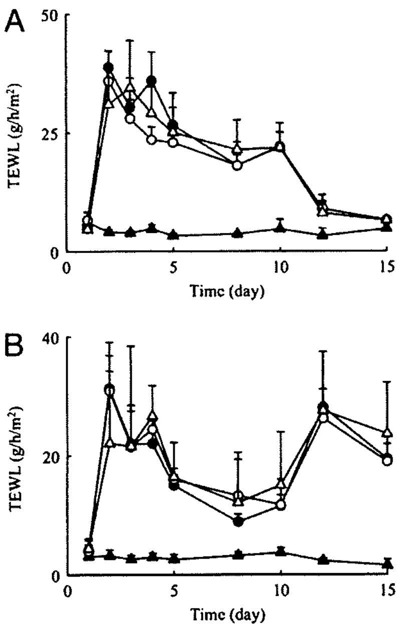

FIGURE 10. Time course of the TEWL of the back skin of mice in a stripped skin and a TNCB-induced dermatitis model. *A*, The back skin of C57BL/6 mice was shaved and tape stripped on days 1 and 8. ▲, untreated mice; △, tape-stripped mice; ○, tape-stripped, saline-treated mice; ●, tape-stripped, pCpG-Muγ–treated mice. *B*, The back skin of C57BL/6 mice was shaved, and TNCB was applied on days 1, 8, 11, and 13. ▲, untreated mice; △, TNCB-treated mice; ○, TNCB-treated, saline-treated mice; ●, TNCB-treated, pCpG-Muγ–treated mice. Results in *A* and *B* are expressed as the mean ± SD of four mice.

there would be few significant changes in the immune system of the patients receiving such treatments (7–10). This can primarily be attributed to the unfavorable pharmacokinetic properties of IFN-γ, which easily passes through the glomerulus of the kidney because it is smaller than the threshold of glomerular filtration, and it disappears from the systemic circulation very quickly (11).

In the current study, a sustained level of IFN-γ in the serum was successfully achieved by injecting pCpG-Muγ. An IFN-γ concentration >300 pg/ml, which is greater than the half maximal effective concentration of the protein needed to inhibit virus replication (100 pg/ml), was obtained throughout the experimental period of 84 d. This profile of IFN-γ in the serum was in marked contrast to that obtained by the injection of pCMV-Muγ, which resulted in a very transient IFN-γ concentration in the serum. Such a large difference in the duration of transgene expression is most likely due to the number of CpG motifs in plasmid DNA (21, 31). A detailed discussion on this sustained expression of IFN-γ from pCpG-Muγ was presented elsewhere (22).

Sustained expression of IFN-γ from pCpG-Muγ induced a variety of immunological changes in NC/Nga mice. In summary, the level of Th1 cytokines IFN-γ and IL-12 (Fig. 2*A*) was increased and that of IL-4, -5, -10, -13, and -17 and TARC tended to be decreased (Fig. 3); thus, the Th1/Th2 balance shifted toward Th1 dominance. In addition, the level of IgE, a hallmark of allergic reactions, was also significantly decreased. These changes can be considered to be due to the biological activity of IFN-γ, which promotes the production of IL-12, a typical Th1 cytokine important for differentiation to the Th1 subset. IL-12 is produced from macrophages or NK cells stimulated with IFN-γ. The reduced expression of the Th2 cytokines and TARC also resulted from the expression of IFN-γ, as reported using PBMCs, keratinocytes, or Langerhans cells (32–34).

A major finding of the current study is that the immunological changes induced by IFN-γ gene transfer differ, depending on the vector used (i.e., on the pharmacokinetics of IFN-γ). A variety of changes, including the increase in the IL-12 concentration and the decrease in the IgE level, were observed only in the mice receiving pCpG-Muγ. These results clearly indicate the importance of a sustained concentration of IFN-γ at a level high enough to allow it to modulate the Th1/Th2 balance. In addition, these results may explain the fact that most challenges using IFN-γ protein for the treatment of atopic dermatitis are not very effective, because IFN-γ protein quickly disappears from the systemic circulation (11).

The modulation of Th1/Th2 imbalance by sustained expression of IFN-γ was significantly effective in preventing the onset of symptoms of atopic dermatitis in NC/Nga mice. Rash, scratching, bleeding on the back or ear, acanthosis, and infiltration of lymphocytes, eosinophils, and mast cells were not very apparent in the pCpG-Muγ–treated mice. Inhibition of cellular infiltration into skin would be the consequence of the changes induced by the sustained concentration of IFN-γ; this directly inhibits the infiltration of eosinophils (35, 36), and it could inhibit Th2 cell migration into skin promoted by Th2 chemokines (37, 38). Because Th2 lymphocytes are the cells that release Th2 cytokines (39), our findings strongly suggest that IFN-γ gene transfer results in the inhibition of the differentiation of Th2 lymphocytes. Based on the results obtained, a mechanism of inhibition of atopic dermatitis by sustained expression of IFN-γ is proposed as follows. A sustained concentration of IFN-γ promotes the production of IL-12 from macrophages and NK cells, and these two Th1 cytokines inhibit the production of the Th2 cytokines/chemokines. These changes inhibit the recruitment of immune cells to the skin and prevent the onset of symptoms typical of atopic dermatitis. It is important to prove whether sustained IFN-γ gene transfer is also effective in individuals with dermatitis. A few NC/Nga mice that developed dermatitis were injected with pCpG-Muγ, and the clinical skin score and TEWL were measured. These parameters were reduced by IFN-γ gene transfer, although the reduction was less significant than in mice that did not develop dermatitis. Further studies are needed to conclude that IFN-γ gene transfer provides efficacy for patients with atopic dermatitis.

There would be concerns about sustained delivery of IFN-γ, because it is a highly potent Th1 cytokine. However, we observed no significant adverse effects of IFN-γ gene transfer on physiological parameters, such as body weight and body temperature. Also, contact dermatitis, a Th1-associated allergic dermatitis, was not aggravated by the injection of pCpG-Muγ. In addition, the improvement in TEWL observed in NC/Nga mice receiving pCpG-Muγ would not be due to the direct effects of IFN-γ gene transfer, because TEWL was minimally affected by IFN-γ gene transfer in other models (Fig. 10).

The hydrodynamic injection method used for gene transfer in the current study is one of the most efficient methods; using this method, >99% of transgenes were expressed in the liver, followed by kidneys, spleen, lung, and other internal organs (25, 40). Although there is concern about the toxicity related to the mode of gene delivery (25, 26, 41, 42), a recent report showed that this method of gene delivery can be applied to humans with few toxic effects when plasmid DNA is delivered to a lobe using a balloon catheter (43). Computer-assisted hydrodynamic gene delivery would also be a less invasive method (44). Other modes for gene delivery could be used to achieve sustained transgene expression of IFN-γ at a relatively high level, because the dose of pCpG-Muγ used was as low as 0.14 μg/mouse (i.e., ~7 μg/kg body weight). In general, greater amounts of plasmid DNA up to 100 μg/mouse (5 mg/kg) have been administered, and an increase in the dose may compensate for the low efficiency of other gene-delivery methods, such as intradermal injection of naked plasmid DNA.

In conclusion, it was proved that an extraordinarily sustained IFN-γ expression induces a variety of immunological changes, leading to a Th1-dominant state in the atopic dermatitis model. A short expression of IFN-γ was not effective in inducing such changes, indicating the importance of a sustained concentration of IFN-γ. These results provide a novel strategy for the treatment of atopic dermatitis, in which biologically active IFN-γ protein is supplied to patients from cells transduced with plasmid vector expressing the protein for a long period of time.

Disclosures

The authors have no financial conflicts of interest.

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Comparison of the Type of Liposome Involving Cytokine Production Induced by Non-CpG Lipoplex in Macrophages

Sachiyo Yasuda, Hiroyuki Yoshida, Makiya Nishikawa, and Yoshinobu Takakura*

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshidashimoadachi-cho, Sakyo-ku, Kyoto, 606-8501, Japan

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Abstract: To improve the transfection efficiency of plasmid DNA (pDNA) into cells, various types of cationic liposome have been used to prepare pDNA/cationic liposome complexes (lipoplexes). It is well-known that lipoplexes induce a large amount of proinflammatory cytokines because unmethylated CpG dinucleotides (CpG motifs) abundantly present in pDNA are recognized by Toll-like receptor-9 (TLR9) expressed in immune cells such as macrophages and dendritic cells. This nonspecific cytokine production is problematic in nonviral gene therapy. Moreover, recent studies have demonstrated that lipoplexes induce not only proinflammatory cytokines but also another type of cytokine, type I interferons (IFNs), irrespective of the frequency of CpG motifs in DNA and the expression of TLR9. To gain more insight into the CpG motif- and TLR9-independent induction of type I IFNs and proinflammatory cytokines by lipoplex, macrophage activation was evaluated *in vitro* using various cationic liposomes complexed with pDNA containing no CpG motifs. The production of IFN-β, TNF-α and IL-6 by lipoplex was confirmed to be induced independently of the interaction between CpG DNA and TLR9 in macrophages from TLR9-knockout mice. Then, the release of the cytokines, the mRNA expression of *Z-DNA binding protein-1* (*Zbp1*), a cytosolic double-stranded DNA sensor, and the cellular uptake of pDNA were examined in a macrophage-like cell line, RAW264.7. The level of cytokine production and the increase in the *Zbp1* mRNA varied depending on the type of cationic liposome used. A good correlation was observed between the cytokine level and the *Zbp1* mRNA. A confocal microscopic study using fluorescently labeled pDNA complexes showed that the complexes that released a lot of cytokines showed an enhanced distribution of pDNA-derived fluorescence into the cytosol. These results suggest that different intracellular trafficking derived from the type of liposomes determines the recognition of pDNA by ZBP1 after uptake of lipoplexes by the macrophages, followed by the release of type I IFNs and inflammatory cytokines. The present study demonstrates that cationic liposomes should be selected based on these findings for optimization of DNA-based therapies using lipoplexes.

Keywords: Cationic liposome; non-CpG plasmid DNA; transfection; inflammatory response; Z-DNA binding protein-1

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, which are abundant in bacterial DNA but not in vertebrate DNA, are recognized by the mammalian immune system as a danger signal and activate innate immunity.[2] Toll-like receptor-9 (TLR9) is a pattern recognition receptor recognizing CpG motifs,[3] and

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* Corresponding author. Mailing address: Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshidashimoadachi-cho, Sakyo-ku, Kyoto, 606-8501, Japan. Phone: +81-75-753-4615. Fax: +81-75-753-4614. E-mail: takakura@pharm.kyoto-u.ac.jp.

