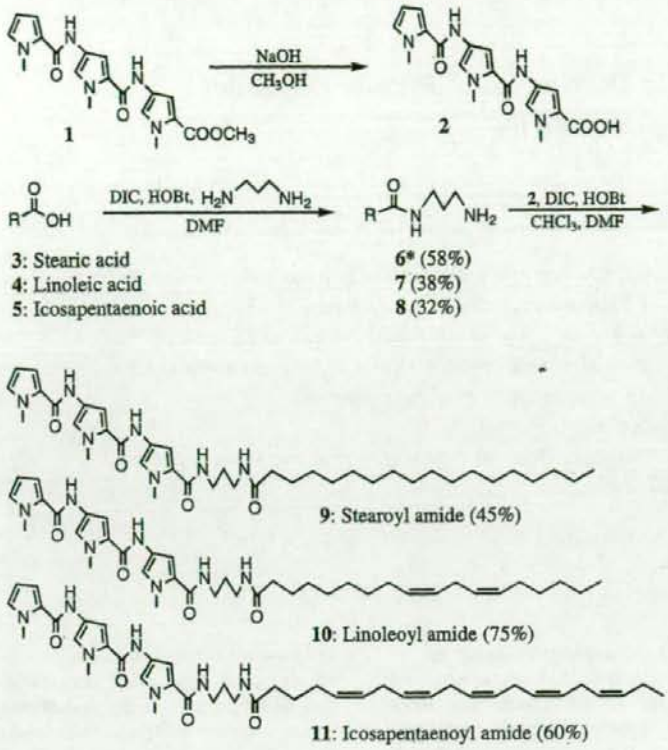


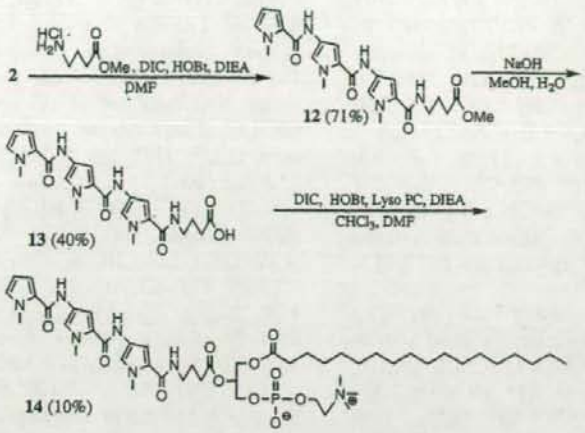
Scheme 1. Synthesis of Fatty Acyl Derivatives (**9**)–(**11**).
*Ref. 21. DIC, Diisopropylcarbodimide; HOBt, Hydroxybenzotriazole

2-carboxylic acid, hydrolysis of the methyl ester to a carboxylic acid, condensation of this acid with *N*-methyl-4-aminopyrrole 2-carboxylic acid methyl ester that had been prepared as already described to afford an intermediate **1**,[18] and finally hydrolysis of the methyl ester to afford **2**.

Acylated pyrrole polyamide (**9–11**) were synthesized by condensation of fatty acid half amides (**6–8**) with the carboxylic acid (**2**) respectively (Scheme 1) as described in the experimental section.

A phospholipid derivative (**14**) was synthesized by using the same intermediate **2** (Scheme 2). In this case, methyl γ-aminobutylate was introduced into **2** as a spacer giving ester **12**, which was hydrolyzed to **13**. Due to its instability, a product **13** was submitted as such to condensation with a lyso PC having a stearoyl group at *sn*-1 position. Silica gel chromatography afforded the desired compound **14** whose structural integrity was confirmed by ^{1}H-NMR and ESI MS data. For further structural confirmation, hog pancreatic phospholipase A2 was applied in an acetate buffer (pH 8.4) to substrate **14** at room temperature for 12 h. A TLC analysis showed that the lyso PC and pyrrole polyamide **13** having the spacer had been liberated by the enzymatic reaction. This finding might give an opportunity to use this enzyme as a molecular switch to liberate the pyrrole polyamide at the right time when it should play some roles in biological systems.[4]

As a preliminary test of biological activity in the present study, lipid derivatives **9–11** and **14** were examined for their *in vitro* cytotoxicity against cultured MT-4 cells. The result showed that the order of their concentration for complete growth inhibition of cultured MT-4 cells was stearoyl derivative **9** (45 μM) < icosapentaenoyl derivative **11** (176 μM) < linoleoyl derivative **10** (≥181 μM) < pyrrole amide methyl ester **1** (≥1304 μM) as a control. This experiment demonstrated for the first time that lipid modification of a pyrrole polyamide remarkedly enhanced its cytotoxicity, and derivative **9** with a saturated acyl group appeared to be more active than the unsaturated type. The same tendency was also observed for the concentration range of partially inhibitive and non-inhibitive cases. Although phospholipid derivative (**14**) enhanced the cytotoxicity (≥260 μM) to some extent, the activity was lower than those by acyl derivatives **9–11**. Combining all the results, lipid modification of the pyrrole polyamide was found to significantly enhance the cytotoxicity against cancer cell line MT-4 cells, and the effect appeared to be higher with the saturated acyl derivative than with the



Scheme 2. Synthesis of Phospholipid Derivatives (**14**).
DIEA, Diisopropylethylamine

unsaturated types. No anti-HIV effect was, however, appearent by the microplate method[19] and Magic 5 method[20] for any of the pyrrole polyamide derivatives synthesized in the present study. The preliminary biological results described here constitute an additional example of the effect of lipid modification for biologically active compounds.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded by a Varian Mercury 300 or VXR 500 using $CDCl_3$, and ESI MS data were recorded by API III (Perkin Elmer) by direct infusion, using a mixture of $THF/CH_3OH/H_2O$ (15:4:1) with 0.1% HCOOH or 0.1% $HCOO^-NH_4^+$ as a solvent in the positive mode.

