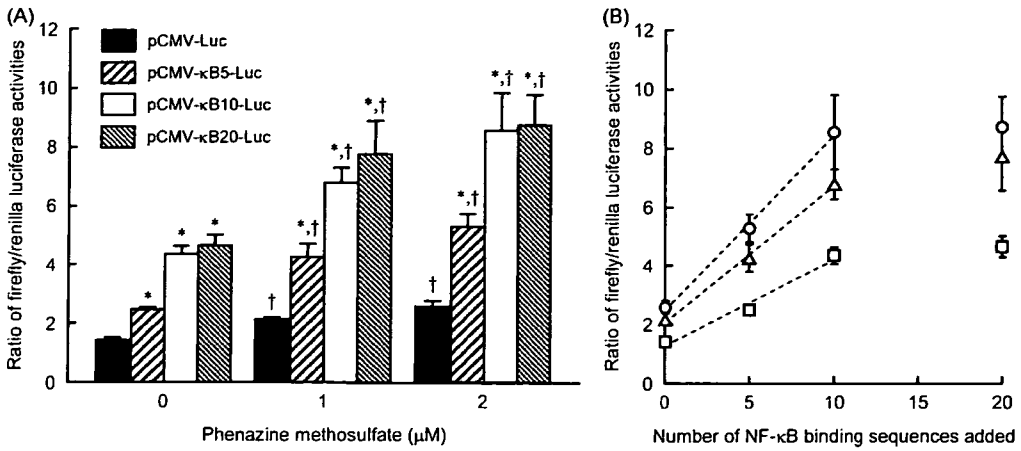

Fig. 4. (A) Effect of PMS on transgene expression in colon 26 cells. Colon 26 cells were transfected with lipoplex consisting of one of the firefly luciferase-expressing pDNAs (0.5 μg per well) and pRL-SV40 (0.1 μg per well) for 4 h. PMS (1 or 2 μM) was added and incubated for 10 h. At 22 h after transfection, firefly and renilla luciferase activities were detected and expressed as the ratio of these activities (mean ± S.D.). *A statistically significant difference ($P<0.05$) compared with the pCMV-Luc group; †a statistically significant difference ($P<0.05$) compared with the no treatment group. (B) Relationship between the transgene expression and the number of NF-κB binding sequences added to pCMV-Luc. Experimental details are described in the legend of (A). The dashed regression lines were obtained using the data, except for those of pCMV-κB20-Luc, and the slopes are 0.29, 0.46 and 0.60 for PMS concentrations of 0 μM (□), 1 (Δ) and 2 μM (○), respectively. Results are expressed as the ratio of the firefly/renilla luciferase activities (mean ± S.D.).

number of NF-κB binding sequences added and the increase in the transgene expression when the number of the NF-κB sequences was 10 or fewer (Fig. 4B). The slope of the regression line became steeper as the PMS concentration increased, suggesting that the advantage of the novel pDNA with many NF-κB binding sequences over a conventional pDNA is marked when the NF-κB in target cells is highly activated.

4. Discussion

Plasmid DNA is unlikely to passively diffuse into the nucleus because its size is much greater than the passive diffusion limit (40–60 kDa) (Ohno et al., 1998). Microinjection of pDNA fragments >2000 bp in length, or any macromolecule >2000 kDa also results in little diffusion into the cytoplasm (Dowty et al., 1995; Lukacs et al., 2000). When pDNA migrates from the cytoplasm to the nucleus, except during mitosis, it must pass through the NPC that is present in the nuclear envelope (Dean et al., 1999). Macromolecules that use the NPC pathway have to be provided with a nuclear localization signal (NLS). However, pDNA does not have any such signals, so this suggests that there may be a certain substance that provides pDNA with NLS. It has been postulated by Dean that binding of transcription factors to their corresponding sequences in pDNA leads to nuclear transport of pDNA through the NPC (Dean et al., 1999). Mesika also reported NF-κB-assisted importation of pDNA into the nuclei of mammalian cells (Mesika et al., 2001). The association of NLS, NF-κB p50, to pDNA not only enhanced the passage of pDNA across the NPC but also facilitated its transport in the cytoplasm (Mesika et al., 2005). Furthermore, we demonstrated by competitive EMSA that the insertion of five repeats of the NF-κB binding sequence into a conventional pDNA increases its binding to NF-κB nuclear protein, resulting in an increased transgene expression (Kuramoto et al., 2006). NF-κB is required for oncogenesis in multiple processes. In addition, some cancer cells depend on NF-κB for their survival (Baldwin, 2001). These findings suggested to us idea that activated NF-κB, which can be found in some cancer cells, can be used as a vehicle for pDNA to enter the nucleus if it contains NF-κB binding sequences. In a previous study, we demonstrated that the insertion of five repeats of the binding sequences into pDNA is effective in increasing the transgene expression in mouse lung after systemic injection as complexes with cationic liposomes. To examine the effect of the number of the binding sequences on transgene expression, we prepared three pDNA preparations in which 5, 10 or 20 additional NF-κB binding sequences were inserted.

The pDNA preparations having additional NF-κB binding sequences showed higher transgene expression than pCMV-Luc in colon 26 cells. There was a trend suggesting that the expression was proportional to the number of added NF-κB binding sequences. However, the expression level by pCMV-κB20-Luc was not significantly higher than that by pCMV-κB10-Luc, suggesting that 10–20 NF-κB binding sequences are optimal for increasing pDNA-based transgene expression. Griesenbach et al. reported that the addition of NF-κB decoy, which is a short double-stranded DNA with an NF-κB binding sequence, into pDNA complex with cationic liposomes reduced the inflammatory response to the complexes (Griesenbach et al., 2000). As demonstrated, NF-κB decoy may prevent the binding of activated NF-κB to its binding sequences in the genome or in pDNA. Because the number of activated NF-κBs is limited, more NF-κB binding sequences in pDNA may act as a decoy and prevent an increase in transgene expression. Ten to 20 NF-κB binding sequences seemed to be optimal for enhanced transgene expression under the present experimental conditions.

The amount of plasmids in the nuclear fraction of transfected cells was measured using real time PCR. A large amount of DNA was detected when the pCMV-κB5-Luc was used for

transfection ($0.89 \pm 0.07\%$ of genome DNA) compared with the pCMV-Luc ($0.72 \pm 0.15\%$), although the difference was not statistically significant ($P = 0.2$) because of the large standard errors and small sample numbers ($n = 3$). These results could support the hypothesis that the nuclear transport is increased by the insertion of the binding sequences into plasmid DNA. However, the differences in transcription efficiency would also contribute to the expression efficiency, because the differences in the transgene expression were much greater than that in the amount of plasmids in the nuclear fraction. The activated NF-κB can bind specifically to its corresponding NF-κB binding sequence in DNA, leading to enhanced transcription and expression of downstream genes (Pahl, 1999). Therefore, the insertion of NF-κB binding sequences into plasmid DNA can increase the nuclear transport of the DNA and the transcription efficiency, both of which will contribute to the increased transgene expression of plasmid DNA with many numbers of the binding sequences.

As demonstrated with several cancer cells, NF-κB is constitutively activated in colon 26 cells under normal culture conditions. The addition of PMS, which is known to induce ROS (superoxide anion and H_2O_2) within cells, will further activate NF-κB. The expression by any pDNA construct was increased when the concentration of PMS added to the cells increased. These results indicate that the transgene expression by pDNA with NF-κB binding sequences is a function of the NF-κB activity of the cells.

In conclusion, we have successfully developed a novel pDNA in which additional NF-κB binding sequences were inserted. We have also clearly demonstrated that insertion of NF-κB binding sequences into plasmid DNA can improve the efficiency of pDNA-based gene transfer in cells where NF-κB activity is high. The level of transgene expression was found to be a function of the level of the NF-κB activity of cells. These results indicate that insertion of NF-κB binding sequences into pDNA is an effective approach to increase transgene expression in cancer cells in which NF-κB is activated

Acknowledgements

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Improved anti-cancer effect of interferon gene transfer by sustained expression using CpG-reduced plasmid DNA

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Plasmid DNA (pDNA) expressing mouse interferon (IFN)-β or IFN-γ (pCMV-Muβ and pCMV-Muγ, respectively) has been shown to be effective in inhibiting the growth of colon carcinoma CT-26 cells in the liver (Kobayashi *et al.*, Molecular Therapy 2002;6:737–44). The therapeutic effect of such IFN gene transfer could be significantly increased by the sustained expression of IFNs. In the present study, CpG-reduced pDNA encoding IFN-β or IFN-γ (pGZB-Muβ and pGZB-Muγ, respectively) was constructed. pCMV-Muβ and pCMV-Muγ were used as conventional CpG-replete pDNAs. Each pDNA was injected into the tail vein of mice by the hydrodynamics-based procedure. An injection of pGZB-Muβ resulted in very high IFN-β activities in the serum for at least 24 hr after injection, whereas the IFN-β activity after pCMV-Muβ injection declined quickly. About a 14-fold greater amount of IFN-β was produced from pGZB-Muβ than from pCMV-Muβ. pGZB-Muβ markedly inhibited the pulmonary metastasis of CT-26 cells. Similar, but more marked results were obtained with pGZB-Muγ: it increased the area under the concentration-time curve by more than a 60-fold and the mean residence time of IFN-γ 4-fold compared with pCMV-Muγ. The survival time of the pGZB-Muγ-treated mice was significantly ($p < 0.05$) longer than that of the saline- or pCMV-Muγ-treated mice. These results indicate that long-term expression of IFN can be achieved by CpG-reduced pDNA and sustained IFN gene expression results in enhanced therapeutic effects of IFN gene transfer against tumor metastasis.
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Key words: interferon; cancer gene therapy; pulmonary metastasis; hydrodynamics; CpG dinucleotides

High and sustained transgene expression is indispensable for effective gene therapy. Generally speaking, persistent gene expression increases the therapeutic benefits of gene transfer and reduces the need for redosing. Although plasmid DNA (pDNA), the most frequently-used nonviral vector, avoids the fatal disadvantages that have been experienced with viral vectors, such as virus-induced acute organ failure and insertional mutagenesis, its transgene expression characteristics need to be improved for effective *in vivo* gene therapy.[1,2] The low level of transgene expression has been unacceptable in pDNA-based nonviral vectors for many years, but the development of highly effective nonviral gene delivery methods has now almost solved the problem. The application of electric pulses or ultrasound can significantly increase the level of transgene expression up to 100-fold or more in various experimental settings. In addition Liu *et al.*[3] and Zhang *et al.*[4] have demonstrated that a very high level of transgene expression can be obtained by an intravenous injection of naked pDNA dissolved in a large volume of saline when injected at a high velocity: the so-called hydrodynamics-based procedure.[3] This method of gene delivery results in a high level of transgene expression in internal organs, particularly the liver, apparently unaccompanied by any severe toxicity.[5,6] Therefore, this method could be applied therapeutically with some modifications.[7] However, the transient nature of transgene expression by nonviral vectors is still a major problem associated with nonviral vector-based gene transfer.

