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RESEARCH PAPER

The Co-Delivery of Oxaliplatin Abrogates the Immunogenic Response to PEGylated siRNA-Lipoplex

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ABSTRACT

Purpose *In vivo* application of siRNA/PEGylated cationic liposome complex (lipoplex) is impeded by two main obstacles: cytokine responses and anti-PEG IgM responses to PEGylated siRNA-lipoplex. Here, we investigated whether co-administration of oxaliplatin (l-OHP) abrogates the cytokine release and anti-PEG IgM production by PEGylated siRNA-lipoplex.

Methods Free l-OHP was administered either simultaneously or 30 min prior to PEGylated siRNA-lipoplex administration, and cytokine response and anti-PEG IgM production were evaluated. In addition, the effect of the liposomal encapsulation of l-OHP on the immunogenic response of PEGylated siRNA-lipoplex was investigated.

Results Simultaneous co-administration of free l-OHP with PEGylated siRNA-lipoplex caused a significant reduction in anti-PEG IgM production, along with an increase in the cytokine response. Free l-OHP injected prior to the lipoplex injection, however, successfully reduced cytokine release and anti-PEG IgM response. Platination of siRNA by simultaneously administered free l-OHP might facilitate the dissociation of double-stranded siRNA to single-stranded siRNA, resulting in the inducement of a potent immuno-stimulation of siRNA *via* endosomal toll-like receptors (TLRs). On the other hand, encapsulation of l-OHP into the siRNA-lipoplex resulted in a reduction of both anti-PEG IgM production and cytokine responses.

Conclusions Our results suggest that, besides the expected therapeutic efficacy of co-administration, encapsulation of l-OHP into the PEGylated siRNA-lipoplex has great potential for minimizing the immunostimulation of PEGylated siRNA-lipoplex, resulting in a safe, applicable, and compliant treatment regimen for sequential clinical administration.

KEY WORDS anti-PEG IgM · cytokines · PEGylated siRNA cationic liposome (lipoplex) · short interfering RNA (siRNA) · β-galctosidase

ABBREVIATIONS

ABC	Accelerated blood clearance
CHOL	Cholesterol
DC-6-14 O,O'	O,O'- ditetradecanoyl-N-(α-trimethylammonio acetyl) diethanolamine chloride
DOPE	Dioleoylphospatidyl-ethanolamine
HRP	Horseradish peroxidase
HSPC	Hydrogenated soy phosphatidyl choline
IL-6	Interleukin 6
IFN-γ	Interferon gamma
l-OHP	Oxaliplatin
$mPEG_{2000}$-DSPE	2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000
NF-κB	Nuclear factor kappa B
POPC	1-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RNAi	RNA interference
siCL	siRNA-lipoplex
siRNA	Short interfering RNA
ssiRNA	Single stranded siRNA

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TLRs	Toll like receptors (TLRs)
Tm	Thermal melting
TNF-α	Tumor necrosis factor alpha

INTRODUCTION

RNA interference, by means of short-interfering RNA (siRNA), is a promising strategy to suppress the expression of target genes and induce post-transcriptional gene silencing (1). Recently, siRNA-mediated gene silencing has gained a lot of interest as a tool for functional genomics studies and as a promising therapeutic agents for the treatment of various diseases, including cancers (2–4). However, poor cellular uptake, a short half-life, rapid systemic clearance, and RNase degradation of naked siRNA impose major impediments against the efficient *in vivo* delivery of siRNA (5,6).

Among nanocarriers, cationic liposomes have proved to be effective tools for gene delivery, both *in vitro* and *in vivo* (7). Cationic liposomes form positively charged complexes with siRNA (siRNA-lipoplexes) and facilitate the intracellular delivery through electrostatic interactions with the membrane of the target cell *in vitro* (8). However, following systemic injection, the positively-charged siRNA-lipoplexes form aggregates with anionic serum proteins (opsonins) and blood cells, which leads to their accumulation in the lungs and in the liver (9).

PEGylation has been frequently employed to increase stability and the circulation time of nanocarrier system (10). Application of this strategy to siRNA-lipoplex is known to enhance the *in vivo* delivery of siRNA to the target tissue *via* a reduction in the adsorption of opsonins and in the recognition of the lipoplex by the cells of the mononuclear phagocyte system (11,12). However, it is now well established that siRNA in some lipoplex formulations, depending on their structure and siRNA sequence, strongly stimulates the innate immune system through the activation of toll-like receptors 3, 7, and 8 (TLRs 3/7/8) (13,14), and through the activation of cytoplasmic receptors (15,16). Stimulation of the innate immune system by siRNA resulted in the release of pro-inflammatory cytokines and interferons, which would lead to significant systemic inflammation (17). In addition, we, along with others, have reported interesting immunoresponses against PEGylated siRNA-lipoplex—in particular, anti-PEG IgM production (18). Such anti-PEG IgM causes the rapid clearance of a second dose of PEGylated siRNA-lipoplex (18–20), and, thereby, would frustrate the therapeutic efficacy of PEGylated siRNA-based therapeutic regimens that require multiple administration. Accordingly, an important factor that may affect the clinical applicability of such siRNA-based therapeutic strategies is the immunogenicity of PEGylated siRNA-lipoplexes.

Recently, the cytotoxicity of chemotherapeutic agents was improved *via* co-treatment with siRNA because the tumor cells were sensitized to the agents (21). In addition, many studies have reported that chemotherapeutic agents show a significant immunosuppressive effect *in vivo* (22,23). In the present study, therefore, we examined the effect of the co-delivery of l-OHP with PEGylated siRNA-lipoplex on the cytokine release and anti-PEG IgM response triggered by PEGylated siRNA-lipoplexes. The results may have important implications for the therapeutic use of the combined treatment of siRNA with chemotherapeutic agent(s) in regimens that required multiple administrations.

