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Original Article

Protection of Mice from LPS-induced Shock by CD14 Antisense Oligonucleotide

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CD14 is a pattern recognition receptor on myeloid cells and plays a pivotal role in an innate immune system that is responsible for Gram-negative and Gram-positive bacteria infection. Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, can induce production of a large quantity of proinflammatory cytokines into the circulation mediated by CD14-mediated macrophages and monocytes. These cytokines eventually cause septic shock. Several *in vitro* and *in vivo* studies have shown that suppression of a CD14 function by a CD14 antibody led to an inhibition of the production of proinflammatory cytokines such as TNF-α, IL-1 β, and IL-8. In the present study, we found that CD14 antisense oligonucleotide (ODN) can prevent lethal LPS shock in D-galactosamine-sensitized mice. This ODN inhibited CD14 expression in a mouse macrophage cell line, RAW264.7, and suppressed production of TNF-α in LPS-stimulated RAW264.7 cells. Furthermore, we designed a consensus antisense ODN that could hybridize human and mouse CD14 RNA, and we evaluated its efficacy. The consensus antisense ODN rescued mice primed with Mycobacterium bovis bacillus Calmette-Guerin (BCG) from the LPS-induced lethal shock. In this model, the CD14 antisense ODN down-regulated LPS-elicited CD14 expression in the liver, resulting in a decrease in LPS-induced TNF-α production. These findings suggest that the CD14 antisense ODN is distributed in the liver and efficiently suppresses LPS-induced TNF-α production by reducing CD14 expression on Kupffer cells. This CD14 antisense ODN may be useful for the development of a therapeutic agent against sepsis and septic shock.

Key words: sepsis, TNF-α, BCG (bacillus Calmette-Guerin)

Gram-negative bacterial infections can cause septic shock. It has been reported that 500,000 people in the United States suffer from sepsis and 175,000 of these die annually [1]. Thus, the disease is highly lethal, yet an effective therapeutic agent has not yet been developed [2].

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CD14 is a glycosyl phosphatidylinositol-linked type glycoprotein expressed on monocytes/macrophages, including Kupffer cells [3]. In 1990, CD14 was found to be a receptor for lipopolysaccharide (LPS), which is a component of the outer membrane of all Gram-negative organisms [4]. Consequently, CD14-deficient mice were found to have strong resistance against LPS [5]. This indicates that CD14 is the major LPS receptor component with high affinity. LPS in the blood is associated with LPS-binding protein (LBP) and is transferred to

CD14 on monocytes/macrophages or forms a complex with soluble CD14. The LPS signal is then transmitted into the cell via a Toll-like receptor (TLR) 4 [6]. Lipoteichoic acid and peptideglycan, components of Gram-positive bacteria, are also associated with CD14 and activate TLR2 on the cell [6-8]. Thus, CD14 plays a key role in infections of Gram-positive as well as negative-bacteria.

It has been reported that the inhibition of CD14 functions can be applied to human sepsis therapy [9]. Anti-CD14 antibody inhibited the transfer of LPS to CD14 on the membrane and to soluble CD14 by binding to the N terminal portion of CD14 protein. As a result, anti-CD14 antibody protected against shock by inhibiting inflammatory cytokine production in primates [9]. In fact, CD14 antibody protected rabbits against organ injury and death even if the antibody was administered 4 h after LPS injection [10]. Similarly, blockade of the interaction of CD14 with bacteria is beneficial for host defense in rabbits injected with bacterium pneumonia [11]. The symptoms of these rabbits were clinically similar to those of humans.

CD14 antisense ODN at the translation start site inhibited IL-1 β expression in mouse embryonic calvarial cells and LPS-stimulated differentiation to osteoclastic cells [12]. Actually, the CD14 antisense ODN dramatically suppressed LPS-stimulated CD14 up-regulation, but sense ODN, c-fos, and c-jun antisense ODNs did not suppress it in macrophages [13]. In addition, *in vivo* studies have shown extensive distribution and accumulation of antisense ODNs in the liver and kidney [14]. Thus, we hypothesized that antisense ODNs could be distributed in the liver to protect the organ more efficiently by blocking LPS signals through CD14 on Kupffer cells.

In this study, we found that CD14 antisense ODN is able to block LPS-elicited lethal shock in an *in vivo* septic shock model using mice. Furthermore, to develop CD14 antisense ODN as a therapeutic agent against septic shock, we attempted to identify the human CD14 active site for antisense ODN and to design a consensus CD14 ODN that hybridizes both human and mouse CD14 genes. The consensus ODN inhibited CD14 up-regulation stimulated by LPS and suppressed serum TNF-α increase in *in vivo* and *in vitro* experiments. The consensus ODN could protect mice from lethal shock even if LPS-shocked mice were pre-infected with BCG. These data suggest the usefulness of CD14 antisense ODN as a therapeutic agent against septic shock.

Methods

Antisense ODNs, antibody, cells and cultures. Antisense ODNs for CD14 were designed against the translational start site. The sequences used were: SM0105A, 5'-CACACGCTCCATGGTCGGTA G-3'; SM0105Amt3B, 5'-CACCCTCGCATGGTCGGT AG-3'; SH0105A, 5'-CGCGCGCTCCATGGTCGATA A-3'; SU0105A and MU0105A, 5'-CICICGCTCCAT GGTCGITAI-3'. The antisense ODNs used in this study were purchased from several suppliers including Sawady Technology (Tokyo, Japan), Amersham Pharmacia Biotech (Upssala, Sweden), and Nisshinbo Industries Inc. (Tokyo, Japan). Antisense ODNs used for *in vivo* evaluations and cell-based assays were modified with phosphorothioate. Antiserum against murine CD14 was developed to immunize rabbits with KLH CNPFLDE-SHSEKFNS, corresponding to the murine CD14 peptide sequence from 308 to 322, by a method described previously [15].