Cytokine-supported tumor immunotherapy is a promising strategy for cancer gene therapy. Interferon (IFN) gene transfer is considered to be useful for immunotherapy because IFNs have antiproliferative and immunomodulatory activities which are capable of contributing to the host's defense against tumors.[8–11] We have reported that IFN-β or IFN-γ gene delivery by the hydrodynamics-based procedure is effective in inhibiting the growth of hepatic metastasis of mouse colon carcinoma CT-26 cells.[12] However, we also found that the IFN gene transfer by this procedure was only marginally effective against pulmonary metastasis of the tumor cells. This was associated with the transient concentration of the IFNs in the lung tissue as well as in plasma after the injection of the conventional pDNA encoding IFN-β or IFN-γ.

Systemic administration of a pDNA/cationic liposome complex, or lipoplex, induces several inflammatory cytokines, hematologic changes, and increases the plasma levels of liver enzymes and acute-phase response proteins.[13,14] Such responses can be used as stimuli for transiently increasing pDNA-based transgene expression through the activation of nuclear factor κB.[15] In addition, the nonspecific induction of inflammatory cytokines would be beneficial for cancer gene therapy. However, inflammatory cytokines are reported to reduce the transgene expression at a later time after gene transfer.[16] Early studies have demonstrated that unmethylated CpG dinucleotides in pDNA play significant roles in the induction of inflammatory cytokines through the Toll-like receptor (TLR)-9 upon administration of lipoplex.[17] The cytosine residue of unmethylated CpG dinucleotides in pDNA can also be a target for methylation by DNA methyltransferases.[18,19] Methylated CpG dinucleotides, in turn, recruit the methyl-CpG binding proteins such as MBD1 and MeCP2, which mediate transcriptional repression of the transgene.[20,21] Therefore, the presence of CpG dinucleotides in pDNA may have two adverse consequences: the generation of an inflammatory cytokine response through TLR-9 and the chronic suppression of transgene expression induced by the methylation of CpG dinucleotides as well as by the binding of methyl-CpG binding proteins.

Several approaches have been reported to reduce the number of CpG dinucleotides in pDNA, including the use of polymerase chain reaction (PCR) fragments of pDNA,[22] methylation of the cysteine in CpG dinucleotides by methylase,[23] and the elimination of the sequences.[24] Yew *et al.*[25–27] have reported that a

Abbreviations: AUC, area under the plasma concentration-time curve; C_{max}, peak plasma concentration; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IFN, interferon; MRT, mean residence time; PCR, polymerase chain reaction; pDNA, plasmid DNA; TLR, Toll-like receptor.

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CpG-reduced pDNA, pGZB, in which about 80% of the CpG sequences were depleted from the original vector, showed sustained and enhanced transgene expression after administration to mice in several formulations, such as the naked pDNA and lipoplex. Therefore, the use of the CpG-reduced pDNA may improve the efficacy of IFN gene therapy in various tumor models. In the present study, we inserted murine IFN-β or IFN-γ cDNA into the pGZB vector and examined first whether the expression of the IFNs could be improved by the vector. The expression of the IFNs was quantitatively evaluated using a moment analysis method and the parameters obtained, such as the area under the plasma concentration-time curve (AUC) and mean retention time (MRT), were compared with those obtained after injection of a conventional CpG-replete vector. Then, we examined the therapeutic efficacy of pGZB-based IFN gene delivery by the hydrodynamics-based procedure against the lung metastasis produced by CT-26 cells in mice.

Material and methods

Cell cultures and mice

A mouse colon carcinoma cell line, CT-26, was cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS). L cells were cultured in 2 g/l glucose-containing Eagle's minimal essential medium supplemented with 6% FCS. Seven-week-old BALB/c mice, purchased from Shizuoka agricultural cooperative association for laboratory animals (Shizuoka, Japan), were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the US National Institutes of health guide for the care and use of laboratory animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences, Kyoto University.

Plasmid DNA

pCMV-Luc, pCMV-Muβ and pCMV-Muγ, which were constructed as previously reported,[28,29] were used as CpG-replete pDNA encoding firefly luciferase, mouse IFN-β and mouse IFN-γ, respectively. pGZB vector,[24–27] a CpG-reduced pDNA that has a backbone different from pCMV vectors, was kindly provided by Dr. Yew (Genzyme Corporation, MA, USA). A fragment of mouse *IFN*-β cDNA was amplified by PCR from pCMV-Muβ, and inserted into the *Sfi*I/*Eco*RI site of the pGZB vector to construct pGZB-Muβ. pGZB-Muγ and pGZB-Luc were also constructed in a similar manner. Each pDNA was injected into the tail vein of mice at the indicated doses dissolved in 1.6 ml saline by the hydrodynamics-based procedure.[3,6] The dose of each pDNA was optimized by preliminary experiments; it was set at 10 μg/mouse for IFN-β-expressing pDNA, which was the same dose as in the previous study.[12] Because the injection of pGZB-Muγ at this high dose was found to be lethal to mice, 3 μg was used for IFN-γ-expressing pDNA.

Measurement of TNF-α concentration

The levels of TNF-α in serum were measured using an ELISA kit (AN'ALYZA™, Genzyme, Cambridge, MA) as reported previously.[15] In brief, mice received an intravenous injection of naked pCMV-Luc or pGZB-Luc at a dose of 25 μg/mouse by the hydrodynamics-based procedure. At 1.5 hr after injection, blood was collected from the vena cava of mice under anesthesia, and allowed to stand for 3 hr at 4°C. Then the samples were centrifuged at 3,000*g* for 30 min at 4°C and the serum obtained was used for the assay.

Measurement of IFN activity

To measure the IFN activity in mouse serum, 50–70 μl blood was collected from the tail vein at indicated times after pDNA injection. The blood samples were kept at 4°C for 2–3 hr to allow clotting and then centrifuged to obtain serum. The antiviral activity of IFN-β in the serum was measured using the cytopathic effect of vesicular stomatitis virus on L cells and expressed in international units (IU) as calibrated against the international reference mouse IFN-α, β preparation (NIH-G002-904-511).[30] The concentration of IFN-γ in the serum was determined by ELISA using a commercial kit (Ready-SET-Go! Mouse IFN-γ ELISA, eBioscience). The peak plasma concentrations (C_{max}) of IFNs were obtained from the actual data recorded after gene transfer. The AUC and MRT were calculated by integration to infinite time.[31] The normal distribution test was performed using the following equation.[32]

$$Z_0 = \frac{\left|\bar{\phi}_1 - \bar{\phi}_2\right|}{\sqrt{SE_1^2 + SE_2^2}}$$

where $\bar{\phi}_1$ and $\bar{\phi}_2$ are the means of pharmacokinetic parameters, and SE_1 and SE_2 are the variances in groups 1 and 2, respectively. If $Z_0 > 1.96$ (confidence interval $p < 0.05$), the difference was assumed to be significant between groups 1 and 2.

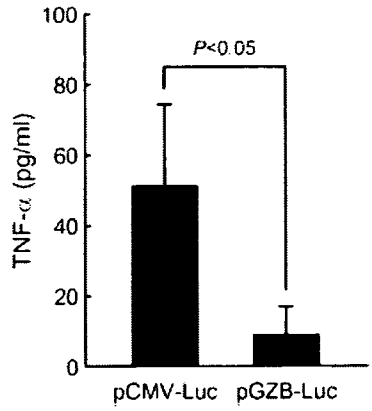

FIGURE 1 – TNF-α level in serum after intravenous injection of pCMV-Luc or pGZB-Luc at a dose of 25 μg/mouse by the hydrodynamics-based procedure. At 1.5 hr after injection, mice were euthanized and blood was collected. Serum concentrations of TNF-α were determined by ELISA. The results are expressed as the mean ± S.D. of 3 mice.

Experimental pulmonary metastasis

CT-26 cells were trypsinized and suspended in Hanks' balanced salt solution (HBSS). The cell suspensions were injected intravenously into syngeneic BALB/c mice at a dose of 1×10^5 cells in 200 μl HBSS/mouse to establish pulmonary metastasis. Mice inoculated with CT-26 cells were injected intravenously with pDNA by the hydrodynamics-based procedure at indicated times. At 14 days after inoculation of the tumor cells, the lung was excised and the number of pulmonary colonies was counted. In addition, the survival of mice was also evaluated in different animals.

Statistical analysis

Data on the number of metastatic colonies were analyzed by one-way ANOVA followed by the Student-Newmann-Keuls multiple comparison test. Survival of mice was analyzed by a Kaplan-Meier survival plot followed by a log-rank (Mantel-Cox) test.

Results

TNF-α production after injection of pCMV-Luc and pGZB-Luc

Figure 1 shows the TNF-α concentration in mouse serum 1.5 hr after intravenous injection of pCMV-Luc or pGZB-Luc at a dose of 25 μg/mouse by the hydrodynamics-based procedure. Previous studies indicated that the TNF-α concentration in serum has a

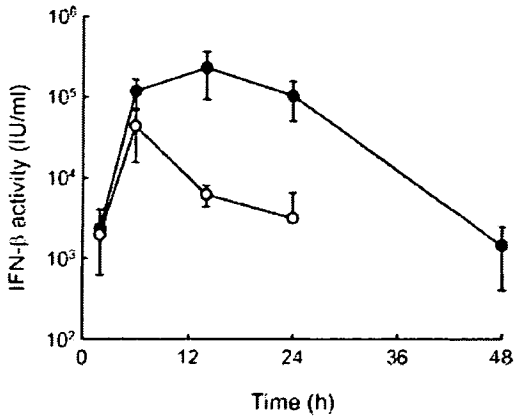

FIGURE 2 – Time-course of the activity of IFN-β in the serum after intravenous injection of pCMV-Muβ (○) or pGZB-Muβ (●) at a dose of 10 μg/mouse by the hydrodynamics-based procedure. The IFN-β activity was measured by bioassay. The activity at 48 hr after injection of pCMV-Muβ was below 400 IU/ml, which is not shown in the figure. The results are expressed as the mean ± SD of 3 mice.