Synthesis of N-linoleoylpropane-1,3-diamine (7). To a solution of linoleic acid (1.89 g, 6.75 mmol) in ethanol-free chloroform (25 ml) were added HOBt (0.95 g, 7.0 mmol) and DIC (0.84 g, 7.0 mmol), and the solution was stirred at r.t. overnight. A solution of 1,3-propanediamine (1.0 g, 13.5 mmol) in ethanol-free chloroform (7 ml) was added dropwise to the reaction mixture which was stirred at r.t. overnight. After evaporating the solvent under reduced pressure, the residue was chromatographed on silica gel, eluting with a mixture of cloroform/methanol/aq.NH_3 (80:20:5) to afford half amide **7**. $R_f = 0.65$ ($CHCl_3$:CH_3OH:NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$)δ(ppm): 0.78 (3H, t, $J = 7.8$, –CH_2–CH_3), 1.16 (14H, m, –$(CH_2)_4$–CH_2–(CH=CH–CH_2)$_2$–$(CH_2)_3$–CH_3), 1.50 (2H, m, –C(O)–CH_2–CH_2–), 1.60 (2H, d, $J = 6.6$ Hz, –NH–CH_2–CH_2–CH_2–NH–), 2.00 (4H, m, –CH_2–CH=CH–CH_2–CH=CH–CH_2–), 2.08 (2H, t, $J = 7.8$, –C(O)–CH_2–), 2.65 (2H, d, $J = 6.9$ Hz, –CH_2–NH_2), 2.75 (2H, t, $J = 6.5$, =CH–CH_2–CH=), 3.16 (2H, d, $J = 6.3$ Hz, –NH–CH_2–), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (4H, m, –(CH=CH–CH_2)$_2$–), 6.13 (1H, m, –CH=CH–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, ($M + H^+$) 337.2; $C_{21}H_{40}N_2O$ requires 336.6.

Synthesis of N-icosapentaenoylpropane-1,3-diamine (8). This intermediate was prepared under the same conditions as those used for the preparation of **7**. $R_f = 0.67$ ($CHCl_3$:CH_3OH: NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$): δ(ppm): 0.95 (3H, t, $J = 7.5$, –CH_2–CH_3), 1.60 (2H, d, $J = 6.6$ Hz, –NH–CH_2–CH_2–CH_2–NH–), 1.75 (2H, m, –C(O)–CH_2–CH_2–), 2.10 (4H, m, –CH_2–CH=CH–, –CH_2CH_3), 2.30 (2H, t, $J = 8.0$, –C(O)–CH_2–), 2.65 (2H, d, $J = 6.9$ Hz, –CH_2–NH_2), 2.80 (8H, m, –(CH=CH–CH_2)$_4$–), 3.16 (2H, d, $J = 6.3$ Hz, –NH–CH_2–), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (10H, m, –(CH=CH–CH_2)$_5$–), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, ($M + H^+$) 359.2; $C_{23}H_{38}N_2O$ requires 358.6.

Synthesis of the stearoyl derivative (9). To a solution of **2** (55.2 mg, 0.15 mmol) and HOBt (24.4 mg, 0.18 mmol) in DMF (3 ml) was added DIC (22.2 mg, 0.18 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Amide **6** (45.6 mg, 0.15 mmol) and distilled ethanol-free chloroform (3 ml) were added to this reaction mixture which was stirred at 50 °C for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was chromatographed in a silica gel column ($CHCl_3$/MeOH, 95:5) to give final compound **9**. $R_f = 0.57$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.80 (3H, t, $J = 7.0$, –CH_2–CH_3), 1.30 (28H, m, –$(CH_2)_{14}$–CH_3), 1.65 (4H, m, –C(O)–CH_2–CH_2–, –NH–CH_2–CH_2–CH_2–NH–), 2.20 (2H, t, $J = 8.0$, –C(O)–CH_2–), 3.35 (4H, m, –NH–CH_2–CH_2–CH_2–NH–), 3.90–4.00 (9H, s, 3 × N–CH_3), 6.15 (1H, m, –CH=CH–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings) ESI MS m/z: found, ($M + H^+$) 692.4; $C_{39}H_{61}N_7O_4$ requires 691.5.

Synthesis of the linoleoyl derivative (10). This product was prepared under the same conditions as those used

for the synthesis of **9**. TLC (Silica gel) $R_f = 0.38$ ($CHCl_3$:CH_3OH, 95:5); ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.85 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.30 (14H, m, $-(CH_2)_4-CH_2-(CH=CH-CH_2)_2-(CH_2)_3-CH_3$), 1.60 (4H, m, $-C(O)-CH_2-CH_2-$, $-NH-CH_2-CH_2-CH_2-NH-$), 2.00 (4H, q, $J = 7.5$, $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$), 2.20 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 2.75 (2H, t, $J = 6.5$, $=CH-CH_2-CH=$), 3.35 (4H, m, $-NH-CH_2-CH_2-CH_2-NH-$), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (4H, m, $-(CH=CH-CH_2)_2-$), 6.60–6.90 (6H, m, protons on the pyrrole rings); ESI MS m/z: found, $(M+H)^+$ 688.5; $C_{39}H_{57}N_7O_4$ requires 687.5.

*Synthesis of the icosapentaenoyl derivative (**11**).* This product was prepared under the same conditions as those used for the synthesis of **9**. $R_f = 0.32$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.95 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.75 (4H, m, $-C(O)-CH_2-CH_2-$, $-NH-CH_2-CH_2-CH_2-NH-$), 2.10 (4H, m, $-CH_2-CH_2-CH=CH-$, $-CH_2-CH_3$), 2.30 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 2.80 (8H, m, $-(CH=CH-CH_2-CH=CH)_4$), 3.40 (4H, m, $-NH-CH_2-CH_2-CH_2-NH-$), 3.90–4.00 (9H, , s, 3 × N–CH_3), 5.35 (10H, m, $-(CH=CH-CH_2)_5-$), 6.13 (1H, m, $-CH=CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M+H)^+$ 710.4; $C_{41}H_{55}N_7O_4$ requires 709.4.