MATERIALS AND METHODS

Materials

Dioleoylphospatidylethanolamine (DOPE), hydrogenated soy phosphatidyl choline (HSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 2- distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000 (mPEG2000-DSPE) were generously donated by NOF (Tokyo, Japan). O,O'-ditetradecanoyl-N-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) as a cationic lipid was purchased from Sogo Pharmaceutical (Tokyo, Japan). Oxaliplatin (l-OHP) was generously donated by Taiho Pharmaceutical (Tokyo, Japan). Cisplatin was purchased from Nippon Kayaku (Tokyo, Japan), Cholesterol (CHOL), sodium cacodylate trihydrate (NaCaO) and sodium chloride (NaCl) all were of analytical grade (Wako Pure Chemical, Osaka, Japan). All other reagents were of analytical grade.

siRNA

siRNA against β-galactosidase (β-gal siRNA), chemically synthesized and purified by HPLC, was purchased from Hokkaido System Science (Hokkaido, Japan). The sequence of siRNA against β-gal was as follows: sense sequence, 5′-CUACACAAAUCAGCGAUUUUU-3′; and, antisense sequence, 5′- AAAUCGCUGAUUUGUGUAGUU-3′. The complentary anti-sense and sense strands in TE buffer (10 μM Tris-HCl, 1 μM EDTA (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. The quality of the duplex was checked by 15% PAGE. The final concentration of the duplex was adjusted to 50 μM with TE buffer.

Animals

Male BALB/cCr Slc mice aged 4–5 weeks (20–25 g) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under pathogen-free conditions with free access

to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12-h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

Preparation of Fluid PEGylated siRNA-lipoplex (fsiCL)

Fluid cationic liposomes, composed of POPC/CHOL/DOPE/DC-6-14 (0.5:0.75:0.75:0.5, molar ratio) were prepared as previously described (24). The mean diameter and zeta potential of the resultant liposomes were 109.2±16.2 nm and 20.1±0.5 mV (n=3), respectively, as determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The phospholipid concentration of the liposomes was determined by colorimetric assay (25).

For the preparation of siRNA/cationic liposome complexes (siRNA-lipoplexes), siRNAs and cationic liposomes were mixed at a charge ratio of 3.81 (+/−), which was equal to 800/1 (lipid/siRNA, molar ratio), and the mixture was vigorously vortexed (3,200 rpm, 10 min, room temperature) to form siRNA-lipoplexes. The mean diameter and zeta potential of siRNA-lipoplexes were 398.6±42.0 nm and 19.6±1.1 mV (n=3), respectively. For PEGylation, siRNA-lipoplexes were surface-modified by polyethylene glycol (PEG)-conjsugated lipid (PEGylation) using a post-insertion technique (26). Briefly, mPEG2000-DSPE (5 mol% of total lipid) in 9% sucrose solution was added to the siRNA-lipoplex solution, and the mixture was gently shaken for 1 h at 37°C. The mean diameter of fluid PEGylated siRNA-lipoplexes (fsiCL) was 385.2±60.7 nm (n=3). To detect the free-siRNA in the prepared fsiCL, electrophoresis was performed on 2% agarose gel in 40 mM Tris-acetate/1 mM EDTA buffer and siRNA visualization was carried out using a UV transilluminator. No bands that related to free siRNA were detected, indicating that virtually 100% of the siRNA was associated with, and/or encapsulated in, the fsiCL.

For the preparation of l-OHP-containing PEGylated fluid siRNA-lipoplexes (l- OHP-containing fsiCL), a lipid film layer, prepared as mentioned above, was dissolved in a mixture of chloroform/ether/l-OHP solution (8 mg/ml) (1:2:1 v/v). The resultant emulsion was sonicated for 15 min at room temp. The organic phase was evaporated from liposomes in a rotary evaporator at 40°C under a reduced pressure of 250 hPa for 1 h. The resultant liposomes were sized as previously described (24). The mean diameters and zeta potentials of the resultant liposomes were 120.2±13.1 nm and 20.3±0.9 mV (n=3), respectively. The PEGylation and siRNA association was carried out as described above. The mean diameter and zeta potential of the l-OHP-containing fsiCL were 392.3±67.5 nm and 22.2±0.5 mV (n=3), respectively. The l-OHP encapsulation efficiency, determined using an atomic absorption photometer (Z-5700, Hitachi, Tokyo), was 10.3±0.4% (n=3).

Preparation of Solid PEGylated siRNA-lipoplex (ssiCL)

Solid PEGylated cationic liposomes composed of HSPC/Chol/DC-6-14/mPEG$_{2000}$-DSPE (2:1:0.2:0.2 molar ratio) were prepared by dissolving the lipids and mPEG2000-DSPE in chloroform. After evaporation of the organic solvent, the resultant PEGylated lipid film was hydrated in 9% sucrose. The resultant liposomes were sized at 65°C, as previously described (24). The mean diameter and zeta potential for solid PEGylated cationic liposomes were 148.9±45.1 nm and 22.1±0.2 mV (n=3), respectively. The phospholipid concentration of the liposomes was determined by colorimetric assay (25).

siRNA interaction was carried out and determined as described above. PEGylation did not prevent siRNA interaction to solid liposome under these conditions. The mean diameter of the resultant solid PEGylated siRNA-lipoplex was 340.5±45.1 nm. l-OHP-containing solid PEGylated siRNA-lipoplex (l-OHP-containing ssiCL) was prepared as described above. The values for mean diameter and zeta potential of l-OHP-containing ssiCL were 342.6±56.1 nm and 24.3±0.4 mV (n=3), respectively. The l- OHP encapsulation efficiency was 18.6±0.7% (n=3).

Detection of Anti-PEG IgM

In order to investigate the effect of free and encapsulated l-OHP on the anti-PEG IgM response induced by an intravenous injection of PEGylated siRNA-lipoplexes (siCL), mice were randomly divided into 9 groups (6 animals in each group). The treatment schemes are presented in Table I.

For the determination of anti-PEG IgM production, on day 5 after treatment as described in Table I, mice were euthanized and blood samples were obtained from the abdominal vein. To obtain serum, the blood was placed at room temperature for 30 min and then centrifuged at 3,000 rpm and 4°C for 15 min. The serum collected from naïve mice was used as the control serum. Anti-PEG IgM determination was carried out using ELISA, as described previously (26).

Measurement of Inflammatory Cytokine

To evaluate the effect of free or encapsulated l-OHP on the cytokine response induced by intravenous injection of siCL, mice were randomly divided into 11 groups (6 animals in each group). The treatment schemes are presented in Table II.

