

Fig. 2. Effect of heat-inactivation of serum on the serum-dependent hepatic uptake of test-dose PEGylated liposomes. Radiolabeled test-dose PEGylated liposomes were infused for single-pass perfusion into the livers of rats that had received HEPES-buffered saline 5 days before. Open column; test-dose liposomes pre-incubated with HEPES-buffered saline. Hatched column; test-dose liposomes incubated with serum from rats that were pre-dosed with HEPES-buffered saline. Filled column; test-dose liposomes incubated with serum from rats pre-dosed with PEGylated liposome. Dotted column; test-dose liposomes incubated with heat-treated serum (30 min 56 °C) from rats pre-dosed with PEGylated liposomes. Each value represents the mean \pm S.D. of 4 or 5 separate experiments. *** $p < 0.005$.

phospholipid/kg), that is capable of causing the ABC phenomenon at a maximal level [9,13]. In the absence of serum in the perfusate, no enhancement in hepatic uptake of radiolabeled test-dose PEGylated liposomes was observed (Fig. 1A). This result obviously shows that the intravenous injection of PEGylated liposomes 5 days before did not increase the intrinsic phagocytic activity of the liver macrophages.

3.2. Effect of opsonization with rat serum on the uptake of test-dose PEGylated liposomes by liver macrophages

The test-dose liposomes were infused in the liver following incubation at 37 °C for 15 min with two types of sera. The serum obtained from rats that had received HEPES-buffered saline did not enhance the uptake of test-dose liposomes by the livers of rats that had received either PEGylated liposomes or HEPES-buffered saline (Fig. 1B). By contrast, serum obtained from rats that were pre-dosed with PEGylated liposomes significantly enhanced the hepatic uptake of test-dose liposomes in the livers of rats that were pre-dosed with either PEGylated liposomes or HEPES-buffered saline (Fig. 1C). These indicate that the first-dose PEGylated liposome induces production of serum factor(s), which is/are responsible for enhanced hepatic uptake of test-dose PEGylated liposomes.

It should be noted that the level of hepatic uptake of test-dose liposomes was significantly higher in the liver of pre-dosed rats than in the liver of rats that had only received HEPES-buffered saline (Fig. 1C). This suggests that the pre-dose PEGylated liposome augments the macrophages' response to properly opsonized liposomes. It is likely that the ABC phenomenon occurs as a result of an immune response induced by the first dose [7–9,19]. Such immunological responses may result in the

production of a variety of cytokines and/or chemokines [20,21]. The cytokines/chemokines thus produced might stimulate the liver macrophages directly and thereby induce the expression of receptors such as complement receptors and Fc receptors, related to phagocytosis and/or endocytosis of opsonized materials.

3.3. Heat-sensitivity of the serum factor(s) causing the serum-dependent hepatic uptake of test-dose PEGylated liposomes

To identify serum factors that are involved in the serum-dependent hepatic uptake (Fig. 1), the serum obtained from rats pre-dosed with PEGylated liposome was pre-treated by heating at 56 °C for 30 min, which is known to inhibit all complement activity. Following incubation for 30 min at 37 °C with this heat-treated serum, test-dose liposomes were infused in the livers of rats that had received HEPES-buffered saline. The heated serum (56 °C, 30 min) completely abolished the serum-dependent hepatic uptake of test-dose liposomes (Fig. 2).

We recently proposed the tentative mechanism for the induction of the ABC phenomenon [7–9]. Anti-PEG IgM, induced by the first injection of PEGylated liposomes, plays a key role in the mechanism. IgM has a strong potential to activate the complement system and consequently enhances the uptake of foreign materials via complement receptor-mediated endocytosis or phagocytosis, but by itself it has no ability to promote endocytosis or phagocytosis directly. It is well known that the complement system loses activity when treated at 56 °C for 30 min [12]. The serum-dependent uptake of test dose by liver was virtually abolished by treatment of the serum at 56 °C for 30 min (Fig. 2). This finding proves that complement activation, probably triggered by selective binding of anti-PEG IgM to test-dose PEGylated liposome [7–9], is responsible for the ABC phenomenon. This is supported by previous findings of Dams et al. [3] showing that the accelerated blood clearance of PEGylated liposomes is mediated by a soluble heat-labile (56 °C, 30 min) serum factor(s). Therefore, we could confirm that the anti-PEG IgM-mediated complement activation induced by the second dose PEGylated liposome is the major cause of the induction of the accelerated blood clearance of PEGylated liposome. Nevertheless, we can not exclude an alternative explanation that the additive or synergistic effect of activated liver macrophages (Fig. 1C) and complement activation enhanced uptake of the test dose PEGylated liposome in the ABC phenomenon.

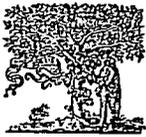
4. Conclusion

This paper highlights that first-dose PEGylated liposomes do not increase the intrinsic phagocytic activity of Kupffer cells and supports the notion that complement activation is essential for induction of the accelerated blood clearance of second-dose PEGylated liposomes.

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Efficient gene expression in megakaryocytic cell line using nucleofection

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Abstract

To clarify the mechanism of platelet production from megakaryocytes, expression of target proteins by gene transfection was examined using various gene delivery techniques. Transfection into hematopoietic cells, including megakaryocytes, by conventional gene delivery techniques such as electroporation and lipofection are known to be difficult. In this study, in addition to electroporation and lipofection, we tested other gene-transfer methods (nucleofection, transfection using inactivated virus envelope, and transferrin-linked cationic polymer) with the green fluorescent protein (GFP) gene into the human megakaryocytic cell line MEG-01. We found that nucleofection, which uses a combination of special electrical parameters and specific solutions, was the best, judging from the expression ratio of GFP-positive cells (approximately 70% of cells) and low toxicity. The efficiency of GFP expression was not related to the amount of pDNA delivered into the MEG-01 cells. To verify the utility of nucleofection, the thrombopoietin (TPO) receptor *c-mpl* was transfected into MEG-01 cells. Transfected cells showed a higher responsiveness to TPO than mock-transfected MEG-01 cells. We propose that nucleofection is a useful method for transfecting target genes to megakaryocytic cells when addressing the mechanism of platelet production.

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Keywords: Transfection; Megakaryocytes; MEG-01; Nucleofection

1. Introduction

Megakaryocytes, which are estimated to constitute approximately 0.4% of the total bone marrow cells (Levine, 1980), are differentiated from hematopoietic stem cells into platelets via many stages. Since various hemorrhagic and thrombotic disorders are strongly attributed to abnormalities in platelet number or function, understanding the mechanism of platelet production is important for establishing new pharmacologic strategies to regulate disordered or inappropriate platelet production. To clarify the molecular mechanisms of megakaryocyte differentiation and platelet production, approaches using gene expression analysis such as cDNA microarray, serial analysis of gene expression, differential display, and cDNA subtraction are available. On the

basis of these analyses, candidate genes involved in the regulation of cellular differentiation have been identified. Knockout or transgenic technology is a powerful tool for validating the functions of candidate genes. Indeed, knockout mice have been established for several genes. Mice deficient in thrombopoietin (TPO) (Bartley et al., 1994), *c-mpl* (TPO receptor) (Carver-Moore et al., 1996), GATA-1 (Shivdasani et al., 1997), or nuclear factor erythrocyte 2 (Shivdasani et al., 1995) have been shown to have decreased numbers of platelets in the blood, suggesting that these proteins participate in platelet production. However, establishing transgenic or knockout mice generally takes a long time, and the number of candidate genes is sometimes too large to knockout all of them at once. Therefore, a simple gene validation system is required to clarify the mechanism of platelet production.

One powerful method is transfection of target genes relating to the target proteins into megakaryocytes. However, transfection into megakaryocyte and megakaryocytic cell lines by conventional methods is notoriously difficult and the efficiency is very low (less than 5%) (Wang et al., 1999). To overcome the

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Table 1
Transfection efficiency of liposome- and cationic polymer-based reagents with manufacturers' recommended conditions

Reagent (μl)	DNA (μg)	Cell number ($\times 10^5$)	Viability (%)	Positivity (%)	Efficiency (%)
LFA2000	4.0	1.0	84.9	1.7	1.4
FuGENE6	1.2	0.2	92.2	0.1	0.1
SuperFect	4.0	2.0	86.2	0.0	0.0
Effectene	10	0.4	88.4	0.0	0.0
TransFast	4.5	0.5	76.8	0.8	0.6
jetPEI	2.0	1.0	81.0	0.2	0.2
GenePORTER2	7.0	2.0	87.9	0.4	0.4
DuoFect	4.5	10	78.5	0.1	0.1

MEG-01 cells were transfected with commercially available liposome- or cationic polymer-based reagents complexed or mixed with pEGFP-C1 pDNA. Viability of the cells and GFP-positive cells were analyzed by flowcytometer 24 h after transfection.

2.3.4. Transferrin-linked cationic polymer

A transfection system (Duofect; Q-Biogene) using the interaction between transferrin (TF)-containing polyethyleneimine and TF receptors on the cell surface was tested. Transfection was performed according to the manufacturer's guidelines. In brief, 20 h before transfection, the cells were collected and re-suspended in RPMI-1640 supplemented with 10% FBS containing deferrioxamine. Deferrioxamine can increase the density of TF receptors on the cell surface, thus further enhancing gene delivery to the cell via TF receptor-mediated endocytosis. DNA/Duofect complex was added to the cells (5×10^5) and they were incubated at 37 °C for 4 h. The complex was then removed by aspiration, washed twice with RPMI-1640 supplemented with 10% FBS, and further cultured with RPMI-1640 supplemented with 10% FBS at 37 °C for 24 h. Twenty-four hour after transfection, GFP expression was determined using a flowcytometer as described below.

2.3.5. Nucleofection

MEG-01 cells were transfected using a Nucleofector Device from Amaxa Biosystems GmbH (Cologne, Germany). MEG-01 cells (1×10^6 cells) were collected by centrifugation ($300 \times g$, 5 min, 4 °C), re-suspended in 100 μl of Nucleofector Solution R, T, or V. Following addition of pEGFP-C1 pDNA (5 μg), the mixture was transferred into an electroporation cuvette. The cuvette was placed in the Nucleofector Device (Amata). Initially, eight programs with different intensities and lengths of electric pulse (A-23, A-27, T-20, T-27, T-16, T-01, G-16, and O-17) were used to obtain an optimal transfection condition. Control experiments were performed by processing MEG-01 cells in the same way, but without adding pDNA to the cells. Immediately after nucleofection, the MEG-01 cell suspension was transferred into a well of a 12-well plate containing 1 ml pre-warmed RPMI-1640 supplemented with 10% FBS. After 24 h, the MEG-01 cells were collected by centrifugation ($300 \times g$, 5 min, 4 °C) and re-suspended in PBS containing 0.4% bovine serum albumin (BSA) and 5 mM EDTA. The ratio of transfected cells was determined by analyzing the expression of GFP by flowcytometry. Following the initial optimization study, another seven programs with higher transfection efficiencies (T-01, T-16, U-08, U-16, U-01, T-17, T-19, T-21, and T-09) are recommended by Amaxa Biosystems. Further details of the programs are proprietary information of Amaxa Biosystems. The transfection

efficiencies were again measured by flowcytometry as described above.