*Synthesis of methyl γ-aminobutylate derivative of pyrrole polyamide (**12**).* A mixture of intermediate acid **2**, (312 mg, 0.8 mmol), HOBt (137 mg, 1.03 mmol) and DIC (130 mg, 1.03 mmol) in DMF (0.68 ml) was stirred at r.t. for 24 h. To this solution were added methyl 4-aminobutylate hydrochloride (130 mg, 0.85 mmol) and DIEA (260 µl, 1.49 mmol), and stirred at r.t. for further 24 h. After an addition of deionized water (10 ml), the product was extracted with chloroform. The product was purified by silica gel chromatography (hexane/ethyl acetate, 3:7) affording an unstable oil. TLC(Silica gel) $R_f = 0.3$ (Hexane:EtOAc, 2:8); ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.91 (2H, m, $-NH-CH_2-CH_2-$), 2.40 (2H, m, $-NH-CH_2-CH_2-CH_2-CO$), 3.39 (2H, m, $-NH-CH_2-$), 3.80 (3H, s, O–CH_3), 3.78–4.00 (9H, s, 3 × N–CH_3), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: Found, $(M+NH_4)^+$ 486.3; $C_{23}H_{28}N_6O_5$ requires 486.0.

*Synthesis of γ-aminobutylate derivative of the pyrrole polyamide (**13**).* A solution of the ester **11** (200 mg, 0.43 mmol) and 2*N*–NaOH (10 ml) in methanol (10 ml) was stirred at 60 °C for 12 h. After removing the solvent under reduced pressure, the residue was acidified with 1*N*-HCl, and the acid was extracted with a mixture of chloroform/methanol (2:1). Silica gel chromatography (chloroform/methanol, 9:1 → 5:5) afforded acid **13** as an unstable oil which was used as such for the next reaction.

*Synthesis of the phospholipid derivative (**14**).* To a solution of **13** (100 mg, 0.22 mmol) and HOBt (34.4 mg, 0.26 mmol) in a mixed solvent of $CHCl_3$ (1 ml) and DMF (1 ml) was added DIC (32.8 mg, 0.26 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Lyso-PC (136.2 mg, 0.26 mmol) and DIEA (100 µl) were added to this reaction mixture which was stirred at room temperature for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was separated by silica gel column chromatography ($CHCl_3$/MeOH, 6:4) monitored by preparative TLC ($CHCl_3$/MeOH/NH_3aq. 65:35:5) to yield final compound **13** as an yellow oil. TLC (silica gel) $R_f = 0.6$ ($CHCl_3$:CH_3OH:NH_3aq, 60:30:5); ^{1}H-NMR ($CDCl_3$:CD_3OD, 8:2) δ(ppm): 0.80 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.05 (2H, m, $-CH_2-CH_3$), 1.20 (26H, m, $-(CH_2)_{13}-CH_2-CH_3$), 1.50 (2H, $-C(O)-CH_2-CH_2-$), 1.90 (2H, m, $-NH-CH_2-CH_2-$), 2.20 (2H, m, $-NH-CH_2-CH_2-CH_2-$), 2.40 (2H, m, $-CH_2-O-C(O)-CH_2-$), 2.90 (2H, m, $-NH-CH_2-$), 3.30 (2H, m, $-CH_2-N-(CH_3)_3$), 3.50 (9H, s, $-N-(CH_3)_3$), 3.70–3.90 (9H, s, 3 × *N*–CH_3 on the pyrrole rings), 4.00 (2H, m, $-CH-CH_2-O-P-$), 4.10 (2H, m, $-CH_2-CH_2-O-P-$), 4.20 (2H, m, $-CH-CH_2-O-C(O)-$), 5.10 (1H, m, $-CH_2-CH-CH_2-$), 6.00 (1H, m, $-CH=CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M+H)^+$ 960.6; $C_{48}H_{78}N_7O_{11}P$ requires 959.6.

In vitro cytotoxicity and anti-HIV assays were respectively conducted by the microplate method and magic-5 method reported by Otake *et al.*[19] and Kimpton *et al.*[20]

Microplate method. Sample solutions (100 µl) were sequentially diluted at 1:2 or 1:5 with an RPMI1640 medium containing 10% FCS in a 96-well plate. For the cytotoxicity experiment, 100 µl of cell a suspension of MT-4 cells (2×10^5/ml) in a stage of exponential growth was added to each well. For the anti-HIV experiment, MT-4 cells (2×10^6) were infected by the addition of a stock solution of HTLV-BIII to a concentration suitable as an infectious dose (100TCID50) to the tissue culture, which was incubated at 37 °C for 1 h. The cells were resuspended in 10 ml of the medium, and the suspension (100 µl) was added to all the wells in the 96-well plate. After incubating for 5 days, the cytotoxicity and cytopathic effect (CPE) were evaluated by counting the cells by optical microscopic observation.

Magic-5 method. Magic-5 cells (10^4 cells) per one well of a 96-well plate were cultured at 37 °C to the stage at which the cells were allowed to adhere to the plastic surface of the plate. After removing the culture medium, a sample solution of the pyrrole polyamide diluted 2 times with the medium was added, this being followed by the addition of HIV-1 Ba-L strain prepared to a concentration of 100–200 BFU/50 µl by using the medium containing DEAE-dextran. The cells were incubated at 37 °C for 48 h in a CO_2 incubator. After removing the medium, 1%-formaldehyde and 0.2%-glutaraldehyde in PBS were added, and the mixture incubated at r.t. for 5 min. After washing the cells, 4 mM-potassium ferrocyanide, 4 mM-potassium ferricyanide, 2 mM $MgCl_2$ and 400 mg/ml of X-gal were added, and the mixture incubated at 37 °C for 1 h. The staining solution was removed and the cells were washed. The

cells stained blue were counted by using optical microscopic observation. In this experiment, TAK-779 and AZT were used as controls for the anti-HIV activity.

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RESEARCH ARTICLE

The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2

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LPS recognition; toll-like receptor signal transduction; cell activation.

Abstract

We analysed the lipopolysaccharide (LPS)-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. When TLR4 and CD14 were transiently expressed in HEK293 cells, cell-surface expression of TLR4 was observed, although the expression level was lower than that in cells coexpressing MD-2. We found that membrane CD14–TLR4 complexes were formed in these cells in response to LPS stimulation even in the absence of MD-2 expression, although NF-κB-dependent reporter activity was not induced. A strong activation of NF-κB was observed when these cells were stimulated with LPS followed by soluble MD-2 in this order, even when excess LPS was removed after formation of the CD14–TLR4 complex by washing cells prior to sMD-2 addition. From these results, we propose an additional LPS-recognition mechanism. In cells expressing TLR4 and CD14 but lacking MD-2, LPS is first transferred to membrane CD14 with the aid of LPS binding protein, which leads to the formation of the TLR4–CD14 complex. Then, the binding of soluble MD-2 to this complex triggers the transmembrane signal transduction. Cells expressing TLR4 and CD14 but lacking MD-2, such as airway epithelial cells, may be activated in response to LPS by this mechanism.