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is present in the intracellular compartments of immune cells, such as macrophages and dendritic cells responsible for the innate immune response.[4–7] These cells secrete a large amount of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-12, upon uptake of the CpG motif-containing DNA. This nonspecific induction of proinflammatory cytokines is problematic because transgene expression from vectors is reduced due to their cytotoxicity to cells expressing the transgene or promoter attenuation activity.[8–10] In order to overcome this problem, CpG-reduced pDNA and CpG-deleted pDNA have been constructed, which offer an improvement in safety and gene expression through reduced CpG motif-dependent inflammatory cytokines induced by pDNA.[11,12]

To improve the transfection efficacy of pDNA, various types of cationic liposomes are often used to obtain pDNA/cationic liposome complexes (lipoplexes) and the resultant complexes have been used both *in vivo* and *in vitro*. However, we and others have demonstrated that DNA complexed with cationic liposomes induces innate immune responses via both TLR9-dependent and -independent pathways using primary cultured cells from $Tlr9^{-/-}$ mice.[13–15] Moreover, it has also been reported that, when a large quantity of DNA is introduced into the cells using cationic liposomes, double-stranded DNA (dsDNA) derived from either pathogens or the host, but not single-stranded DNA, activates both immune and nonimmune cells, such as thyroid cells and mouse embryonic fibroblasts (MEFs).[14,16] It has been reported that type I interferons (IFNs), most notably IFN-β, play a key role in the immunostimulatory cascade triggered by dsDNA.[17,18] Takaoka et al. have shown that Z-DNA binding protein-1 (ZBP1) is a candidate for a cytosolic dsDNA receptor leading to the induction of type I IFN signals and other immune responses, and renamed as a DNA-dependent activator of IFN-regulatory factors (DAI).[19] In addition, the authors of this previous study have demonstrated that a synthetic linear dsDNA containing no CpG motifs activates RAW264.7, murine macrophage-like cell lines, and L929 followed by not only the upregulation of *Ifn-β* and *Il-6* mRNA but also the production of their proteins via interaction with ZBP1.[19]

In addition to proinflammatory cytokines, type I IFNs also markedly affect various aspects of pDNA-based approaches. It has been reported that type I IFNs potently suppress gene expression from plasmid vector delivered in the form of lipoplex.[20] On the other hand, DNA-induced type I IFNs have been shown to be essential for the effect of DNA vaccination.[21] Therefore, it is extremely important to understand the mechanism of the pDNA-mediated production of both inflammatory

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cytokines and type I IFNs in pDNA-based therapies, such as gene therapy and DNA vaccination. In a series of basic *in vitro* studies on cytokine production induced by dsDNA, DNA has been complexed with various cationic reagents, such as Lipofectamine2000 (LA2000),[15,19,21–24] FuGENE 6,[17,18,25] 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP)[26] and LAplus.[13,27] However, only a single cationic reagent was used for efficient DNA delivery to the cells of interest in each study. To our knowledge, there is no report directly comparing the characteristics of immune responses to different types of lipoplexes.

In the present study, in order to gain more insight into the production of type I IFNs and inflammatory cytokines induced by lipoplex, we examined the production of IFN-β, TNF-α and IL-6 in macrophages upon stimulation with lipoplexes composed of various cationic liposomes focusing on ZBP1. From therapeutic point of view, it would be very important to reveal the molecular mechanisms underlying the induction of these cytokines by lipoplex, a representative pDNA delivery system, because they could affect the outcome of DNA-based therapies, gene therapy and DNA vaccination. We used pCpG-ΔLuc, a pDNA containing no CpG motifs, for lipoplex preparation to clearly analyze the cytokine production independent of the interaction of CpG motifs and TLR9. Here, we show that the non-CpG pDNA/cationic liposome complex (non-CpG lipoplex) activates macrophages in a TLR9-independent manner and that ZBP1 is significantly involved in the cytokine production. We also demonstrate the importance of the type of cationic liposome in the cytokine production induced by non-CpG lipoplexes, because it markedly influences ZBP1 expression and the amount of pDNA delivered to the cytosol.

Experimental Section

Chemicals. Lipofectamine 2000 (LA2000), Lipofectamine (LA), and Plus reagent were purchased from Invitrogen (Carlsbad, CA). According to the manufacturer's information, LA is composed of 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/dioleoylphosphatidylethanolamine (DOPE), and LAplus was prepared by adding the Plus reagent to LA. LA2000 contained various components although no details were given. *N*-[1-(2,3-Dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was obtained from Tokyo Kasei (Tokyo, Japan). DOPE was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol and Triton X-114 were obtained from Nacalai Tesque (Kyoto, Japan). LPS, polymyxin B sulfate salt, and poly(dA) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFN-β was obtained from PBL InterferonSource (Piscataway, NJ).

Cell Cultures. Male C57BL/6 mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). *Tlr9*$^{-/-}$ mice with a C57BL/6 genetic background were used at 9−11 weeks of age. Peritoneal macrophages were collected and cultured on 24-well culture plates as previously reported.[28] The murine macrophage-like cell line, RAW264.7 cells, was cultured in RPMI-1640 supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (292 μg/mL). The cells were plated on 96- or 24-well culture plates at a density of 5×10^4 cells/well or 2.5×10^5 cells/well, respectively, and cultured for 24 h. Colon26/NF-κB-Luc, a clone of murine colon carcinoma colon26 cells stably expressing NF-κB responsive firefly *luciferase* reporter gene,[29] were cultured in DMEM supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (292 μg/mL). They were then plated on 96-well culture plates at a density of 2.5×10^4 cells/well and cultured for 24 h.

Plasmid DNA. pcDNA3.1 vector (Invitrogen) has 27 5′-Pur-Pur-CpG-Pyr-Pyr-3′ sequences including two GACGTT sequences reported to be the most potent CpG motifs for

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mice.[30] pCpG-ΔLuc vector was constructed by inserting the *BglII/NheI* firefly *luciferase* cDNA fragment from pORF-Luc::Sh-ΔCpG (InvivoGen, San Diego, CA), which contains no CpG, into the *BglII/NheI* site of the pCpG-mcs vector (InvivoGen). pNF-κB-Luc containing five tandem repeats of an NF-κB-binding site was purchased from Stratagene (La Jolla, CA). pCMV-Luc was amplified in the *Escherichia coli* strain DH5α and pCpG-ΔLuc was amplified in the *E. coli* strain GT115 and then isolated and purified using a JETSTAR 2.0 Plasmid GIGA Purification Kit (Genomed GmbH, Bad Oyenhausen, Germany). Purified pCpG-ΔLuc was sequenced by the Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan), and it was confirmed that the vector had no CpG dinucleotides.

Purification of DNA. After purification using the JETSTAR 2.0 Plasmid GIGA Purification Kit, DNA samples were extensively purified with Triton X-114 to minimize cellular activation by contaminated LPS as previously reported.[28,31,32] The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako, Tokyo, Japan) and was found to be reduced below the detection limit of 0.001 EU/μg pDNA by the Triton X-114 extraction.

Preparation of Cationic Liposomes and Lipoplexes.[33] Cationic liposomes were prepared as previously reported. In brief, DOTMA and neutral helper lipid (DOPE or cholesterol) were mixed in chloroform at a molar ratio of 1:1, then the mixture was dried, vacuum-desiccated, and resuspended in 5% dextrose solution in sterile test tubes. After hydration for 30 min at room temperature, the dispersion was sonicated for 2.5 min in a tip sonicator to form liposomes, and then three cycles of freezing and thawing treatment were performed. Finally, the dispersion was sterilized by passing through a Ministart 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). DOTMA/DOPE liposomes or DOTMA/cholesterol liposomes and DNA were mixed at a charge ratio of +2.0 and left at room temperature for 30 min to form a lipoplex. The DNA/LA2000 complex and DNA/LAplus complex were prepared according to the manufacturer's instructions. LA2000, LA, and Plus reagent were added at a concentration of 2, 1, and 1.2 μL volume per 1 μg of DNA, respectively. The size and zeta-potential of lipoplexes were measured using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) (Table 1). Condensation of pDNA and positively charged lipoplex formation were confirmed.

Table 1. Physicochemical Properties of pDNA and Lipoplexes Used in This Study

	particle size (nm)	zeta-potential (mV)
naked pCpG-ΔLuc	1870 ± 476	
lipoplex		
LA2000	107.8 ± 26.5	35.1 ± 2.6
LAplus	78.7 ± 40.1	33.3 ± 3.6
DOTMA/DOPE	69.4 ± 15.8	32.7 ± 3.7
DOTMA/cholesterol	72.1 ± 3.9	29.6 ± 2.0

Cytokine Release from Macrophages. Cells were incubated with polyI:C, LPS, or pcDNA3.1 for 8 h, and the supernatants were collected for ELISA and kept at −80 °C. In the case of polyI:C, 5 μg/mL polymyxin B was added to avoid activation by contaminated LPS. Separately, cells were incubated with lipoplex 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 incubation time of lipoplex was slightly shorter than the standard transfection time described in the manufacturer's instruction of LA2000 and LAplus to avoid the cytotoxicity induced by the high concentration of lipoplexes. The level of TNF-α and IL-6 in the supernatants was determined by ELISA using the OptEIA set (BD Biosciences Pharmingen, San Diego, CA). The IFN-β level in the supernatants was measured by ELISA as described previously.[20] In the IFN-β pretreatment experiment, cells were incubated with IFN-β at the indicated concentration for 6 h, and the IFN-β in the medium was washed out before addition of the lipoplex. In our preliminary experiments, 1000 U/mL IFN-β was approximately equal to 20 ng/mL IFN-β. Preapplied IFN-β was negligible in the ELISA assay because no IFN-β was detected in medium-treated cells.

NF-κB Activation in Colon26/NF-κB-Luc Cells. Colon26/NF-κB-Luc cells were incubated with the lipoplex for 2 h. Then, cells were washed and incubated with growth medium for an additional 6 h. The activation of NF-κB was determined by measuring the luciferase activity of cell lysates in a luminometer (Lumat LB9507; EG & G Berthold, Bad Wildbad, Germany) as previously reported.[29]

Uptake of Lipoplex in RAW264.7 Cells. pDNA was labeled using a LabelIT Cy3 Labeling Kit (Mirus Bio, Madison, MI) according to the manufacturer's instructions. Cy3-labeled pDNA was used for the preparation of the lipoplex. RAW264.7 cells were incubated with naked Cy3-labeled pDNA or Cy3-labeled lipoplex for 30 min or 2 h at 4 or 37 °C and washed three times with phosphate-buffered saline. Then, the intensity of the cell fluorescence was analyzed by flow cytometry (FACScan; BD Biosciences, San

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Jose, CA) using CellQuest software (version 3.1; BD Biosciences).

mRNA Quantification. Total RNA was isolated using MagExtractor MFX-2100 and a 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, Shiga, Japan). Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer following the manufacturer's protocol. For quantitative mRNA expression analysis, real-time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The oligodeoxynucleotide primers used for amplification were as follows: *Zbp1*-sense 5′-GAC GAC AGC CAA AGA AGT GA-3′, *Zbp1*-antisense 5′-GAG CTA TGT CTT GGC CTT CC-3′; and *Gapdh*-sense 5′-CTG CCA AGT ATG ATG ACA TCA AGA A-3′, *Gapdh*-antisense 5′-ACC AGG AAA TGA GCT TGA CA-3′. Amplification products were detected online via intercalation of the fluorescent dye SYBR green (SYBR *Premix Ex Taq*; Takara Bio).

Confocal Microscopy. pCpG-ΔLuc was fluorescently labeled with YOYO-1 iodide (Molecular Probes, Eugene, OR) or using a LabelIT Fluorescein Labeling Kit (Mirus Bio) according to the manufacturer's instructions. Cells were incubated with fluorescently labeled (FL-pDNA) complexed with LA2000 or DOTMA/cholesterol. After a 15 min or 2 h incubation at 37 °C, cells were washed and incubated with the medium containing 10 μg/mL of transferrin-Alexa Fluor 594 (Molecular Probes) or 100 nM LysoTracker Red DND-99 (Molecular Probes), for 15 min. Cells were washed and fixed with 4% paraformaldehyde for 15 min at room temperature. The images of the cells were captured digitally using a laser scanning confocal microscope (LSM5 Pascal; Zeiss, Jena, Germany) and Pascal LSM software (Zeiss).