TABLE I – C_{MAX}, AUC AND MRT OF SERUM IFN-β AFTER INTRAVENOUS INJECTION OF PCMV-MUβ AND PGZB-MUβ INTO MICE BY THE HYDRODYNAMICS-BASED PROCEDURE

pDNA	C_{max} (IU/ml)	AUC (IU hr/μl)	MRT (hr)
pCMV-Muβ	43,000 ± 27,800	334 ± 126	8.1 ± 2.6
pGZB-Muβ	227,000 ± 134,000	4,520 ± 650*	16.7 ± 3.6*

The C_{max} values were determined at 6 and 14 h after intravenous injection of pCMV-Muβ and pGZB-Muβ, respectively, by the hydrodynamics-based procedure at a dose of 10 μg/mouse, and are expressed as the mean ± SD of three mice. The AUC and MRT were calculated by integration to infinite time, and are expressed as the calculated mean ± SE.

*Statistically significant ($p < 0.05$) compared with pCMV-Muβ.

peak value around 1.5 hr after injection of various pDNA formulations. The injection of the CpG-replete pCMV-Luc resulted in the peak TNF-α concentration of 51 ± 23 pg/ml, which was significantly greater than that of pGZB-Luc (8.9 ± 8.0 pg/ml). However, these levels of TNF-α production were much lower than those obtained with pDNA/cationic liposome complexes, some of which resulted in a peak TNF-α concentration of 500 pg/ml or greater at the same dose of pDNA.[12,15]

IFN-β activity after injection of pCMV-Muβ and pGZB-Muβ

Figure 2 shows the time-courses of the IFN-β concentrations in serum after intravenous injection of pCMV-Muβ or pGZB-Muβ at a dose of 10 μg/mouse. High IFN-β activities were detected after injection of pCMV-Muβ by the hydrodynamics-based procedure with a peak level of 43,000 ± 27,800 IU/ml at 6 hr after injection. However, the activity declined quickly with time and was below 400 IU/ml by 48 hr. In contrast, the IFN-β activities after injection of pGZB-Muβ were significantly greater than those of pCMV-Muβ at any sampling point except for 2 hr. About a 5-fold greater peak level (227,000 ± 134,000 IU/ml) was obtained at 14 hr after injection, and a significant level of activity could be detected at 48 hr after injection. Table I summarizes the pharmacokinetic parameters of serum IFN-β after injection of each IFN-β-expressing pDNA. The AUC values were calculated to be 334 ± 126 and 4,520 ± 650 IU hr/l after injection of pCMV-Muβ and pGZB-Muβ ($p < 0.05$), respectively, indicating that about a 14-fold greater amount of IFN-β was produced from pGZB-Muβ than from pCMV-Muβ. A long MRT (16.7 ± 3.6 hr) was obtained with pGZB-Muβ, which was statistically ($p < 0.05$) significantly different from that of pCMV-Muβ (8.1 ± 2.6 hr).

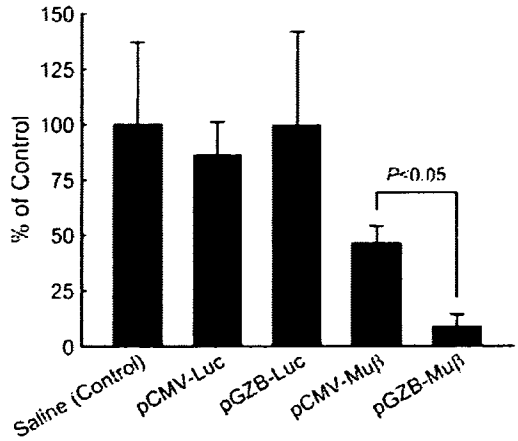

FIGURE 3 – Effect of IFN-β gene transfer on the pulmonary metastasis of CT-26 cells in mice. Mice were inoculated with CT-26 cells by a tail vein injection at a dose of 1×10^5 cells/mouse (day 0). At 24 hr after inoculation, mice were injected intravenously with saline (control), pCMV-Luc, pGZB-Luc, pCMV-Muβ or pGZB-Muβ at a dose of 10 μg/mouse by the hydrodynamics-based procedure. On day 14, mice were euthanized and the number of metastatic colonies on the lung surface was counted. The results are normalized to the control value (226 ± 84, the saline-treated group) and are expressed as the mean ± SD of at least 4 mice.

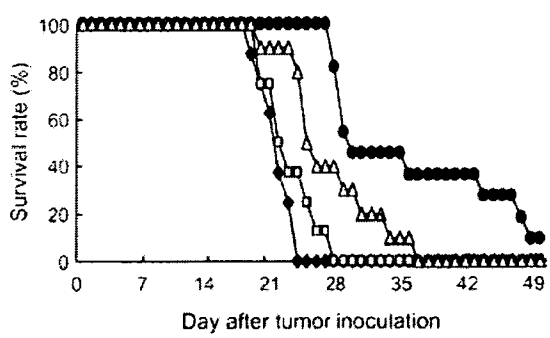

FIGURE 4 – Effect of IFN-β gene transfer on the survival rate of mice bearing pulmonary metastasis of CT-26 cells. Mice inoculated with 1×10^5 CT-26 cells were treated with saline (control, ◆), pCMV-Luc (□), pCMV-Muβ (△) or pGZB-Muβ (●) at a dose of 10 μg/mouse by the hydrodynamics-based procedure at 24 hr after tumor inoculation. Each group consisted of at least 9 mice.

Effects of IFN-β-expressing pDNA on pulmonary metastasis in mice

Inoculation of CT-26 cells into the tail vein of mice resulted in the formation of 226 ± 84 metastatic colonies on the lung surface at 14 days (Fig. 3). pCMV-Muβ or pGZB-Muβ (10 μg/mouse) significantly reduced the number of metastatic colonies following a single injection at 24 hr after the inoculation of CT-26 cells: 46.3% ± 7.9% and 8.6% ± 5.6% of the colonies were found in pCMV-Muβ-treated and pGZB-Muβ-treated mice, respectively, compared with the number in the saline-treated controls. pCMV-Luc (86.0% ± 15.1%) or pGZB-Luc (99.3% ± 42.5%) had little effect on the number of metastatic colonies in mouse lung at the same dose (10 μg), suggesting that the reduction in the number of metastatic colonies is mediated by IFN-β activity.

Figure 4 shows the survival rate of CT-26-bearing mice receiving a single injection of each pDNA (10 μg/mouse). The pCMV-Muβ- or pGZB-Muβ-treated mice survived significantly ($p < 0.05$) longer than the pCMV-Luc-treated mice. The mean survival

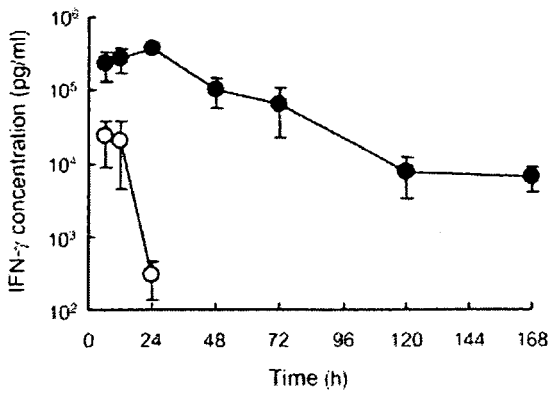

FIGURE 5 – Time-course of the concentration of IFN-γ in serum after intravenous injection of pCMV-Muγ (○) or pGZB-Muγ (●) at a dose of 3 μg/mouse by the hydrodynamics-based procedure. The IFN-γ concentration was measured by ELISA. The concentrations at 36 hr or later after injection of pCMV-Muγ were below the detection limit of 15 pg/ml. The results are expressed as the mean ± SD of at least 3 mice.

TABLE II – C_{MAX}, AUC AND MRT OF SERUM IFN-γ AFTER INTRAVENOUS INJECTION OF PCMV-MUγ AND PGZB-MUγ INTO MICE BY THE HYDRODYNAMICS-BASED PROCEDURE

pDNA	C_{max} (pg/ml)	AUC (pg hr/μl)	MRT (hr)
pCMV-Muγ	23,300 ± 14,700	262 ± 38	8.9 ± 1.8
pGZB-Muγ	382,000 ± 50,600*	15,900 ± 900*	34.8 ± 3.7*

The C_{max} values were obtained at 6 hr and 24 hr after intravenous injection of pCMV-Muβ and pGZB-Muβ, respectively, by the hydrodynamics-based procedure at a dose of 3 μg/mouse, and are expressed as the mean ± SD of at least three mice. The AUC and MRT were calculated by integration to infinite time, and are expressed as the calculated mean ± SE.

*Statistically significant ($p < 0.05$) compared with pCMV-Muγ.

times of mice treated with pCMV-Luc, pCMV-Muβ and pGZB-Muβ were 20.8 ± 1.5, 22.8 ± 3.1 and 26.6 ± 5.1 days, respectively. No statistically significant difference was obtained between the pCMV-Muβ and pGZB-Muβ-treated groups as far as the survival of CT-26-bearing mice was concerned.

IFN-γ concentration after injection of pCMV-Muγ and pGZB-Muγ

Figure 5 shows the time-courses of the IFN-γ concentration in serum after intravenous injection of pCMV-Muγ or pGZB-Muγ at a dose of 3 μg/mouse. Again, sustained IFN-γ concentrations were observed in mice receiving CpG-reduced pGZB-Muγ. More than 10,000 pg IFN-γ/ml was detected in the serum from 6 hr to 3 days after injection of pGZB-Muγ, whereas the concentrations were below the detection limit at 36 hr or later after injection of pCMV-Muγ. The initial IFN-γ concentration after injection of pGZB-Muγ was much greater than that after pCMV-Muγ ($p < 0.05$). In addition, the IFN concentration declined with a longer half-life in mice receiving pGZB-Muγ than in those receiving pCMV-Muγ, suggesting prolonged expression of IFN-γ by pGZB-Muγ. These expression properties of both vectors resulted in significant differences ($p < 0.05$) in the pharmacokinetic parameters (Table II): more than a 60-fold greater AUC and an about 4-fold longer MRT were obtained when pGZB-Muγ was injected in place of pCMV-Muγ.