Introduction

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and plays a major role in septic shock. LPS induces the production of various cytokines by the activation of transcription factors, including NF-κB and IRF-3 (Schletter *et al.*, 1995; Hatada *et al.*, 2000; Sakaguchi *et al.*, 2003). It is known that LPS binding protein (LBP), CD14, MD-2 and Toll-like receptor 4 (TLR4) are required for the recognition of LPS. Both CD14 and MD-2 exist in membrane-anchored and soluble forms, and it has been suggested that both forms are important for LPS recognition (Schromm *et al.*, 2001; Visintin *et al.*, 2001; Muroi *et al.*, 2002; Nagai *et al.*, 2002; Ohnishi *et al.*, 2003; Van Amersfoort *et al.*, 2003). How these factors recognize LPS on the cell surface has been discussed, and some models have been proposed. The most widely accepted model is as follows. LPS is transferred to membrane CD14 (mCD14) by LBP, and then the LPS–CD14 complex is recognized by the TLR4–membrane MD-2 (mMD-2) complex on the cell surface (da Silva Correia *et al.*, 2001; Gioannini *et al.*, 2004). This mCD14–TLR4–mMD-2 complex is able to transmit the activation signal of LPS to cytosol via the intracellular domain of TLR4.

The role of soluble CD14 (sCD14) in the LPS response has been widely studied. sCD14 exists in human plasma and its level increases on infection (Landmann *et al.*, 1995). It has been reported that, in cells expressing TLR4 and mMD-2, sCD14 confers responsiveness to LPS (Landmann *et al.*, 1996), indicating that sCD14 is able to be a substitute for mCD14. It has also been reported that artificially created LPS–sMD-2 complexes *in vitro*, with the assistance of sCD14, are able to activate cells expressing only TLR4 (Gioannini *et al.*, 2004). This suggests that sMD-2 is also able to substitute for mMD-2. In fact, it has been reported that cells expressing TLR4 and CD14 but lacking mMD-2 respond to LPS when sMD-2 is added (Jia *et al.*, 2004; Pugin *et al.*, 2004). However, how LPS is recognized in these cells has yet to be elucidated. In this paper, we investigate the LPS-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2.

Materials and methods

Cells and reagents

The human embryonic kidney 293 cell line (obtained from the Human Science Research Resource Bank, Tokyo, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco BRL), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). The anti-serum (no.1060) against the equine infectious anemia virus (EIAV)-tag epitope (amino acid sequence: ADRRIPGTAEE) was a kind gift from Dr Nancy Rice (NCI-Frederick Cancer Research and Development Center). Sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate, sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3′-dithiopropionate and immobilized streptavidin agarose came from Pierce (Rockford, IL). Anti-FLAG M2 agarose and anti-FLAG M2 antibody were obtained from Sigma-Aldrich Co. (St Louis, MO). *Escherichia coli* O111:B4 LPS (Sigma-Aldrich Co.) was re-purified according to Hirschfeld *et al.* (2000). Unless otherwise noted, all other chemicals were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Plasmid construction

cDNA encoding EIAV-tagged human CD14, TLR4 and MD-2 were separately cloned into a mammalian expression vector as previously described (Ohnishi *et al.*, 2001). A plasmid for FLAG-tagged TLR4 was constructed by inserting a coding sequence minus its predicted signal peptide sequences into the pFLAG-CMV-1 expression vector (Sigma-Aldrich Co.).

Biotinylation of cell-surface proteins

After plating 293 cells in 6 cm dishes, the cells were transfected with EIAV-tagged TLR4 (5 μg), EIAV-tagged CD14 (0.25 μg) and EIAV-tagged MD-2 (5 μg) expression plasmids using the calcium phosphate precipitation method. Twenty-four hours later, the cells were washed twice with PBS and treated with 2 mL of 0.5 mg mL^{-1} sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate, a membrane-impermeable biotinylation reagent according to a previously described method (Ohnishi *et al.*, 2001). Cellular extracts were prepared using 200 μL of a lysis buffer (20 mM HEPES-KOH, 5 mM EDTA, 0.5% Nonidet P-40 and 10 mM KCl; pH7.9) containing a protease inhibitor mix (Roche Diagnostics, Basel, Switzerland). The cellular extracts were diluted with PBS containing 0.5% Nonidet P-40 to a final volume of 500 μL and then incubated with immobilized streptavidin agarose at 4 °C for 1 h. After washing three times with PBS containing 0.5% Nonidet P-40, the agarose was boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The resulting supernatant was subjected to SDS-PAGE and Western blot analysis. TLR4, MD-2 and CD14 were detected using a rabbit anti-EIAV antiserum.

Analysis of TLR4–CD14 association on a membrane surface

After plating 293 cells in 10 cm type I collagen-coated dishes (Corning Inc., Corning, NY), the cells were transfected with FLAG-tagged TLR4 (24 μg) and EIAV-tagged CD14 (0.2 μg) expression plasmids using the calcium phosphate precipitation method. Twenty-four hours later, the cells were washed three times with PBS and then stimulated with 0.01 μg mL^{-1} LPS in serum-free DMEM containing 0.1 μg mL^{-1} LBP at 37 °C for 3 h. After incubation, the cells were washed twice with PBS and collected into 1.5-mL microcentrifuge tubes. The cells were exposed to 1 mg mL^{-1} membrane-impermeable bifunctional cross-linking reagent, sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3′-dithiopropionate in PBS, at 15 °C in the dark for 30 min with rocking. After UV irradiation at 25 °C for 1 min, the cells were washed with PBS, and the cell extracts were prepared with PBS containing 0.5% Nonidet P-40 and a protease inhibitor mix. SDS was added to the cell extracts to a final concentration of 1%. The cell extracts were boiled for 5 min and diluted to 5 mL with PBS containing 0.5% Nonidet P-40. The cell extracts were incubated with anti-FLAG M2 agarose at 4 °C for 1 h. The agarose was washed three times with PBS containing 0.5% Nonidet P-40 and incubated with 0.1 M glycine-HCl buffer, pH 2.5, for 5 min. The supernatants were subjected to SDS-PAGE and Western blot analysis. CD14 that cross-linked with TLR4 was detected using a rabbit anti-EIAV antiserum after the cross-linking reagent was cleaved with a reducing agent.