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

Results

TLR9- and CpG Motif-Independent Cytokine Production Induced by Lipoplexes in Peritoneal Macrophages from *Tlr9*$^{-/-}$ Mice. To confirm the TLR9- and CpG motif-independent cytokine production by pDNA, the production of IFN-β and IL-6 was examined in peritoneal macrophages from *Tlr9*$^{-/-}$ mice. A CpG replete pDNA, pcDNA3.1, or a non-CpG pDNA, pCpG-ΔLuc, was mixed with LA2000, one of the most widely used cationic liposomes for *in vitro* transfection, to obtain lipoplexes. Both pcDNA3.1/LA2000 and pCpG-ΔLuc/LA2000 complexes induced IFN-β (Figure 1A) and IL-6 (Figure 1B) production in peritoneal macrophages. The presence of CpG motifs in pDNA did not increase the production of these cytokines induced by the pDNA/LA2000 complex, probably because the cells did not express TLR9. Naked CpG ODN 1668, a single-stranded phosphorothioate DNA and a typical ligand for TLR9, was a weak inducer of cytokines in *Tlr9*$^{-/-}$ macrophages. Therefore, the cytokine production in *Tlr9*$^{-/-}$ macrophages can be considered to be mediated by mechanisms other than the interaction of CpG DNA and TLR9. These results clearly demonstrate that pDNA complexed with LA2000 induces the production of IFN-β and IL-6 in macrophages in a TLR9- and CpG motif-independent manner.

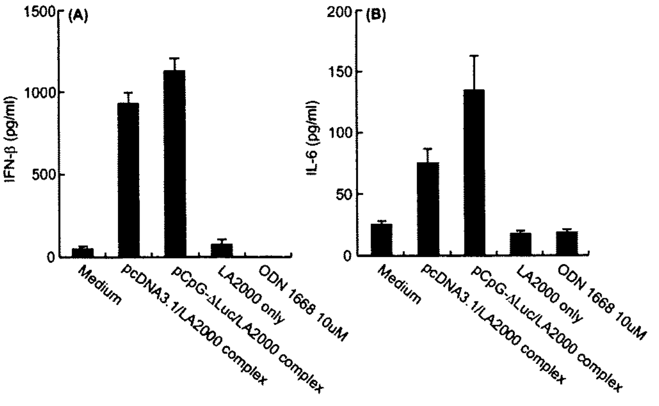

Figure 1. Cytokine production induced by pDNA/LA2000 complexes in peritoneal macrophages from *Tlr9*$^{-/-}$ mice. The pDNA/LA2000 complex (5:10 μg/mL) was added to the cells. After an 8 h incubation, supernatants were collected and the level of IFN-β (A) or IL-6 (B) was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

Cytokine Production Induced by Non-CpG Lipoplexes Composed of Various Cationic Liposomes in RAW264.7 Cells. In the following experiments, RAW264.7, a murine macrophage cell line, was selected, and the CpG motif-independent immune response was examined using non-CpG lipoplexes. Figure 2 shows the cytokine production in RAW264.7 cells upon addition of non-CpG lipoplexes composed of various cationic liposomes. The incubation with

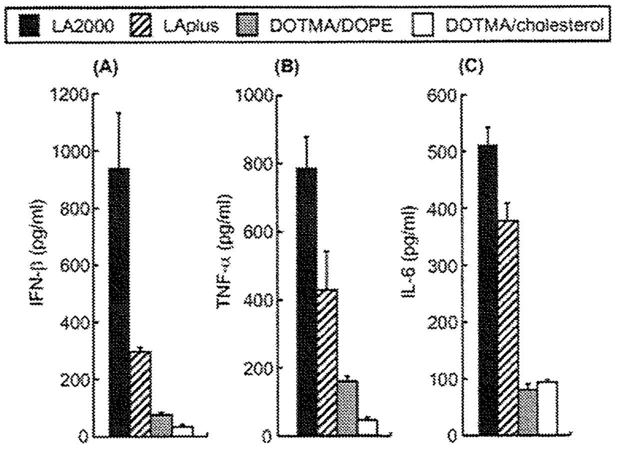

Figure 2. Cytokine production induced by non-CpG lipoplexes composed of various cationic liposomes in RAW264.7 cells. The cells were incubated with pCpG-ΔLuc complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5: 42.7 μg/mL) or DOTMA/cholesterol (white bars) (5: 31.9 μg/mL). After an 8 h incubation, the supernatants were collected, and the concentration of IFN-β (A), TNF-α (B) or IL-6 (C) was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

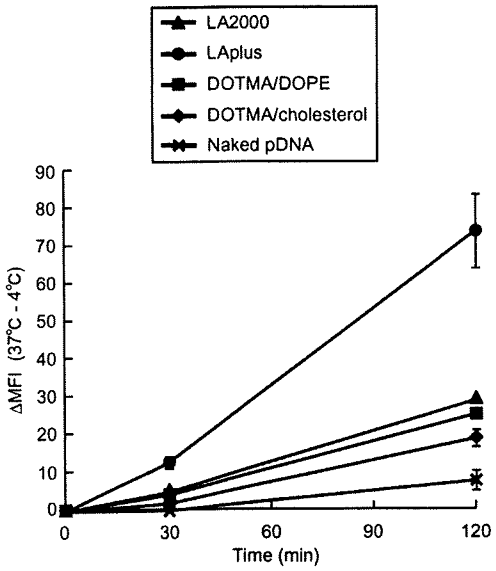

Figure 3. Uptake of Cy3-labeled pCpG-ΔLuc/cationic liposome complexes by RAW264.7 cells. The cells were incubated with naked Cy3-labeled pDNA (cross) or the Cy3-labeled pCpG-ΔLuc complexed with LA2000 (triangle) (5:10 μg/mL), LAplus (circle) (5:10 μg/mL), DOTMA/DOPE (square) (5: 42.7 μg/mL), DOTMA/cholesterol (diamond) (5: 31.9 μg/mL) for 30 min or 2 h at 4 °C or 37 °C and the amount of pDNA associated with the cells was measured by flow cytometry. The MFI is expressed as the mean ± SD ($n = 2$).

liposomes alone induced no cytokine production, suggesting that cationic liposomes are immunologically inert in the cells (data not shown). pCpG-ΔLuc/LA2000 complex induced the production of IFN-β (Figure 2A) and inflammatory cytokines, TNF-α (Figure 2B) and IL-6 (Figure 2C), in RAW264.7 cells, indicating that CpG motif-independent cytokine production can also be observed in the cells. To examine whether the type of cationic liposome affects this CpG motif-independent cytokine production from macrophages, lipoplexes were prepared using various cationic liposomes: LA2000, LAplus, DOTMA/DOPE, and DOTMA/cholesterol liposomes. All the non-CpG lipoplexes prepared induced the production of IFN-β (Figure 2A), TNF-α (Figure 2B) and IL-6 (Figure 2C). However, the level of cytokine production was markedly different among the four types of lipoplexes composed of different cationic liposomes. The highest level of cytokines was observed in the LA2000 complex-treated cells, followed by the LAplus complex and the DOTMA/DOPE complexes, and the DOTMA/cholesterol complex scarcely induced any production of cytokines in the cells. In a different set of experiments, we measured the *luciferase* gene expression in RAW264.7 cells following incubation with lipoplexes under the same conditions. The luciferase activities in the cells treated with LA2000 lipoplex and LAplus lipoplex were significantly higher than those of DOTMA/DOPE lipoplex and DOTMA/cholesterol lipoplex (data not shown). Therefore, there was a similar trend in the *luciferase* gene expression to the cytokine production by lipoplex.

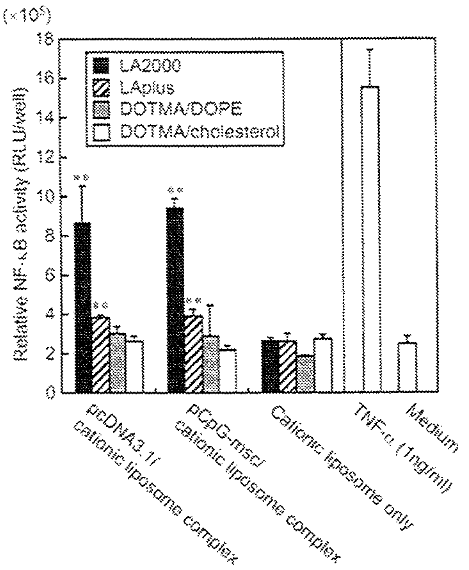

Figure 4. NF-κB activation induced by lipoplexes composed of various cationic liposomes in colon26/NF-κB-Luc cells. The cells were incubated with the pDNA complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5:42.7 μg/mL) or DOTMA/cholesterol (white bars) (5:31.9 μg/mL). After an 8 h incubation, the activity of NF-κB was determined by measuring the luciferase activity of cell lysates. Each result represents the mean ± SD ($n = 3$). Significantly different (**$P < 0.01$) from medium-treated cells.

Uptake of pCpG-ΔLuc Complexed with Various Cationic Liposomes by RAW264.7 Cells. To investigate whether the cytokine level induced by non-CpG lipoplexes is dependent on the amount of DNA taken up by cells, the uptake of Cy3-labeled pCpG-ΔLuc/cationic liposome complexes was examined in RAW264.7 cells by measuring the fluorescence intensity of the cells using a flow cytometer, and the difference in the mean fluorescence intensity (MFI) at 4 and 37 °C (ΔMFI) was plotted. Figure 3 shows the time courses of the ΔMFI of cells incubated with naked pDNA and various non-CpG lipoplexes. For all lipoplexes examined, the uptake of pDNA was higher than that of naked pDNA and increased with time up to 2 h of incubation with DNA samples. The cellular uptake of pDNA was the highest when LAplus complex was added to cells. The ΔMFI values were almost identical for the complex using LA2000, DOTMA/DOPE and DOTMA/cholesterol. These results indicate that the cytokine production by non-CpG lipoplex is not proportional to the apparent amount of DNA taken up by cells.

NF-κB Activation by Lipoplexes Composed of Various Cationic Liposomes in Colon26/NF-κB-Luc Cells. It has been reported that dsDNA-induced cytokine production is accompanied by the activation of NF-κB, which is a key molecule leading to the production of proinflammatory cytokines. The determination of NF-κB activity can be used as one of the valuable tools for the evaluation of the immune activation mechanism at a molecular level.[19] Therefore, we investigated whether NF-κB is activated by lipoplexes by

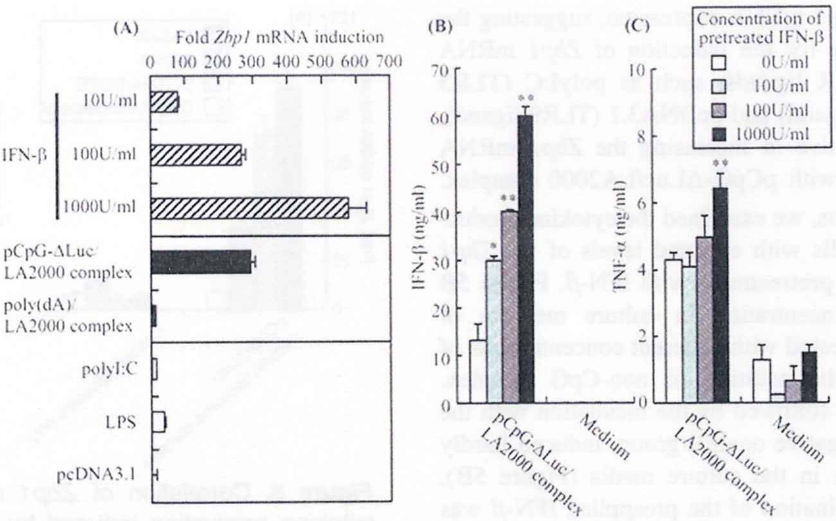

Figure 5. Involvement of *Zbp1* mRNA expression in type I IFN and inflammatory cytokine production induced by non-CpG lipoplex in RAW264.7 cells. (A) *Zbp1* mRNA expression was induced by IFN-β, DNA/LA2000 complexes, and TLR ligands in RAW264.7 cells. The cells were incubated with IFN-β (indicated concentration), DNA/LA2000 complexes (5:10 μg/mL), and TLR ligands, polyI:C (TLR3 ligand, 5 μg/mL), LPS (TLR4 ligand, 1 ng/mL), and pcDNA3.1 (TLR9 ligand, 10 μg/mL). *Zbp1* mRNA levels were measured 6 h later by real-time PCR. Each result represents the mean ± SD ($n = 3$) in *Zbp1/Gapdh* mRNA levels compared with untreated cells. (B, C) Cytokine production induced by the pCpG-ΔLuc/LA2000 complex in IFN-β-pretreated RAW264.7 cells. The cells were pretreated with IFN-β at indicated concentrations. After a 6 h incubation, IFN-β was washed out, and then the cells were incubated with the pCpG-ΔLuc/LA2000 complex (5:10 μg/mL). After an incubation of more than 8 h, supernatants were collected, and the concentration of IFN-β (B) or TNF-α (C) was determined by ELISA. Each result represents the mean ± SD ($n = 3$). Significantly different (*$P < 0.01$, **$P < 0.001$) from 0 U/mL of IFN-β pretreated cells.