Effects of IFN-γ-expressing pDNA on pulmonary metastasis in mice

Mice receiving CT-26 cells by intravenous injection were injected intravenously with pGZB-Muγ, pCMV-Muγ, pGZB-Luc or pCMV-Luc (3 μg/mouse) by the hydrodynamics-based procedure at 1 day after tumor inoculation. Figure 6 shows the number of metastatic colonies on the lung surface measured at 14 days

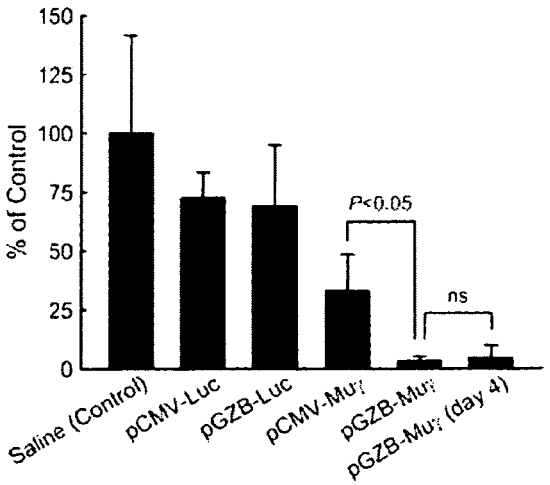

FIGURE 6 – Effect of IFN-γ gene transfer on the pulmonary metastasis of CT-26 cells in mice. Mice were inoculated with CT-26 cells by a tail vein injection at a dose of 1×10^5 cells/mouse (day 0). At 24 hr after inoculation, mice were injected intravenously with saline (control), pCMV-Luc, pGZB-Luc, pCMV-Muγ or pGZB-Muγ at a dose of 3 μg/mouse by the hydrodynamics-based procedure. On day 14, mice were euthanized and the number of metastatic colonies on the lung surface was counted. The results are normalized to the control value (215 ± 89, the saline-treated group) and are expressed as the mean ± SD of at least 4 mice.

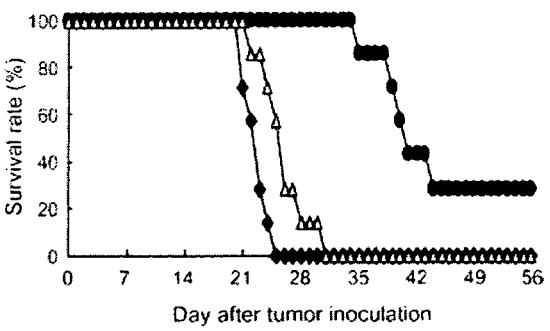

FIGURE 7 – Effect of IFN-γ gene transfer on the survival rate of mice bearing pulmonary metastasis of CT-26 cells. Mice inoculated with 1×10^5 CT-26 cells were treated with saline (control, ◆), pCMV-Muγ (△) or pGZB-Muγ (●) at a dose of 3 μg/mouse by the hydrodynamics-based procedure at day 1, 14, and 28 after tumor inoculation. Each group consisted of at least 7 mice.

after inoculation. pCMV-Muγ and pGZB-Muγ reduced the number of colonies to 32.8% ± 15.7% and 3.4% ± 1.7% of those in the saline-treated group (215 ± 89 colonies). To examine the effect of the interval of tumor inoculation and IFN-γ gene transfer, pGZB-Muγ was injected 4 days after tumor inoculation. This treatment also reduced the number of colonies to 4.4% ± 5.5% of those in the saline-treated group, which was not significantly different from the pGZB-Muγ-treated group injected 1 day after tumor inoculation. No significant reduction was obtained by pCMV-Luc (72.7% ± 10.7%) or pGZB-Luc (69.1% ± 25.9 %). Then, the survival of CT-26-bearing mice was examined in different sets of mice, which received 3 injections of pCMV-Muγ or pGZB-Muγ (3 μg/mouse/shot) at day 1, 14 and 28 after tumor inoculation (Fig. 7). The mean survival times of the saline-treated group and the pCMV-Muγ-treated group were 21.7 ± 1.5 and 25.0 ± 2.9 days, respectively, and the difference was not statistically significant. In contrast, the survival time of the pGZB-Muγ-treated mice

was significantly ($p < 0.05$) longer than that of the other groups. Furthermore, 2 out of 7 mice survived more than 56 days.

Discussion

We have already reported that IFN-β or IFN-γ gene delivery by the hydrodynamics-based procedure results in a significant antitumor effect against hepatic metastasis, but a weaker effect against pulmonary metastasis, of mouse colon carcinoma CT-26 cells.[12] The hydrodynamics-based procedure results in transgene expression in various internal organs, but the expression in the lung is much lower than that in the liver.[3] In a previous study, we could detect high IFN activities in the liver after gene transfer by this procedure, but failed to detect significant concentrations of IFNs in the lung. In addition, the plasma concentration of IFNs after gene transfer declined very quickly because of the transient nature of the gene expression driven by the conventional CMV promoter. These characteristics of the hydrodynamic delivery of IFN genes using conventional pDNA with CMV promoter explain the low efficacy of IFN gene transfer against pulmonary metastasis in mice. Consequently, to increase the therapeutic efficacy of IFN cancer gene therapy, we tried to increase and prolong the IFN expression by reducing CpG dinucleotides in pDNA.

As reported with other reporter and therapeutic genes,[25–27,33] the expression of IFN-β and IFN-γ from the pGZB vectors, which was estimated from their serum concentration profiles, was much longer than that from conventional pDNA after intravenous injection by the hydrodynamics-based procedure. A similar prolonged expression with pGZB vector was also obtained with firefly *luciferase* gene (unpublished data). Early studies have claimed that inflammatory cytokines, such as TNF-α produced after pDNA administration, reduce the transgene expression. In the present study, we found that the serum TNF-α level was significantly lower in mice receiving pGZB-Luc than in mice receiving pCMV-Luc (Fig. 1), suggesting that a reduced production of inflammatory cytokines is involved in the prolonged expression of IFNs from the pGZB vector. However, the low and transient nature of the induction of inflammatory cytokines after the injection of naked pDNA by the hydrodynamics-based procedure suggests that sustained gene expression from the pGZB vector is due not only to a reduction of the inflammatory response, but also to other mechanisms that have so far not been investigated.[33]

Transcriptional repression of endogenous genes is often associated with a higher frequency of methylated cytosine residues in the 5′ flanking region of the genes: promoter or enhancer.[34,35] Moreover, CpG methylation has been reported to be associated with the absence of integrated viral gene expression[36] and it has been suggested that *de novo* methylation of foreign DNA represents a cellular defense mechanism against the transcription of a foreign gene.[37] Because all CpG dinucleotides, except for those in the replication origin region, were converted to TpG dinucleotides in the pGZB vector, there was no substrate for DNA methyltransferase in the regions that were relevant to transgene expression. Therefore, sustained gene expression from CpG-reduced pDNA might be closely related to CpG methylation in addition to the reduced inflammatory response.

It has already been reported that the expression of reporter genes, such as chloramphenicol acetyltransferase or a secreted form of human placental alkaline phosphatase, is prolonged by the removal of CpG dinucleotides from the vectors.[25–27] In the present study, we also obtained a sustained transgene expression of either mouse IFN-β or IFN-γ by using CpG-reduced pDNA. However, the expression of IFN-β was not as persistent as that of IFN-γ and other proteins in the literature, suggesting that some of the pharmacological activities of IFN-β shut off the expression. Recently, Sellins *et al.*[38] reported that type I IFNs, such as IFN-α and IFN-β, play a key role in suppressing transgene expression following systemic *in vivo* gene transfer. To examine the effects of IFN-β as well as IFN-γ on transgene expression, the transgene expression in the liver was examined in mice using the pDNA encoding firefly *luciferase* gene (pGZB-Luc). Preadministration of pGZB-Muβ or pGZB-Muγ 48 hr prior to the hydrodynamic delivery of pGZB-Luc reduced the luciferase activity in the liver, but the reduction was significantly greater with pGZB-Muβ (data not shown). Therefore, it is probable that a moderate persistence of IFN-β activity after injection of pGZB-Muβ is a consequence of the ability of IFN-β to affect transcription of the transgene.

The increase in the IFN-β activity, which was quantitatively evaluated by a pharmacokinetic analysis to be a 14-fold increase in the AUC, led to a drastic reduction in the number of metastatic colonies in the lung. However, there was no significant difference in the survival time of the tumor-bearing mice between pCMV-Muβ-treated and pGZB-Muβ-treated groups. On the other hand, the use of pGZB-Muγ produced a significantly greater increase in the AUC (60-fold) and MRT (4-fold), resulting in a significant reduction in the number of tumor colonies as well as a significant increase in the survival time. Taken together, these results demonstrate that long-term expression of either IFN can be achieved by CpG-reduced pDNA and that sustained IFN gene expression results in enhanced therapeutic effects against lung metastasis of tumor cells. IFN-γ gene transfer was much more effective in increasing the survival of CT-26-bearing mice than IFN-β gene transfer, probably reflecting the greater increase in the serum IFN concentrations following the use of pGZB vector. Although multiple injections of conventional pDNA, such as pCMV-Muγ, could be effective in increasing the anti-cancer effect of IFNs, high and sustained serum concentrations of IFN-γ achieved by the pGZB-Muγ will not be obtained by increasing the frequency of administration of pCMV-Muγ. However, too high levels of IFN could induce toxic effects as observed in mice receiving 10 μg of pGZB-Muγ (data not shown). Therefore, the administration dose of pDNA should be carefully adjusted for future clinical applications.

Transfection of CT-26 cells with the IFN genes may occur by the hydrodynamic delivery of pDNA, which could be responsible for the antitumor effects of IFN gene transfer as reported in previous studies.[39,40] However, the level of transgene expression in the liver is far greater than those in other organs,[3] including the lung, the organ where metastatic tumor cells colonize in the present study. We also demonstrated a similar organ expression spectrum of the transgene following injection of pCMV-Muβ and pCMV-Muγ.[12] Therefore, direct transfection of tumor cells with the IFN genes, if it occurs, could have little contribution to the antitumor effects of hydrodynamic IFN gene transfer.

