pDNA were used as CpG motif-containing DNA. pCMV-Luc was treated with *Sss*I CpG methylase (New England Biolabs, Ipswich, MA), which methylates only the cytosine of CpG dinucleotides, to obtain methylated pCMV-Luc.[3] The methylation was confirmed by digesting the DNA with *Hpa*II. Calf thymus DNA (CT DNA; Sigma) and methylated pCMV-Luc were used as DNA with few or no CpG motifs.

Preparation of Cationic Liposomes and Lipoplex

Cationic liposomes containing DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin film in a round-bottomed flask using a rotary evaporator, and then hydrating in 5% w/v dextrose by gentle vortexing. After hydration, the dispersions were sonicated for 3 min and passed through a MILLEX®-HV 0.45 μm filter unit (Millipore, Bedford, MA). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako kit (Wako Pure Chemical, Osaka, Japan). Cationic liposomes and DNA in 5% dextrose were mixed at a charge ratio +2.24 and the mixture was left for at least 30 min before use as lipoplex.

Animals

Male ICR and male C3H/HeJ mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and maintained on a standard food and water diet under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Institutional Animal Experimentation Committee, Kyoto University.

Tissue Distribution of [^{32}P]pDNA

pDNA (pCMV-Luc) was radiolabeled with [α-^{32}P] dCTP by nick translation, and diluted with non-radiolabeled pDNA for injection. ICR mice received naked or lipoplexed [^{32}P]pDNA by tail vein injection at indicated doses. Under anesthesia, blood was withdrawn from the vena cava and urine was collected from the urinary bladder. Then mice were euthanized at 3, 10, and 60 min after injection and the heart, lung, liver, spleen, and kidney were harvested, rinsed with saline, and weighed. Samples of blood, urine and small pieces of tissue were dissolved in 0.7 mL Solene-350 (Packard, Netherlands) at 55°C, and 0.2 mL isopropanol, 0.2 mL H_2O_2, 0.1 mL 5 M HCl, and 5 mL Clear-sol I (Nacalai Tesque) were added to each sample. The radioactivity was counted in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan).

Transgene Expression

Male ICR mice received pDNA at a dose of 25 μg pDNA/mouse in 1.6 mL saline (the so-called hydrodynamics-based procedure) or pDNA lipoplex at a dose of 25 μg pDNA/mouse in 300 μL 5% dextrose by tail vein injection. In addition, naked pDNA in 50 μL saline was injected into the gastrocnemius muscle at a dose of 25 μg pDNA/mouse. At indicated times after injection, tissues including the heart, lung, liver, spleen, kidney, and gastrocnemius muscle were harvested, washed with ice-cold saline, and homogenized in 5 mL/g (liver) or 4 mL/g (other tissues) of lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and subjected to three cycles of freezing in liquid nitrogen for 3 min and thawing in a water bath at 37°C for 3 min. The homogenates were centrifuged at 10000*g* for 10 min at 4°C. Then, 10 μL of the supernatant was mixed with 100 μL luciferase assay buffer (Pikkagene, Toyo Ink, Tokyo, Japan) and the chemiluminescence was measured with a luminometer (Lumat LB 9507, EG&G Bethhold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined using a protein quantification kit (Dojindo Laboratories, Kumamoto, Japan). Luciferase activity in each organ was normalized to relative light units (RLU) per milligram extracted protein. Luciferase activities in lungs of untreated or 5% dextrose-injected mice were 15 RLU/mg protein or lower.

Cytokine Assay

Naked DNA was injected into the tail vein by a normal procedure or the hydrodynamics-based procedure, or into the gastrocnemius muscle. Lipoplex was injected into the tail vein. Blood was collected in plastic tubes from the vena cava of mice under anesthesia, and allowed to stand for 3 h at 4°C. Then the samples were centrifuged at

3000*g* for 20 min at 4°C and the serum obtained was used for cytokine assay. Organs such as heart, lung, liver, spleen, and kidney were isolated at 1.5 h after injection of DNA, washed with ice-cold saline, blotted dry and stored at −80°C until use. The frozen organs were slowly thawed on ice, then homogenized in 2 mL phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors for assay. The cytoplasmic fractions were isolated as the supernatant following centrifugation at 15000*g* for 10 min at 4°C. The levels of TNF-α in serum and cytoplasmic fractions of organs were measured using an ELISA kit (AN'ALYZA™, Genzyme). Experiments were performed at least in duplicate, and a typical set of data was indicated.

Clodronate Liposomes

Phosphatidylcholine and cholesterol were dissolved in chloroform and a thin lipid film was formed by low-vacuum rotary evaporation. This film was dispersed in 10 mL PBS in which clodronate was dissolved. The suspension was maintained at room temperature for 2 h followed by sonication for 3 min. After another 2 h at room temperature, the suspension was centrifuged at 22000*g* for 1 h at 10°C to remove free clodronate, and then washed four times using centrifugation at 22000*g* for 25 min at 10°C. The liposomes were then resuspended in 4 mL PBS and stored at 4°C until use. Clodronate liposomes were used without removal of free clodronate.

Transient Depletion of Macrophages

Macrophages were transiently depleted by intravenous injection of clodronate liposomes as previously reported.[19] Clodronate liposomes or PBS (200 μL) was injected into the tail vein 24 h prior to the injection of naked DNA or pDNA lipoplex. This treatment of mice with clodronate liposomes was reported to transiently deplete phagocytes, including Kupffer cells, because clodronate induces apoptosis of cells taking up the liposomes. To avoid possible effects of empty liposomes on macrophage functions, PBS was injected to the control group of mice.

Inhibition of NF-κB Activation

NF-κB activation was inhibited by pre-administration of dexamethasone.[20] Dexamethasone was dissolved in PBS, and injected via the tail vein 1 h prior to the injection of lipoplex at a dose of 5 mg/kg body weight. The dosing and timing of dexamethasone for inhibition of the lipoplex-induced NF-κB activation were determined by according to a previous paper.[21] Control mice received PBS instead of dexamethasone.

Statistical Analysis

Differences were statistically evaluated by Student's *t*-test. *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TNF-α Production by CpG Replete DNA

Lipoplex consisting of pCMV-Luc induced a large amount of TNF-α in serum after intravenous injection at a dose of 25 μg DNA/mouse (Fig. 1A). No detectable TNF-α was found in serum after intravenous (Fig. 1B) or intramuscular (data not shown) injection of naked pCMV-Luc at the same dose. Increasing the dose of naked pCMV-Luc to 250 μg induced TNF-α production in serum after intravenous injection (Fig. 1B). To show that the TNF-α production was not due to contaminated lipopolysaccharide (LPS) in DNA samples, similar experiments were carried out in C3H/HeJ mice, a mouse strain lacking a response to LPS. Naked pCMV-Luc at a high dose of 250 μg induced TNF-α even in C3H/HeJ mice (104 ± 22 pg/mL), indicating that the DNA, pCMV-Luc, is the source of the TNF-α production even in the naked form. In both cases of the pCMV-Luc lipoplex and the high-dose naked pCMV-Luc, serum TNF-α levels increased rapidly and reached a peak at 1.5 h, then gradually decreased and had almost disappeared at 12 h post injection (data not shown). These results indicate that pCMV-Luc, a conventional CpG replete pDNA, induces TNF-α production regardless of its form, naked, or lipoplex. In addition, it was found that the immunostimulatory activity of pDNA is dependent on the method of administration.