2.4. Determination of transfection efficiency

2.4.1. Measurement for GFP

GFP-expression in MEG-01 cells was determined by flow-cytometric analysis at 24 h post-transfection. Harvested cells were re-suspended in PBS containing 0.4% BSA and 5 mM EDTA, filtered through a 40- μm nylon cell strainer (BD Falcon, MA, USA), and analyzed using EPICS ELITE ESP equipped with a 488 nm argon laser (Beckman Coulter). The results are shown as positivity, viability, and efficiency. For each sample, 1×10^4 cells were analyzed and the live cell number was counted by the live gate (determined by forward scatter versus side scatter). Viability was calculated from the number of live and dead cells. To determine the GFP expression gate setting, mock (pCMV-Script)-transfected cells were used as a negative control. Positivity was the percentage of GFP-positive cells among the viable cells. The transfection efficiency was calculated as follows:

$$\text{Efficiency (\%)} = \frac{[\text{Positivity (\%)}] \times [\text{Viability (\%)}]}{100 (\%)}$$

2.4.2. Determination of luciferase activity

Twenty-four hour after transfection, the cells were collected by centrifugation ($300 \times g$, 5 min, 4 °C) and lysed in 100 μl of Glo Lysis Buffer (Promega) for 5 min at room temperature. The cellular debris was spun down at $10,000 \times g$ for 10 min at 4 °C in a microcentrifuge and the supernatant was transferred into a new microtube and kept -20 °C until measurement of luciferase activity. This was done by mixing 50 μl of the supernatant with 50 μl of substrate solution (Bright Glo Luciferase Assay System; Promega), followed by measurement of light emission using ArvoSX (Perkin-Elmer, MA, USA). All luciferase activities were calibrated for viable cell numbers determined by trypan blue dye exclusion assay.

2.5. Determination of cell-associated pDNA by PCR

The pDNA delivered to cells following transfection was determined using the method of Tachibana et al. (2002a) with

low transfection efficiency, transgene-expressing cells have to be enriched to validate the function of the transgenes, and this is a time- and labor-consuming process. To overcome this problem, Burstein et al. (1999) used a retrovirus vector and showed that 41–82% of megakaryocytes were positive. In addition, Gillitzer et al. (2005) showed successful gene-transfer into CD34⁺ stem cells using a retrovirus vector, and that the transfected cells differentiated into megakaryocytes in response to stimulation by TPO. However, the utility of viral vectors is limited by the time required, facilities, the overall expense, and safety considerations. A simple method of transfecting genes to megakaryocytes should be a great advantage.

In studies of megakaryocyte differentiation, K562 cells are among those most commonly used (Drexler et al., 2004). However, this cell line has both pro-erythroidic and megakaryocytic properties. In contrast, MEG-01 cells (a human megakaryocytic leukemia cell line) (Ogura et al., 1985) are committed to the megakaryocytic lineage and can produce platelet-like particles (Takeuchi et al., 1998). In addition, this cell line is frequently used in megakaryocyte differentiation studies and in gene function validation studies in megakaryocytopoiesis, platelet-like particle production, ploidy, and in vivo tumorigenesis (Zunino et al., 2001). In this study, we examined various gene-transfer methods with MEG-01 cells.

2. Materials and methods

2.1. Cell line

MEG-01 cells were purchased from American Type Culture Collection (VA, USA) and cultured in RPMI-1640 medium (Sigma, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH, KS, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, CA, USA) at 37 °C in a 5% CO₂ humidified atmosphere.

2.2. Plasmid DNA (pDNA)

pEGFP-C1, encoding green fluorescent protein (GFP), was purchased from Clontech (CA, USA). pGL3-Control, encoding luciferase, was purchased from Promega (WI, USA). pCMV-Script, a negative control plasmid, was purchased from Stratagene (CA, USA). pcDNA3 was purchased from Invitrogen. All plasmids were purified using an EndoFree Plasmid DNA Purification Kit (QIAGEN, CA, USA). A TPO receptor (c-mpl)-expressing plasmid was constructed as follows. Briefly, human c-mpl cDNA was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) using M-MLV Reverse Transcriptase (Invitrogen), KOD-plus DNA polymerase (Toyobo, Osaka, Japan), and a specific primer set (sense, 5'-CG-CCACCATGCCCTCCTGGGCCCTCTTCAT-3'; antisense, 5'-TCAAGGCTGCTGCCAATAGCTTAGTGGTAG-3'). In the sense primer, the kozak consensus sequence (underlined) was included as the start codon. Full-length cDNA was subcloned into a pCR-Blunt vector (Invitrogen) and it was cloned into the EcoRI site of the expression vector pcDNA3. The orientation of the insert was determined by restriction mapping. The sequence

of c-mpl was confirmed as accession number NM_005373 using a CEQ2000 DNA sequencer (Beckman Coulter, CA, USA).

2.3. Transfection

2.3.1. Electroporation

Electroporation was performed with an Electro Square Porator T820 electroporation system (BTX Inc., CA, USA) according to the manufacturer's recommended method. In brief, 1×10^6 cells suspended in 0.1 ml Dulbecco phosphate-buffered saline (PBS) were mixed with 15 µg of pGL3-Control plasmid. The mixture was transferred into a 2 mm gap electroporation cuvette and incubated on ice for 10 min. Then, electroporation was performed with various field strengths, pulse lengths, and pulse numbers. After electroporation, the cells were incubated on ice for 10 min and transferred into RPMI-1640 supplemented with 10% FBS. Ten minute or 24 h after electroporation, cell viability was determined by trypan blue dye exclusion assay. Twenty-four hour after electroporation, expression was determined by luciferase assay.

2.3.2. Hemagglutinating virus of Japan envelope (HVJ-E) vector

HVJ-E vector is a unique transfection tool that employs the cell fusion ability of the envelope of Sendai virus (HVJ) originally described by Kaneda (2003) (see review). Transfection with HVJ-E was performed according to the manufacturer's recommendations. Briefly, HVJ-E vector (25 µl) (GenomONE or GenomONE-Neo; Ishihara Sangyo, Osaka, Japan) was mixed with DNA (5 µg) and reagent B supplied in the kit (1 µl). The mixture was centrifuged at $10,000 \times g$ for 5 min at 4 °C. The pellet was suspended with the buffer supplied in the kit (30 µl). Then, the supplied reagent C (5 µl) was added. An aliquot of the mixture (1–4 µl) was added to cells (2.5×10^5 cells/0.5 ml) in a microcentrifuge tube, and the cells were centrifuged at $10,000 \times g$ for 30 min at 35 °C. The cell pellet was re-suspended with RPMI-1640 supplemented with 10% FBS and cultured in a 24-well plate. Twenty-four hour after transfection, GFP expression was determined using a flowcytometer as described below.

2.3.3. Lipid or cationic polymer

MEG-01 cells were transfected using Lipofectamine2000 (LFA2000; Invitrogen), FuGENE6 (Roche Diagnostics, Basel, Switzerland), GenePORTER2 (Gene Therapy Systems, CA, USA), Effectene (Qiagen), SuperFect (Qiagen), jetPEI (Q-Biogene, CA, USA), or TransFast (Promega). All transfections were performed according to the manufacturers' guidelines. Representative conditions for each reagent are shown in Table 1. Briefly, the reagents and pDNA were mixed to form the DNA–reagent complex. Then, the mixture was added to the cell suspension in the culture plate and cultured for 24 h. According to the manufacturers' recommendations, for all reagents, withdrawal of the transfection reagents from the culture was not necessary. Twenty-four hour after transfection, GFP expression was determined using a flowcytometer as described below.

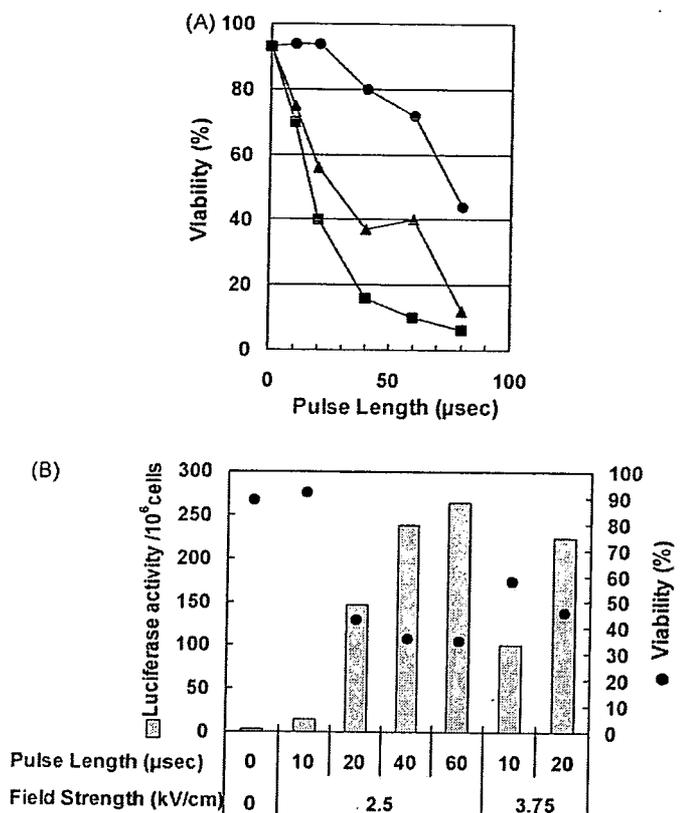


Fig. 1. Effect of electroporation and transfection efficiency with electroporation in MEG-01 cells. (A) MEG-01 cells (1×10^6) were treated with various field strengths and pulse lengths. Three pulses were applied at field strengths of 1.25 (●), 2.5 (▲), and 3.75 kV/cm (■) with various pulse lengths (0–80 μ s). (B) MEG-01 cells (1×10^6) were transfected with pGL3-Control (15 μ g) plasmid. Cell viability and luciferase activity were determined 20 h after transfection. The pulse number was kept at three in all electroporations.

while cell viability was inversely related to this amount. Dilution factors largely affected GFP expression and cell viability, but did not affect the positivity of GFP expression. To improve both the efficiency of GFP expression and cell viability, we tried to optimize the transfection conditions by modification of the preparation conditions, adding the volume and dilution ratio

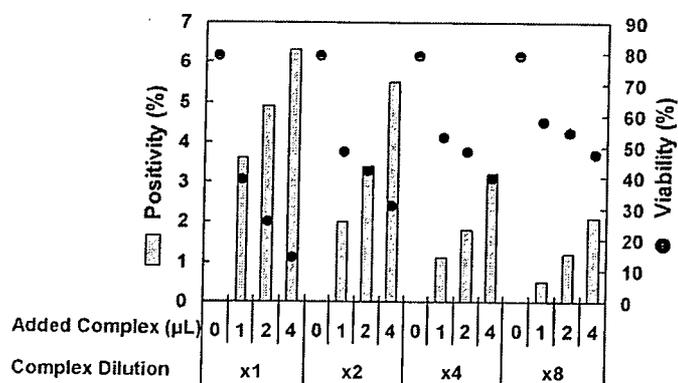


Fig. 2. Transfection efficiency of HVJ-envelope vector, GenomONE-Neo, in MEG-01 cells. MEG-01 cells (2.5×10^5) were treated with various amounts of GenomONE-Neo (25 μ L)-pEGFP-C1 (5 μ g) complex. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

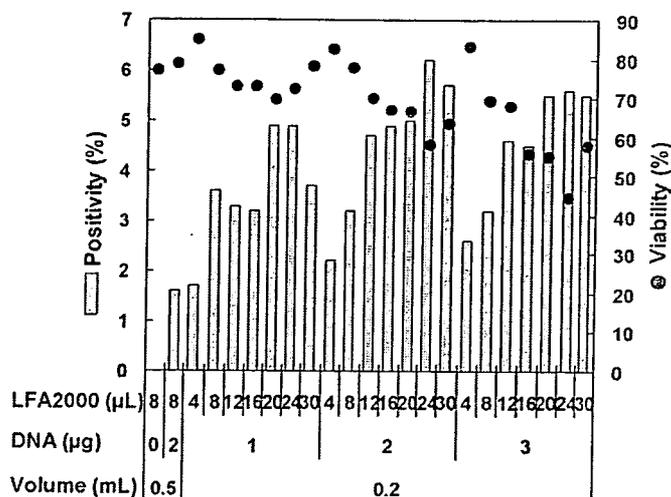


Fig. 3. Transfection efficiency of LFA2000 in MEG-01 cells. MEG-01 cells (5×10^5) were transfected with 1, 2, or 3 μ g of pEGFP-C1 with various amounts of LFA2000. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

of the HVJ-E–DNA complex. However, no improvement was achieved (data not shown).

