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Research Paper

Global Gene Expression Profiling in Cultured Cells Is Strongly Influenced by Treatment with siRNA–Cationic Liposome Complexes

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Purpose. The purpose of this study is to determine if the treatment with siRNA-lipoplexes significantly influences on global gene expression in the treated cells.
Methods. We investigated global gene expression in a HT1080 cell line by a cDNA microarray. We also evaluated the effect of lipofection on global gene expression by determining the change of the expression of an exogenous gene, green fluorescence protein (GFP), and also determined treatment-related cytotoxicity.
Results. Treatment of the cells with either siRNA-lipoplexes or cationic liposomes altered the expression of approximately 2,500 genes. When lipoplexes containing non-specific siRNAs were used, GFP expression was enhanced. In this case the effect was independent on the dose and type of siRNA in the formulation. By contrast, when lipoplexes containing a specific siRNA against GFP was used, GFP expression was markedly diminished. These results clearly indicate that an efficient reduction of a targeted gene expression by a specific siRNA is accompanied by a significant alteration of the expression of numerous non-targeted genes. In addition, treatment-related cytotoxicity increased with siRNA- and cationic lipid-doses, but was not dependent on siRNA type.
Conclusion. Non-specific effects of siRNA-lipoplexes may either enhance, attenuate or even fully mask the desired outcomes of siRNA-based biochemical studies and therapies.

KEY WORDS: cationic liposome; lipofection; lipoplex; RNAi; siRNA.

INTRODUCTION

Small interfering RNAs (siRNAs) were first detected as products of RNA interference (RNAi). The field of RNAi, although in its infancy, shows promise for the development of novel therapeutics. siRNAs induce the sequence-specific hydrolysis of mRNA in mammalian cells without triggering an interferon-inducible response (1, 2) and have stronger and more durable gene-knockdown effects than antisense oligodeoxynucleotide (asODN) (3). Therefore, siRNAs are currently widely used as a powerful gene-knockdown tool in the biochemical field (4–6). In addition, siRNA-based treatments are being developed to treat acquired illnesses such as viral infection, neurological disease, ocular disease, inflammatory disease and cancer (4–6).

Various non-viral vectors have been developed as a carrier of nucleic acids such as plasmid DNA, asODN, ribozyme, aptamer and siRNA, since nucleic acids can not by themselves permeate through the plasma membrane of cells. The non-viral vectors are generally superior to viral vectors due to lower immunogenicity but higher productivity and safety (7, 8). However, it has been reported recently that unmodified siRNA's can be potent triggers of the innate immune response, particularly when associated with delivery vehicles that facilitate their intracellular uptake (9, 10). This represents a significant barrier to the development of siRNA as a potential therapeutic due to toxicity and off-target gene effects associated with this inflammatory response. Such toxicity and off-target effects may affect the expression of a variety of genes in the treated cells, other than the siRNA-targeted gene. However, there is little information about such global gene expression effect following treatment with siRNA-lipoplexes, formulated from siRNA and cationic liposomes (CL) which are widely used as a non-viral vector.

In order to obtain the desired outcome of siRNA-based biochemical experiments and therapies, the risks associated with the exposure of cells to siRNA lipoplexes, should be well understood. In this study, we examined the alteration of global gene expression in cells that were treated with siRNA-lipoplexes or with CL only by means of a cDNA microarray assessment. The treatments substantially affected global gene expression in HT1080 cells, regardless of the type of siRNA used. Such non-specific effects of siRNA-lipoplex treatment

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ABBREVIATIONS: asODN, antisense oligodeoxynucleotide; CL, cationic liposome; GFP, green fluorescence protein; IFN, interferon; Lf2000, LipofectAMINE 2000; mRNA, messenger RNA; RNAi, RNA interference; siRNA, small interfering RNA.

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on global gene expression could either amplify, attenuate or even fully mask the desired outcome of siRNA-based biochemical studies and therapies.

MATERIALS AND METHODS

Cell Culture

Human fibrosarcoma cells (HT1080) were maintained in DMEM (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Bioserum, Tokyo, Japan), 10 mM L-glutamine (MP Biomedicals, OH, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (ICN Biomedical, OH, USA). HT1080 cells stably expressing green fluorescence protein (GFP) (HT1080-GFP cells) were kindly donated by Drs. T. Asai and N. Oku (University of Shizuoka). Geneticin (G418, CalBiochem, CA, USA) was added to the medium at a concentration of 0.2 mg/ml to maintain stable GFP-gene expression.