using colon26/NF-κB-Luc cells. The cells did not express TLR9 but were stably transfected with pNF-κB-Luc encoding firefly *luciferase* cDNA driven by a basic element plus five tandem copies of NF-κB binding elements [(TGGGGACTTTCCGC)$_5$],[34] so NF-κB activation induced by lipoplexes can easily be evaluated by measuring the luciferase activity in the cells. To avoid the expression of firefly luciferase from pDNA added as lipoplexes, empty pDNAs, i.e., pcDNA3.1 (CpG-replete pDNA) and pCpG-mcs (non-CpG pDNA), were used. Figure 4 shows the luciferase activity of the lysates of colon26/NF-κB-Luc cells 8 h after addition of CpG or non-CpG lipoplex. Cationic liposome alone did not increase the luciferase activity. The addition of pCpG-mcs/LA2000 complexes as well as TNF-α, a well-known activator of NF-κB, greatly increased the luciferase activity of the cells, indicating that NF-κB was activated by pCpG-mcs/LA2000 complexes. pCpG-mcs/LAplus complex showed only weak activation, and no significant activation was observed for DOTMA-based complexes. On the other hand, lipoplexes containing pcDNA3.1 increased the luciferase activity of the cells as much as lipoplexes containing pCpG-mcs did, which can be explained by the fact that TLR9 is not expressed in the cells. Therefore, CpG lipoplexes and non-CpG lipoplexes equally activate NF-κB through mechanisms different from the TLR9 pathway, when pDNA is added to cells in the complexed form with the same cationic liposomes.

Involvement of *Zbp1* mRNA Expression in Cytokine Production Induced by Non-CpG Lipoplex in RAW264.7 Cells. As one of the DNA receptors other than TLR9, ZBP1 was recently identified to be a candidate molecule for a cytosolic dsDNA receptor contributing to cytokine production.[19,35] Therefore, we examined the relationship between the cytokine production and ZBP1 expression. The expression of ZBP1 is known to be modulated by cytosolic dsDNA and IFN-β treatment. At first, we confirmed the effect of these treatments on the *Zbp1* mRNA expression in RAW264.7 cells (Figure 5A). RAW264.7 cells were treated with different concentrations of IFN-β, DNA/LA2000 complex or TLR ligands. The mRNA expression of *Zbp1* in RAW264.7 cells was significantly increased by IFN-β treatment in a concentration-dependent manner (Figure 5A, hatched bar), which was in good agreement with the previous report using MEFs.[19] Furthermore, the *Zbp1* mRNA expression was also greatly increased by pCpG-ΔLuc/LA2000 complex (Figure 5A, black bar). On the other hand, poly(dA)/LA2000, a complex consisting of a single-stranded DNA, induced hardly

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any increase in the *Zbp1* mRNA expression, suggesting the importance of dsDNA for the induction of *Zbp1* mRNA expression. Other TLR ligands, such as polyI:C (TLR3 ligand), LPS (TLR4 ligand) and pcDNA3.1 (TLR9 ligand), were much less effective in increasing the *Zbp1* mRNA expression compared with pCpG-ΔLuc/LA2000 complex.

Based on these results, we examined the cytokine production in RAW264.7 cells with elevated levels of the *Zbp1* mRNA expression by pretreatment with IFN-β. Figure 5B shows the IFN-β concentration in culture medium of RAW264.7 cells pretreated with different concentrations of IFN-β followed by the addition of non-CpG lipoplex. Pretreatment of IFN-β followed by the incubation with the medium alone, the negative control group, induced hardly any IFN-β production in the culture media (Figure 5B), indicating that contamination of the preapplied IFN-β was negligible in the ELISA assay for IFN-β in the cells. The IFN-β production induced by the addition of non-CpG lipoplexes increased depending on the concentration of pretreated IFN-β. On the other hand, the effect of pretreatment with IFN-β on the TNF-α production was relatively minor: only the highest concentration (1000 U/mL) significantly increased the level of TNF-α (Figure 5C). Taken together with the results showing that pretreatment with IFN-β increased the mRNA expression of *Zbp1* in an IFN-β concentration-dependent manner (Figure 5A), these results suggest that cytokine production induced by non-CpG lipoplexes correlates well with the level of mRNA expression of *Zbp1*.

Correlation of *Zbp1* mRNA Expression with Cytokine Production Induced by Non-CpG Lipoplexes Composed of Various Cationic Liposomes in RAW264.7 Cells. Next, we investigated whether the *Zbp1* mRNA expression is induced by the addition of non-CpG lipoplexes composed of different cationic liposomes. The expression of *Zbp1* mRNA in RAW264.7 cells was increased by the addition of any of the four types of complex (Figure 6A), although the level was very dependent on the type of liposome. In Figure 6B, IFN-β and TNF-α production induced by non-CpG lipoplexes in RAW264.7 cells shown in Figures 2A and 2B were plotted against the *Zbp1* mRNA expression shown in Figure 6A. A good correlation was observed between the production of these cytokines and the level of *Zbp1* mRNA expression among the four cationic liposomes.

Intracellular Localization of pDNA Complexed with LA2000 or DOTMA/Cholesterol in RAW264.7 Cells. Based on the above results, we assumed that the difference in the cytokine production by each non-CpG lipoplex would be due to the difference in the intracellular distribution of pDNA because ZBP1 is localized in the cytosolic compartment of cells. To test this hypothesis, confocal microscopic studies were carried out using the FL-pCpG-Luc complexed with LA2000 or DOTMA/cholesterol in RAW264.7 cells. At 15 min after addition of pDNA complexes, FL-pCpG-ΔLuc complexed with LA2000 and DOTMA/cholesterol were colocalized with the early endosomal marker transferrin-Alexa Fluor 594 (Figure 7A,B). After a 2 h incubation, while FL-pCpG-ΔLuc complexed with DOTMA/cholesterol was colocalized with LysoTracker, a marker for the lysosomes (Figure 7C), colocalization of FL-pCpG-ΔLuc/LA2000 with LysoTracker was significantly less (Figure 7D), suggesting that pDNA complexed with DOTMA/cholesterol, but not with LA2000, was efficiently sorted to the late endosomes or the lysosomes.

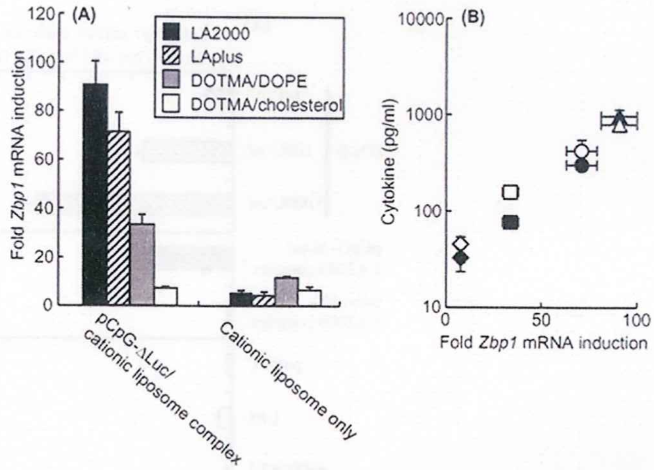

Figure 6. Correlation of *Zbp1* mRNA expression with cytokine production induced by non-CpG lipoplexes in RAW264.7 cells. (A) RAW264.7 cells were incubated with the pCpG-ΔLuc complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5: 42.7 μg/mL) or DOTMA/cholesterol (white bars) (5: 31.9 μg /mL). After a 12 h incubation, the *Zbp1* mRNA level in the cells was measured by real-time PCR. Each result represents the mean ± SD (n = 3) in *Zbp1*/*Gapdh* mRNA levels compared with untreated cells. (B) IFN-β (closed mark) and TNF-α (open mark) in Figure 2 were plotted against the level of *Zbp1* mRNA expression in RAW264.7 cells upon stimulation of pCpG-ΔLuc complexed with LA2000 (triangle), LAplus (circle), DOTMA/DOPE (square) and DOTMA/cholesterol (diamond).

Discussion

A number of studies using lipoplexes have reported that the efficiency of gene expression varies depending on the type of cationic liposome used for complex formation with pDNA. Cationic liposomes containing DOPE as a neutral helper lipid, which has a high ability to destabilize lipid bilayers, exhibit a high transfection activity *in vitro*.[36,37] It is suggested that cationic liposomes which contain DOPE destabilize the endosomal membrane and allow the release of DNA into the cytosol. On the other hand, cationic

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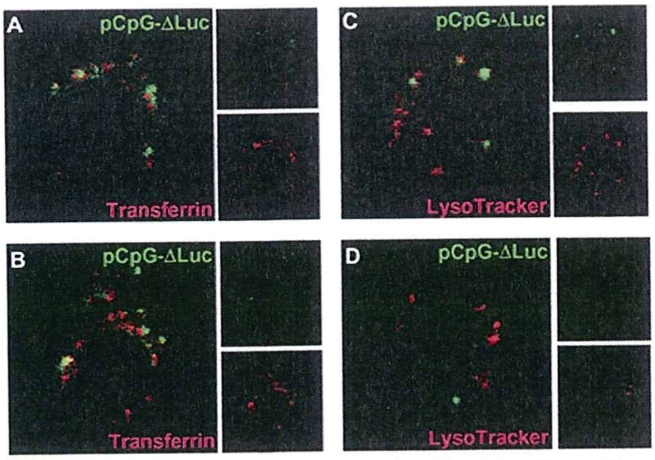

Figure 7. Confocal images of the FL-pCpG-ΔLuc complexed with LA2000 or DOTMA/cholesterol in RAW264.7 cells. The FL-pCpG-ΔLuc complexed with DOTMA/cholesterol (A, C) (1:6.4 μg/mL) or LA2000 (B, D) (1:2 μg/mL) was incubated with the cells for 15 min (A, B) or 2 h (C, D) and washed, and the medium containing transferrin-Alexa Fluor 594 (10 μg/mL) or LysoTracker Red (100 nM), respectively, was added. After a 15 min incubation, the cells were fixed and scanned by confocal microscopy.

liposomes containing cholesterol, which is known to stabilize lipid bilayers, exhibit high transfection efficiency *in vivo* because they produce hardly any fusion with erythrocytes within the blood circulation.[38] However, in the basic studies of cytokine production induced by pDNA complexed with cationic liposome, only a single cationic liposome was used for efficient DNA delivery into the cells. Therefore, we examined macrophage activation characteristics by stimulation with non-CpG pDNA complexed with four kinds of cationic liposome, i.e., two types of LA-based cationic liposomes (LA2000 and LAplus) and two types of DOTMA-based cationic liposomes (DOTMA/DOPE and DOTMA/cholesterol liposome). In the present study, lipoplexes have a tendency to induce IFN-β, IL-6 and TNF-α production dependent on the complexes in RAW264.7 cells; the complexes that induced large amounts of IFN-β exhibited a significant release of proinflammatory cytokines. pDNA complexed with LA2000 or LAplus showed more potent immunostimulatory activity than pDNA complexed with DOTMA/DOPE or DOTMA/cholesterol (Figure 2). In addition, using the TLR9-lacking cell line, colon26/NF-κB-Luc cells, we have demonstrated that pDNA complexed with LA2000 or LAplus strongly activated NF-κB in a TLR9-independent manner (Figure 4). These findings suggest that cellular activation by lipoplex is highly dependent on the type of cationic liposome complexed with pDNA. In the *in vivo* study using cholesterol-based liposomes, Sakurai et al.[39] have reported that inflammatory cytokine production was hardly induced after intravenous injection of non-CpG lipoplex composed of DOTAP/cholesterol into mice. Our very recent *in vivo* study has also demonstrated that non-CpG lipoplex composed of DOTMA/cholesterol does not produce any significant induction of IFN-β or IL-6 after intravenous injection into mice.[40] These *in vivo* results are in good agreement with the results of the present *in vitro* study using cholesterol-based liposomes and suggest that the predominant immune responses induced by lipoplexes are dependent on both the CpG motif and TLR9. However, our present study has shown that CpG motif- and TLR9-independent cytokine production may occur *in vivo* when different cationic liposomes are used. Further *in vivo* studies will be required to clarify the cytokine production by other types of lipoplexes.