Electrophoresis and Western blotting

A discontinuous SDS-10% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29 : 1) was prepared in an AE-6400 electrophoresis cell (ATTO Corp., Tokyo, Japan) according to the method of Laemmli (1970). Following the addition of Laemmli's reducing sample buffer, samples were boiled for 5 min and then electrophoresed. Electophoretically separated samples were transferred to a polyvinyliden difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) with a semidry blot electrophoretic transfer system (EB-150; Toyo Roshi Kaisha Ltd, Tokyo, Japan). After blocking with 5% nonfat dry milk dissolved in TBST (10 mM Tris, 100 mM NaCl, 0.2% Tween 20; pH 7.5), the membrane was probed with the indicated antibody and washed with TBST three times. The membrane was then probed with a peroxidase-labelled second antibody and washed with TBST three times. The signals were visualized using an enhanced chemiluminescence plus system (Amersham, Arlington Height, IL).

Purification of recombinant human LBP, MD-2 and CD14

Human LBP was expressed in yeast and purified as previously described (Ohnishi *et al.*, 2001). The coding regions of human MD-2 and CD14 minus their signal sequences were subcloned into yeast expression vector pGAPZα (Invitrogen, Carlsbad, CA) with an N-terminal × 6 histidine tag sequence. MD-2 and CD14 were expressed in a Pichia expression system (Invitrogene) and purified with a Ni^{2+}-column (Novagen, Madison, WI) under denaturing conditions according to the manufacturer's recommendation.

NF-κB reporter assay

After plating 293 cells in 12-well plates (2×10^5 well^{-1}), the cells were transfected by the calcium phosphate precipitation method with the expression plasmids indicated in the figure, 0.1 μg of pELAM-L luciferase reporter plasmid, and 0.0025 μg of phRL-TK (Promega, Madison, WI) for normalization. After 24 h, cells were stimulated as indicated in the figure in serum-free DMEM. The reporter gene activity was measured according to the manufacturer's (Promega) recommendation.

Results

TLR4 was expressed on the cell surface in the absence of MD-2

To study the mechanism of LPS recognition in cells expressing TLR4 and CD14 but lacking MD-2, we used 293 cells transiently transfected with TLR4 and CD14. As it has been indicated that the association of TLR4 with MD-2 stimulates the expression of TLR4 on the cell surface (Nagai *et al.*, 2002), we first examined whether 293 cells express TLR4 on the cell surface in the absence of MD-2. We expressed TLR4 with/without CD14 and MD-2, and labelled the cell-surface proteins with a membrane-impermeable biotinylation reagent. The biotinylated proteins were collected with streptavidin-agarose, and electrophoresed by SDS-PAGE. Finally, cell-surface TLR4 was detected by Western blotting (Fig. 1). When cells were not treated with the biotinylation reagent, the expression of TLR4 on the cell surface was not detected, although a nonspecific band whose molecular weight was smaller than that of TLR4 was detected (Fig. 1, fourth lane from left). The cells transiently transfected with TLR4 and MD-2 strongly expressed TLR4 on the cell surface (Fig. 1, third and fifth lanes from left). Apparent cell-surface TLR4 expression was also observed in the cells transfected with both TLR4 and CD14 (Fig. 1, second lane from left), or with TLR4 alone (Fig. 1, first lane from left) at comparable levels, although the expression level was lower than that in cells expressing both TLR4 and MD-2.

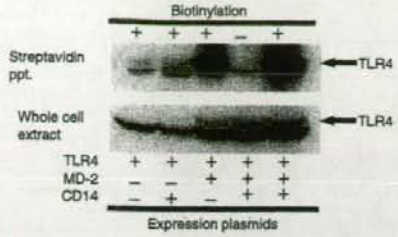

Fig. 1. Cell-surface expression of TLR4 in the absence or presence of MD-2. After plating 293 cells in 6 cm dishes, cells were transfected with the expression plasmids indicated (5 μg for EIAV-tagged TLR4 and MD-2, 0.25 μg for CD14). After 24 h, the cells either were left untreated or were treated with a membrane-impermeable biotinylation reagent, and cell extracts were prepared. The cell extracts were divided into two portions. One portion was analysed directly (lower panel) for TLR4 by Western blotting with an anti-EIAV tag antibody. Biotinylated proteins were collected from the second portion with streptavidin-agarose and boiled in SDS-PAGE sample buffer. The resulting supernatant (upper panel) was analysed for TLR4 by Western blotting with anti-EIAV tag antibody.

LPS induced the association of mCD14 with TLR4 in the absence of MD-2

Because cell-surface expression of TLR4 was observed even in the absence of MD-2, we next examined the formation of cell-surface TLR4–CD14 complexes in cells expressing TLR4 and CD14 but lacking MD-2. FLAG-tagged TLR4 and EIAV-tagged CD14 expression plasmids were transfected into 293 cells. After stimulation with LPS in the presence of LBP, cell-surface proteins were cross-linked with a membrane-impermeable bifunctional cross-linking reagent. After washing, cellular extracts were prepared and boiled in the presence of SDS to disrupt noncovalent protein–protein association. Following dilution, FLAG-tagged TLR4 was precipitated with anti-FLAG M2 agarose. The precipitated proteins were subjected to SDS-PAGE, and CD14 cross-linked with FLAG-tagged TLR4 was analysed by Western blot (Fig. 2). When cells were not treated with the cross-linking reagent, CD14 was not detectable, indicating that noncovalently associated CD14 was not coprecipitated with TLR4 under this experimental condition (Fig. 2, first and third lanes from left in upper panel). CD14 was also not precipitated with TLR4 in unstimulated cells (Fig. 2, second lane from left in upper panel). In contrast, when cells were stimulated with LPS and cell-surface proteins were cross-linked, CD14 was co-precipitated with TLR4 (Fig. 2, fourth lane from left in upper panel). The total expression levels of TLR4 and CD14 were not changed by treatment with LPS (middle and lower panels). These results indicate that LPS stimulation induces the formation of CD14–TLR4 complexes on the cell surface even in cells without MD-2 expression.

