the liver section of mice receiving 0.02 μg pEGFP-F, an equal amount of pEGFP-F used as a template for EGFPF-mini preparation (Fig. 2A). On the other hand, significant numbers of EGFPF-expressing cells were detected in the liver sections of mice receiving pEGFP-F (Fig. 2B) and EGFPF-mini (Fig. 2C). There were no marked differences in the number and distribution of EGFPF-expressing cells in the liver sections. These results suggest that the hydrodynamic delivery of these DNA preparations is hardly affected by their shape and size, although they were quite different from each other, that is, circular 4.8 kb-plasmid DNA and linear 1.7 kb-DNA fragment.

Transgene Expression by Luciferase-Expressing DNA Preparations in Cultured Cells

Figure 3A shows the luciferase activity in B16-BL6 cells after transfection of pLuc or Luc-mini complexed with Lipofectamine2000. All Luc-mini preparations gave significant levels of transgene expression, although the expression levels were about 100-fold less than that of pLuc. No apparent relationship was observed between the level of transgene expression and the size of the PCR-amplified DNA fragments. Figure 3B shows the luciferase activity in B16-BL6 cells after electric transfer of pLuc or Luc-mini. Again, all Luc-mini preparations gave high levels of luciferase activity, but the levels were significantly smaller than that of pLuc. However, in the case of electric gene transfer, the difference in the luciferase activity between pLuc and Luc-mini preparations was much smaller than that for cationic liposome-mediated transfection (Fig. 3B). In both experiments, Luc-mini2.6PS showed almost identical levels of luciferase activity to Luc-mini2.6, suggesting that the PS substitution have little effects on transgene expression at least under the conditions examined.

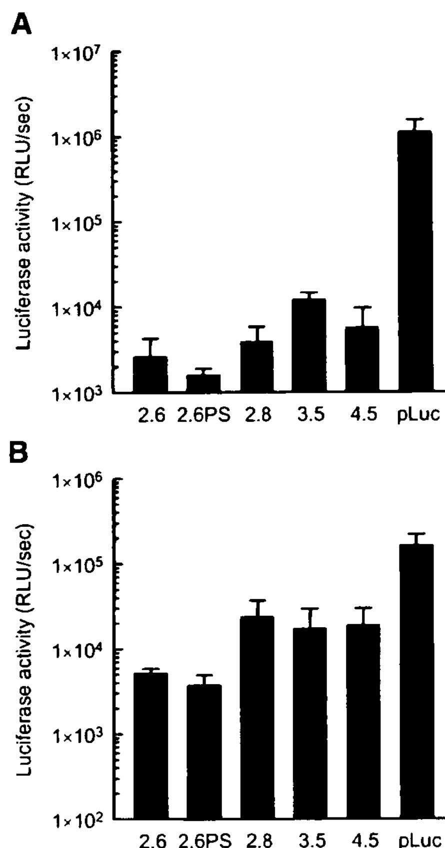

Figure 3. Luciferase activity in B16-BL6 cells after transfection *in vitro*. (A) pLuc or Luc-mini preparation (0.2 pmol/well) was mixed with Lipofectamine2000 and resulting complexes were added to cells. (B) pLuc or Luc-mini preparation (0.2 pmol/well) was added to B16-BL6 cells as the naked form and electric pulses (100 V, 4 ms, 10 Hz, 12 pulses) were applied. Cells were collected at 24 h after transfection and lysed in a lysis buffer, and the luciferase activity in the supernatant was measured in a luminometer. PS, phosphorothioated primers were used to obtain PCR-amplified DNA fragments. Results are expressed as mean ± SD of three determinations.

Transgene Expression in Mouse Liver Following Hydrodynamic Delivery of Luciferase-Expressing DNA Preparations

Figure 4 shows the luciferase activity in the liver 6 h after intravenous injection of each luciferase-expressing DNA preparation into mice by the hydrodynamics-based procedure at a dose of 0.23 pmol/mouse, together with 0.1 μg of pRL-CMV. The relative firefly luciferase activity (Pp-Luc) increased as the length of the DNA fragments became longer. The expression level of Luc-mini4.5 was not significantly different from that of pLuc, but the levels of DNA fragments with the length of 3.5 kb or smaller were significantly ($p < 0.05$) lower than those of Luc-mini4.5 and pLuc. Again, there were no significant effects of the PS substitution in the level of luciferase activity. Pyrobest, a high-fidelity DNA polymerase, was used to prepare a 3.5 kb-DNA fragment, Luc-mini3.5Pyro, but no significant difference in transgene expression was

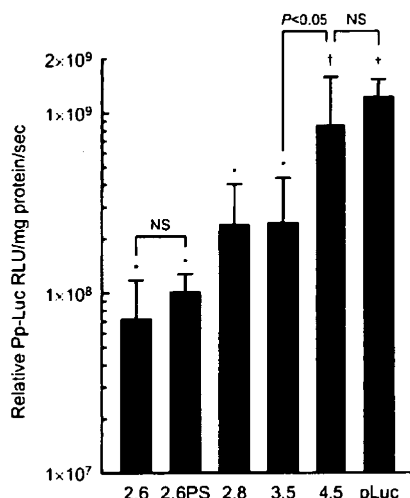

Figure 4. Relative luciferase activity in mouse liver after intravenous injection of pLuc or Luc-mini preparation by the hydrodynamics-based procedure. Mice received pLuc or Luc-mini (0.23 pmol/mouse) along with pRL-CMV (0.1 μg/mouse). At 6 h after injection, luciferase activities in the liver were determined. The ratio of the activity of firefly luciferase (Pp-Luc) to renilla luciferase (Rr-Luc) was calculated to correct for differences in transfection efficiency among mice. The results are expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals. PS, phosphorothioated primers were used to obtain PCR-amplified DNA fragments. Results are expressed as mean ± SD of at least three mice. $^{*}p < 0.05$ versus pLuc; $^{\dagger}p < 0.05$ versus Luc-mini2.6.

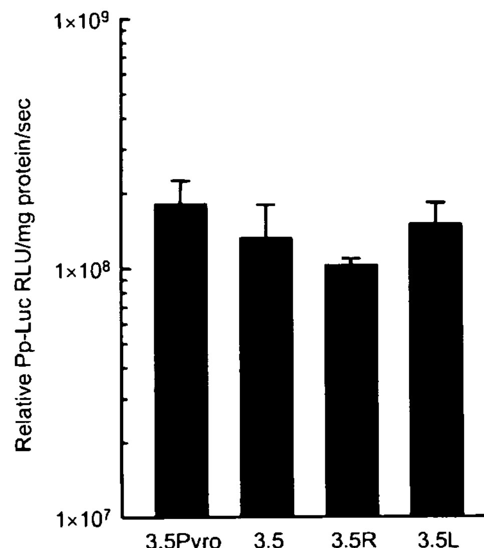

Figure 5. Effect of polymerases and the extension of 5′- and 3′-side of the expression unit in DNA fragment on transgene expression. Mice received Luc-mini (0.23 pmol/mouse) along with pRL-CMV (0.1 μg/mouse). At 6 h after injection, luciferase activities in the liver were determined. The results are expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals. Pyro, a high-fidelity DNA polymerase Pyrobest, which was used to prepare a 3.5 kb-DNA fragment; L, the 5′-side (left-hand side) of the expression unit was extended; R, the 3′-side (right-hand side) of the expression unit was extended. Results are expressed as mean ± SD of at least three mice.

observed between Luc-mini3.5Pyro and Luc-mini3.5 after intravenous injection in mice by the hydrodynamics-based procedure (Fig. 5).

Effect of the Extension of 5′- and 3′-Side of the Expression Unit in DNA Fragment on Transgene Expression

It was found that the elongation of DNA fragments is effective in increasing the level of transgene expression after *in vivo* gene transfer. Then, we examined the effects of extension of 5′- or 3′-side of the minimal expression unit on transgene expression after hydrodynamic delivery to mouse liver. For Luc-mini3.5, both sides of the expression unit were extended. Only the 5′- or 3′-side of the unit was extended for Luc-mini3.5L and Luc-mini3.5R, respectively. Figure 5 shows the relative firefly luciferase activity of these DNA fragments with an identical length of 3.5 kb. The expression of these three DNA fragments with the same length of 3.5 kb showed similar transgene expression, although Luc-mini3.5R, which has no additional nucleotides on the outside of the promoter, was a little lower than that of the other two preparations, Luc-mini3.5 and Luc-mini3.5L. These results suggest that the length of DNA fragments is a primary factor for the level of transgene expression, and that the extension of the 5′-side of expression units is slightly favorable to some extent over the extension of the other side.