Effect of CpG Motifs on TNF-α Production

Methylated pCMV-Luc, pGZB-Luc (CpG reduced pDNA), and CT DNA (linear mammalian DNA) were injected into the tail vein of mice in the naked or lipoplex form. pGZB-Luc lipoplex

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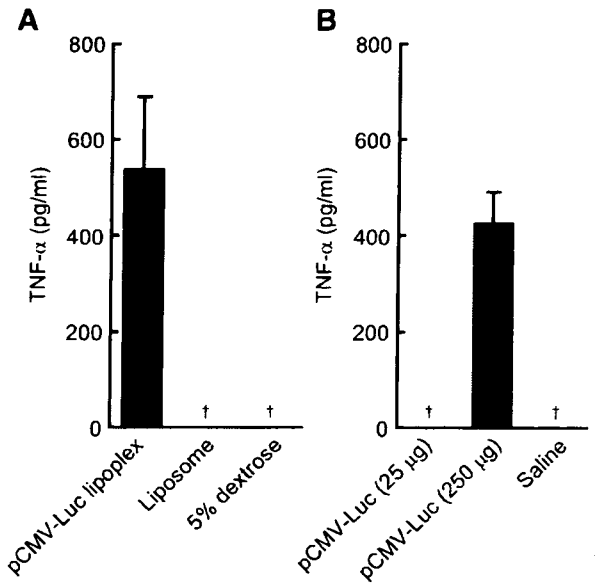

Figure 1. TNF-α level in serum after injection of naked pCMV-Luc or pCMV-Luc lipoplex. Mice were intravenously injected with (A) pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse, (B) naked pCMV-Luc at indicated doses. Vehicles, 5% dextrose and saline, were used as controls. At 1.5 h after injection, serum was collected and the TNF-α level in serum was measured by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected.

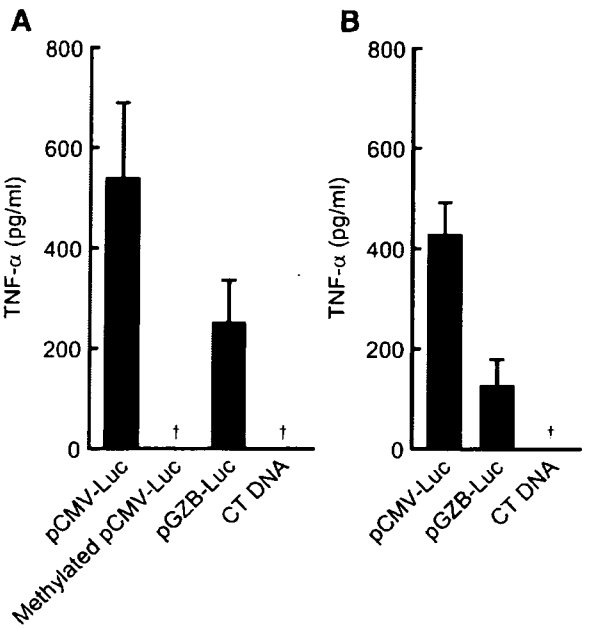

Figure 2. TNF-α level in serum after intravenous injection of naked DNA and DNA lipoplex. Mice were injected with (A) DNA lipoplex at a dose of 25 μg DNA/mouse, or (B) naked DNA at a dose of 250 μg DNA/mouse. At 1.5 h after injection, serum was collected and the TNF-α level in serum was determined by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected.

induced TNF-α production in serum, but the level was almost half that of pCMV-Luc lipoplex (Fig. 2A). Methylated pCMV-Luc lipoplex or CT DNA lipoplex induced hardly any TNF-α production. Naked DNA-induced TNF-α production also showed similar results to those with DNA lipoplex (Fig. 2B). Naked CT DNA induced no detectable TNF-α production even at the high dose of 250 μg. These results indicate that CpG motifs in DNA are essential for the induction of inflammatory cytokines in mice, and the level of cytokines is proportional to the number of CpG motifs in DNA.

Effects of Macrophage Depletion on Tissue Distribution of DNA and TNF-α Production

As reported previously, clodronate liposomes were injected into the tail vein of mice to deplete macrophages and other phagocytes. Figure 3 shows the effects of macrophage depletion on the tissue distribution of [^{32}P]pCMV-Luc lipoplex after intravenous injection. In untreated mice, ^{32}P-radioactivity rapidly disappeared from the

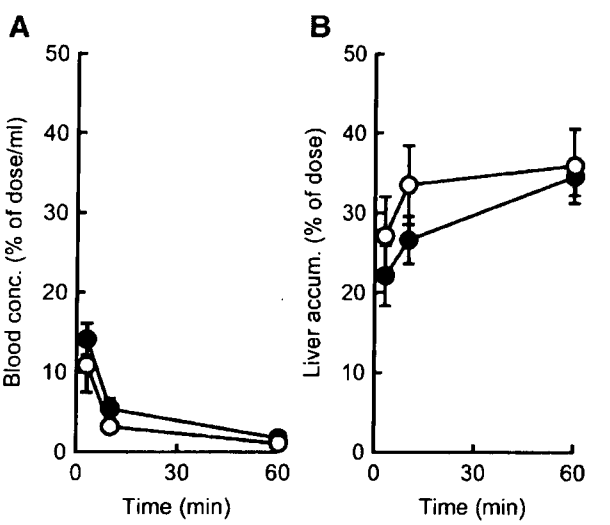

Figure 3. Blood concentration (A) and liver accumulation (B) of ^{32}P-radioactivity after intravenous injection of [^{32}P]pCMV-Luc lipoplex into mice pretreated with clodronate liposomes or PBS. Mice were injected with [^{32}P]pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse 24 h after intravenous injection of clodronate liposomes (closed circle) or PBS (open circle). The results are expressed as the mean ± SD of three mice.

systemic circulation, was trapped in the lung and then was gradually delivered to the liver. Pre-administration of clodronate liposomes slightly altered the distribution of radioactivity after intravenous injection of [^{32}P]pCMV-Luc lipoplex. In marked contrast, DNA lipoplex-induced TNF-α in serum and organs were greatly reduced by the pre-administration of clodronate liposomes (Fig. 4). Taking into account the fact that clodronate liposomes transiently deplete phagocytes, such as liver Kupffer cells and splenic macrophages, these results indicate that these cells are the major contributor to the TNF-α production by pDNA lipoplex.

Effects of Macrophage Depletion on Transgene Expression

As reported previously, intravenous injection of pCMV-Luc lipoplex resulted in transgene expression in various organs, with the highest in the lung (Fig. 5A). Pre-administration of clodronate liposomes significantly reduced the levels of transgene expression by the lipoplex. The transgene expression in the lung by pNF-κB-Luc lipoplex was also reduced by the clodronate liposome treatment (Fig. 5B). Although a similar level of TNF-α was produced by administration of pGL3-control vector lipoplex, the expression by the pGL3-control vector lipoplex was hardly affected by clodronate liposomes (Fig. 5C). As listed in Table 1, there are two NF-κB binding sites in the enhancer region of pGL3-control, whereas pCMV-Luc and pNF-κB-Luc have 4 and 5 NF-κB binding sites, respectively. TNF-α is one of the most potent stimulators of NF-κB, so it is possible that pDNA-induced TNF-α activates

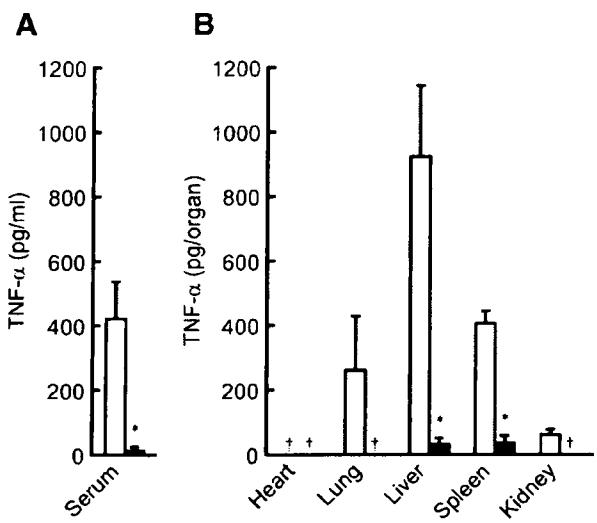

Figure 4. Effect of clodronate liposomes on TNF-α production after intravenous injection of pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse. Mice were injected with pCMV-Luc lipoplex 24 h after intravenous injection of clodronate liposomes (closed bars) or PBS (open bars). At 1.5 h after injection of pCMV-Luc lipoplex, (A) serum and (B) organs were collected and the TNF-α levels were determined by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected. *Significantly different ($p < 0.05$) from the PBS-treated mice.