A murine macrophage cell line (RAW264.7), a human promonocytic cell line (THP-1), and HeLa cells were obtained from ATCC. RAW264.7 and THP-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 (Life Technologies Oriental, Inc., Tokyo, Japan) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA).

Establishment of a stable transformant expressing CD14/luciferase fusion protein. The human CD14 gene was cloned from the total RNA extracted from THP-1 cells treated with 1α, 25-dihydroxyvitamin D_3 (Funakoshi, Tokyo, Japan), by RT-PCR using the primers 5'-ACGCGTCGACGAGTT CACAAGTGTGAAGCCTG-3' (sense) and 5'-ACATG CATGCTTAATAAAGGTGGGGCAAAGGG-3' (antisense). The 5'-noncoding and coding regions of CD14, which consisted of 102 bp and 316 bp in length, respectively, were inserted into a pGEMluc plasmid carrying the luciferase reporter gene (Promega Corp., Madison, WI, USA). This human CD14/luciferase fusion fragment was then subcloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) using an hCD14/luciferase fusion expression vector, pM1651.

To establish a transformant expressing human CD14/luciferase fusion protein, HeLa cells were co-transfected with pM1651 and pRV-CMV plasmid (Promega) using the modified calcium phosphate method

(Mammalian Transfection Kit, Stratagene, La Jolla, CA, USA). After incubation in DMEM containing 10% FBS overnight, the cells were seeded to a 96-well plate at concentrations of 100 to 500 cells/well. Starting the next day, the cells were cultured in growth medium containing 0.6 mg/ml G-418 (Life Technologies Oriental, Inc.). An He1651d3-20 clone exhibiting both firefly and renilla luciferase activity was used for cell-based assay by antisense ODNs.

HeLa transformant cell-based assay. He1651d3-20 cells were inoculated at 1×10^5 cells/well in a 24-well plate in DMEM containing 10% FBS and 0.6 mg/ml of G418, and incubated at 37 °C overnight. After the cells had been washed twice with saline, the culture medium was replaced with 450 μl/well of Opti-MEM (Life Technologies Oriental, Inc.). Subsequently, according to the manufacturer's protocol, 50 μl of a mixture of lipofectin (50 μg/ml) reagent (Life Technologies Oriental, Inc.) and ODN at various concentrations was added to the culture. The cells were incubated at 37 °C for 6 h, and the medium was then replaced with DMEM containing 10% FBS and 0.6 mg/ml of G418, followed by overnight incubation. After being washed twice with saline, the cells were harvested into 80 μl of passive lysis buffer (Promega). According to the manufacturer's protocol, 20 μl of cell lysate was mixed with 100 μl of luciferase assay reagent II in a plate for fluorescence measurement (Dynatec Labs., VA, USA). Luminescence as firefly luciferase activity was measured for 10 sec a luminometer (LB96B, Berthold). Then to this reaction solution 100 μl of Stop & Glo solution was added, and renilla-luciferase activity was measured in the same way. The luciferase activity was estimated by normalizing firefly-luciferase activity in terms of renilla-luciferase activity.

RAW 264.7 cell-based assay for antisense ODNs. RAW264.7 cells were seeded at 1×10^5 cells/well in a 6-well plate with CD14 ODNs and cultured in a serum-free medium. This was followed by 3 additions of CD14 ODNs from 1 μM to 30 μM at 24 h intervals. The cells were treated with 6 ng/ml of LPS (*E. coli* 055: B5) in the presence of 2% mouse serum. The supernatants were collected 4 h after treatment. TNF-α was measured using a mouse TNF ELISA kit (Amersham Pharmacia Biotech). The cells were washed 3 times with PBS and lysed directly in 200 μl of 1 × laemmli buffer. These cell lysates were used for Western analysis.

THP-1 cell-based assay for antisense ODNs. THP-1 cells were seeded at 1×10^5 cells/well into 24-well plates and cultured in RPMI1640 medium containing 10% inactivated FBS in the presence of 10 ng/ml of phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO, USA) for 24 h. After the cells had been washed twice with saline, the culture medium was replaced with 450 μl/well of Opti-MEM (Life Technologies Oriental, Inc.). Subsequently, according to the manufacturer's protocol, 50 μl of a mixture of lipofectin (50 μg/ml) reagent (Life Technologies Oriental, Inc.) and ODN (100 nM) was added to the culture. After the cells had been incubated at 37 °C for 4 h, the medium was replaced with RPMI1640 medium containing 10% inactivated FBS in the presence of 40 ng/ml of 1α, 25-dihydroxyvitamin D3 (Funakoshi) for 20 h. After washing the cells, the medium was replaced with RPMI1640 containing 2% human serum to which 1 ng/ml of LPS was added. After 4 h incubation, TNF-α in the culture supernatant was measured using a human TNF-α ELISA kit (Amersham Pharmacia Biotech).

D-Galactosamine-sensitized endotoxin shock model. All studies were performed in accordance with guidelines described in the NIH *Guide for the Care and Use of Laboratory Animals* (DHHS publication No. [NIH] 85-23, revised 1996). Six-week-old-Balb/c male mice (Charles River Japan, Kanagawa, Japan) were divided into 7 groups (each group consisting of 10 animals) based on body weight. Subsequently, 0.3 mg/kg to 3 mg/kg of SM0105A, 0.3 mg/kg to 3 mg/kg of control ODN (21 mer oligonucleotide with a randomizing sequence), or 10 ml/kg of saline were injected into the tail vein in one administration. At 24 h after the administration, 5 μg/kg of LPS and 700 mg/kg of D-galactosamine hydrochloride (Wako, Osaka, Japan) were injected into the tail vein to induce shock. Immediately before the LPS injection, 0.3 mg/kg of methylprednisolone (Shionogi Pharmaceuticals, Osaka, Japan) was administered to the mice. The survival rate was periodically evaluated until 24 h after the shock induction. At 1 h and 24 h after LPS administration, blood was collected from the eyeground vein using a glass capillary pretreated with heparin solution (1000 IU/ml) (Mochida, Tokyo, Japan). Plasma samples were obtained by centrifuging 50 μl of the blood samples. GPT activity was determined using a GPT activity measurement slide, GPT/ALT-P, and Fuji DRI-CHEM 5000 (Fuji Film, Tokyo, Japan).