Preparation of siRNAs

siRNAs, chemically synthesized and purified by HPLC, were purchased from Hokkaido Systems Sciences (Hokkaido, Japan). The following sequences were synthesized: siRNA for Argonaute2 which associates with small RNAs that guide mRNA degradation, translational repression, or a combination of both (11) (sense sequence): 5′-GCA CGG AAG UCC AUC UGA AUU-3′, (anti-sense sequence): 5′-UUC AGA UGG ACU UCC GUG CUU-3′ (11): firefly luciferase (sense sequence): 5′-CUU ACG CUG AGU ACU UCG ATT-3′, (anti-sense sequence): 5′-UCG AAG UAC UCA GCG UAA GTT-3′ (12): GFP (sense sequence): 5′-GGC UAC GUC CAG GAG CGC ATT-3′, (anti-sense sequence): 5′-UGC GCU CCU GGA CGU AGC CTT-3′ (13); inverted sequence of firefly luciferase (sense sequence): 5′-AGC UUC AUA AGG CGC AUG CTT-3′, (anti-sense sequence): 5′-GCA UGC GCC UUA UGA AGC UTT-3′ (12): Lamin A/C which is nucleoskeletal constituent (14) (sense sequence): 5′-CUG GAC UUC CAG AAG AAC ATT-3′, (anti-sense sequence): 5′-UGU UCU UCU GGA AGU CCA GTT-3′ (12) and mouse VEGF (sense sequence): 5′-CAU GGG ACU UCU GCU CUC CTT-3′, (anti-sense sequence): 5′-GGA GAG CAG AAG UCC CAU GTT-3′ (15).

The complementary antisense and sense strands in TE buffer (10 μM Tris-HCl, 1 μM ethylenediaminetetraacetic acid (pH 8.0), DNase and RNase free grade, Nippon Gene, Tokyo, Japan) were mixed in equal amounts, followed by heating at 90°C for 1 min. The reaction mixture was then allowed to cool at room temperature. Quality of duplex siRNA was checked by 15% PAGE. The final concentration of the duplexes was adjusted to 50 μM with TE buffer.

Treatment with CL and siRNA-Lipoplexes

HT1080 or HT1080-GFP cells were seeded in a well of a six-well plate at a density of 5.0×10^4 cells or of a 96-well plate at a density of 2.5×10^3 cells, and then cultured for 24 h before each experiment. siRNA was transfected using a commercially available CL, LipofectAMINE 2000 (Lf 2000, Invitrogen, CA, USA), which is widely used as non-viral vector system for pDNA and siRNA. Duplex siRNA was mixed with CL in Opti-MEM I (Invitrogen, CA, USA) to prepare the lipoplex and allowed to stand for 20 min at room temperature. The ratio of siRNA (μg) to CL (μl) in the siRNA-lipoplex and the absolute siRNA concentration in the incubation mixture were varied by mixing the appropriate amounts of siRNA with the CL. Cells were incubated for 24 h with the CL or the siRNA-lipoplex. Then the medium was replaced by fresh medium, and the incubation continued for another 24 h, following which subsequent assays were performed.

Microarray Analysis

Whole Human Genome Oligo Microarray 44Kx4 pack (Agilent Technologies, CA, USA) was used. The quality of three total RNA samples, based on the 28S/18S rRNA ratio, was assessed by using the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNAs were extracted from the cells that were treated with siRNA for firefly luciferase/Lf 2000 complex (siRNA-lipoplex) or Lf 2000 only (CL only) by means of the RNeasy Mini Kit with RNase-Free DNase Set (Qiagen, Hilden, Germany). The RNAs were amplified, converted to complementary DNAs and labeled with Cy3-CTP using Low RNA Fluorescent Agilent Linear Amplification kit (Agilent Technologies). As a result, the amplified complementary RNA products were labeled with Cy3. Then the labeled cRNAs were fragmented and hybridized using Agilent's *in situ* hybridization plus kit on Human1A ver.2 Oligo Microarray (Agilent Technologies). Microarrays were scanned with the Agilent Technologies Microarray Scanner (Agilent Technologies) at 5 μm resolution. By comparative analysis of the cells that were treated with siRNA-lipoplex or with CL only, the genes which, using a two sample *t*-test, were found to be significant different from the ones in untreated cells ($p<0.05$) were categorized by gene ontology. The experiment was performed according to the manufacturer's protocol. The data were analyzed with the GeneSpring software (Agilent Technologies).

Determination of GFP Fluorescence in HT1080-GFP Cells

HT1080-GFP cells (5.0×10^4/2 ml) were seeded in a well of six-well plates. Lipofection was performed according to the method described above. At 48 h post-lipofection, cells were removed by treatment with trypsin, collected into microtubes, and then centrifuged (300×*g*, 5 min, 4°C). The precipitated cells were resuspended in phosphate buffered saline (PBS, pH 7.4). The fluorescence intensity of the cells was immediately determined by a flow cytometer (Guava EasyCyto Mini (GE Healthcare, CA, USA)). During measurement, the apoptotic and dead cells were filtered out. In each sample 1.0×10^4 cells were counted and the data stored in the list mode. Data analysis was performed using Cytosoft ware (GE Healthcare).

Cell Proliferation Assay

The cytotoxicity of siRNA-lipoplex or CL towards HT1080 cells was determined by the 3-(4,5-dimethylthiazol-2-yl 2,5-diphenyl) tetrazolium bromide (MTT) assay. Cells (2.5×10^3/100 μl of culture medium) were seeded in a well of a 96-well plate. At 48 h after lipofection, cells were washed

twice with PBS and 50 μl of a stock solution of 3-(4,5-dimethylthiazol-2-yl 2,5-diphenyl) tetrazolium bromide (Nacalai Tesque, Kyoto, Japan) (5 mg/ml in PBS) was added to each well. After a 4-h incubation, the formazan crystals produced in the medium were dissolved by addition of 150 μl of an acidic isopropanol solution (containing 0.04N HCl). The absorption of the solution was read in a plate reader, Wallac 1420 ARVOsx multi-label counter (PerkinElmer, MA, USA) at 590 nm.

