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「経口型抗がん剤の metronomic dosing による腫瘍内微小環境変化を利用した革新的 siRNA デリバリー技術の開発とがん治療への応用」に関する研究

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研究要旨　siRNA を薬剤として利用する場合、生体内動態を量的・空間的に制御することが重要である。表在性の腫瘍を除き、siRNA の静脈内投与は不可避である。100nm 程の粒子径を持つナノキャリアは腫瘍内新生血管内皮の隙間を通過して腫瘍に集積する。しかし、血管周囲に留まるだけで腫瘍深部まで到達できない。これを改善するには、キャリアの改変だけでは不可能であり、腫瘍内の３次元的空間を人為的に変化させる必要があることに気づいた。研究代表者は当該研究期間において、外来化学療法で用いられている経口フッ化ピリミジン系抗がん剤(S-1)の繰り返し投与と siRNA・ナノキャリア複合体を併用することで siRNA・ナノキャリア複合体の腫瘍内移行性および拡散性が亢進され、このような腫瘍内移行性変化に付随して顕著な抗腫瘍効果が得られることを確認した。本検討で使用した S-1 は既に臨床応用されており、このような薬剤と組み合わせるだけで siRNA の効果が向上しうる本戦略は、siRNA を用いたがん治療の試みを実現しうる優れた戦略である。

A. 研究目的

siRNAを薬剤として利用する場合、その生体内動態を量的・空間的に制御することが重要である。表在性の腫瘍を除き、siRNAの静脈内投与は不可避である。100nm程の粒子径を持つナノキャリアは腫瘍内新生血管内皮の隙間を通過して腫瘍に集積する。しかし、血管周囲に留まるだけで腫瘍深部まで到達できない。これを改善するには、キャリアの改変だけでは不可能であり、腫瘍内の３次元的空間を人為的に変化させる必要がある。研究代表者は既に、外来化学療法で汎用されている経口フッ化ピリミジン系抗がん剤(S-1)の繰り返し投与と併用することでsiRNA・キャリア複合体の腫瘍内移行性および拡散性の亢進と、それに基づく顕著な抗腫瘍効果が得られることを平成21-22年度の研究で確認している。最終年度にあたる本年度は、臨床応用可能な抗がん剤とsiRNAの組み合わせとして、5-FUのprodrugであるtegafulを含むS-1と5-FUの標的でもあるthymidylate synthase (TS)に対するsiRNAをデザインし、本検討課題によって確立したsiRNAデリバリー技術の有用性に関して更に検討を行った。

B. 研究方法

(1) 担がんモデル動物の作成と siRNA 搭載リポソームの調製

I. 担がんマウスの作成

BALB/c *nu/nu* 雄性マウスの背部皮下に DLD-1 あるいは 5-FU に耐性を持つ DLD-1/FU 細胞懸濁液 (2×10^6 cells/100 μL) を接種した。5 日後、腫瘍体積が約 100 mm^3 に達したマウスを実験に用いた。

II. siRNA 搭載リポソームの調製

PEG 修飾 cationic liposome は DOPE:POPC:CHOL:DC-6-14=3:2:3:2 (モル比) の脂質組成で、II と同様に調製した。得られたリポソームの粒子径は約 110 nm であった。Liposome への PEG 修飾は、ポストインサーション法で行った。調製した PEG 修飾 CL の粒子径及びゼータ電位は、112.3 ± 8.9 nm 及び 20.1 ± 1.1 mV であった。

PEG 修飾 lipoplex は、PEG 修飾 CL と siRNA とを N/P 比が 3.81 となるように混合し、10 分間激しく攪拌することで調製した。調製した PEG 修飾 lipoplex の粒子径及びゼータ電位は、450.3 ± 141.2 nm 及び 18.8 ± 2.1 mV であった。

(2) TS-siRNA による RNAi 効果の確認(in vitro)

ウェスタンブロッティング：DLD-1 担がんマウスから摘出した腫瘍を冷 PBS (-) で洗浄した後、液体窒素に入れて凍結させた。その後、粉砕し、重量の 5-10 倍 (20 mg の腫瘍ならば、100-200 μL) の

冷 Lysis buffer を加え腫瘍を完全に溶解させた。サンプルを 20 μL (40 μg protein/レーン) となるように SDS-PAGE した。ニトロセルロースに転写後、一次抗体として mouse monoclonal anti-human TS antibody を用いて TS を検出した。また、目的タンパク質のバンドを ECL Plus Chemiluminescence Reagent を用いて LAS-4000 EPUVmini (FUJIFILM) により検出した。

(3) 抗腫瘍効果の検討

I. PEG 修飾 siRNA 搭載リポソーム(TS 標的 siRNA 含有) と S-1 の併用療法による抗腫瘍効果の検討

腫瘍体積が約 100 mm^3 以上に達した DLD-1 担がんマウスに対し、1) PEG 修飾 siRNA 搭載リポソーム(siTS もしくはコントロールとして siGFP 含有) を siRNA 量で 80 μg/200μL ずつ 1 日間隔で合計 8 回マウス尾静脈より投与し、同時に 2) S-1 を tegafur 量で 6.9 mg/kg ずつ毎日経口より投与を行い、腫瘍体積変化と体重変化について検討を行った。さらに、PEG 修飾 siRNA 搭載リポソームの最終投与 2 日後に、DLD-1 担がんマウスから腫瘍を摘出し、重量を測定した。

II. PEG 修飾 siRNA 搭載リポソーム(TS 標的 siRNA 含有) と S-1 の併用療法によるアポトーシス誘導

アポトーシスの検出：担がんマウスから摘出した腫瘍を 4% PFA 溶液で固定後、30% スクロース溶液中で置換した。その後、腫瘍を Tissue-Tek O.C.T. Compound に包埋・凍結させ、Cryostat (Leica Microsystems、Solms、Germany) を用いて厚さ 5 μm の腫瘍切片を得た。この腫瘍切片に対して TUNEL 染色を行った。その後、Hoechest33342 溶液 (5 μg/mL) を添加して室温で 10 分間インキュベートすることで核染色を行った。その後、蛍光顕微鏡 (Axiovert 200M) を用いてアポトーシス細胞を観察した。また、Analyze Software (AxioVision、Zeiss) を用いてアポトーシス細胞の割合を定量評価した。

・倫理面への配慮

当該研究に関して、全ての動物実験プロトコールは所属機関における動物実験委員会による審査・承認を受けている。また、動物愛護の精神に乗っ取り、実験により派生する恐怖・苦痛をできるかぎり軽減できる方法を選択し、用いた。

C. 研究結果

(1) 抗腫瘍効果の検討

I. DLD-1 腫瘍 (5-FU 感受性) 移植モデルでの検討

PEG修飾TS-siRNA搭載リポソーム(全身投与)とS-1 (metronomic投与) とを組み合わせることによる抗腫瘍効果について検討を行った。

治療スケジュールとしては、PEG修飾TS-siRNA搭載リポソームを1日おきに計8回、siRNA量で80 μg/200 μLずつ尾静脈より投与し、一方S-1はtegafur量で6.9 mg/kgとなるように毎日経口投与した。その結果、スクロース投与群(Control)に対してPEG修飾TS-siRNA搭載リポソームとS-1単独投与でもそれぞれ高い腫瘍成長抑制効果を示したが、PEG修飾TS-siRNA搭載リポソームとS-1とを併用することによって極めて高い相乗的な腫瘍成長抑制効果が得られた。

さらに、治療後の腫瘍を用いて腫瘍内アポトーシス細胞の比率を評価したところ、PEG 修飾 TS-siRNA 搭載リポソーム単独投与群、S-1 単独投与群よりも併用投与群で顕著に高いアポトーシス誘導が確認された。

ついで、併用処置後の腫瘍内における新生血管とアポトーシス部位の評価を行った。結果として、PEG修飾siRNA-lipoplex単独処置とS-1単独処置、そしてそれらを併用処置したものとではアポトーシスが起こる部位に違いがあることが明らかとなった。PEG 修飾 TS-siRNA-lipoplex 単独処置により誘導されたアポトーシス部位は、腫瘍新生血管とほぼ同じ位置に観察された。S-1 単独処置により誘導されたアポトーシス部位は、新生血管とほとんど一致せず、血管外でアポトーシスを起こしていることが明らかとなった。一方で併用群では、血管、そして血管外の両方においてアポトーシスが顕著に誘導されていることが分かった。

また、治療後の TS 遺伝子ノックダウンに関して、腫瘍内の TS タンパク発現状況をウェスタンブロッティングによって評価したところ、PEG 修飾 TS-siRNA 搭載リポソーム単独投与群では顕著な発現の抑制は観察されなかったが、併用投与群においては顕著な発現抑制効果を観察することができた。

II. DLD-1/FU 腫瘍 (5-FU 耐性－TS 高発現腫瘍) 移植モデルでの検討

5-FU耐性腫瘍においても、PEG修飾TS-siRNA搭載リポソーム(全身投与) とS-1 (metronomic投与) とを組み合わせることで、高い抗腫瘍効果が得られるか検討を行った。

治療スケジュールとしては、I.の検討と同様に、PEG修飾TS-siRNA搭載リポソームを1日おきに計8回、siRNA量で80 μg/200 μLずつ尾静脈より投与し、一方S-1はtegafur量で6.9 mg/kgとなるように毎日経口投与した。

その結果、極めて残念であるが、5-FU耐性のDLD-1/FU移植マウスモデルにおいては、I.と同様の高い抗腫瘍効果を獲得することはできなかった。

D. 考察

これまでの siRNA の DDS 研究は、siRNA を運ぶナノキャリア (送り手) に焦点を当てた研究が主流であった。しかし、ナノキャリアは血行性に腫瘍組織へ到達するため、腫瘍内への移行はおのずと腫瘍内血管ネットワークに依存する。ところが、腫瘍内の無秩序な血管パターンや高い間質圧が原因となり、腫瘍深部の細胞まで siRNA を送達させることは現状の DDS では困難である。このことを改善するにはキャリアを改変するだけでは不十分であり、腫瘍内 (受け手) の三次元的空間を人為的に変化させる必要があると思われる。従って、siRNA の効率的な DDS の開発研究を進める上で、キャリアの改変のみならず、腫瘍内の微小環境を改変させて siRNA の腫瘍移行性・腫瘍内拡散性をもたせることは、革新的な siRNA デリバリー技術の開発につながるものと考えられる。

研究代表者が見出した S-1 の metronomic 投与による EPR 効果の増強メカニズムに関しては、以前から以下のように考察してきた。即ち、S-1 の metronomic 投与によって、A) 腫瘍新生血管の内皮細胞が傷害され、内皮細胞間の間隙が広がり、より漏出性の高い血管が形成されたこと、B) 腫瘍内血管系が正常化し、腫瘍内の広範囲に血液が分布するようになったこと、さらに C) 血管近傍のがん細胞が傷害され、血管外スペースが増加したこと、など腫瘍内の微小環境が改変されたことを想定してきた。本年度の成果として、5-FU に耐性を示す DLD-1/FU 細胞を用いた際、併用効果による高い抗腫瘍効果が得られなかった。この事から、S-1 の metronomic 投与による EPR 効果の増強メカニズムは、主として C)の血管近傍のがん細胞が傷害され、血管外スペースが増加したこと、に起因する可能性が極めて高い事が分かった。