Cytosolic dsDNA has recently been reported to be an immunostimulatory molecule which triggers the induction of type I IFNs and other genes involved in innate immunity through a cytosolic DNA sensor, ZBP1.[14,16,19,25] Moreover, a very recent report demonstrated that innate immune activation by cytosolic dsDNA is also observed in MEFs and bone marrow-derived dendritic cells from *Zbp1*$^{-/-}$ mice.[21] Furthermore, the existence of other DNA-sensing molecules for recognition of cytosolic dsDNA other than ZBP1 is implied, and the relative contribution of ZBP1 to the immune response induced by dsDNA depends on the type of cell.[35] In our previous studies, lipoplexes were mainly taken up by phagocytic cells such as resident macrophages after intravenous administration into mice.[40,41] Therefore, we have evaluated the cytokine production induced by non-CpG lipoplex using macrophages and clearly indicated that non-CpG lipoplex-induced cytokine production is closely related to the expression of ZBP1 in RAW264.7 cells (Figure 6B). However, taking other recent studies into consideration, DNA-sensing molecules other than ZBP1 could also be involved in the cellular activation by pDNA in RAW264.7 cells. In addition, very recent studies have identified another novel cytosolic DNA sensor involved in different innate

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immune responses in macrophages.[42−44] Further studies using various types of immune and nonimmune cells of the immune responses to cytosolic DNA are needed to obtain a better understanding of dsDNA-induced innate immunity.

Among the four types of complexes, pDNA/LA2000 complex and pDNA/LAplus complex were strong inducers of cytokine production and *Zbp1* mRNA expression. The cellular uptake of pDNA/LA2000 complex was almost equal to that of pDNA/DOTMA/DOPE complex and pDNA/DOTMA/cholesterol complex (Figure 3), indicating that the apparent amount of pDNA taken up by the cells is not a determinant of cellular activation. Therefore, we focused on the intracellular trafficking of pDNA. Confocal microscopic analysis showed that intracellular localization of pDNA complexed with LA2000 was different from that of pDNA complexed with DOTMA/cholesterol. In the latter case, fluorescent signals were observed in the late endosomal or lysosomal compartment after being localized in the early endosomes, indicating that most pDNA complexed with DOTMA/cholesterol follows the classical endosome−lysosome pathway, and then the DNA is efficiently degraded in the lysosomes. On the other hand, pDNA complexed with LA2000 was not clearly detected in the lysosomal compartment after localization in the early endosomes. These results imply that pDNA complexed with LA2000 escaped from the lysosomal compartment, perhaps into the cytosol, more efficiently than that complexed with DOTMA/cholesterol. This speculation is supported by the results showing that the LA2000 complex exhibited higher reporter gene expression than the DOTMA/cholesterol complex. More efficient delivery to the cytosol may be one of the reasons why the pDNA/LA2000 complex induces potent cellular activation through the recognition of DNA by ZBP1 and other dsDNA receptors present in the cytosol.

In conclusion, we have investigated the cytokine production induced by non-CpG lipoplex in macrophages and found that the types of cationic liposome affect cytokine production, indicating that cationic liposomes should be selected taking this into consideration. Moreover, we have shown the possibility of the involvement of ZBP1 in the cytosolic compartment in the cellular activation induced by non-CpG pDNA efficiently escaping from the lysosomal compartment with the help of cationic liposomes. The present studies suggest that the efficient trafficking of pDNA to the cytosolic compartment is generally important for effective gene expression, although this results in CpG motif-independent cytokine production by dsDNA. The results of the present study provide useful information about DNA-induced innate immune response for the achievement of optimal DNA-based therapies using nonviral plasmid vectors.

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Induction of Tumor-specific Immune Response by Gene Transfer of Hsp70-cell-penetrating Peptide Fusion Protein to Tumors in Mice

Makiya Nishikawa[1], Takayuki Otsuki[1], Atsushi Ota[1], Xin Guan[1], Seiji Takemoto[1], Yuki Takahashi[1] and Yoshinobu Takakura[1]

[1]*Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Japan*

To induce a tumor-specific immune response by delivering tumor-associated antigens in tumor cells to antigen-presenting cells (APCs), we designed a fusion protein which consists of heat-shock protein 70 (Hsp70) and the C-terminal 34 amino acids of herpes simplex virus VP22 protein ($VP22_{268-301}$), the former having a peptide binding domain and an ability to be recognized by APCs, and the latter able to achieve cell penetration. $Hsp70\text{-}VP22_{268-301}$, the fusion protein, was efficiently taken up by mouse dendritic cell (DC) line DC2.4. Major histocompatibility complex (MHC) class I–restricted presentation of an epitope peptide of ovalbumin (OVA) was examined in DC2.4, and $Hsp70\text{-}VP22_{268-301}$ significantly increased the presentation of the peptide compared with Hsp70. Electroporation-assisted injection of naked plasmid vector expressing $Hsp70\text{-}VP22_{268-301}$ ($pHsp70\text{-}VP22_{268-301}$) into subcutaneous tumors of EG7-OVA, a mouse lymphoma–expressing OVA, significantly increased the survival of mice compared with the same treatment with pHSp70, a plasmid expressing Hsp70. Splenocytes from the $pHsp70\text{-}VP22_{268-301}$-treated mice exhibited cytolytic activity against both EG7-OVA and the parent EL4, but not against mouse melanoma B16-F10, suggesting that not only OVA-derived antigens but those common to EG7-OVA and EL4 are delivered to APCs. These results provide a new therapeutic method to induce tumor-specific antitumor immunity without identifying nor isolating tumor-associated antigens.

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INTRODUCTION

Cancer immunotherapy, which requires the stimulation of the immune system, is expected to be a safe and effective strategy that shows promise in the treatment of cancer patients.[1–3] Effective induction of a tumor-specific immune response requires efficient delivery of antigens to antigen-presenting cells (APCs) followed by presentation of antigen-derived peptides on major histocompatibility complex (MHC) class I molecules to naive $CD8^+$ T cells.[4] Therefore, controlling the *in vivo* distribution of antigens will increase antigen-specific immune responses, including cytotoxic T lymphocyte (CTL) response.

Heat-shock protein 70 (Hsp70), a molecular chaperon induced under stress conditions, can present a variety of tumor antigens to APCs and elicit innate immunity.[5–10] A previous study has reported that tumor-derived Hsps initiate protective and tumor-specific CTL responses.[11] The mechanisms involved in these processes have been partially identified as follows. Hsps noncovalently form complexes with tumor antigen-derived peptides[12–14] and bind to dendritic cells (DCs) and macrophages through CD91 and other Hsp receptors,[15–17] followed by colocalization with the MHC class I molecule in endosomes. In addition, Hsps also activate the innate immunity through interaction with CD40 and Toll-like receptor-2 on DCs, which eventually leads to cytokine release.[12,18–21] These events result in the migration of mature DCs to draining lymph nodes where they present antigens to T cells and initiate the T-cell response.

Because Hsps can deliver antigen peptides to APCs and efficiently activate the immune response to tumor-associated antigens, their application to cancer immunotherapy has been extensively investigated. One of the most common strategies is the use of purified tumor-derived Hsp-peptide complexes, including gp96-peptide complex.[11,22–25] Recently, an Hsp-peptide vaccine (Oncophage; Antigenics, Lexington, MA) has been approved in Russia for the treatment of kidney cancer patients. Such complexes can be reconstituted using several types of Hsps and synthetic peptides.[26] All challenges using synthetic or tissue-isolated peptides require the purification and identification of antigen peptides. However, such processes are very expensive, time consuming, and labor intensive. Because tumor tissues are a depot for tumor antigens, any approach to delivering these antigens outside tumor cells to APCs would induce a tumor-specific immune response without exogenous administration of antigens.

In recent years, several peptides and proteins have been reported to translocate across the membranes of mammalian cells.[27] These molecules, collectively called cell-penetrating peptides (CPPs) or protein transduction domains, have been applied for the intracellular delivery of a large variety of compounds, including proteins, liposomes, and plasmid DNA complex. Although the precise mechanism of their cellular uptake has not yet been fully identified, cytosolic distribution of CPP-containing

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Correspondence: Makiya Nishikawa, E-mail: makiya@pharm.kyoto-u.ac.jp

compounds has been reported after their addition to cultured cells. VP22, a structural protein of human simple herpes virus-1, has been reported to possess a cell-penetrating activity and to be secreted from cells in which it is produced, thereafter entering surrounding cells.[28] Recently, Lemken *et al.*[29] have demonstrated intercellular trafficking of VP22 in living cells. The C-terminal peptide consisting of 34 amino acids (VP22$_{268-301}$) has been shown to be responsible for this activity. Therefore, conjugation of this peptide with Hsp70 may facilitate the transmembrane transport of Hsp70 and its cargos, such as antigen peptides. Once taken up by APCs, VP22$_{268-301}$ may accelerate the cytosolic delivery of the complex through the activity of VP22$_{268-301}$, an important process for inducing Hsp70/peptide-mediated CTL responses.[30]

These lines of evidence led us to hypothesize that gene delivery of a fusion protein consisting of Hsp70 and VP22$_{268-301}$ to tumor cells would induce a tumor-specific immune response through the delivery of tumor-associated antigens existing within tumor cells to APCs. To prove this, plasmid vectors encoding mouse Hsp70 or Hsp70-VP22$_{268-301}$ fusion protein were constructed, and the distribution and antitumor activity of these proteins were examined after gene transfer to cultured cells and to subcutaneous tumors in mice.

RESULTS

Physicochemical properties of Hsp70-VP22$_{268-301}$

Samples from B16-F10 melanoma cells transfected with pcDNA3.1 (mock), pHsp70, or pHsp70-VP22$_{268-301}$ were evaluated by western blot analysis (**Figure 1**, lanes 2–4). All samples showed a weak band for endogenous Hsp70, and cells transfected with pHsp70 (lane 3) or pHsp70-VP22$_{268-301}$ (lane 4) showed a strong band with a molecular weight representative of Hsp70 and Hsp70-VP22$_{268-301}$, respectively. There were a few bands in the lanes of lysates from cells expressing pHsp70 (lane 3) or pHsp70-VP22$_{268-301}$ (lane 4), suggesting that their interaction with cellular proteins. The purified Hsp70-VP22$_{268-301}$ from bacteria (lane 1) showed a single band with a molecular weight slightly greater than Hsp70 (lane 2), so that the increase in molecular weight of Hsp70 by fusion with the VP22 peptide with a molecular weight of about 3,000 was confirmed.