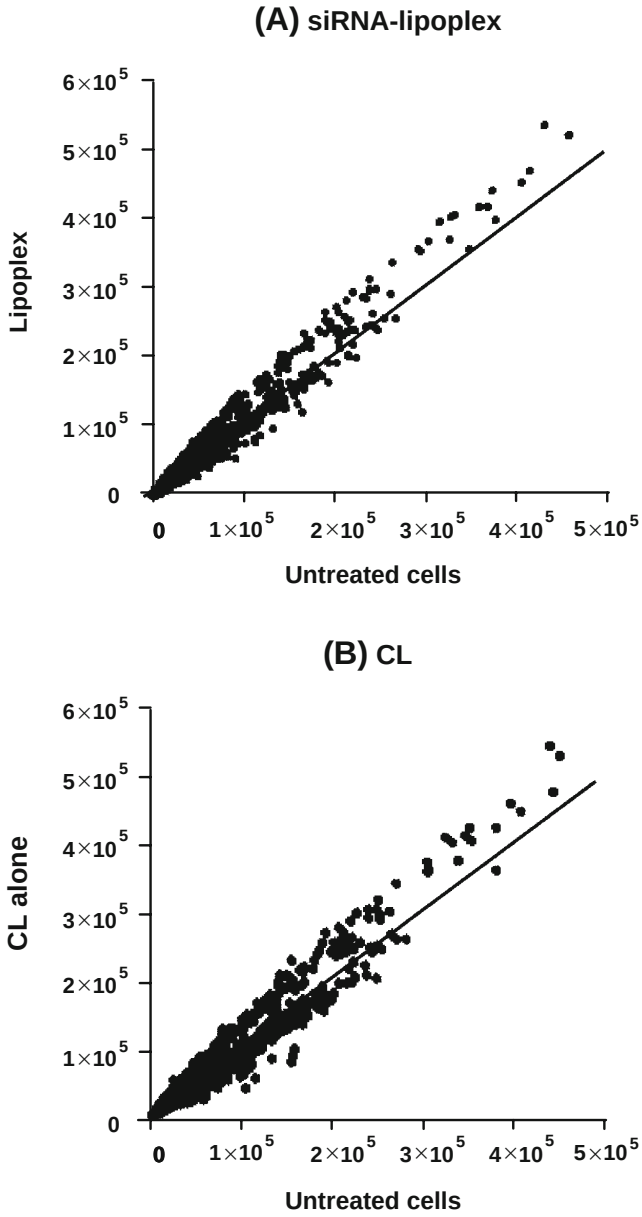

Fig. 1. Raw data scatter plot of array signals of HT1080 cells treated with **A** siRNA-lipoplex or **B** CL. Genes (25655) are shown. The cells (5×10^4) were treated with CL or siRNA-lipoplex (12.5 nM siRNA, corresponding to 0.5 μg/well in a well of six-well plate) for 24 h. The ratio of siRNA (μg) to CL (μl) was 1 to 5 (0.5 μg/2.5 μl), that is in accordance with the manufacturer instructions. Total RNAs were extracted at 48 h post-treatment, and subjected to the microarray experiment. The *drawn reference lines* shown in the figure indicate intensity ratios of genes exhibiting no changes.

Table I. Numbers of Significantly Up-regulated and Down-regulated Genes in HT1080 Cells Treated with siRNA-Lipoplex or CL. The Total Number of Genes Selected Was 25655

Treatment	siRNA-lipoplex	CL
	Number of genes	
Up-regulated ($p<0.05$)	63	79
Down-regulated ($p<0.05$)	22	13
Total	85	92

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 $p<0.05$.

RESULTS

Microarray-Based Gene-Expression Profiling Following Treatment with siRNA-Lipoplex or CL

cDNA microarray was performed with the cells treated with siRNA-lipoplex or CL only. Approximately 25,000 genes were validated. Both treatments induced a similar individual gene expression-pattern (Fig. 1). The total number of genes changed >1.5-fold and <0.67 (2/3)-fold was 2,622 (10.22% of the total number of reliable genes) for cells treated by siRNA-lipoplex and 2,402 (9.36%) for cells treated by CL. The number of significantly up-regulated or down-regulated genes ($p<0.05$) was 63 (up) or 22 (down) in siRNA-lipoplex-treated cells and 79 (up) or 13 (down) in CL-treated cells, respectively (Table I). This resulted in 45 overlapping genes altered by treatment with siRNA-lipoplex and CL only. Among these, several structure-function categories were represented, e.g. immune response (nine genes), metabolism (eight genes), apoptosis (five genes), cell adhesion (five genes), cell cycle (four genes), transcriptional regulation (four genes), signal transduction (three genes) (Table II). The nine genes relating to immune response were identified as *CCL2*, *CEBPB*, *ICAM1*, *IFI27*, *IFIT2*, *IL6*, *PTX3*, *SPP1* and *TLR4*.