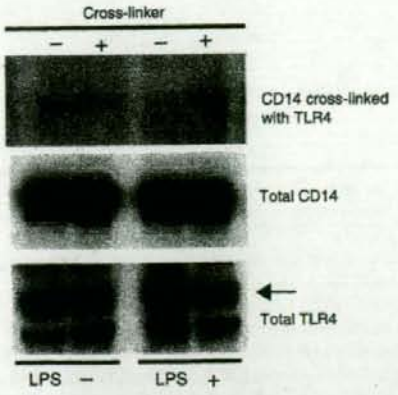

Fig. 2. LPS-induced formation of the cell-surface TLR4–CD14 complex in the absence of MD-2. After plating 293 cells in type I collagen-coated 10 cm dishes, cells were transiently transfected with FLAG-tagged TLR4 and EIAV-tagged CD14 expression plasmids (24 μg for TLR4 and 0.2 μg for CD14). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. The cells either were left unstimulated (left two columns) or were stimulated with LPS (0.01 μg mL^{-1}) in the presence of LBP (0.1 μg mL^{-1}) for 3 h (right two columns). The cells were then either left untreated or treated with a membrane-impermeable cross-linking reagent, and cell extracts were prepared. The cell extracts were divided into three portions. SDS was added to one portion of the extract to a final concentration of 1%, and the extract was boiled for 5 min. Then, FLAG-tagged TLR4 was precipitated with anti-FLAG M2 agarose, and CD14 that had been cross-linked with TLR4 was detected by Western blotting with an anti-EIAV tag antibody after the cross-linking reagent was cleaved with a reducing agent (upper panel). The other two cell-extract portions were analysed for CD14 (middle panel) and TLR4 (lower panel) by Western blotting with an anti-EIAV tag antibody and anti-FLAG M2 antibody, respectively.

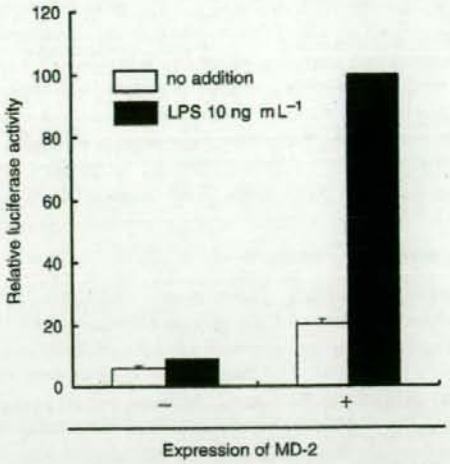

Fig. 3. Effect of MD-2 expression on LPS-induced activation of NF-κB in 293 cells. After plating 293 cell in 12-well plates, cells were transfected with TLR4 (0.02 μg), CD14 (0.02 μg) expression plasmids, pELAM-L (0.1 μg) and phRL-TK (0.0025 μg), and with/without MD-2 (0.02 μg) expression plasmid. After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. The cells were treated for 6 h with LPS (0.01 μg mL^{-1}) in the presences of LBP (0.1 μg mL^{-1}), and luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in 293 cells expressing MD-2. Values are means ± SE from at least three independent experiments.

The recognition of LPS in cells expressing TLR4 and CD14

Because CD14–TLR4 complex formation was observed in response to LPS in cells expressing TLR4 and CD14 but lacking MD-2, we studied whether LPS stimulation led to the activation of NF-κB (Fig. 3). When we stimulated these cells in the presence of LBP, no activation of NF-κB was observed (Fig. 3, left). In contrast, when the cells expressing TLR4, CD14 and MD-2 were stimulated with LPS, strong activation of NF-κB was observed (Fig. 3, right), as expected. These results indicate that, although LPS stimulation induces the formation of CD14–TLR4 complexes, intracellular signal transduction does not occur in the absence of MD-2.

We then stimulated 293 cells expressing TLR4 and CD14 with LPS in the presence of sMD-2 and LBP (Fig. 4). Surprisingly, no activation of NF-κB was observed, even in the presence of sMD-2 (Fig. 4a). We hypothesized that excess sMD-2 may inhibit LPS signal transduction by associating with LPS. We therefore first treated cells with LPS and LBP, and then added sMD-2 after 3 h. The delayed addition of sMD-2 strongly induced the activation of NF-κB (Fig. 4b). We also preincubated the cells with sMD-2 first, and after 3 h LPS and LBP were added to the medium (Fig. 5). No response to LPS stimulation was observed in this case (Fig. 5a). However, if sMD-2 was removed by washing prior to the addition of LPS and LBP, the cells responded strongly to LPS stimulation (Fig. 5b). These results indicate that the coexistence of LPS and sMD-2 leads to an inactivation of LPS signal transduction in cells. These results also suggest that, once LPS is transferred to mCD14 and the TLR4–CD14 complex is formed, the binding of sMD-2 to the TLR4–CD14 complex enables the activation of 293 cells expressing TLR4 and CD14. To confirm this, we preincubated 293 cells expressing TLR4 and CD14 with LPS in the presence of LBP, and after 3 h LPS and LBP were removed by washing. Cells were then treated with sMD-2, and reporter activity was measured. A strong activation of NF-κB was observed in the cells with the addition of sMD-2, even in the absence of LPS (Fig. 6).