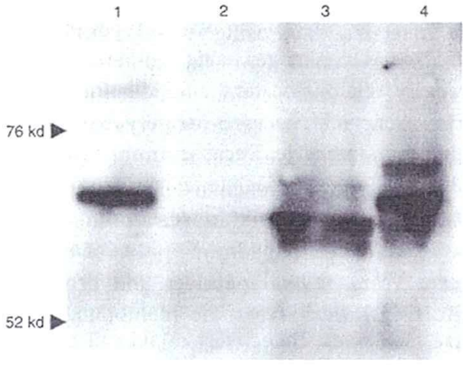

Figure 1 Western blot analysis of Hsp70 and Hsp70-VP22$_{268-301}$ expressed in B16-F10 cells. B16-F10 cells were transfected with pcDNA3.1 (2, mock), pHsp70 (3) or pHsp70-VP22$_{268-301}$ (4) using Lipofectamine 2000, and cell lysates were subjected to 10% SDS-PAGE. After transfer to a membrane, Hsp70 and Hsp70-VP22$_{268-301}$ were detected using mouse anti-Hsp70 monoclonal antibody. Lane 1 shows Hsp70-VP22$_{268-301}$ expressed and purified from bacteria.

Hsp70-VP22$_{268-301}$ had a slightly lower electrophoretic mobility ($-1.29 \pm 0.10 \times 10^{-4}$ cm^2/V·s) compared with Hsp70 (-1.60 ± 0.05), indicating that the surface charge of the fusion protein was slightly less negative due to the presence of many basic amino acids in the VP22 peptide.

Intracellular distribution of Hsp70 and Hsp70-VP22$_{268-301}$ in tumor cells

The intracellular distribution of Hsp70 and Hsp70-VP22$_{268-301}$ was observed in B16-F10 cells after transfection with plasmids expressing each protein. **Figure 2** shows the confocal images of B16-F10 cells, in which Hsp70 and Hsp70-VP22$_{268-301}$ were detected using anti-Hsp70 antibody. Mock (pcDNA3.1) transfected cells showed no significant signals of Hsp70 (**Figure 2a**), indicating that the level of endogenous Hsp70 was too low to be detected under the conditions used. Cells transfected with pHsp70 exhibited a uniform distribution of Hsp70 within cells (**Figure 2b**). On the other hand, cells transfected with pHsp70-VP22$_{268-301}$ showed localized fluorescent signals close to cell membranes (**Figure 2c**). To compare the intracellular distribution of Hsp70 and Hsp70-VP22$_{268-301}$, the fluorescent images of cells expressing these proteins were quantitated by fluorescent microscopy. The intensity of cells indicated dotted lines in **Figure 2b,c** was summarized in **Figure 2d**. The distribution in cells expressing Hsp70 was rather uniform over the cells. On the other hand, the signal of Hsp70-VP22$_{268-301}$ showed high distribution to the edge of cells, suggesting their high affinity for the membranes.

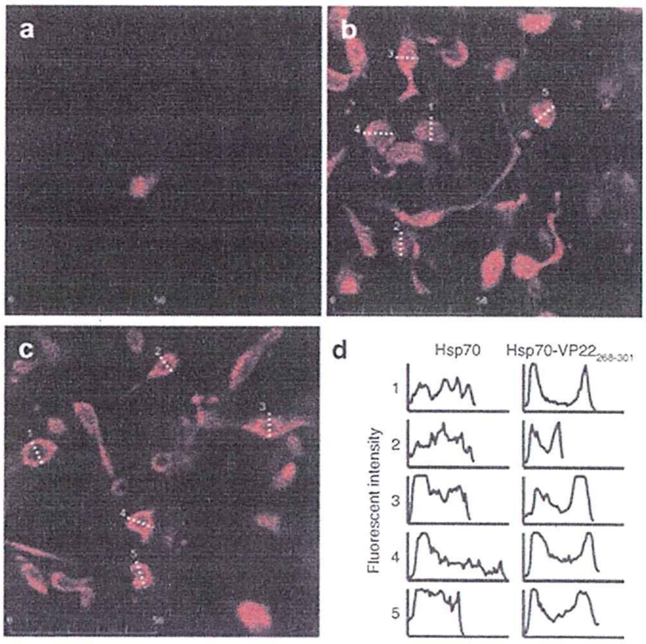

Figure 2 Confocal microscopic images of Hsp70 and Hsp70-VP22$_{268-301}$ transiently expressed in B16-F10 cells. B16-F10 cells were transfected with (**a**) pcDNA3.1 (mock), (**b**) pHsp70 or (**c**) pHsp70-VP22$_{268-301}$ using Lipofectamine 2000. After being permeabilized with Triton X-100, cells were stained with mouse anti-Hsp70 monoclonal antibody, followed by Alexa 594-conjugated secondary antibody. Images were obtained by confocal laser scanning microscopy (MRC-1024, Bio-Rad, Hercules, CA). (**d**) The fluorescent intensity of cells indicated by dotted lines in **b** and **c** was quantitated by fluorescent microscopy.

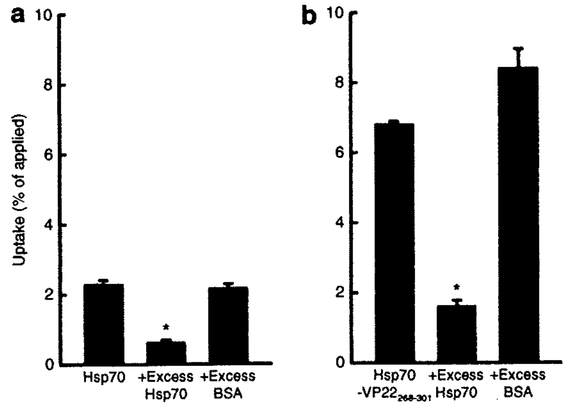

Figure 3 Uptake of radioactivity in DC2.4 cells after addition of ^{111}In-Hsp70 or ^{111}In-Hsp70-VP22$_{268-301}$. DC2.4 cells were incubated with (**a**) 2.5 µg ^{111}In-Hsp70 or (**b**) ^{111}In-Hsp70-VP22$_{268-301}$ for 1 hour at 37 °C, with or without excess (125 µg) Hsp70 or bovine serum albumin (BSA). Results are expressed as mean ± SD of three determinations. *$P < 0.05$ compared with the group without any competitor.

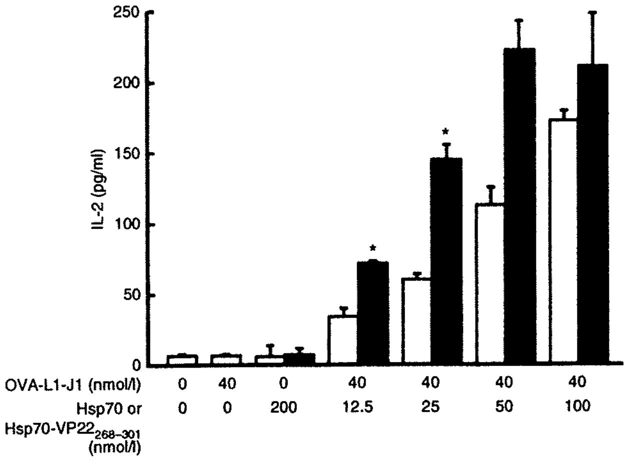

Figure 4 MHC class I–restricted presentation of OVA-derived peptide in DC2.4 cells. DC2.4 cells were added with OVA-L1-J1 (40 nmol/l), Hsp70 (200 nmol/l), Hsp70-VP22$_{268-301}$ (200 nmol/l), or OVA-L1-J1 (40 nmol/l) mixed with a varying amount of Hsp70 (open bars) or Hsp70-VP22$_{268-301}$ (closed bars). At 24 hours after incubation, CD8OVA1.3 T hybridoma cells were added and the IL-2 concentration in supernatants was measured by ELISA. Results are expressed as mean ± SD of three determinations. *$P<0.05$ compared with the OVA-L1-J1/Hsp70 group.

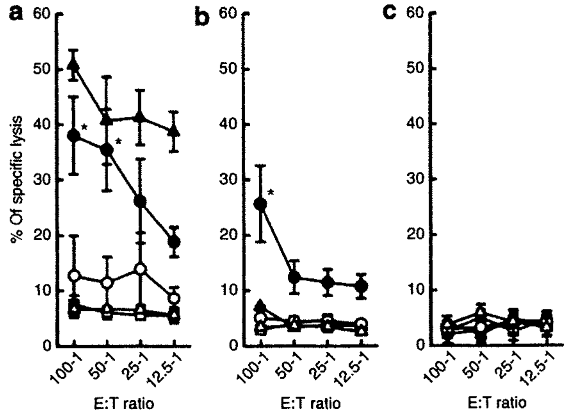

Figure 5 OVA-specific CTL response after injection of pHsp70 or pHsp70-VP22$_{268-301}$ into EG7-OVA tumors in mice. EG7-OVA-bearing mice were left untreated (open triangles), or received a subcutaneous injection of 100 µg OVA in Freund's complete adjuvant (closed triangles), or three injections (4 days apart) of pcDNA3.1 (mock, open squares), pHsp70 (open circles), or pHsp70-VP22$_{268-301}$ (closed circles) into the subcutaneous tumor followed by electroporation. Four days after the last gene transfer, spleen cells were isolated and a standard ^{51}Cr release assay was carried out against (**a**) EG7-OVA, (**b**) EL4, and (**c**) B16-F10 cells. Results are expressed as mean ± SD of at least three determinations. *$P < 0.05$ compared with the pcDNA3.1-treated and pHsp70-treated groups.

Uptake of Hsp70 and Hsp70-VP22$_{268-301}$ in DCs

Figure 3 shows the level of radioactivity in DC2.4 cells 1 hour after addition of ^{111}In-Hsp70 or ^{111}In-Hsp70-VP22$_{268-301}$. As reported in our previous study,[30] ^{111}In-Hsp70 was efficiently taken up by DC2.4 cells (**Figure 3a**). This uptake was significantly inhibited by an excess of unlabeled Hsp70, but not by the same amount of bovine serum albumin, suggesting that the uptake of Hsp70 by DC2.4 cells is mediated by some mechanisms specific to Hsp70, such as LOX-1 and other Hsp receptors. Compared with ^{111}In-Hsp70, ^{111}In-Hsp70-VP22$_{268-301}$ was more effectively taken up by DC2.4 cells (**Figure 3b**). Again, the uptake was significantly inhibited by an excess of Hsp70, indicating that Hsp70-VP22$_{268-301}$ is also recognized by the same uptake mechanism as Hsp70. Even when the uptake was inhibited by an excess of Hsp70, the amount of ^{111}In-Hsp70-VP22$_{268-301}$ taken up by DC2.4 cells was significantly greater than that of ^{111}In-Hsp70. Therefore, it can be speculated that the cellular uptake of Hsp70-VP22$_{268-301}$ through mechanisms other than the Hsp70-specific ones is more efficient compared with that of Hsp70.

MHC class I–restricted presentation of OVA-derived peptide in DC2.4 cells with added Hsp70 or Hsp70-VP22$_{268-301}$

Figure 4 shows the interleukin-2 (IL-2) levels in the supernatant of CD8OVA1.3 T hybridoma cells mixed with DC2.4 cells. The addition of OVA-L1-J1 peptide to the cells resulted in a very low induction of IL-2, indicating that this peptide is hardly presented by itself under the conditions used. The Hsp70/OVA-J1-L1 complex prepared using the same amount of peptide was more effective than the free peptide alone for IL-2 production. These results suggest that the MHC class I–restricted presentation of the peptide was significantly increased by the complex formation with Hsp70, which was in good agreement with the previous results.[31] The addition of Hsp70-VP22$_{268-301}$/OVA-J1-L1 complex resulted in a significantly greater production of IL-2 than that of Hsp70/OVA-J1-L1 complex.

