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	2.5	84.9	1.7	1.4
FuGENE6	1.2	0.2	5	92.2	0.1	0.1
SuperFect	4.0	2.0	5	86.2	0.0	0.0
Effectene	10	0.4	5	88.4	0.0	0.0
TransFast	4.5	0.5	2	76.8	0.8	0.6
jetPEI	2.0	1.0	2.5	81.0	0.2	0.2
GenePORTER2	7.0	2.0	5	87.9	0.4	0.4
DuoFect	4.5	10	5	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 flowcytometor 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 (Amaxa). 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 flowcytometric 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 × 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

minor modifications. Briefly, the cells were transfected by LFA2000 or nucleofection. After 4 h of transfection, the cells were collected by centrifugation (300 × *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 × *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 Ultrospec3000 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′-CTGCAGAATTCGAAGCTTGAGCTCG-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,

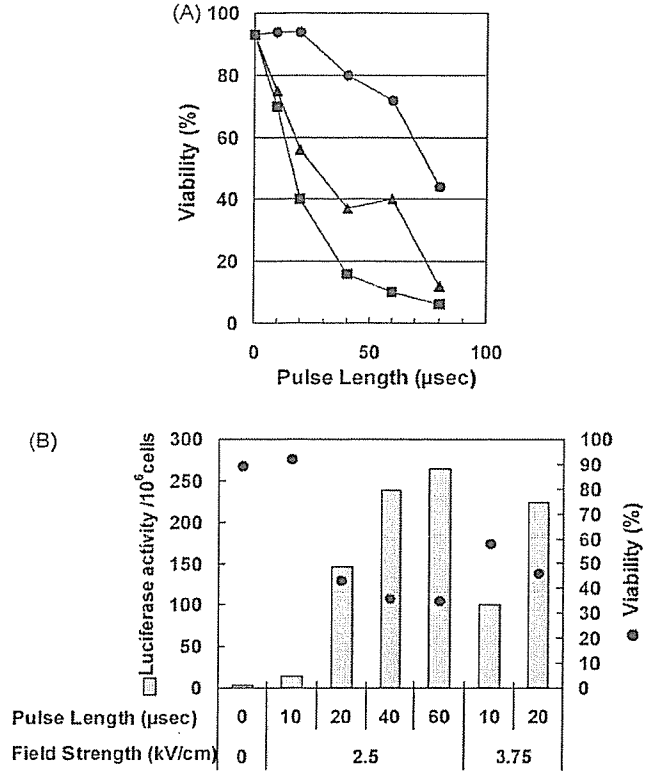

Fig. 1. Effect of electroporation and transfection efficiency with electroporation in MEG-01 cells. (A) MEG-01 cells (1×10^6) were treated with various field strengths and pulse lengths. Three pulses were applied at field strengths of 1.25 (●), 2.5 (▲), and 3.75 kV/cm (■) with various pulse lengths (0–80 μs). (B) MEG-01 cells (1×10^6) were transfected with pGL3-Control (15 μg) 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

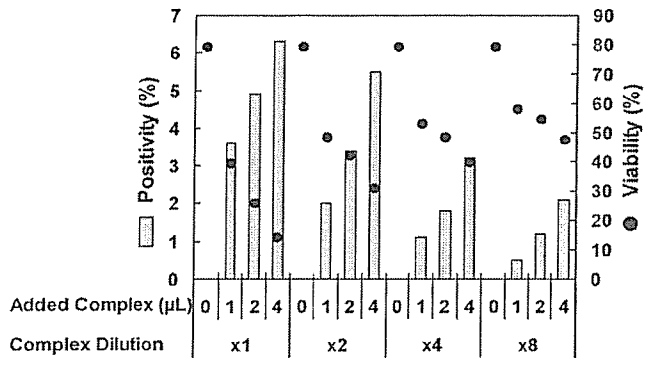

Fig. 2. Transfection efficiency of HVJ-envelope vector, GenomONE-Neo, in MEG-01 cells. MEG-01 cells (2.5×10^5) were treated with various amounts of GenomONE-Neo (25 μl)-pEGFP-C1 (5 μg) complex. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

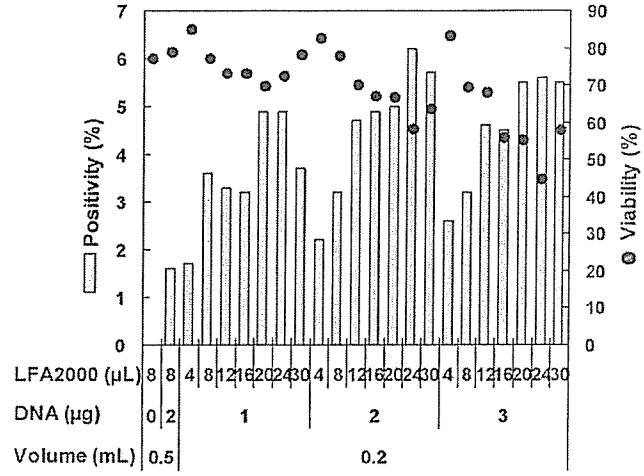

Fig. 3. Transfection efficiency of LFA2000 in MEG-01 cells. MEG-01 cells (5×10^5) were transfected with 1, 2, or 3 μg of pEGFP-C1 with various amounts of LFA2000. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

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

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 μg 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 phospholylated 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 phospholylated (activated) than that in mock-transfected cells (arrowheads) following incubation with TPO. STAT5 phospholylation in c-mpl-transfected cells was enhanced 10 and 30 min after TPO treatment. The prolif-

Table 3
Transfection efficiency of nucleofection (II)

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

For each condition, MEG-01 cells (1×10^6) were suspended with Nucleofector Solution R together with 5 μg 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 ± standard error of three independent experiments.

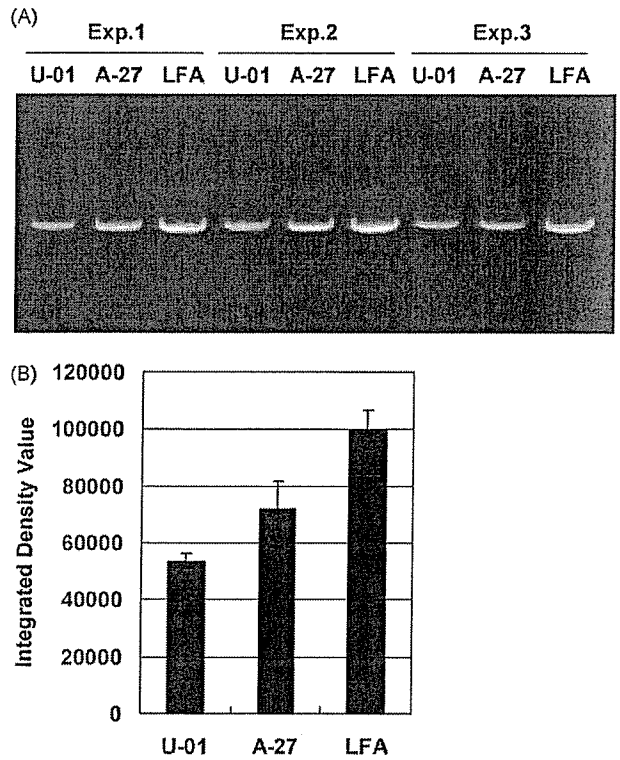

Fig. 4. Amount of cell-associated pDNA following nucleofection and lipofection. MEG-01 cells (1×10^6) were transfected with pEGFP-C1 plasmid using nucleofection (solution R, program U-01 or A-27) and LFA2000. At 4 h after transfection, pDNA was isolated from the cells and the amount was determined by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