Enhancement of GFP-Gene Expression, as a Model of Global Gene Expression, by Treatment with siRNA-Lipoplex or CL

We employed a cell line stably expressing GFP (HT1080-GFP) to investigate in a more quantitative manner if and to what extent the siRNA-lipoplex or CL affects global gene expressions. For the purpose of this, we prepared siRNA-lipoplex at the ratio of siRNA to CL, 1:10 and treated the cells with the prepared siRNA-lipoplex, 0.5 μg siRNA/5.0 μl CL, which contains larger volume of CL than that the manufacturer instructions of Lf2000 recommended. The siRNA-lipoplex contained various siRNAs (firefly luciferase, inverted sequence of firefly luciferase, mouse VEGF, human Lamin A/C, human Argonaute2) and GFP against exogenous or endogenous mRNAs. The treatment with siRNA-lipoplex

Table II. Common Genes Which Were Significantly Up- or Down-regulated ($p<0.05$) by Treatment with siRNA-Lipoplex or CL

Symbol	GenBank	Gene name	Category	Lipoplex		CL	
CCL2	NM_002982	Small inducible cytokine A2 precursor	Immune response	1.27	(↑)	1.60	(↑)
CEBPB	NM_005194	CCAAT/enhancer binding protein beta	Immune response	1.25	(↑)	1.27	(↑)
ICAM1	NM_000201	Intercellular adhesion molecule 1 precursor	Immune response	1.41	(↑)	1.43	(↑)
IFI27	NM_005532	Interferon, alpha-inducible protein 27	Immune response	1.75	(↑)	2.15	(↑)
IFIT2	NM_001547	Interferon-induced protein with tetratricopeptide repeats 2	Immune response	3.62	(↑)	3.42	(↑)
IL6	NM_000600	Interleukin 6 (interferon, beta 2)	Immune response	2.28	(↑)	3.05	(↑)
PTX3	NM_002852	Pentraxin-related gene, rapidly induced by IL-1 beta	Immune response	0.59	(↓)	0.67	(↓)
SPP1	NM_000582	Secreted phosphoprotein 1 isoform b	Immune response	1.95	(↑)	1.97	(↑)
TLR4	NM_138554	Toll-like receptor 4 precursor	Immune response	1.60	(↑)	1.38	(↑)
AKR1B10	NM_020299	Aldo-keto reductase family 1, member B10	Metabolism	3.35	(↑)	3.49	(↑)
GCNT3	NM_004751	Glucosaminyl (N-acetyl) transferase 3, mucin type	Metabolism	4.13	(↑)	3.71	(↑)
GSTM1	NM_146421	Glutathione S-transferase M1 isoform 2	Metabolism	1.36	(↑)	1.47	(↑)
LRP1	NM_002332	Low density lipoprotein-related protein 1	Metabolism	1.78	(↑)	1.98	(↑)
MTHFR	NM_005957	5,10-methylenetetrahydrofolate reductase (NADPH)	Metabolism	1.30	(↑)	1.71	(↑)
PLA2G4A	NM_024420	Cytosolic phospholipase A2, group IVA	Metabolism	2.24	(↑)	2.32	(↑)
ST3GAL6	NM_006100	Alpha2,3-sialyltransferase VI	Metabolism	1.57	(↑)	1.54	(↑)
TDO2	NM_005651	Tryptophan 2,3-dioxygenase	Metabolism	4.47	(↑)	5.17	(↑)
APOE	NM_000041	Apolipoprotein E precursor	Apoptosis	1.35	(↑)	1.60	(↑)
BIRC5	NM_001012271	Baculoviral IAP repeat-containing protein 5 isoform 3	Apoptosis	0.70	(↓)	0.70	(↓)
BMF	NM_001003940	Bcl2 modifying factor isoform bmf-1	Apoptosis	3.34	(↑)	4.55	(↑)
CASP8	NM_033356	Caspase 8 isoform C	Apoptosis	0.72	(↓)	0.72	(↓)
FAS	NM_000043	Tumor necrosis factor receptor superfamily, member 6 isoform 1 precursor	Apoptosis	1.58	(↑)	1.81	(↑)
FN1	NM_212482	Fibronectin 1 isoform 1 preproprotein	Cell adhesion	1.82	(↑)	2.96	(↑)
ITGB3	NM_000212	Integrin beta chain, beta 3 precursor	Cell adhesion	1.60	(↑)	1.52	(↑)
MMP1	NM_002421	Matrix metalloproteinase 1 preproprotein	Cell adhesion	1.71	(↑)	1.88	(↑)
MMP9	NM_004994	Matrix metalloproteinase 9 preproprotein	Cell adhesion	1.67	(↑)	1.85	(↑)
THBS1	NM_003246	Thrombospondin 1 precursor	Cell adhesion	0.66	(↓)	3.41	(↑)
CDKN1A	NM_000389	Cyclin-dependent kinase inhibitor 1A	Cell cycle	1.42	(↑)	1.84	(↑)
CPR8	AF011794	Cell cycle progression restoration 8 protein	Cell cycle	1.62	(↑)	1.70	(↑)
E2F1	NM_005225	E2F transcription factor 1	Cell cycle	0.60	(↓)	0.62	(↓)
MDM2	NM_002392	Mouse double minute 2 homolog isoform MDM2	Cell cycle	1.26	(↑)	1.51	(↑)
BRCA2	NM_000059	Breast cancer 2, early onset	Transcriptional regulation	1.23	(↑)	1.38	(↑)
HMOX1	NM_002133	Heme oxygenase (decyclizing) 1	Transcriptional regulation	1.48	(↑)	2.09	(↑)
POLR2A	NM_000937	DNA directed RNA polymerase II polypeptide A	Transcriptional regulation	0.52	(↑)	0.44	(↓)
SUMO1	NM_001005781	SMT3 suppressor of mif two 3 homolog 1 isoform a precursor	Transcriptional regulation	1.48	(↑)	1.29	(↑)
AGT	NM_000029	Angiotensinogen preproprotein	Signal transduction	3.82	(↑)	7.62	(↑)
IGF2	NM_001007139	Insulin-like growth factor 2	Signal transduction	3.65	(↑)	4.70	(↑)
RND3	NM_005168	ras homolog gene family, member E	Signal transduction	1.35	(↑)	1.61	(↑)
ACP5	NM_001611	Tartrate resistant acid phosphatase 5 precursor	Others	4.08	(↑)	3.73	(↑)
ETFB	NM_001014763	Electron-transfer-flavoprotein, beta polypeptide isoform 2	Others	1.31	(↑)	1.32	(↑)
FAM64A	NM_019013	Hypothetical protein LOC54478	Others	0.66	(↓)	0.69	(↓)
PTGS2	NM_000963	Prostaglandin-endoperoxide synthase 2 precursor	Others	1.44	(↑)	2.54	(↑)
SERPIND1	NM_000185	Heparin cofactor II precursor	Others	2.77	(↑)	1.24	(↑)
SERPINE1	NM_000602	Plasminogen activator inhibitor-1	Others	2.01	(↑)	1.75	(↑)
TERT	NM_198253	Telomerase reverse transcriptase isoform 1	Others	0.67	(↓)	0.63	(↓)