Tumor-specific immune response after injection of pHsp70 or pHsp70-VP22$_{268-301}$ into EG7-OVA tumors

The CTL response was measured to evaluate whether the antigen-specific CTLs were elicited by intratumoral gene transfer of Hsp70 or Hsp70-VP22$_{268-301}$ (**Figure 5**). Three cell lines were selected as target cells in the assay to examine whether the CTL response is specific to the type of tumor cells: EG7-OVA, the cell line used to inoculate mice; EL4, the parent cell line of EG7-OVA that shares antigen peptides except for those derived from OVA; and B16-F10, a melanoma cell line that has no relationship to the

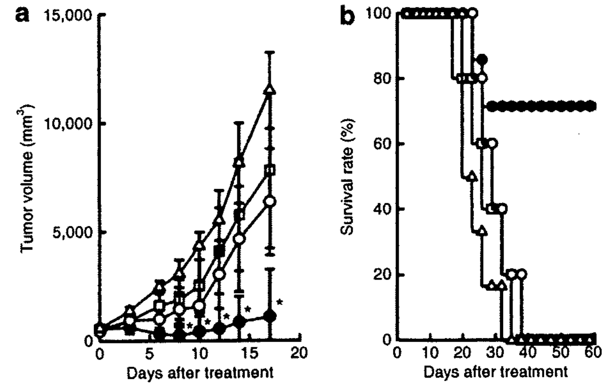

Figure 6 Tumor growth and survival of EG7-OVA-bearing mice after injection of pHsp70 or pHsp70-VP22$_{268-301}$ into EG7-OVA tumors in mice. EG7-OVA-bearing mice were left untreated (open triangles), or injected three times (5 days apart) with pcDNA3.1 (mock, open squares), pHsp70 (open circles), or pHsp70-VP22$_{268-301}$ (closed circles) into the subcutaneous tumor followed by electroporation. (**a**) The tumor size was measured periodically and (**b**) the survival of mice was monitored until 60 days after the start of the treatment. $^*P < 0.05$ compared with the pcDNA3.1-treated and pHsp70-treated groups.

former two lymphoma cell lines. Splenocytes from mice treated with pHsp70-VP22$_{268-301}$ showed a higher level of CTL activity against EG7-OVA cells compared with those treated with pHsp70 (**Figure 5a**). The activity was close to that obtained with OVA in Freund's complete adjuvant, which is a highly effective but very toxic formulation. Splenocytes from the pHsp70-VP22$_{268-301}$-injected mice were also cytotoxic to EL4 cells (**Figure 5b**), but not to B16-F10 cells (**Figure 5c**). These results suggest that not only highly antigenic OVA-derived peptides but antigens common to EG7-OVA and EL4 cells are present on the MHC class I molecules of APCs in mice receiving intratumoral injections of pHsp70-VP22$_{268-301}$. The absence of CTL activity against B16-F10 cells indicated that the cytotoxic activity produced is specific to EG7-OVA and EL4 cells.

Antitumor effect of intratumoral injection of pHsp70 or pHsp70-VP22$_{268-301}$

Intratumoral injections of a control pcDNA3.1 plasmid followed by electroporation slightly retarded the tumor growth (**Figure 6a**). pHsp70 had little additional effects on the tumor growth compared with pcDNA3.1. On the other hand, pHsp70-VP22$_{268-301}$ significantly inhibited tumor growth. In accordance with the profile of tumor growth, intratumoral injections of pHsp70-VP22$_{268-301}$ significantly increased the survival of EG7-OVA-bearing mice (**Figure 6b**).

DISCUSSION

Theoretically, the induction of an antigen-specific immune response is achieved by delivering tumor-related antigens to APCs. In addition to the delivery of antigens, many studies have emphasized the importance of the activation of APCs for an efficient induction of such responses. Of the various candidates used as antigen delivery systems, Hsp proteins are considered to be highly effective because they exhibit both delivery and immune stimulation functions.[5–10] The present study proposes a new approach for delivering endogenous antigen peptides expressed in tumor cells by using Hsp70-VP22$_{268-301}$ fusion protein after being expressed in tumor cells by *in vivo* gene transfer. The VP22 peptide, one of the CPPs that has been reported to be translocated through biological membranes, was selected and fused to the C-terminal of Hsp70 to increase the release of Hsp70-antigen peptide complex from tumor cells.

Although the precise mechanism of transmembrane transport of CPPs has not been fully identified, such peptides have been widely used in the delivery of drugs with a variety of physicochemical properties.[29,32] However, few challenges have involved the delivery of any compounds present inside cells to their outside. This is, at least partly, due to the fact that many CPPs also have a nuclear transport ability. HIV Tat peptides are frequently used as CPPs but, after entering cells, they localize in the nuclei. On the other hand, HSV-1 VP22 and its derivatives have been reported to be able to deliver transgene products to untransfected cells present around the transfected cells.[28,33–35] In preliminary experiments, we compared the whole VP22 protein and the VP22$_{268-301}$ fragment in terms of the induction of CTL responses in tumor-bearing mice, and found that the plasmid expressing Hsp70-VP22$_{268-301}$ showed better results than that expressing Hsp70-VP22 (data not shown). These findings may be explained by the difference in the size of the fusion proteins, although further studies are needed to prove this.

Hsp70, as well as other Hsps, is believed to possess properties suitable for applications as cancer vaccines. We and others have tried to use Hsps as delivery vehicles for tumor antigens to APCs because they can be recognized by Hsp receptors expressed on APCs.[9,10,12–17,36] In these approaches, antigens are bound to Hsps through their peptide binding domains, or covalently conjugated. In clinical trials, Hsps binding endogenous tumor antigens have been isolated and administered to cancer patients as vaccines.[11,22–25] Antigens bound to Hsps were reported to be internalized then, through unknown mechanisms, enter the cytosol where antigens can be processed to be presented to MHC class I molecules. In this study, it was suggested that Hsp70 and Hsp70-VP22$_{268-301}$ interact with some cellular proteins when expressed in melanoma cells (**Figure 1**). Although the details of such interaction need further study, these results as well as preclinical and clinical observations of Hsp-peptide complexes[11,22–25] would support the hypothesis that Hsp70 and Hsp70-VP22$_{268-301}$ can be used to deliver endogenous tumor antigens. In addition to these properties of tissue distribution and intracellular trafficking, Hsps can activate APCs through receptors, such as toll-like receptors. These lines of evidence strongly support the usefulness of Hsps as inducers of tumor-specific immune responses.

Fusion of the VP22$_{268-301}$ peptide to Hsp70 will alter various processes in the distribution of the fusion protein after *in vivo* gene transfer. Intracellular localization of Hsp70-VP22$_{268-301}$ is different from that of Hsp70, and the fusion protein showed a facilitated distribution to plasma membranes (**Figure 2**). The change in the hydrophilic/hydrophobic balance and apparent surface charge of the Hsp70-VP22$_{268-301}$ could explain the localized distribution of the Hsp70-VP22$_{268-301}$ to the membranes. Because neither Hsp70 nor Hsp70-VP22$_{268-301}$ was secreted from cells transduced, the first step in the release from transduced cells is the association with the plasma membranes. Therefore, the localized distribution of Hsp70-VP22$_{268-301}$ would be beneficial for the release from the cells transduced, the next step in the induction of a tumor-specific immune response.

Once released, the Hsp70 and Hsp70-VP22$_{268-301}$, especially those complexed with antigen peptides, should be taken up by APCs. Hsp70-VP22$_{268-301}$ showed a greater uptake in DC2.4 cells than Hsp70 (**Figure 3**). The uptake of ^{111}In-Hsp70 and ^{111}In-Hsp70-VP22$_{268-301}$ was specifically inhibited by an excess of Hsp70. Therefore, both would be recognized by cells through receptors specific to Hsp70. It seems that the fusion of VP22$_{268-301}$ to Hsp70 increases the interaction with those receptors, because the uptake of ^{111}In-Hsp70-VP22$_{268-301}$ to DC2.4 cells was much higher than that of ^{111}In-Hsp70 and the uptake was efficiently inhibited by an excess of Hsp70. Under conditions where an excess of Hsp70 was added, the amount of ^{111}In-Hsp70-VP22$_{268-301}$ associated with cells was two- to threefold greater than that of ^{111}In-Hsp70, suggesting the cellular interaction of Hsp70 through mechanisms other than the Hsp70-specific ones is also increased by the fusion of VP22$_{268-301}$. Furthermore, the fusion protein induced a significantly greater amount of IL-2 than Hsp70 in the antigen presentation assay (**Figure 4**). Similar to the results of a previous study,[31] OVA-L1-J1 alone induced very little IL-2 production and this confirmed that the production of IL-2 is a result of the intracellular delivery of this peptide by delivery systems, i.e., Hsp70 or Hsp70-VP22$_{268-301}$. Both were found effective in inducing the production of IL-2 from T-cell hybridoma (**Figure 4**), but the level was significantly higher when Hsp70-VP22$_{268-301}$-peptide complex was added to the cells. Although the binding of the peptide to Hsp70 proteins was not directly examined in this study, the results of the IL-2 production strongly support the notion that OVA-L1-J1 binds to Hsp70 and Hsp70-VP22$_{268-301}$ and is delivered efficiently into the cytosol of the cells, then presented on the MHC class I molecules, as demonstrated in the previous study using peptide and Hsp70 (ref. 31). These results suggest that, once released from tumor cells, Hsp70-VP22$_{268-301}$ more efficiently deliver antigens than Hsp70.

In a previous study, we confirmed that electroporation-assisted *in vivo* gene transfer is an effective approach for achieving high levels of transgene expression irrespective of the tissues or organs involved.[37] In addition, we compared several plasmid-based gene transfer methods to solid tumor tissues in terms of the level of transgene expression.[38,39] Based on these studies, we concluded that electroporation-assisted gene transfer is also highly effective for transgene expression in tumor tissues over other methods, including naked plasmid DNA injection without electroporation and injection of plasmid DNA/cationic liposome complex. Therefore, we first examined whether the parameters of electroporation that had been optimized in the previous studies were effective for gene transfer to EG7-OVA tumors. Preliminary experiments demonstrated that EG7-OVA tumor tissues were not an exception and a significant level of transgene expression was obtained after direct tissue injection of naked plasmid DNA followed by electroporation (data not shown). The treatment using control plasmid DNA produced some inhibitory effects on tumor growth. This is probably due to the tissue damage created by both injections and electric pulses. Some cells would be destroyed due to the heat created by the pulses. In addition, the injection of plasmid DNA may induce inflammatory responses against unmethylated CpG dinucleotides, or CpG motifs.[40–42] Such responses would activate nonspecific antitumor responses. Taken together, the control treatment in which control plasmid DNA was injected in the naked form followed by electroporation has some effect in inhibiting tumor growth. However, such effects are marginal and make hardly any difference to the survival of tumor-bearing mice. In addition, Hsp70 gene transfer was not very effective in inhibiting tumor growth compared with the injection of control plasmid DNA, indicating that a simple supplementation of Hsp70 was not effective in inducing antitumor effects. It has been reported that Hsp70 localizes in the cytosol,[8,43,44] and we also confirmed that the Hsp70 expressed in B16-F10 cells evenly distributes within the cytosol (**Figure 2**). Thus, even although Hsp70-antigen peptide complexes are generated within tumor cells transduced, they have little effect on inhibiting tumor growth probably because they are not significantly released from the cells. However, once released, they should be effective in inducing an antigen-specific immune response as observed in the presentation assay (**Figure 4**).