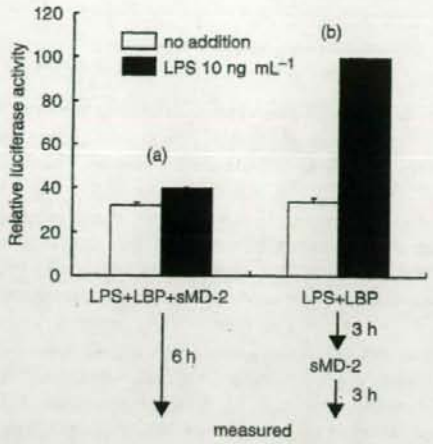

Fig. 4. Effect of the delayed addition of sMD-2 on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. (a) The cells either were left untreated (open column) or were treated for 6 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of sMD-2 and LBP (0.1 μg mL^{-1} each), and luciferase activity was measured. (b) The cells either were left untreated (open column) or were treated for 3 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of LBP (0.1 μg mL^{-1}). sMD-2 was then added to a final concentration of 0.1 μg mL^{-1}. After incubation had been continued for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

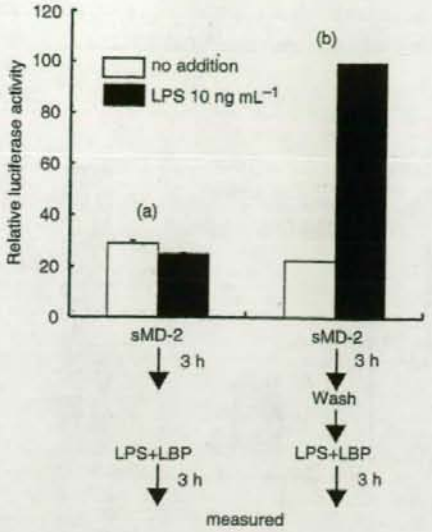

Fig. 5. Effect of the removal of sMD-2 prior to the addition of LPS and LBP on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. Then, the cells were treated with sMD-2 (0.1 μg mL^{-1}) for 3 h. The cells either were left unwashed (a) or were washed three times with PBS (b), and then either left unstimulated (open column), or stimulated with LPS (0.01 μg m^{-1}, closed column) in the presence of LBP (0.1 μg mL^{-1}). After incubation for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

Discussion

In this study, we investigated the LPS-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. We used 293 cells transiently transfected with both TLR4 and CD14 expression plasmids. First, we studied the cell-surface expression of TLR4 in the absence of MD-2 expression. It has been demonstrated that the expression of MD-2 with TLR4 induces the translocation of TLR4 to the cell surface (Nagai *et al.*, 2002). Other studies have shown that MD-2 is necessary for the glycosylation of TLR4, which enhances the cell-surface expression of TLR4 (da Silva Correia & Ulevitch, 2002; Ohnishi *et al.*, 2003). In contrast, Viriyakosol *et al.* (2001) reported that TLR4 is translocated to the cell surface even in the absence of MD-2. Our results clearly demonstrated that the cell-surface expression of TLR4 is greatly enhanced by coexpression of MD-2, but apparent TLR4 expression was observed on the cell surface even in the absence of MD-2. It is therefore likely that MD-2 is not essential for the translocation of TLR4, although its expression strongly enhances the cell-surface expression of TLR4.

In cells expressing TLR4, CD14 and MD-2, it has been suggested that mCD14 and TLR4–mMD-2 complexes exist separately on the cell surface, and that the binding of LPS to mCD14 induces the formation of mCD14–TLR4–mMD-2 complexes (Jiang *et al.*, 2000). We examined the formation of TLR4–mCD14 complexes in cells expressing TLR4 and CD14 but lacking MD-2. Our results showed that the complex of TLR4 and mCD14 was formed by LPS stimulation even in the absence of mMD-2 (Fig. 2). This indicates that mMD-2 is not necessary for the formation of TLR4–mCD14 complexes, and that the binding of LPS to mCD14 can lead to the formation of TLR4–mCD14 complex without mMD-2. However, this complex formation did not lead to the activation of NF-κB, and MD-2 was required for activation (Fig. 3). In cells that expressed TLR4 and CD14 but lacked MD-2, it was thought that MD-2 was

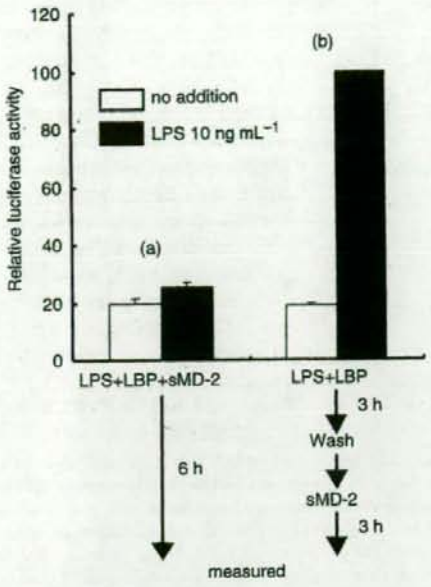

Fig. 6. Effect of the removal of LPS and LBP prior to the addition of sMD-2 on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. (a) The cells either were left untreated (open column) or were treated for 6 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of sMD-2 and LBP (0.1 μg mL^{-1} each), and luciferase activity was measured. (b) The cells either were left untreated (open column) or were treated with LPS (0.01 μg mL^{-1}, closed column) for 3 h in the presence of LBP (0.1 μg mL^{-1}). The cells were washed three times with PBS and then treated with sMD-2 (0.1 μg mL^{-1}). After incubation for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

supplied as sMD-2 (Jia *et al.*, 2004). In a situation in which sMD-2 exists at 0.1 μg mL^{-1}, it is expected that LPS will interact with sMD-2 (Viriyakosol *et al.*, 2001) before it interacts with mCD14 because of the overwhelming amount of sMD-2 compared with mCD14. LPS loses its activity if it interacts with sMD-2 before it interacts with CD14 (Fig. 5, and Viriyakosol *et al.*, 2001). The loss of activity probably depends on the formation of an inactive complex of aggregated LPS–sMD-2 (Gioannini *et al.*, 2004) or may partly depend on the increased aggregation of TLR4 in patches on the endosomes (Husebye *et al.*, 2006). There are thus two timings of addition of sMD-2 for the activation of cells expressing TLR4 and CD14 but lacking MD-2, namely before or after LPS interacts with CD14. When cells were pretreated with sMD-2 and excess sMD-2 was removed by washing prior to the addition of LPS and LBP, these cells responded strongly to LPS stimulation (Fig. 5). This result suggests that sMD-2 is transferred to TLR4, that the TLR4–sMD-2 complex is formed, and that finally the binding of LPS to mCD14 induces the formation of the TLR4–sMD-2–mCD14 complex. The formation of this complex leads to the activation of NF-κB. Because the TLR4–sMD-2 complex is formed first, this is a similar mechanism of LPS-recognition to that in cells expressing TLR4 and mMD-2. However, when cells were treated first with LPS in the presence of LBP, the strong activation of NF-κB was induced by the subsequent addition of sMD-2, even if LPS and LBP were removed by washing prior to the addition of sMD-2 (Figs 4 and 6). This result indicates that LPS is first transferred to mCD14 and that this transfer induces the formation of the TLR4–CD14 complex (Fig. 2); subsequently, the delayed binding of sMD-2 to the TLR4–CD14 complex is able to induce the activation of NF-κB.