5-FU による細胞死の誘導による腫瘍細胞の減少は、先に述べたような腫瘍内圧の低下による細胞間隙内の自由水の拡散性の向上とこれに伴う siRNA 搭載ナノキャリアの拡散を導くだけでなく、腫瘍細胞の圧力から解放された血管の開放とこれに伴う血流の改善（ナノキャリア自体の腫瘍移行量が増加する）、さらには障害の反動としての活発な新生血管の造成（ナノキャリアの血管外への漏出が増加する）などを誘起することも考えられ、結局、前段落で示した A)-C)の全ての過程を連鎖的に誘起しているものと想定される。

昨今のがん化学療法は多剤併用療法が主流となっており、5-FU は多くのがん種における first line regimen の構成薬剤となっている。5-FU に対する直接的な耐性機構は明らかになっていないが、5-FU の標的分子である TS の発現亢進が、耐性化の一つの機構として言われている。5-FU による治療は 5-FU に感受性のある（TS 発現が相対的に低い）細胞に有効であり、治療サイクルに依存してこれらの細胞は死滅し、代わって 5-FU 治療で死なかった（TS の発現が比較的高い）細胞が多くを占めるようになるものと考えられる。この細胞の置き代わりが耐性化に他ならず、治療初期から shRNA によって TS の発現を抑制できれば、このような耐性化を阻止する事が可能になるものと期待され、臨床上の意義も高まるものと考えられる。さらに、今回、TS 強発現株を用いて S-1 と siRNA 搭載ナノキャリアの併用投与を行ったが、残念ながら高い抗腫瘍効果を得る事はできなかった。これは、S-1 が今回の投与条件では TS に障害を与える事が出来なかったからであると考えている。一方、臨床においては 5-FU に感受性を示さない症例であっても、必ずしも全ての細胞が TS 高発現になっているわけではなく、また TS 発現もまちまちであると考えるのが妥当である。したがって、今回の併用療法は、臨床において高い効果を示す可能性が高く、この事を実証するような実験系を構築し、今後検討していく予定である。

E. 結論

抗がん剤のような低分子化合物の場合、漏れ出た薬剤自体が腫瘍内を拡散して効果を発揮するのに対し、siRNA は siRNA 単体では細胞膜不透過性であるため、キャリアを介して細胞内に導入する必要がある。したがって、siRNA が

薬効を発現できる領域は必然的にナノキャリアの分布領域に依存するため、siRNA の薬効が及ぶ領域を拡大させるためには、腫瘍内においてナノキャリア自体の拡散性を向上させ、その分布領域を拡大させることが非常に重要となる。本研究課題を遂行した結果、経口型抗がん剤 S-1 の繰り返し投与が腫瘍内の微小環境を変化させ、siRNA 含有ナノキャリアの腫瘍内移行性を亢進させると共にその腫瘍内分布領域を拡大させ、結果として高い抗腫瘍効果を発揮させる事を世界で初めて確認することができた。

一方、TS の発現が亢進した腫瘍に関しては、S-1+TS-siRNA 搭載ナノキャリアは腫瘍増殖抑制効果を確認する事はできなかった。しかし、この事は S-1 が腫瘍血管から漏出し、腫瘍細胞に障害を与える事が引き金となり、siRNA 搭載ナノキャリアの腫瘍移行性および腫瘍内拡散性が改善される事を示唆しており、期せずして S-1 の腫瘍内微小環境変化誘導機構を明らかにすることもできた。

昨今のがん化学療法は多剤併用療法が主体であり、これら遊離型抗がん剤の投与は S-1 のように腫瘍内の微小環境をナノキャリアが移行しやすいものに変化させる可能性が高い。この性質を利用し、これらの抗がん剤の効果を亢進させうるような siRNA を送達すれば、より高い抗腫瘍効果を臨床で獲得することができるものと期待できる。

本研究で確立した siRNA デリバリーシステムは、抗がん剤による治療時に生ずる腫瘍内微小環境変化を利用しており、臨床応用へのバリアは極めて低く、siRNA を用いたがん治療の実現に大きく貢献する優れた成果であると確信している。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

(1)Barichello, J.M., Kizuki, S., Tagami, T., Luiz Alberto Lira Soares, L.A.L., Ishida, T., Kikuchi, H., Kiwada, H., Agitation during lipoplex formation harmonizes the interaction of siRNA to cationic liposomes. Int. J. Pharm., 430, 359-365 (2012)

(2)Tagami, T., Abu Lila, A.S., Matsunaga, M., Moriyoshi, N., Nakamura, H., Nakamura, K., Suzuki, T., Doi, Y., Ishida, T., Kiwada, H., Improved intratumoral delivery of PEG-coated siRNA-lipoplexes by combination with metronomic S-1 dosing in a murine solid tumor model. Drug Deliv. Transl. Res., 2, 77-86 (2012)

(3)Tagami, T., Suzuki, T., Matsunaga, M., Nakamura, K., Moriyoshi, N., Ishida, T., Kiwada, H., Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery. Int. J. Pharm., 422, 280-289 (2012)

(4)Nakamura, K, Abu Lila A., Matsunaga, M., Doi, Y., Ishida, T., Kiwada H., A double-modulation strategy in cancer treatment with a chemotherapeutic agent and siRNA. Mol. Ther., 19, 2040-2047 (2011)

(5)Tagami, T., Suzuki, T., Hirose, K., Barichello, J.M., Yamazaki, N., Asai, T., Oku, N., Ishida, T., Kiwada, H., Argonaute2 is a potential target for siRNA-based cancer therapy for HT1080 human fibrosarcoma. Drug Deliv. Transl. Res., 1, 277-288 (2011)

(6)Barichello, J.M., Kizuki, S., Tagami, T., Asai, T., Ishida, T., Kikuchi, K., Oku, N., Kiwada, H., Agitation during lipoplex formation improves the gene knockdown effect of siRNA. Int. J. Pharm., 410, 153-160 (2011)

(7)Tagami T., Uehara Y., Moriyoshi N., Ishida T., Kiwada H., Anti-PEG IgM production by siRNA encapsulated in a PEGylated lipid nanocarrier is dependent on the sequence of the siRNA. J. Control. Release, 151, 149-154 (2011)

2. 学会発表

(1) 石田 竜弘、際田 弘志、腫瘍内微小環境の能動的制御に基づく siRNA デリバリー技術の開発とがん治療への展開、日本薬学会第 132 年会（札幌）、2012 年 3 月 30 日

(2) 岡田 知子、土井 祐輔、Amr ABU LILA、石田 竜弘、際田 弘志、フッ化ピリミジン系経口抗癌剤 S-1 が PEG 修飾カチオニックリポソームの腫瘍移行性に与える影響、日本薬学会第 132 年会（札幌）、2012 年 3 月 30 日

(3) 中村 浩之、土井 祐輔、石田 竜弘、際田 弘志、S-1 とオキサリプラチン封入 PEG 修飾リポソームの併用療法におけるリポソームの体内動態の変化に関する検討、日本薬学会第

132 年会（札幌）、2012 年 3 月 30 日

(4) Moriyoshi, N., Matsunaga, M., Nakamura, K., Ishida, T., Kiwada, H., Development of new cancer treatment strategy that combines PEG-coated siRNA-lipoplex with metronomic S-1 dosing. International Liposome Society 2011 meeting, University of London, London, UK, Dec. 11 (2011)

(5) 石田竜弘、腫瘍内微小環境変化を利用した核酸デリバリー戦略、遺伝子・デリバリー研究会第 11 回夏期セミナー（高知）、2011 年 8 月 9 日

(6) 岡田 知子、土井 祐輔、Amr S. Abu Lila、石田 竜弘、際田 弘志、フッ化ピリミジン系経口抗癌剤が PEG 修飾カチオニックリポソームの腫瘍移行性に与える影響、第 20 回 DDS カンファランス（静岡）、2011 年 9 月 16 日

(7) 松本 春菜、土井 祐輔、中村 浩之、西尾 美穂、石田 竜弘、原田 雅史、際田 弘志、PEG 修飾リポソームの腫瘍内分布領域可視化の試み、第 20 回 DDS カンファランス（静岡）、2011 年 9 月 16 日

(8) 橋本 洋佑、上原 友美、石田 竜弘、際田 弘志、siRNA リポプレックス投与時の TLR 刺激が及ぼす anti-PEGIgM 分泌誘導への影響、アンチセンス・遺伝子・デリバリーシンポジウム 2011（大阪）、2011 年 9 月 1 日

(9) 森吉 直人、中村 和也、松永 真理子、石田 竜弘、際田 弘志、Tegaful 製剤 S-1 と siRNA 含有 liposome の併用による新規がん治療法の開発、アンチセンス・遺伝子・デリバリーシンポジウム 2011（大阪）、2011 年 9 月 1 日

(10) 橋本 洋佑、森吉 直人、上原 友美、田上 辰秋、石田 竜弘、際田 弘志、siRNA 含有 PEG 修飾ナノキャリアによる anti-PEG IgM 誘導におけるトール様受容体（TLR）の関与、第 27 回日本 DDS 学会学術集会、東京、2011 年 6 月 9 日

H. 知的財産権の出願・登録状況

なし

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Barichello, J.M., Kizuki, S., Tagami, T., Asai, T., Ishida, T., Kikuchi, K., Oku, N., Kiwada, H.,	Agitation during lipoplex formation improves the gene knockdown effect of siRNA.	*Int. J. Pharm.*	410	153-160	2011
Tagami, T., Suzuki, T., Hirose, K., Barichello, J.M., Yamazaki, N., Asai, T., Oku, N., Ishida, T., Kiwada, H.,	Argonaute2 is a potential target for siRNA-based cancer therapy for HT1080 human fibrosarcoma.	*Drug Deliv. Transl. Res.,*	1	277-288	2011
Nakamura, K, Abu Lila A., Matsunaga, M., Doi, Y., Ishida, T., Kiwada H.,	A double-modulation strategy in cancer treatment with a chemotherapeutic agent and siRNA.	*Mol. Ther.*	19	2040-2047	2011
Tagami, T., Suzuki, T., Matsunaga, M., Nakamura, K., Moriyoshi, N., Ishida, T., Kiwada, H.,	Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery.	*Int. J. Pharm.*	422	280-289	2012
Tagami, T., Abu Lila, A.S., Matsunaga, M., Moriyoshi, N., Nakamura, H., Nakamura, K., Suzuki, T., Doi, Y., Ishida, T., Kiwada, H.,	Improved intratumoral delivery of PEG-coated siRNA-lipoplexes by combination with metronomic S-1 dosing in a murine solid tumor model.	*Drug Deliv. Transl. Res.*	2	77-86	2012
Barichello, J.M., Kizuki, S., Tagami, T., Luiz Alberto Lira Soares, L.A.L., Ishida, T., Kikuchi, H., Kiwada, H.	Agitation during lipoplex formation harmonizes the interaction of siRNA to cationic liposomes.	*Int. J. Pharm.*	430	359-365	2012

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Anti-PEG IgM production by siRNA encapsulated in a PEGylated lipid nanocarrier is dependent on the sequence of the siRNA

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ABSTRACT

We recently reported that the prolonged circulation property of PEGylated cationic liposomes containing nucleic acids disappears, if the second dose is injected within a few days later, due to the production of anti-PEG IgM. This accelerated blood clearance is a concern for treating diseases which require repeated treatment with a PEGylated formulation containing nucleic acids. In this study, we investigated the effect of encapsulation of siRNA in a recently introduced PEGylated lipid nanocarrier for which the term "wrapsome" (PEGylated wrapsome, PEG-WS) was proposed as well as the sequence of the encapsulated siRNA on anti-PEG IgM production. siRNA encapsulated in PEG-WS produced little anti-PEG IgM relative to siRNA in conventional PEGylated lipoplexes. The sequence of siRNA in the PEG-WL dramatically affected the anti-PEG IgM production; a potent immune stimulatory siRNA induced a higher anti-PEG IgM production. Such enhanced effect was abrogated by incorporation of 2′-*O*-methyl (2′-OMe) uridine into the sequence of siRNA, probably via inhibiting cytokine induction such as IL-6 and TNF-α. Our results strongly indicate that the use of an encapsulation-type lipid nanocarrier with a low immuno-stimulatory siRNA may allow repeated dosing of siRNA containing PEGylated formulations without the induction of a strong immune reaction against PEG and thus may advance synthetic siRNA into a broad range of therapeutic applications.