3.3. Liposome or cationic polymer-based reagents

LFA2000, FuGENE6, SuperFect, Effectene, TransFast, jet-PEI, GenePORTER2, and DuoFect were tested with pEGFP-C1 pDNA. Representative results based on the manufacturers' recommended protocols are given in Table 1. Among these reagents, LFA2000 showed the highest transgene positivity. This finding is consistent with the results of Chuang and Schleef (2001), who showed that the transfection efficiency with FuGENE6, SuperFect, Effectene, or GenePORTER2 was very low for MEG-01 cells (<0.001%). To obtain a higher positivity with LFA2000, parameters such as the amount of pDNA, the transfection volume, and the culture time for transfection or after transfection were changed. As shown in Fig. 3, increasing the amount of LFA2000 used to prepare the lipoplex with pDNA improved the positivity without an increase in cytotoxicity. Positivity was also improved by decreasing the incubation volume. Finally, we obtained conditions that achieved a transfection positivity of around 6% (Fig. 3).

3.4. Nucleofection

Nucleofection was tested with pEGFP-C1 pDNA. The results of preliminary experiments to optimize the transfection conditions are summarized in Table 2. Although there were some combinations of solution and program that gave a high positivity (up to 90%), the viabilities were lower. In terms of viability, solution R showed slight lower cytotoxicity than solutions T and V. We then tested the combination of solution R with another eight programs recommended by Amaxa Biosystems: T-01, T-16, U-08, U-16, U-01, T-17, T-19, T-21, and T-09. The results are shown in Table 3. Consequently, program U-01 was selected

minor modifications. Briefly, the cells were transfected by LFA2000 or nucleofection. After 4 h of transfection, the cells were collected by centrifugation ($300 \times g$, 5 min, 4°C) and washed twice with cold PBS. The collected cells were suspended in 0.5 ml of DNA extraction buffer (10 mM Tris-HCl (pH 7.4), 3 mM MgCl_2 , 10 mM NaCl). Then, 10 μl of 0.5 M EDTA, 10 μl of 10% SDS, and 5 μl of proteinase K (20 mg/ml) were added to the cell suspension. After incubation at 37°C overnight, proteins were eliminated by phenol/chloroform treatment and the DNA was precipitated by the addition of ethanol. The precipitate obtained by centrifugation ($10,000 \times g$ for 10 min) was dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl; pH 8.0) and used as a DNA sample. DNA concentrations were determined by measurement of the absorbance at 260 nm with an Ultraspec3000 spectrophotometer (Amersham Pharmacia, Uppsala, Sweden). A part of the GFP region of pEGFP-C1 in the DNA samples was then amplified by PCR using TaKaRa Ex Taq DNA polymerase with the primers 5'-GACGTAAACGGCCACAAGTTCAGCG-3' and 5'-CTGCAGAATTTCGAAGCTTGAGCTCG-3'. The PCR comprised denaturation at 94°C for 2 min, followed by 26 cycles at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. After PCR, 10 μl of the reaction mixture was subjected to agarose gel electrophoresis, and the signal intensities of the amplified DNA bands were quantified using a FluorChem image analyzer (Alpha Innotech, CA, USA).

2.6. Evaluation of transgene function

2.6.1. Detection of phosphorylated STAT5 by Western blot

A c-mpl-encoding pDNA was transfected by nucleofection. To check whether the transgene was successfully expressed, Western blot analysis for phosphorylated STAT5, which is known to appear as a signal of TPO through the TPO receptor, c-mpl, was performed. MEG-01 cells transfected with the c-mpl expression plasmid or the control plasmid (pcDNA3) were cultured in RPMI 1640 containing 0.5% BSA for 16 h. The cells were stimulated with 100 ng/ml recombinant human TPO (Peprotech, NJ, USA) for 0, 10, and 30 min, and then directly lysed by SDS sample buffer (100 μl per 1×10^6 cells). The samples were separated on a 10% denaturing polyacrylamide gel (SDS-PAGE) and transferred to a 0.45- μm nitrocellulose membrane (Hybond ECL; Amersham, Uppsala, Sweden). The membranes were blocked with blocking buffer (Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% skim milk) for 1 h at room temperature. Subsequently, the membranes were incubated for 16 h at 4°C with anti-STAT5 and phospho-STAT5 (Tyr 694) antibodies (Cell Signaling Technology, MA, USA) using 1:2000 and 1:1000 dilutions with primary antibody dilution buffer (TBS containing 0.1% Tween-20 and 5% BSA), respectively. After washing three times with TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, CA, USA) using a 1:5000 dilution. After washing three times with TBS-T, immunocomplexes were

visualized with a SuperSignal West Pico Kit (Pierce, IL, USA).

2.6.2. Cell proliferation assay

MEG-01 cells transfected with c-mpl-encoding pDNA or control pDNA (pcDNA3) were suspended with RPMI 1640 (serum free), seeded at 10,000 cells/well in a 96-well plate, and pre-cultured at 37°C in 5% CO_2 . After 16 h, recombinant human TPO (1, 10, and 100 ng/ml) was added to the wells and culture was continued for a further 2 days. A WST-8 assay was performed according to the manufacturer's recommendations (Dojindo, Kumamoto, Japan). Briefly, 10 μl of WST-8 reagent was added to each well following all treatments and the plates were then further incubated for 4 h at 37°C in 5% CO_2 . Subsequently, the color development was read at 450 nm using a plate-reader (E-Max; Molecular Devices, CA, USA).

2.7. Statistics

Data were analyzed using Student's *t*-test for unpaired samples. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Electroporation

An initial transfection experiment was performed using an Electro Square Porator T820 electroporation system (BTX). According to the manufacturer's guidelines, MEG-01 cells were electroporated using a broad range of different electrical conditions to check the effects of pulse field strength and pulse length. Fig. 1A shows cell viability as a function of pulse length and field strength. Cell viability declined steadily with increasing pulse length and field strength. It appears that cell are affected by both field strength and pulse length.

pGL3-Control pDNA, which can express luciferase, was transfected under various conditions, with viabilities above 30%. The higher the luciferase activity, the lower the cell viability (Fig. 1B). Luciferase activities increased with increasing pulse length and field strength. However, the activities were relatively lower (<300 , cf. 27,000 in the case of LFA2000). Use of the lowest field strength (1.25 kV/cm), which showed higher viability (Fig. 1A), led to no luciferase activity at any pulse length or field strength (data not shown).

3.2. HVJ-E vector GenomONE and GenomONE-Neo

The inactivated HVJ-E vector systems GenomONE and GenomONE-Neo were tested. Unfortunately, GenomONE was very highly cytotoxic to MEG-01 cells. Thus, it was impossible to detect luciferase activity under the manufacturer's recommended conditions (data not shown). We examined another HVJ-E vector, GenomONE-Neo, with the pEGFP-C1 plasmid, which expresses GFP. Fig. 2 shows the relation between GFP positivity and viability following transfection. GFP-positive cells increased in response to the amount of complex added,

Table 2
Transfection efficiency of nucleofection (I)

Program	DNA	Solution R			Solution T			Solution V		
		Viability (%)	Positivity (%)	Efficiency (%)	Viability (%)	Positivity (%)	Efficiency (%)	Viability (%)	Positivity (%)	Efficiency (%)
A-23	+	73.2	38.1	27.9	52.1	60.4	31.5	59.3	49.7	29.5
A-27	+	81.5	6.7	5.5	77.4	15.3	11.8	79.4	10.4	8.3
T-20	+	38.1	61.2	23.3	32.3	95.0	30.7	37.1	94.8	35.2
T-27	+	53.1	90.7	48.2	35.1	92.6	32.5	49.1	93.2	45.8
T-16	+	63.8	90.6	57.8	48.1	92.5	44.5	57.6	92.5	53.3
T-01	+	82.1	74.6	60.6	76.8	75.0	57.6	78.8	77.2	60.8
G-16	+	67.3	57.0	38.4	54.9	69.5	38.2	49.9	68.9	34.4
O-17	+	63.1	75.5	47.6	51.2	81.3	41.6	65.2	91.3	59.5
–	–	88.1	0.0	0.0	88.6	0.0	0.0	86.5	0.0	0.0
T-16	–	78.4	0.0	0.0	77.6	0.0	0.0	79.0	0.0	0.0

For each condition, MEG-01 cells (1×10^6) were suspended with Nucleofector Solution R, T, or V, together with 5 μ g of pEGFP-C1 plasmid, and processed using various programs. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

for further experiments because of the higher efficiency and viability.

3.5. Amount of pDNA delivered to cells by nucleofection

To examine the reason for the high efficiency using nucleofection program U-01, we determined the amount of pDNA delivered by nucleofection. Program U-01 was compared with program A-27, which showed high viability and low transgene efficiency (Table 2). As a negative control, transfection with LFA2000 was selected. At 4 h after transfection, cell-associated pDNA was determined according the method described in Section 2. Interestingly, the amount of delivered pDNA by nucleofection was lower than that by LFA2000 irrespective of the use of program U-01 or A-27 (Fig. 4).

3.6. Nucleofection of *c-mpl*-encoding pDNA

To verify the utility of nucleofection, MEG-01 cells were transiently transfected with *c-mpl*-encoding pDNA. The effect of treatment with the ligand TPO on the transfected cells was

then evaluated by Western blotting of phosphorylated STAT5, which is activated by the TPO-*mpl* signal cascade, and by cell proliferation assay. As shown in Fig. 5B, STAT5 in *c-mpl*-transfected cells was more phosphorylated (activated) than that in mock-transfected cells (arrowheads) following incubation with TPO. STAT5 phosphorylation in *c-mpl*-transfected cells was enhanced 10 and 30 min after TPO treatment. The prolifer-

Table 3
Transfection efficiency of nucleofection (II)

Program	Viability (%)	Positivity (%)	Efficiency (%)
T-01	72 \pm 2	44 \pm 9	32 \pm 7
T-16	46 \pm 7	82 \pm 4	38 \pm 7
U-08	51 \pm 4	81 \pm 5	41 \pm 4
U-16	43 \pm 4	87 \pm 5	37 \pm 5
U-01	68 \pm 2	70 \pm 7	48 \pm 6
T-17	42 \pm 4	87 \pm 3	37 \pm 3
T-19	41 \pm 2	85 \pm 6	35 \pm 4
T-21	41 \pm 3	83 \pm 5	34 \pm 4
T-09	47 \pm 4	83 \pm 4	39 \pm 5
–	80 \pm 4	0 \pm 0	0 \pm 0

For each condition, MEG-01 cells (1×10^6) were suspended with Nucleofector Solution R together with 5 μ g of pEGFP-C1 plasmid, and processed using various programs. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection. Data are mean \pm standard error of three independent experiments.