BCG-sensitized endotoxin shock model. We established a mouse model by modifying the previous

method [16]. Six-week-old-Balb/c male mice (n = 5) (Charles River Japan) intravenously received 200 μl of BCG suspension (approximately 7×10^7 organisms) (Nihon BCG Co., Ltd., Tokyo, Japan). After 11 days, MU0105A was administrated intravenously to the mice at doses of 30, 10, 3, 1, or 0.3 mg/kg. Finally, 3 μg/mouse of LPS was injected intravenously on day 12. The mice were observed for 2 days after LPS injection. The survival rate was evaluated at 24 and 48 h after LPS injection.

Preparation of liver homogenate from BCG-sensitized mice. Six-week-old male mice were treated with BCG and 12 days later intravenously given 3 μg of LPS. The mice were then sacrificed by bleeding 6 h after LPS injection. One g of the liver on ice was sliced and homogenized in 5 ml of homogenate buffer containing 10 mM Tris-HCl (pH 7.2), 0.3 M sucrose, and 2 mM EGTA using a Polytron homogenizer (PT-1200C, Kinematica). After centrifugation at 1,800 rpm for 10 min at 4 °C, the supernatant was frozen at − 80 °C until analysis. The protein concentration was determined with BSA as a standard using a protein assay kit (BioRad). Two hundred μl of the supernatant was denatured for 4 min in boiling water and mixed with 200 μl of 2 × Laemmli buffer. Ten μl of each denatured sample was analyzed by electrophoresis on SDS-polyacrylamide gels, followed by Western blotting. The signal intensity was determined using a densitometer (Amersham Pharmacia Biotech, Image Master). The relative intensity was estimated by using the arbitrary intensity of CD14 signals from RAW264.7 cell lysates as a standard.

Results

Reduction in the rate of mortality of D-galactosamine-sensitized mice injected with LPS by CD14 antisense ODN. D-galactosamine-sensitized mice were used to evaluate CD14

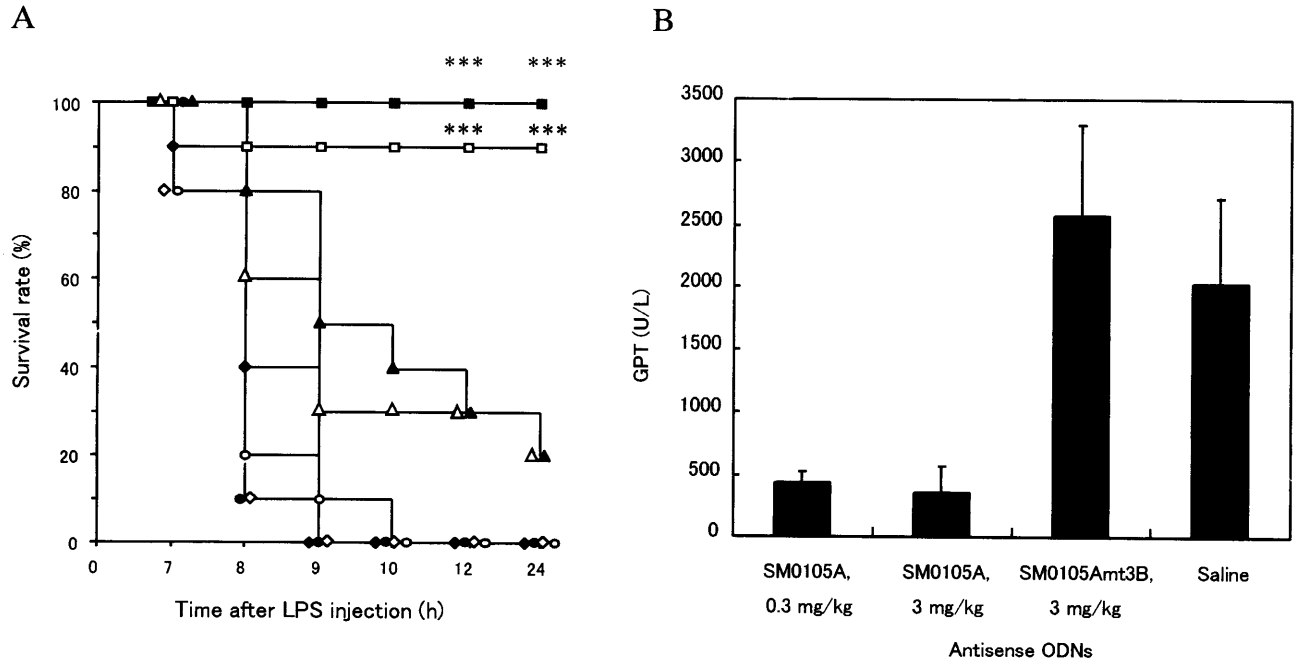

Fig. 1 Protective effects of CD14 antisense ODN on the survival rate and hepatic injury of mice sensitized with D-galactosamine and LPS. **A**, Six-week-old Balb/c male mice (n = 10) intravenously given 0.3 mg/kg (△), 1 mg/kg (□) and 3 mg/kg (■) of SM0105A, 0.3 mg/kg (◇), 1 mg/kg (●) and 3 mg/kg (○) of control ODN, 10 ml/kg of saline (◆) or 0.3 mg/kg of methylprednisolone (▲). Twenty-four hours later, 5 g/kg of LPS and 700 mg/kg of D-Galactosamine were given to induce shock. The survival rate was periodically evaluated until 24 h after LPS injection. **B**, Six-week-old Balb/c male mice (n = 9) were given SM0105A (mouse CD14 antisense ODNs), SM0105Amt3B (control ODN with 3 mismatched bases introduced in SM0105A) or saline. D-galactosamine (700 mg/kg) and LPS (5 μg/mouse) were injected 24 h after the ODN treatment. GPT activity in plasma 6 h after LPS injection was measured using a Fuji DRY-CHEM 5000 kit. * * *, $P < 0.0001$ compared with untreated group (LPS + saline) using Fisher's exact test.