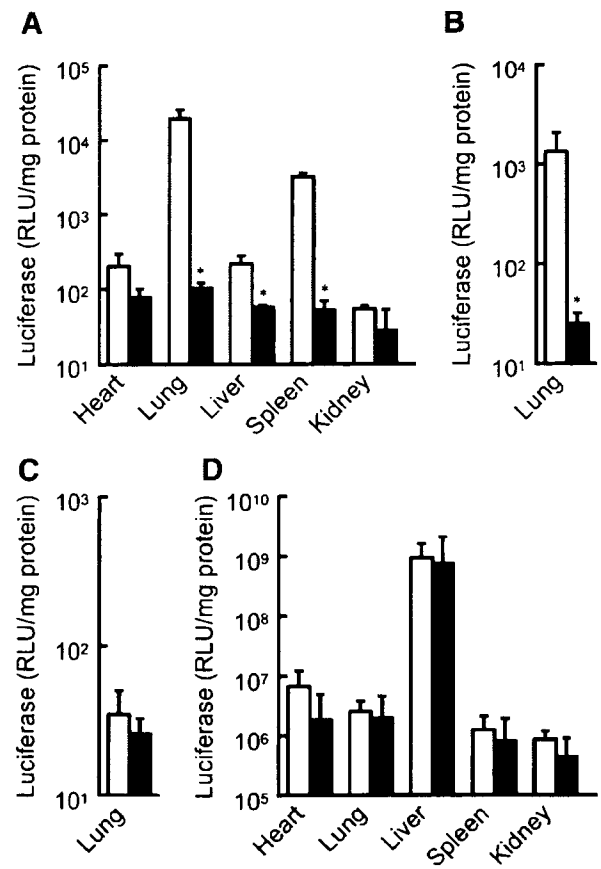

Figure 5. Effect of clodronate liposomes on transgene expression after intravenous injection of pDNA lipoplex. Mice were injected with lipoplex containing 25 μg (A) pCMV-Luc, (B) pNF-κB-Luc, (C) pGL3-control vector, or (D) 25 μg naked pCMV-Luc by the hydrodynamics-based procedure 24 h after intravenous injection of clodronate liposomes (closed bars) or PBS (open bars). At 6 h after injection of pDNA lipoplex, mice were euthanized and the luciferase activities in organs were measured. The results are expressed as the mean ± SD of three mice. * Significantly different ($p < 0.05$) from the PBS-treated mice.

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Table 1. Properties of pDNA Used in This Study

Plasmid	Length (kbp)	Number of CpG (% to pCMV-Luc)	Promoter	Number of NF-κB Binding Sites
pCMV-Luc	7.1	846 (100)	CMV	4
pGZB-Luc	4.5	282 (53)	CMV	4
pGL3-control	5.1	604 (100)	SV40	2
pNF-κB-Luc	5.7	Undisclosed	TATA box + NF-κB binding sites	5
pCpG-Luc	4.8	192 (34)	EF1α	8
pLuc-mcs	5.7	500 (68)	TATA box	0

NF-κB which, in turn, increases the level of transgene expression from pDNA containing many NF-κB binding sites. The relationship between the number of the NF-κB binding sites and TNF-α-induced increase in transgene expression was further examined using two additional pDNA: pCpG-Luc, a plasmid containing 8 NF-κB binding sites, and pLuc-mcs with no binding sites. Macrophage depletion significantly reduced the peak level of transgene expression in the lung 6 h after administration of pCpG-Luc lipoplex (10100 ± 1600 and 1950 ± 830 RLU/mg protein in the PBS-treated-, and clodronate liposome-treated mice, respectively). No significant transgene expression was observed in both the PBS- and clodronate liposome-treated mice when pLuc-mcs lipoplex was injected, because of the very weak promoter activity of the plasmid. Then, to confirm the importance of TNF-α, not of macrophage depletion, on transgene expression, pCMV-Luc was injected into the tail vein of mice by the hydrodynamics-based procedure (Fig. 5D), which induced little TNF-α.[22] Pre-administration of clodronate liposomes hardly changed the levels of transgene expression. These findings strongly support the hypothesis that TNF-α produced by pDNA lipoplex increases transgene expression from the lipoplex through the activation of NF-κB.

Inhibition of NF-κB Activation by Dexamethasone

Dexamethasone inhibits NF-κB activation by inducing the NF-κB inhibitor, IκB.[20] Mice receiving dexamethasone 1 h prior to the injection of pCMV-Luc or pNF-κB-Luc lipoplex exhibited a significantly lower level of transgene expression at 6 h than those pre-injected with PBS (Fig. 6A and B). These findings suggest a positive correlation between TNF-α production and NF-κB activation. Although transgene expression at later time points did not show impressive increases, it was slightly greater in dexamethasone-treated mice than in control mice. A dexamethasone-induced increase in transgene expression was observed in mice receiving pGL3-control plasmid lipoplex (Fig. 6C), or naked pCMV-Luc by the hydrodynamics-based procedure (Fig. 6D). As reported in previous studies, these results suggest that TNF-α reduces transgene expression at later time points regardless of the type of pDNA.

DISCUSSION

Recent studies have demonstrated that pDNA administration induces significant inflammatory responses. The inflammatory properties of bacteria-derived pDNA limit many current pDNA-based gene therapy strategies. However, the cellular basis for the inflammatory response to pDNA and the effects of the inflammatory response on pDNA-induced transgene expression are poorly understood.

In the present study, we focused on TNF-α as the inflammatory cytokine induced by pDNA administration, because this cytokine is initially secreted by macrophages recognizing a foreign substrate. We confirmed that a significant level of TNF-α was detected in the serum after intravenous injection of pCMV-Luc lipoplex or a high dose of naked pCMV-Luc (Fig. 1). These results indicate that pDNA containing CpG motifs induces TNF-α production regardless of the naked or complexed form. The inflammatory responses observed in mammalian cells have been shown to arise in part from the recognition of the CpG motifs present in bacterial DNA or pDNA.[1] The importance of the CpG motif on TNF-α production was confirmed by using CpG-reduced pGZB-Luc, CpG-methylated pCMV-Luc, and CT DNA. In many nonviral *in vivo* gene transfer studies using mice, several tens of micrograms pDNA are generally administered. Because of the less toxic nature of naked pDNA than lipoplex, the dose for naked pDNA can be as high as a few hundreds

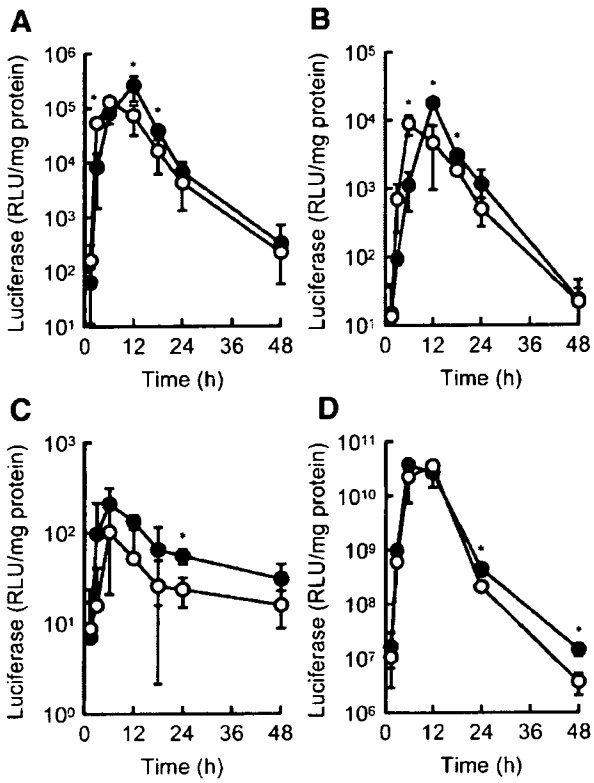

Figure 6. Effect of dexamethasone on transgene expression after intravenous injection of pDNA lipoplex. Mice were injected with pDNA lipoplex consisting of 25 μg (A) pCMV-Luc, (B) pNF-κB-Luc, (C) pGL3-control vector, or (D) 25 μg naked pCMV-Luc by the hydrodynamics-based procedure 1 h after intravenous injection of dexamethasone (closed circle) or PBS (open circle). At the indicated times after injection, mice were euthanized and the luciferase activities in lung were measured. The results are expressed as the mean ± SD of three mice. *Significantly different ($p < 0.05$) from the PBS-treated mice.

of micrograms. Thus, we selected the dose of lipoplex to 25 μg DNA per mouse, and those of naked DNA to 25 and 250 μg per mouse. The results of the present study clearly demonstrated that more naked pDNA is required to induce TNF-α production than lipoplex, suggesting that complexation with cationic liposomes greatly enhances the pDNA-induced TNF-α production.