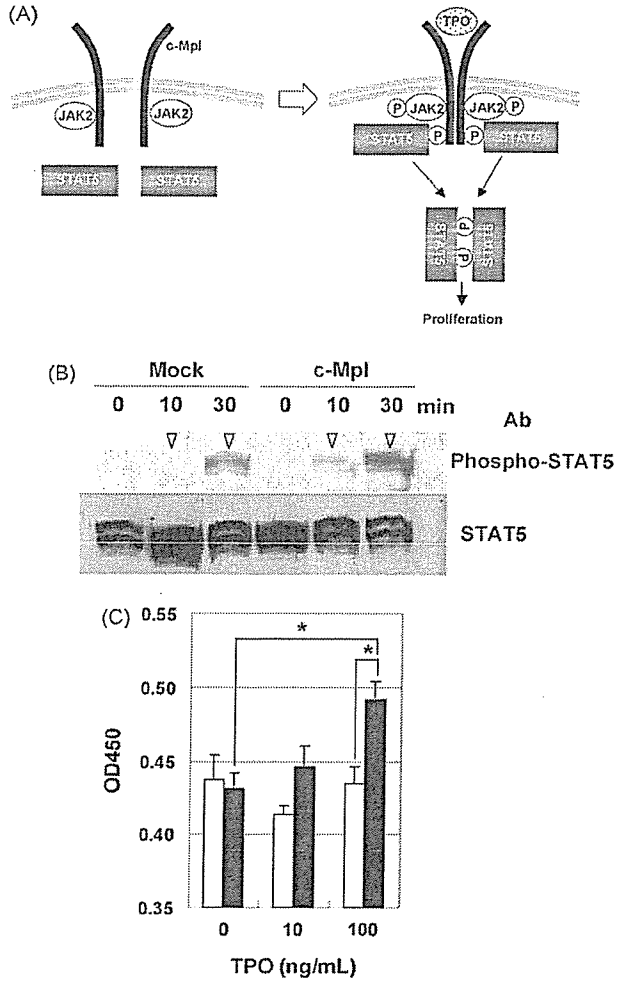

Fig. 5. TPO response in c-mpl-transfected cells. (A) Signal transduction cascades of TPO-induced STAT5 activation and proliferation of megakaryocytes. (B) Western blot of phospho-STAT5 and STAT5. MEG-01 cells (3×10^6) were transfected with c-mpl-encoding pDNA (5 μg) by nucleofection. Transfected cells 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 Ruysschaert, 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 hematopoictic cells.

References

Bartley, T.D., Bogenberger, J., Hunt, P., Li, Y.S., Lu, H.S., Martin, F., Chang, M.S., Samal, B., Nichol, J.L., Swift, S., 1994. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 77, 1117–1124.

Burstein, S.A., Dubart, A., Norol, F., Debili, N., Friese, P., Downs, T., Yu, X., Kincade, P.W., Villeval, J.L., Vainchenker, W., 1999. Expression of a foreign protein in human megakaryocytes and platelets by retrovirally mediated gene transfer. Exp. Hematol. 27, 110–116.

Carver-Moore, K., Broxmeyer, H.E., Luoh, S.M., Cooper, S., Peng, J., Burstein, S.A., Moore, M.W., de Sauvage, F.J., 1996. Low levels of erythroid and myeloid progenitors in thrombopoietin-and c-mpl-deficient mice. Blood 88, 803–808.

Chuang, J.L., Schleef, R.R., 2001. Recombinant Semliki Forest virus enhanced plasminogen activator inhibitor 1 expression and storage in the megakaryocytic cell line MEG-01. J. Cell. Biochem. 82, 277–289.

Distler, J.H., Jungel, A., Kurowska-Stolarska, M., Michel, B.A., Gay, R.E., Gay, S., Distler, O., 2005. Nucleofection: a new, highly efficient transfection method for primary human keratinocytes*. Exp. Dermatol. 14, 315–320.

Drexler, H.G., Matsuo, Y., MacLeod, R.A., 2004. Malignant hematopoietic cell lines: in vitro models for the study of erythroleukemia. Leuk. Res. 28, 1243–1251.

Elouahabi, A., Ruysschaert, J.M., 2005. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol. Ther. 11, 336–347.

Gillitzer, A., Peluso, M., Laugwitz, K.L., Munch, G., Massberg, S., Konrad, I., Gawaz, M., Ungerer, M., 2005. Retroviral infection and selection of culture-derived platelets allows study of the effect of transgenes on platelet physiology ex vivo and on thrombus formation in vivo. Arterioscler. Thromb. Vasc. Biol. 25, 1750–1755.

Graf, G., Zaborski, M., Quentmeier, H., Drexler, H.G., 1996. Phorbol ester PMA induces expression of the thrombopoietin receptor MPL in leukemia cells. Leuk. Lymphoma 24, 149–157.

Hama, S., Akita, H., Ito, R., Mizuguchi, H., Hayakawa, T., Harashima, H., 2006. Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. Mol. Ther. 13, 786–794.

Hamm, A., Krott, N., Breibach, I., Blindt, R., Bosserhoff, A.K., 2002. Efficient transfection method for primary cells. Tissue Eng. 8, 235–245.

Kaneda, Y., 2003. New vector innovation for drug delivery: development of fusigenic non-viral particles. Curr. Drug Targets 4, 599–602.

Kaushansky, K., 2003. Thrombopoietin: accumulating evidence for an important biological effect on the hematopoietic stem cell. Ann. N. Y. Acad. Sci. 996, 39–43.

Levine, R.F., 1980. Isolation and characterization of normal human megakaryocytes. Br. J. Haematol. 45, 487–497.

Maasho, K., Marusina, A., Reynolds, N.M., Coligan, J.E., Borrego, F., 2004. Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system. J. Immunol. Methods 284, 133–140.

Ogura, M., Morishima, Y., Ohno, R., Kato, Y., Hirabayashi, N., Nagura, H., Saito, H., 1985. Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome. Blood 66, 1384–1392.

Shivdasani, R.A., Fujiwara, Y., McDevitt, M.A., Orkin, S.H., 1997. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J. 16, 3965–3973.

Shivdasani, R.A., Rosenblatt, M.F., Zucker-Franklin, D., Jackson, C.W., Hunt, P., Saris, C.J., Orkin, S.H., 1995. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell 81, 695–704.

Tachibana, R., Harashima, H., Ide, N., Ukitsu, S., Ohta, Y., Suzuki, N., Kikuchi, H., Shinohara, Y., Kiwada, H., 2002a. Quantitative analysis of correlation between number of nuclear plasmids and gene expression activity after transfection with cationic liposomes. Pharm. Res. 19, 377–381.

Tachibana, R., Harashima, H., Ishida, T., Shinohara, Y., Hino, M., Terada, H., Baba, Y., Kiwada, H., 2002b. Effect of cationic liposomes in an in vitro transcription and translation system. Biol. Pharm. Bull. 25, 529–531.

Takeuchi, K., Satoh, M., Kuno, H., Yoshida, T., Kondo, H., Takeuchi, M., 1998. Platelet-like particle formation in the human megakaryoblastic leukaemia cell lines, MEG-01 and MEG-01s. Br. J. Haematol. 100, 436–444.

Trompeter, H.I., Weinhold, S., Thiel, C., Wernet, P., Uhrberg, M., 2003. Rapid and highly efficient gene transfer into natural killer cells by nucleofection. J. Immunol. Methods 274, 245–256.

Wang, Z., Zhang, Y., Lu, J., Sun, S., Ravid, K., 1999. Mpl ligand enhances the transcription of the cyclin D3 gene: a potential role for Sp1 transcription factor. Blood 93, 4208–4221.