For measurement of the cytokine response, blood samples were withdrawn from the abdominal vein of each treated mouse at 4 h after injection. Serum was obtained as described above. Interleukin 6 (IL-6), interferon gamma

Table I The Treatment Scheme for Anti-PEG IgM Determination

Group	Treatment
1	9% sucrose solution (naïve)
2	Fluid siCL[a] (fsiCL)
3	l-OHP-containing fluid siCL[a,b] (l-OHP-containing fsiCL)
4	Simultaneous intravenous administration of free l-OHP[b] and fluid siCL[a] (fsiCL+l-OHP (mix.))
5	Intravenous injection of free l-OHP solution[b]30 min prior fluid siCL[a] administration (l-OHP➔fsiCL (sep.))
6	Solid siCL[a] (ssiCL)
7	l-OHP-containing solid siCL[a,c] (l-OHP-containing ssiCL)
8	Simultaneous intravenous administration of free l-OHP[c] and solid siCL[a] (ssiCL+l-OHP (mix.))
9	Intravenous injection of free l-OHP solution[c] 30 min prior solid siCL[a] administration (l-OHP➔ssiCL (sep.))

[a] 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg

[b] 0.3 mg l-OHP/kg

[c] 0.56 mg l-OHP/kg

(IFN-γ) and tumor necrosis factor alpha (TNF-α) in the serum were measured using Quantikine Immunoassay Kits (R&D Systems, MN, USA) according to the manufacturer's instructions.

Isolation of Splenic B Cells

Spleen cell suspensions were prepared as described previously (24). Briefly, spleen slices were squeezed through a Cell Strainer (100 μm, Becton Dickinson, NJ,USA). The cells were suspended in cold PBS (37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4; pH 7.4) containing 0.5 mM EDTA (EDTA-PBS). Red blood cells in the suspension were lysed by treatment with ammonium chloride lysis buffer (0.83% NH_4Cl) for 5 min on ice. Cells were washed twice with EDTA-PBS and filtered with a Cell Strainer to remove clumps. Then, spleen cells were suspended in RPMI-1640 medium, and the splenic B cells were separated from the non-B cells using a B-Cell Isolation Kit and an autoMACS® Pro Separator (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer's instructions.

Table II The Treatment Scheme for Inflammatory Cytokine Measurement

Group	Treatment
1	9% sucrose solution (naïve)
2	Fluid siCL[a] (fsiCL)
3	Free l-OHP[b]
4	l-OHP-containing fluid siCL[a,b] (l-OHP-containing fsiCL)
5	Simultaneous intravenous administration of free l-OHP[b] and fluid siCL[a] (fsiCL+l-OHP (mix.))
6	Intravenous injection of free l-OHP solution[b] 30 min prior fluid siCL[a] administration (l-OHP➔fsiCL (sep.))
7	Free l-OHP[c]
8	Solid siCL[a] (ssiCL)
9	l-OHP-containing solid siCL[a,c] (l-OHP-containing ssiCL)
10	Simultaneous intravenous administration of free l-OHP[c] and solid siCL[a] (ssiCL+l-OHP (mix.))
11	Intravenous injection of free l-OHP solution[c] 30 min prior solid siCL[a] administration (l-OHP➔ssiCL (sep.))

[a] 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg

[b] 0.3 mg l-OHP/kg

[c] 0.56 mg l-OHP/kg

NF-κB Activation Assays

To investigate the effect of encapsulated l-OHP on the activation of nuclear factor-kappa B (NF-κB) induced by an intravenous injection of PEGylated siRNA-lipoplexes (siCL), the isolated splenic B cells were cultured in 12-well plates at a density of 5×10^6 cells/well in RPMI-1640 medium, and then were incubated for 1 h with either siRNA-lipoplex (0.05 μmol phospholipid/well corresponding to 0.8 μg siRNA), a mixture of free l-OHP (0.8 μg/well) and siRNA lipoplex (0.05 μmol phospholipid/well corresponding to 0.8 μg siRNA) or l-OHP-containing siRNA lipoplex (0.05 μmol phospholipid/well corresponding to 0.8 μg siRNA and 0.8 μg l-OHP/well). Then, the expression level of IkBα, an indicator of NF-κB activation, was determined using Western blotting. Briefly, 30 micrograms of splenic cells protein extracts were resolved on 10% SDS-PAGE gel (ATTO Corp., Tokyo, Japan). After electrophoresis, the proteins were electro-transferred onto Hybond-ECL nitrocellulose membrane (GE Healthcare, Cleveland, OH, USA), blocked with 5% non-fat milk, and probed with antibody against IkBα (Cell Signaling Technology, Tokyo, Japan) according to manufacturer's protocol. Thereafter, the blot was washed with Tris-buffered saline containing 0.05% Tween 20, exposed to HRP-conjugated secondary antibodies (MP Biomedicals, Solon, OH, USA) for 1 h, and finally detected by using the ECL Plus Chemiluminescence Reagent (GE Healthcare UK, Little Chalfont, UK), and the obtained images were analyzed using LAS-4000 EPUVmini and Multi Gauge v.3.2 (FujiFilm, Tokyo, Japan).

Thermal Melting Study

To investigate the effect of the platination of β-gal siRNA by l-OHP on the stability of β-gal siRNA, a thermal melting (Tm) study was carried out as described previously (27). Briefly, β-gal siRNA duplex was mixed with a 10-fold molar excess of l-OHP. The siRNA sample was precipitated using an ethanol precipitation technique. Thermal melting of the siRNA sample was performed in 1 mM NaCl and 100 mM NaCaO buffer (pH=6.3) on a UV-vis-NIR spectrophotometer UV-

1800 (Shimadzu) equipped with a temperature control unit. The data were analyzed using a TMSPC Tm analysis system (Shimadzu, Kyoto, Japan). The results were analyzed by both 1st derivative and hyperchromicity calculations.

Statistical Analysis

All values are expressed as the mean±S.D. Statistical analysis was performed with a two-tailed unpaired Student's *t*-test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p<0.05$.