In conclusion, the CpG depletion from pDNA has been proved to be a useful approach for IFN cancer gene therapy. Because the sustained expression from pGZB vector may not be specific for its intravenous injection by the hydrodynamics-based procedure, less invasive methods of administration may be possible for future applications of IFN gene transfer to patients.

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IMMUNOLOGY ORIGINAL ARTICLE

DNA and its cationic lipid complexes induce CpG motif-dependent activation of murine dendritic cells

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Summary

Unmethylated CpG motifs in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response of antigen-presenting cells (APC). In this study, we investigated the cytokine release from murine dendritic cells (DC) by the addition of various types of DNA in the free or complexed form with cationic lipids. Naked plasmid DNA and *Escherichia coli* DNA with immunostimulatory unmethylated CpG motifs induced pro-inflammatory cytokine secretion from granulocyte–macrophage colony-stimulating factor (GM-CSF)-cultured bone marrow-derived DC and the DC cell-line, DC2.4 cells, though vertebrate calf thymus DNA (CT DNA) with less CpG motifs did not. These characteristics differed from mouse peritoneal resident macrophages that do not respond to any naked DNA. The amount of cytokines released from the DC was significantly increased by complex formation with cationic lipids when CpG-motif positive DNAs were used. Unlike murine macrophages or Flt-3 L cultured DC, GM-CSF DC did not release inflammatory cytokines in response to the addition of CT DNA/cationic lipid complex, suggesting that the activation is completely dependent on CpG motifs. Taken together, the results of the present study demonstrate that murine DC produce pro-inflammatory cytokines upon stimulation with CpG-containing DNAs and the responses are enhanced by cationic lipids. These results also suggest that DC are the major cells that respond to naked CpG DNA *in vivo*, although both DC and macrophages will release inflammatory cytokines after the administration of a DNA/cationic lipid complex.

Keywords: CpG motifs; dendritic cells; TLR9; DNA and DNA uptake

Introduction

It is well known that unmethylated CpG sequences (CpG motifs) in bacterial DNA, but not in vertebrate DNA, are recognized by the immune system as a danger signal.[1] Cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12 and interferon-α (IFN-α) are secreted from antigen presenting cells, especially macrophages or dendritic cells (DC), upon stimulation with CpG DNA and synthetic oligodeoxynucleotides (ODN) containing CpG motifs. These cytokines significantly modify the therapeutic effects of DNA-based therapies in different ways.[2] For example, in gene therapy, cytokine production generally seems inappropriate because these inflammatory cytokines significantly reduce transgene expression in target cells through their direct

Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; BMDC, bone-marrow derived dendritic cell; CT DNA, calf thymus DNA; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; IL-12, interleukin-12; IFN-α, interferon-α; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IRF, interferon regulatory factor; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; MHC, major histocompatibility complex; TLR, Toll-like receptor; JNK, c-Jun NH_2-terminal kinase; Flt-3 L DC, Flt-3ligand cultured bone-marrow dendritic cells; EC DNA, *Escherichia coli* DNA, pDNA, plasmid DNA; FL-pDNA, fluorescein labelled plasmid DNA; GM-CSF, DC; granulocyte–macrophage, colony-stimulating factor cultured dendritic cells; DNase II, deoxyribonuclease II.

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cytotoxicity and promoter attenuation.[3–5] On the other hand, it is essential for DNA vaccination because these cytokines can enhance the immune responses and profoundly affect the balance of these cytokines and the nature of the immune responses.[6–9]

DC are one of the most important cell populations as far as both innate and acquired immunity are concerned. They influence a variety of immunological responses associated with the therapeutic use of CpG DNA.[10,11] In addition to cytokine secretion, the expression of surface major histocompatibility complex (MHC) class I and II molecules as well as costimulatory molecules increases, and the maturation of DC is induced upon stimulation with CpG motifs.[12] The initial important step for all these processes associated with CpG DNA is cellular uptake because the receptor of CpG DNA, Toll-like receptor-9 (TLR9), is expressed within cells.[13,14] Our previous *in vitro* study using a DC cell line, DC2.4 cells, in mice demonstrated that DC take up pDNA via a mechanism specific to some defined polyanions[15] similar to cultured mouse peritoneal macrophages.[16,17]

There is a rapidly growing body of information about the mechanism of antigen-presenting cell (APC) activation by CpG DNA. This activation requires endosomal acidification and recognition by TLR9.[18–20] CpG DNA appears to use a TLR9 signaling pathway for NF-κB and c-Jun NH_2-terminal kinase (JNK) and IRF-7 through MyD88.[19,21] However, these proposed mechanisms are mainly based on studies using synthetic phosphorothioate CpG ODN, and there is little information about the activation induced by native DNA. Our previous study has demonstrated that, in contrast to macrophage cell lines, primary cultured mouse peritoneal macrophages secrete almost no inflammatory cytokines upon stimulation with pDNA, in spite of extensive uptake of the CpG DNA[22]. However, DNA/cationic lipid complex can activate the murine macrophages to induce inflammatory cytokines, whether they have replete CpG motifs or not.[23] Flt-3-ligand cultured bone-marrow DC (Flt-3 L DC) exhibit a different type of activation.[24,25] Upon stimulation with naked DNA, bacterial pDNA and CpG ODN stimulate Flt-3 L DC to induce cytokines IFN-α or IL-6 although vertebrate CT DNA does not. However, TLR9 in Flt-3 L DC can react when CT DNA is combined with cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP).[24] Methylated CpG motifs or non-canonical CpG motifs complexed with DOTAP induce the activation of TLR9 in Flt-3 L DC. Further experiments have proved that the other sequences also induce the activation of TLR9 when ODNs are translocated to endosomes by DOTAP.[25] While receptor-mediated endocytosis restricts the uptake of DNA, adsorptive endocytosis by cationic lipids does not. Thus, enhancement of DNA uptake seems to control the activation of TLR9 by vertebrate DNA. In the present study, we used a different type of DC and showed that the cells could respond to only DNA with CpG motifs even if the DNA was translocated to endosomes by cationic lipids.

Materials and methods

Chemicals

RPMI-1640 medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). *Escherichia coli* DNA (EC DNA) and calf thymus DNA (CT DNA) were purchased from Sigma (St Louis, MO). Lipofectin reagent and Opti-MEM were purchased from Invitrogen (Rockville, MD). Mouse recombinant GM-CSF (rGM-CSF) and Triton-X-114 were purchased from Nacalai Tesque (Kyoto, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Amersham, UK). Fetal Bovine Serum (FBS) was purchased from Thermo Trace (Melbourne, Australia).

Cell culture

Male ICR mice (5 weeks) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). After bone marrow was flushed out of the bones of the hind legs of the mice, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml rGM-CSF. After a 4–5 day incubation at 37° in 5% CO_2-95% air, cells were collected and centrifuged at 200 ***g*** for 10 min. After removal of the supernatant, the cells were resuspended in 400 μl phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA) per 10^8 total cells. The cell suspension was mixed thoroughly with 100 μl magnetic-activated cell sorting (MACS) CD11c MicroBeads (Miltenyi Biotec, Germany), and incubated for 15 min at 4°. After incubation, the cells were washed, centrifuged at 200 ***g*** for 10 min, and resuspended in 500 μl PBS containing 0·5% BSA. Then, magnetic separation with MACS was carried out to isolate the DC by selecting CD11c-positive cells from the cultured cells. These isolated cells were washed and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well and cultured for 24 hr. The murine DC2.4 cells were a gift from Dr Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA). DC2.4 cells display dendritic morphology, express dendritic cell-specific markers, MHC molecules, and costimulatory molecules, and exhibit phagocytic activity and an antigen-presenting capacity.[26] DC2.4 cells were cultured with RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 50 μM 2-mercaptoethanol, and antibiotics. They were then plated on a 24-well culture plate at a density of 5×10^5 cells/well and cultured for 24 hr.

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DNA

pCMV-Luc encoding firefly luciferase gene was constructed, as described previously.[27] pDNA was purified using an Endo-free plasmid Giga kit (Qiagen, Valencia, CA). For the cellular association experiments, pDNA was radiolabelled with [α-^{32}P]dCTP by nick translation.[28] For the activation experiments, all DNA samples were extensively purified with Triton-X-114, a non-ionic detergent, to minimize the activation by contaminated lipopolysaccharide (LPS). Extraction of endotoxin from pDNA, EC DNA, and CT DNA samples was performed according to reviously published methods[29,30] with slight modifications. DNA samples were purified by extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and ethanol precipitation. Then, 10 mg DNA was diluted with 20 ml pyrogen-free water, followed by the addition of 200 μl Triton-X-114 and mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55°. Subsequently, the solution was centrifuged for 20 min at 25°, 600 *g*. The upper phase was transferred to a new tube, 200 μl Triton-X-114 was added, and the previous steps were repeated at least three times. The activity of LPS was measured by *Limulus* amoebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free plasmid Giga kit, 1 μg/ml pDNA contained 0·01–0·05 EU/ml endotoxin. After Triton-X-114 extraction, the endotoxin levels of the DNA samples could no longer be determined by LAL assay, i.e. 1 μg/ml DNA contained less than 0·001 EU/ml. Without extraction of endotoxin by Triton-X-114, 100 μg/ml naked pDNA, which contains 1–5 EU/ml endotoxin, could release 521 ± 73 pg/ml TNF-α at 24 hr from peritoneal macrophages.

Cationic liposome formation

Lipofectin complexes were prepared according to the manufacturer's instructions. In brief, DNA was diluted in 100 μl Opti-MEM per 1 μg DNA (solution A) and 5 μl Lipofectin reagent was diluted in another 100 μl Opti-MEM (solution B). Then solutions A and B were combined and mixed gently. After a 15 min incubation at room temperature, complex was added to the cells.

Cellular association experiments

DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml Hanks' balanced salt solution (HBSS) without phenol red and 0·5 ml HBSS containing 0·1 μg/ml naked [^{32}P]pDNA or 0·1 μg/ml [^{32}P]pDNA/Lipofectin complex was added. After incubation at 37 or 4° for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1·0 ml 0·3 N NaOH with 0·1% Triton-X-100. Aliquots of the cell lysate were taken for the determination of ^{32}P radioactivity using an LSA-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content was measured using the modified Lowry method with BSA as a standard.