containing siRNA against GFP caused marked reduction of the GFP gene (Fig. 2). By contrast, treatment with siRNA-lipoplexes containing non-specific siRNAs rather increased the GFP expression in the cells (Fig. 2). Interestingly, CL not containing siRNA also significantly increased GFP expression to an extent that was similar to those of non-specific siRNA-lipoplexes. Treatment with free siRNA at same concentration as in the lipoplex did not show any enhancement effect on the GFP expression.

As presented in Fig. 3A, the enhancing effect of non-target siRNA-lipoplex (siLuciferase) on GFP expression increases, at a fixed CL/siRNA ratio of 5, with the concentration of the lipoplex. At the same time, the silencing power of the target-specific siRNA-lipoplex on GFP expression remained virtually unchanged within the concentration range applied. This might be related to biological half-life of the target GFP protein, amount of target mRNA and amount of a multi-protein RNA-inducing silencing complex in the cells stably expressing GFP. Interestingly, at a constant amount of siRNA, the enhancing effect of the non-target lipoplexes on GFP expression increases with increasing amount of CL in the lipoplexes (Fig. 3B), while the silencing potential of the GFP-specific siRNA lipoplexes is insensitive to the amount of CL in the lipoplex. Treatment with free siRNA against GFP and Luciferase did not show any effect on the GFP expression within the concentration range applied for siRNA-lipoplex treatment.

Finally, also in absence of any type of siRNA, GFP expression increased with increasing CL concentration (Fig. 3C), to the same extent as when non-target siRNA was present in the lipoplex (Fig. 3A). This strongly suggests that it is the CL in the siRNA/CL lipoplexes that is predominantly if

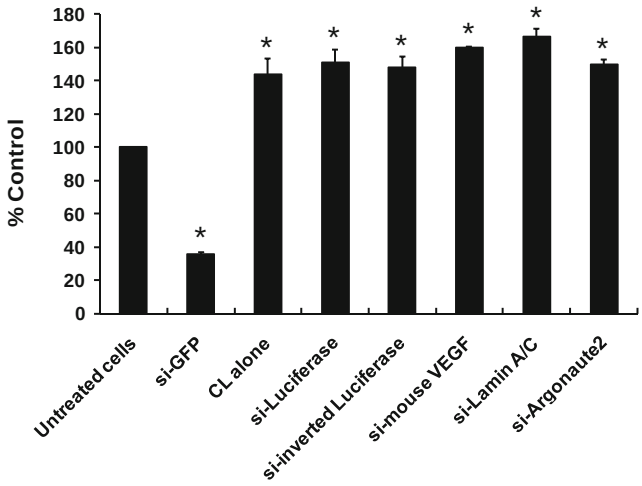

Fig. 2. GFP-gene expression in HT1080-GFP cells after treatment with different siRNA-lipoplexes or CL. The cells, stably expressing the GFP gene (5×10^4), were treated with CL or siRNA-lipoplex (12.5 nM siRNA, corresponding to 0.5 μg/well in a well of six-well plate) for 24 h. A siRNA against GFP (si-GFP) was used as a specific siRNA. A siRNA against Luciferase was used as a non-specific siRNA. The ratio of siRNA (μg) to CL (μl) was 1:10 (0.5 μg/5.0 μl). At 48 h post-treatment, the cells were collected and analyzed by flow cytometry. Data are expressed as mean±SD of three separate experiments. *$p<0.05$, significant difference from GFP-expression in the untreated cells.