Zunino, R., Li, Q., Rose, S.D., Romero-Benitez, M.M., Lejen, T., Brandan, N.C., Trifaro, J.M., 2001. Expression of scinderin in megakaryoblastic leukemia cells induces differentiation, maturation, and apoptosis with release of platelet like particles and inhibits proliferation and tumorigenesis. Blood 98, 2210–2219.

Please cite this article in press as: Isakari, Y. et al., Efficient gene expression in megakaryocytic cell line using nucleofection, Int. J. Pharmaceut. (2007), doi:10.1016/j.ijpharm.2007.01.042

Complex Formation with Plasmid DNA Increases the Cytotoxicity of Cationic Liposomes

Lap Thi NGUYEN,[a,b] Kazutaka ATOBE,[a] Jose Mario BARICHELLO,[a,c] Tatsuhiro ISHIDA,[a] and Hiroshi KIWADA*,[a]

[a] *Department of Pharmacokinetics and Biopharmaceutics, Subdivision of Biopharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima; 1–78–1 Sho-machi, Tokushima 770–8505, Japan:* [b] *Vietnam National Institute of Drug Quality Control; 48-Hai Ba Trung Street, Hanoi, Vietnam: and* [c] *Japan Association for the Advancement of Medical Equipment; Tokyo 113–0033, Japan.* Received November 27, 2006; accepted January 11, 2007

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 cytoplasma.[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

* To whom correspondence should be addressed. e-mail: hkiwada@ph.tokushima-u.ac.jp

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 μg/well) while the LF2000 amount was varied (0.25, 0.5, 1, and 2 μl/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 μg/ml streptomycine. Cultures were maintained at 37 °C in a 5% CO_2 air incubator. Different amounts of LF2000 alone or lipoplexes were added to the wells and the final volume was set to 100 μl/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 μl/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 μg/well), LF2000-treated cells (2 μl/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 μl/well) with constant pDNA amount (0.2 μg/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 (μg) : CL (μl) 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

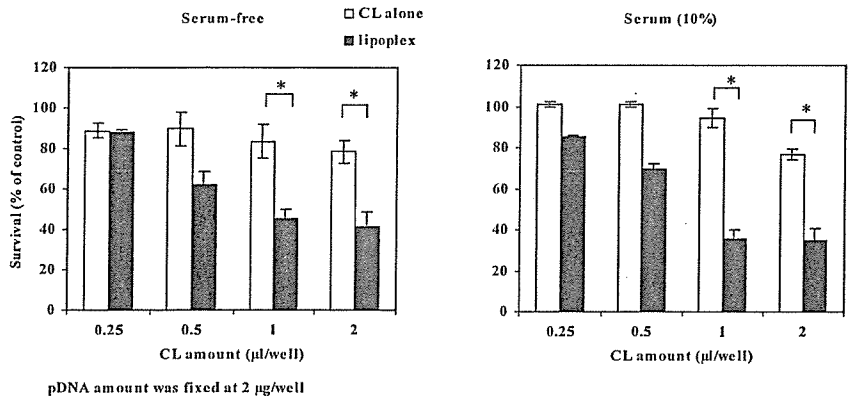

Fig. 1. Cytotoxicity Induced by CL Alone or Lipoplex

Cells were treated with CL alone (open column) or lipoplex (pGL-3-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±S.D. ($n=3$) from three independent experiments. Significant statistical difference of cytotoxicity between lipoplexes and CL alone are reported as $*p<0.01$.

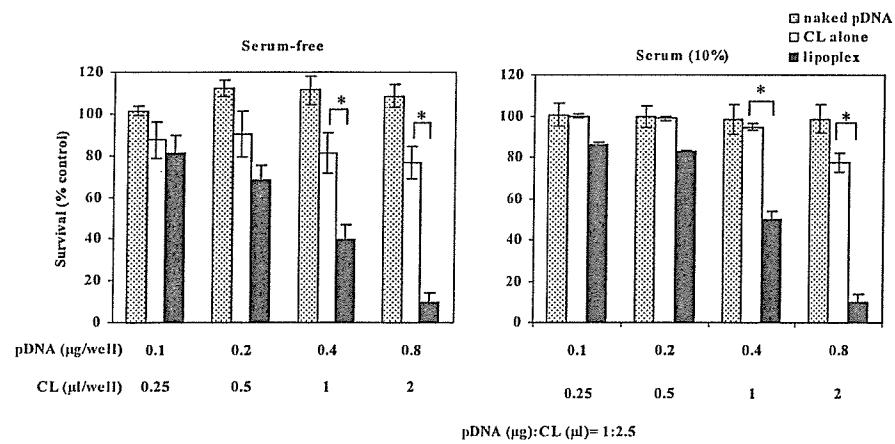

Fig. 2. Effect of Lipoplex-Dose on the Induced Cytotoxicity

Cells were treated with naked pDNA (pGL-3-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±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 μl/well and 0.8 μg/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 μl/well (Fig. 5B). The apoptosis percentage increased as the pDNA content was reinforced in the lipoplex formulations. Naked pDNA (0.8 μg/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-

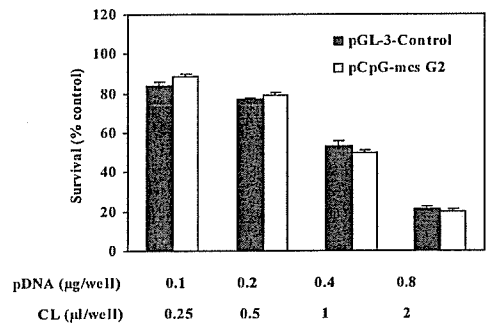

Fig. 3. Effect of Different pDNAs in the Lipoplex on the Induced Cytotoxicity

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±S.D. (n=3) from three independent experiments.

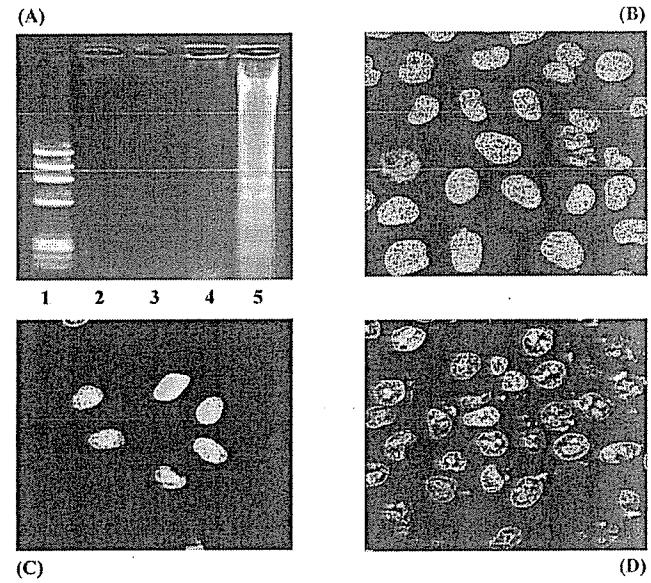

Fig. 4. DNA Fragmentation and Morphological Analysis

(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 (ΦX174 DNA-Hae III Digest (New England BioLabs, MA, U.S.A.), 1 μg/well). Lane 2: Non-treated cells. Lane 3: Naked pDNA (pGL-3-Control), Lane 4: CL alone (2 μl/well). Lane 5: Lipoplex (0.8 μg pGL-3-Control/2 μl CL /well). (B—D) Morphological analysis in HeLa cells after 8 h treatment was done: (B): non-treated cells, (C): CL alone (2 μl/well), (D): lipoplexes (0.8 μg pGL-3-Control/2 μl CL /well). A similar result was observed in three independent experiments and a typical result was shown.