Hsp70-VP22$_{268-301}$ gene transfer to EG7-OVA tumor tissue was effective in inducing CTL responses against EG7-OVA cells. These cells highly express an antigenic protein, OVA, and have been used in vaccine studies in which OVA or its peptides were administered in protein/peptide or DNA form. The specificity of the CTL response induced by Hsp70-VP22$_{268-301}$ was validated using B16-F10 cells; no significant cell lysis was observed when B16-F10 cells were used as target cells in the CTL assay. Interestingly, a detectable level of CTL response was observed when splenocytes of mice treated with Hsp70-VP22$_{268-301}$ were mixed with EL4 cells, the parent cells of EG7-OVA. Although the shared antigens remain to be identified, these results indicate that not only OVA-derived peptides but also peptides common to EL4 and EG7-OVA cells are delivered to APCs by gene transfer of Hsp70-VP22$_{268-301}$ to EG7-OVA-tumor tissues. The difference in the CTL activity between EG7-OVA and EL4 cells may be explained by a fact that the model antigen, OVA, is highly immunogenic compared with other endogenous antigen peptides derived from EG7-OVA and EL4 cells. The importance of Hsp70 in inducing antitumor immune responses has been repeatedly reported in previous publications, but it was not directly examined in the present study. The VP22$_{268-301}$ part of the fusion protein was 34 amino acids in length and about 3,000 in molecular weight, so that it could be less efficient in forming complexes with antigen-derived peptides than Hsp70, a full-length protein with a molecular weight of about 70,000. Further studies are needed to confirm the roles of each part of Hsp70-VP22$_{268-301}$ in the induction of antitumor immune responses.

Another approach to inducing CTL responses could be the complex formation of Hsp70 or Hsp70-VP22$_{268-301}$ with tumor-associated antigens outside cells, not inside cells, which was used in the present study. This possibility needs to be investigated, but we think that gene transfer of Hsp70-VP22$_{268-301}$ to tumor cells would greatly increase the opportunity for the fusion protein to interact with tumor-associated antigens, which could lead to high CTL responses as observed in this study. Immune reaction to the VP22 peptide could also be elicited after gene transfer of Hsp70-VP22$_{268-301}$ to tumor cells, because the fusion protein was effectively taken up by immune cells, such as DCs. The effects of such reaction need additional studies to guarantee the safety of this approach.

In conclusion, gene transfer of Hsp70-VP22$_{268-301}$ to tumor cells has been shown to be a novel approach to inducing a tumor-specific immune response. The fusion of VP22$_{268-301}$ to Hsp70 was found to be important to obtain the immune response, suggesting that the peptide helps the fusion protein complexed with intracellular antigens to be released from the cells transduced. The increase in the positive charge of the fusion protein increased its uptake by immune cells, which would also be involved in the effective induction of the response. This system does not require identification or purification of tumor antigens, so that it can be applied to a variety of tumor immune therapies without identification or purification of tumor antigens.

MATERIALS AND METHODS

Plasmid DNA constructs. The pTrc99A expression vector containing genomic mouse clone Hsp70.1 cDNA was kindly supplied by Paul Slusarewicz (Mojave Therapeutics, Hawthorne, NY). The Hsp70.1 cDNA amplified by Ex Taq polymerase (Takara, Tokyo, Japan) was cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) at the *Bam*HI and *Hind*III restriction sites. To construct plasmid encoding Hsp70-VP22$_{268-301}$, the PCR product of Hsp70 and double strand oligonucleotides for VP22$_{268-301}$ (5′-aat tct gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag tga ggt-3′ and 5′-cta gac ctc act cga cgg gcc gtc tgg ggc gag aag cgg agc ggg ctg ggg ctc gag gtc gct cgg tgg ggc gcg acg ccg cag aac gcc ctc gag tcg ccg tgg ccg cgt cag-3′) were cloned in pcDNA3.1(+). Both plasmid constructs were confirmed by DNA sequencing.

Cell culture. EL4 (C57BL/6, H-2b, T lymphoma), EG7-OVA (EL4 cells transfected with OVA cDNA),[45] and B16-F10 (C57BL/6, melanoma) were purchased from American Type Culture Collection (Manassas, VA). EL4 and B16-F10 were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactive fetal bovine serum. EG7-OVA was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 µmol/l 2-mercaptoethanol, 2 mmol/l L-glutamine, glucose, sodium pyruvate, HEPES, and G418. The murine DC line, DC2.4 (ref. 46), was kindly supplied by Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA). DC2.4 cells were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 µmol/l nonessential amino acids, 50 µmol/l 2-mercaptoethanol, and antibiotics.

Purification of Hsp70 and Hsp70-VP22$_{268-301}$. Hsp70 and Hsp70-VP22$_{268-301}$ proteins were isolated by a method reported previously.[36] In brief, the expression of Hsp70 and Hsp70-VP22$_{268-301}$ in *Escherichia coli* DH5α cells was induced by 1 mmol/l isopropyl-β-D-thiogalactopyranoside, and the harvested cells were disrupted. The clarified supernatants of Hsp70 and Hsp70-VP22$_{268-301}$ were loaded onto a glutathion sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden), and these proteins were eluted by applying pre-scission protease (Amersham Pharmacia Biotech) in 50 mmol/l Tris-HCl solution (pH 7.5). Each eluate was subjected to 8% SDS-PAGE. Then, proteins were transferred to a polyvinyldiene difluoride membrane (Immobilon PTM, Millipore, Bedford, MA), blotted with antibody, and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Mouse anti-Hsp70 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection. After washing with phosphate-buffered saline (PBS) containing 0.1% Tween-20, horseradish peroxidase–conjugated mouse anti-IgG antibody was incubated for 1 hour. After washing with PBS containing 0.1% Tween-20, the labeled spots were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The electrophoretic mobility of Hsp70 and Hsp70-VP22$_{268-301}$ was measured with a laser electrophoresis-ζ potential analyzer (Zetasizer 3000HS, Malvern Instruments, Worcestershire, UK), as an indicator of the net charge of these proteins.[47]

Western blot analysis of Hsp70 and Hsp70-VP22$_{268-301}$ expressed in B16-F10 cells. B16-F10 cells were seeded at 2×10^5 per well of six-well plates. After a 24-hour incubation in 5% CO_2/95% air, transfection of cells with pHsp70 or pHsp70-VP22$_{268-301}$ was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. At 2 hours after transfection, the medium was replaced with fresh culture medium. The cells were collected at 24 hours after transfection and were lysed, and the lysates were subjected to 10% SDS-PAGE. Then, Hsp70 proteins were assayed by western blot analysis as described above.

Immunofluorescence of Hsp70, Hsp70-VP22$_{268-301}$ and Hsp70-VP22 in B16-F10 cells. B16-F10 cells were plated on coverplates into 12-well plates at a density of 5×10^5 cells/well, and transfected with pHsp70 or pHsp70-VP22$_{268-301}$ as described above. Then, cells were washed with PBS and fixed for 15 minutes at room temperature with 4% paraformaldehyde in PBS. After washing with PBS, cells were treated with 0.2% Triton X-100 for permeabilization, and stained with mouse anti-Hsp70 monoclonal antibody, followed by an Alexa 594-conjugated secondary antibody. Images were obtained by confocal laser scanning microscopy (MRC-1024, Bio-Rad, Hercules, CA). The fluorescent intensity of cells expressing Hsp70 or Hsp70-VP22$_{268-301}$ was quantitated by fluorescent microscopy (Biozero BZ-8000, KEYENCE, Osaka, Japan).

Uptake of Hsp70 and Hsp70-VP22$_{268-301}$ in DC2.4 cells. Hsp70 and Hsp70-VP22$_{268-301}$ were radiolabeled with ^{111}In using the bifunctional chelating agent, diethylenetriaminepentaacetic dianhydride, as reported previously.[36] DC2.4 cells (1×10^6 cells/well) cultured on 24-well plates were incubated with 2.5 µg ^{111}In-labeled proteins in Hanks' balanced salt solution for 1 hour at 37 °C. Then, the protein solution was removed, and cells were washed and solubilized with 0.3 mol/l NaOH with 0.1% Triton X-100. Radioactivity in cell lysate was measured using a well-type NaI-scintillation counter (ARC-500, Aloka, Tokyo, Japan). To evaluate the involvement of the Hsp70-specific uptake mechanism in the cellular uptake of ^{111}In-Hsp70 and ^{111}In-Hsp70-VP22$_{268-301}$, a 50-fold excess (125 µg/well) of Hsp70 or bovine serum albumin (Sigma Chemical, St Louis, MO) was added to the ^{111}In-labeled samples.

Antigen presentation assay. The efficacy of the MHC class I presentation activity of Hsp70 and Hsp70-VP22$_{268-301}$ was examined using DC2.4 cells and T hybridoma cells that specifically recognize the SIINFEKL complex with mouse MHC class I molecule Kb (SIINFEKL-Kb) and release IL-2, a simple method for the evaluation of OVA vaccination systems.[48] To avoid direct binding of the SIINFEKL peptide to MHC class I molecules on DC2.4 cells, OVA-L1-J1, a hybrid peptide,[31] was selected and obtained from Hokkaido System Science (Sapporo, Japan). This peptide was mixed with Hsp70 or Hsp70-VP22$_{268-301}$ in PBS, and the mixture was incubated for 1 hour at 25 °C. Then, an amount of 17 ng OVA-L1-J1 peptide with or without Hsp70 derivatives was added to DC2.4 cells (5×10^4 cells/well) cultured on 96-well plates. At 24 hours after incubation, CD8OVA1.3 T hybridoma cells (1×10^5/well) were added to each well and further incubated for 20 hours at 37 °C. Then, supernatants were collected and freeze-thawed. The response of CD8OVA1.3 T cells was determined by measuring IL-2 levels in supernatants with an enzyme-linked immunosorbent assay (ELISA; AN'ALYZA mouse IL-2, Genzyme-techne, Minneapolis, MN).

Animals. Female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions and received humane care according to the criteria outlined in the National

Institutes of Health's Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

CTL assay. Mice were inoculated with EG7-OVA (1×10^6 cells/mouse) in the dorsal skin. When the tumor volume approached 40 mm^3, 100 μg plasmid/50 μl was injected into the subcutaneous tumor, and electric pulses (250 V/cm, 5 ms, 4 Hz, 12 pulses) were delivered through forceps-type electrodes (CUY21, Nepagene, Chiba, Japan). The conditions for electroporation were optimized using a plasmid vector expressing firefly luciferase (data not shown). The intratumoral injection and following electroporation were performed three times at intervals of 4 days. A positive control group of mice received a subcutaneous injection of 100 μg OVA protein (Sigma, St Louis, MO) emulsified in Freund's complete adjuvant (ICN Biomedicals, Aurora, OH). At 4 days after the last gene transfer, splenocytes were isolated and restimulated *in vitro* for 4 days with mitomycin C–treated EG7-OVA. Separately, EG7-OVA, EL4 and B16-F10 cells were labeled with ^{51}Cr by incubation with $Na_2{}^{51}CrO_4$ in culture medium for 45 minutes at 37 °C. After washing, 2×10^4 of ^{51}Cr-labeled cells and serially diluted splenocytes were coincubated for 4 hours at 37 °C. Then, cells were centrifuged for 5 min, and 100 μl supernatant was collected for radioactivity measurement. The maximal and spontaneous release of ^{51}Cr from ^{51}Cr-labeled cells was measured in the presence or absence of 1% Nonidet-P40. The cytolytic activity of CTL was calculated as: % of killing = (observed release-spontaneous release)/(maximal release-spontaneous release) × 100.

Antitumor effect of intratumoral gene transfer. EG7-OVA tumor-bearing mice were treated with each plasmid as described above. The intratumoral injection and following electroporation was performed three times at intervals of 5 days. The tumor size was measured with a slide caliper and expressed as a tumor index, determined as the square root of (major axis × minor axis). The survival of tumor-bearing mice was also recorded.

Statistical analysis. Differences were statistically evaluated by one-way ANOVA followed by the Student–Newmann–Keuls multiple comparison test and Kaplan–Meier analysis with a log-rank test to determine survival, and the level of statistical significance was $P < 0.05$.

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