Time Course of Transgene Expression of Luciferase-Expressing DNA Preparations

Figure 6 shows the time courses of firefly luciferase activity in mouse liver after hydrodynamic delivery of pLuc, Luc-mini2.6, and Luc-mini3.5. The expression levels of Luc-mini2.6 and

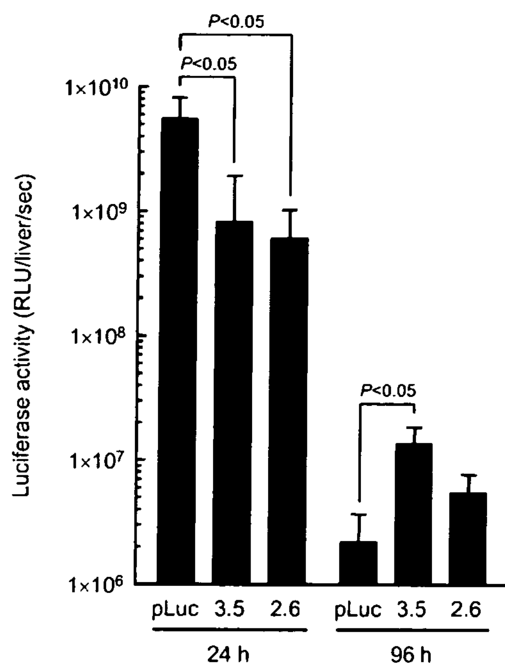

Figure 6. Luciferase activity in mouse liver after intravenous injection of pLuc, Luc-mini2.6, and Luc-mini3.5 by the hydrodynamics-based procedure. Mice were injected with pLuc or Luc-mini (0.23 pmol/mouse), and luciferase activities in the liver were determined at 24 and 96 h after injection. Results are expressed as the mean ± SD of three mice.

Luc-mini3.5 at 24 h were lower than that of pLuc as well as at 6 h (Fig. 4). However, the level of Luc-mini3.5 was significantly ($p < 0.05$) higher than that of pLuc at 96 h after intravenous injection by the hydrodynamics-based procedure. These results suggest that the PCR-amplified DNA fragments are effective in achieving prolonged transgene expression than conventional plasmid DNA.

Stability of PCR-Amplified DNA Fragments

To examine the possibility whether the differences in the transgene expression is due to the stability of DNA preparations, we examined the degradation of Luc-mini preparations by an exo- (Fig. 7) or endonuclease. All DNA preparations were degraded with time in the presence of an exonuclease, Bal31. The disappearance of DNA bands was the slowest with pLuc. All DNA fragments showed a reduction in the DNA size with time, suggesting that they are gradually degraded by the exonuclease. In the case of pLuc

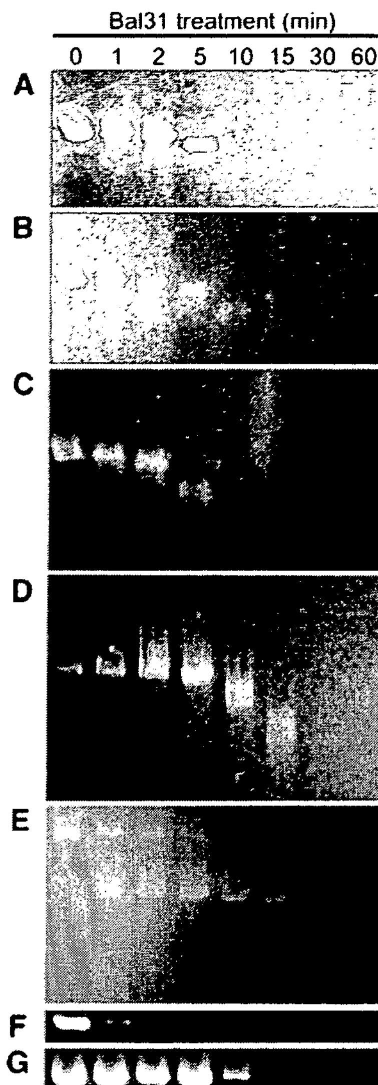

Figure 7. Gel electrophoresis of pLuc and Luc-mini after incubation with an exonuclease Bal31. (A–E) Each luciferase-expressing DNA preparation was incubated with Bal31 (1 U/µg DNA) at 37°C. Aliquots were removed at each sampling point and added with EDTA to stop the degradation of DNA. Each aliquot was mixed with a loading buffer and loaded on an agarose gel. (A) Luc-mini2.6; (B) Luc-mini2.6PS; (C) Luc-mini2.8; (D) Luc-mini3.5; (E) pLuc. (F,G) Bal31-treated Luc-mini2.6 and Luc-mini3.5 were amplified by PCR using the primers for Luc-mini2.6, and the PCR products were loaded on an agarose gel. (F) Luc-mini2.6; (G) Luc-mini3.5.

(Fig. 7E), two to three bands were detected, suggesting a time-dependent conversion of plasmid DNA from its supercoiled form to other forms. To confirm whether the minimal

expression unit in DNA fragments remains during the incubation with the nuclease, the unit of Luc-mni2.6 and Luc-mini3.5 was amplified by PCR using the primers used for the preparation of Luc-mini2.6 (Fig. 7F, G). The PCR product from Luc-mini2.6 was detectable only at 1 min after incubation, whereas that from Luc-mini3.5 was able to be detected at least for the first 10 min of incubation. Digestion with DNase I, an endonuclease, also gave similar results to those obtained with Bal31 (data not shown).

DISCUSSION

Effective *in vivo* gene therapy relies on the controlled expression of transgenes after *in vivo* gene transfer. In spite of the safety of plasmid DNA as a nonviral vector,[1] its clinical applications has significantly been obstructed by the low and transient nature of its transgene expression. Of numbers of problems on plasmid DNA-based gene transfer, inefficient delivery of the DNA inside target cells is the most important one. This is mainly attributed to the large size of plasmid DNA,[30] which is in the range of several millions in molecular weight. One approach to reducing the size is the use of PCR-amplified DNA fragments, which would also have advantages over plasmid DNA, such as small numbers of immunostimulatory CpG motifs and little contamination of LPS. Although errors in PCR amplification could be an inherent problem of this approach for large scale synthesis, previous studies have shown that PCR-amplified DNA fragments are potent vectors to achieve significant transgene expression both *in vitro* and *in vivo*,[23,24] but it has hardly been examined whether the size of such DNA fragments affects the transgene expression. In the present study, we prepared various PCR-amplified DNA fragments and examined their efficiency on transgene expression. Transgene expression from a vector like plasmid DNA would be determined by the accessibility of the vector to the nucleus of target cells, the rate of transcription, and the stability of the vector. In addition to these parameters, promoter attenuation[31] and integration into genome would also determine the duration of transgene expression. First, we used cationic liposome-mediated delivery to cultured cells. The expression of any DNA fragment was almost one hundredth of that of plasmid DNA (Fig. 3A), indicating that the DNA fragments are not as effective for transgene expression as plasmid DNA. It was reported that DNA fragments larger than 1 kb remained in the cytoplasm,[32] suggesting that DNA fragments have little advantages over plasmid DNA as far as the size-dependent nuclear transport is considered. In nondividing cells like hepatocytes, such large DNAs are transported into the nucleus through the nuclear pore complex.[33] Dean et al.[34,35] reported that the process of nuclear transport of DNA is sequence specific and SV40 enhancer is all that is necessary for this DNA nuclear import. Therefore, pLuc might be more efficiently transported into the nucleus than DNA fragments via sequences outside the fragments. In addition, the stability of the DNA and rate of transcription can also be the reasons for the inefficient transgene expression of DNA fragments when delivered as cationic complexes.

Electroporation creates pores on cell membranes, and charged molecules, such as DNA, can be delivered inside cells by electrophoresis.[11] The hydrodynamic delivery method greatly increases the membrane permeability of macromolecules including plasmid DNA in the liver, probably through the creation of membrane pores.[12,13] Because the passage through such pores is a function of molecular size of solutes, the efficiency of cellular entry of DNA preparations could be a function of molecular size. We found that the DNA fragments prepared showed less transgene expression than plasmid DNA, when delivered to cells by electroporation (Fig. 3B). However, the difference between plasmid DNA and DNA fragments was much smaller when they were delivered by electroporation than by cationic liposomes (Fig. 3A). These results suggest that the limited delivery of plasmid DNA through membrane pores is somewhat improved by the reduction in size by PCR amplification of the expression unit of plasmid DNA. This improvement in the delivery of DNA inside cells seemed to be more pronounced in mouse liver after intravenous injection by the hydrodynamics-based procedure. In addition to this hypothesis, the hydrodynamic or electroporative delivery of DNA will take much shorter time than lipofection as far as the delivery of DNA into target cells is concerned. Therefore, a reduced nuclease degradation of DNA may also contribute to the improved gene expression from the PCR-amplified DNA fragments by hydrodynamic delivery and electroporation. No significant differences were observed in the luciferase assay between pLuc

and Luc-mini4.5 (Fig. 4). Similar results were obtained with EGFPF-expressing DNA preparations (Fig. 2). These results indicate that the reduction in size of DNA preparations would be promising for an enhanced delivery, although the level of transgene expression may be low because of the other factors than delivery.