RESULTS

Effect of l-OHP on Anti-PEG IgM Production by Fluid PEGylated siRNA-lipoplex (fsiCL)

The anti-PEG IgM production was assessed on day 5 after a single injection of fluid PEGylated siRNA-lipoplex (fsiCL), because we had confirmed in a previous study that the level of serum anti-PEG IgM peaks at day 5 (20). siRNA against β-galactosidase (β-gal) was used in the present study, because it is well-known that the sequence exerts a potent immune stimulatory effect, including the production of anti-PEG IgM (28) and various inflammatory cytokines (13). As shown in Fig. 1, a single intravenous injection of fsiCL significantly induced anti-PEG IgM production, which was consistent with our previous reports (20,26,28). Simultaneous administration of free l-OHP (0.3 mg/kg mouse) with fsiCL (l-OHP+fsiCL (mix)) caused a significant reduction in the anti-PEG IgM response. Interestingly, a further significant reduction was induced by the intravenous injection of free l-OHP 30 min prior to fsiCL injection (l-OHP➔fsiCL (sep)). The effect of l-OHP encapsulation into fluid siCL (l-OHP-containing fsiCL) on the anti-PEG IgM levels was also investigated. Liposomal l-OHP encapsulation significantly suppressed anti-PEG IgM production, compared to either fsiCL or simultaneous injection of free l-OHP and fsiCL (l-OHP+fsiCL (mix)). No significant difference in the anti-PEG IgM production was observed between the treatment with l-OHP-containing fsiCL and fsiCL 30 min after free l-OHP injection (l- OHP➔fsiCL (sep)).

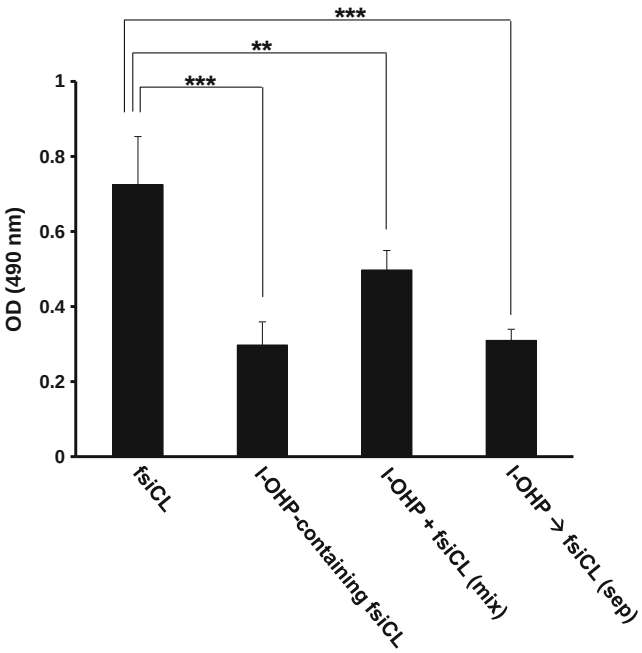

Fig. 1 Effect of l-OHP on the anti-PEG IgM response induced by i.v. Injection of fluid siCL. BALB/c mice were intravenously injected on day 0 with either fluid siCL (fsiCL; 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), l-OHP-containing fluid siCL (l-OHP-containing fsiCL; 0.3 mg l-OHP/kg, 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), a mixture of free l-OHP (0.3 mg/kg) and fluid siCL (l-OHP+fsiCL (mix.)), or l-OHP 30 min prior to fluid siCL (l-OHP➔fsiCL (sep.)). Mice receiving 9% sucrose instead of fluid siCL served as controls. On day 5, the serum anti-PEG IgM level was detected using ELISA, as described in the "Materials and Methods" section. Each value represents the mean±S.D. ($n=6$). **$p<0.01$ and ***$p<0.005$.

Effect of l-OHP on Cytokine Response Induced by Fluid PEGylated siRNA-lipoplex (fsiCL)

A large number of reports have demonstrated that inflammatory cytokines, particularly IL-6, TNF-α and IFN-γ, participate in the potent immune stimulatory effect of siRNA-lipoplexes (13,17,28). Therefore, to gain more insight into the effect of free l-OHP on the immunostimulation of fluid PEGylated siRNA-lipoplexes (fsiCL), production levels of the inflammatory cytokines, IL-6, TNF-α, and INF-γ, were determined at 4 h after injection (when the production of inflammatory cytokines by β-gal siRNA-lipoplex is maximum (28)) (Fig. 2). Intravenous injection of fsiCL alone caused a significant production of IL-6, TNF-α, and IFN-γ. A single injection of free l-OHP at our low dose caused no inflammatory cytokines. Co-treatment of free l-OHP with fsiCL altered the induction pattern of inflammatory cytokines depending on the order of treatment. Intravenous injection of free l-OHP 30 min prior to fsiCL injection (l-OHP➔fsiCL (sep)) significantly reduced all tested cytokines induction by intravenous injection of fsiCL. Surprisingly, the simultaneous injection of l-OHP with fsiCL (l-OHP+fsiCL (mix)) rather increased the release of IL-6 and TNF-α. In terms of IFN-γ induction, co-treatment with free l-OHP (l-OHP+fsiCL (mix), and (l-OHP➔fsiCL (sep)) entirely reduced the production. The effect of l-OHP encapsulation within fluid PEGylated siRNA-lipoplex (l-OHP-containing fsiCL) on the inflammatory cytokine production was also evaluated. l-OHP-containing fsiCL caused a significant reduction in the production of IL-6, TNF-α, and IFN-γ compared to fsiCL. The reduction level was nearly the same as that by free l-OHP followed by fluid siCL (l-OHP➔fsiCL (sep)) (Fig. 2).