antisense activity *in vivo*. SM0105A, control ODN, and saline were each administered at various concentrations before LPS injection. All mice administered saline as a negative control died within 9 h after the LPS injection. All mice administered the control ODN at any dose died within 10 h after the LPS injection. On the other hand, the survival rates of mice given 3 mg/kg, 1 mg/kg, and 0.3 mg/kg SM0105A were 100%, 90%, and 20%, respectively (Fig. 1A). The survival rate of mice injected with 0.3 mg/kg of SM0105A was equivalent to that of the group injected with methyl prednisolone (Fig. 1A).

We then examined the grade of liver injury of mice treated with D-galactosamine and LPS by CD14 antisense ODN. GPT activity remarkably increased to more than 2000 U/l with simultaneous injections of D-galactosamine and LPS. GPT activity in normal mice was less than 100 U/l (data not shown). CD14 antisense ODN at doses of 0.3 mg/kg and 3 mg/kg caused average GPT activity in plasma to decrease to 437 U/l and 360 U/l, respectively. On the other hand, SM 0105 Amt 3B with 3 mismatched bases in SM0105A showed an increase in GPT activity (Fig. 1B). Thus, CD14 antisense ODN exerted a protective effect on hepatic injury in D-galactosamine-sensitized mice injected with LPS. Taken together, protection of liver functions by SM0105A eventually contributed to the mice's survival.

Identification of the active sequences in the 5'-non-coding region of the human CD14 gene. Since the antisense ODN against the translational start site of mouse CD14 effectively alleviated LPS shock, we attempted to determine what parts of the human CD14 antisense ODNs were effective. Twenty-eight ODNs of 21 mer in length complementary to the 5'-non-coding region were evaluated for TNF-α inhibitory activity in LPS-induced THP-1 cells treated with 1α, 25-dihydroxyvitamine D_3. We found 2 active regions in the 5'-non-coding region (Fig. 2). The one was from the 23 rd cytosine to the 62 nd adenine, and the other was from the 93 rd guanine to the 145 th uridine. Since the major start site of CD14 transcription is the 13 th adenine [17], the first one existed near the capping site of the CD14 transcript, while the second one was over the translation initiation site. Furthermore, in order to determine whether antisense ODNs with TNF-α inhibitory activity could suppress CD14 expression, SH0105A and SH0109A in the second active region were

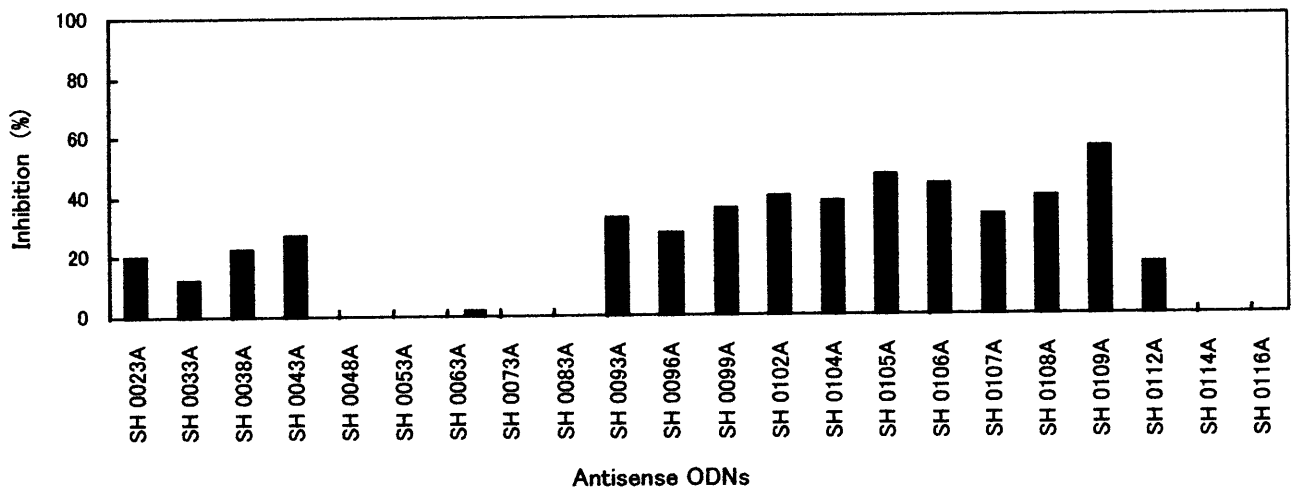

Fig. 2 Antisense ODNs complementary to human CD14 mRNA around the translation initiation site inhibits TNF-α production in LPS-induced THP-1 cells treated with 1α, 25-dihydroxyvitamine D_3.

The inhibitory activities of antisense ODN complementary to the 5'-non-coding regions of human CD14 were compared. Inhibitory activity of TNF-α production was determined by using 100 μM SH0023A, SH0033A, SH0038A, SH0043A, SH0063A, SH0093A, SH0096A, SH0099A, SH0102A, SH0104A, SH0105A, SH0106A, SH0107A, SH0108A, SH0109A, and SH0112A. Two active regions were found. One region was the CUGGAAGCCGCCGGGUGCCGCUGUGUAGGAAAGAAGCUAAA sequence as indicated by the ODNs complementary to a part of the sequence. The other region was the GGUUCGGAAGACUUAUCGACCAUGGAGCGCGCGUCCUGC sequence as indicated by the oligonucleotides complementary to a part of the sequence. The values were calculated using the equation: Inhibition % = (1-TNF-α concentration in ODN-treated sample/TNF-α concentration in untreated sample) × 100.

tested in a HeLa/luciferase assay. In this assay, these human antisense ODNs showed strong inhibitory activity at 100 nM, but the control ODN did not (Fig. 3). In conclusion, the antisense ODNs around the translation initiation site exhibited strong inhibitory activity in the human cell line.