The size of the DNA fragments significantly affected transgene expression in mouse liver after intravenous injection by the hydrodynamics-based procedure. The longer the fragment was injected the higher transgene expression was. The incubation of DNA preparations with nucleases clearly demonstrated that the expression unit in Luc-mini3.5 remained much longer than that in Luc-mini2.6 (Fig. 7), suggesting that the presence of additional nucleotides retards the degradation of the expression unit and the following transgene expression. Because PCR-amplification is highly versatile, there would be least limitations on selecting the size and location for amplification. Loss of some nucleotides in the promoter region would largely reduce the transcriptional activity of DNA preparations. We found that the extension of 5′-side of the expression unit, that is, the promoter side, could be a little better in increasing transgene expression (Fig. 5). However, the effects of the location of the expression unit in DNA fragments were much smaller than those of their length. A possible concern about using PCR amplification is that errors occur during the amplification. We used two polymerases with different fidelities: Taq and Pyrobest polymerases, but observed no significant differences in transgene expression between Luc-mini3.5 and Luc-mini3.5Pyro (Fig. 5). These results do not exclude the possibility of errors in PCR amplification of DNA fragments, but the frequency could be very low.

In spite of the lower expression by DNA fragments at 6 h after injection, Luc-mini2.6 and Luc-mini3.5 showed significantly greater expression at 96 h after injection than pLuc (Fig. 6). Duration of transgene expression may be affected by several factors, including the stability of vectors, promoter attenuation, and integration into genome.[31,36] CMV promoter, the one used in the present study, has been reported to be attenuated in various experimental settings.[37,38] The methylation of CpG dinucleotides in the promoter would be involved in this attenuation.[39] In addition, the presence of CpG motifs triggers the induction of inflammatory cytokines upon administration to animals,[16] because mammalian TLR-9 recognizes unmethylated CpG dinucleotides and induce such cytokines.[15] Although this feature of plasmid DNA can be a drawback for sustained transgene expression, the induction of the cytokines or the attenuation of CMV promoter by itself may not entirely account for the difference in the duration of transgene expression between pLuc and Luc-minis. Another possibility is the difference in the integration into genome of DNA preparations, as reported by Nakai et al.[40] which may cause toxic side effects. However, Chen et al.[41] reported that linear DNA-treated mice showed a 10-fold drop in transgene expression after partial hepatectomy, which suggests that transcriptionally active liner DNAs remained predominantly extra-chromosomal in the liver. Similar results were also reported by Kameda et al.[42] using PCR-amplified DNA fragments expressing erythropoietin. These previous reports would support an idea that PCR-amplified DNA fragments are hardly integrated into the genome. Further studies are needed to understand the reason for the prolonged transgene expression by PCR-amplified DNA fragments.

In conclusion, we demonstrated that PCR-amplified DNA fragments are effective vectors to achieve significant transgene expression both *in vitro* and *in vivo*. They do not require bacteria for production, and they can be manipulated without the possible contamination of LPS once template DNA is prepared. Fragmentation tended to reduce the stability and transcriptional activity of expression units, but a relatively prolonged transgene expression can be obtained with DNA fragments. An extension of DNA fragments would retard their degradation, resulted in high level of transgene expression. This effect seems to be enhanced in the present study, because the dose was set at low levels. The stabilization of DNA fragments against nucleases will further increase the potential of such short, structure-controlled, and synthetic DNA fragments for *in vivo* gene delivery.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by grants form the Ministry of Health, Labour and Welfare, Japan, by a grant from Uehara Memorial Foundation.

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Plasmid DNA Uptake and Subsequent Cellular Activation Characteristics in Human Monocyte-Derived Cells in Primary Culture

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Received 30 May 2006; revised 21 August 2006; accepted 22 September 2006

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20816

ABSTRACT: Plasmid DNA (pDNA) uptake and subsequent cellular activation characteristics were studied in three types of human monocyte-derived cells, that is, human monocytes, macrophages, and dendritic cells (DCs) in primary culture. Naked pDNA was bound to and taken up by the macrophages and DCs while only significant binding occurred in the monocytes. pDNA binding to these monocyte-derived cells was significantly inhibited by polyinosinic acid (poly[I]), dextran sulfate, maleylated bovine serum albumin (Mal-BSA) and to a lesser extent by polycytidylic acid (poly[C]), but not by dextran or galactosylated BSA (Gal-BSA), mannosylated BSA (Man-BSA), suggesting that a specific mechanism for polyanions is involved in the pDNA binding. In cellular activation studies, naked pDNA could not induce TNF-α production from any monocyte-derived cells, regardless of the abundant presence of CpG motifs in the pDNA. However, when complexed with cationic liposomes, pDNA produced a significant amount of TNF-α from the human macrophages. TNF-α induction was not observed in the monocytes or DCs. Moreover, calf thymus DNA (CT DNA) complexed with cationic liposomes also induced TNF-α production to a similar extent in the human macrophages. These results indicate that, among human monocyte-derived cells, macrophages are activated by DNA when complexed with cationic liposomes in a CpG motif-independent manner.
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Keywords: gene therapy; plasmid DNA; cellular uptake; degradation; monocyte-derived cells

INTRODUCTION

Plasmid DNA (pDNA) is an important macromolecular agent in gene therapy as well as DNA vaccination.[1–3] In spite of the lower transfection efficiency compared with viral vectors, pDNA has advantages in terms of its safety and productivity. Because pDNA is a bacterial DNA that is generally replete with unmethylated CpG dinucleotides (CpG motifs) in contrast to vertebrate DNA, it can activate mammalian immune cells through recognition by TLR9. Upon activation, the cells release inflammatory cytokines, which may influence the efficacy of pDNA-based therapy. To achieve effective and safe pDNA-based therapy, it is essential to understand the mechanism by which pDNA is taken up by macrophages and dendritic cells (DCs), the most important cells responsible for the uptake of pDNA *in vivo* and subsequent cytokine production.

Abbreviations: pDNA, plasmid DNA; CT DNA, calf thymus DNA; DC, dendritic cell; TLR, toll-like receptor; poly[I], polyinosinic acid; poly[C], polycytidylic acid.

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Journal of Pharmaceutical Sciences, Vol. 96, 1576–1584 (2007)
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Previously, we have demonstrated that pDNA is rapidly removed from the circulation and taken up by the liver, preferentially by the liver nonparenchymal cells, after intravenous administration to mice.[4] Recently, we confirmed that Kupffer cells, liver resident macrophages, and liver sinusoidal endothelial cells have a significant role in the hepatic uptake and degradation of pDNA.[5] *In vitro* studies using cultured mouse peritoneal macrophages demonstrated that pDNA uptake was dramatically inhibited by defined poylanions, such as polyinosinic acid (poly[I]) and dextran sulfate, but not by polycytidylic acid (poly[C]).[6] A similar inhibition profile was observed for the *in vivo* hepatic uptake of pDNA following intravenous administration.[4] These results suggested that a specific receptor, like the class A scavenger receptor (SRA), could be involved in the endocytotic uptake of pDNA by macrophages. However, the pDNA uptake was not significantly altered in macrophages isolated from SRA-knockout mice and in SRA-knockout mice *in vivo*.[7] More recently, it was found that a murine DC cell-line, DC2.4, takes up pDNA by a similar specific mechanism more efficiently and rapidly than macrophages.[8]

Macrophages and DCs are known to secrete a large amount of inflammatory cytokines following recognition of unmethylated CpG motifs in pDNA via TLR9 as a danger signal.[9] These inflammatory cytokines significantly reduce transgene expression of pDNA, because they may lead the transfected cells to undergo cell death or shut off the viral promoter-based expression.[10,11] Meanwhile, these cytokines are essential for increasing the pDNA-induced immune response following DNA vaccination.[3,12] Our previous study has demonstrated that, in contrast to macrophage cell-lines, primary cultured mouse peritoneal macrophages secreted almost no inflammatory cytokines upon stimulation with pDNA, in spite of extensive uptake of the CpG DNA.[13] However, not only pDNA but also vertebrate DNA, and calf thymus DNA (CT DNA) can activate the murine macrophages to induce inflammatory cytokines, when complexed with cationic liposomes.[14] Similar results were obtained in murine bone marrow DCs cultured with Flt-3 ligand,[15] indicating that endosomal translocation of vertebrate DNA can activate macrophages and DCs in a CpG-independent manner. Furthermore, recent studies using macrophages and DCs isolated from TLR9-knockout mice have demonstrated that both pDNA and CT DNA can activate the TLR9-independent pathway.[15–17]