The liver and spleen are known to play central roles in the removal of foreign particles and invasive microorganisms from the circulation, mainly via Kupffer cells and splenic macrophages.[23,24] Kupffer cells are liver macrophages and their functions are activated by a variety of particles, viruses, LPS, and TNF-α.[25] The phagocytosis of parasites by Kupffer cells is accompanied by the release of TNF-α. Splenic macrophages are also activated in addition to liver Kupffer cells. We and other groups have also reported that lipoplex accumulates in the liver and spleen, as well as in the lung after intravenous injection, and liver Kupffer cells and splenic macrophages are directly involved in the uptake of pDNA lipoplex and inflammatory cytokine production.[26–30] We functionally depleted these cells by injecting clodronate liposomes[19] and examined the tissue distribution and TNF-α production after intravenous injection of pDNA. Pretreatment with clodronate liposomes slightly reduced the liver accumulation of pDNA lipoplex, but significantly reduced TNF-α production. These results suggest that, even when liver Kupffer cells are depleted, pDNA lipoplex is largely delivered to the liver, but where it induces hardly any inflammatory responses. Other liver-constituting cells, such as hepatocytes and sinusoidal endothelial cells, are responsible for the hepatic uptake of the lipoplex.

To examine the effects of TNF-α on transgene expression, we compared the level of transgene expression in mice, with or without pretreatment of clodronate liposomes. Pretreatment resulted in a significant reduction in the level of transgene expression by pCMV-Luc lipoplex and pNF-κB-Luc lipoplex (Fig. 5A and B). Clodronate liposomes hardly affected the level of transgene expression by hydrodynamically delivered naked pCMV-Luc (Fig. 5D), which excludes the possibility that the treatment reduces transgene expression in a nonspecific manner. pCMV-Luc and pNF-κB-Luc contain 4 and 5 NF-κB binding sites in their promoter/enhancer regions,[31] whereas pGL3-control vector has only two NF-κB binding sequences in the enhancer region. The results of the present study using several types of pDNA with different properties suggest that the number and/or position of NF-κB binding sites affect the responsibility of pDNA to NF-κB. A recent study by Kuramoto et al.[15] showed that the TNF-α-induced increase in transgene expression correlated with the number of NF-κB binding sites in pDNA. Therefore, the fewer numbers of the binding sites in the pGL3-control vector may explain why the expression by the pGL3-control lipoplex was not significantly affected by the treatment.

To investigate the relationship between the transgene expression and NF-κB activation, dexamethasone, an inhibitor of NF-κB activation,[20] was injected prior to pDNA administration. Again, pretreatment with dexamethasone signifi-

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cantly reduced transgene expression by pCMV-Luc and pNF-κB-Luc lipoplex during the early period of injection. However, transgene expression by pGL3-control vector lipoplex was slightly increased by dexamethasone at any time point examined (Fig. 6C). Pretreatment with dexamethasone also significantly increased transgene expression by naked pCMV-Luc and pNF-κB-Luc at later time periods. These findings suggest that there is a positive correlation between TNF-α production and NF-κB activity-dependent transgene expression when pDNA contains effective NF-κB binding sites. Other studies also support these findings. CMV promoter activity was increased by inflammation in a rat arthritis model, where a positive correlation between transgene expression and the dose of LPS was obtained.[32] Reactivation of the previously silenced CMV promoter was observed after administration of LPS in mouse liver.[33] It is possible that pDNA containing NF-κB binding sites can be translocated into the nucleus through NF-κB activation. Mesika et al. reported an NF-κB-assisted import of pDNA into the nuclei of mammalian cells *in vitro*.[34] At the same time, however, the results of dexamethasone treatment also suggested that TNF-α reduces transgene expression in the long-term even at low concentrations. Tan et al.[35] reported that dexamethasone increased the pDNA complex-induced transgene expression in mouse lung via the suppression of TNF-α production. They also demonstrated that co-delivery of an NF-κB decoy increased the pDNA complex-mediated transgene expression. pDNA lipoplex has been shown to induce apoptosis of lung endothelial cells via TNF-α.[36] Thus, it is possible that TNF-α induces apoptosis of the transfected target cells and the expression is eventually lost.

In conclusion, we have demonstrated that tissue macrophages involving liver Kupffer cells and splenic macrophages are closely involved in TNF-α production after pDNA administration. TNF-α production was dependent on the number of CpG motifs in DNA regardless of the form of the DNA administered. TNF-α can increase transgene expression through NF-κB activation during the early phase after gene transfer, but it reduces the expression, probably through the apoptosis of transfected target cells or other mechanisms. These findings provide useful basic information for the development of more efficient *in vivo* gene delivery systems.

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Cellular Activation by Plasmid DNA in Various Macrophages in Primary Culture

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ABSTRACT: Macrophages are an important group of cells responsible for the inflammatory response to unmethylated CpG dinucleotide (CpG motif) in plasmid DNA (pDNA) via Toll-like receptor 9 (TLR9). This finding is primarily based on *in vitro* studies. Previous *in vivo* studies also have suggested that tissue macrophages are involved in inflammatory cytokine release in the circulation following intravenous administration of pDNA to mice. However, the relationship between the *in vitro* and *in vivo* studies has not been sufficiently clarified. To gain insight into which types of cells are responsible for the production of cytokines upon interaction with pDNA, peritoneal macrophages, splenic macrophages, hepatic nonparenchymal cells (NPCs) including Kupffer cells and mesangial cells were isolated from mice. All types of primary cultured cells, except for mesangial cells, express TLR9 at varying levels. Splenic macrophages and hepatic NPCs were activated to produce tumor necrosis factor-α (TNF-α) by naked pDNA, whereas peritoneal macrophages and mesangial cells were not. pDNA complexed with *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride/cholesterol liposome induced TNF-α in the splenic macrophages but not in the other cell types. These results indicate that splenic macrophages and hepatic NPCs are closely involved in TNF-α production in response to pDNA. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: plasmid DNA; lipoplexes; liposomes; DNA delivery; immunology; tissue macrophage; naked DNA; inflammatory cytokine; CpG DNA

INTRODUCTION

Plasmid DNA (pDNA) has become an important macromolecular agent suitable for nonviral gene therapy as well as DNA vaccination.[1] It is well known that unmethylated CpG dinucleotides, or CpG motifs, in bacterial DNA, but not in vertebrate DNA, are recognized by the mammalian immune system as a danger signal.[2] Toll-like receptor 9 (TLR9) is a pattern recognition receptor recognizing CpG motifs,[3] and is present in the intracellular compartments of immune cells, such as macrophages and dendritic cells.[4] These cells secrete inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), and IL-12, upon uptake of CpG motif-containing DNA. These cytokines reduce transgene expression in target cells through direct cytotoxicity and/or promoter attenuation.[5,6] Consequently, reduction or prevention of pDNA-induced cytokine production is important for increasing the efficiency of *in vivo* gene transfer.