1. Introduction

Small interfering RNA (siRNA) has a sequence-specific and potent gene silencing effect based on a RNA interference mechanism. In a growing number of studies the application of siRNAs as potential therapeutic agents for treating a variety of diseases, including cancers, genetic disorders and viral infections [1–4]. However, due to the instability of siRNA as well as their poor cellular uptake and pharmacokinetic profiles *in vivo*, the therapeutic application of siRNA is largely dependent on the development of a delivery vehicle which must be efficient, safe and allow repeated administration. Cationic liposomes have been studied as a promising non-viral carrier of nucleic acids including pDNA, antisense oligonucleotides and recently siRNA [5,6]. These cationic carriers strongly interact electrostatically with nucleic acids yielding a net positively charged particle. This cationic lipoplex is believed to be efficiently taken up by cells due to electrostatic interaction with the negatively charged membrane, resulting in *in vitro* transfection efficiencies. However, the cationic lipoplex makes aggregates easily with anionic serum proteins following systemic injection, resulting in accumulations in first-pass organ such as lungs and livers.

To circumvent such problem, strategies have been developed. These include the use of polyethylene glycol (PEG) to shield the positive charge on the lipoplex surface as well as the use of neutral lipids to deliver siRNA systemically. Cationic liposome-polycation-DNA (LPD) [6], stable nucleic acid lipid particles (SNALP) [7] and wrapsome (WS) [8], mainly coating of a core containing siRNA with a lipid layer including PEG-conjugated lipid, were introduced and achieve high encapsulation efficiency and long circulation of siRNA, resulting in *in vivo* therapeutic effects. These are modified by PEG-conjugated lipid (PEGylation) to achieve their prolonged *in vivo* circulation time. It is generally believed that liposome surface-grafted PEG can attract a water shell around the particle and results in reduced adsorption of opsonins and the recognition of the liposomes by the cells of the mononuclear phagocyte system [9,10]. The technology has already been utilized for anti-cancer drugs in clinical settings, exploiting the prolonged blood circulation time of PEGylated lipid nanocarriers of approximately 100 nm in diameter and their preferential accumulation in solid tumors due to the enhanced permeability and retention (EPR) effect [11]. However, we and others have reported that an intravenous injection of PEG-coated liposomes causes a second dose of PEG-coated liposomes, injected a few days later, to lose its long-circulating characteristics and to accumulate extensively in the liver. This effect is known as the "accelerated blood

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clearance (ABC) phenomenon" [12–14]. On the basis of our recent results [15,16], we proposed the following tentative mechanism for the cause of this phenomenon: anti-PEG IgM, which is produced in the spleen in response to a first dose, selectively binds to the PEG of the second dose liposomes injected several days later and subsequently activates the complement system, as a consequence, the liposomes are taken up by the Kupffer cells in the liver.

The ABC phenomenon involving anti-PEG IgM production may also represent a major concern in designing an efficient siRNA delivery system. In fact, we very recently reported that the intravenous injection of a PEG-coated siRNA/cationic liposome complex (PEGylated conventional lipoplex), prepared according to conventional methods, caused anti-PEG IgM production and thereby led to accelerated blood clearance of a second dose [17]. Many papers acknowledge that siRNA is a potent activator of the innate immune system and consequently produces inflammatory cytokines [18,19]. In addition, it is shown that the induction of innate immunity by siRNA is dependent on siRNA structure and sequence [20]. Accordingly, it is assumed that siRNA in the PEG-coated conventional lipoplex might affect anti-PEG IgM production via activation of innate immunity. Therefore, in this study, we attempted to confirm the effect of siRNA, either containing immune stimulatory sequences or not, in the lipoplex on anti-PEG IgM production. In addition, we investigated whether encapsulation of siRNA into the core of PEGylated WS (PEG-WS) attenuates anti-PEG IgM production. Our findings raise important concerns regarding the development of a potent siRNA delivery system allowing for safe and repeated administrations.

2. Materials and methods

2.1. Materials

2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000 (PEG_{2000}-DSPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were generously donated by NOF (Tokyo, Japan). A cationic lipid, *O,O'*-ditetradecanoyl-*N*-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical Co. Ltd. (Tokyo, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

2.2. Animals

Male Std-ddY mice aged 4–5 weeks (20–25 g) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under pathogen-free conditions. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

2.3. siRNAs

The unmodified siRNAs and the 2'-*O*-methyl uridine-modified (2'-OMe) siRNA were chemically synthesized by Nippon EGT (Toyama, Japan). The sequences of the siRNAs [17,20,25] were listed as follows: siRNA for GFP; sense sequence, 5'-GGCUACGUCCAGGAGCGCATT-3'; anti-sense sequence, 5'-UGCGCUCCUGGACGUAGCCTT-3': VEGF; sense sequence, 5'-CAUGGGACUUCUGCUCUCCTT-3'; anti-sense sequence, 5'-GGAGAGCAGAAGUCCCAUGTT-3': Argonaute2, which associates with small RNAs that guide mRNA degradation, translational repression, or a combination of both; sense sequence, 5'-GCACGGAAGUCCAUCUGAAUU-3'; anti-sense sequence, 5'-UUCAGAUGGACUUCCGUGCUU-3': firefly luciferase; sense sequence, 5'-CUUACGCUGAGUACUUCGATT-3'; anti-sense sequence, 5'-UCGAAGUACUCAGCGUAAGTT-3': inverted sequence of firefly luciferase (inverted luciferase); sense sequence, 5'-AGCUUCAUAAGGCGCAUGCTT-3'; anti-sense sequence, 5'-GCAUGCGCCUUAUGAAGCUTT-3': ApoB-1; sense sequence, 5'-GUCAUCACACUGAAUACCAAU-3'; anti-sense sequence, 5'-AUUGGUAUUCAGUGUGAUGACAC-3': β-galactosidase (β-gal); sense sequence; 5'-CUACACAAAUCAGCGAUUUUU-3'; anti-sense sequence, 5'-AAAUCGCUGAUUUGUGUAGUU-3'.

In this study, the sequences of Inverted luciferase, ApoB-1 and β-gal were 2'-OMe modified, and then, these all nucleotides except for overhung sequences were modified.

The complementary 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. Quality of duplex siRNA was checked by 15% PAGE. The final concentration of the duplexes was adjusted to 50 μM with TE buffer.

2.4. Preparation of cationic liposome and PEGylation on the prepared liposome

Cationic liposomes were prepared as described before [17]. Briefly, the lipids (DC-6-14/POPC = 50/50, molar ratio) were dissolved in chloroform. After evaporation of organic solvent, the resulting thin lipid film was hydrated in 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with consecutive pore sizes of 400, 200, 100 and 80 nm. The mean diameters and zeta potentials of the resulting liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The mean diameter and zeta potential of cationic liposomes were 80.2 ± 3.2 nm and 23.4 ± 3.5 mV (n = 3). For PEGylation of cationic liposomes, a post-insertion technique was used [21]. PEG_{2000}-DSPE (5 mol% of total lipid) in 9% sucrose solution was mixed with the cationic liposomes, vortexed for 15 s and gently shaken for 1 h at 37 °C. The mean diameter and zeta potential of PEGylated cationic liposomes were 84.3 ± 2.1 nm and 18.2 ± 1.9 mV (n = 3), respectively. The concentration of phospholipids was determined by colorimetric assay [22].

2.5. Preparation of core-lipoplex

Core-lipoplex was prepared as described previously with minor modification [17]. To formulate the core-lipoplex, siRNA solution (17 μg siRNA diluted in 170 μl of 9% sucrose solution) was mixed with an equal volume of PEGylated cationic liposomes (7.14 μmol) prepared as described above and incubated for 20 min at room temperature. The mean diameter and zeta potential of core-lipoplexes were 284.1 ± 9.2 nm and 11.5 ± 4.1 mV, respectively.

2.6. Preparation of lipid-wrapped core-lipoplex (PEG-WS)

PEG-WS containing siRNA were prepared as described previously with minor modification [7,23]. To wrap the core-lipoplex with a neutral lipid layer containing PEGylated lipid, the core-lipoplex solution (340 μl) prepared as described above was mixed with 566 μl of an ethanol solution containing POPC (34 μmol) and PEG_{2000}-DSPE (1.7 μmol). The lipid ratio of the core-lipoplex to outer lipid layer was set at 1:5 (w/w) and the prepared PEG-WS contained on average 17 μg of siRNA in 42.84 μmol total lipid. The solution was diluted with 19 ml distilled water with stirring. Following centrifugation (30,000 × *g*, 4 °C, 1 h) and subsequent removal of supernatant containing free lipid, the pellet (siRNA-containing PEG-WS) was suspended in a small amount of 9% sucrose solution to provide an appropriate preparation. The mean diameter and zeta potential of the prepared PEG-WS were 304.6 ± 7.3 nm and −12.4 ± 1.8 mV (n = 3), respectively.