Design of CD14 antisense ODN. The sequence in the proximity of the AUG is relatively conservative between humans, monkeys, and mice. The most commonly conserved sequence in the second active region was from the 109 th to the 121 st on the CD14 gene (Fig. 4). Since antisense ODNs such as SH0105A complementary to the sequence from the 105 th to the 125 th on the CD14 gene exhibited inhibitory activity both in mice and humans, we applied the 0105 sequence for CD14 antisense ODN to mouse and human cells.

The 0105 sequence contains 4 mismatched bases between mice and humans. These base changes were the transition of pyrimidines on the CD14 gene. Inosine is known to form a base pairing with all 4 bases. The order of duplex stability is I:C > I:A > I:T > I:G [18-20]. Therefore, we designed MU0105A having inosine in place of adenine and guanine (Fig. 4). The Tm value of MU0105A was 66.3 °C to mouse and 71 °C to human complementary DNA, respectively (Table 1). These data suggest that MU0105A may be effective for both mice

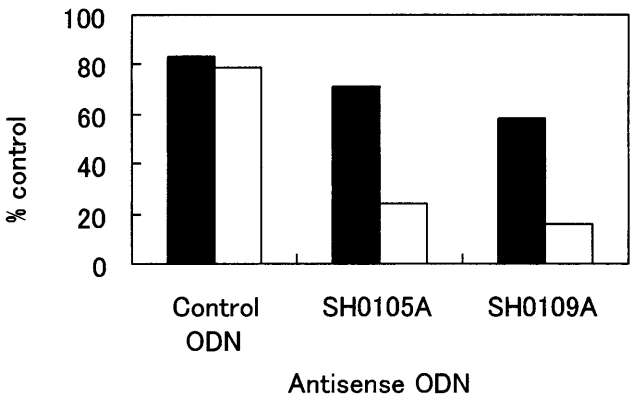

Fig. 3 Inhibition of luciferase activity in HeLa/Luciferase assay by antisense ODNs around AUG.
He1651d3-20 cells established as described in Methods were seeded at 1×10^5 cells/well in 24-well plates and incubated at 37 °C overnight. After washing the cells twice with the saline, the medium was replaced by Opti-MEM, and a mixture of antisense ODN (30 nM, closed bars; 100 nM, open bars) and lipofectin was added to the cultures. The cells were refed with the growth medium at 6 h after treatment and cultured overnight. The cells were harvested in lysis buffer, and luciferase activity was measured as described in Methods. Each value is the average of duplicate measurements.

Table 1 Thermal stability of consensus ODN/target RNA duplexes

ODNs	Tm (°C)	
	Targets	
	Mouse CD14RNA	Human CD14RNA
SM0105A	63.5	NT
SH0105A	NT	67.0
SU0105A	53.2	64.8
MU0105A	66.3	71.0
SM0105Amt3B	49.0	NT

The sequence of human CD14 RNA is 5'-r(UUAUCGACCAUGGAGCGCG CGUCC)-3' and that of mouse CD14 RNA is 5'-r(CUACCGACCAUGGAG CGUGUGCUU)-3'. The ODN and the target (each 3 μM) were dissolved in 400 μl of hybridization buffer containing 5 mM Na_2HPO_4 (pH 7.0), 5 mM $MgCl_2$, 40 mM KCl and denatured at 99 °C for 5 min, followed by gradually cooling to 30 °C for 1 h. Tm values of the samples were measured at 260 nm over a range 30–90 °C at 0.5 °C/min with a Beckman DU-7500 and were calculated using a micro Tm analysis system.

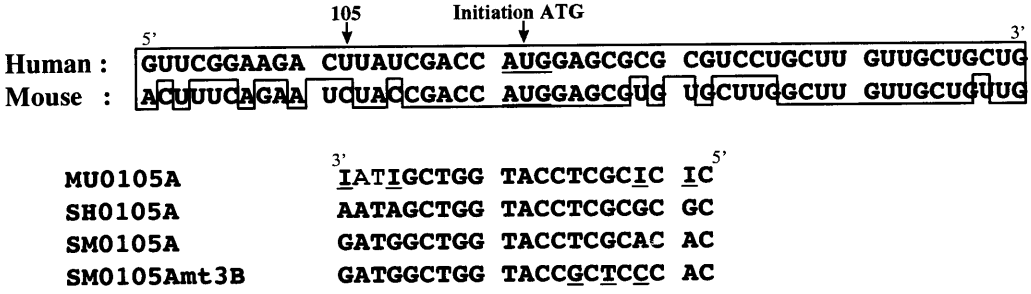

Fig. 4 Human and mouse sequences of CD14 mRNA around the translation initiation site and the structure of ODNs with 0105A sequence.

and humans.