In spite of a growing body of information on pDNA uptake and cellular activation characteristics in mouse cells, there is insufficient information about human cells. Very recently, we studied the cellular uptake and activation characteristics of naked pDNA and its cationic liposome complex in human macrophagelike cell-lines, U937 cells and THP-1 cells.[18] The present study was carried out to expand the knowledge to human cells in primary culture, which would provide us with more important information for DNA-based therapy. In the present study, we used three types of human monocyte-derived cells, that is, monocytes, macrophages, and DCs, to examine the uptake and subsequent cellular activation by pDNA in both naked and complexed forms.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) and Ficoll-Paque were obtained from Amersham (Buckinghamshire, England). poly[I] (MW 103.3 kDa), poly[c] (MW 99.5 kDa), and dextran (MW 70 kDa) were purchased from Pharmacia (Uppsala, Sweden). Dextran sulfate (MW 150 kDa) was purchased from Nacalai Tesque (Kyoto, Japan). pCMV-Luc encoding firefly luciferase gene constructed previously was used as a model pDNA. CT DNA, used as DNA with less CpG motifs, was purchased from Sigma (St Louis, MO). Synthetic phosphorothioate ODN were purchased from Hokkaido System Science Co. Ltd (Sapporo, Japan). The sequence of CpG S-ODN2006 is 5′-TCGTCGTTTTGTCGTTTTGT-CGTT-3′, a proven activator of human immune cells.[19] Phosphorothioate non-CpG ODN 2006GC (5′-TGCTGCTTTTGTGCTTTTGTGCTT-3′) was used as a control. LipofectAMINE 2000 (LA) and Opti-MEM were purchased from Lifetechnologies (Rockville, MD). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). GM-CSF and IL-4 were purchased from Pepro-Tech. Bovine serum albumin (BSA) derivatives, that is, galactosylated BSA (Gal-BSA), mannosylated BSA (Man-BSA), and maleylated bovine serum albumin (Mal-BSA) were synthesized as described previously.[4] All other chemicals used were of the highest purity available.

Harvesting and Culture of Primary Human Monocyte-Derived Cells

After obtaining informed consent, fresh whole blood was withdrawn from healthy donors and transferred into tubes. Peripheral blood mononuclear cells (PBMC) were obtained from the buffy coat of healthy donors through Ficoll-Hypaque centrifugation in order to prepare monocytes, macrophages, [20] and DCs.[21] Briefly, $CD14^+$ monocytes were isolated from PBMC by positive selection using a MACS system, according to the manufacturer's protocol. These cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (1.2 μg/mL). Monocytes were cultured in the presence of GM-CSF (50 ng/mL) to induce monocyte-derived macrophages. For the preparation of monocyte-derived immature DCs, GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) were added to the culture medium. After 6 days, the cells were recovered by washing with PBS to remove all nonadherent DCs and adherent macrophages before starting the experiments. These cells were seeded at 5×10^5 cells in 24-well plates for the experiments.

Plasmid DNA

For the cellular association experiment, pCMV-Luc was radiolabeled using [α-^{32}P]dCTP by nick translation.[22] For the confocal microscopic study, pCMV-Luc was labeled using a Fasttag FL labeling kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). DNA/LA complexes were prepared in a ratio of DNA:LA 1:2 according to the manufacturer's instructions.

Purification of DNA

To minimize the activation by contaminated LPS, each DNA sample was extensively purified by washing with a nonionic detergent, Triton X-114, according to the previously published methods[23,24] with slight modifications as previously described.[12] DNA samples were purified by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and ethanol precipitation. DNA (10 mg) was diluted with 20 mL pyrogen-free water, and then 200 μL Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55°C. Subsequently, the solution was centrifuged for 20 min at 25°C, 600*g*. The upper phase was transferred to a new tube, 200 μL Triton X-114 was added, and the previous steps were repeated three or more times. The activity of LPS was measured by limulus amebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After Triton X-114 extraction, the endotoxin levels of DNA samples could no longer be determined by LAL assay; that is, 1 μg/mL DNA contained less than 0.001 EU/mL. Without extraction of endotoxin by Triton X-114, 100 μg/mL naked pDNA contained 1–5 EU/mL endotoxin.

Cellular Association Experiments

Cells were washed with 0.5 mL HBSS without phenol red and 0.5 mL HBSS containing 0.1 μg/mL naked [^{32}P]pDNA or [^{32}P]pDNA/LA complex was added. After incubation at 37°C or 4°C for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1 mL 0.3 M NaOH containing 0.1% Triton X-100. Aliquots of the cell lysate were taken for the determination of ^{32}P radioactivity using an LAS-500 scintillation counter (Beckman, Tokyo, Japan) and protein content was measured using a modified Lowry method with BSA as a standard. Unlabeled macromolecules, such as poly[I], poly[C], dextran, and dextran sulfate, were added to the incubation wells concomitantly with [^{32}P]pDNA to examine the effects of those macromolecules on the binding of [^{32}P]pDNA.

Precipitation of Degraded pDNA

After the cells were incubated with [^{32}P]pDNA in the naked form in the cellular association experiments at 37°C, the medium and the cell lysate were separately collected and subjected to trichloroacetic acid (TCA) precipitation experiments to assess the degradation of [^{32}P]pDNA following cellular uptake by monocyte-derived cells as previously described.[8] A portion of the supernatant was used directly for radioactivity counting as described above. After extraction with 10 mM Tris-HCl, 1 mM EDTA (TE)-saturated phenol buffer (pH 7.8), aliquots of the supernatant were mixed with TCA to give a final concentration of 5% (w/v), kept on ice for 10 min, and then

DOI 10.1002/jps

centrifuged at 9000*g* for 30 min at 4°C. The supernatant (TCA-soluble fraction) was used for radioactivity counting and the amount of degradation products of ^{32}P-labeled pDNA was calculated. The TCA-soluble degradation products are small DNA fragments (short oligonucleotides) since 50% precipitation occurs with the 16-mer oligonucleotides in the case of TCA.

Cytokine Secretion

Prior to the addition of naked or complexed DNA, monocyte-derived cells were washed with PBS. Naked DNA and DNA/LA complex were diluted in Opti-MEM. Following the addition of DNA/LA complexes to cells, the cells were incubated for 2 h and then the solution was removed and the cells were incubated with RPMI 1640 with 10% FBS medium continuously for specified periods up to 24 h. When naked pDNA was added to cells, the cells were incubated with Opti-MEM up to 24 h. After incubation, the medium was collected to measure the cytokines. We measured TNF-α, one of the typical inflammatory cytokines, which was reported to reduce transgene expression significantly,[10,19] as an indicator of cellular activation, although type I interferon response is also indicative to the activation in human cells.[15,19] The levels of TNF-α in the conditioned medium were measured using a Human TNF-α ELISA set (eBioscience, San Diego, CA) according to the manufacturer's instructions.

RESULTS

Cellular Uptake and Degradation of Naked pDNA in Human Primary Monocyte-Derived Cells

Figure 1 shows the time-courses of the cellular association of pDNA in three types of human monocyte-derived cells. A extensive cellular association of [^{32}P]pDNA was observed for monocytes (Fig. 1A), however, there was no significant difference between the amounts at 37°C and 4°C, suggesting that only binding to the cell surface occurred. On the other hand, macrophages and DCs appeared to significantly take up [^{32}P]pDNA in a temperature-dependent manner (Fig. 1B and C). TCA-soluble products increased with time in the culture medium of macrophages and DCs incubated with naked [^{32}P]pDNA in the cellular association experiments at 37°C (Fig. 2B and C) whereas this was not the case in the culture medium of monocytes (Fig. 2A). DCs degraded [^{32}P]pDNA more efficiently than macrophages. These results indicate that cellular association profiles are different between human monocyte-derived cells and macrophages and that DCs, but not monocytes, significantly take up and degrade pDNA.

Cellular Association of pDNA/LA Complex in Human Monocyte-Derived Cells

Figure 3 shows the time-courses of the cellular association of [^{32}P]pDNA/liposome complex in the

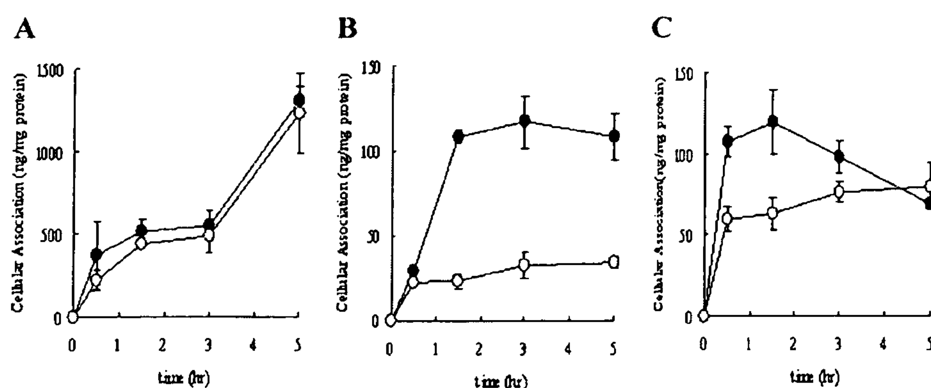

Figure 1. Time-courses of the cellular association of [^{32}P]pDNA with (A) monocytes, (B) macrophages, and (C) DCs. The cells were incubated with [^{32}P]pDNA (0.1 μg/mL) at 37°C (closed circle) or 4°C (open circle). Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small. The percentages of the cellular association with monocytes, macrophages, and DCs were 27.1%, 5.0%, and 9.5% of added pDNA at 5 h, respectively.