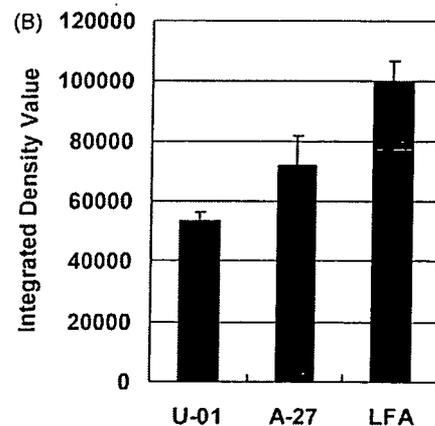
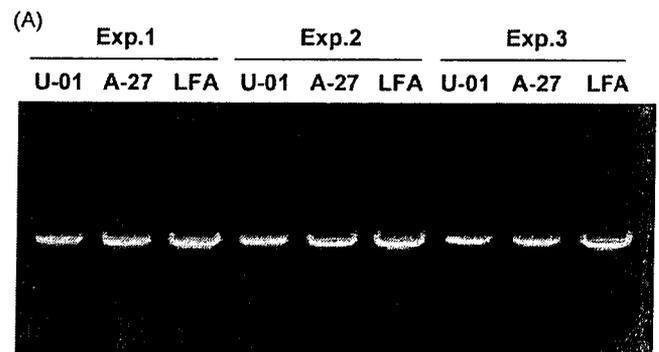


Fig. 4. Amount of cell-associated pDNA following nucleofection and lipofection. MEG-01 cells (1×10^6) were transfected with pEGFP-C1 plasmid using nucleofection (solution R, program U-01 or A-27) and LFA2000. At 4 h after transfection, pDNA was isolated from the cells and the amount was determined by PCR. (A) Agarose gel image of PCR products. (B) Integrated density value (IDV) of DNA bands. Data are averages of three independent experiments.

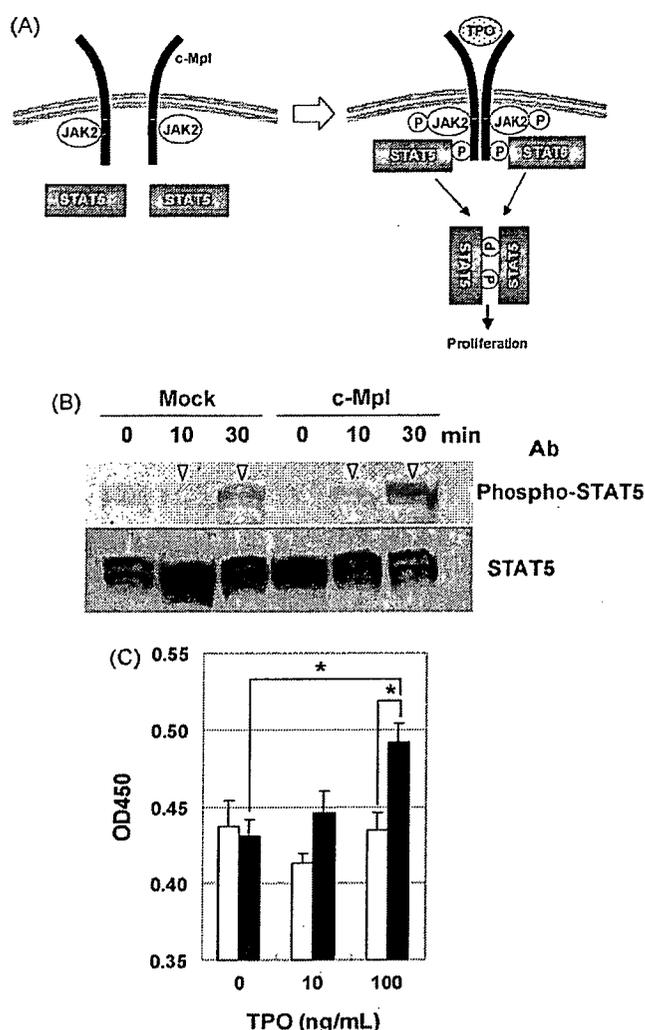


Fig. 5. TPO response in c-mpl-transfected cells. (A) Signal transduction cascades of TPO-induced STAT5 activation and proliferation of megakaryocytes. (B) Western blot of phospho-STAT5 and STAT5. MEG-01 cells (3×10^6) were transfected with c-mpl-encoding pDNA (5 μ g) by nucleofection. Transfected cells were stimulated with TPO (100 ng/ml) for 0, 10, or 30 min. (C) Cell proliferation of c-mpl- (■) or mock (□)-transfected cells following incubation in the presence of TPO (0, 10, or 100 ng/ml) for 2 days.

eration assay indicated that c-mpl-transfected cells proliferated extensively after treatment with TPO (100 ng/ml) for 2 days (Fig. 5C). These results show the high utility of nucleofection in validating the function of genes in MEG-01 related to differentiation.

4. Discussion

Efficient transfection of megakaryocytes is required to study the mechanism of platelet production from megakaryocytes. However, conventional transfection methods are generally insufficient. In this study, we investigated several transfection methods (electroporation, commercially available liposomal reagents, transferrin-linked cationic polymer, inactivated virus envelope vectors, and nucleofection) to obtain efficient transgene expression in MEG-01 cells. The results indicate that, among the methods we tested, nucleofection was the only

one to achieve efficient transgene expression in MEG-01 cells.

To clarify part of mechanism of efficient transgene expression by nucleofection, we determined the amount of pDNA delivered by transfection using two different nucleofection programs (U-01, most efficient; A-27, less efficient) and LFA2000 (negative control). The amount of pDNA delivered by nucleofection with U-01 was lower than that by nucleofection with A-27 and by LFA2000 (Fig. 4). It appears that there is not necessarily a good correlation between the amount of pDNA delivered by nucleofection and transgene expression. This observation is supported by the recent report of Hama et al. (2006). Even when transfected pDNA is incorporated into cells, there are many hurdles to clear, such as lysosomal traps and nuclear entry, before significant amounts of the gene product are expressed (Elouahabi and Ruyschaert, 2005). In nucleofection, electroporation based gene delivery technique, pDNA is assumed to be introduced directly into the cytoplasm and efficiently used for gene expression. In contrast, with LFA2000, lipofection based gene delivery reagent, pDNA forms complexes with the LFA2000, and the genes seem to be introduced into cells via endocytosis. This means that the genes are more likely to be trapped in endosomes and lysosomes, and cannot easily enter the nucleus. Tachibana et al. (2002b) reported that liposomes themselves inhibit transcription using a cell-free transcription and translation system. Hence, it is assumed that, though the amount of pDNA delivered to the cells by LFA2000 was more than that by nucleofection (U-01 or A-27), a relatively lower transfection efficiency was observed. Further investigation is needed to clarify the exact mechanism and achieve efficient gene expression with the relatively small amount of pDNA delivered by nucleofection.

To demonstrate the utility of nucleofection, we selected the c-mpl gene because c-mpl expression is very low (Graf et al., 1996) and the TPO signaling cascade is well investigated in megakaryocytes (Kaushansky, 2003). As shown in Fig. 5, there was a significant difference between c-mpl- and control pDNA-transfected cells in their responsiveness to TPO. This result strongly suggests that nucleofection is practical for validating gene function in MEG-01 cells.

Among the various gene-transfer methods, it has been reported that only methods using viral vectors show high transgene expression in megakaryocytes. Burstein et al. (1999) achieved 41–82% transgene-expressing megakaryocytes using a retroviral vector. Recently, Gillitzer et al. (2005) reported the use of a GFP-expressing retrovirus with CD34⁺ cells; the infected cells were cultured in the presence of TPO and differentiated into megakaryocytes. After differentiation, GFP expression on the culture-derived platelets was 40%. This method may be suitable for transgene expression in megakaryocytes. However, viral methods are not suitable for large numbers of validations of transgene function. This is because, for each target gene, the procedure requires many steps: viral vector construction, transfection to package cells, virus production, virus purification, preparation, and titer check for efficient infection. Moreover, there is a safety problem when using the virus, which can infect human cells and must be handled with appropriate facilities. In contrast, nucleofection is a relatively fast and easy method.

Nucleofection has been reported as an efficient technique of pDNA delivery to primary cultured cells and non-adhered hematopoietic cells (Hamm et al., 2002). For example, primary keratinocytes was successfully transfected without any change in their cellular properties (Distler et al., 2005). Natural killer cells, a kind of hematopoietic cell, and cell lines were also successfully transfected with high efficiency (Trompeter et al., 2003; Maasho et al., 2004). This is the first study to achieve efficient transgene expression in a megakaryocytic cell line, MEG-01, by nucleofection.

In conclusion, we have obtained an efficient transfection method for a megakaryocytic cell line, MEG-01. This low cytotoxicity, higher efficiency, non-viral method may be useful for clarifying the kinds of genes that are involved in the mechanism of platelet production from megakaryocytes. Moreover, nucleofection may be a useful technique, not only for megakaryocytes but also for other hematopoietic cells. This method may help in clarifying the mechanisms of the functions of hematopoietic cells.

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Complex Formation with Plasmid DNA Increases the Cytotoxicity of Cationic Liposomes

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Cationic liposomes (CL) are one of the most widely studied non-viral vectors for gene delivery. It is well-known that CL induces cytotoxicity following lipofection. However, little is known regarding the mechanism involved in the cytotoxicity. In this study, the *in vitro* cytotoxicity of CL and its complex with pDNA (lipoplex) was investigated, and a part of the mechanism of induction as well. While free pDNA did not show any cytotoxicity, pDNA increased the cytotoxicity of CL *via* the formation of lipoplex. In addition, the lipoplex-induced cytotoxicity increased in a lipoplex dose-dependent manner, irrespective of the type of pDNA, cell line and the absence or presence of serum. An assay showed that apoptosis was largely induced by treatment with the lipoplex (lipofection), but not with CL alone, in the tested range of concentration of CL and pDNA. Furthermore, following treatment with lipoplexes, the cells exhibited the morphological features of apoptosis and DNA fragmentation. A cDNA microarray study showed that the lipofection up-regulated 45 genes related to apoptosis, transcription regulation and immune response. These results clearly indicate that pDNA in the lipoplex increases the cytotoxicity of CL as a result of inducing apoptosis. The fundamental principle for gene therapy is to deliver gene-based therapeutics to target cells for specific gene targeting with minimal cytotoxicity. Our results suggest the possibility that cytotoxicity induced by lipofection, accompanied by gene changes, could intrinsically exacerbate, attenuate or even mask the desired effects of gene-based therapy.

Key words cationic liposomes; lipofection; cytotoxicity; plasmid DNA; transfection

Gene therapy has emerged as one of the most potent and promising strategies for the development of a treatment for inherited and acquired disorders with underlying genetic defects or malfunctions.^{1,2} Gene-based therapies require acceptable and efficient delivery along with minimal toxicity. Hence, viral and non-viral vectors have been extensively studied for *in vivo* and *ex vivo* gene transfer with the goal of developing a strategy that could lead to a large number of cells showing higher gene expression and minimum cytotoxic effects.