CD14 antisense ODNs inhibited mouse CD14 expression and suppressed TNF-α production in LPS-induced RAW264.7 cells. We evaluated the efficacy of CD14 antisense ODNs in an *in vitro* assay using RAW264.7 cells. TNF-α released from LPS-stimulated RAW264.7 cells was measured in the supernatant of the cells treated with antisense ODNs 24 h before LPS stimulation. SM0105A and MU0105A having a high affinity to both mouse and human CD14 equally inhibited TNF-α production in a dose-dependent manner (Fig. 5). More than 10 μM of these ODNs completely inhibited TNF-α production. To determine whether the inhibition of TNF-α production depends on the decrease in CD14 expression by these antisenses, we carried out Western analysis of the above cells. MU0105A and SM0105A specifically inhibited CD14 expression at doses of more than 3 μM. Mouse CD14 protein was no longer detected when treated with more than 10 μM of these antisense ODNs. On the other hand, SM0105Amt3B did not suppress TNF-α production or CD14 expression (Figs. 5, 6). An obvious dose-response relationship was observed between the suppression of CD14 expression and the inhibition of TNF-α release by MU0105A and SM0105A.

CD14 antisense ODN rescued mice from lethal shock in the BCG-sensitized LPS shock model. Next, we used a pre-sensitized shock model to determine the effectiveness of MU0105A. ODNs were given to mice 11 days after BCG challenge, and LPS was injected into those mice 24 h later to determine the effects of ODNs on mortality. MU0105A protected the mice from lethal shock in a dose-dependent manner. The effective dose of MU0105A for 100% survival was 10 mg/kg (Fig. 7). Although SM0105A had already caused a remarkable reduction in the mortality rate in the D-galactosamine-sensitized septic shock model, this ODN also protected the mice from lethal shock in the BCG-sensitive model (data not shown). In this model, the TNF-α level in plasma increased to 0.7 ng/ml by sensitization with BCG. LPS injection augmented the TNF-α level by about 20 fold. On the other hand, MU0105A dramatically suppressed LPS-elicited TNF-α down to 22 % within 6 h after the injection and diminished production of BCG-induced TNF-α in a dose-dependent manner (Fig. 8A). CD14 expression increased remarkably in the BCG-sensitized mouse liver, but its expression was not detected in the normal mouse liver. Although LPS injection increased CD14 expression by 1.6 fold, MU0105A reduced CD14 up-regulation down to 62% within 6 h

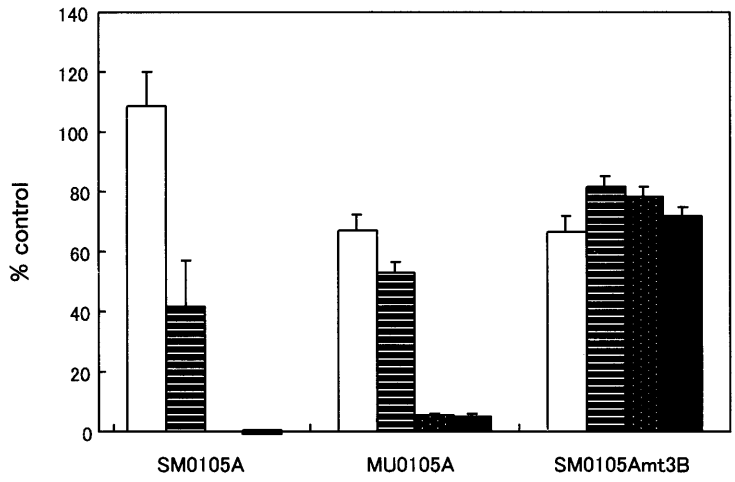

Fig. 5 Inhibition of TNF-α production in LPS-induced RAW264.7 cells by CD14 antisense ODNs.
SM0105A is mouse CD14 antisense ODN, MU0105A is the consensus CD14 antisense and SM0105Amt3B with 3 mismatched bases. RAW264.7 cells were treated 3 times every day with the antisense ODNs at various concentrations: 1 μM (open bars), 3 μM (hatched bars), 10 μM (shaded bars), and 30 M (closed bars). Finally, 10 ng/ml of LPS was added to the culture after the medium had been replaced with fresh medium containing 2% mouse serum, and the cells were incubated at 37 °C for 4 h. The murine TNF-α in the supernatant was measured using a murine TNF-α-specific ELISA kit. The values (n = 5, mean ± SEM) were calculated using the equation: % control = TNF-α concentration in ODN-treated sample/the average of TNF-α concentration in untreated sample × 100.