To improve the transfection efficacy with pDNA, DNA/cationic liposome complexes are often used both *in vivo* and *in vitro*. Several

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recent studies have shown that intravenous (i.v.) administration of pDNA/cationic liposome complexes leads to effective gene expression although such complexes also induce high amounts of inflammatory cytokines.[5–10]

In a series of investigations on the tissue disposition of naked pDNA we have shown that the hepatic nonparenchymal cells (NPCs), such as Kupffer cells and sinusoidal endothelial cells, play important roles in the clearance of pDNA.[11,12] In addition, it has been suggested that tissue macrophages, such as Kupffer cells (liver resident macrophages) and splenic macrophages, are responsible for cytokine production following intravenous injection of DNA/cationic liposome complexes.[13] On the other hand, there is very little production of cytokines by cultured peritoneal macrophages following stimulation with naked pDNA,[14] even although they exhibit extensive DNA uptake via scavenger receptorlike mechanisms.[15,16] Surprisingly, peritoneal macrophages produce inflammatory cytokines upon incubation with DNA/cationic liposome complexes irrespective of the presence of the CpG motif.[17] Moreover, we found that this response was induced via a TLR9-independent pathway, because primary cultured cells from TLR9 knockout mice also released cytokines upon incubation with such complexes.[18,19]

In spite of extensive studies on the cellular activation induced by CpG DNA including pDNA, the relationship between *in vitro* and *in vivo* studies requires further investigation because specific types of immune cells including cell lines were generally used in these *in vitro* studies. In order to understand the pDNA-mediated cellular activation *in vivo*, it is necessary to evaluate the capability of various types of macrophages and macrophage-like cells to induce inflammatory cytokines because pDNA is distributed to some organs after i.v. administration. In the present study, we examined whether primary cultured cells from mouse organs, including peritoneal macrophages, are activated by naked DNA or DNA/cationic liposome complexes in order to identify the roles of these cells in the immune response at the whole-body level.

MATERIALS AND METHODS

Chemicals

N-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol and Triton X-114 was purchased from Nacalai Tesque (Kyoto, Japan). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS), a cocktail of protease inhibitors, calf thymus (CT) DNA and type I-A collagenase were purchased from Sigma (St. Louis, MO). ITS™ was purchased from BD Bioscience (San Diego, CA). Monoclonal mouse anti-mouse TLR9 antibody was purchased from InvivoGen (San Diego, CA) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was purchased from Amersham Biosciences Inc. (Piscataway, NJ).

Plasmid DNA

Plasmid vector pCMV-Luc, which encodes firefly *luciferase* gene, was constructed based on pcDNA3 as described previously.[20] pCMV-Luc has 33 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT, one of the most potent CpG motifs for mice.[21] pCMV-Luc was amplified in the *E. coli* strain DH5α and then isolated and purified using a Qiagen Endofree™ Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany).

Purification of DNA

To minimize the activation by contaminated LPS, DNA samples were extensively purified with Triton X-114, a nonionic detergent. Extraction of LPS from pDNA and CT DNA samples was performed according to previously published methods.[14,22,23] The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free™ plasmid Giga kit, the level of LPS was found to be 0.01–0.05 EU/μg pDNA, which was reduced below the detection limit of 0.001 EU/μg pDNA by the Triton X-114 extraction.

Preparation of Cationic Liposomes and DNA/Cationic Liposome Complexes

In the present study, DOTMA/cholesterol liposomes were used to prepare the DNA complexes because these complexes are widely used in *in vivo* studies due to their high *in vivo* transfection efficacy.[24,25] Cationic liposomes consisting of DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin

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film in a round-bottomed flask using a rotary evaporator, and then hydrating in 5% (w/v) dextrose by gentle mixing. After hydration, the dispersions were sonicated for 3 min and passed through a Minisart® 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako (Wako Pure Chemical, Osaka, Japan). Cationic liposomes and DNA in 5% dextrose were mixed at a charge ratio of +2.24 and left for 30 min at 37°C to form DNA lipoplex.

Isolation of Primary Cultured Cells

Male ICR (5–6-week-old) mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan).

Resident Peritoneal Macrophages

Peritoneal macrophages were collected aseptically from the peritoneal cavity of unstimulated mice with cold phosphate-buffered saline (PBS). Cells were washed, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (1.2 μg/mL), and then plated on 24-well culture plates (Falcon®, Becton-Dickinson, Franklin Lakes, NJ) at a density of 5×10^5 cells/well for the activation experiments. After a 2 h incubation at 37°C in 5% CO_2–95% air, adherent macrophages were washed twice with RPMI-1640 medium to remove nonadherent cells and then cultured for 24 h.

Splenic Macrophages

Spleens were removed aseptically from ICR mice, divided up with a spatula and filtered through a cell-strainer (mesh size 100 μm, Falcon®, Becton-Dickinson, Franklin Lakes, NJ) to obtain single-cell suspensions. Red blood cells were lysed by exposure to a hypo-osmotic solution of ammonium chloride 0.1% for 5 min at 4°C. After washing three times, splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), 10^{-5} M 2-mercaptoethanol, and plated in 10 cm culture dishes. After a 3 h incubation at 37°C in 5% CO_2–95% air, adherent macrophages were washed twice with RPMI-1640 medium to remove nonadherent cells and plated again on 24-well culture plates at a density of 5×10^6 cells/well for the activation experiments.

Hepatic Nonparenchymal Cells

Isolation of hepatic NPCs was performed as previously described[26] with slight modifications. Briefly, mouse liver was perfused under anesthesia via the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) at 37°C for 5 min at a flow rate of 4–5 mL/min. Then, the liver was perfused with HBSS containing 5 mM Ca^{2+} and 0.05% (w/v) collagenase for 7 min. The digested liver was minced and filtered through a cotton gauze and cell-strainer (mesh size 100 μm) and then fractioned into hepatocytes and hepatic NPCs by differential centrifugation. Hepatic NPCs were suspended in RPMI-1640 medium supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (2.5 μg/mL), and then plated on 24-well culture plates at a density of 5×10^5 cells/well. After a 3 h incubation at 37°C in 5% CO_2–95% air, adherent hepatic NPCs were washed twice with RPMI-1640 medium to remove nonadherent cells and then cultured for 24 h for the activation experiments.

Mesangial Cells

Isolation of glomerular cores was performed by the method of Mori et al.[27] with a slight modification. Briefly, the kidneys of ICR mice were removed aseptically and cut into slices. Each slice was pressed through a 425 μm pore-size metal sieve. The resulting material was rinsed three times in HBSS and pellets were centrifuged at 1700*g* for 3 min. The pellets were then incubated for 27 min at 37°C with 750 units/mL collagenase in 10 mL HBSS, followed by washing three times with HBSS and passing through two sieves with pore-sizes of 100 and 40 μm. The glomerular cores remaining on the finest sieve were cultured at 37°C for 2 weeks in RPMI-1640 medium with 20% FBS, penicillin G (100 units/mL), streptomycin (100 μg/mL) and ITS in 75 cm^2 plastic tissue culture flasks in 5% CO_2–95% air. The mesangial cells obtained from the 6th to 10th passage were plated on 24-well culture plates at a density of 5×10^5 cells/well and cultured for 24 h for the activation experiments.

Cell Line

Murine macrophagelike cell lines, RAW264.7 cells, were cultured in RPMI-1640 supplemented with 10% FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL). They were plated on 24-well culture plates at a density of 2.5×10^5 cells/well and cultured for 24 h.

mRNA Quantification

Total RNA was extracted from RAW264.7 cells and primary cultured cells using a MagExtractor MFX-2100 and MagExtractor RNA kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Before reverse transcription, the total RNA was treated with DNase I (Takara Bio). Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer according to the manufacturer's protocol. For quantitative mRNA expression analysis, real time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basle, Switzerland). The oligodeoxynucleotide primers used for amplification were TLR9-sense: 5′-AGC TCA ACC TGT-3′, TLR9-antisense: 5′-ATG CCG TTC ATG TTC AGC TCC TGC-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-sense: 5′-CTG CCA AGT ATG ATG ACA TCA AG AA-3′, GAPDH-antisense: 5′-ACC AGG AAA TGA GCT TGA CA-3′. The size of the TLR9 and GAPDH products was 313 and 186 bp, respectively. Amplification products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics, Basle, Switzerland). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, 58°C for 5 s, and 72°C for 10 s for TLR9 and GAPDH. All cycling reactions were performed in the presence of 3.5 mM $MgCl_2$. Gene specific fluorescence was detected at 72°C.