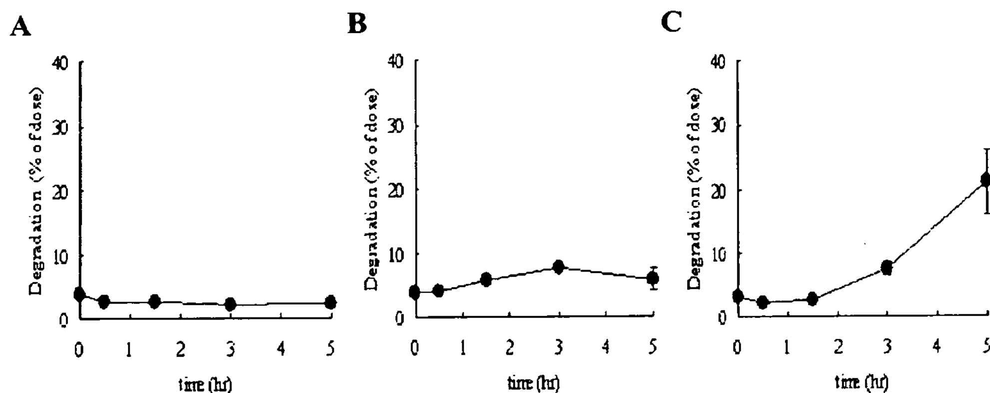

Figure 2. Time-courses of TCA soluble radioactivity in (A) monocytes, (B) macrophages, and (C) DCs in culture medium. The cells were incubated with [^{32}P]pDNA (0.1 μg/mL) at 37°C. Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small.

human monocyte-derived cells. In contrast to naked pDNA, similar cellular association profiles were observed. [^{32}P]pDNA/liposome complex was taken up by all types of cells in a temperature-dependent manner. The apparent uptake rate for the initial period was higher in DCs (Fig. 3C) and lower in macrophages (Fig. 3B), compared with that in monocytes (Fig. 3A).

Effect of Various Polyanions on the Binding of Naked pDNA to Human Primary Monocyte-Derived Cells

The binding characteristics of naked [^{32}P]pDNA were examined by competition experiments using various macromolecules. As shown in Figure 4, the binding of [^{32}P]pDNA was significantly inhibited by poly[I], dextran sulfate, or poly[C], but not by dextran in all types of monocyte-derived cells at 4°C. Figure 5 shows the effects of various modified BSAs on the cellular binding of [^{32}P]pDNA to the three types of cells. The binding was significantly inhibited by Mal-BSA with negative charges while synthetic glycoproteins, such as Man-BSA and Gal-BSA, did not show any inhibition. These results also suggest that the recognition of pDNA by these monocyte-derived cells is based on the negative charges of the molecule.

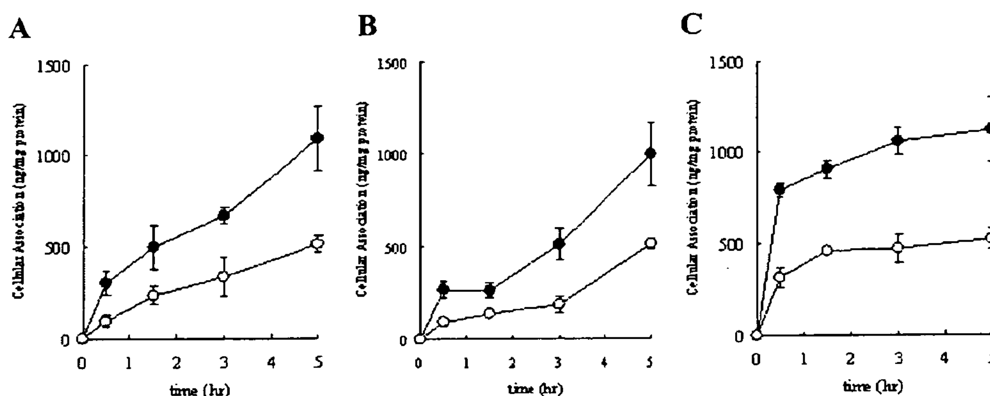

Figure 3. Time-courses of cellular association of [^{32}P]pDNA/LA complex with (A) monocytes, (B) macrophages, and (C) DCs. The cells were incubated with [^{32}P]pDNA/LA (0.1 μg/mL: 0.2 μg/mL) at 37°C (closed circle) or 4°C (open circle). Each point represents the mean ± SD ($n = 3$). The SD was included in the symbol when it was very small. The percentages of the cellular association with monocytes, macrophages, and DCs were 34.7%, 34.4%, and 72.0% of added pDNA at 5 h, respectively.

DOI 10.1002/jps

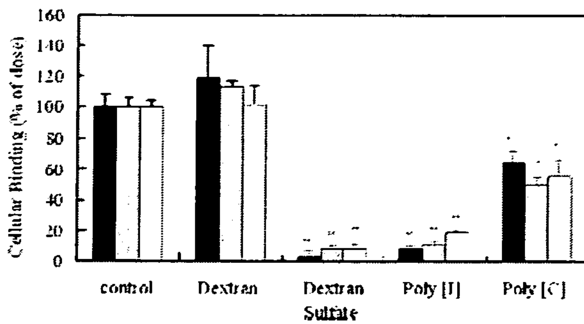

Figure 4. Inhibition of cellular binding of [^{32}P]pDNA with monocytes (black bar), macrophages (gray bar), and DCs (white bar) by macromolecules at 4°C. The cells were incubated with [^{32}P] pDNA (0.1 μg/mL) for 3 h in the presence or absence of the various macromolecules (50 μg/mL). The results are expressed as mean ± SD ($n = 3$). Statistical significance was analyzed by the Student t-test. *$p < 0.05$, **$p < 0.01$ versus control.

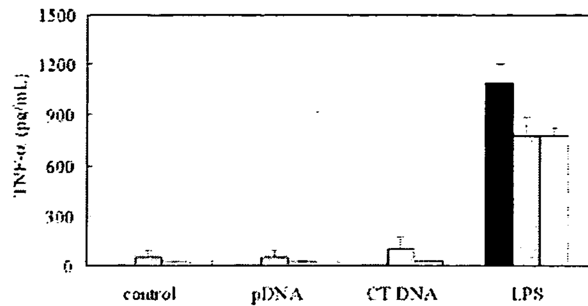

Figure 6. TNF-α release induced by naked DNA and from monocytes (black bar), macrophages (gray bar), and DCs (white bar). The cells were incubated with pDNA or CT DNA (10 μg/mL) or LPS (10 ng/mL a positive control). The control cells were treated with medium alone. The supernatants were collected after 24 h. TNF-α levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

Cellular Activation Stimulated by Naked DNA or DNA/LA Complex in Human Monocyte-Derived Cells

To evaluate the activation of human monocyte-derived cells by pDNA, the cells were incubated with various naked pDNAs and CT DNAs or their cationic liposome complexes. Figure 6 illustrates TNF-α production following stimulation with naked pDNA, CT DNA, and LPS as a positive control. Neither pDNA nor CT DNA induces a significant amount of TNF-α in any of the cells even at a high concentration of 100 μg/mL. A synthetic CpG S-ODN2006 (10 μM) having active sequence for human cells [19] also failed to induce TNF-α production from the human monocyte-derived cells (data not shown), suggesting these cells express no significant TLR9. Only LPS showed cellular activation to secrete a significant amount of TNF-α. We also carried out the experiments using pDNA or CT DNA complexed with LA. The pDNA/LA complex stimulated macrophages to produce a large amount of TNF-α whereas no significant TNF-α production was observed for monocytes and DCs (Fig. 7). Cationic liposomes alone were unable to stimulate the

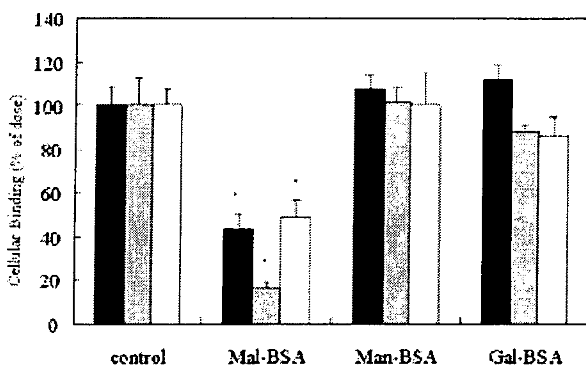

Figure 5. Inhibition of cellular binding of [^{32}P]pDNA to monocytes (black bar), macrophages (gray bar), and DCs (white bar) by modified BSA at 4°C. The cells were incubated with [^{32}P] pDNA (0.1 μg/mL) for 3 h in the presence or absence of the various forms of modified BSA (250 μg/mL). The results are expressed as mean ± SD ($n = 3$). Statistical significance was analyzed by the Student t-test. *$p < 0.01$ versus control.