Effect of l-OHP on NF-κB Activation by Fluid PEGylated siRNA-lipoplex (fsiCL)

To elucidate the probable mechanism underlying the modulatory effect of encapsulated l-OHP on inflammatory cytokine production by PEGylated siRNA-lipoplex (siCL), the influence of l-OHP encapsulation on NF-κB activation by fluid PEGylated siRNA-lipoplex (fsiCL) was investigated. In this experiment, the expression level of IkBα by splenic B cells, regulated by NF-κB, was used as an indicator of NF-κB

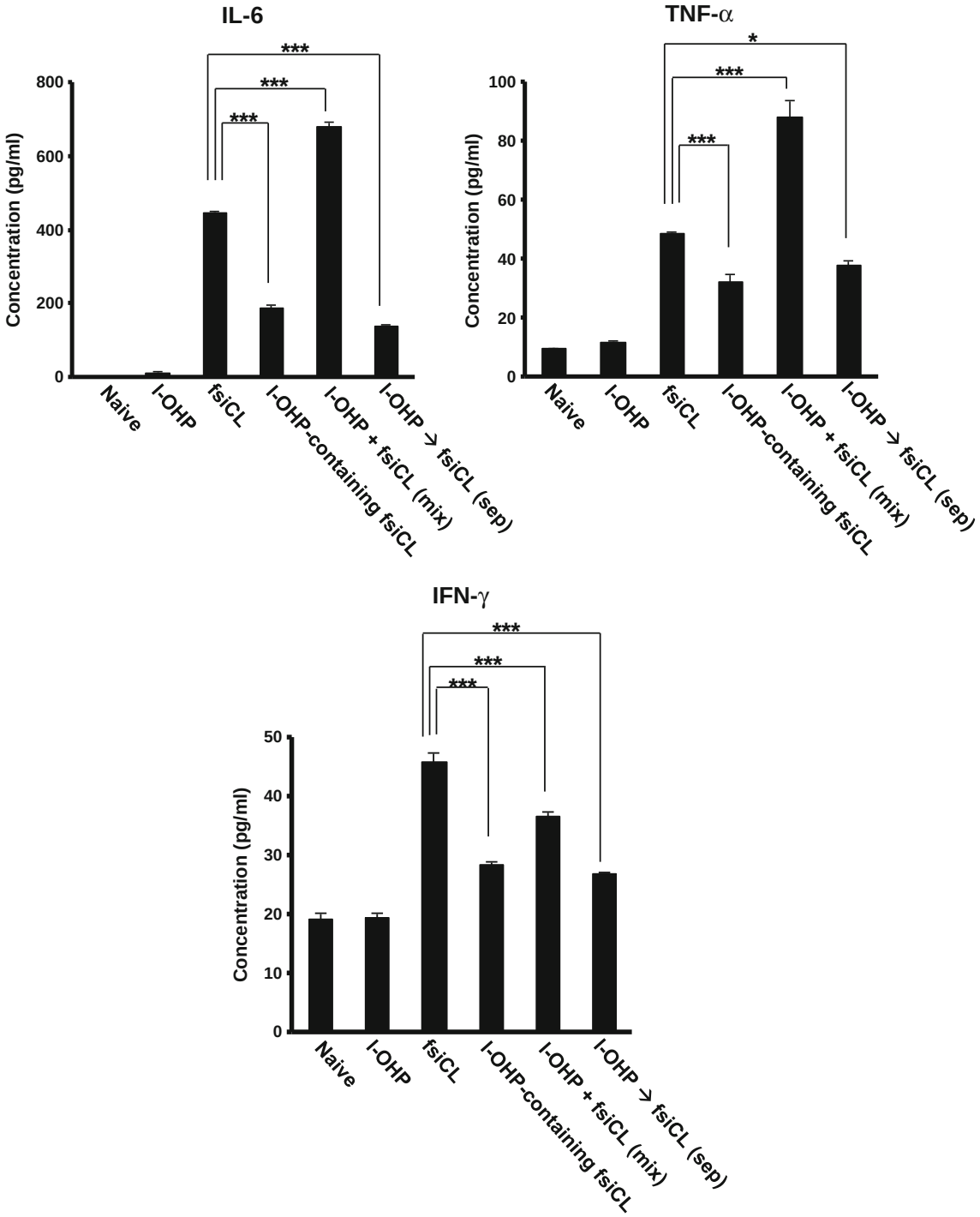

Fig. 2 Effect of l-OHP on cytokine response induced by i.v. Injection of fluid siCL. BALB/c mice were intravenously injected with either fluid siCL (fsiCL; 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), l-OHP-containing fluid siCL (l-OHP-containing fsiCL; 0.3 mg l-OHP/kg, 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), a mixture of free l-OHP (0.3 mg/kg) and fluid siCL (l-OHP+fsiCL (mix.)), or l-OHP 30 min prior to fluid siCL (l-OHP➔fsiCL (sep.)). Mice receiving 9% sucrose instead of fluid siCL served as controls. At hour 4, post injection of fluid siCL, cytokine levels (IL-6, TNF-α, and IFN-γ) in the serum were determined. Each value represents the mean±SD ($n=6$). *$p<0.05$ and ***$p<0.005$.

activation by fsiCL. As shown in Fig. 3, fsiCL increased the expression level of IkBα. l-OHP encapsulated within fsiCL (l-OHP-containing fsiCL) strongly suppressed the increase of IkBα expression level by fsiCL treatment, nearly to the negative control (naïve) level. On the other hand, a mixture of free l-OHP and fsiCL (l-OHP+fsiCL) failed to suppress the increased IkBα expression level. These results indicate that the encapsulation of l-OHP within siRNA-lipoplex suppress the NF-κB activation, strongly correlates with the production of inflammatory cytokines, by siRNA-lipoplex.

Effect of Membrane Fluidity of siRNA-lipoplex on Anti-PEG IgM and Cytokine Response

Assuming that the fluidity of the liposomal membrane may affect the rate of release of l-OHP, and as a consequence, the pattern of anti-PEG IgM and cytokine response induced by PEGylated siRNA-lipoplex, the production of anti-PEG IgM and cytokines by solid PEGylated siRNA-lipoplex in the presence or absence of l-OHP was investigated.