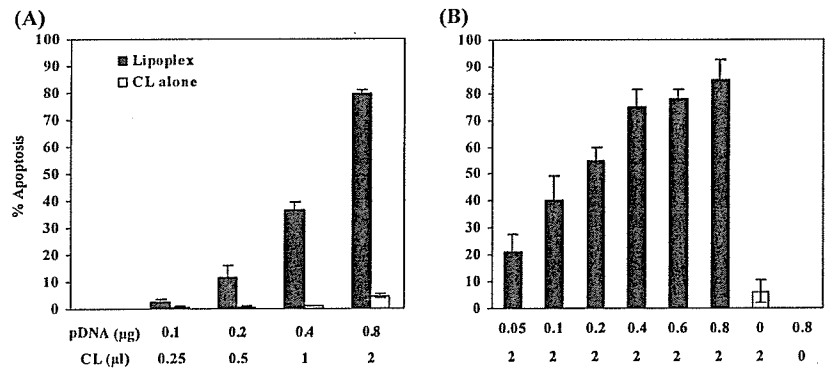

Fig. 5. Effect of Lipoplex-Dose (A) or Amount of pDNA in the Lipoplex (B) on the Lipoplex-Induced Apoptosis

(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±S.D. of at least 200 cells from randomly selected fields were counted by two independent observations.

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 tress 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 oncogen 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, inteferon, alpha-inducible protein 15 kDa	1.1798	19.6599
NM_001548	Immune response	IFIT1, inteferon, 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		PHLDA1, PHLDA1 protein	2.6866	12.1289

45 genes up- or down-regulated by treatment with lipoplex or CL alone (Fig. 6) were identified 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), KL6 (suppression of tumorrigenecity 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.

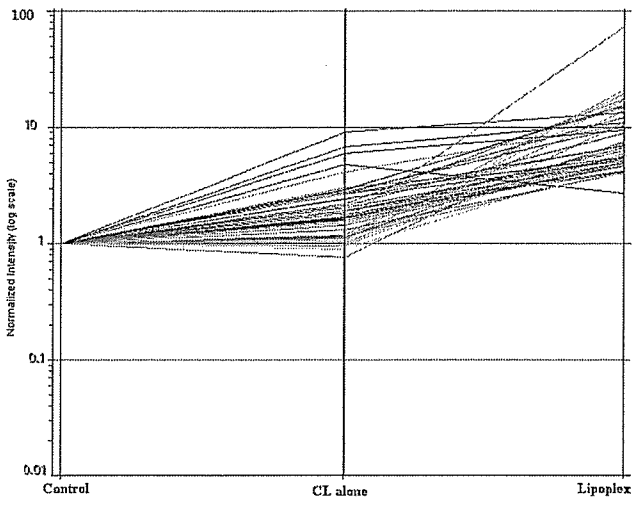

Fig. 6. Up or Down-Regulation of Genes by Treatment with Lipoplex or CL Alone

Comparative microarray was carried out following treatment with CL alone (2 μl/well) or lipoplex (0.8 μg pGL-3-Control/2 μl 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 dinucleotids.[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 ATP/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 JNK[32] and that JNK and ATF3 are activated through ER stress.[33] AFT3 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.

Acknowledgments The authors wish to thank Kinuka Yamane (Hokkaido System Science) for her technical assistance and helpful discussion and Dr. Milton S. Feather for his helpful comments in writing the English manuscript. This study was supported, in part, by Health and Labour Sciences Research Grants, on Advanced Medical Technology from Ministry of Health, Labour and Welfare, Japan.

REFERENCES

1) Patil S. D., Burgess D. J., "AAPS Newsmagazine," Vol. 6, No. 27, 2003.
2) Romano G., Micheli P., Pacilio C., Giordano A., *Stem Cells*, **18**, 19—39 (2000).
3) Zhdanov R. I., Podobed O. V., Vlassov V. V., *Bioelectrochemistry*, **58**, 53—64 (2002).
4) Brown M. D., Schatzlein A. G., Uchegbu I. F., *Int. J. Pharm.*, **229**, 1—21 (2001).
5) Felgner P. L., Gadek T. R., Holm M., Roman R., Chan H. W., Wenz M., Northrop J. P., Ringold G. M., Danielsen M., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413—7417 (1987).
6) Nabel G. J., Nabel E. G., Yang Z. Y., Fox B. A., Plautz G. E., Gao X., Huang L., Shu S., Gordon D., Chang A. E., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 11307—11311 (1993).
7) Hui K. M., Ang P. T., Huang L., Tay S. K., *Gene Ther.*, **8**, 783—790 (1997).
8) Caplen N. J., Alton E. W., Middleton P. G., Dorin J. R., Stevenson B. J., Gao X., Durham S. R., Jeffery P. K., Hodson M. E., Coutelle C., *Nat. Med.*, **1**, 39—46 (1995).
9) McLachlan G., Ho L. P., Davidson-Smith H., Samways J., Davidson H., Stevenson B. J., Carothers A. D., Alton E. W., Middleton P. G., Smith S. N., Kallmeyer G., Michaelis U., Seeber S., Naujoks K., Greening A. P., Innes J. A., Dorin J. R., Porteous D. J., *Gene Ther.*, **3**, 1113—1123 (1996).
10) Gill D. R., Southern K. W., Mofford K. A., Seddon T., Huang L., Sorgi F., Thomson A., MacVinish L. J., Ratcliff R., Bilton D., Lane D. J., Littlewood J. M., Webb A. K., Middleton P. G., Colledge W. H., Cuthbert A. W., Evans M. J., Higgins C. F., Hyde S. C., *Gene Ther.*, **4**, 199—209 (1997).
11) Filion M. C., Phillips N. C., *Biochim. Biophys. Acta*, **1329**, 345—356 (1997).
12) Aramaki Y., Takano S., Tsuchiya S., *FEBS Lett.*, **460**, 472—476 (1999).
13) Omidi Y., Hollins A. J., Benboubetra M., Drayton R., Benter I. F., Akhtar S., *J. Drug Target*, **11**, 311—323 (2003).
14) Lappalainen K., Urtti A., Soderling E., Jaaskelainen I., Syrjanen K., Syrjanen S., *Biochim. Biophys. Acta*, **1196**, 201—208 (1994).
15) Friend D. S., Papahadjopoulos D., Debs R. J., *Biochim. Biophys. Acta*, **1278**, 41—45 (1996).
16) Yew N. S., Wang K. X., Przybylska M., Bagley R. G., Stedman M., Marshall J., Scheule R. K., Cheng S. H., *Hum. Gene Ther.*, **10**, 223—234 (1999).
17) Dow S. W., Fradkin L. G., Liggitt D. H., Willson A. P., Heath T. D., Potter T. A., *J. Immunol.*, **163**, 1552—1561 (1999).
18) Bird A. P., *Nature* (London), **321**, 209—213 (1986).
19) Afshari C. A., Nuwaysir E. F., Barrett J. C., *Cancer Res.*, **59**, 4759—4760 (1999).
20) Oda E, Ohki R., Murasawa H., Nemoto J., Shibue T., Yamashita T., Tokino T., Taniguchi T., Tanaka N., *Science*, **288**, 1053—1058 (2000).
21) Yakovlev A. G., Di Giovanni S., Wang G., Liu W., Stoica B., Faden A. I., *J. Biol. Chem.*, **279**, 28367—28374 (2004).
22) Li J., Lee B., Lee A. S., *J. Biol. Chem.*, **281**, 7260—7270 (2006).
23) McCullough K. D., Martindale J. L., Klotz L. O., Aw T. Y., Holbrook N. J., *Mol. Cell Biol.*, **21**, 1249—1259 (2001).
24) Oyadomari S., Mori M., *Cell Death Differ.*, **11**, 381—389 (2004).
25) Marciniak S. J., Yun C. Y., Oyadomari S., Novoa I., Zhang Y., Jungreis R., Nagata K., Harding H. P., Ron D., *Genes Dev.*, **18**, 3066—3077 (2004).
26) Zinszner H., Kuroda M., Wang X., Batchvarova N., Lightfoot R. T., Remotti H., Stevens J. L., Ron D., *Genes Dev.*, **12**, 982—995 (1998).
27) Karin M., Liu Z., Zandi E., *Curr. Opin. Cell Biol.*, **9**, 240—246 (1997).
28) Guyton K. Z., Xu Q., Holbrook N. J., *Biochem. J.*, **314**, 547—554 (1996).
29) Tang J. R., Nakamura M., Okura T., Takata Y., Watanabe S., Yang Z. H., Liu J., Kitami Y., Hiwada K., *Biochem. Biophys. Res. Commun.*, **290**, 1255—1259 (2002).
30) Qiao D., Im E., Qi W., Martinez J. D., *Biochim. Biophys. Acta*, **1583**, 108—116 (2002).
31) Hai T., Hartman M. G., *Gene*, **273**, 1—11 (2001).
32) Cai Y., Zhang C., Nawa T., Aso T., Tanaka M., Oshiro S., Ichijo H., Kitajima S., *Blood*, **96**, 2140—2148 (2000).
33) Zhang C., Kawauchi J., Adachi M. T., Hashimoto Y., Oshiro S., Aso T., Kitajima S., *Biochem. Biophys. Res. Commun.*, **289**, 718—724 (2001).
34) Wolfgang C. D., Chen B. P. C., Martindale J. L., Holbrook N. J., Hai T., *Mol. Cell. Biol.*, **17**, 6700—6707 (1997).
35) Huang R. P., Darland T., Okamura D., Mercola D., Adamson E. D., *Oncogene*, **9**, 1367—1377 (1994).
36) Huang R. P., Liu C., Fan Y., Mercola D., Adamson E. D., *Cancer Res.*, **55**, 5054—5062 (1995).
37) Muthukkumar S., Nair P., Sells S. F., Maddiwar N. G., Jacob R. J., Rangnekar V. M., *Mol. Cell Biol.*, **15**, 6262—6272 (1995).
38) Nair P., Muthukkumar S., Sells S. F., Han S. S., Sukhatme V. P., Rangnekar V. M., *J. Biol. Chem.*, **272**, 20131—20138 (1997).
39) Bottone F. G., Jr., Moon Y., Alston-Mills B., Eling T. E., *J. Pharmacol. Exp. Ther.*, **315**, 668—677 (2005).
40) Spets H., Georgii-Hemming P., Siljason J., Nilsson K., Jernberg-Wiklund H., *Blood*, **92**, 2914—2929 (1998).
41) Summersgill J. T., Sahney N. N., Gaydos C. A., Quinn T. C., Ramirez J. A., *Infect. Immune*, **63**, 2801—2803 (1995).
42) Kito M., Akao Y., Ohishi N., Yagi K., *Biochem. Biophys. Res. Commun.*, **257**, 771—776 (1999).
43) Ishihara T., Aga M., Hino K., Ushio C., Taniguchi M., Iwaki K., Ikeda M., Kurimoto M., *Biomed. Res.*, **26**, 179—185 (2005).

Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 333 (2007) 62–69
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

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

Tatsuaki Tagami [a], Jose Mario Barichello [a,c], Hiroshi Kikuchi [b], Tatsuhiro Ishida [a,*], Hiroshi Kiwada [a]

[a] *Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, 1-78-1 Sho-machi, Tokushima 770-8505, Japan*
[b] *Drug Metabolism and Physicochemistry Research Laboratory R&D Division, Daiichi Pharmaceutical Co. Ltd., Tokyo 134-8630, Japan*
[c] *Japan Association for the Advancement of Medical Equipment, Tokyo 113-0033, Japan*

Received 17 August 2006; received in revised form 22 September 2006; accepted 26 September 2006
Available online 10 October 2006

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.

Keywords: RNA interference; Small interfering RNAs; Cationic liposomes; Gene knockdown; Lipofection; Co-transfection

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 therapeutic 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.

* Corresponding author. Tel.: +81 88 633 7260; fax: +81 88 633 7260.
E-mail address: ishida@ph.tokushima-u.ac.jp (T. Ishida).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.09.057

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′-CUUACGCUGAGUACUUCGATT-3′; anti-sense sequence, 5′-UCGAAGUACUCAGCGUAAGTT-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 μM) was prepared by mixing complementary antisense-stranded RNA and sense-stranded RNA in TE buffer (10 μM Tris–HCl, 1 μM 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 ddH_2O (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 μg/μl) was prepared by mixing 45 μl of pGL3 solution (1 μg/μl in TE buffer) and 5 μl of pRL-TK (1 μg/μl 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 μg/μl) were mixed with 100 μl 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 μM) 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 μl of the diluted siRNA (312.5, 625, 1250, 2500, 5000 nM) was further diluted with 1150 μl of OPTIMEM I. The siRNA solution was mixed with 1250 μl of TFL-3 (20 μM) in OPTIMEM I. For the pDNA/TFL-3 lipoplex, 1250 μl of pDNAs (1 μg/1 μl) were mixed with equal volume of TFL-3 (20 μM) 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.

2.4. Cell culture and transfection procedure

B16BL6, a murine melanoma cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a 5% CO_2 air incubator at 37 °C. The cells were maintained in exponential growth. Two luciferase plasmids (*Photinus* (firefly) luciferase, pGL-3 and *Renilla* (sea pansy) luciferase, pRL-TK) were used as reporter and control genes, respectively, by a reported transfection protocol with modifications (Elbashir et al., 2001). The cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well 24 h before the transfection was carried out. Prior to transfection, the medium was removed. The cells were co-transfected with pDNA/siRNA/TFL-3 lipoplex or with the mixture of pDNA/TFL-3 lipoplex and siRNA/TFL-3 lipoplex in OPTIMEM I (500 μl/well). The siRNA concentrations for transfection were 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 nM. The transfection medium was exchanged for culture medium (500 μl) after 4 h. All experiments were performed in triplicate and repeated at last twice.

2.5. Dual-luciferase reporter assay

Cells were harvested 20 h after transfection and lysed by passive lysis buffer (100 μl/well), according to the instruction of the Dual-Luciferase Reporter Assay System (Promega) with modifications. The luciferase activities of the samples were measured using a luminometer (BLR-301, Aloka, Tokyo, Japan), with a delay time of 2 s and an integrate time of 10 s. The volumes of sample (20 μl), luciferase assay reagent II (100 μl), and Stop & Glo Reagent (100 μl) were reduced to half the amount when the activity of the reporter or control gene saturated the luminometer. The inhibitory effects generated by siRNA were expressed as normalized ratios between the activities of reporter (firefly) luciferase gene and control (sea pansy) luciferase gene (Elbashir et al., 2001; Xu et al., 2003).

2.6. Effect of non-transcriptional pDNA on the gene-silencing effect of siRNA

Non-transcriptional pDNA, pGEM-luc, was purchased from Promega (WI, USA). pDNA (pGL-3 and pRL-TK, 0.5 μg)/TFL-3 lipoplex (2.5 nmol) was prepared in OPTIMEM I as described above. siRNA/pDNA (pGEM-luc)/TFL-3 lipoplex was also prepared in OPTIMEM I as described above. For preparation of the siRNA/pDNA/TFL-3 lipoplex, siRNA was kept at 25 nM and pDNA (pGEM-luc) was varied from 0.0625 to 0.5 μg. The cells were co-transfected as described above and the dual-luciferase assay was performed.