2.7. Detection of anti-PEG IgM

At day 5 after injection of siRNA-containing PEG-WS, the peripheral blood was withdrawn from each treated mouse by heart puncture. To obtain serum, the blood was placed for 30 min at room temperature and then centrifuged at 3000 rpm at 4 °C for 15 min. The serum collected from naïve mice was used as control serum. A simple ELISA as described previously [17] was employed to detect anti-PEG IgM in the serum. Briefly, 10 nmol of PEG_{2000}-DSPE in 50 μl ethanol was added to each well of a 96-well plate. The plate was allowed to air dry completely for 2 h. The lipid-coated plates were then blocked for 1 h with Tris-buffered saline (pH 7.4) containing 1% BSA and were subsequently washed three times with phosphate buffered saline (PBS, pH 7.4). Diluted serum samples (1:100) (100 μl) were then applied in the well, incubated for 1 h and washed three times with PBS. Horseradish peroxidase (HRP)-conjugated antibody (100 μl, 1 μg/ml, Goat anti-mouse IgM IgG-HRP conjugate; Bethyl Laboratories, TX, USA) was added to the wells. After 1 h incubation, the wells were washed three times with PBS. The coloration was initiated by adding 100 μl of *o*-phenylenediamine (1 mg/ml) (Sigma, MO, USA). After 15 min incubation, the reaction was stopped by adding 100 μl of 1 M H_2SO_4. The absorbance was measured at 490 nm using a microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were performed at room temperature.

2.8. Measurement of inflammatory cytokine

At appropriated times after injection of siRNA-containing PEG-WS (10 μg siRNA/mouse), the peripheral blood was withdrawn from each treated mouse by heart puncture. The serum was obtained as described above. Interleukin 6 (IL-6) and interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF-α) in the serum was measured with Quantikine Immunoassay Kit (R&D Systems, MN, USA) according to the manufacturer's instructions.

2.9. 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 $p<0.05$.

3. Results

3.1. Effect of encapsulation of siRNA on anti-PEG IgM production

Anti-PEG IgM production was assessed on day 5 after a single injection, by which time the ABC phenomenon is markedly manifest [17]. We confirmed that a low-dose single injection of siRNA-containing PEGylated lipoplex, regardless of the complex type (PEGylated lipoplex (core-lipoplex)) and the encapsulation type (PEG-WS), causes a significant induction of anti-PEG IgM production (Fig. 1). Consistent with our earlier observations [17,24], the level of specific IgM induction was reversely related to the dose of both siRNA-containing lipoplexes. But, the responses against encapsulation type (WS) was completely diminished at higher dose as previously observed in the empty PEGylated cationic liposome [17,24] causing immunological tolerance, while the responses against the complex type (PEGylated lipoplex) was not declined upon increasing the dose. It appears that encapsulation of siRNA in the core of lipoplex is sufficient to attenuate activation of innate immunity by siRNA which induces the anti-PEG IgM production.

3.2. Effect of siRNA-sequence on anti-PEG IgM production

We then investigated whether the sequence of siRNA in the PEG-WS affects the anti-PEG IgM production. Two siRNAs were compared:

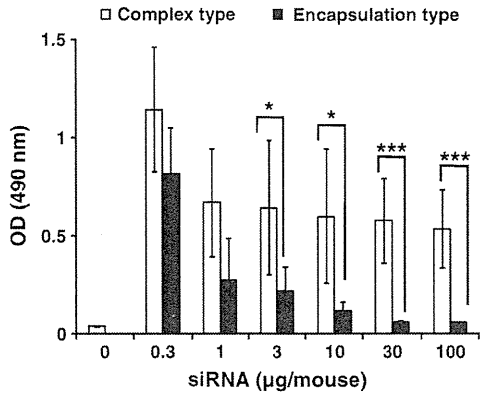

Fig. 1. Effect of encapsulation of siRNA in PEGylated lipoplex on anti-PEG IgM production. PEGylated lipoplex (core-lipoplex (complex type)) or WS (encapsulation type) containing GFP-siRNA was intravenously injected into mice. Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$ and *** $p<0.005$.

siRNA against β-galactosidase (β-gal) which has an immune stimulatory effect and produces many inflammatory cytokines [20] and siRNA against GFP which does not show a strong immune stimulatory effect in Fig. 1 and our earlier study [17].

As is shown in Fig. 2, at a low dose of 0.3 μg siRNA per mouse, GFP-siRNA-containing PEG-WS and β-gal-siRNA-containing PEG-WL produced nearly identical amounts of anti-PEG IgM. However, at doses higher than 1 μg siRNA, anti-PEG IgM production by the β-gal-siRNA-containing PEG-WL exceeded that produced by the GFP counterpart by a factor of 3 to 5. To extend the study, we assayed anti-PEG IgM levels elicited by PEG-WSs containing a range of different siRNAs. As demonstrated in Fig. 3, the potential of PEG-WS-encapsulated siRNAs to elicit anti-PEG IgM varies largely with the RNA sequence. Therefore, it is to be expected that the severity of the ABC phenomenon upon repeated administration of this type of siRNA formulation will also strongly depend on the sequence of the used siRNA. Furthermore, it is worthy here to note that an immune stimulatory siRNA such as β-gal siRNA can strongly stimulate the immune system to produce anti-PEG IgM despite the condition that the siRNA was encapsulated in the delivery vehicle.

3.3. Effect of 2′-OMe siRNA on anti-PEG IgM production

There is reason to believe that the response of innate immunity to synthesized siRNA is Toll-like receptor (TLR)-mediated [19]. Recently, some reports demonstrated that chemical modifications of the 2′-OH

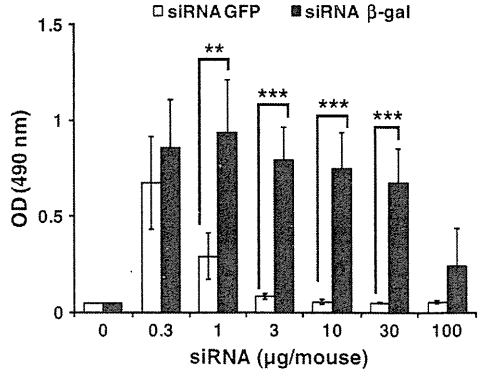

Fig. 2. Effect of immune stimulatory siRNA in PEG-WS on anti-PEG IgM production. WS containing beta-gal siRNA or GFP-siRNA was intravenously injected into mice. Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). ** $p<0.01$ and *** $p<0.005$.

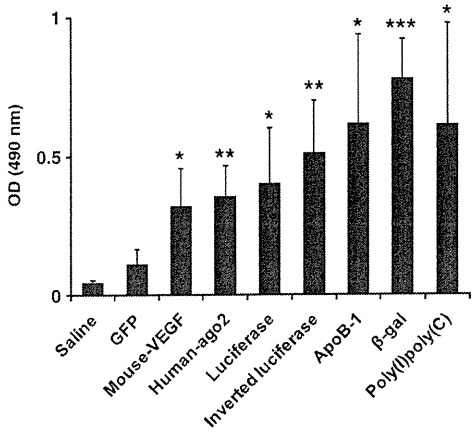

Fig. 3. Effect of sequence of siRNA in the PEG-WS on anti-PEG IgM production. WS containing various sequences of siRNA was intravenously injected into mice (10 μg siRNA/mouse). Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$, ** $p<0.01$, and *** $p<0.005$.

group in the ribose sugar backbone such as 2′-*O*-methylytion (2′-OMe) prevent recognition of the siRNA by the innate immune system without loss of RNAi activity [25,26]. Robbins et al. [27] clearly demonstrated that 2′-OMe siRNA acts as TLR7 antagonist. Therefore, we obtained 2′-OMe siRNAs against inverted luciferase, ApoB-1 and β-gal and investigated the effect of this modification of siRNA on the anti-PEG IgM production induced by siRNA-containing PEG-WS. The immune stimulatory β-gal siRNA [20] in the WS strongly induced anti-PEG IgM production, while the chemically modified β-gal siRNA, notably 2′-OMe β-gal siRNA, in the PEG-WS did not induce IgM production (Fig. 4). Similar tendency was observed with inverted luciferase siRNA and ApoB-1 siRNA. The extent of immune activation of 2′-OMe β-gal siRNA was similar to that of less immune stimulatory GFP-siRNA (Fig. 3).

To check the strength of the immune stimulatory effect of siRNA on anti-PEG IgM production, the inflammatory cytokines, IL-6, INF-γ and TNF-α, were determined. PEG-WL containing β-gal siRNA induced IL-6 at 4 h after injection, while no such induction was observed for other siRNA formulations including 2′-OMe β-gal siRNA (Fig. 5A). Similar IL-6 induction was observed for Apo B-1 siRNA, while no such induction was observed for 2′-OMe Apo B-1 siRNA (Fig. 5B). 2′-OMe

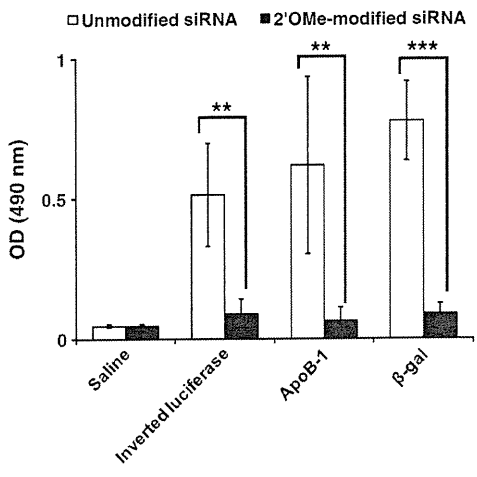

Fig. 4. Effect of 2′-*O*-methyl uridine-modification to siRNA on the anti-PEG IgM production. siRNA containing PEG-WS was intravenously injected into mice (10 μg siRNA/mouse). Five days later, anti-PEG IgM in the serum was determined. Each value represents the mean ± SD (n = 4). * $p<0.05$ and *** $p<0.005$.

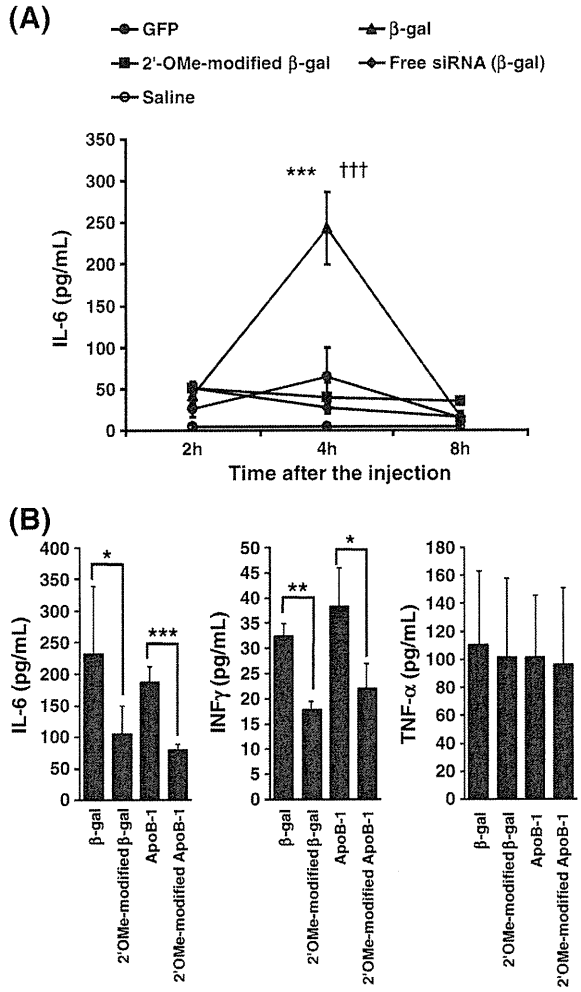

Fig. 5. Detection of inflammatory cytokine (IL-6, INF-γ, TNF-α) induced by siRNA-containing PEG-WS. (A) Time course change of IL-6 secretion. Each siRNA formulation was intravenously injected into mice (10 μg siRNA/mouse), and then, at 2, 4 or 8 h after injection, blood was withdrawn and IL-6 in the serum was determined with ELISA. Each value represents the mean ± SD (n = 4). *** $p<0.005$, β-gal siRNA versus GFP siRNA; ††† $p<0.005$, β-gal siRNA versus 2′-OMe β-gal siRNA. (B) The secretion of different types of inflammatory cytokines. At 4 h after injection of each siRNA formulation (10 μg siRNA/mouse), blood was withdrawn and inflammatory cytokines (IL-6, INF-γ, TNF-α) in the serum was determined with ELISA. Each value represents the mean ± SD (n = 4). * $p<0.05$, ** $p<0.01$, and *** $p<0.005$.

modification of siRNA reduced INF-γ production induced by PEG-WL containing β-gal siRNA and Apo B-1 siRNA. Interestingly, similar TNF-α inductions were observed in PEG-WS containing siRNA and 2′-OMe siRNA (Fig. 5B), suggesting that TNF-α may not contribute to anti-PEG IgM response against PEG-WS. These observations clearly indicate that chemical modification of siRNA to avoid potent activation of innate immunity abrogates the inductive effect of siRNA on anti-PEG IgM response against PEG-WS.