Nonviral vectors such as cationic liposomes (CL) have been shown to be generally preferred over viral-vectors because they are thought to be non-immunogenic, relatively easy to assemble, and amenable to scale-up for industrial production.^{3,4} Unlike viruses, they have no restrictions on the size of DNA to be delivered; CL can deliver nucleic acids of essentially unlimited size ranging up to large mammalian artificial chromosomes. CL can also be covalently grafted to receptor-specific ligands for targeted gene delivery. Therefore, since their first introduction as a potential delivery system in 1987,⁵ DNA-cationic lipid complexes (lipoplexes) have been used in numerous research protocols for the delivery of genes, antisense oligonucleotides and siRNA in a range of cell types. Several lipoplex formulations have been evaluated in clinical treatment of cancer^{6,7} and cystic fibrosis.^{8–10}

One of the prerequisites for the success of gene therapy is a safe and efficient gene delivery system. Thus, the risk associated with the exposure and the molecular mechanism of any cytotoxicity need to be well understood. But the related information is scant. Some *in vitro* studies have been shown that CL induces cytotoxic effects or apoptosis in several cell

types, for example, in phagocytic macrophages or immune cells.^{11,12} In addition, other studies have demonstrated that some cationic lipid-based transfection agents induced gene-expression changes in human epithelial cells.¹³ However, these investigations are limited owing to the fact that they were concerned only with material-related intrinsic cytotoxicity.

Concerning lipoplex-induced toxicity, lipoplexes cause changes to cells, including cell shrinking, reduced number of mitoses, and vacuolization of the cytoplasm.¹⁴ This toxicity may, in part, result from the large size or high positive zeta potential of the lipoplexes required for their uptake.¹⁵ However, the detailed molecular mechanisms leading to cell death caused by lipoplexes are still not fully understood, thus it is important for us to decipher the origin and nature of the cytotoxicity and related molecular mechanisms.

In this study, we investigated the *in vitro* cytotoxicity of one of the most widely used CL, Lipofectamine 2000, and its complex with pDNA as well as a part of the mechanism of its induction. Furthermore, we examined the relationship between gene expression profile changes and the induced cytotoxicity. Our investigation may help to develop a novel CL mediated-gene delivery lipoplex formulation with a very low cytotoxicity.

MATERIALS AND METHODS

Materials Lipofectamine 2000 (LF2000) was purchased from Invitrogen (CA, U.S.A.). The luciferase assay kit and the cell culture lysis reagent (CCLR) were purchased from Promega (WI, U.S.A.). APOPercentage™ apoptosis assay kit was obtained from Biocolor (Northern Ireland, U.K.). Syto

24 Green Fluorescence nucleic acid stain was purchased from Molecular Probes (Oregon, U.S.A.). Opti-MEM I medium was purchased from Life Technologies (MD, U.S.A.). The other cell culture reagents were obtained from Nissui Pharmaceutical (Tokyo, Japan). Other reagents were of analytical grade.

Cells HeLa cells (human cervix) were obtained from Dr. Yasuo Shinohara (Division of Gene Expression, Institute of Genome Research, the University of Tokushima, Japan). B16BL6 cells (mouse murine melanoma) were generous gift from Dr. Naoto Oku (Department of Medical Biochemistry, University of Shizuoka, Japan). RGC-6 cells (rat brain, glioma) were obtained from Cell Resource Center for Biomedical Research (Tohoku University, Japan).

Plasmid The pDNAs, pGL3-Control containing the cDNA of firefly luciferase (Promega, WI, U.S.A.), pORF9-mPTEN containing the cDNA of mPTEN (InvivoGen, CA, U.S.A.) and pEGFP-N1 containing the cDNA of EGFP (BD Biosciences, CA, U.S.A.) were propagated in *Escherichia coli* and isolated using a plasmid Giga kit (Qiagen, Hilden, Germany). The DNA concentration was determined by measuring the UV absorbance at 260 nm. Purity was confirmed by agarose gel electrophoresis. Another pDNA, pCpG-mcs G2 containing an MCS (Multiple Cloning Site) with several commonly used restriction sites, for convenient cloning of a CpG-free gene, was purchased from InvitroGen and kindly propagated by Dr. Naoshi Yamazaki (Department of Medicinal Biochemistry, Institute of Health Biosciences, the University of Tokushima, Japan).

Preparation of Lipoplexes Lipoplexes of CL with pDNA were prepared as recommended by the manufacturer. Briefly, LF2000 in Opti-MEM I medium was added to vials containing pDNA in Opti-MEM I. They were vortexed at low speed for 20 s to avoid pDNA shearing and the lipoplexes were allowed to form for 20 min at room temperature. Four lipoplex formulations were prepared by keeping constant the pDNA amount (0.2 $\mu\text{g}/\text{well}$) while the LF2000 amount was varied (0.25, 0.5, 1, and 2 $\mu\text{l}/\text{well}$). Four other formulations were prepared by keeping the optimal charge ratio recommended by the manufacturer constant (pDNA (μg): LF2000 (μl) ratio at 1 : 2.5) and proportionally increasing the amount of both pDNA and LF2000.

Lipofection Cells were seeded in 96 well-plates at a density of 1×10^4 cells/well 24 h prior to the lipofection in growth medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM glutamine, and 100 U/ml penicilline and 100 $\mu\text{g}/\text{ml}$ streptomycine. Cultures were maintained at 37 °C in a 5% CO₂ air incubator. Different amounts of LF2000 alone or lipoplexes were added to the wells and the final volume was set to 100 $\mu\text{l}/\text{well}$ with serum free Opti-MEM I or with Opti-MEM I containing 10% FBS. After 4 h of lipofection, the medium was replaced with fresh growth medium after washing twice with cold phosphate buffered saline (PBS).

Evaluation of Cytotoxicity An MTT assay was employed to determine the cytotoxicity of cells as a result of 4 h exposure to naked pDNA, LF2000 alone and lipoplexes. The growth medium was removed 24 h-post lipofection and the cells were washed with PBS. Fifty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.5 mg/ml) was added to each well and further incubated for 4 h.

The formazan was dissolved in 150 μl of 2-propanol/HCl and incubated for 30 min at 37 °C. The absorbance of each well was read at 570 nm on a microplate reader Wallac 1420 ARVOsx (PerkinElmer, Trukn, Finland). Cytotoxicity data were expressed as the percentage absorbance relative to untreated control cells.

Apoptosis Assay

Assessment of Morphological Changes To assess the morphological changes, untreated cells, LF2000-treated cells (2 $\mu\text{l}/\text{well}$) and lipofected-cells (0.8 μg pGL-3-Control/2 μl CL/well), were washed once with PBS at 8 h post-lipofection and the cells were then stained with Syto 24 Green Fluorescence Nucleic acid stain. Apoptotic cells were detected by fluorescence confocal microscopy (Carl Zeiss, Oberkochen, Germany).

DNA Fragmentation Assay At 24 h post-lipofection, untreated cells, naked pDNA-treated cells (0.8 $\mu\text{g}/\text{well}$), LF2000-treated cells (2 $\mu\text{l}/\text{well}$) and lipofected cells (0.8 μg pGL-3-Control/2 μl CL/well) were washed once with PBS. Approximately 5×10^7 cells were then harvested by trypsinization and lysed by mixing with a 100 μl of lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8), 1% Triton X-100) at 4 °C for 10 min. The lysate was centrifuged at 16000 rpm for 5 min to separate the fragment DNA (supernatant) from intact chromatin (pellet). The solution containing low molecular mass DNA was treated with 200 μg RNase A for 10 min at 37 °C to digest RNA and with 200 μg proteinase K for 1 h at 50 °C to digest protein. DNA was precipitated with 20 μl 5 M NaCl and 120 μl isopropanol for 2 h at -20 °C, and then centrifuged at 16000 rpm for 15 min and washed once with 0.5 ml ice cold 70% ethanol. The pellets were dissolved in 30 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8)) and subjected to 2% agarose gel electrophoresis at 50 V in TAE buffer for about 1.5 h. DNA was visualized by ethidium bromide staining under UV light (ATTO, Tokyo, Japan).

Quantitative Measurement of Apoptosis The APOPercentage™ apoptosis assay kit was used to quantify apoptosis according to the manufacturer's instructions. The APOPercentage dye was added to the cells at 8 h post-lipofection and incubated for 1 h at 37 °C. The cells were washed twice with PBS, and immediately examined under fluorescent microscopy. The percentage apoptosis was determined by counting the cells under light microscopy. At least 200 cells were counted from randomly selected fields in two independent observation sessions.

Microarray Analysis Total RNA was extracted from cells that had been treated with naked pDNA, LF2000 alone, or lipoplexes by means of RNeasy micro kit (Qiagen). The RNAs were amplified, converted to complementary DNAs and labeled with Cy-5-CTP using the Low RNA Fluorescent Linear Amplification kit (Agilent Technologies, CA, U.S.A.). As a result, the amplified complimentary RNA products were labeled with Cy-5. As a control, Cy-3 labeled reference human RNA was used. Labeled cRNAs were then fragmented and hybridized using Agilent's *in situ* hybridization plus kit on Human1A ver.2 Oligo Microarray (Agilent Technologies). The arrays were scanned using an Agilent Technologies Microarray Scanner (Agilent Technologies). The microarray consisted of 20227 genes including genes related to cell cycle regulation, apoptosis-related, DNA transcription

factors/damage response/repair and recombination, metabolism, translation/cytokines. The experiment was performed according to the manufacturer's protocol. The data were analyzed with the GeneSpring software (Agilent Technologies).

In this study, data from each gene were typically reported as a base 2 logarithm of the expression ratio of the control. One way analysis of variance (ANOVA) followed by multiple comparison test (*post-hoc*) and/or unpaired two-tailed *t*-test were used with an assumption of *p* value less than 0.05 for significant differences.

RESULTS

Lipoplexes Induced Marked Cytotoxicity Compare to CL Alone

The effect of treatment with CL alone on cytotoxicity was compared to the effect of treatment with their lipoplex in HeLa cells (Fig. 1). Cells were treated with various amounts of CL alone or with different lipoplexes prepared by varied CL amount (0.25, 0.5, 1, 2 μ l/well) with constant pDNA amount (0.2 μ g/well). CL alone induced only a slight cellular toxicity in the range of CL tested, irrespective of the absence (Fig. 1 left) or presence (Fig. 1 right) of

serum. While, in the similar experimental conditions, lipoplexes induced a significant cytotoxicity compared to CL alone and the cytotoxicity increased as the CL content in the lipoplex increased ($p < 0.01$).

The effect of the amount of lipoplex on cytotoxicity was examined using lipoplexes in which the pDNA (μ g) : CL (μ l) ratio was kept at 1 : 2.5 (Fig. 2). The induced cytotoxicity increased in a lipoplex dose-dependent manner, irrespective of the absence (Fig. 2 left) or presence (Fig. 2 right) of serum. The cytotoxicity in the lipoplex-treated cells was markedly higher than either CL- or pDNA-treated cells. Through this experiment, pDNA itself did not show any cytotoxicity in the range of concentrations used, while CL alone showed a slight cytotoxicity. A similar tendency in terms of cytotoxicity was observed for the other two cell lines (RGC-6 and B16BL6) (data not shown).