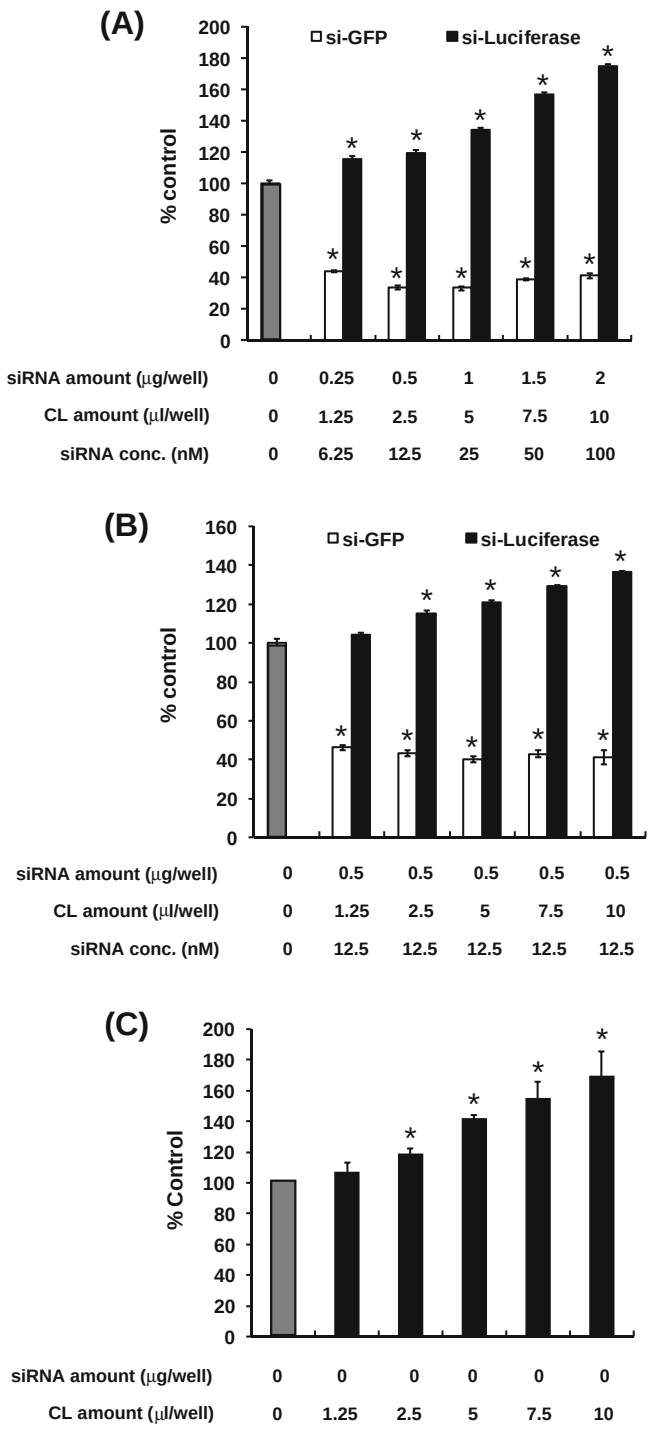

Fig. 3. GFP-gene expression in HT1080-GFP cells after treatment with different doses of siRNA-lipoplex or CL. A siRNA against GFP (si-GFP) or Luciferase were used as a specific and a non-specific siRNA, respectively. The cells (5×10^4) per well in a six-well plate were treated with siRNA-lipoplexes for 24 h. The ratio of siRNA (μg) to CL (μl) was fixed at 1:5. **A** Effect of dose of siRNA-lipoplex on GFP expression. **B** Effect of dose of CL in the siRNA-lipoplex on GFP expression. **C** Effect of dose of CL without siRNA on GFP expression following treatment. Data are expressed as mean±SD of 3 separate experiments. *$p<0.05$, significant difference from GFP-expression in the untreated cells.

not only responsible for the gene enhancing effect of non-target lipoplexes.

Cytotoxity Induced by Treatment with siRNA-Lipoplex or CL

It is conceivable that enhanced global gene expression, as observed in the preceding paragraphs, is accompanied by or even causes cell damage. Hence, we examined the cytotoxicity of the formulations used in the preceding sections. Treatment of cells with siRNA-lipoplexes prepared with various siRNA amounts (12.5, 25, 50, 75, 100 ng/well) but with a constant amount of CL (125 nl/well) caused a marked cytotoxicity in a siRNA dose-dependent manner (Fig. 4A). Treatment with siRNA-lipoplexes containing a constant siRNA/CL ratio also showed substantial cytotoxicity in a siRNA-lipoplex dose dependent manner (Fig. 4B). Finally, treatment with CL only (no siRNA) also caused cytotoxicity in a CL dose dependent manner (0 to 600 nl/well) (Fig. 4C). Treatment with free siRNA did not show any cytotoxicity in the range of concentrations tested (12.5–100 ng/well) (data not shown).