4. Discussion

A major key to successful siRNA-based therapy is the development of an efficient siRNA delivery system. For this purpose, several nanocarrier systems have been explored extensively. PEGylation is generally preferred to achieve increased stability and prolonged residence time of a nanocarrier system in the circulation. In an earlier report we showed that intravenous injection of siRNA-containing PEG-coated lipoplex gives rise to anti-PEG IgM production [24] that is

responsible for rapid clearance of a second dose of liposomes, injected a few days later [13]. This can become a major concern in designing a siRNA delivery system for *in vivo* use. In this study, we investigated the effect of siRNA encapsulation into the core of PEG-WS as well as the effect of siRNA sequence and incorporation of 2′-OMe uridine into the sequence of siRNA on the anti-PEG IgM response. Encapsulation of siRNA dramatically attenuated the production level of anti-PEG IgM (Fig. 1). The use of siRNA containing an immune stimulatory sequence (e.g. siRNA against β-gal [20]) strongly promoted anti-PEG IgM production (Fig. 2). Thus, such siRNA somewhat overpowers the inhibitory effect of lipid encapsulation of siRNA on recognition by TLRs. In addition, the incorporation of 2′-OMe uridine abrogated such adjuvant effect on the anti-PEG IgM production (Fig. 4). These results suggest that the design of a better siRNA delivery system allowing for safe and repeated administration can be achieved by combining less immune stimulatory siRNA sequences, adequate chemical modification and masking the siRNA by lipid encapsulation into the core of PEGylated liposomes.

Nucleic acids are essentially potent activators that can promote an innate immune response, inducing high levels of inflammatory cytokines such as TNF-α, IL-6 and interferons (IFNs) [18,20,28,29]. Activation of the innate immune system via synthesized siRNA is assumed to represent a significant undesirable side effect *in vivo*, due to the toxicities associated with excessive cytokine release and associated inflammatory syndromes. In addition, we here indicated that immune stimulatory siRNA in the PEG-WS functions as an adjuvant which promotes anti-PEG IgM production (Fig. 2). Thus, an understanding of how to minimize such adjuvant effect of siRNA will be helpful to researchers as they attempt to develop a safe and efficient siRNA delivery system with PEGylated lipid nanocarriers for repeated administration. As described above, in addition to choosing less immune stimulatory siRNA sequences, the lipid encapsulation of siRNA was sufficient to attenuate the adjuvant effect of siRNA on the production of anti-PEG IgM at higher dose (Fig. 1). Judge et al. [20] proposed that the recognition of siRNA in their system (encapsulation type SNALP) occurs within the endosomal pathway in a manner similar to that of other immune stimulatory nucleic acids, including ssRNAs known to activate plasmacytoid dendritic cells [30,31]. The endosome is the very cellular compartment where the siRNA-sensing pattern recognition receptors such as Toll-like receptors (TLR) exist [19]. Sioud et al. [32] also emphasized the importance of endosomal localization of siRNA for the innate immunity activation. In the acidic environment of the endosome, double strand siRNA is separated into single strand RNA. Single and double strand RNAs likely activate different TLRs, and thereby induce various immune responses. Our complex type-lipoplex (conventional lipoplex) displayed large amount of siRNA on the outer surface. By contrast, our PEG-WS (encapsulation type) entrapped siRNA within its inner core and, as a result, siRNA is fully masked. Although the exact mechanism by which the WS resulted in less engagement of immune response than the conventional lipoplex is still uncertain. The WS might prevent encapsulated siRNA from interaction with TLRs in the endosome. Lipid encapsulation (not association) of siRNA is frequently used to provide protection from intravascular nuclease degradation and passive targeting to disease sites and can enhance *in vivo* delivery of siRNA [6–8,33–35]. Our current study presents an additional advantage of a lipid encapsulation type delivery strategy for siRNA.

It is well known that nucleic acids can activate mammalian innate immunity and that the strength of the response is sequence dependent. For instance, CpG motifs in the pDNA sequence activate the TLR 9 signaling pathway in immune competent cells and induce the production of a variety of inflammatory cytokines and interferons [28]. We also recently reported that pDNA containing CpG motif strongly facilitates an anti-PEG IgM response upon intravenous injection of PEGylated pDNA-lipoplexes [24]. Heil et al. [30] showed that in siRNA a GU-rich sequence activates innate immunity via TLR7 and TLR8. Judge et al., [20] indicated that the GU contents in the siRNA motif are important for its immune stimulatory effect. By contrast, Hornung et al., [18] indicated that GU contents are not necessarily required for activation of innate immunity. Taken together, the exact immune stimulatory sequences in synthesized siRNA are unclear at present [19]. In fact, the adjuvant effect of siRNAs on the anti-PEG IgM production was random and not necessarily related to a specific siRNA sequence such as GU rich motif (Fig. 2). The siRNA sequence (β-gal 728) against β-gal [20] we employed in this study has been shown to be a potent inducer of inflammatory cytokines and this siRNA is more potent at inducing anti-PEG IgM production than other siRNAs (Fig. 3). Therefore, further work will be required to confirm the precise mechanism of siRNA-mediated immune activation before development of safe and efficient siRNA delivery system can be fully achieved.

Chemical modification of synthesized siRNA has been extensively applied to increase stabilization of siRNA against rapid nuclease degradation. These modifications include modifications of the 2′-OH group in the ribose sugar backbone, such as 2′-OMe and 2′-fluoro (2′-F) substitutions, that are readily introduced as modified nucleotides during siRNA synthesis. A number of reports have shown that siRNA containing 2′-OMe [36], 2′-F [36], 2′-deoxy [37], or locked nucleic acid [38] modifications can retain functional RNAi activity, indicating that these chemical conversions can be compatible with the RNAi machinery. Judge et al. [25] showed that incorporation of 2′-OMe uridine into one strand of the siRNA duplex completely abrogated the immune stimulatory property of siRNA without disrupting its gene silencing effect. In this study, we showed that the promoted anti-PEG IgM production by β-gal siRNA was significantly reduced by using 2′-OMe β-gal siRNA (Fig. 4), suggesting that selective modification of siRNA attenuates its adjuvant effect on anti-PEG IgM production and thus would moderate the induction of the ABC phenomenon. This brings along a novel advantage in the strategy of chemical modification of siRNA with 2′-OMe uridine.

It has been reported that inflammatory cytokines and chemokines are generally correlated with the promotion of IgM production [39,40]. Especially, IL-6 and INF-γ are closely related with IgM production in B cells [41]. We showed that 2′-OMe β-gal siRNA abrogated the IL-6 and TNF-γ secretion compared to unmodified immune stimulatory β-gal siRNA [20] (Fig. 5). Robbins et al. [27] defined 2′-OMe siRNA as an antagonist of TLR 7. This led to the assumption that the adjuvant effect of β-gal siRNA (β-gal 728) on anti-PEG IgM production is closely related with the TLR 7 signaling pathway associated with induction of IL-6 and TNF-γ secretion. In the case of pDNA, we recently reported that PEG-coated pDNA-lipoplexes also enhance anti-PEG IgM production compared with PEG-coated cationic liposomes without pDNA. In the same experiment we showed that the removal of the CpG motif from pDNA, which is the ligand of TLR9, significantly reduces anti-PEG IgM production [24]. In addition, our preliminary study indicates that PEGylated liposomes containing lipopolysaccharide (LPS), which is the ligand of TLR 4, enhances anti-PEG IgM production in the same manner as siRNA and pDNA (personal communication of T.T.). These results indicate that the adjuvant effect of nucleic acids on anti-PEG IgM production is mediated via TLRs-related signaling pathways. However, since TLRs are present in various immune competent cells such as macrophages, dendritic cells and B cells, the entire event relating to the adjuvant effect of immune stimulatory nucleic acids can be expected to be very complex. Further extensive research will be required to open this black box.

5. Conclusion

We showed that the encapsulation of siRNA in PEGylated lipoplexes (PEG-WS) and the use of less immune stimulatory siRNA as a payload can achieve the abrogation of anti-PEG IgM response against the delivery vehicle, presumably due to preventing siRNA-mediated

activation of the innate immune system. Our results may have important implications for the development of a safe and efficient *in vivo* siRNA delivery system that mediates RNAi but with minimal induction of immune activation.