Confocal microscopy

DC2.4 cells were washed three times with 1·0 ml HBSS and incubated with HBSS containing fluorescein-labelled pDNA (FL-pDNA) or FL-pDNA/Lipofectin complex. After a 3 hr incubation, the cells were washed five times and fixed with 4% paraformaldehyde for 10 min.

Cytokine secretion

BMDC or DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml RPMI-1640 before use. Naked DNA was diluted in 0·5 ml Opti-MEM. The cells were incubated with the naked DNA solution continuously for 8 hr. In the case of DNA/Lipofectin complexes, cells were incubated for 2 hr with 0·5 ml of the solutions containing the complexes. Then, the cells were washed with RPMI-1640 and incubated with RPMI-1640 with 10% FBS. After a 6 hr incubation, the supernatant was collected for ELISA and kept at −80°. The levels of TNF-α, IL-6, and IL-12p70 in the supernatants were determined by the OptEIA Set (BD Biosciences, San Diego, CA).

Results

Uptake of DNA with cationic lipid complexes is not saturated, although normal uptake is saturated in GM-CSF DC

TLR9 exists in the endosomal-lysosomal compartment.[13,14] The amount of naked DNA in the compartment can be limited because naked DNA is supposed to be taken up by DC via receptor-mediated endocytosis.[15] However, DNA/cationic lipid complexes are supposed to be taken up by DC via a non-specific mechanism based on electrostatic interaction, so-called adsorptive endocytosis. Therefore, cationic lipid Lipofectin was used to deliver DNA efficiently to the compartment. To examine the binding and uptake of naked pDNA and pDNA/cationic lipid complexes in DC, we carried out cellular uptake experiments using naked [^{32}P]pDNA and [^{32}P]pDNA/Lipofectin complexes. As expected, the uptake of naked [^{32}P]pDNA by DC2.4 cells at 37° was increased up to 2 hr (Fig. 1a). Following an incubation of 2–5 hr, the amount of DNA remained unchanged, probably due to continued uptake and degradation.[15] On the other hand, complexation with cationic lipids enhanced the DNA uptake. Cationic lipids enhanced [^{32}P]pDNA binding and

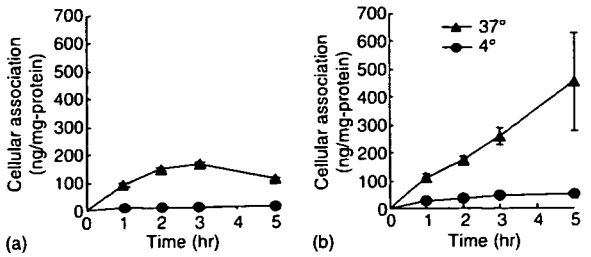

Figure 1. Cellular association time courses of naked [^{32}P]pDNA (a) or [^{32}P]pDNA/Lipofectin complex (b) in DC2.4 cells. Cells were incubated at 37° (closed triangle) or 4° (closed circle). Each point represents the mean ± SD (n = 3).

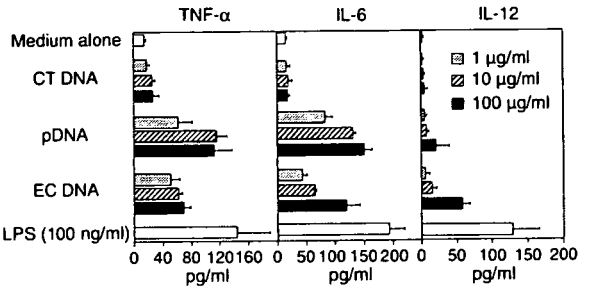

Figure 3. Cytokine secretion induced by naked DNA from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

uptake in DC2.4 cells and the amount of [^{32}P]pDNA increased in a time-dependent manner (Fig. 1b).

Next, we examined the localization of fluorescence-labelled DNA (FL-pDNA). In the confocal microscopy experiments, the fluorescence derived from naked FL-pDNA is bound to the cellular membrane at 4° (Fig. 2a). At 37°, FL-pDNA was observed inside the cells after 1 hr and it appeared to accumulate in the nucleus after a 3 hr incubation. On the other hand cationic lipids completely changed the localization of DNA. The fluorescence of the FL-pDNA/Lipofectin complex was observed in a punctuated pattern at 1 hr, then diffused into the cells after a 3 hr incubation (Fig. 2b).

The activation of GM-CSF DC by DNA

Next, cytokine production from DC by naked DNA was examined. Plasmid DNA and *E. coli* DNA were used as models of bacterial CpG DNA, and calf thymus DNA was used as a model of vertebrate DNA. As shown in Fig. 3, naked bacterial plasmid DNA and *E. coli* DNA with replete immunostimulatory CpG motifs induced TNF-α, IL-6 and IL-12 secretions from bone marrow-derived DC. The results are consistent with previous studies demonstrating that plasmid DNA stimulates GM-CSF DC to induce TNF-α and IL-12.[18] Vertebrate calf thymus DNA (CT DNA) containing less CpG motifs did not. LPS induced small amounts of cytokines, probably because of relatively short-term incubation (8 hr). Similar results were observed in the experiment using DC2.4 cells, although the cells released a higher amount of cytokines (Fig. 4). These results demonstrate that the cytokine secretion from the DC corresponds to the difference between endogenous DNA and exogenous DNA.

Next, cellular activation in DC by DNA/cationic lipid complexes was examined. The *E. coli* DNA/Lipofectin complexes stimulated GM-CSF cultured DC to produce cytokines, TNF-α, IL-6 and IL-12 in a dose-dependent manner (Fig. 5). Similar results were observed with pDNA/Lipofectin complex. The amounts of cytokines released from the DC were significantly increased by complex formation with cationic lipids compared with naked DNA (Fig. 3). The DC were unable to produce a significant amount of pro-inflammatory cytokines following

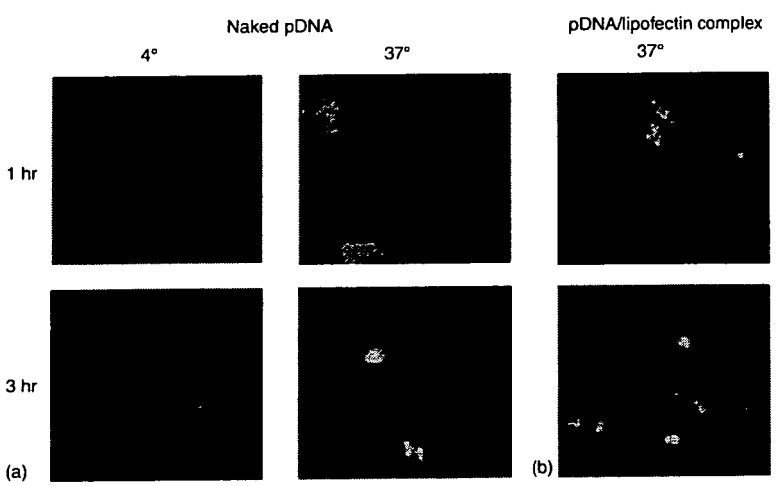

Figure 2. Uptake of naked FL-pDNA (a) or FL-pDNA/Lipofectin complex (b) by DC2.4 cells. The cells were incubated with 5·0 μg/ml naked FL-pDNA or 30 μg/ml FL-pDNA/Lipofectin complex.

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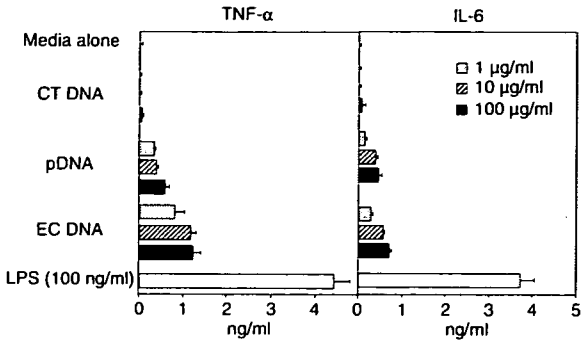

Figure 4. Cytokine secretion induced by naked DNA from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

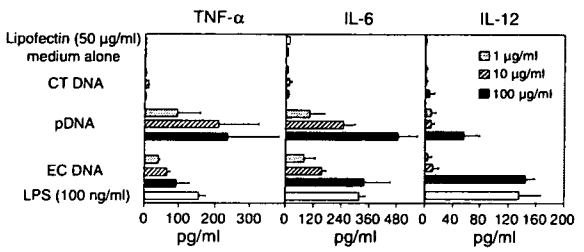

Figure 5. Cytokine secretion induced by DNA/Lipofectin complex from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 hr after the incubation with liposomes. The amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

stimulation with vertebrate calf thymus DNA (CT DNA) containing less CpG motifs when DNA is complexed to Lipofectin. Lipids alone were unable to stimulate the DC sufficiently to release pro-inflammatory cytokines. Similar results were obtained in DC2.4 cells (Fig. 6). These results demonstrate that GM-CSF DC discriminate between bacterial DNA and mammalian DNA.

Discussion

The most important role of immune system is to distinguish between 'self' and 'non-self'. Although the TLR9 subfamily (TLR7, 8 and 9) recognizes non-self nucleic acids[31] under special conditions, such as systemic lupus erythematosus, these TLRs are stimulated in response to self nucleic acids. For example chromatin–immunoglobulin complexes trigger DC activation in a TLR9-dependent and TLR9-independent manner.[32] Recently, Barton *et al.* have demonstrated that the fusion protein of

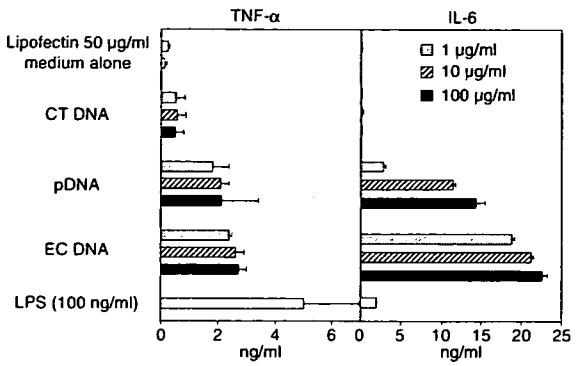

Figure 6. Cytokine secretion induced by DNA/Lipofectin complex from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after the incubation with liposomes. The amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

TLR4/9, which is delivered to cellular membranes, is activated by vertebrate DNA.[33] One proposed hypothesis is that compartmentalization of TLR9 prevents the response induced by endogenous DNA.