Western Blotting

Western blot analysis of TLR9 was performed by a standard method. Briefly, cells were lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 0.2% deoxycholic acid, 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride) containing a cocktail of protease inhibitors. Fifty micrograms of protein were dissolved in a loading buffer, denatured at 95°C for 3 min, and loaded on 4.75 or 9% SDS–polyacrylamide gels, respectively. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride microporous membrane (Immobilon-P; Millipore Corp., Bedford, MA) by semidry-blotting with Transblot SD (Bio-Rad, Hercules, CA). Membrane was blocked with 5% skimmed milk in Tris buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature, washed, and incubated with primary anti-mouse TLR9 monoclonal antibody (1/1000 dilution) overnight at 4°C. Then, the membrane was washed with TBS-T and incubated with the HRP-conjugated secondary antibody (1/1000 dilution) for 1 h at 37°C. Detection of antigen was performed using chemiluminescent HRP substrate (Immobilon Western; Millipore Corp., Bedford, MA).

Cytokine Secretion

Cells plated on 24-well culture plates were washed twice with 0.5 mL RPMI-1640 before use. Cells were incubated with naked DNA or LPS for 8 hr, and the supernatants were colleted for ELISA and kept at −80°C. Separately, cells were incubated with DNA/DOTMA/cholesterol complex for 2 h. Then, cells were washed with RPMI-1640 and incubated with each growth medium for an additional 6 h, and the supernatants were collected for ELISA and kept at −80°C. The level of TNF-α in the supernatant was determined by the OptEIA™ set (BD Biosciences, San Diego, CA).

RESULTS

All Primary Cultured Cells, Except for Mesangial Cells, Express TLR9

Figure 1A shows the level of TLR9 mRNA expression in primary cultured cells and RAW264.7 cells. TLR9 mRNA was detected in peritoneal macrophages, splenic macrophages, hepatic NPCs and RAW264.7 cells, but not in mesangial cells. Splenic macrophages expressed at least thirty times more TLR9 mRNA than the other cells. The amount of TLR9 protein correlated well with the level of the mRNA (Fig. 1B). Splenic macrophages showed the highest level of TLR9 protein, followed by peritoneal macrophages and hepatic NPCs. No TLR9 protein was detected in RAW264.7 cells.

Splenic Macrophages Secrete TNF-α Upon Stimulation with DNA in Both Naked and Complexed Form in a CpG Motif-Dependent Manner

When highly purified naked plasmid DNA (<0.001 EU LPS/μg pDNA), CpG-replete DNA, was added to splenic macrophages, TNF-α was secreted from splenic macrophages in a

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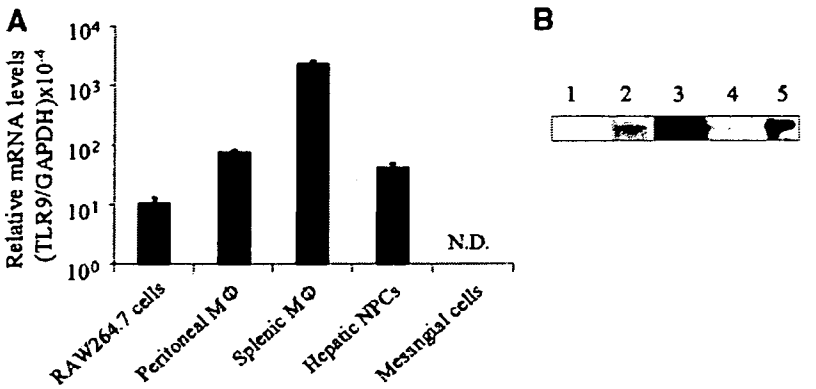

Figure 1. Expression of TLR9 in primary cultured cells and RAW264.7 cells. (A) Expression of TLR9 mRNA was examined by real-time polymerase chain reaction (real-time PCR) using extracted total RNA. Each result represents the mean and standard deviation of three measurements. (B) TLR9 protein expression was evaluated by Western blotting on cell lysate as described in Materials and methods. Lane 1, RAW264.7 cells; lane 2, peritoneal macrophages; lane 3, splenic macrophages; lane 4, hepatic NPCs; lane 5, size marker representing 120 kDa.

dose-dependent manner. A very low level of cytokines was detected by CT DNA, a mammalian DNA containing many fewer CpG motifs (Fig. 2A). TNF-α was also secreted from splenic macrophages when DNA complexed with DOTMA/cholesterol liposome was added. However, with DOTMA/cholesterol liposomes, only pDNA induced TNF-α production and CT DNA did not (Fig. 2B). LPS, a TLR4 ligand, induced significant TNF-α in the splenic macrophages in primary culture. These results suggest that splenic macrophages are able to produce TNF-α by addition of pDNA which contains many CpG motifs.

Hepatic NPCs Secrete TNF-α Upon Stimulation Only with Naked pDNA

Next, we used hepatic NPCs in which Kupffer cells and sinusoidal endothelial cells are the major components. Similar to the results using splenic

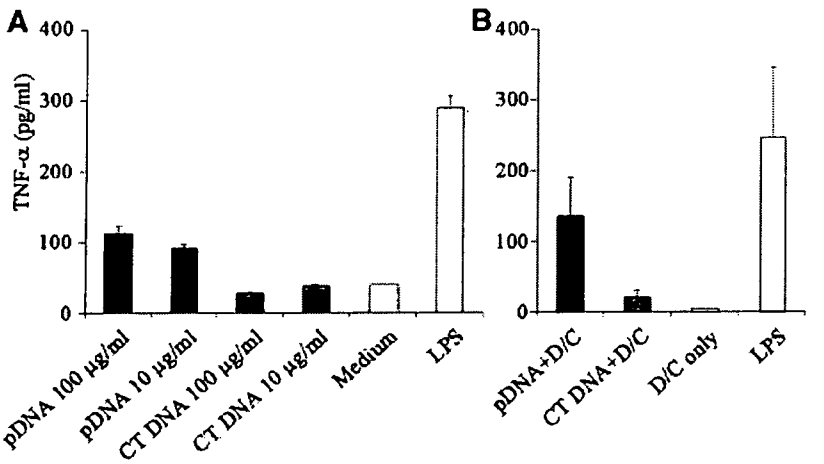

Figure 2. TNF-α secretion by DNA from splenic macrophages. Cells were incubated with naked DNA (100 or 10 μg/mL) (A) or DNA/cationic liposome complex (10:70.18 μg/mL) (B). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 1000 ng/mL. The concentration of TNF-α secreted from splenic macrophages was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

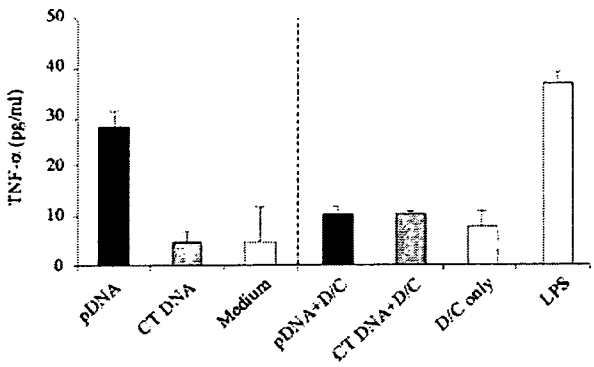

Figure 3. TNF-α secretion by DNA from hepatic NPCs. Cells were incubated with naked DNA (100 μg/mL) or DNA/cationic liposome complex (10:70.18 μg/mL). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 1000 ng/mL. The concentration of TNF-α secreted from NPCs was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

macrophages, hepatic NPCs secreted TNF-α when incubated with naked pDNA, but not with CT DNA (Fig. 3). Addition of the DNA/cationic liposome complexes produced very little TNF-α release from hepatic NPCs irrespective of the type of DNA used. TNF-α production was observed following LPS stimulation, but the level was very low.

Peritoneal Macrophages and Mesangial Cells Do Not Secret TNF-α Following Addition of DNA

Peritoneal macrophages did not secrete any TNF-α following addition of pDNA (Fig. 4A), which was in a good agreement with our previous findings. Moreover, the pDNA/cationic liposome complexes also did not induce TNF-α production in the peritoneal macrophages. The responsiveness of the macrophages to LPS was confirmed by TNF-α secretion. Mesangial cells, which expressed no detectable TLR9 mRNA, did not induce TNF-α following DNA addition to the cells in naked or complex form (Fig. 4B). LPS stimulation also failed to induce TNF-α secretion in the cells.