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References

[1] S. Akhtar, I.F. Benter, Nonviral delivery of synthetic siRNAs in vivo, J. Clin. Invest. 117 (2007) 3623–3632.
[2] M.A. Behlke, Progress towards in vivo use of siRNAs, Mol. Ther. 13 (2006) 644–670.
[3] P. Kumar, H. Wu, J.L. McBride, K.E. Jung, M.H. Kim, B.L. Davidson, S.K. Lee, P. Shankar, N. Manjunath, Transvascular delivery of small interfering RNA to the central nervous system, Nature 448 (2007) 39–43.
[4] Y. Sato, K. Murase, J. Kato, M. Kobune, T. Sato, Y. Kawano, R. Takimoto, K. Takada, K. Miyanishi, T. Matsunaga, T. Takayama, Y. Niitsu, Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone, Nat. Biotechnol. 26 (2008) 431–442.
[5] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl Acad. Sci. USA 84 (1987) 7413–7417.
[6] Y.C. Tseng, S. Mozumdar, L. Huang, Lipid-based systemic delivery of siRNA, Adv. Drug Deliv. Rev. 61 (2009) 721–731.
[7] A.D. Judge, M. Robbins, I. Tavakoli, J. Levi, L. Hu, A. Fronda, E. Ambegia, K. McClintock, I. MacLachlan, Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice, J. Clin. Invest. 119 (2009) 661–673.
[8] N. Yagi, I. Manabe, T. Tottori, A. Ishihara, F. Ogata, J.H. Kim, S. Nishimura, K. Fujiu, Y. Oishi, K. Itaka, Y. Kato, M. Yamauchi, R. Nagai, A nanoparticle system specifically designed to deliver short interfering RNA inhibits tumor growth in vivo, Cancer Res. 69 (2009) 6531–6538.
[9] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl Acad. Sci. USA 88 (1991) 11460–11464.
[10] W. Li, F.C. Szoka Jr., Lipid-based nanoparticles for nucleic acid delivery, Pharm. Res. 24 (2007) 438–449.
[11] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[12] E.T. Dams, P. Laverman, W.J. Oyen, G. Storm, G.L. Scherphof, J.W. van Der Meer, F.H. Corstens, O.C. Boerman, Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes, J. Pharmacol. Exp. Ther. 292 (2000) 1071–1079.
[13] T. Ishida, H. Kiwada, Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes, Int. J. Pharm. 354 (2008) 56–62.
[14] T. Ishida, R. Maeda, M. Ichihara, K. Irimura, H. Kiwada, Accelerated clearance of PEGylated liposomes in rats after repeated injections, J. Control. Release 88 (2003) 35–42.
[15] T. Ishida, M. Ichihara, X. Wang, H. Kiwada, Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes, J. Control. Release 115 (2006) 243–250.
[16] T. Ishida, M. Ichihara, X. Wang, K. Yamamoto, J. Kimura, E. Majima, H. Kiwada, Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes, J. Control. Release 112 (2006) 15–25.
[17] T. Tagami, K. Nakamura, T. Shimizu, T. Ishida, H. Kiwada, Effect of siRNA in PEG-coated siRNA-lipoplex on anti-PEG IgM production, J. Control. Release 137 (2009) 234–240.
[18] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7, Nat. Med. 11 (2005) 263–270.
[19] M. Robbins, A. Judge, I. MacLachlan, siRNA and innate immunity, Oligonucleotides 19 (2009) 89–102.
[20] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.
[21] T.M. Allen, P. Sapra, E. Moase, Use of the post-insertion method for the formation of ligand-coupled liposomes, Cell. Mol. Biol. Lett. 7 (2002) 889–894.
[22] G.R. Bartlett, Colorimetric assay methods for free and phosphorylated glyceric acids, J. Biol. Chem. 234 (1959) 469–471.
[23] M. Yamauchi, H. Kusano, E. Saito, T. Iwata, M. Nakakura, Y. Kato, T. Uochi, S. Akinaga, N. Aoki, Improved formulations of antisense oligodeoxynucleotides using wrapped liposomes, J. Control. Release 114 (2006) 268–275.
[24] T. Tagami, K. Nakamura, T. Shimizu, N. Yamazaki, T. Ishida, H. Kiwada, CpG motifs in pDNA-sequences increase anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes, J. Control. Release 142 (2010) 160–166.
[25] A.D. Judge, G. Bola, A.C. Lee, I. MacLachlan, Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo, Mol. Ther. 13 (2006) 494–505.
[26] L. Cekaite, G. Furset, E. Hovig, M. Sioud, Gene expression analysis in blood cells in response to unmodified and 2′-modified siRNAs reveals TLR-dependent and independent effects, J. Mol. Biol. 365 (2007) 90–108.
[27] M. Robbins, A. Judge, L. Liang, K. McClintock, E. Yaworski, I. MacLachlan, 2′-O-methyl-modified RNAs act as TLR7 antagonists, Mol. Ther. 15 (2007) 1663–1669.
[28] D.M. Klinman, A.K. Yi, S.L. Beaucage, J. Conover, A.M. Krieg, CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma, Proc. Natl Acad. Sci. USA 93 (1996) 2879–2883.
[29] H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA, Nature 408 (2000) 740–745.
[30] F. Heil, H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, S. Bauer, Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8, Science 303 (2004) 1526–1529.
[31] S.S. Diebold, T. Kaisho, H. Hemmi, S. Akira, C. Reis e Sousa, Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA, Science 303 (2004) 1529–1531.
[32] M. Sioud, Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization, J. Mol. Biol. 348 (2005) 1079–1090.
[33] Y.C. Tseng, L. Huang, Self-assembled lipid nanomedicines for siRNA tumor targeting, J. Biomed. Nanotechnol. 5 (2009) 351–363.
[34] S.C. Semple, A. Akinc, J. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srinivasulu, M.J. Weinstein, Q. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan, M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, Nat. Biotechnol. 28 (2010) 172–176.
[35] T.S. Zimmermann, A.C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornlocher, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, Nature 441 (2006) 111–114.
[36] T.P. Prakash, C.R. Allerson, P. Dande, T.A. Vickers, N. Sioufi, R. Jarres, B.F. Baker, E.E. Swayze, R.H. Griffey, B. Bhat, Positional effect of chemical modifications on short interference RNA activity in mammalian cells, J. Med. Chem. 48 (2005) 4247–4253.
[37] Y.L. Chiu, T.M. Rana, siRNA function in RNAi: a chemical modification analysis, Rna 9 (2003) 1034–1048.
[38] J. Elmen, H. Thonberg, K. Ljungberg, M. Frieden, M. Westergaard, Y. Xu, B. Wahren, Z. Liang, H. Orum, T. Koch, C. Wahlestedt, Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality, Nucleic Acids Res. 33 (2005) 439–447.
[39] M.C. Glaum, S. Narula, D. Song, Y. Zheng, A.L. Anderson, C.H. Pletcher, A.I. Levinson, Toll-like receptor 7-induced naive human B-cell differentiation and immunoglobulin production, J. Allergy Clin. Immunol. 123 (2009) 224–230, e224.
[40] J.A. Hanten, J.P. Vasilakos, C.L. Riter, L. Neys, K.E. Lipson, S.S. Alkan, W. Birmachu, Comparison of human B cell activation by TLR7 and TLR9 agonists, BMC Immunol. 9 (2008) 39.
[41] A.K. Yi, J.H. Chace, J.S. Cowdery, A.M. Krieg, IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides, J. Immunol. 156 (1996) 558–564.

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Agitation during lipoplex formation improves the gene knockdown effect of siRNA

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ABSTRACT

The successful delivery of therapeutic siRNA to the designated target cells and their availability at the intracellular site of action are crucial requirements for successful RNAi therapy. In the present study, we focused on the siRNA-lipoplex preparation procedure and its effect on the gene-knockdown efficiency of siRNA *in vitro*. Agitation (vortex-mixing) during siRNA-lipoplex (vor-LTsiR) preparation and its effect on the gene-knockdown efficiency of stably expressed cell GFP was investigated, and their efficiency was compared with that of spontaneously formed lipoplex (spo-LTsiR). A dramatic difference in size between lipoplexes was observed at the N/P ratio of 7.62 (siRNA dose of 30 nM), even though both lipoplexes were positively charged. With the siRNA dose of 30 nM, vor-LTsiR accomplished a 50% gene-knockdown, while spo-LTsiR managed a similar knockdown effect at the 120 nM level, suggesting that the preparation procedure remarkably affects the gene-knockdown efficacy of siRNA. The uptake of vor-LTsiR was mainly via clathrin-mediated endocytosis, whereas that of spo-LTsiR was via membrane fusion. In addition, by inhibiting clathrin-mediated endocytosis, the gene-knockdown efficiency was significantly lowered. The size of the lipoplex, promoted by the preparation procedure, is likely to define the entry pathway, resulting in an increased amount of siRNA internalized in cells and an enhanced gene-knockdown efficacy. The results of the present study definitively show that a proper siRNA-lipoplex preparation procedure makes a significant contribution to the efficiency of cellular uptake, and thereby, to the gene-knockdown efficiency of siRNA.

1. Introduction

RNA interference (RNAi), a naturally occurring biological process of gene regulation conserved in mammalian cells (Elbashir et al., 2001), has recently shown great potential as a novel therapeutic strategy (Li et al., 2006; Behlke, 2006). The target sites of the negatively charged small interfering RNA (siRNA) used in RNAi therapy are inside the cytoplasm. Therefore, it is essential that these molecules traverse the plasma membrane to reach their target sites (Li et al., 2006; Behlke, 2006). The plasma membrane of living cells is a dynamic structure that is relatively lipophilic and negatively charged in nature, and it restricts the entry of large, hydrophilic, or negatively charged, molecules (Leung and Whittaker, 2005). Thus, an appropriate delivery system is required to achieve an efficient cellular uptake and to release the siRNA inside the cell, cytoplasm (Soutschek et al., 2004; Leung and Whittaker, 2005).

Several delivery methodologies based on viral and nonviral vectors have been developed to circumvent this problem and have been successfully used for the introduction of siRNA into cells both *in vitro* and *in vivo* (Leung and Whittaker, 2005; Li et al., 2006; Behlke, 2006). Among these methodologies, cationic liposome has shown simplicity of use and ease of large-scale production, which makes it particularly promising and potentially useful for the delivery of siRNA (Behlke, 2006; Khalil et al., 2006a). The current cationic liposomes used for siRNA delivery have been adapted from those developed to deliver plasmid DNA (pDNA) and oligodeoxynucleotide to cells. Thus, conventional siRNA-lipoplex is formed through the spontaneous electrostatic interaction between the positively charged lipid in the liposome membrane and the negatively charged phosphate backbone of the siRNA (Lasic and Templeton, 1996). Despite a certain degree of success, spontaneous

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formation is a static method that allows little control over the interaction process, leading to a very heterogeneous lipoplex size distribution and to excessive size (Lasic and Templeton, 1996; Kawakami et al., 2002; Faneca et al., 2004). However, although some data demonstrating the gene-knockdown efficiency of siRNA-lipoplex has been accumulated, little is known about the effect of varying the condition during lipoplex formation and to what extent this affects the gene knockdown efficiency of siRNA.

Therefore, in the present study, we focused on the preparation procedure for siRNA-lipoplex. The effect of agitation (vortexing) during siRNA-lipoplex formation on the knockdown efficiency against green fluorescence protein (GFP) stably expressed in HT-1080 cells was investigated. Also, the size distribution of lipoplex, its attached amount of siRNA, and its uptake mechanism were investigated, along with the amount of siRNA internalized in cells. Herein, we show that the uptake and promoting pathway of siRNA-lipoplex, which is modulated by the preparation procedure, influences the gene-knockdown efficiency of siRNA *in vitro*.

2. Material and methods

2.1. Materials

The cationic liposome, LipoTrust™-SR (LT), composed of *O,O'*-ditetradecanoyl-*N*-(α-trimethyl ammonioacetyl) diethanolamine chloride (DC-6-14), dioleoylphosphatidylethanolamine (DOPE) and cholesterol in the molar ratio of 1.00/0.75/0.75 was purchased from Hokkaido System Science (Hokkaido, Japan). The Hoechst 33342 was purchased from Molecular Probes (OR, USA). Z-Phe-Phe-Gly (ZfFG) and filipin complex were purchased from Sigma–Aldrich (MO, USA). The 1% Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-containing LipoTrust™-SR (RhLT) was a generous gift from Daiichi-Sankyo Pharmaceutical (Tokyo, Japan). All other chemicals were reagent grade and used as received.