2.7. Amount of siRNA transferred into the cells by transfection

Lipoplexes containing fluorescence (FAM)-labeled siRNA, custom synthesized by Hokkaido System Science, were transfected as described above. At 4 h after transfection, the cells were washed twice with ice-cold PBS and treated with 100 μl/well of a lysis buffer (ice-cold PBS containing 2% (w/v) Triton-X 100, 1 μl of protease inhibitor (×100 protease inhibitor cocktail (50 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 15 μM aprotinin (from bovine lung), 100 μM E-64, 100 μM leupeptin hemisulfate salt, 50 μM EDTA, Nacalai Tesque, Kyoto, Japan))) on ice for 1 h. The cell lysate was collected and centrifuged (20,000 × *g*, 5 min, 4 °C) to give a clear supernatant. The fluorescence of samples was determined using a fluorometer (F-4500, Hitachi, Tokyo, Japan). The protein content in the samples was determined with the DC protein assay kit (Bio-Rad Laboratories, CA, USA). The data are expressed as the fluorescence/mg of protein.

2.8. Confocal microscopy

The cells were transfected with lipoplexes containing FAM-labeled siRNA and rhodamine-labeled TFL-3 (supplied by Daiichi Pharmaceutical). For preparation of the siRNA/pDNA/TFL-3 lipoplex, FAM-labeled siRNA (25 pmol), pDNA (2 μg) and rhodamine-labeled TFL-3 (5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. For preparation of the siRNA/TFL-3 lipoplex, FAM-labeled siRNA (25 pmol) and rhodamine-labeled TFL-3 (2.5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. For preparation of the pDNA/TFL-3 lipoplex, pDNA (2 μg) and rhodamine-labeled TFL-3 (2.5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. At 4 h after transfection, the cells were washed twice with ice-cold PBS. Then the cells were immediately examined using a Zeiss LSM5 inverted confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) without fixation. FAM-siRNA was imaged using a 488 nm filter for excitation and a 510–530 nm filter for emission, respectively. Rhodamine-labeled TFL-3 was imaged using a 540 nm filter for excitation and a 650 nm filter for emission, respectively.

2.9. Statistics

All values are expressed as the mean ± S.D. Statistical analysis was performed with a two-tailed unpaired *t*-test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Characterization of lipoplexes

After preparation, all lipoplexes (siRNA/pDNA/TFL-3, siRNA/TFL-3 and pDNA/TFL-3) were checked for free nucleic acids by an agarose electrophoresis. No free siRNA and/or pDNA were detected (not shown). The mean diameters and zeta potentials of all lipoplexes were analyzed (Table 1). The mean particle sizes of siRNA/pDNA/TFL-3 lipoplexes produced by the co-preparation method (Method I) increased with

Table 1
Characterization of the lipoplexes

siRNA/pDNA/TFL-3			Diameter (nm)	Zeta potential (mV)
siRNA (μM)	pDNA (μg/ml)	TFL-3 (μM)		
Co-preparation (Method I)				
12.5	2	20	305.3	+29.26
25	2	20	374.2	+29.07
50	2	20	392.4	+29.44

siRNA/TFL-3		Diameter (nm)	Zeta potential (mV)
siRNA (μM)	TFL-3 (μM)		
Separate preparation (Method II)			
12.5	10	301.9	+36.59
25	10	279.9	+37.13
50	10	307.7	+30.35

pDNA/TFL-3		Diameter (nm)	Zeta potential (mV)
pDNA (μg/ml)	TFL-3 (μM)		
Separate preparation (Method II)			
20	10	317.5	+27.54

TFL-3 (μM)	Diameter (nm)	Zeta potential (mV)
Control		
10	266.5	+37.49

Mixture of pGL-3 and pRL-TK (9:1, w/w) was used as pDNA. siRNA for *Renilla* (firefly) luciferase was used.

increasing amount of siRNA, while zeta potential values did not change. The mean particle size of the siRNA/TFL-3 complexes produced by separate preparation (Method II) did not change regardless of the amount of siRNA, while the zeta potential values of these lipoplexes decreased with increasing amounts of siRNA. The mean diameter of TFL-3 liposomes increased by complexing with pDNA (separate preparation according to Method II), while the zeta potential value decreased.

3.1.1. Suppression of Renilla (firefly) luciferase expression in B16BL6 cells

The ratio of firefly and sea pansy luciferase activities was determined after co-transfection (Fig. 1). The siRNA in the lipoplexes produced by co-preparation (Method I) was far more effective than the siRNA in the separately prepared lipoplexes (Method II). Even at a concentration as low as 1.5625 nM, the siRNA in the co-prepared lipoplexes induced more than 80% inhibition. To obtain a similar inhibition with the separately prepared lipoplexes, more than 100 nM of siRNA was required. The gene-silencing effect of siRNA in the lipoplexes produced by co-preparation was 60-fold higher than that in the lipoplexes produced by separate preparation. It is to be noted that the activities of sea pansy luciferase (control) were similar throughout the experiments (Table 2). Furthermore, unrelated siRNA did not show any significant gene-silencing effect at any of the applied concentrations regardless of the preparation method (data not shown).

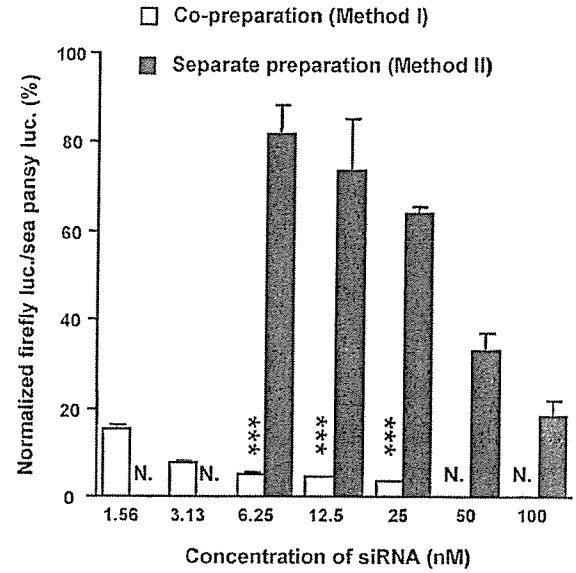

Fig. 1. Effect of the lipoplex preparation method (Method I or Method II) on the gene-silencing potential of transfected siRNA. Results presented are from three independent experiments. $^{***}p < 0.005$; N., not determined.

3.2. Amount of siRNA associated with the cells

To evaluate whether intracellular uptake of siRNA was related to the induced gene-silencing effect, the amount of siRNA transferred into the cells was determined by using FAM-labeled siRNA. The amount of siRNA associated with the cells increased with increasing the siRNA concentration (Fig. 2). Although the amount of siRNA transferred by lipoplexes produced by co-preparation was larger than that transferred by separately prepared lipoplexes, there was no significant difference in the amount of transferred siRNA.

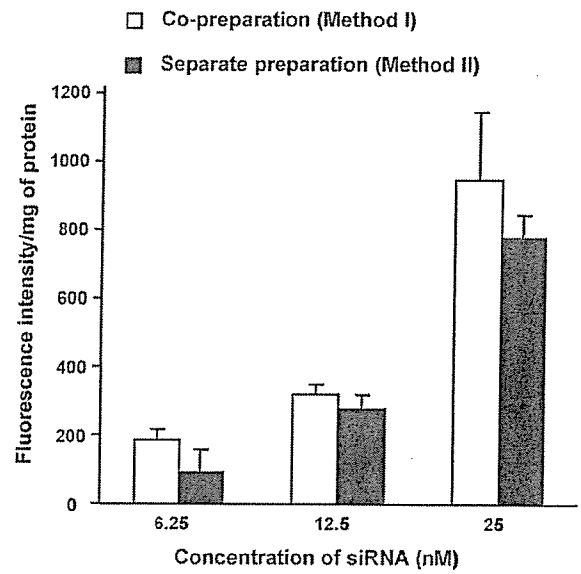

Fig. 2. Amount of siRNA associated with the cells following transfection. Cells were incubated with lipoplexes containing a fluorescently labeled siRNA. After 4 h the cells were lysed and fluorescence in the lysates was quantified. For details see Section 2. Results presented are from three independent experiments.