DISCUSSION

Safety and efficacy are important factors in achieving successful gene therapy with nucleic acids such as pDNA and siRNA. Non-viral vectors have been developed as relatively safe nucleic acid delivery systems (8, 16). Undesired side effects relating to the use of a non-viral vector, such as CL, might strongly affect the outcome of gene-therapeutic treatments based on such delivery devices. In this study, we investigated the extent and nature of the gene-expression changes induced by treatment with siRNA-lipoplexes or the lipid vector alone, i.e. CL. cDNA microarray analysis revealed that more than 10% of total genes were non-specifically affected by treatment with siRNA-lipoplexes, while a little less were affected by treatment with CL alone (Table I). This suggests that, while specific gene-knockdown may be achieved by target-specific siRNA-lipoplexes, this may be accompanied by substantial non-specific up- or down regulation of expression of other genes. This notion is strongly supported by the result of our experiments on cells stably expressing an endogenous model gene (GFP), demonstrating pronounced enhancement of GFP expression following treatment with non-specific siRNA-lipoplexes (Figs. 2 and 3).

As the siRNA/CL lipoplex and the CL alone were shown to be similarly active in enhancing global gene expression, assayed either with the cDNA micro-array assay or with the exogenous GFP model gene (Fig. 1; Table I), it is likely that it is not the siRNA in the lipoplex but rather the CL that is the major cause of the gene-expression changes following the treatment. In contrast to these observations, we recently demonstrated that pDNA-lipoplexes notably up- or down-regulated gene expression as compared to CL only (17). This difference may be due to differences in physicochemical properties of siRNA and pDNA or the lipoplexes formulated with CL. pDNA has several thousands of base pairs, while siRNA only has 21. The lipoplexes prepared from siRNA and of pDNA had very different sizes and zeta potentials and different mechanisms of interaction with the cells (18–21). It

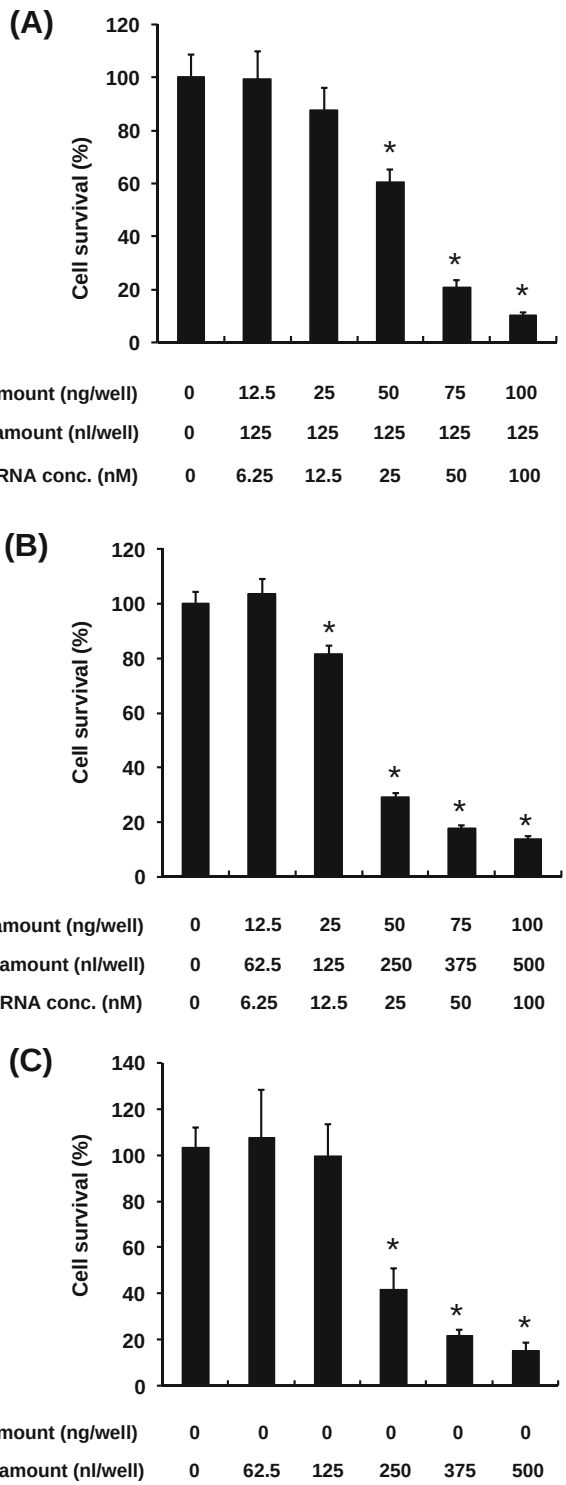

Fig. 4. Cytotoxicity following treatment of HT1080-GFP cells with siRNA-lipoplexes or CL. The cells (2.5×10^3) per a well in a well of 96-well plate were treated for 24 h with siRNA-lipoplexes (**A**, **B**) or CL (**C**). At 48 h post-treatment, the cytotoxicity was determined with the MTT assay. **A** siRNA-lipoplexes with a constant amount of CL and various amounts of siRNA. The siRNA/CL ratio varied from 8:10 to 1:10. (**B**) siRNA-lipoplexes with a constant siRNA/CL ratio of 1:5. **C** CL only. No siRNA. Data are the mean±SD of three independent experiments. *$p<0.05$, significant difference from GFP expression in the untreated cells.