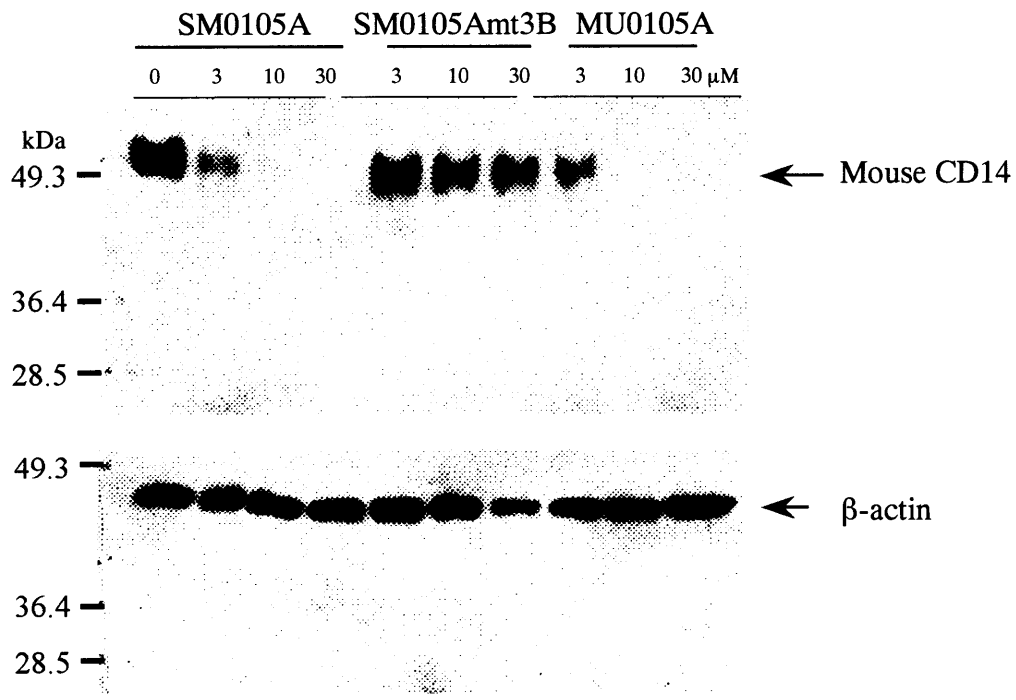

Fig. 6 Reduction of mouse CD14 protein expression in RAW264.7 cells by CD14 antisense ODNs.
RAW264.7 cells treated with ODNs were lysed directly in 1 × laemmli buffer. After boiling, the cell lysates (1×10^5 cells per lane) were separated by SDS-PAGE (4–20% gradient polyacrylamide gel), transferred to a PVDF membrane, and then treated with anti CD14 antibody. CD14 protein was visualized using an enhanced chemiluminescence detection system.

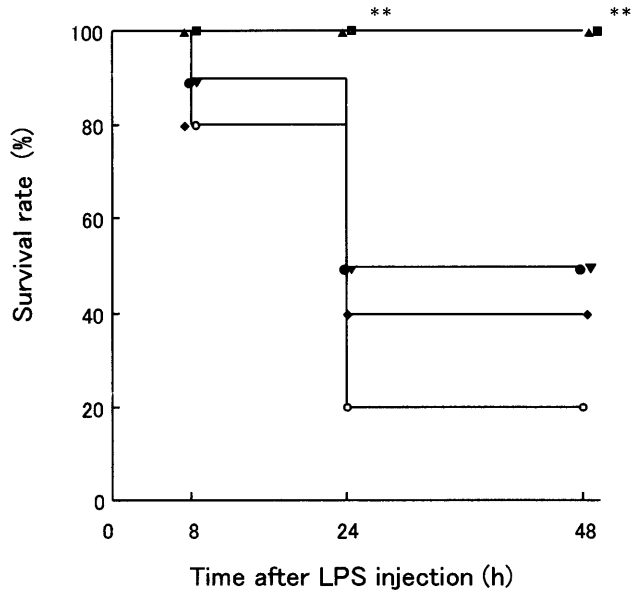

Fig. 7 Protection of BCG-primed mice injected with LPS by CD14 antisense ODNs.
Six-week-old male BALB/c mice (n = 5) were intravenously injected with 2 mg/mouse of BCG 11 days before LPS injection. Thirty mg/kg (■), 10 mg/kg (▲), 3 mg/kg (●) 1 mg/kg (▼), or 0.3 mg/kg (◆) of MU0105A, or 200 μl of saline (○) was intravenously given to mice 1 day before LPS injection. LPS (3 μg/mouse) intravenously-injected mice were observed for 24 to 48 h. The survival rates of the 30 mg/kg MU0105A and 10 mg/kg MU0105A groups were significantly different. **, $P < 0.001$ compared with the untreated group (LPS + saline) using Fisher's exact test.

after LPS injection. Like the suppression of TNF-α induction, MU0105A decreased CD14 up-regulation in BCG-sensitized mice in a dose-dependent manner (Fig. 8B).

Discussion

This is the first study to show that CD14 antisense ODN works well *in vivo* to prevent septic shock. The aim of this study was to develop therapeutic agents against septic shock. Herein, we reported that CD14 antisense ODN was able to protect mice from lethal shock and diminished hepatocyte damage. The mechanism of CD14 antisense effects is thought to involve the inhibition of LPS-elicited proinflammatory cytokine production by the suppression of CD14 expression in the liver.

In several clinical studies, the blockade of targets such as TNF-α, IL-1, and IL-8 were not sufficiently efficient to induce therapeutic benefits for sepsis and septic shock [21]. The LPS signal mediated by CD14 is located upstream of the cytokine cascade and other mediators [22]. Thus, blockade of CD14 function by anti-CD14 antibody improved hemodynamics and decreased systemic release of proinflammatory cytokines *in vivo* [22].

Unlike an antibody, antisense ODN was found to be exclusively distributed in the liver and kidney [14]. The liver is an important organ for detoxifying LPS, and Kupffer cells in the liver play a crucial role in LPS-induced proinflammatory cytokine production. TNF-α derived from Kupffer cells can injure sinusoidal endothelial cells by triggering the off-sticking of polymorphonuclear neutrophilic granulocytes and activating procoagulant activity. This potentiates the release of reactive oxygen intermediates and lytic enzymes such as elastase [23], resulting in septic shock.