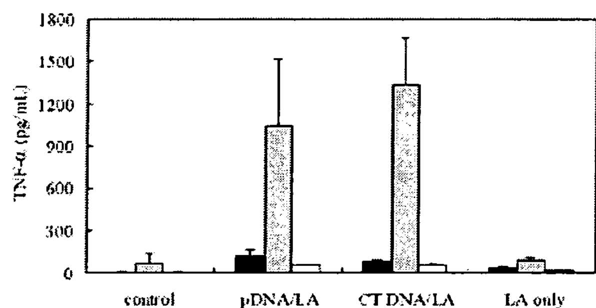

Figure 7. TNF-α release induced by DNA/LA complex from monocytes (black bar), macrophages (gray bar) and DCs (white bar). The cells were incubated with pDNA or CT DNA/LA complex (2.5 μg: 5 μg/well) or LA only (5 μg/well). After 2 h incubation, liposomes were removed and Opti-MEM was added to the cells. The control cells were treated with medium alone. The supernatants were collected 24 h after incubation with liposomes. TNF-α levels were determined by ELISA. Each result represents the mean ± SD ($n = 3$).

macrophages sufficiently to release TNF-α, suggesting that pDNA is indispensable for TNF-α production by the liposome formulation. Interestingly, CT DNA complexed with cationic liposomes also induced TNF-α secretion from the macrophages. These results suggest that the DNA/cationic liposome complex activates human macrophages independent of the amount of CpG motifs contained in DNA.

DISCUSSION

The present study demonstrated that the naked pDNA uptake characteristics were different among human monocyte-derived cells in primary culture (Figs. 1 and 2). An efficient uptake and subsequent degradation of naked pDNA was observed in DCs. Macrophages also showed a similar profile with less degradation activity than DCs, while only significant binding occurred in monocytes. pDNA was more efficiently taken up and rapidly degraded by DCs than by macrophages. In mouse macrophages and DCs, endosomal acidification inhibitors, such as bafilomycin A and chloroquine, significantly inhibited degradation of naked [^{32}P]pDNA (unpublished results), suggesting that the degradation would occur in acidic milieu in the cells, probably in lysosomes after endocytosis.[25] A similar phenomenon might take place in the case of human macrophages and DCs.

In spite of the different characteristics involving pDNA uptake, the effect of polyanions on naked pDNA binding to the cellular surface was similar in all types of monocyte-derived cells (Figs. 4 and 5). pDNA binding to these monocyte-derived cells was significantly inhibited by an excess of some polyanions, such as poly[I] or dextran sulfate, but not by dextran (Fig. 4). poly[C] showed a significant but much lower degree of inhibition. Among BSA derivatives, only Mal-BSA with strong negative charges showed an inhibitory effect (Fig. 5). This inhibition profile by polyanions was almost identical to our previous observations in mouse macrophages,[7] DCs,[9] and a human macrophagelike cell-line.[18] poly[G], poly[I], and other particular polynucleotides are known to form a base-quartet-stabilized four-strand helix (quadruplex),[26] which would give a high density of negative charges. These negative charges might be important for pDNA binding in all types of monocyte-derived cells. These results suggest that a mechanism, by which the polyanionic nature of pDNA is recognized by the putative receptor, might be involved in the uptake of pDNA by the monocyte-derived macrophages and DCs in the primary culture. However, pDNA binding of a similar nature in terms of inhibition by polyanions did not result in uptake by the monocytes. One possible explanation is involvement of fibronection in DNA binding. Fibronection is a ubiquitous protein comprising extracellular matrix, which is reported to bind DNA with a similar affinity to RNA (poly [I] vs. poly [C]) observed in this study (Fig. 4).[27] We consider both putative receptor(s) and fibronection would be involved in DNA uptake in three types of monocyte-derived cells. We speculate that contribution of binding to fibronection, which does not result in internalization, might be higher in monocytes than DCs and macrophages. Further studies are required to clarify the uptake characteristics of naked pDNA.

In contrast to naked pDNA, the cellular association profiles of pDNA/LA complex were similar in all types of monocyte-derived cells (Fig. 3). These results suggest that the pDNA/cationic lipid complex is taken up by a nonspecific mechanism based on electrostatic interaction.

pDNA is a bacterial DNA that can be distinguished from the vertebrate form as far as replete unmethylated CpG dinucleotides (CpG motifs) are concerned. Mouse macrophages, DCs, and B cells are known to recognize unmethylated CpG motifs in pDNA or bacterial DNA via TLR9 as a danger signal to release inflammatory cytokines.[9] With regard to human cells, extensive studies have been carried using synthetic oligonucleotides (ODN) containing a variety of CpG motifs. However, the effects of CpG motifs contained in pDNA on human cells are not fully understood.

In the present study, the cellular activation induced by naked DNA or DNA/cationic liposome complex in the human monocyte-derived cells was investigated. None of the types of monocyte-derived cells released a significant amount of TNF-α by stimulation of naked DNA with replete CpG motifs or DNA with significantly fewer CpG motifs. In human immune cells, it is known that plasmacytoid DCs (pDCs) and B cells, but not monocytes or monocyte-derived immature DCs, express TLR9 and mainly recognize CpG motifs in DNA.[28,29] Siren et al.[30] reported that TLR9 mRNA was not detected in human monocyte-derived macrophages while it was reported that the response to pDNA disappeared in DCs from TLR9 knockout mice.[31] Probably, the lack of response to CpG DNA in human monocyte-derived

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cells in the present study (Fig. 6) is due to the absence or very limited expression of TLR9. The results of synthetic CpG ODN also support this speculation.

DNA/cationic liposome complex is also used as a gene vector or carrier in human clinical trials of nonviral gene therapy. However, the pDNA/cationic liposome complex is well known to induce a high amount of inflammatory cytokines in *in vivo* studies in mice.[32–34] In the present study, when pDNA was complexed with cationic liposomes, a significant amount of TNF-α was released from human macrophage, although TNF-α was not induced by naked pDNA. Moreover, CT DNA containing less CpG motifs could also induce cellular activation in the complexed form. Recently, macrophages and DCs obtained from TLR9$^{-/-}$ mice were activated by DNA/cationic liposomes in a CpG motif-independent manner,[15–17] indicating that cellular activation can be induced through a TLR9-independent pathway. Our recent study using U937 cells also demonstrated that pDNA complexed with cationic liposomes activates the human macrophagelike cells to produce TNF-α independent of the amount of CpG motifs.[18] Together with this finding, the results obtained in the present study suggest that, among monocyte-derived cells, human macrophages are activated by DNA/cationic liposome complex independent of the amount of CpG motifs. This cellular activation might take place through a TLR9-independent pathway based on the literature information on TLR9 expression [30] and inability of CpG ODN in our cellular activation experiments, although we did not confirm the expression of TLR9 in this study.

In conclusion, we have demonstrated that the mechanisms involved in the uptake of pDNA by these cells has similar characteristics in terms of the specificity of ligand recognition among monocyte-derived cells, although the uptake properties of [^{32}P]pDNA were different. The pDNA/cationic liposome complex was recognized as a danger signal by human macrophages, although the underlying mechanism remains to be elucidated. These findings represent an important basis for the development of safer DNA-based therapy including gene therapy and DNA vaccination.

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 DOI 10.1002/jps

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Journal of Controlled Release 115 (2006) 226–233

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Inhibition of peritoneal dissemination of tumor cells by single dosing of phosphodiester CpG oligonucleotide/cationic liposome complex ☆

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Received 24 April 2006; accepted 1 August 2006

Available online 12 August 2006

Abstract

Although unmethylated CpG dinucleotide-containing oligodeoxynucleotides (CpG ODN) are able to inhibit tumor metastasis through the induction of antitumor immunity, their stability and delivery to antigen presenting cells needs to be improved. In this study, we formulated a CpG ODN complex with cationic liposomes (CpG ODN-lipoplex) and its antitumor activity was evaluated in peritoneal dissemination models of tumor cells stably labeled with firefly *luciferase* gene. A single intraperitoneal administration of CpG ODN-lipoplex greatly reduced the number of tumor cells to 0.01% or lower compared with that detected in untreated mice, which may be associated with increased production of TNF-α and IL-12. CpG ODN-lipoplex increased the survival time of the tumor-bearing mice, and most long-term survivors rejected rechallenged tumor cells. These results indicate that a single dosing of CpG ODN-lipoplex is effective in inhibiting peritoneal dissemination and inducing long-lasting antitumor immunity.

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Keywords: CpG ODN; Cationic liposomes; Peritoneal cavity; TNF-alpha; IL-12

1. Introduction

The vertebrate immune system has evolved to recognize certain patterned nucleotide sequence motifs present in the bacterial genome and to elicit immunological reactions to counteract bacterial infections [1]. Previous studies have demonstrated that CpG dinucleotides are present at the expected frequency (1/16 dinucleotides) in bacterial DNA, but they are only about one-quarter as prevalent in vertebrates, and are mostly methylated [2,3].