RAW264.7 Cells Secrete Large Amounts of TNF-α Following Addition of Naked DNA and DNA/Cationic Liposome Complexes

Finally, we investigated the cellular response of a murine macrophagelike cell line, RAW264.7, to DNA. In spite of the low expression of TLR9 (Fig. 1), a large amount of TNF-α was produced by naked DNA in a CpG motif-dependent manner from RAW264.7 cells (Fig. 5A). Naked CT DNA hardly induced any TNF-α. On the other hand, TNF-α was induced in a CpG motif-independent manner when DNA/DOTMA/cholesterol liposome complexes were added to the cells (Fig. 5B).

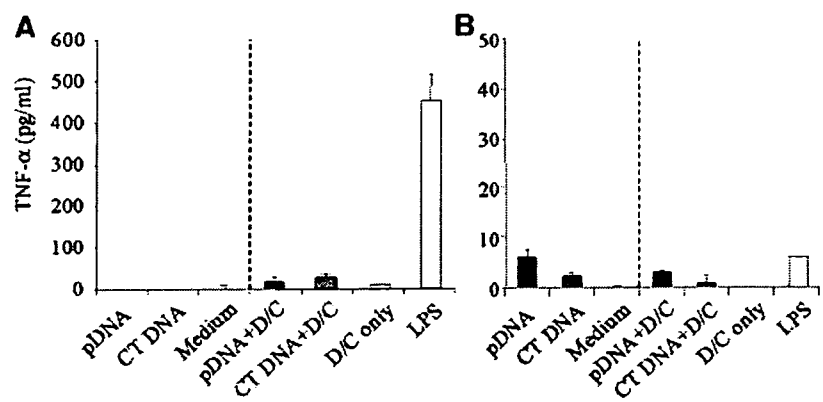

Figure 4. TNF-α secretion by DNA from peritoneal macrophages (A), and mesangial cells (B). Cells were incubated with naked DNA (100 μg/mL) or DNA/cationic liposome complex (10:70.18 μg/mL). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 100 ng/mL. The concentration of TNF-α secreted from the cells was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

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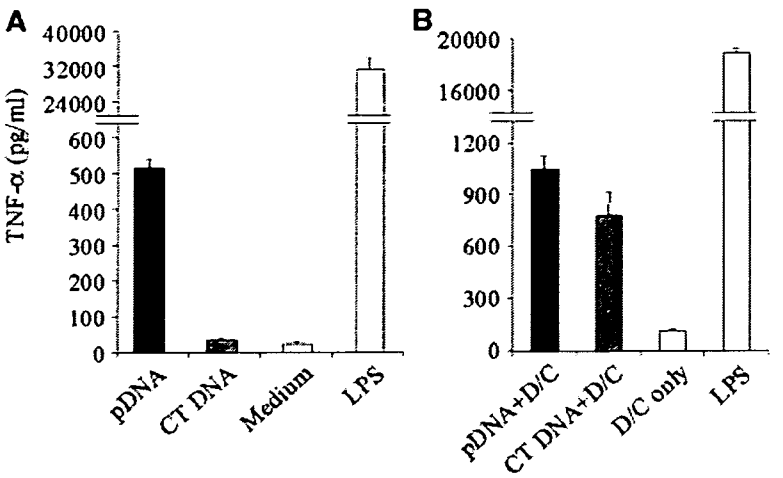

Figure 5. TNF-α secretion by DNA from RAW264.7 cells. Cells were incubated with naked DNA (5 μg/mL) (A) or DNA/cationic liposome complex (5:35.09 μg/mL) (B). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 1 ng/mL. The concentration of TNF-α secreted from RAW264.7 cells was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

DISCUSSION

Many *in vivo* studies using mice have shown that naked pDNA and pDNA/cationic liposome complexes stimulate significant cytokine production.[9,28–30] Although there is a growing body of information about macrophage activation by CpG DNA, most studies have been performed using murine macrophage cell lines, such as RAW264.7. Few studies have been reported using primary cultured macrophages freshly isolated from animals, which would be a better model than immortalized cell lines.

In the present study, we used primary cultured cells, including hepatic NPCs, splenic macrophages, mesangial cells and peritoneal macrophages. Liver and spleen play a central role in the removal of foreign particles from the circulation mainly via Kupffer cells and splenic macrophages.[31,32] In the kidney, mesangial cells, which play a central role in the physiology and pathophysiology of the glomerulus, are also reported to exhibit phagocytic or endocytic activity.[33] CpG DNA requires endocytosis for specific recognition by intracellular TLR9 for immunoactivation.[4,14,34,35] Therefore, we first quantitatively evaluated the TLR9 expression in the mRNA and protein levels (Fig. 1). We confirmed that primary cultured cells, peritoneal macrophages, splenic macrophages and hepatic NPCs, expressed TLR9. No TLR9 mRNA was detected in mesangial cells, as previously reported.[36,37] It has been reported that the expression level of TLR was altered not only when macrophages were exposed to inflammatory cytokines but also when cells were isolated from inflammatory disease patients,[38,39] suggesting that the TLR9 expression is regulated by various factors, including cytokines and pathogens. Therefore, the level of TLR9 expression in various types of macrophages might reflect the immunological milieu of tissues where the cells are isolated.

Next, we examined the cellular activation of primary cultured cells by the ligand of TLR9, naked CpG DNA at initial concentrations of 10 and/or 100 μg/mL. Nichols et al.[40] reported that pDNA remains intact for several hours after intramuscular injection in mice. In addition, naked pDNA can induce inflammatory cytokines after intravenous injection at a high dose of 250 μg/mouse.[41] Under these conditions, the initial plasma concentration of pDNA can be as high as 200 μg/mL so that the concentration could be over 10 μg/mL, the concentration used in the present study, for some time. Selective uptake of DNA by macrophages would take place after its systemic administration, which would also increase the local concentration of DNA around the cells. The dose of 250 μg pDNA seems to be high, but pDNA up to a few hundred micrograms

have been used for gene transfer to mice. In addition, the diffusion of pDNA within the tissues injected is severely limited because of its macromolecular nature, and the local concentration can be higher at the injection sites.

Binding of TLRs to their ligands leads to cellular activation and inflammatory cytokine production, and this responsiveness corresponded to the level of TLRs expression.[42] Therefore, it was expected that TNF-α would be produced in parallel with the level of TLR9 expressed in the cells. Predictably, splenic macrophages and hepatic NPCs secreted TNF-α by naked DNA in a CpG motif-dependent manner (Figs. 2 and 3). When splenic macrophages and hepatic Kupffer cells were functionally depleted by preloading clodronate liposomes into mice, TNF-α levels in spleen and liver were significantly reduced after intravenous injection of naked DNA.[41] The results in the present study clearly indicate the involvement of splenic macrophages and hepatic NPCs in the inflammatory responses to pDNA *in vivo*. In addition to Kupffer cells, liver sinusoidal endothelial cells (LSECs), which are another major component of hepatic NPCs, have been reported to express TLR9 mRNA and produce IL-1β and IL-6 following the addition of CpG-oligonucleotides.[43] Not only Kupffer cells but also LSECs play an important role in the immune response to CpG DNA because both types of cells are major contributors to the hepatic uptake of pDNA after intravenous injection.[26]

Some differences have been noticed in the CpG DNA-mediated cellular activation among the several types of macrophages cell lines.[44] Of various types of cell lines, we selected RAW264.7 cells as a reference type of macrophagelike cells in the present study, because RAW264.7 cells (i) release cytokines upon addition of CpG DNA and (ii) have been extensively used in previous studies. To our surprise, RAW264.7 cells expressed a lower level of TLR9 than the primary macrophages even although they were activated to produce a large amount of TNF-α upon addition of plasmid DNA. A high responsiveness of RAW264.7 cells was also noticed when added with LPS, the TLR4 ligand, compared with primary macrophages. These findings suggest that the cytokine production of RAW264.7 cells is efficient compared with that of primary macrophages. Differences in the expression of molecules involved in the cytokine production except for the TLRs may explain such differences in TNF-α production observed between RAW264.7 cells and primary macrophages. These results also indicate the importance of the use of primary macrophages, not macrophage cell lines, for estimating the events occurring in the body after administration of DNAs, such as pDNA.