Table 2
Firefly and sea pansy luciferase activities in B16BL6 cells

	siRNA concentration							
	0 nM (Control)	1.56 nM	3.13 nM	6.25 nM	12.5 nM	25 nM	50 nM	100 nM
Co-preparation (Method I)								
Firefly luciferase activity (cpm)	266137.3 ± 41567.5	37204.3 ± 1214.6	19677.5 ± 1974.9	15127.3 ± 1869.0	12394.3 ± 1175.7	9367. 7 ± 2710.1	N.	N.
Sea pansy luciferase activity (cpm)	4187.8 ± 491.4	4256.3 ± 198.1	4583.3 ± 321.9	5270.0 ± 349.8	4562.7 ± 299.2	3823.0 ± 1039.9	N.	N.
Separate preparation (Method II)								
Firefly luciferase activity (cpm)	256359.2 ± 29810.1	N.	N.	180235.5 ± 13314.2	155066.8 ± 16901.1	144200.5 ± 19648.0	44268.8 ± 4925.7	25888.0 ± 2866.2
Sea pansy luciferase activity (cpm)	5141.4 ± 728.5	N.	N.	5621.8 ± 518.5	5867.0 ± 772.4	5893.3 ± 215.9	4216.5 ± 380.9	3919.8 ± 343.6

The luciferase activities were determined according to the method described in Section 2. Results presented are from three independent experiments. N., not determined.

Fig. 3. Intracellular distribution of the lipoplexes produced by the (A) co-preparation method and (B) separate preparation method. Cells were incubated for 4 h with lipoplexes containing rhodamine-labeled TFL and FAM-labeled siRNA and immediately thereafter observed by confocal microscopy. Rhodamine, red; FAM, green.

3.3. Intracellular distribution of lipoplexes

The intracellular trafficking of the lipoplexes containing FAM-labeled siRNA, pDNAs and rhodamine-labeled TFL-3 was examined by means of confocal microscopy. Pictures were taken immediately after the transfection was terminated. Fig. 3A shows the intracellular distribution of lipoplexes (FAM-siRNA/pDNA/rhodamine-TFL-3) produced by co-preparation (Method I). Co-localization of siRNA and TFL-3 (merging green and red producing yellow) was observed. Fig. 3B shows the intracellular distribution of lipoplexes (FAM-siRNA/TFL-3 and pDNA/rhodamine-TFL-3) produced by separate preparation (Method II). siRNA (green) and TFL-3 (red) can be distin-

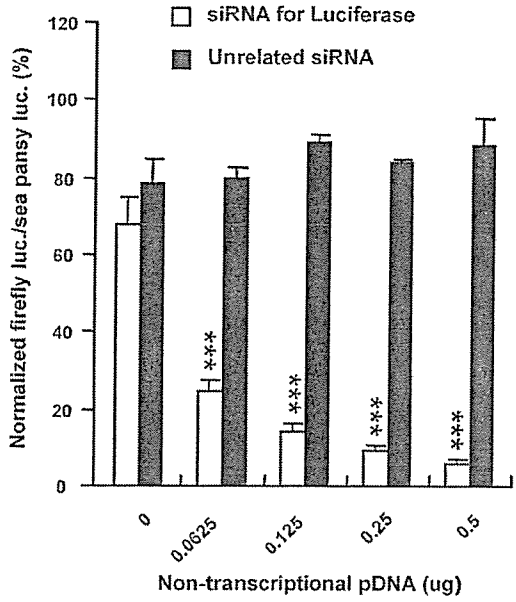

Fig. 4. Effect of the presence of non-transcriptional pDNA in the lipoplex on the gene-silencing potential of siRNA. See Section 2 for details. Results presented are from three independent experiments. $^{***}p < 0.005$.

guished as individual spots in the cells. It should be noted that the amount of rhodamine-labeled TFL-3 for the preparation of lipoplexes with siRNA and pDNA was twice as large as that for the preparation of lipoplexes with pDNA. The fluorescence intensity of siRNA/pDNA/rhodamine-labeled TFL-3 lipoplexes will therefore be higher than that of pDNA/rhodamine-labeled TFL-3.

3.4. Induction of the gene-silencing effect of siRNA by a non-transcriptional pDNA

From the results of the confocal microscopy, we assumed that pDNA in the lipoplexes produced by co-preparation (Method I) has somehow the ability to bring the siRNA to an optimal position in the cytoplasm allowing it to induce a higher gene-silencing effect. To test this, we prepared lipoplexes containing a non-transcriptional pDNA and siRNA for *Renilla* (firefly) luciferase and incubated them with cells after transfection with pDNAs/TFL-3 lipoplexes. Inhibition of gene expression increased with the amount of non-transcriptional pDNA (Fig. 4). It is noteworthy that the sea pansy luciferase activities (control) were similar throughout the experiments (data not shown).

4. Discussion

Gene silencing by siRNA can be assayed by studying its inhibitory effect on the expression of an exogenous gene co-transfected into the cell simultaneously with the siRNA. In this study, we compared two principally different approaches for this co-transfection, either by accommodating the pDNA in the same complex together with the siRNA (Method I), or by incorporating the pDNA and the siRNA in two separate complexes (Method II).

The siRNA lipoplexes produced by Method I showed a much stronger reporter-gene silencing effect than those produced by Method II (Fig. 1). This observation clearly calls for caution when drawing conclusions concerning the activity of synthesized siRNAs and/or the effectiveness of the delivery system used. It may be necessary to first establish a reliable evaluation method. Aiming at an endogenous gene or a stably expressed exogenous gene rather than at a transiently expressed gene would probably provide more reliable results.

During transfection with lipoplexes produced by Method II, one lipoplex might competitively inhibit the cellular uptake of the other. The consequently lower amount of transferred siRNA might be insufficient to silence the larger exogenous gene expression. In addition, lower target gene-expression due to lower cellular uptake of pDNA might lead to an over-estimation of the gene-silencing effect by transferred siRNA. We observed, however, that the expression of the control gene (*Renilla* (sea pansy) luciferase) as well as the amount of siRNA associated with the cells were nearly constant, irrespective of the preparation method (Fig. 2). This strongly suggests that the lower gene-silencing effect of lipoplexes prepared by Method II cannot be attributed to mutually competitive inhibition of the cellular uptake of pDNA or siRNA.

Although pDNA and siRNA both have anionic phosphodiester backbones with identical negative charge/nucleotide ratios and should therefore readily interact electrostatically with cationic liposomes to form lipoplexes, they are very different from each other in terms of molecular weight and molecular topography. This may bring along potentially important consequences. The resulting lipoplexes, i.e. complexes with only siRNA or only pDNA or complexes with both siRNA and pDNA, are each likely to display unique physicochemical properties and, consequently, to lead to different types of interaction with the cells. In fact, in the case of pDNA, it has been reported that larger lipoplexes tend to give better transfection results than smaller lipoplexes (Kreiss et al., 1999; Ross and Hui, 1999; Simberg et al., 2001; Almofti et al., 2003). In contrast to the pDNA delivery, no difference in gene-silencing potential was observed between smaller lipoplexes (50–100 nm) and larger lipoplexes (200–600 nm) (Spagnou et al., 2004). In our experiments we observed no significant differences between complexes prepared by the two different methods in terms of particle diameter and zeta potential (Table 1), presumably due to the small amounts of siRNA and pDNA that were used to form the lipoplexes. We therefore believe that it is also not a difference in physicochemical properties of the lipoplexes that is the major cause of the different gene-silencing effects of siRNA observed in our study. This view is supported by the constant values of the control exogenous gene expression levels we observed and by the similar amounts of siRNA associated with the cells, irrespective of the preparation method.