Recognition of CpG dinucleotides by the innate immune system requires engagement of Toll-like receptor (TLR)-9 on antigen presenting cells [4]. In general, CpG DNA stimulates B cells, dendritic cells (DCs), and monocytes/macrophages to produce cytokines that polarize the T-cell response towards Th-1. However, the level of immune stimulatory effects of DNA depends to a great degree on the precise bases flanking the CpG dinucleotide. Together with the one or two bases on its 5′ and 3′ sides, the CpG dinucleotide comprises a CpG motif. Immunostimulatory activities of bacterial DNA can be mimicked by synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) [5–8]. By examining many possible base combinations, the optimal CpG motif for activating murine immune cells was found to be GACGTT [3,9,10].

The efficacy of CpG ODN in preventing or treating tumor progression or metastasis has been examined in several experimental models [15]. For cancer immunotherapy, the phosphodiester (PO) backbone of CpG ODN is generally substituted by phosphorothioate (PS) to increase the stability against nuclease degradation [11]. PS-type ODN, however, induces potent systemic toxic effects, such as transient anticoagulant activity, activation of complement cascade, and inhibition of basic fibroblast growth factor binding to surface receptors [12–14]. Therefore, we use cationic liposome to prevent enzymatic degradation

☆ Grant sponsor: Ministry of Health, Labour and Welfare, Japan.

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doi:10.1016/j.jconrel.2006.08.002

of PO-type CpG ODN as substitute for PS modification. Cationic liposomes have been extensively employed as a vehicle for plasmid DNA in gene therapy trials, because they protect plasmid DNA from degradation, enhance its cellular uptake, and greatly improve its transgene expression activity [15]. They are also thought to be useful for the delivery of PO-type ODN, so they are capable of stabilizing PO-type ODN without severe toxic effects. A previous study demonstrated that intravenous injection of lipopolyplex consisting of cationic liposomes, protamine sulfate and PO-CpG ODN inhibited tumor growth in mouse lung [16]. However, there has been no report about the antitumor effect of PO-type CpG ODN complexed with cationic liposomes on peritoneal dissemination. Because immune cells will be involved in the antitumor effects of the ODN complex, its antitumor effects would be dependent on the route of its administration and the metastatic sites of the tumor cells.

In this study, we designed a 26-mer PO-type CpG ODN based on several reports examining the requirements of CpG ODN for immune activation. Then, the CpG ODN/cationic liposome complex, or lipoplex, was formulated and its antitumor activity was examined in experimental peritoneal dissemination models in mice. Using the clones of murine melanoma B16-BL6 cells and colon26 cells stably transfected with firefly *luciferase* gene, the process of tumor metastasis was examined in mice by measuring the luciferase activity in the peritoneal organs after intraperitoneal injection of the luciferase-expressing tumor cells (Hyoudou et al, unpublished data). Our data show that a single administration of CpG ODN-lipoplex induces antitumor Th-1 type cytokines in the abdominal cavity, resulting in the suppression of tumor cell growth, and rejection of peritoneal dissemination.

2. Materials and methods

2.1. Animals

Male C57/BL6 (6-week-old) mice and male CDF1 (6-week-old) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

2.2. Chemicals

Dulbecco's modified Eagle's minimum essential medium (DMEM), RPMI1640 medium, phosphate buffered saline (PBS), and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from GIBCO-Invitrogen (Tokyo, Japan). All other chemicals were of the highest grade available. *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan) and cholesterol was from Nacalai Tesuque (Kyoto, Japan). Oligonucleotides with phosphodiester backbones were purchased from Operon (Tokyo, Japan). The sequences of ODNs were 5′-TCGACGTTTTGACGTTTTGACGTTTT-3′ (CpG ODN), 5′-CCCAGGGATCTCCACCTCCTGACACT-3′ (random ODN) and 5′-TGCAGCTTTTGAGCTTTTGAGCTTTT-3′ (GpC ODN). The levels of TNF-α, and IL-12 (p70) in the peritoneal lavage fluids and culture supernatant were determined by the OptEIA™ set (BD Pharmingen, San Diego, CA).

2.3. Cell lines

Murine melanoma B16-BL6 tumor cells [18], obtained from the Cancer Institute of Japan (Tokyo, Japan), were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.15% $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in humidified air containing 5% CO_2. Murine adenocarcinoma colon26 tumor cells [19] and murine macrophage-like RAW264.7 cells were also grown in 5% CO_2 in humidified air at 37 °C with RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. B16-BL6 cells that stably express the firefly *luciferase* gene (B16-BL6/Luc) were established as previously reported [17]. Colon26 cells expressing the same gene (colon26/Luc) were also established by the same protocol as that for B16-BL6/Luc cells.

2.4. Preparation of lipoplex

Mixtures of DOTMA and cholesterol (1:1 in molar ratio) were dissolved in chloroform, vacuum-desiccated, and hydrated by vortexing in 3 ml sterilized 5% dextrose to give a final concentration of 6 mg/ml total lipids. After hydration, the dispersion was sonicated for 3 min in a bath sonicator at 37 °C. The lipid solution was frozen in liquid nitrogen for 2 min and thawed in a water bath for 6 min at 37 °C. The dispersion was passed through a MILLEX®-GV 0.45 μm filter unit (Millipore, Bedford, MA, USA). Liposomes with a diameter of 214.7± 6.7 nm were obtained using dynamic light scattering (Zetasizer nano ZS, Malvern Instruments, UK). Cationic liposomes and CpG ODN in 5% dextrose were mixed at a charge ratio of 1:2.3 (−/+), i.e., 7.5 μg total lipid per 1 μg ODN, and left at 37 °C for 30 min to form a cationic liposome/CpG ODN complex (CpG ODN-lipoplex) [20]. Random ODN and GpC ODN were also mixed with cationic liposomes to obtain random ODN-lipoplex and GpC ODN-lipoplex, respectively.

2.5. Induction of peritoneal dissemination in mice

B16-BL6/Luc cells and colon26/Luc cells were trypsinized and suspended in HBSS. B16-BL6/Luc cells (5.0×10^4 cells in 100 μl HBSS) or colon26/Luc cells (1.0×10^5 cells in 100 μl HBSS) were inoculated intraperitoneally into male C57BL/6 mice or male CDF1 mice, respectively.

2.6. Antitumor effect of CpG ODN administration

Soon after tumor inoculation (within 1 min), saline, 5% dextrose, CpG ODN (10 μg), CpG ODN-lipoplex (ODN: liposome = 1 μg:7.5 μg or 10 μg:75 μg), random ODN-lipoplex

(ODN:liposome = 10 μg:75 μg), GpC ODN-lipoplex (ODN: liposome = 10 μg:75 μg), or cationic liposomes (75 μg) were administered into the peritoneal cavity of mice (200 μl 5% dextrose solution/mouse). Seven days after tumor inoculation, the mice were euthanized by cervical dislocation and the intraperitoneal organs (greater omentum, peritoneum, spleen, kidney, and liver) were excised and washed with ice-cold saline. For investigation of the administration route-dependent activity of CpG ODN-lipoplex, CpG ODN-lipoplex was administered intraperitoneally, intravenously, or intradermally (100 μl 5% dextrose solution/mouse). Then the organs were homogenized in a lysis buffer (0.05% Triton X-100, 2 mmol/l EDTA, 0.1 mol/l Tris pH 7.8), and centrifuged at 10,000 g for 10 min. Ten milliliter of the supernatant was mixed with 100 μl of a luciferase assay buffer (Picagene, Toyo Ink, Tokyo Japan), and the light produced was measured with a luminometer (Lumat LB 9507, EG and G Berthold, Bad Wildbad, Germany). The luciferase activity of the peritoneal organs was converted to the number of B16-BL6/Luc cells using a regression line as previously reported [16]. The luciferase activity derived from colon26/Luc cells was also converted to a cell number using an established regression line. The regression line gave a constant of 3 to 5 RLU/sec/cell for the quantification of the number of B16-BL6/Luc cells and colon26/Luc cells. Measuring the *luciferase* activity of greater omentum homogenates mixed with B16-BL6/Luc cells or colon26/Luc cells showed that ≥300 cells were enough for the detection of these cells (data not shown).

2.7. Effect of CpG ODN on survival of tumor-bearing mice

For investigation of the survival rate of tumor-bearing mice, CDF1 mice were injected intraperitoneally with colon26/Luc cells. Soon after tumor inoculation (within 1 min), mice were treated with saline, liposome only, CpG ODN, CpG ODN-lipoplex, and random ODN-lipoplex intraperitoneally (the number of mice was at least 8 in each group). On day 80, survivors were inoculated again with colon26/Luc cells intraperitoneally. Those surviving then received an injection of colon26/Luc cells into the tail vein 144 days after the first challenge.