We studied the activation of NF-κB as one of the activation markers for LPS signalling because the activation of NF-κB is the most common and well-studied type of signalling. It is also known that LPS stimulation leads to the activation of the MAPK kinase signalling pathway as well as to the activation of NF-κB (Kawai *et al.*, 1999). It would therefore be interesting to evaluate the role of MD-2 in the activation of the MAPK kinase signalling pathway.

The mechanism of LPS recognition on the cell surface has been discussed, and some models have been proposed. One widely accepted model is shown in Fig. 7a. The mCD14 molecule and the TLR4–mMD-2 complex exist separately on the cell surface (Jiang *et al.*, 2000). LPS is transferred to mCD14 by LBP, and the LPS–mCD14 complex is formed. The formation of this complex leads to the association of the LPS–CD14 complex with the TLR4–mMD-2 complex (Jiang *et al.*, 2000), and LPS is transferred to the TLR4–MD-2 complex (da Silva Correia *et al.*, 2001). Finally, the oligomerization of TLR4 occurs and the intracellular signalling is generated (Saitoh *et al.*, 2004). In contrast, the mechanism of LPS recognition in cells expressing TLR4 and CD14 but lacking MD-2 has not received much attention. Gioannini *et al.* have reported that the artificially created LPS–sMD-2 complexes with the assistance of sCD14 *in vitro* were able to activate cells expressing only TLR4 (Gioannini *et al.*, 2004), it is predicted that the transfer of LPS–sMD-2 complexes created in the medium to TLR4 may induce transmembrane signal transduction (Fig. 7b). In addition to these mechanisms, we propose a further LPS-recognition mechanism in these cells in the present study (Fig. 7c). mCD14 and TLR4 exist separately on the cell surface and LPS is transferred to mCD14 by LBP. This binding of LPS to mCD14 induces the association of mCD14 with TLR4, and the mCD14–TLR4

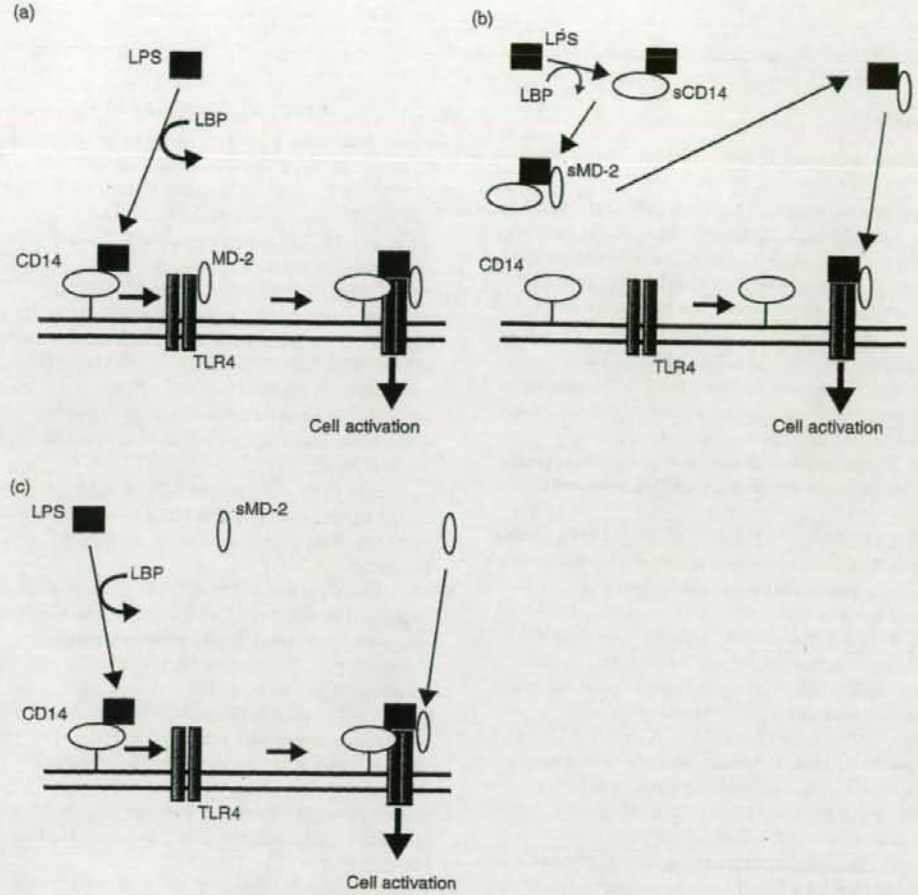

Fig. 7. Schematic representation of conventional (a, b) and proposed additional (c) LPS-recognition mechanisms. (a) LPS is transferred to mCD14 by LBP, and then the LPS–mCD14 complex is recognized by the TLR4–mMD-2 complex in cells expressing TLR4, MD-2 and CD14. (b) The LPS–sMD-2 complex is formed in the medium with the assistance of sCD14, and the LPS–sMD-2 complex is transferred to TLR4. (c) LPS is transferred to mCD14 by LBP, and this association induces the formation of the LPS–mCD14–TLR4 complex in cells expressing TLR4 and CD14. The binding of sMD-2 to this complex leads to the activation of the cells.

complex is formed. The binding of sMD-2 to this complex triggers the transmembrane signal transduction. The presence/absence and dynamics of sMD-2 in human plasma have been controversial. It has been reported that cells expressing TLR4 and CD14 but lacking MD-2 responded to LPS following the addition of plasma containing sMD-2 (Pugin *et al.*, 2004). We hypothesize that, if cells expressing TLR4 and CD14 but lacking mMD-2, such as airway epithelial cells, are exposed to LPS when the plasma sMD-2 level is already high, no activation of NF-κB may occur. However, if these cells are exposed to LPS followed by sMD-2 successively, such as in the case of infection (Pugin *et al.*, 2004), these cells will respond to LPS using our LPS-recognition mechanism.

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研究代表者 小川 温子

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