In order to determine whether different pDNAs lead to different levels of cytotoxicity, lipoplexes were prepared with CL and three different pDNAs (pGL3-Control, pORF9-mPTEN or pEGFP-N1). All lipoplexes induced a similar level of cytotoxicity (data not shown). In addition, to circumvent the possibility that CpG motifs in the pDNA sequence

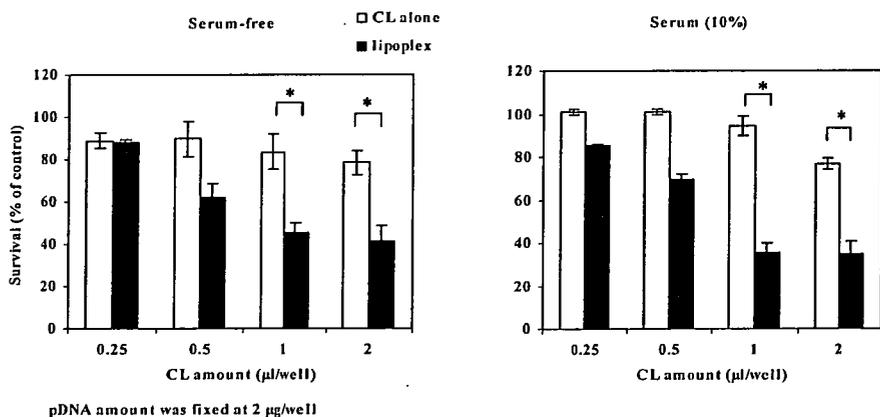


Fig. 1. Cytotoxicity Induced by CL Alone or Lipoplex

Cells were treated with CL alone (open column) or lipoplex (pGL3-Control/CL) (closed column) in the absence of serum (left) or presence of 10% FBS (right) for 4 h. Twenty four hours post-lipofection, cytotoxicity was determined by an MTT assay according to the methods described in Materials and Methods. Data are the mean \pm S.D. ($n=3$) from three independent experiments. Significant statistical difference of cytotoxicity between lipoplexes and CL alone are reported as $*p < 0.01$.

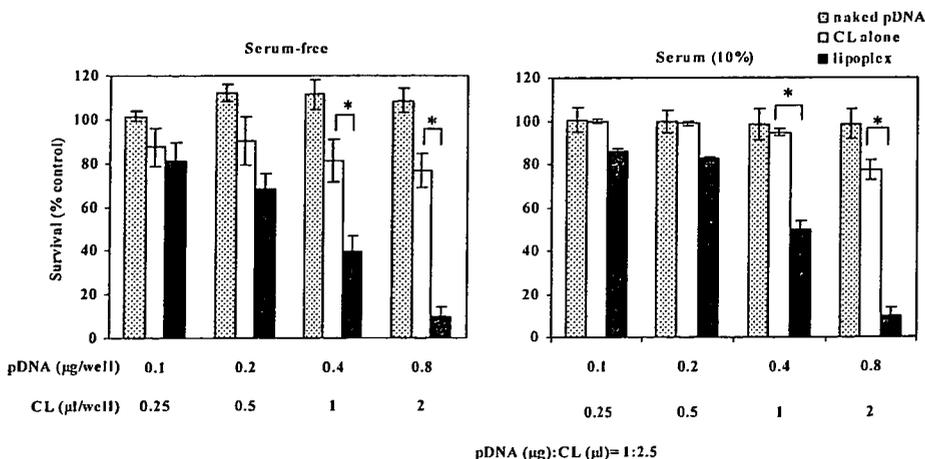


Fig. 2. Effect of Lipoplex-Dose on the Induced Cytotoxicity

Cells were treated with naked pDNA (pGL3-Control) (dotted column), CL alone (open column) or lipoplex (closed column) in the absence of serum (left) or presence of 10% FBS (right) for 4 h. At 24 h post-lipofection, cytotoxicity was determined by an MTT assay according to the methods described in Materials and Methods. Data are mean \pm S.D. ($n=3$) from three independent experiments. Significant statistical difference of cytotoxicity between lipoplexes and CL alone are reported as $*p < 0.01$.

may lead to cell death associated with the inflammatory toxicity, further investigations were carried out (Fig. 3). The cytotoxicity induced by lipoplexes including CpG-free pDNA was similar to that induced by lipoplexes including CpG motif-containing pDNA.

Apoptosis Induced in Lipofected Cells To investigate if the cell death induced by lipoplex or CL alone was caused *via* apoptosis, apoptosis was evaluated with two different methods: agarose gel electrophoresis to determine DNA fragmentation (the hallmark of apoptosis) and cellular morphological analysis. Treatment with lipoplex induced substantial DNA fragmentation, while treatment with CL alone induced only minimal DNA fragmentation and treatment with pDNA failed to induce any DNA fragmentation (Fig. 4A). A fluorescence microscopic image of non-treated cells and CL-treated cells showed a normal nuclear morphology with a large nuclei and evenly distributed chromatin (Fig. 4B and Fig. 4C, respectively). In contrast, nuclei with condensation chromatin were clearly observed in the cells that had been treated with lipoplex (Fig. 4D).

In addition, the induced apoptosis was quantitatively assessed using the APOPercentage apoptosis assay. This assay reflects the translocation of plasma membrane phosphatidylserine that is produced in the early apoptosis process. Following treatment with various amounts of CL alone and

lipoplex, the assay was carried out at 8 h post-lipofection. Apoptosis was caused by treatment with lipoplex in a lipoplex-dose dependent manner (Fig. 5A). More than 80% of the cells were apoptotic at the highest lipoplex dose (CL and pDNA amounts were 2 μ l/well and 0.8 μ g/well, respectively) used in the treatment. In addition, the effect of the pDNA content in lipoplex in causing apoptosis was investigated as the level of CL was kept at 2 μ l/well (Fig. 5B). The apoptosis percentage increased as the pDNA content was reinforced in the lipoplex formulations. Naked pDNA (0.8 μ g/well) did not lead to any apoptosis,

Up or Down-Regulated Genes as a Result of Treatment with Lipoplex or CL Alone In order to understand what occurred in cells following lipofection, gene expression pro-

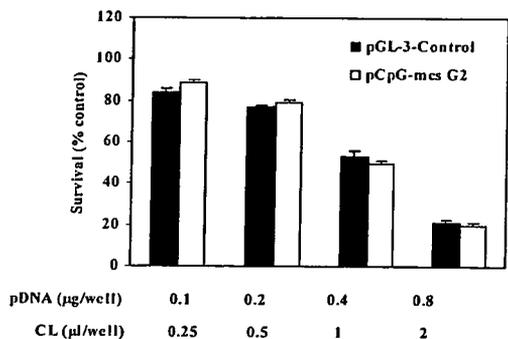


Fig. 3. Effect of Different pDNAs in the Lipoplex on the Induced Cytotoxicity

Cells were treated with lipoplex containing pGL-3-Control (closed column) or lipoplex containing pCpG-mcs G2 (opened column) for 4 h. After 24 h lipofection, cytotoxicity was determined using an MTT assay according to the methods described in Materials and Methods. Data are mean \pm S.D. ($n=3$) from three independent experiments.

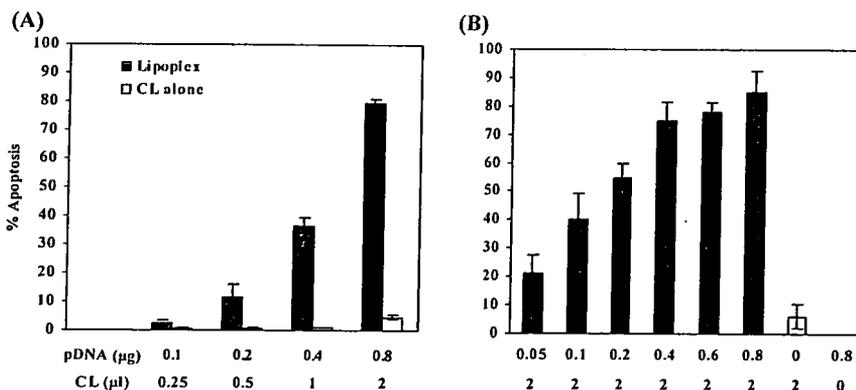


Fig. 5. Effect of Lipoplex-Dose (A) or Amount of pDNA in the Lipoplex (B) on the Lipoplex-Induced Apoptosis

(A) Percent apoptosis of HeLa cells after a 4 h treatment with various amounts of CL (opened column) and their lipoplexes (pGL-3-Control/CL) (closed column) were determined as described in the Materials and Methods. (B) Apoptosis percentages of HeLa cells after 4 h treatment with lipoplexes containing different amount of pDNA (pGL-3-Control) but a constant amount of CL was determined. Values are expressed as mean \pm S.D. of at least 200 cells from randomly selected fields were counted by two independent observations.

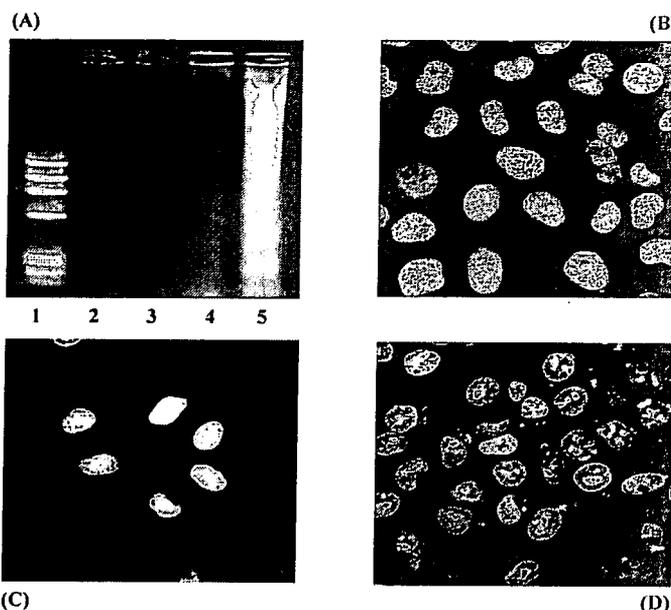


Fig. 4. DNA Fragmentation and Morphological Analysis

(A) Agarose gel electrophoresis of DNA extracted from HeLa cells at 12 h after treatment with naked pDNA, CL alone or lipoplex was carried out as described in Materials and Methods. Lane 1: DNA marker (Φ X174 DNA-Hae III Digest (New England BioLabs, MA, U.S.A.), 1 μ g/well). Lane 2: Non-treated cells. Lane 3: Naked pDNA (pGL-3-Control), Lane 4: CL alone (2 μ l/well). Lane 5: Lipoplex (0.8 μ g pGL-3-Control/2 μ l CL /well). (B–D) Morphological analysis in HeLa cells after 8 h treatment was done: (B): non-treated cells, (C): CL alone (2 μ l/well), (D): lipoplexes (0.8 μ g pGL-3-Control/2 μ l CL /well). A similar result was observed in three independent experiments and a typical result was shown.