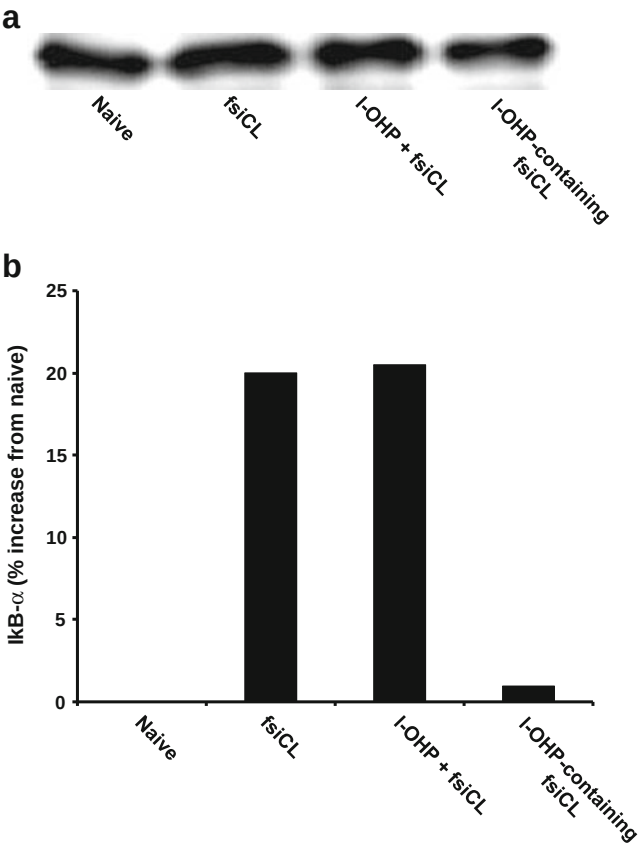

Fig. 3 NF-κB inhibition by l-OHP encapsulated within PEGylated siRNA-lipoplex. (**a**) IkBα protein expression by isolated splenic B cells after treatment with either fsiCL, a mixture of free l-OHP and fsiCL or l-OHP-containing fsiCL, as determined by Western blotting. (**b**) Quantitative evaluation of the percent increase in expression levels of IkBα protein in the treated B cells against naïve (non-treated) B cells.

In a similar pattern to fsiCL, ssiCL induced significant anti-PEG IgM production (Fig. 4). Co-treatment with free l-OHP (l-OHP+ssiCL (mix.)) significantly reduced the anti-PEG IgM production induced by empty ssiCL. Injection of free l-OHP 30 min prior to ssiCL (l-OHP➔ssiCL (sep.)) induced a more significant reduction in anti-PEG IgM production induced by ssiCL ($p<0.005$). The anti-PEG IgM reduction level by l-OHP-containing ssiCL was more significant than that caused by the co-injection of free l-OHP with ssiCL (l-OHP+ssiCL (mix.)). The reduction in the anti-PEG IgM production by l-OHP-containing ssiCL was more significant than that caused by free l-OHP 30 min prior to solid siCL injection (l-OHP➔ssiCL(sep.)) (Fig. 4).

In terms of cytokine induction, ssiCL induced significant cytokine release. A single injection of our low dose of l-OHP did not induce a cytokine response. Co-treatment with free l-OHP (l-OHP+ssiCL (mix.)) significantly increased the release of IL-6 and TNF-α, but reduced the IFN-γ release induced by ssiCL (Fig. 5). Injection of free l-OHP 30 min prior to ssiCL injection (l-OHP➔ssiCL (sep.)) significantly reduced the cytokine release induced by ssiCL. Encapsulation of l-OHP into ssiCL (l-OHP-containing ssiCL) significantly reduced the production of cytokines induced by ssiCL. The decrease in cytokine release by solid encapsulated l-OHP was equal to that

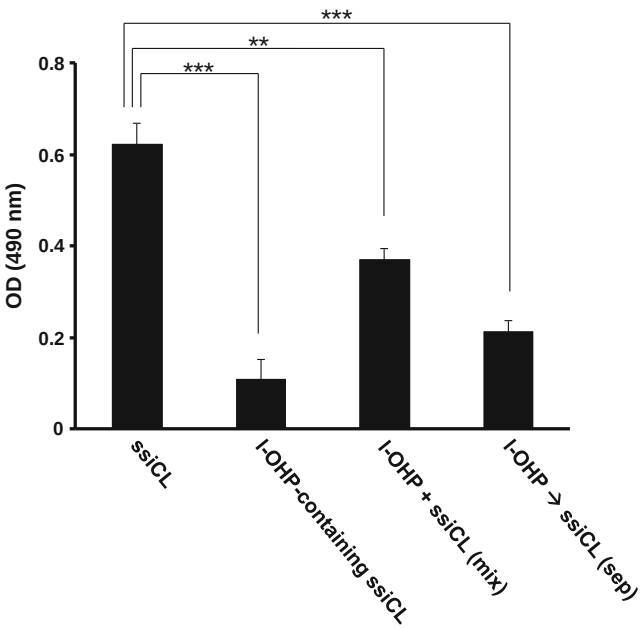

Fig. 4 Effect of membrane fluidity of siRNA-lipoplex on anti-PEG IgM response. BALB/c mice were intravenously injected on day 0 with either solid siCL (ssiCL; 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), l-OHP-containing solid siCL (l-OHP-containing ssiCL; 0.56 mg l-OHP/kg, 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), a mixture of free l-OHP (0.56 mg/kg) and solid siCL (l-OHP+ssiCL (mix.)), or l-OHP 30 min prior to solid siCL (l-OHP➔ssiCL (sep.)). Mice receiving 9% sucrose instead of solid siCL served as controls. On day 5, the serum anti-PEG IgM level was detected using ELISA, as described in the "Materials and Methods" section. Each value represents the mean±S.D. ($n=6$). **$p<0.01$ and ***$p<0.005$.