We showed that the consensus antisense ODNs could

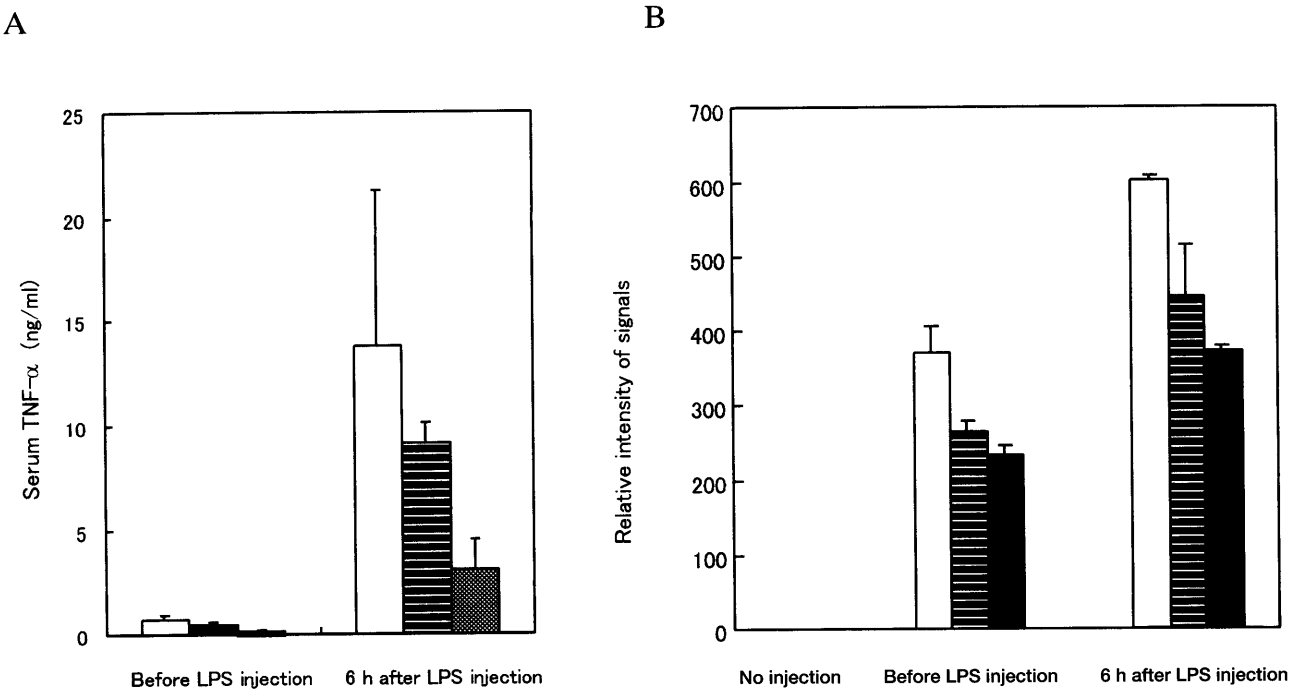

Fig. 8 Suppression of TNF-α production and liver CD14 expression in BCG-primed mice injected with LPS by CD14 antisense ODN. **A**, MU0105A inhibited TNF-α production (mean $\pm$ SEM) in BCG-primed 6-week-old male Balb/c mice (n = 3). The mice were sensitized with 2 mg/mouse of BCG 12 d before LPS injection, administered with MU0105A 1 d before the injection, and received 3 μg/kg of LPS. Plasma TNF-α was measured just before LPS injection (left) and at 6 h after LPS injection (right). Saline (open bars), 3 mg/kg (hatched bars) or 30 mg/kg (closed bars) of MU0105A was injected. **B**, Liver homogenates were prepared from BCG-sensitized mice (n = 3) and a control mouse (n = 1, left). Mice were given saline (open bars), 3 mg/kg of MU0105A (hatched bars), or 30 mg/kg of MU0105A (closed bars). CD14 expression (means $\pm$ SEM) before LPS injection (n = 3, middle) and at 6 h after the injection (n = 3, right) was analyzed by Western blotting. The signal intensity was determined using a densitometer as described in Methods.

hybridize both human and mouse CD14 with high affinity. These CD14 antisense ODNs prevented mortality in D-galactosamine-sensitized mice injected with LPS by reducing the extent of liver injury. These ODNs also suppressed LPS-induced TNF-α production in bacteria-primed mice, suggesting that CD14 antisense ODN inhibits LPS-elicited production of proinflammatory cytokines, which could be released from Kupffer cells, by suppressing CD14 up-regulation [24]. In fact, the antisense ODN reduced induction of CD14 expression in the liver. However, this was a partial inhibition. In BCG-sensitized mice, CD14 in the liver was already up-regulated remarkably before LPS injection, but only a small amount of TNF-α was produced. On the other hand, after LPS injection, TNF-α increased dramatically by 20 fold, but CD14 expression increased by only 1.6 fold. These findings imply that the increased basal CD14 level in the liver of BCG-primed mice did not contribute to TNF-α production as much as did LPS-induced CD14. CD14 in the liver was observed not only in Kupffer cells but also in other cells [25]. In fact, CD14 was constitutively expressed in human liver cells as well [24, 26]. Furthermore, CD14 transgenic mice whose CD14 was specifically expressed in hepatic cells were more persistant to LPS than were normal mice [27]. Taken together, these results suggest that CD14 in hepatic cells plays a role in LPS clearance. We speculate that the basal CD14 level is expressed in hepatic cells and that the expressed CD14 can work for LPS clearance without producing inflammatory cytokines. When mice were treated with antisense ODN, LPS-induced CD14 up-regulation was suppressed and the increase in TNF-α production was reduced by 22%. Thus, CD14 antisense ODN is likely to work only for the inhibition of CD14 expression on Kupffer cells, contributing to TNF-α production. Presumably, ODN is distributed in Kupffer cells and can suppress the inducible expression of CD14 in these cells.

Finally, 2 CpGs present in CD14 antisense ODN have been reported to express some biological effects [28]. A major effect of CpG ODN is activation of the innate immune system [29], because CpG ODN induces cytokines such as IL-6, IL-12, IFN-γ, and TNF-α to stimulate monocytes/macrophages, NK cells, and B cells. ODN CpG induced a large amount of TNF-α to activate NF-κB in macrophages and caused lethal shock in D-galactosamine-sensitized mice by inducing fulminant apoptosis of liver cells [30]. CD14 antisense CpG, which was non-palindromic CpG and non-typical 5'-Pu-Pu-CpG-Py-Py-3' [31], protected D-galactosamine-sensitized mice from lethal shock. Furthermore, although SM0105Amt3B also has 2 CpGs in the sequence, SM0105Amt3B did not express any activity *in vivo* as well as *in vitro*. These results indicate that CD14 antisense ODN may work via an antisense mechanism. This possibility can be confirmed by using CD14 antisense ODN modified with methylation of cytidine at the C5 position on CpG. Through the use of clinical application, we plan to clarify the tissue distribution using an isotope labeled compound.

In any event, further studies on CD14 antisense ODN will be instrumental in developing safe and potent therapeutic agents for sepsis and septic shock.

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