It is generally assumed that the cellular entry and intracellular trafficking of siRNA are the key processes in determining the efficiency of gene-silencing effects with exogenous siRNA following transfection (Hammond et al., 2000; Martinez et al.,

2002; Schwarz et al., 2002). Our confocal microscopy study demonstrated that the siRNA delivered by siRNA/pDNA/TFL-3 lipoplexes co-localized in the cell with TFL-3 (Fig. 3A), implying that siRNA also co-localized with pDNA. By contrast, the observation that the siRNA delivered by siRNA/TFL-3 lipoplexes did not co-localize with pDNA/rhodamine-labeled TFL-3 lipoplexes (Fig. 3B) indicated that siRNA and pDNA localize separately after entry in the cells. These observations may indicate that the cellular entry and the intracellular trafficking of the siRNA/pDNA/TFL-3 lipoplexes differ from that of other lipoplexes (siRNA/TFL-3 and pDNA/TFL-3). We recently reported that the cellular entry of pDNA/TFL-3 lipoplex occurs via the endocytosis pathway (Almofti et al., 2003). The mechanisms of siRNA entry into the cells and their intracellular trafficking mediated by the TFL-3 have not yet been established, however. Miller and co-workers (Keller et al., 2003; Spagnou et al., 2004) demonstrated that intracellular uptake and localization of siRNA and pDNA complexed in their carrier system (CDAN (N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine)/DOPE (dioleoylphosphatidylethanolamine) liposomes) differ substantially between siRNA lipoplexes and pDNA lipoplexes. Uptake and intracellular trafficking of siRNA/pDNA/TFL-3 lipoplex likewise may differ from those of siRNA/TFL-3 and pDNA/TFL-3 lipoplexes.

Although we do not yet understand the underlying mechanism, we clearly demonstrated that pDNAs, also if non-transcriptional, can increase the gene-silencing effect of the associated siRNA provided that it is present with the siRNA in the same lipoplex (Figs. 1 and 4). Recently, Schiffelers et al. (2004) reported similar findings in *in vivo* experiments. They compared co-injection (pDNA and siRNA injected simultaneously) with sequential injections (siRNA injected 2 h after pDNA) with respect to exogenous-gene-silencing potential siRNA-RGD-PEI-PEG-nanocomplex, targeted to tumor neovasculature expressing integrins, *in vivo*. siRNA was efficiently delivered by the nanocomplex using either injection protocol, but the induced gene-silencing effect was lower with the sequential injection protocol than with the co-injection protocol. The pDNA in the same lipoplex with siRNA may guide the siRNA to the correct position to function in the cells. This may open up new perspectives for gene therapy by means of pDNA and siRNA. The silencing of (aberrant) genes linked to human disorders and at the same time expressing genes producing a therapeutic protein with specific functions in the same cells may synergistically increase the efficacy of the gene therapy, when lipoplexes containing both a pDNA encoding functional gene and a gene-specific siRNA are injected. To achieve this ambitious goal suitably targeted carrier systems will have to be developed which are capable of delivering their nucleic acid cargos to target tissues or cells.

Acknowledgements

We thank Dr. G.L. Scherphof for his help in writing the manuscript. This study was supported in part by Health and Labour Sciences Research Grants on Advanced Medical Technology from the Ministry of Health, Labour and Welfare.

References

Almofti, M.R., Harashima, H., Shinohara, Y., Almofti, A., Li, W., Kiwada, H., 2003. Lipoplex size determines lipofection efficiency with or without serum. Mol. Membr. Biol. 20, 35–43.

Bitko, V., Musiyenko, A., Shulyayeva, O., Barik, S., 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat. Med. 11, 50–55.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293–296.

Keller, M., Harbottle, R.P., Perouzel, E., Colin, M., Shah, I., Rahim, A., Vaysse, L., Bergau, A., Moritz, S., Brahimi-Horn, C., Coutelle, C., Miller, A.D., 2003. Nuclear localization sequence templated non-viral gene delivery vectors: investigation of intracellular trafficking events of LMD and LD vector systems. Chembiochem 4, 286–298.

Khoury, M., Louis-Plence, P., Escriou, V., Noel, D., Largeau, C., Cantos, C., Scherman, D., Jorgensen, C., Apparailly, F., 2006. Efficient new cationic liposome formulation for systemic delivery of small interfering RNA silencing tumor necrosis factor alpha in experimental arthritis. Arthritis Rheum. 54, 1867–1877.

Kikuchi, A., Aoki, Y., Sugaya, S., Serikawa, T., Takakuwa, K., Tanaka, K., Suzuki, N., Kikuchi, H., 1999. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. Hum. Gene Ther. 10, 947–955.

Kreiss, P., Cameron, B., Rangara, R., Mailhe, P., Aguerre-Charriol, O., Airiau, M., Scherman, D., Crouzet, J., Pitard, B., 1999. Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. Nucleic Acids Res. 27, 3792–3798.

Landen Jr., C.N., Chavez-Reyes, A., Bucana, C., Schmandt, R., Deavers, M.T., Lopez-Berestein, G., Sood, A.K., 2005. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res. 65, 6910–6918.

Li, W., Ishida, T., Tachibana, R., Almofti, M.R., Wang, X., Kiwada, H., 2004. Cell type-specific gene expression, mediated by TFL-3, a cationic liposomal vector, is controlled by a post-transcription process of delivered plasmid DNA. Int. J. Pharm. 276, 67–74.

Li, W., Ishida, T., Okada, Y., Oku, N., Kiwada, H., 2005. Increased gene expression by cationic liposomes (TFL-3) in lung metastases following intravenous injection. Biol. Pharm. Bull. 28, 701–706.

Lundstrom, K., Boulikas, T., 2003. Viral and non-viral vectors in gene therapy: technology development and clinical trials. Technol. Cancer Res. Treat. 2, 471–486.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., Tuschl, T., 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110, 563–574.

Morrissey, D.V., Lockridge, J.A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C.S., Jeffs, L.B., Judge, A., MacLachlan, I., Polisky, B., 2005. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat. Biotechnol. 23, 1002–1007.

Muratovska, A., Eccles, M.R., 2004. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett. 558, 63–68.

Nguyen, L.T., Ishida, T., Ukitsu, S., Li, W., Tachibana, R., Kiwada, H., 2003. Culture time-dependent gene expression in isolated primary cultured rat hepatocytes by transfection with the cationic liposomal vector TFL-3. Biol. Pharm. Bull. 26, 880–885.

Nguyen, L.T., Ishida, T., Kiwada, H., 2005. Gene expression in primary cultured mouse hepatocytes with a cationic liposomal vector, TFL-3: comparison with rat hepatocytes. Biol. Pharm. Bull. 28, 1472–1475.

Nogawa, M., Yuasa, T., Kimura, S., Tanaka, M., Kuroda, J., Sato, K., Yokota, A., Segawa, H., Toda, Y., Kageyama, S., Yoshiki, T., Okada, Y., Maekawa, T., 2005. Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. J. Clin. Invest. 115, 978–985.