2.2. siRNA preparation

All RNA sequences were chemically synthesized and purified with HPLC by Hokkaido System Science. The siRNA for GFP (siGFP) composed of the sense strand 5′-GGCUACGUCCAGGAGCGCACC-3′ and the antisense strand 5′-UGCGCUCCUGGACGUAGCCUU-3′ (Song et al., 2003) and the siRNA against firefly luciferase (siLuc) composed of a sense strand 5′-AGCUUCAUAAGGCGCAUGCTT-3′ and an antisense strand 5′TTUCGAAGUAUUCCGCGUACG-3′ were used as the control siRNA (Elbashir et al., 2001). The siGFP labeled at the 5′-end of the sense strand with carboxyfluorescein (FAM) (siFAM) and labeled at the 5′ end of the antisense strand with alexa Fluor 488 (siALEXA) were used as fluorescent siRNA probes, respectively. For siRNA preparation, the complementary antisense and sense strands were mixed in TE buffer (1×10^{-2} mM Tris–HCl, 1×10^{-3} mM EDTA, pH 8.0, DNase and RNase free grade, Nippon Gene, Tokyo, Japan) in a 1:1 molar ratio followed by heating at 95 °C for 1 min. The reaction was then allowed to cool at room temperature. The quality of siRNA was checked by 15% PAGE. The final concentration of the duplexes was 50 μM in TE buffer.

2.3. Preparation of siRNA lipoplexes

Various aliquots (0.1875–6.000 μl) of the siRNA solution (50 μM) were diluted to a final volume of 100 μl with fresh Opti-MEM (Invitrogen, CA, USA). A 25 μl aliquot of LT suspension (2.4 mM) was also diluted to a final volume of 100 μl with fresh Opti-MEM. The diluted siRNA solutions were then mixed with the diluted LT suspension. The N/P ratios were set at 1.90, 3.81, 7.62, 15.24, or 30.45. The LT-siRNA lipoplexes (LTsiR) were allowed to form in two ways: the lipoplex was formed spontaneously (spo-LTsiR) by standing samples for 10 min, and the lipoplex was formed under application of a high vortex-mixing (2500 rpm) (vor-LTsiR) (Vortex-Genie 2, Scientific Industries, NY, USA) for 10 min.

2.4. Particle size and zeta potential of siRNA lipoplexes

The particle size and zeta-potential of siRNA lipoplexes formed at the N/P ratio of 7.62 (9.6 μM/30 nM of cationic lipid/siRNA, respectively) were determined on a Nicomp 380 Submicron Particle Sizer (Particle Sizing System, CA, USA). To determine the particle size, LTsiR lipoplexes (200 μl) were prepared in either Opti-MEM or 9% sucrose, as described above, and were diluted with 200 μl of the same medium (Opti-MEM or 9% sucrose). The mean particle size represents the average of three different preparations of the same lipoplex. For zeta-potential determination, lipoplexes were formed in 9% sucrose, and the volume was adjusted with the same medium to 2.2 ml. The zeta potential represents the average of three different preparations of the same lipoplex. Zeta-potential could not be determined in Opti-MEM due to the large amount of salts this medium contains.

2.5. Correlation between the relative size of the siRNA lipoplex and the amount of siRNA attached to the lipoplexes

To examine the correlation between the relative size of the siRNA lipoplex and the amount of siRNA attached to the lipoplex, LT-siFAM lipoplexes (200 μl) were prepared at an N/P ratio of 7.62 (9.6 μM/30 nM per well), as described above in Opti-MEM. Then, a flow cytometer Guava EasyCyte mini system (Guava Technology, CA, USA) equipped with an argon laser exciting at a wavelength of 488 nm was used to analyze 20,000 lipoplexes. Forward scatter and fluorescence emission was centered at 525 nm (Green fluorescence). The fluorescence was collected using a logarithmic scale. Data were analyzed using WinMDI 2.7 software (Scripps Institute, CA, USA).

2.6. Cells and cell culture

A human fibrosarcoma (HT-1080) cell line was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan). The stably expressed green fluorescence protein (GFP) in HT-1080 cells (HT-1080GFP) was established previously by Drs. T. Asai and N. Oku (Department of Medical Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, Japan) (Yamakawa et al., 2000). The cells were cultured in DMEM (Sigma, St. Louis, MO, USA) and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Bioserum, Tokyo, Japan), 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN Biomedical, OH, USA) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were maintained in exponential growth.

2.7. In vitro GFP gene knockdown

HT-1080GFP cells were seeded in 24-well plates at a density of 1.25×10^4 cells/well 24 h prior to siRNA lipofection. For lipofection, the amount of the lipoplex was fixed at 9.6 μM per well while the siRNA amounts were varied at 7.5, 15, 30, 60, and 120 nM per well. The growth medium was removed and replaced with 410 μl of Opti-MEM, 50 μl of FBS and 40 μl of LT-siGFP or LT-siLuc, followed by incubation for 24 h. Then, the medium was replaced with 500 μl of fresh DMEM containing 10% FBS and the cells again were incubated for another 24 h. To assess gene knockdown efficiency, HT-1080GFP cells were lysed in 2% Triton X-100 in PBS (100 μl/well) for 1 h on ice, following removal of the incubation media. Plates were then gently agitated in a shaker for 10 min

and 1 ml of PBS/well was added. The samples were transferred to microtubes and centrifuged at 10,000 rpm for 5 min at 4 °C. The fluorescence of the GFP in the clear lysates was measured using a standard fluorimetric method for GFP (excitation at 495 nm and emission at 510 nm) in a F-4500 Fluorescence Spectrophotometer (HITACHI, Tokyo, Japan).

2.8. Confocal microscopy

HT-1080 cells were seeded in a 35-mm glass-bottom dish (Iwaki Glass, Tokyo, Japan) at a density of 5×10^3 cells/dish and incubated for 24 h. For lipofection, the growth medium was replaced with 164 μl of Opti-MEM, 20 μl of FBS and 16 μl of RhLT-siFAM at the N/P ratio of 7.62 (LT/siFAM = 9.6 μM/30 nM) per well. Cells were then incubated for 4 h with RhLT-siFAM in OptiMEM. After lipofection, the cells were washed with 200 μl of PBS and then were incubated with Hoechst 33342 DMEM (10 μM) supplemented with 10% FBS for 20 min at room temperature. Confocal images were acquired using a Zeiss LSM5 inverted confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) without fixation. LSM510 (Version 3.2 SP2) software was used to process and analyze the images.

2.9. Interaction and internalization of siRNA lipoplexes with cells

To investigate the cellular association and internalization of both vor-LTsiR and spo-LTsiR in HT-1080 cells, RhLT-siGFP and LT-siFAM were prepared spontaneously or with high-vortex mixing at an N/P ratio of 7.62 (9.6 μM/30 nM, respectively). HT-1080 cells were seeded at a density of 5.0×10^4 cells/well in 6-well plates and were incubated for 24 h at 37 °C. After 4 h of lipofection with LTsiR, the lipoplexes were removed and the cells were washed twice with PBS. Then, the cells were trypsinized and collected in a 1.5 ml tube. After removal of the supernatant by centrifugation (1,000 rpm, 5 min, 4 °C), the cell pellet was resuspended in 0.5 mM EDTA-PBS. The total fluorescence intensity of siRNA lipoplexes (surface-bound and internalized ones) in a cell was directly analyzed in a flow cytometer Guava EasyCyte mini system. The fluorescence intensity-related to the internalized lipoplex was analyzed after quenching the extracellular fluorescence by incubating the cells in a 0.3% trypan blue PBS solution (Zuhorn et al., 2002).

2.10. The uptake mechanism of siRNA lipoplexes

To examine the uptake mechanism of vor-LTsiR and spo-LTsiR by cells, the effect of the following uptake inhibitors on the internalization of the lipoplexes was investigated: a hypertonic sucrose medium, which can inhibit clathrin-mediated endocytosis through dissociation of the clathrin lattice; amiloride, which can specifically inhibit macropinocytosis by inhibiting the Na^+/H^+ exchange required for macropinocytosis; filipin complex, which can specifically inhibit caveolar uptake through cholesterol depletion; and, the tri-peptide ZfFG, which can inhibit membrane fusion (Khalil et al., 2006a,b). Cells were incubated with Opti-MEM supplemented with 10% FBS in the absence or presence of the inhibitors for 30 min: sucrose (0.45 M), ZfFG (200 μM), amiloride (0.5 mM), or filipin complex (5 μg/ml). LT-siFAM lipoplex (LT/siFAM = 7.2 μM/22.5 nM) was then added, followed by incubation for 1 h. The cells then were trypsinized, centrifuged at 1,000 rpm for 5 min and 4 °C, and collected in 0.3% trypan blue PBS solution to quench the extracellular fluorescence (Zuhorn et al., 2002). Trypan blue-treated cells were then washed twice with PBS, and resuspended in 0.5 mM EDTA-PBS. Samples (5,000 cells) were analyzed in a flow cytometer Guava EasyCyte mini system.

2.11. Influence of the uptake pathway on the gene knockdown effect of siRNA

This experiment was designed to examine the influence of the uptake pathway on the gene knockdown effect of siRNA. In this experiment, an exogenous gene instead of an endogenous gene model was used in order to shortening the time for siRNA transfection, since some of the inhibitors we used are toxic for the cell, and could lead to cell death. HT-1080 cells were seeded in a 12-well plate at a density of 2.5×10^4 cells/well in DMEM supplemented with 10% FBS prior to the experiment, then were cultured for 24 h at 37 °C. One microgram of pDNA (pEGFP-N1 vector) was transfected for 1 h with 2 μl of LipofectAMINE 2000 (Lf 2000, Invitrogen, CA, USA), according to the manufacturer's instruction. The transfection medium was then replaced with DMEM supplemented with 10% FBS and incubated for 30 min at 37 °C. After removal of the medium, the cells were incubated with OptiMEM supplemented with 10% FBS in the absence or presence of the uptake inhibitors sucrose (0.45 M), amiloride (0.5 mM), and ZfFG (200 μM) for 30 min at 37 °C. After removal of the medium, the cells were transfected with LT-siGFP or LT-siLuc (LT/siRNA = 4.8 μM/15 nM) for 2 h. The lipofection medium was then removed and replaced with fresh DMEM containing 10% FBS, and cells were incubated for a further 42 h. The cells were trypsinized, transferred to a 1.5 ml tube and centrifuged at 100 × g for 5 min at 4 °C. The collected cells were resuspended in 0.5 mM EDTA-PBS. A flow cytometer Guava EasyCyte mini system was then used to analyze 5,000 cells in each sample.

2.12. Statistical analysis

Statistical analyses (Unpaired *t*-test with Welch correction) were performed using Graph Pad Stat View software (Abacus Concepts, Inc., CA). For the GFP gene silencing effect data, a non-parametric ANOVA (Kruskal–Wallis test) with post hoc Dunn's multiple comparisons was applied using the same software. The level of significance was set at $p < 0.05$.