Table 1. Summary of Genes Changed by the Treatments

GenBank	Gene category	Gene title	CL alone FC ^{a)}	Lipoplex FC ^{a)}
NM_005347	ATP binding; protein binding, stress response	HSPA5, heat shock protein 70 kDa protein 5	1.305	4.05704
NM_017510	Protein carrier	HSGP25L2G, gp25L2 protein	1.1633	4.24495
NM_004419	Cell cycle/protein modifier	DUSP5, dual specificity phosphatase 5	1.5863	4.65321
NM_002923	Cell cycle	RGS2, regulator of G-protein signaling 2	1.9277	4.94052
NM_004417	Cell cycle/protein modifier	DUSP1, dual specificity phosphatase 1	1.6989	6.69753
NM_014685	Protein modifier	HERPUD1, endoplasmic reticulum stress response-inducible	2.8895	15.4729
X89399	GTPase activator activity	GAP1 IP4BP, RAS p21 protein activator 3	0.9516	21.1763
NM_002133	Anti-apoptosis, growth factor	HO-1, heme oxygenase (decyclizing) activity; oxidoreductase activity; signal transducer activity	4.7743	2.71169
NM_001657	Anti-apoptosis, growth factor	AREG, amphiregulin preproprotein	1.6699	4.97542
NM_003407	DNA binding, pro-apoptosis	TTP, zinc finger protein 36	1.676	5.45199
NM_001300	Pro-apoptosis	KLf6, suppression of tumorigenicity 12	2.0236	5.52261
NM_021127	Apoptosis	NOXA, phorbol-12-myristate-13-acetate-induced protein 1	1.8397	6.34913
NM_004083	Cell cycle arrest, pro-apoptosis	CHOP, DNA damage-inducible transcript 3	2.3735	7.08512
NM_004024	Transcription factor/pro-apoptosis	ATF3, transcription factor activity	2.0929	8.95974
NM_002228	Transcription factor/pro-apoptosis	AP1, activator protein 1; JUN	2.7726	10.0429
NM_005252	DNA binding, pro-apoptosis	FOS, v-fos FBJ murine osteosarcoma viral oncogene homolog	2.9243	14.9736
NM_001964	Transcription factor/pro-apoptosis	EGR1, early growth response 1	2.1906	17.4851
NM_021052	Transcription factor/DNA binding protein	HIST1H2AE, H2A histone family, member A	1.777	4.11556
NM_003530	Transcription factor/DNA binding protein	HIST1H3D, H3 histone family, member B	3.0309	5.09793
NM_021968	Transcription factor/DNA binding protein	HIST1H4J, H4 Histone family, member E	2.4303	5.29728
NM_004414	Transcription factor/DNA binding protein	DSCR1, calcipressin 1 aciform a	2.6586	5.54703
NM_003518	Transcription factor/DNA binding protein	HIST1H2BG, H2B histone family, member A	1.9303	5.58707
S62138	Transcription factor/DNA binding protein	TLS/CHOP, fusion protein, TLS/CHOP	1.6422	5.64676
NM_003537	Transcription factor/DNA binding protein	HIST1H3B, H3 histone family, member L	2.6696	6.85893
NM_003548	Transcription factor/DNA binding protein	HIST2H4, histone 2, H4	5.9915	9.4456
NM_003509	Transcription factor/DNA binding protein	HIST1H2AI, H2A histone family, member C	4.1403	10.2784
NM_003543	Transcription factor/DNA binding protein	HIST1H4H, H4 Histone family, member H	6.8413	10.8678
NM_003540	Transcription factor/DNA binding protein	HIST1H4F, H4 histone family, member C	9.0812	13.2378
NM_003955	Immune response	SSI-3, suppressor of cytokine signaling 3	1.6258	4.72137
NM_001511	Immune response	CXCL1; GRO1, chemokine ligand 1	1.5064	4.871
NM_002089	Immune response	CXCL2, chemokine ligand 2	1.4233	4.87239
NM_006417	Immune response	IFI44, interferon induced, hepatitis C-associated microtubular aggregate	1.0206	7.33255
NM_001549	Immune response	IFIT3, interferon induced protein with tetratricopeptide repeats 3	0.9638	7.34021
NM_002201	Immune response, cell proliferation	ISG20, interferon stimulated gene 20 kDa	1.1346	7.4357
NM_000600	Immune response, apoptosis	IL6, interferon, beta 2	1.1069	7.50226
NM_003733	Immune response	p59OASL, 2'-5'-oligoadenylate synthetase-like	0.752	11.1649
NM_005101	Immune response, protein binding	IFI15, interferon, alpha-inducible protein 15 kDa	1.0911	13.934
NM_014314	Immune response	DDX58, DEAD/H(Asp-Glu-Ala-Asp/His) box polypeptide RIG-I	1.0536	17.2823
BC009507	Immune response, protein binding	IFI15, interferon, alpha-inducible protein 15 kDa	1.1798	19.6599
NM_001548	Immune response	IFIT1, interferon, alpha-inducible protein 56	1.1199	20.9507
NM_001547	Immune response	IFIT2, interferon induced protein with tetratricopeptide repeats 2	1.6092	72.5171
A_23_P57836	Unknown	Unknown	1.9254	4.2029
AB040917	Unknown	KIAA1484, KIAA1484 protein	0.8935	4.92871
A_23_P21293	Unknown	Unknown	1.1315	6.00392
BC018929	Unknown	PHLDA1, PHLDA1 protein	2.6866	12.1289

45 genes up- or down-regulated by treatment with lipoplex or CL alone (Fig. 6) were identified and are listed. Each gene is denoted by the GeneBank accession number and the Gene Ontology (<http://www.geneontology.org/>), the annotations for Biological process and Molecular function. ^{a)} FC: Fold changes in gene expression represented the ratio of the samples treated with either CL alone or lipoplex divided by those of untreated cells.

files were examined by a microarray analysis. The microarray study showed that 45 gene-expressions were significantly up- or down-regulated following treatment with CL alone and lipoplex (Fig. 6). The exact identity of 45 genes changed are listed in Table 1. Several structure-function categories were represented in the list of differently expressed genes, *i.e.*, cell cycle, apoptosis-related, DNA-binding, transcription, and immune response. Among them, a great number of pro-apoptotic genes including TTP (zinc finger protein 36), KLf6 (suppression of tumorigenicity 12), NOXA (phorbol-12-

myristate-13-acetate-induced protein 1), CHOP (DNA damage-inducible transcript 3), ATF3 (transcription factor activity), AP1 (activator protein 1), FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog), and EGR1 (early growth response 1) were observed. This proves that lipoplex induced cell death *via* the apoptosis pathway. In addition, some genes related to the regulation of DNA binding, transcription factors and pro-inflammatory cytokines including interleukin (IL6) and interferon- α , were up-regulated.

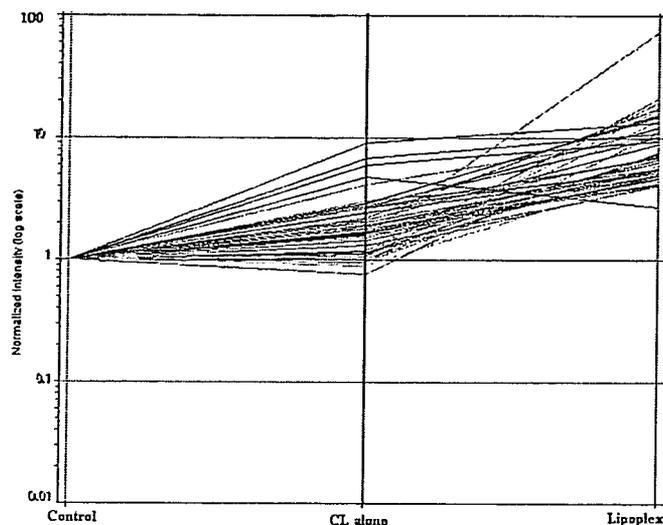


Fig. 6. Up or Down-Regulation of Genes by Treatment with Lipoplex or CL Alone

Comparative microarray was carried out following treatment with CL alone ($2 \mu\text{l/well}$) or lipoplex ($0.8 \mu\text{g pGL-3-Control/2} \mu\text{l CL/well}$).

DISCUSSION

A fundamental principle of gene therapy is to deliver gene-based therapeutics into target cells for specific gene targeting with minimal cytotoxicity. CL has been extensively investigated as a potentially safer alternative for gene delivery. However, the results described here indicated that the cytotoxicity of CL is further increased by the formation of a complex with non-toxic pDNA (lipoplex) and that this lipoplex results in cellular apoptosis. In addition, 45 genes were up-regulated as a result of lipofection. Such synergistic cytotoxicity induced by CL and pDNA, accompanied by gene-expression up-regulation, may intrinsically affect the outcome of gene therapy.

The remarkable cytotoxicity was caused as lipoplex was exposed to the cells, while no distinct cytotoxicity was found for the uncomplexed material (either CL or pDNA alone) (Figs. 1, 2). In addition, the cytotoxicity increased with increasing lipoplex dose (Figs. 2, 5A) or pDNA content in the lipoplex (Fig. 5B). These results clearly indicate that there is a synergism between CL and pDNA in causing cytotoxicity. It is likely that such induced cytotoxicity is not necessarily related to cell type or pDNA type. Some reports have demonstrated the pDNA carrying therapeutic genes is associated with the inflammatory toxicity of lipoplex.^{16,17} This is thought to be due to the fact that the non-mammalian genes are recognized as foreign DNA due to the presence of a specific motif, CpG dinucleotides.¹⁸ Our results strongly suggest that the induced cytotoxicity is not as a consequence of inflammation-like stimulation by CpG motif of pDNA in the lipoplex (Fig. 3).

The application of functional genomics technologies—meaning the profiling of differential expression for many genes simultaneously by cDNA microarrays has proven to be very promising over recent years and was used to predict the toxic properties of one compound.¹⁹ The potential benefit of using microarray analysis may provide insights into the mechanisms responsible for the lipoplex-induced cytotoxicity observed in this study. We demonstrated that 45 genes

were significantly up-regulated in HeLa cells as a result of treatment with lipoplex, which are involved in a wide spectrum of biological processes and molecular functions, such as apoptosis, cell cycle control, DNA binding/transcription factors, and immune response. Lipoplex much strongly induced up-regulation of genes than that of CL alone (Fig. 6, Table 1). There seems to be a good correlation between cytotoxicity and up-regulation of gene-expression induced by lipoplex.