In the present study, we have demonstrated that GM-CSF-derived DC activation is triggered by exogenous naked DNA. Bacterial DNA induces cytokine secretion from DC, although vertebrate DNA does not. Flt-3 L cultured murine DC (Flt-3 L DC) also induce activation of TLR9 in response to naked bacterial DNA, but not naked vertebrate DNA.[24] Therefore, these studies imply that both GMCSF-DC and Flt-3 L DC can discriminate between bacterial non-self DNA and vertebrate self DNA.

On the other hand, these characteristics are different from murine macrophages.[22] Primary macrophages do not respond to naked DNA in spite of TLR9 expression, although the macrophage-like cell line RAW264.7 cells do. Both primary macrophages and DC take up DNA via a similar mechanism.[15–17] The mechanism of unresponsiveness of macrophages to DNA has not been elucidated, although TLR9 is present in the cells. Macrophages have deoxyribonuclease II (DNase II) in the lysosomal compartment, and they are responsible for apoptotic cell engulfment, DNA digestion and erythroid cell differentiation.[34] In erythropoiesis, macrophages take up nuclei and digest DNA. In DNase II-deficient mice, undigested DNA in macrophages causes IFN-β production via unknown receptors.[35] The cytokine production is mediated by the TLR9/MyD88 pathway and novel pathways that have been identified recently.[36,37] Therefore, the mechanism of the unresponsiveness of macrophages to naked DNA may involve the limited uptake and degradation by DNase II. However, further investigation is required.

The TLR4/9 fusion protein on the cell membrane is activated by vertebrate DNA.[33] This research indicates that compartmentalization into cells avoids TLR9 responses to endogenous DNA. Therefore, we forced DNA to internalize into cells using cationic lipids. In fact, vertebrate DNA/cationic lipid complexes can induce cytokine secretion from murine macrophages and Flt-3 L DC.[23,24] Following enhancement of DNA uptake by cationic lipids, these cells cannot distinguish between 'self' and 'non-self' DNA. In peritoneal macrophages, complexation of calf thymus DNA with cationic lipids elicited a similar level of inflammatory cytokine production to that obtained with bacterial *E. coli* DNA using cationic lipids.[23] In addition, calf thymus DNA with cationic lipid DOTAP causes a high degree of IFN-α release from murine Flt-3 L cultured DC or human peripheral blood mononuclear cells.[24] The amount of IFN-α induced by calf thymus DNA with DOTAP is similar to that induced by bacterial plasmid DNA. However, the result with GM-CSF DC is different from that in these cells. The cells only recognize bacterial DNA. Vertebrate DNA/cationic lipid complexes do not stimulate GM-CSF DC, although bacterial DNA does. There are two possibilities to explain these observations. One is the possibility that different types of cationic lipids lead to different forms of delivery of DNA, and result in different responses. For example, murine macrophages release inflammatory cytokines in response to the addition of vertebrate CT DNA/cationic lipid complexes.[23] Lipofectamine was used for this research. Synthetic double-stranded DNA containing no CpG motif can stimulate macrophage cell lines when DNA is complexed with the cationic lipid Fugene 6.[38] In addition, vertebrate CT DNA/cationic lipid Lipofectamine complexes induce macrophage activation via TLR9-dependent and -independent mechanisms.[39] Flt-3 L cultured DC (Flt-3 L DC) also responds to vertebrate DNA/cationic lipid DOTAP complexes via TLR9-dependent and -independent pathways.[24] TLR9-independent activation is also observed following transfection using Lipofectamine 2000.[37] Honda *et al.* showed that different cellular distributions of DNA result in different cytokine responses.[40] CpG-B ODN normally do not induce IFN-α release from plasmacytoid DC. However, following complexation with DOTAP, the same ODNs trigger IFN-α. Confocal microscopy reveals that DOTAP retains DNA in early endosomes, although ODNs without DOTAP are immediately transferred to lysosomal vesicles. Taken together, enhancement of the DNA uptake may not explain the response of TLR9 to vertebrate DNA and TLR9 may be present in specific compartments.

The other possibility is that GM-CSF DC, Flt-3 L DC and macrophages may contribute to the immune systems in different ways, by producing different types or degrees of induction. TLR9 is mainly expressed in B cells and plasmacytoid DC in humans.[31] On the other hand, mouse TLR9 is also present in myeloid DC and macrophages. Although further studies are required to clarify the contribution of DC or macrophages to immune responses *in vivo*, the present study suggests that DC are the main cells that respond to naked bacterial DNA, although both DC and macrophages will release inflammatory cytokines after the administration of bacterial DNA/cationic lipid complexes.

Very recently Martin *et al.* have shown that GM-CSF DC release type I IFN upon stimulation of mammalian DNA complexed with Fugene, another kind of lipid for transfection.[41] Interestingly, the cells do not produce TNF-α, IL-6 or IL-12. The activation is independent of TLR9 because GM-CSF DC from $TLR9^{-/-}$ deficient mice respond to mammalian DNA/Fugene complexes to secrete type I IFN. Another group has also demonstrated that non-CpG DNA/lipofectamine complexes stimulate GM-CSF DC to induce type I IFN.[42] The activation is not dependent on the MyD88 or TRIF pathways. Based on these observations, one can hypothesize that, distinct from Flt-3 L DC, GM-CSF DC respond to only bacterial or viral DNA via TLR9-dependent pathway, and release cytokines, such as TNF-α IL-6 and IL-12. However when mammalian DNAs are translocated into cells, GM-CSF DC may not induce these cytokines. Instead, the cells may release IFN-α through a TLR9-independent pathway. Further studies are required for these TLR9-dependent and -independent mechanisms.

In conclusion, the present study has demonstrated that murine GM-CSF DC or the DC cell line, DC2.4, produce pro-inflammatory cytokines following stimulation with CpG-containing DNAs and this production is increased when the DNAs are added to the cells in a complex form with cationic lipids. These findings form an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

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Design of PCR-Amplified DNA Fragments for *In Vivo* Gene Delivery: Size-Dependency on Stability and Transgene Expression

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ABSTRACT: PCR-amplified DNA fragments can be more efficient and safer vectors than conventional plasmid DNA because of their smaller size and fewer numbers of immunostimulatory cytosine-phosphate-guanine (CpG) motifs. In the present study, the expression unit of plasmid DNA encoding farnesylated enhanced green fluorescent protein (EGFPF; pEGFP-F) or firefly luciferase (pLuc) was amplified by polymerase chain reaction (PCR) to obtain DNA fragments (EGFPF-mini, Luc-mini). EGFPF-mini was as effective as pEGFP-F on the basis of the number of EGFPF-expressing cells after intravenous injection into mice by the hydrodynamics-based procedure. Then, the effects of the length of DNA fragments on transgene expression were examined using luciferase-expressing DNA preparations. Luc-mini preparations showed high levels of luciferase activity in cultured cells as well as in mouse liver, even although the levels did not exceed that of pLuc. An elongation of the DNA fragment on either side of the minimal expression unit was effective in increasing the transgene expression and the stability against nucleases. PCR-amplified DNA fragments showed a sustained luciferase activity in mouse liver compared with pLuc, indicating that they are effective in achieving a prolonged expression. Their stabilization against nucleases will further increase the potential of such short, structure-controlled and synthetic DNA fragments for *in vivo* gene delivery. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:2251–2261, 2007

Keywords: hydrodynamics-based procedure; plasmid DNA; PCR-amplified fragment; gene transfer

INTRODUCTION

For an effective *in vivo* gene therapy, therapeutic genes should be efficiently delivered to target cells within the body. Of various gene delivery methods developed thus far,[1] the use of naked plasmid DNA without any lipids or polymers is the simplest and safest method. The hydrodynamic delivery of naked plasmid DNA, first reported by Liu et al.[2] and Zhang et al.,[3] which involves a large-volume and high-speed intravenous injection of naked plasmid DNA, gives a significantly high level of transgene expression in the liver and other major internal organs.[2–4] A large-volume injection of naked plasmid DNA solution has also been applied to gene transfer to specified organs, including the liver,[5] kidney,[6] and skeletal muscle.[7] In addition, *in vivo* electroporation increased transgene expression by naked plasmid DNA after its injection into the blood circulation[8,9] or tissues.[10] Hydrodynamic pressure or electric fields will allow plasmid DNA to enter cells, probably through pores created on cell membranes.[9,11–13]

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About 75% of cytosine-phosphate-guanine (CpG) dinucleotides in mammalian DNA are methylated at the cytosine position, whereas CpGs are largely unmethylated in bacterial DNA.[14] Because of the presence of unmethylated CpG dinucleotides, or CpG motifs, bacterial and plasmid DNAs stimulate immune responses in mammals.[15] It has been reported that systemically produced TNF-α reduces transgene expression in the lung after intravenous injection of plasmid DNA/cationic liposome complex.[16] Therefore, methylation of CpG motif is a useful method to reduce such immune responses against plasmid DNA, but it generally inhibits the transcriptional activity of most promoters.[17] Reducing the number of CpG motifs from plasmid DNA has been challenged by replacing cytosine of CpG to thimidine[18] or by recombination of plasmid backbone to give minicircles.[19–21] Another concern about the use of plasmid DNA is contamination of lipopolysaccharide (LPS) in DNA preparations, which would also reduce transgene expression.[22] Therefore, it would be useful to develop an alternative method that constructs therapeutic gene-coding DNA preparations without amplification in bacteria.