2.8. Detection of cytokines produced by RAW264.7 cells

RAW264.7 cells were plated on 24-well culture plates at a density of 1×10^6 cells/ml and cultured for 24 h. Cells were washed three times with 0.5 ml PBS before use. Naked CpG ODN (2.5 μg) CpG ODN-lipoplex and GpC ODN-lipoplex (ODN:liposome = 2.5 μg:18.75 μg)were diluted in 0.5 ml Opti-MEM (Invitrogen). The cells were incubated with Opti-MEM containing lipoplex for 2 h, then, washed three times with PBS and incubated with Opti-MEM for 8 h. The supernatants were collected for ELISA to determine TNF-α.

2.9. Detection of cytokines in peritoneal lavage

C57BL/6 mice intraperitoneally inoculated with B16-BL6/Luc cells received an intraperitoneal injection of CpG ODN-lipoplex, GpC ODN-lipoplex (ODN:liposome = 10 μg:75 μg). One, six, twelve, and eighteen hours later, peritoneal lavage was performed by the injection of 3 ml cold sterile PBS intraperitoneally. The levels of TNF-α and IL-12 (p70) in the peritoneal lavage were determined by ELISA.

2.10. Statistical analysis

Differences were statistically analyzed by Student's *t*-test and Kaplan–Meier analysis with a log-lank test to determine survival.

3. Results

3.1. Inhibition of the proliferation of B16-BL6/Luc cells and colon26/Luc cells in peritoneal organs by CpG ODN-lipoplex

We first examined the number of B16-BL6/Luc cells in the peritoneal organs after inoculation of 5×10^4 cells. About 2×10^6 cells were found in the greater omentum of the saline-treated mice 7 days after tumor inoculation. The number of the cells in the greater omentum accounted for more than 90% of the total number of tumor cells detected in the peritoneal organs (Fig. 1). Because the proliferation of tumor cells in the greater omentum is life-threatening in peritoneal dissemination patients [21], the number of the cells in the greater omentum was used as an indicator of the antitumor activity of each treatment in the following studies.

CpG ODN-lipoplex significantly reduced the number of tumor cells in the greater omentum at a dose of 1 μg ODN (data not shown). When the dose was increased to 10 μg ODN/mouse, the number of tumor cells was further reduced to about 0.1% of that detected in the saline-treated controls (Fig. 2). Unlike the CpG ODN-lipoplex, the random ODN-lipoplex or GpC ODN-lipoplex slightly reduced the number of cells, suggesting an induction of CG sequence-dependent antitumor response. Naked CpG ODN or cationic liposomes hardly reduced the

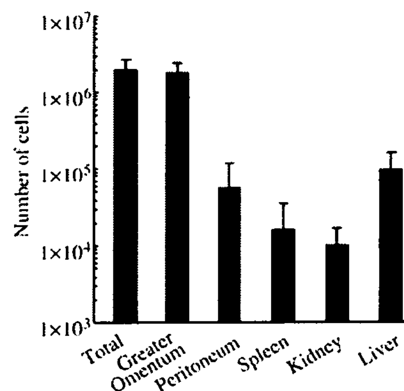

Fig. 1. Number of B16-BL6/Luc cells in peritoneal organs 7 days after inoculation into the peritoneal cavity of mice. C57BL/6 mice were inoculated intraperitoneally with 5×10^4 B16-BL6/Luc cells. Seven days after inoculation, mice were sacrificed and luciferase activity in the greater omentum was measured. Results are expressed as mean+SD of five mice. The experiment shown is representative of two experiments with similar results.

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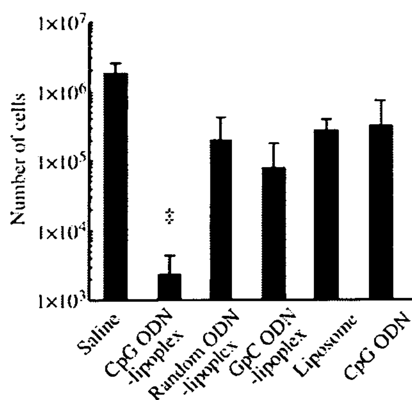

Fig. 2. Effect of CpG ODN-lipoplex on the number of B16-BL6/Luc cells in the greater omentum of mice. C57BL/6 mice were inoculated intraperitoneally with B16-BL6/Luc cells. Soon after tumor inoculation, saline, naked CpG ODN, CpG ODN-lipoplex, random ODN-lipoplex, GpC ODN-lipoplex, and cationic liposomes were administrated into the peritoneal cavity. Seven days after injection, mice were sacrificed and the luciferase activity of the greater omentum was measured. Results are expressed as mean + SD of at least four mice. The experiment shown is representative of two experiments with similar results. ‡ $p<0.01$; The number of cells was significantly different from saline i.p. group.

number of tumor cells. The treatment with 5% dextrose, the vehicle of CpG ODN-lipoplex, showed no significant effects on the number of tumor cells in the greater omentum (data not shown). These results indicate that complexation with cationic liposomes is essential for CpG ODN to inhibit the proliferation of tumor cells in the peritoneal cavity.

Similar experiments were performed using colon26/Luc tumor cells. No luciferase activity was detected in the greater omentum (<300 tumor cells) of mice receiving CpG ODN-lipoplex (10 μg ODN), suggesting that the adhesion and proliferation of the tumor cells was almost completely inhibited by the treatment (data not shown).

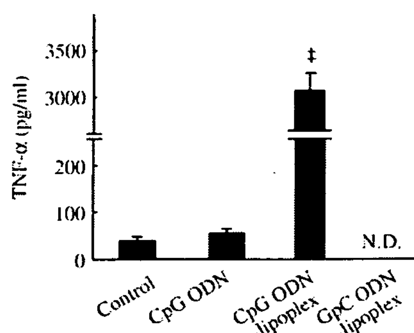

Fig. 3. Secretion of TNF-α from RAW264.7 cells produced by CpG ODN-lipoplex. Naked CpG ODN, CpG ODN-lipoplex or GpC ODN-lipoplex were added to RAW264.7 cells, and incubated for 2 h. Then, the medium was removed and the cells were washed with PBS, and cultured in new medium for 8 h. The presence of cytokines in culture medium was detected by ELISA. Results are presented as mean + SD. The experiment shown is representative of two experiments with similar results. ‡ $p<0.01$; The concentration of TNF-α was significantly different from the control group (t-test). N.D.; not detected (< 16 pg/ml).

3.2. In vitro cytokine production from macrophage-like cell lines

To examine whether macrophages are responsible for the production of the TNF-α by CpG ODN-lipoplex, RAW264.7, mouse macrophage-like cells, were treated with CpG ODN-lipoplex and the concentration of TNF-α and IL-12 (p40) in the supernatants were examined. RAW264.7 cells treated with CpG ODN-lipoplex produced a significant amount of TNF-α (3024± 300 pg/ml), while naked CpG ODN produced less TNF-α (56± 4 pg/ml) (Fig. 3). However, cells treated with GpC ODN-lipoplex produced a level of cytokines that was below the limit of detection (< 16 pg/ml).

3.3. In vivo cytokine production by administration of CpG ODN-lipoplex

The levels of TNF-α and IL-12 (p70) in the peritoneal cavity were below the limits of detection (TNF-α < 16 pg/ml, IL-12 (p70) < 62 pg/ml) at 1 h after intraperitoneal injection of CpG

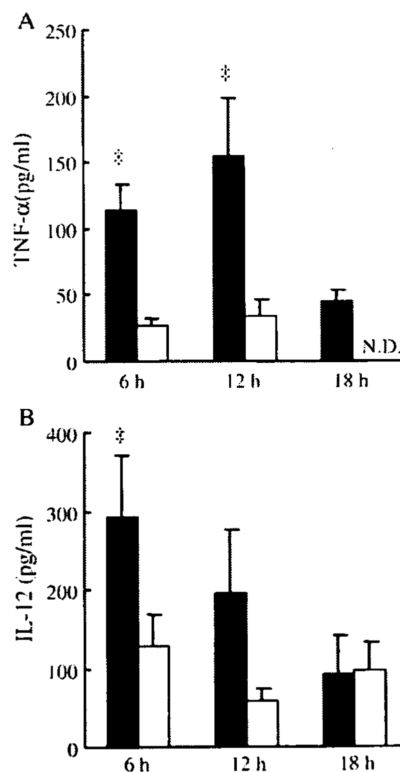

Fig. 4. TNF-α and IL-12 (p70) in peritoneal lavage fluid of mice given an intraperitoneal injection of CpG ODN-lipoplex. B16-BL6/Luc tumor cells were inoculated into the peritoneal cavity of C57BL/6 mice. Soon after tumor inoculation, CpG ODN-lipoplex (closed bars), and GpC ODN-lipoplex (open bars) were administered to the peritoneal cavity. Six, twelve, and eighteen hours later, ascitic fluid was collected and the levels of TNF-α (A) and IL-12 (p70) (B) were measured by ELISA. Results are presented as mean + SD of at least four mice. The experiment shown is representative of two experiments with similar results. ‡ $p<0.01$; The concentration of cytokine was significantly different from GpC ODN-lipoplex i.p. group. N.D.; not detected (TNF-α < 16 pg/ml, IL-12 (p70) < 62 pg/ml).