is well recognized that pDNA has a great potential to affect mammalian cells because of its polyanionic nature (22, 23). But the much smaller siRNA may have less biological potency in this respect than the relatively large pDNA. Probably, a much higher siRNA concentration would be required to induce biological alterations additively or synergistically than that used in the treatment.

As summarized in Table II, treatment of the cells with either siRNA-lipoplex or CL alone significantly altered the expression of 45 overlapping genes. These genes are related to immune response, metabolism, apoptosis, cell adhesion, cell cycle, transcriptional regulation and signal transduction. Three of four genes relating to transcriptional regulation were up-regulated, suggesting that cellular transcriptional activity was enhanced by both treatments. This probably accounts for the enhancement of GFP expression by both treatments (Fig. 3). We assumed that the enhancement of expression of an exogenous protein, GFP, reflects that of global gene expressions. If this assumption is correct, the gene-knockdown induced by siRNA-lipoplexes is accompanied by non-specific over-expression of other genes. This might imply, as a consequence, desired outcomes of siRNA-based biochemical studies or therapies might be non-specifically enhanced, attenuated or even fully masked.

It is to be noted that eight out of the nine affected genes relating to immune responses were up-regulated (Table II). Some of the up-regulated genes were IFN- or cytokine-related. This strongly suggests that gene-therapeutic treatment with siRNA-lipoplexes may induce a non-specific cellular immune response. Recently, it was reported that siRNA could non-specifically activate cells of the immune system including the IFN system and induce the production of cytokines both *in vitro* and *in vivo* (9, 24–28). Furthermore, very recently, it was reported that the expression level of several endogenous genes related to cytokines and apoptosis was enhanced by siRNA, transfected by a non-viral vector, Oligofectamine (29) or Lf2000 (30). These reports are consistent with our results reported here. In addition, Judge *et al.* (10) recently reported that siRNAs formulated in a non-viral delivery vehicle (stabilized PEGylated liposomes) induced IFNs and inflammatory cytokine-responses *in vitro* and *in vivo*. This is also consistent with our observation that IFN- or cytokine-relating genes were up-regulated (Table II), although the physicochemical properties and composition of the liposomes were quite different from those of the lipoplexes. Furthermore, reports that siRNA-lipoplexes are recognized by toll-like receptors following internalization by cells (31, 32) and activate natural immunity accompanied by secretion of cytokines such as IFN, IL-6 and TNF-α (33, 34) may account for the siRNA-lipoplex induced immune response. In contrast to these, there were no reports demonstrating that free siRNA (siRNA alone) causes immune response such as up-regulation of IFNs and cytokines (10, 35). In this study, we also demonstrated that free siRNA did not affect the expression of GFP protein and it did not cause any cytotoxicity in the range of concentrations tested. Taken together, these strongly suggest that lipoplex formation and internalization of siRNA together with lipoplex into cells is required for causing non-specific cellular immune responses.

We assumed that cell death could be one of the consequences of cellular immune responses. As shown in Fig. 4B, siRNA-lipoplexes caused cell death in a dose-dependent manner. A similar tendency was observed in terms of GFP-expression following lipoplex or CL treatments (Fig. 3A). These observations may suggest that cell death is a consequence of non-specifically enhanced global gene expression, including the genes of immune response, by siRNA-lipoplexes as presented in Table II. It is known that double-stranded RNAs of 30 base pairs or more (long double-stranded RNA) can trigger global shutdown of gene expression in mammalian cells. This seems to occur through activation of RNase L, that degrades cellular mRNAs, and shutdown of protein translation, ultimately resulting in cell death (36). However, since Elbashir *et al.* (12) reported that the use of synthetic siRNAs of 21 nucleotides circumvents such response, siRNAs are widely used as specific and potent gene silencing tools in mammalian cell lines. From our results obtained in this study it emerges, however, that even the 21-nucleotide siRNA, when accommodated in a cationic lipoplex can induce expression of IFN-inducible genes (Table II), raising serious safety concerns about the use of siRNAs for therapeutic purposes.

Our results clearly indicate that the use of siRNA-mediated therapy could be more complex than originally expected because of non-specific effects on the immune system. However, there should be ways to bypass these siRNA-induced side effects on the immune responses (and possibly resulting cell death) while preserving gene-silencing activity. Further studies will be required to achieve the proper engineering of both siRNAs and delivery systems to minimize immune activation. Moreover, activation of the immune system together with siRNA-mediated gene silencing might actually be desired in the treatment of viral infections and tumors, leading to enhanced therapeutic effects, if only we learn how to control the siRNA-induced responses of the immune system. The studies reported here may provide an important contribution to this endeavor.

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