In cells exposed to lipoplex, pro-apoptosis related genes were significantly over-expressed, including NOXA, CHOP, ATF3, AP1, FOS, and EGR1 (Table 1). The identification of the function of these genes may be important in understanding the lipoplex-induced cytotoxic mechanism. It has been shown that the over-expression of NOXA induces apoptosis.^{20,21} NOXA was identified as a novel BH-3 only protein that is activated by endoplasmic reticulum (ER) stress at the transcript level.²² CHOP, Growth arrest- and DNA damage-inducible gene 153 (GADD153), which belongs to a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcriptional factors. The over-expression of CHOP results in cell growth arrest and apoptosis, its function is antagonized by BCL-2.²³ CHOP is one of the components of the ER stress-mediated apoptosis pathway and much is known on the roles of this molecule on apoptosis.^{23–26} AP-1 is known to modulate apoptosis.²⁷ Some reports have indicated that AP-1 plays an important role in the induction of CHOP gene expression.^{28–30} FOS is also a major component of the AP-1 transcription factor complex. The expression of FOS may result from up-regulation of AP-1 and CHOP (Table 1). Activating transcription factor 3 (ATF3), a member of the ATF/CREB family, is rapidly induced by a wide range of stresses including genotoxic stresses and is involved in cell growth, apoptosis and invasion.³¹ It has been reported that ATF3 is activated by JNK³² and that JNK and ATF3 are activated through ER stress.³³ ATF3 can also function as a stress-inducible transcriptional repressor for CHOP gene.³⁴ The results of previous functional studies suggest that the early growth response 1 gene (EGR-1) is an anti-proliferative signal for tumor cells^{35,36} and that the gene acts as to increase the potency of apoptotic agents.^{37,38} Another report indicated that the overexpression of EGR-1 protein induced ATF3 expression,³⁹ which is consistent with our results showing that EGR1 expression was significantly increased, leading to an increased up-regulation of ATF3 (Table 1). It should be noted that most of the pro-apoptosis genes that are up-regulated by lipofection are related to the ER stress-mediated apoptosis pathway. The ER is the site for synthesis, folding, modification and trafficking of secretory and cell-surface proteins. It has been reported that ER stress could be elicited in the cell culture system by pharmacological agents, severe or prolonged ER stress can lead to cell death through apoptosis.²⁵ Therefore, it appears that a stronger induction in ER stress due to lipofection leads to a greater apoptosis, although further experiments will be necessary to confirm the exact mechanism for causing apoptosis by lipofection.

From our results, the lipoplex appears to be an extremely potent inducer of the immune response (Table 1). Interferon α (IFI15 and IFI1) and interleukin 6 (IL6), up-regulated by lipofection in this study were reported to induce apoptosis.⁴⁰ It is also well-known that interferons cause an inhibition of

cell proliferation.^{41–43} Thus, it is possible that interactions with the lipoplex, but not pDNA or CL alone, modulates and triggers some gene expressions, which are involved in programmed cell death or cytokine generation of innate or adaptive immune reactions. Consequently, cells treated with lipoplex die *via* apoptosis.

To our knowledge, the results presented here show, for the first time, that lipoplex induces a much stronger cytotoxicity than CL, consequently leading to cell death *via* the apoptosis pathway in non-phagocytic or immune cells. Our results clearly indicate that CL and pDNA have a synergism in causing cytotoxicity, although both are non-toxic when used in treating cells. Such synergistic cytotoxicity, accompanied by gene-expression changes, may intrinsically affect the outcome of gene therapy. Further investigations of other types of CL and targeted cells both *in vitro* and *in vivo* will be necessary to elucidate the comprehensive toxicogenomic impact of CL-based formulations for gene therapy.

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The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex

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Abstract

Efficient delivery is a key issue in translating interference RNA technology into a feasible therapy. The efficiency of carrier systems used for this technology is commonly tested by co-transfection, i.e. simultaneous transfection with an exogenous gene and with the siRNA. Two approaches can be distinguished: (1) with the two transfectants in the same carrier complex (siRNA/pDNA/carrier) and (2) with the two transfectants in different carrier complexes (pDNA/carrier and siRNA/carrier). The process to prepare the nucleic acid(s)–carrier complexes and the transfection procedure may affect the effectiveness of the gene-silencing process. In this study, two preparation methods were compared, namely the co-preparation of an siRNA/pDNA/liposome lipoplex (Method I) and the separate preparation of an siRNA/liposome lipoplex and a pDNA/liposome lipoplex (Method II). siRNA in the lipoplex produced by Method I showed a stronger gene-silencing effect than that in the lipoplexes prepared by Method II. There was no significant difference between the two methods in the amount of siRNA delivered to cells. Cellular entry and intracellular trafficking of siRNA/pDNA/liposome lipoplex is likely to differ from those of the separate lipoplexes. When in Method II non-transcriptional pDNA was included in the complex with siRNA, the gene-silencing effect was significantly enhanced. If and to what extent the experimental design is suitable to quantify RNA interference remains to be demonstrated.

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1. Introduction

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing produced by double-strand RNAs. This is a multiple-step process that involves the generation of 21–23-nt small interfering RNA (siRNA) and results in the degradation of the homologous RNA (Elbashir et al., 2001). RNAi has enormous potential not only as an invaluable tool in biological research and drug development but also as a possible approach to the *in vivo* inactivation of gene products linked to human disease and pathology. The primary obstacle in translating RNAi technology from an effective research tool into a feasible ther-

apeutic strategy remains the efficient delivery of siRNA to the targeted cell *in vivo*. It is well recognized that non-viral vectors not only produce simpler transfection devices than viruses but they also provide a safer alternative than viral vectors, especially because of low host immunogenicity (Lundstrom and Boulikas, 2003). Among the non-viral vectors, several cationic liposomes have been reported to effectively transfer of siRNAs *in vitro* and *in vivo* (Sorensen et al., 2003; Sioud and Sorensen, 2003, 2004; Spagnou et al., 2004; Yano et al., 2004; Bitko et al., 2005; Landen et al., 2005; Morrissey et al., 2005; Nogawa et al., 2005; Khoury et al., 2006). So far, however, the achievements in terms of clinical outcome are very limited. This lack of success may be blamed in part to the wide-spread misconception that all nucleic acids are alike and to a general lack of notion that different delivery systems may deliver RNA and DNA to different intracellular pathways and thus bring about different transfection efficiencies.

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The gene-silencing effect (Paddison et al., 2002; Xu et al., 2003, 2004; Muratovska and Eccles, 2004) of siRNA as well as the ability of cationic liposomes to deliver siRNA into cells (Spagnou et al., 2004), are preferably assessed as knockdown of exogenous rather than of endogenous gene expression. In our study, simultaneous transfection with the target gene pDNA and the gene-silencing siRNA was accomplished by means of cationic liposomes. The co-transfection was achieved by two different approaches: Method I in which pDNA and siRNA were in the same carrier complex, and Method II in which a mixture of pDNA/carrier complex and siRNA/carrier complex was used for transfection. The physicochemical properties of the three complexes are likely to be significantly different, because of the substantial chemical and structural differences between pDNA and siRNA. This, in turn, is likely to lead to essential differences in the interaction of the complexes with cells, their cellular uptake and the intracellular distribution and ultimate fate of siRNA and pDNA, which will largely determine the effectiveness of the gene silencing effect.

We developed TFL-3, a cationic liposome composed of the cationic lipid, DC-6-14, with the helper lipids dioleoylphosphatidylethanolamine (DOPE) and cholesterol (CHOL), for pDNA delivery (Kikuchi et al., 1999). TFL-3 has shown high *in vitro* transfection efficiency in serum-containing media (Nguyen et al., 2003; Li et al., 2004; Nguyen et al., 2005) and effective *in vivo* gene transfection activity in a murine lung metastasis model (Li et al., 2005). Based on these features, TFL-3 may prove to be a profitable carrier not only for pDNA but also for siRNA, although systematic investigations with respect to siRNA transfer have not yet been performed.

In this study, we addressed two issues: (1) the potential of TFL-3 to transfer siRNA and (2) the suitability of the co-transfection method to determine the RNAi effect and the efficacy of carriers to transfer siRNA into cells. Lipoplexes were prepared from pDNA, siRNA and TFL-3 by two different methods. Method I involved the preparation of one single complex, siRNA/pDNA/TFL-3 lipoplex, and Method II the preparation of two separate complexes, siRNA/TFL-3 and pDNA/TFL-3. The lipoplexes were used to transfect B16BL6 cells, a murine melanoma, with the firefly luciferase gene and to assess the gene-silencing effect of siRNA on the expression of the exogenous gene.

2. Materials and methods

2.1. Preparation of siRNAs

A siRNA for firefly luciferase (sense sequence, 5'-CUUA-CGCUGAGUACUUCGATT-3'; anti-sense sequence, 5'-UCG-AAGUACUCAGCGUAAGTT-3') and an unrelated siRNA (sense sequence, 5'-AGCUUCAUAAGGCGCAUGCTT-3'; anti-sense sequence, 5'-GCAUGCGCCUUAUGAAGCUTT-3') (Elbashir et al., 2001) were chemically synthesized and purified by means of HPLC by Hokkaido Systems Sciences (Hokkaido, Japan). A siRNA duplex (50 μ M) was prepared by mixing complementary antisense-stranded RNA and sense-stranded RNA in TE buffer (10 μ M Tris-HCl, 1 μ M EDTA, pH 8.0,

DNase and RNase free grade) (Nippon Gene, Tokyo, Japan) at equal molar concentrations. The mixture was incubated in boiling water for 1 min and cooled slowly to room temperature. The quality of the siRNA duplexes siRNA was checked by 15% PAGE. The prepared siRNA duplexes were stored at -80°C .

2.2. Preparation of lipoplex

A cationic lipid mixture, TFL-3, composed of DC-6-14/DOPE/CHOL (1/0.75/0.75 mol/mol) was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). TFL-3 liposomes were prepared at a concentration of 2.4 mM by addition of ddH₂O (milliQ) to the lyophilized lipid mixture under vortexing, to give unilamellar liposomes. Two luciferase pDNAs (*Photinus* (firefly) luciferase, pGL-3 and *Renilla* (sea pansy) luciferase, pRL-TK) were purchased from Promega (WI, USA). A mixture of a reporter gene and a control gene (1 μ g/ μ l) was prepared by mixing 45 μ l of pGL3 solution (1 μ g/ μ l in TE buffer) and 5 μ l of pRL-TK (1 μ g/ μ l in TE buffer). A stock solution of siRNA was serially diluted with TE buffer (DNase and RNase free grade, pH 8.0) to obtain concentrations of 5000, 2500, 1250, 625, 312.5, 156.25 and 78.125 nM, respectively.

2.2.1. Co-preparation of the siRNA/pDNA/TFL-3 lipoplex (Method I)

Five microliter of pDNAs (pGL-3 and pRL-TK) solution (1 μ g/ μ l) were mixed with 100 μ l of the diluted siRNA (78.125, 156.25, 312.5, 625, 1250 nM). The final volume of the mixture was adjusted to 2.5 ml with OPTIMEM I. To the mixtures of pDNA and siRNA 2.5 ml of TFL-3 (20 μ M) was added, diluted by OPTIMEM I. The final mixture was allowed to stand for 20 min at room temperature.

2.2.2. Separate preparation of siRNA/TFL-3 and pDNA/TFL-3 lipoplexes (Method II)

For the siRNA/TFL-3 complex, 100 μ l of the diluted siRNA (312.5, 625, 1250, 2500, 5000 nM) was further diluted with 1150 μ l of OPTIMEM I. The siRNA solution was mixed with 1250 μ l of TFL-3 (20 μ M) in OPTIMEM I. For the pDNA/TFL-3 lipoplex, 1250 μ l of pDNAs (1 μ g/ μ l) were mixed with equal volume of TFL-3 (20 μ M) in OPTIMEM I. The mixtures were allowed to stand for 20 min at room temperature.

After preparation, all lipoplexes were checked for the presence of free pDNA and siRNA by agarose electrophoresis (0.8% and 2%, respectively). Throughout this study, the amounts of cationic liposomes for transfection were kept constant to avoid saturation or competitive inhibition of the delivery of siRNA and pDNA by the liposomes.

2.3. Mean diameter and zeta potential of the lipoplexes

The mean diameter and the zeta potential of the lipoplexes were determined using a laser particle analyzer and a laser electrophoresis zeta potential analyzer device, NICOMP 380 (Particle Sizing System, CA, USA). A 5% dextrose solution was used to dilute the samples.