A unique property of DNA is its reproductive nature, and its amplification by polymerase chain reaction (PCR) has extensively been used in a variety of experimental settings. Because of the convenient nature of PCR, the applications of PCR-amplified DNA fragments to *in vivo* gene transfer have been attempted. Li et al.[23] used PCR-amplified DNA fragments as a model for double-stranded synthetic genes in gene therapy and demonstrated that the DNA fragments could be efficiently transferred into 293-T7 cells. Hofman et al.[24] also examined the transgene expression of such DNA preparations in the liver after intravenous injection into mice by the hydrodynamics-based procedure. These previous studies have revealed that the PCR-based amplification of an expression unit from plasmid DNA gives a therapeutic gene-expressing DNA preparation, which would have a reduced number of CpG motifs and less LPS. Another possible advantage of this approach is that the length of DNA fragments can be easily controlled by selecting appropriate primers for amplification. Because the tissue distribution, cellular entry, and nuclear transport of macromolecular compounds are molecular size-dependent processes,[25,26] smaller ones generally have an advantage in these processes that are involved in plasmid DNA-based *in vivo* gene transfer.

Although the length of DNA fragments may affect their stability, transfection efficiency, and delivery, its importance on gene transfer has hardly been explored. In the present study, we prepared PCR-amplified DNA fragments with varying lengths and examined their transgene expression in cultured cells as well as in mouse liver after intravenous injection by the hydrodynamics-based procedure.

MATERIALS AND METHODS

Plasmid DNA

Plasmid DNA encoding firefly luciferase (pLuc) was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA) as reported previously.[27] pLuc amplified in the DH5α strain of *E. coli* was extracted and purified by a QIAGEN Endofree™ Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining. DNA concentration was measured by UV absorption at 260 nm. pEFGP-F encoding farnesylated enhanced green fluorescent protein (EGFPF) was purchased from BD Biosciences Clontech (Palo Alto, CA). pRL-CMV, a plasmid vector encoding renilla luciferase, was purchased from Promega.

Primers and PCR Amplification

The sequences of primers used for PCR amplification are as follows: EGFPF-mini (sense primer, CCGTATTACCGCCATGCATT; antisense primer, CCACAACTAGAATGCAGTG); Luc-mini2.6 (sense primer, TTCGCGATGTACGGGCCAGA; antisense primer, CATCCCCAGCATGCCTGC-TA); Luc-mini2.8 (sense primer, GGAGGTCGCTGAGTAGTGCG; antisense primer, TAGCGGTCACGCTGCGCGTA); Luc-mini3.5 (sense primer, GGGAATAAGGGCGACACGGA; antisense primer, CCACACCCTAACTGACACAC); Luc-mini4.5 (sense primer, AGCGGTTAGCTCCTTCGGTC; antisense primer, TGCGCTGACAGCCGGAACAC). Phosphorothioated primers for the Luc-mini2.6, in which 4 linkages at the 3′-terminal were phosphorothioated, were also used to obtain Luc-mini2.6PS. Except for the Luc-mini3.5R and Luc-mini3.5L, the both sides of

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the expression unit were extended to obtain DNA fragments of 2.8 kb (Luc-mini2.8) or greater. Only the 3′- or 5′-side of the expression unit was extended for Luc-mini3.5R and Luc-mini3.5L, respectively, using the primers for Luc-mini2.6 and Luc-mini4.5. Figure 1 shows the schematic presentation of the PCR-amplified DNA fragments developed in this study. For PCR, 10 ng of plasmid template, 50 pmol of each primer, and 2.5 U of DNA polymerase (Takara Ex Taq™ or Pyrobest DNA polymerase, Takara Bio Inc., Otsu, Japan), 0.2 mM dNTP mixture were used. The PCR products were extracted with GenElute™ PCR Clean-Up Kit (Sigma, St. Louis, MO).

In Vitro Transfection

Murine melanoma cell line B16-BL6[28] was cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/L-glutamine at 37°C and 5% CO_2. B16-BL6 cells were plated on 24-well culture plates (at a density of 1×10^5 cells/well). After an overnight incubation, pLuc or Luc-mini preparation (0.2 pmol/well) was mixed with Lipofectamine2000 (Invitrogen) in Opti-MEM (Invitrogen) and the mixture was incubated for more than 20 min at room temperature. Then, resulting complexes were added to cells. In a separate set of experiments, each DNA preparation (0.2 pmol/well) was added to B16-BL6 cells on 6-well culture plates (5×10^5 cells/well) as the naked form and electric pulses (100 V, 4 ms, 10 Hz, 12 pulses) were applied using a rectangular direct current generator (CUY-21, Nepagene, Chiba, Japan). In both cases, cells were collected at 24 h after transfection and lysed in a lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and the luciferase activity was determined.

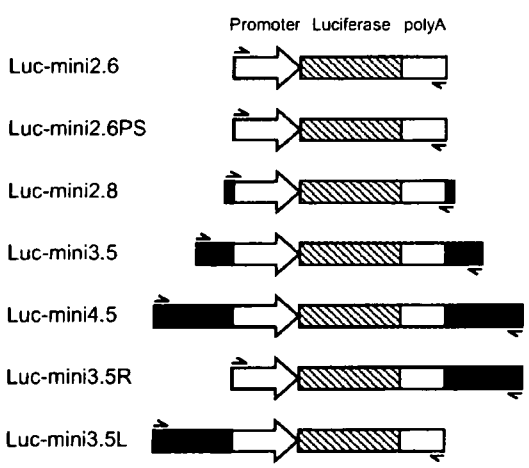

Figure 1. Schematic presentation of DNA fragments amplified from plasmid DNA expressing firefly luciferase. DNA fragments, including the minimal expression unit, were amplified by PCR using specific primers (arrows). For Luc-mini2.6PS, primers, in which 4 linkages at the 3′-terminal were phosphorothioated, were used.

Mice and *In Vivo* Gene Transfer to the Liver by the Hydrodynamics-Based Procedure

Four-week-old female ddY mice (approximately 20 g body weight) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University. Mice under light anesthesia by inhalation of diethyl ether received an injection of naked DNA preparations dissolved in 1.6 mL of saline into the tail vein over less than 5 s using a 26-gauge needle.[2] The doses of DNA preparations were determined based on the sensitivity of the assay systems: 0.23 pmol/mouse for luciferase-expressing DNA preparations (1 μg/mouse for pLuc) and 8.4 pmol/mouse for EGFPF-expressing DNA preparations (25 μg/mouse for pEGFP-F). pRL-CMV (0.1 μg/mouse), which encodes renilla luciferase cDNA, was simultaneously delivered with firefly luciferase-expressing pLuc or Luc-mini preparations.

Confocal Microscopic Observation of Mouse Liver Sections

Mice were euthanized at 6 h after injection of EGFPF-expressing DNA preparations. The liver was then embedded in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen and stored in 2-methyl butanol at −80°C until use. Frozen liver sections of 8 μm in thickness were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by a routine procedure. The liver sections were fixed with Mildform 20 N (8% paraformaldehyde, Wako, Osaka, Japan) for 4 min at 4°C followed by confocal microscopic observation (MRC-1024, BioRad, Hercules, CA).

Luciferase Assay

Mice were euthanized at 6 h after injection of luciferase-expressing DNA preparations unless otherwise indicated. The liver was excised and homogenized in the lysis buffer. The homogenate was subjected to three cycles of freezing (−190°C) and thawing (37°C) and centrifuged at 13000*g* for 10 min at 4°C. Appropriately diluted supernatant of the lysate of B16-BL6 cells or liver homogenate was mixed with luciferase assay buffer (Picagene-Dual, Toyo Ink, Tokyo, Japan), and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany). For liver samples, 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,[29] and the results were expressed as relative Pp-Luc RLU/s/mg protein, which represents Pp-Luc activity normalized to Rr-Luc activity of the individual animals.

Stability of DNA Fragments

Bal31 and DNase I were purchased from Takara Bio Inc. Each luciferase-expressing DNA preparation was incubated with Bal31 (1 U/μg DNA) or DNase I (0.005 U/μg DNA) at 37°C. Aliquots (15 μl) were removed at each sampling point and added with EDTA (a final concentration of 50 mM) to stop the degradation of DNA. Each aliquot was mixed with a loading buffer and loaded on a 1.3% agarose gel and electrophoresed in TBE buffer (89 mM Tris-Borate, 25 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide and DNA bands were visualized by fluorescence in an ultraviolet transilluminator system. To determine whether the minimal expression unit remains after the incubation with Bal31, the nuclease-treated samples of Luc-mini2.6 and Luc-mini3.5 were amplified by PCR using the primers for Luc-mini2.6. Then, the PCR product was examined on an agarose gel described above.

Statistical Analysis

Differences were statistically evaluated by one-way ANOVA followed by the Student-Newmann-Keuls multiple comparison test, and the level of statistical significance was $p < 0.05$.

RESULTS

Distribution of EGFPF Expressing Cells in the Liver after Hydrodynamic Delivery of Plasmid DNA and a DNA Fragment

Figure 2 shows the liver sections of mice receiving an intravenous injection of pEGFP-F or EGFPF-mini by the hydrodynamics-based procedure. The dose was set at 8.4 pmol/mouse, that is, 25 and 8.9 μg/mouse for pEGFP-F and EGFPF-mini, respectively. No positive cells were found in

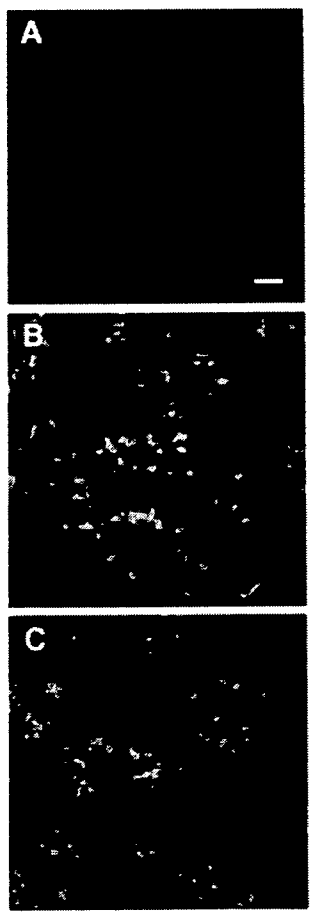

Figure 2. Confocal microscopic images of the liver sections following intravenous injection of pEGFP-F or EGFPF-mini in mice by the hydrodynamics-based procedure. Mice were euthanized at 6 h after injection and the liver sections were made. The images shown are typical of those observed in several visual fields of three mice per group. (A) pEGFP-F (0.02 μg/mouse, equal to the amount of template plasmid DNA included in the EGFPF-mini preparation); (B) pEGFP-F (25 μg or 8.4 pmol/mouse); (C) EGFPF-mini (8.9 μg or 8.4 pmol/mouse).

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