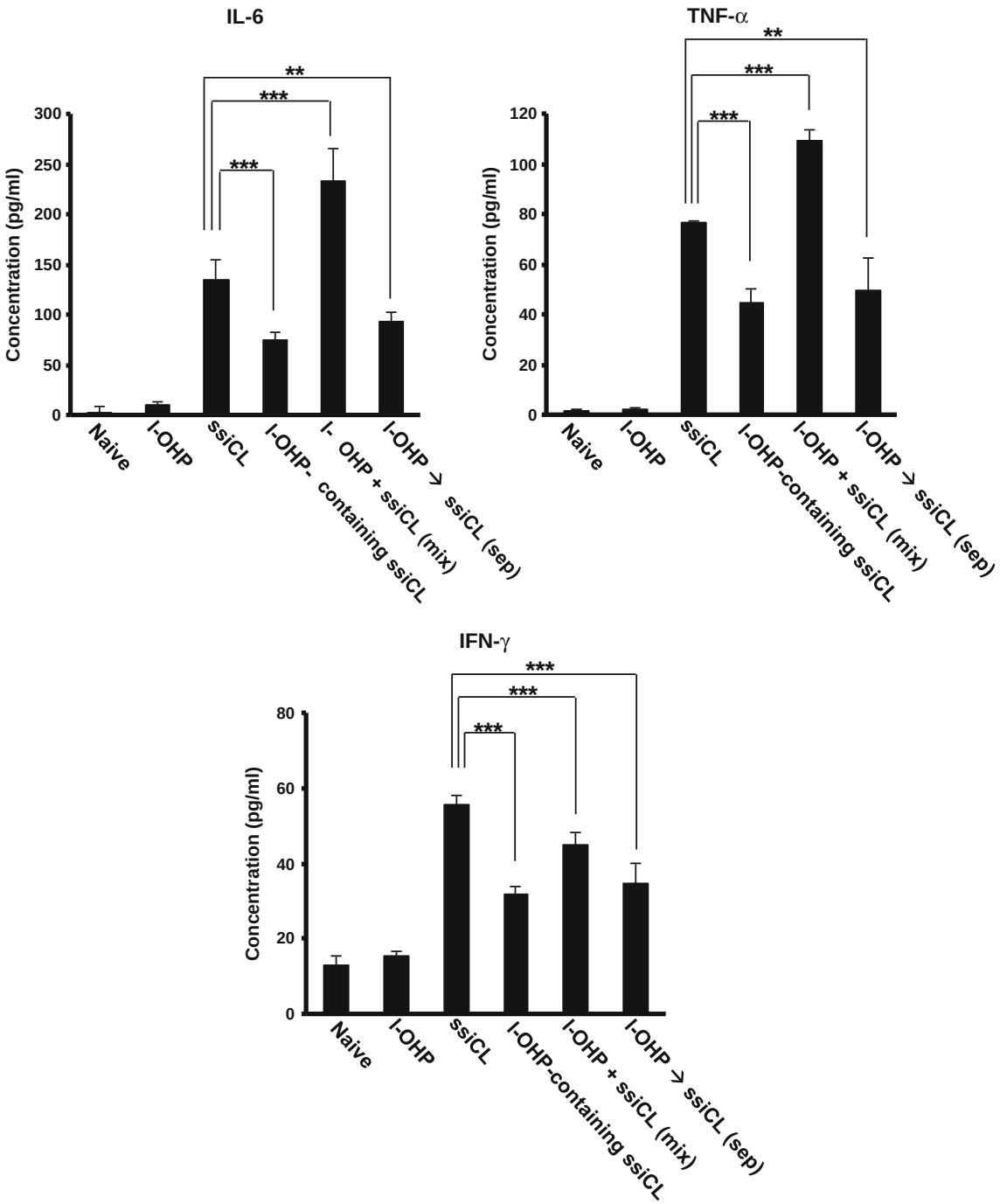

Fig. 5 Effect of membrane fluidity of siRNA-lipoplex on cytokine response. BALB/c mice were intravenously injected with either solid siCL (ssiCL; 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), l-OHP-containing solid siCL (l-OHP-containing ssiCL; 0.56 mg l-OHP/kg, 18.852 μmol phospholipids/kg and 312.5 μg siRNA/kg), a mixture of free l-OHP (0.56 mg/kg) and solid siCL (l-OHP+ssiCL (mix.)), or l-OHP 30 min prior to solid siCL (l-OHP➔ssiCL (sep.)). Mice receiving 9% sucrose instead of solid siCL served as controls. At hour 4, post injection of solid siCL, cytokine levels (IL-6, TNF-α, and IFN-γ) in the serum were determined. Each value represents the mean±SD ($n=6$). $^{**}p<0.01$ and $^{***}p<0.005$.

produced by the separate treatment of l-OHP and ssiCL (l-OHP➔ssiCL (sep.)). Taken together, it seems that the membrane fluidity of PEGylated siRNA-lipoplex affected neither the immunostimulative effect of the lipoplex nor the immunosuppressive effect of l-OHP (free or liposomal formulation) under our experimental conditions.

Thermal Melting (Tm) Study

A thermal melting experiment was performed to study the effect of the platination of β-gal siRNA with l-OHP on siRNA duplex stability. The mixing of β-gal siRNA with l-OHP induced an approximate 14°C reduction in the Tm of

siRNA (Table III). To ensure the effect of siRNA platination on duplex stability, the same experiment was carried out using cisplatin. Cisplatin induced remarkable Tm reduction (approximately 30°C). The observed lowering of the Tm of the siRNA platinated adduct compared to native siRNA indicated a lower stability and a higher tendency for the dissociation of the siRNA-duplex into a single-stranded siRNA.

DISCUSSION

A combination therapy of siRNA with chemotherapeutic agents was expected to confer advantages over conventional chemotherapy (21). However, the activation of the innate immune system with a subsequent excessive cytokine release (IL-6, TNF-α, and interferons) *via* the stimulation of toll-like receptors (TLRs) by siRNA may lead to toxic inflammation (17,29–31). Recent studies have shown that besides the cytotoxic effect on the tumor cells, chemotherapeutic agents abrogate the immune system (32). In the present study, we demonstrated that l-OHP has a schedule-dependent modulating effect against the siRNA immunogenic response. We observed that a separate treatment by free l-OHP and PEGylated siRNA-lipoplex (free l-OHP 30 min prior to PEGylated siRNA-lipoplex) resulted in a decrease in the cytokine release induced by PEGylated siRNA-lipoplex (Figs. 2 and 5). Surprisingly, co-treatment by free l-OHP with PEGylated siRNA-lipoplex significantly increased cytokine release (mainly IL-6 and TNF-α) rather than treatment with PEGylated siRNA-lipoplex (Figs. 2 and 5). These observations may have important implications for the design of a therapeutic strategy of co-treatment with siRNA and chemotherapeutic agents.