A positive relation was clearly observed between the level of TLR9 expression and the level of TNF-α production when primary cultured macrophages were used. Despite of the expression of TLR9, resident peritoneal macrophages did not produce any TNF-α. Previous studies reported that thioglycollate-elicited, activated macrophages effectively induce inflammatory cytokines by CpG-ODN.[3,42] We also found that resident peritoneal macrophages release TNF-α by addition of CpG-ODN 1668, a highly potent stimulator of murine TLR9.[14] These results suggest that nonactivated peritoneal macrophages have low responsiveness to naked pDNA compared with splenic macrophages and hepatic NPCs. In addition, it was also confirmed that the immunostimulatory activity of pDNA is very low compared with that of phosphorothioate-type CpG-ODN. Again, other factors than the TLR9 involved in the TLR9 pathway would also be responsible to the level of cytokine production in TLR9-positive cells.

In the case of cationic liposomes, only splenic macrophages were activated by pDNA/DOTMA/cholesterol liposome complexes in a CpG motif-dependent manner and other primary cultured cells were not activated at all. We and other groups have reported that DNA/cationic liposome complexes accumulate in the liver and spleen after intravenous administration and hepatic Kupffer cells and splenic macrophages are directly involved in the uptake of DNA/cationic liposome complexes and inflammatory cytokine production.[13,45–48] In this respect, the results obtained from the present *in vitro* study did not agree completely with those obtained from the *in vivo* studies. No significant TNF-α production was observed by stimulation with the DNA/cationic liposome complexes in the liver NPC in primary culture (Fig. 3). In the present study, the level of induced TNF-α in the NPC preparations was very low even after treatment with a high concentration of LPS (1 μg/mL). Previous studies have also reported a low responsiveness of mouse Kupffer cells in primary culture to LPS.[49,50] The preparations might have a very low responsiveness to the DNA complexes via TLR9.

In this study, pDNA/DOTMA/cholesterol liposome complexes did not induce TNF-α production in peritoneal macrophages in spite of having many

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CpG motifs. On the other hand, we have already reported that TNF-α is produced from resident peritoneal macrophages by another DNA/cationic liposome, Lipofectamine plus reagent (LAplus), complex. Moreover, this cytokine production is independent of the presence of not only CpG motifs but also TLR9. The discrepancy between the present and previous studies is due to the difference in the composition of cationic liposomes; the type of lipid contained in cationic liposomes alters the cellular trafficking of the DNA complexed with the liposome.[51,52] We showed that splenic macrophages are activated to produce TNF-α by pDNA/DOTMA/cholesterol liposome complexes (Fig. 2B), but not by pDNA/LAplus complexes (data not shown). On the other hand, RAW264.7 cells produced TNF-α by both DNA/cationic liposome complexes independent of CpG motifs (Fig. 5B, Ref.[17]). Other groups have demonstrated that double-stranded DNA complexed with cationic liposomes could induce type I interferon independent of CpG motifs in mouse embryonic fibroblasts or HEK293 cells which did not express TLR9.[53,54] Most recently, Takaoka et al. reported a cytoplasmic DNA sensor DAI, which recognizes double-stranded DNA and activates innate immune responses independent of TLR9.[55] However, it remains to be determined whether DAI is ubiquitously expressed in various types of tissue macrophages. The results in the present study indicate that the immune responses induced by DNA/cationic liposome complexes are dependent on the cell types and/or lipid composition of the cationic liposomes. Further studies are required to clarify TLR9-independent immune responses to DNA complexes.

In conclusion, we have demonstrated that primary cultured splenic macrophages and hepatic NPCs are able to produce inflammatory cytokines by naked DNA in a CpG motif-dependent manner *in vitro*. Moreover, TNF-α production induced by DNA/cationic liposome complexes is partially independent of the CpG motifs and there are differences in inflammatory response between various primary cultured cells. These findings provide valuable information to increase our understanding of macrophage activation by pDNA *in vivo*.

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ORIGINAL ARTICLE

Inhibition of tumor cell growth in the liver by RNA interference-mediated suppression of HIF-1α expression in tumor cells and hepatocytes

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Hypoxia-inducible factor-1 (HIF-1) is a ubiquitously expressed oxygen-regulated transcription factor composed of α and β subunits. HIF-1 activates transcription of various genes including those involved in metastatic tumor growth. In the present study, HIF-1α expression in tumor-bearing mouse liver was examined after inoculation of tumor cells into portal vein. We found that tumor-bearing liver showed greatly increased HIF-1α expression. Plasmid DNA (pDNA) expressing short hairpin RNA targeting HIF-1α (pshHIF-1α) was effective in suppressing protein expression of HIF-1α in vitro. Intravenous injection of pshHIF-1α by hydrodynamics-based procedure reduced the HIF-1α protein expression in both normal and tumor cells and tumor cell number in the liver. Pre-injection of pshHIF-1α to mice, by which pDNA was delivered only to liver cells, not to tumor cells, was also effective in reducing the number of tumor cells inoculated 3 days after pDNA injection. These findings indicate that HIF-1α expression is increased in normal liver cells as well as tumor cells, and HIF-1α expression plays an important role in tumor progression. Use of the RNA interference (RNAi) of HIF-1 is an effective strategy for inhibiting tumor cell growth, and both tumor and normal cells can be the target for RNAi-based anticancer treatment.
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Keywords: *RNAi; HIF-1α; gene delivery; hydrodynamics-based procedure; hepatic metastasis*

Introduction

Metastasis, which is the transfer of cancer cells from one organ to other organs, is the most distinctive feature of malignant tumors and is the cause of approximately 90% of human cancer deaths.[1,2] Tumor metastasis is an exceedingly complex process, which occurs through a series of sequential steps that include dissociation from the primary tumor, invasion of adjacent tissues, intravasation, transport through the circulatory system, arrest in small vessels, adhesion to endothelial cells, extravasation and growth in secondary organs.[3] It can be hypothesized that components of the secondary organ, such as endothelial cells, stromal cells, fibroblasts and parenchymal cells, are functionally organized to promote survival and proliferation of metastasizing cancer cells and generate a favorable microenvironment for cancer cells in metastatic sites.[4,5]

Hypoxia initiates a variety of cellular responses including the activation of hypoxia-inducible factor-1 (HIF-1).[6,7] HIF-1 is a ubiquitously expressed heterodimeric transcription factor composed of a constitutively expressed β subunit and an oxygen-regulated α subunit. Under normal oxygen tension, the α subunit is continuously hydroxylated at conserved prolyl and asparaginyl residues and is targeted for degradation by the von Hippel–Lindau ubiquitin E3 ligase complex.[8] In hypoxia, inhibition of hydroxylation results in the stabilization of HIF-1α and its subsequent nuclear entry, which leads to transcriptional activation of target genes that stimulates angiogenesis, such as vascular endothelial growth factor (VEGF), that controls invasion of cancer cells, such as matrix metalloproteinases (MMPs), and promotes metabolic adaptation to hypoxia.[9] In general, tumor cells grow faster than the rate of angiogenesis so that tumor tissues are characterized by internal hypoxia. Therefore, activation of HIF-1 has been described in a variety of human cancers and their metastases.[10,11] Moreover, although the role of HIF-1α in tumor cell growth has not been fully elucidated, our results and those from other groups have demonstrated that HIF-1α expression in tumor tissues is likely to help tumor cell survival and growth.[12,13]

RNA interference (RNAi) is an evolutionary conserved sequence-specific gene silencing mechanism, which can be triggered by small 21- to 25-nt double-stranded small interfering RNA (siRNA) or short hairpin RNA (shRNA) that is processed in the cell to form siRNA.[14,15] Intravascular injection of a large-volume isotonic solution at a high speed is a very efficient method for delivering any solutes, including siRNA- and shRNA-expressing plasmid DNA (pDNA), to liver cells. This procedure, the so-called hydrodynamics-based procedure, has been applied to suppress expression of target genes in the liver.[16,17] In addition to such application, we found that the hydrodynamic

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