Many studies have confirmed that the immunostimulatory effects of siRNA are mediated mainly through the up-regulation of the expression of transcription factors such as nuclear factor (NF)-κB, which is a critical mediator of macrophage inflammatory responses, including the release of cytokines such as IL-6, TNF-α, and IFN-γ (17,33). Reduction of the siRNA-induced cytokine release by free l-OHP, on separate treatment, may be attributed to the cytotoxic effect of l-OHP against macrophages (34) and B-cells (22) with the subsequent attenuation of the NF-κB-mediated response (Fig. 3), and TLR-induced STAT1 and STAT3 expression. However, there could be other possible mechanisms justifying the l-OHP-mediated abrogation of the siRNA-induced cytokine release.

Interestingly, co-treatment of l-OHP with PEGylated siRNA-lipoplex resulted in increased cytokine responses, mainly IL-6 and TNF-α. This might be due to the platination of siRNA caused by free l-OHP. The mixing with free l-OHP resulted in a Tm value for siRNA that was lower than that of native siRNA (Table III). This indicates a lower stability for the platinated adduct of siRNA (27,35), and thereby an enhanced dissociation of the siRNA-duplex into single-stranded siRNA (ss siRNA). It is known that ss siRNAs are more effectively recognized in the lysosome by TLRs 7 and 8 than are their corresponding duplexes and are more effective in the production of pro-inflammatory cytokines and interferons (36). Accordingly, the platination of siRNA by l-OHP might be a major cause for the potent release of IL-6 and TNF-α by the co-treatment of l-OHP with PEGylated siRNA-lipoplex. Surprisingly, IFN-γ was decreased by the co-injection of free l-OHP with PEGylated siRNA-lipoplex. A recent study has reported the engagement of TLR3 and TLR7 in controlling the release of IFN-γ (37). It is well known that ss siRNA is a strong agonist of TLR7 but not of TLR3 (36). Taken together, we assumed that ss siRNA, produced upon a co-injection of free l-OHP with siRNA-lipoplex, induced a partial release of IFN-γ *via* TLR7 but did not affect the IFN-γ release *via* TLR3. However, due to the overlapping mechanisms of cytokine pathways, further studies are required to ensure our assumption.

Encapsulation of l-OHP into the PEGylated siRNA-lipoplex (either fluid or solid) entirely reduced the cytokines release. PEGylated siRNA-lipoplex containing l-OHP probably increased cellular l-OHP concentration in immune cells because the lipoplex was aggressively taken up by those cells. The l-OHP released from the formulation efficiently suppressed the immunostimulative effect of siRNA (Fig. 3) because of its immunosuppressive effect. Consequently, the formulation of l-OHP-containing PEGylated lipoplex might have an additional advantage in terms of attenuating the systemic inflammation triggered by siRNA in the formulation.

l-OHP entirely reduced anti-PEG IgM production regardless of the treatment schedule and/or formulation (Figs. 1 and 4). This is consistent with previous studies by Laverman *et al.* (38) and by our study (22), which reported that Doxil, PEGylated liposomes containing doxorubicin, did not induce the ABC phenomenon in rats. Based on our previous study (22), we assumed that l-OHP would down-regulate or otherwise inhibit the proliferation of B-cells in the spleen, resulting in a reduction in the anti-PEG IgM production in response to PEGylated siRNA-lipoplex. It is noteworthy, that TLRs are known to be involved in the production of antibodies to components of delivery vehicles,

Table III Melting Temperatures (Tm) of Native and l-OHP Platinated ß-gal siRNA Duplexes

Duplex	Tm (°C)
Native ß-gal siRNA	66.2 ± 0.4
Platinated ß-gal siRNA by l-OHP	52.6 ± 0.8
Platinated ß-gal siRNA by cisplatin	36.6 ± 8.2

linking innate and adaptive immunity (38). However, the potent induction of cytokines (IL-6 and TNF-α) *via* ss siRNA-stimulated TLRs, due to the co-treatment of free l-OHP and PEGylated siRNA-lipoplex (Fig. 2), did not increase the anti-PEG IgM production (Fig. 1). This may be attributable to the inability of stimulated TLRs, as a secondary pathway affecting anti-PEG IgM production, to counteract the inhibitory effect of l-OHP on B-cells, which is a major source of anti-PEG IgM production (transient production of anti-PEG IgM by ss siRNA-stimulated TLRs is counteracted by the potent and persistent inhibitory effect of B-cells by l-OHP).

Despite the expected different release rate of l-OHP from the lipoplex, the liposomal membrane fluidity of the l-OHP-containing PEGylated siRNA-lipoplex did not affect the immunomodulatory effect of l-OHP against PEGylated siRNA-lipoplex. Both solid and fluid l-OHP-containing PEGylated siRNA-lipoplex induced a significant reduction in both cytokine release and anti-PEG IgM production (Figs. 2 and 5). However, the solid l-OHP-containing PEGylated siRNA-lipoplex induced a more significant reduction in anti-PEG IgM production compared with that of the fluid l-OHP-containing PEGylated siRNA-lipoplex. Probably, the expected slower release rate of l-OHP from rigid lipoplex, coupled with a longer circulation time than that of fluid l-OHP-containing PEGylated siRNA-lipoplex (39), explains the enhanced reduction in anti-PEG IgM production.

Many reports have demonstrated that the combination theory of chemotherapeutic agents with siRNA may have an additive or even a synergistic chemotherapeutic efficacy in animal models (40–42). However, the immunostimulation triggered by siRNA and/or the delivery vehicle, is a major obstacle to clinical use. Here, we propose a promising therapeutic strategy for combination therapy. Injection of either l-OHP prior to PEGylated siRNA-lipoplex injection with a short-term duration or l-OHP encapsulated in the PEGylated siRNA-lipoplex, achieves the expected enhanced therapeutic effect and realizes the great potential of minimizing the immunostimulation elicited by the PEGylated siRNA-lipoplex. This provides a safe and compliant treatment regimen for sequential clinical administration.

CONCLUSION

Combination therapy of siRNA with chemotherapeutic agents has proven to be an efficient anticancer therapeutic regimen *via* sensitizing the tumor cells to chemotherapeutic agents. Immunostimulation by siRNA and a delivery vehicle may frustrate the clinical application of this combination therapy. Our study revealed that the effect of either free or encapsulated l-OHP strongly modulates the immunostimulatory effect of PEGylated siRNA-lipoplex. Our results may have important implications for the development of a more efficient and applicable siRNA delivery system with a minimized ABC phenomenon and minimized induction of immune activation.

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