3. Results

3.1. The effect of agitation (vortexing) on the gene knockdown efficiency of siRNA

A different method to achieve knockdown efficiency of the GFP gene in an HT-1080GFP cell was investigated during lipoplex formation. For spo-LTsiR, an effective gene knockdown was observed at the higher dose of 120 nM of siRNA (Fig. 1A). Interestingly, for vor-LTsiR, prepared by applying vortex-mixing during lipoplex formation, a significant gene knockdown effect was observed at a dose of only 30 nM of siRNA (Fig. 1B). Alteration of the viability of HT-1080GFP cells after lipoplex treatment was not observed for any siRNA concentration we tested (data not shown). These indicate that application of agitation during lipoplex formation effectively improves the gene knockdown efficiency of siRNA.

3.2. Particle size and zeta-potential of siRNA lipoplexes

The mean particle size and the zeta-potential of vor-LTsiR and spo-LTsiR are summarized in Table 1. The mean sizes for the lipoplexes vor-LTsiR and spo-LTsiR when prepared in 9% sucrose were 0.300 ± 0.045 and 0.270 ± 0.008 μm, respectively. The zeta-potential of lipoplexes prepared in the same medium was 22.4 ± 3.9 and 18.5 ± 0.4 for vor-LTsiR and spo-LTsiR, respectively. In both cases, no significant difference was observed between the preparation procedures. On the other hand, when lipoplexes were formed in OptiMEM, the transfection medium that contains counter ions, there was an abrupt increase in the population of larger

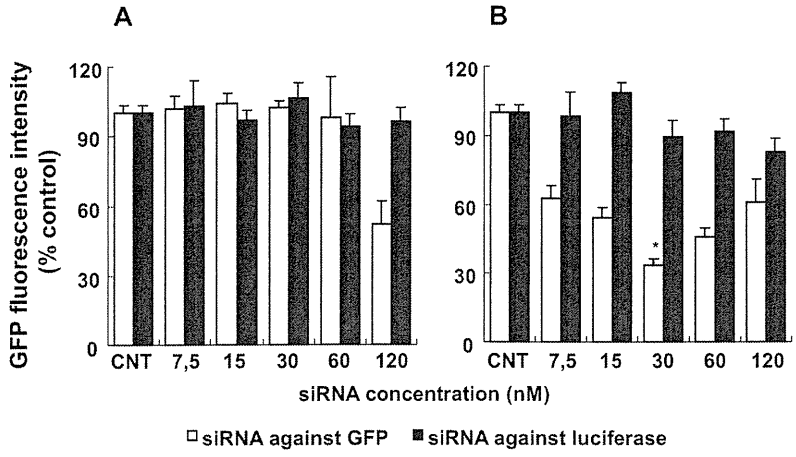

Fig. 1. Effect of siRNA-lipoplex preparation method on gene knockdown efficiency. (A) Lipoplex formed spontaneously (spo-LTsiR) and (B) lipoplex formed under agitation (vortex-mixing) (vor-LTsiR). Amount of cationic liposome was fixed at 9.6 μM while the N/P charge ratios were changed at 15.24, 7.62, 3.81, 1.90 and 0.95 corresponding to siRNA doses. *Legend:* CNT, control (untreated cells). *$P < 0.05$, significant difference in the mean knockdown effect of vor-LTsiR compared to spo-LTsiR at an siRNA dose of 120 nM.

lipoplexes for spo-LTsiR ($p < 0.001$). It appears that lipoplex formation under vortex-mixing allows control over the association process of cationic liposome with siRNA, leading to lipoplexes of less aggregative properties than those formed spontaneously.

3.3. Correlation between the size of siRNA lipoplex and the amount of siRNA attached to it

Despite the wide distribution of particle size, the preparation procedure seems to induce siRNA association to lipoplex populations of a distinct size. In vor-LTsiR (Fig. 2A), the fluorescence intensity of siRNA was detected in a narrow population of small lipoplexes (30–400 nm), indicating that each lipoplex contains a similar amount of associated siRNA. However, in spo-LTsiR (Fig. 2B), the fluorescence of siRNA was detected in relatively larger lipoplexes (150–1500 nm). Interestingly, in spo-LTsiR, the siRNA fluorescence increased in proportion to an increase in lipoplex size, indicating that larger lipoplexes contain larger amounts of siRNA. Agitation during lipoplex formation may somehow alter the association behavior of siRNA and cationic liposomes, resulting in lipoplexes that are homogeneous in terms of size distribution and amount of associated siRNA.

3.4. The effect of agitation (vortexing) on the internalization of lipoplexes

The cellular association of both vor-LTsiR and spo-LTsiR in HT-1080 cells was visualized by confocal microscopy (Fig. 3). Both lipoplexes were internalized, and the green fluorescence related to siRNA was co-localized with the red fluorescence related to the LT. In addition, the red fluorescence of the LT internalized in the cells was proportional between the lipoplexes, whereas the green fluorescence of the siRNA was more intense from the cells treated with vor-LTsiR than from those treated with spo-LTsiR. In addition, the amount of siRNA associated and internalized in the cells was evaluated by flow cytometry (Fig. 4). In spite of the LT and siRNA, the total (surface-bound + internalized) fluorescence intensity in the cells was comparable between vor-LTsiR and spo-LTsiR. In the internalized fraction of lipoplexes, the fluorescence intensity of the LT was also comparable between lipoplexes, while that of the siRNA was 3-fold higher for vor-LTsiR in comparison with spo-LTsiR. These findings indicate that a large amount of siRNA transfected with the spo-LTsiR simply was associated with the cellular surface, and was not being internalized in the cells.

Table 1
Effect of the lipoplex formation method on particle size and zeta potential of the siRNA lipoplex.

	Size		Zeta-potential (mV)
	Opti-MEM (μm)	Sucrose 9% (μm)	
LTrust alone	0.233 ± 0.006	0.239 ± 0.003	21.6 ± 2.0
spo-LTsiR	8.290 ± 1.560	0.270 ± 0.008	18.5 ± 0.4
vor-LTsiR	2.040 ± 0.500*	0.300 ± 0.045	22.4 ± 3.9

The cationic liposome/siRNA ratio was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. The data represent the mean ± S.D. of three independent experiments performed in triplicate.

* $P < 0.001$, significant difference between the mean particle size of vor-LTsiR and spo-LTsiR.

3.5. The uptake mechanism of siRNA transfected by cell lipoplexes

The internalization pathways of both vor-LTsiR and spo-LTsiR were evaluated as an indication of fluorescence intensity for siRNA in the presence of various inhibitors (Fig. 5). The uptake of vor-LTsiR was inhibited at around 42% by the 0.45 M sucrose (clathrin-mediated endocytosis inhibitor), 22% by the ZfFG (fusion inhibitor), and 22% by the amiloride (macropinocytosis inhibitor). The uptake of spo-LTsiR was inhibited 16% by 0.45 M sucrose, 45% by ZfFG, and 22% by amiloride. The treatment with filipin complex (caveolae-mediated endocytosis inhibitor) did not inhibit the uptake of either lipoplex. These results clearly indicate that vor-LTsiR is mainly internalized via clathrin-mediated endocytosis, whereas spo-LTsiR is mainly internalized through membrane fusion.

3.6. Effect of the uptake pathway on the gene knockdown efficiency of vor-LTsiR

The contribution of each uptake pathway of vor-LTsiR to the gene knockdown effect was evaluated (Fig. 6). The knockdown efficiency of the GFP gene was inhibited 25.7 ± 3.5% by 0.45 M sucrose (clathrin-mediated endocytosis) and 10.4 ± 2.8% by amiloride (macropinocytosis), but was not affected by the filipin complex (caveolae-mediated endocytosis inhibitor). The ZfFG, however, enhanced the knockdown efficiency of vor-LTsiR (membrane fusion). These suggest that clathrin-mediated endocytosis and macropinocytosis is the major contributing pathway of

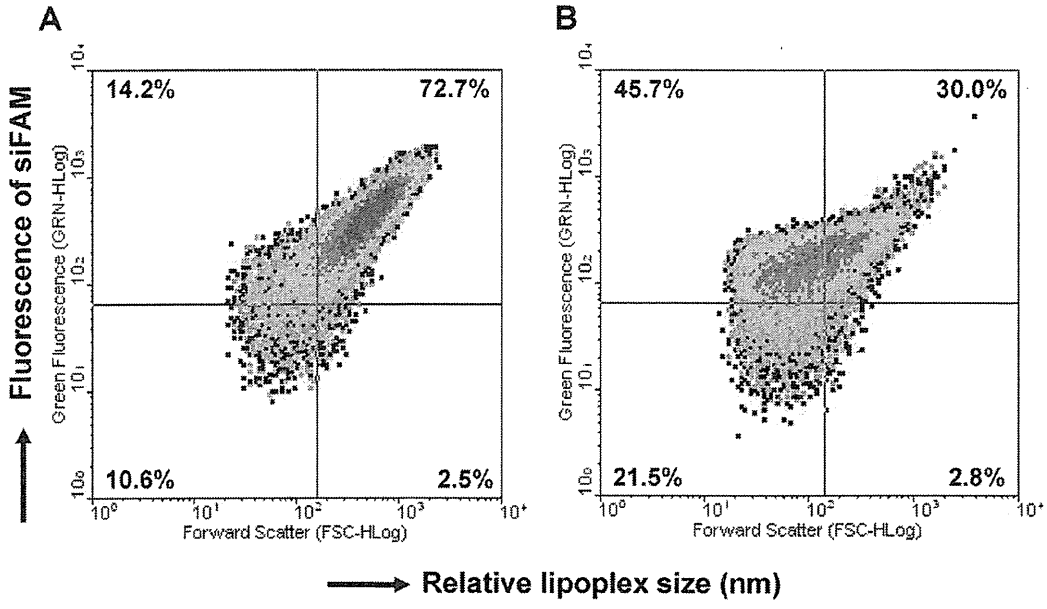

Fig. 2. Effect of lipoplex preparation method on siRNA-lipoplex size distribution. (A) Lipoplex formed spontaneously (spo-LTsiR) and (B) lipoplex formed under agitation (vortex-mixing) (vor-LTsiR). Cationic liposome/siRNA ratio was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. The size distribution and fluorescence intensity of the lipoplexes were measured by flow cytometry.

vor-LTsiR to achieve effective gene knockdown in HT-1080GFP cells.

4. Discussion

siRNA lipoplex usually forms spontaneously, and allows for little control over the process of cationic liposome interaction, which leads to both a wide size distribution and excessive sizes (Lasic and Templeton, 1996; Kawakami et al., 2002; Faneca et al., 2004). In the present study, the initial focus was on investigating the effect of agitation during siRNA lipoplex formation (vor-LTsiR) on the *in vitro* knockdown efficiency of GFP gene stably expressed in HT-1080 cells. This system can partially simulate the downregulation of an endogenous gene. The present study demonstrated that vor-LTsiR could efficiently knockdown the GFP gene using a dose of siRNA against GFP that was 4-fold lower than the dose of spo-LTsiR (Fig. 1).

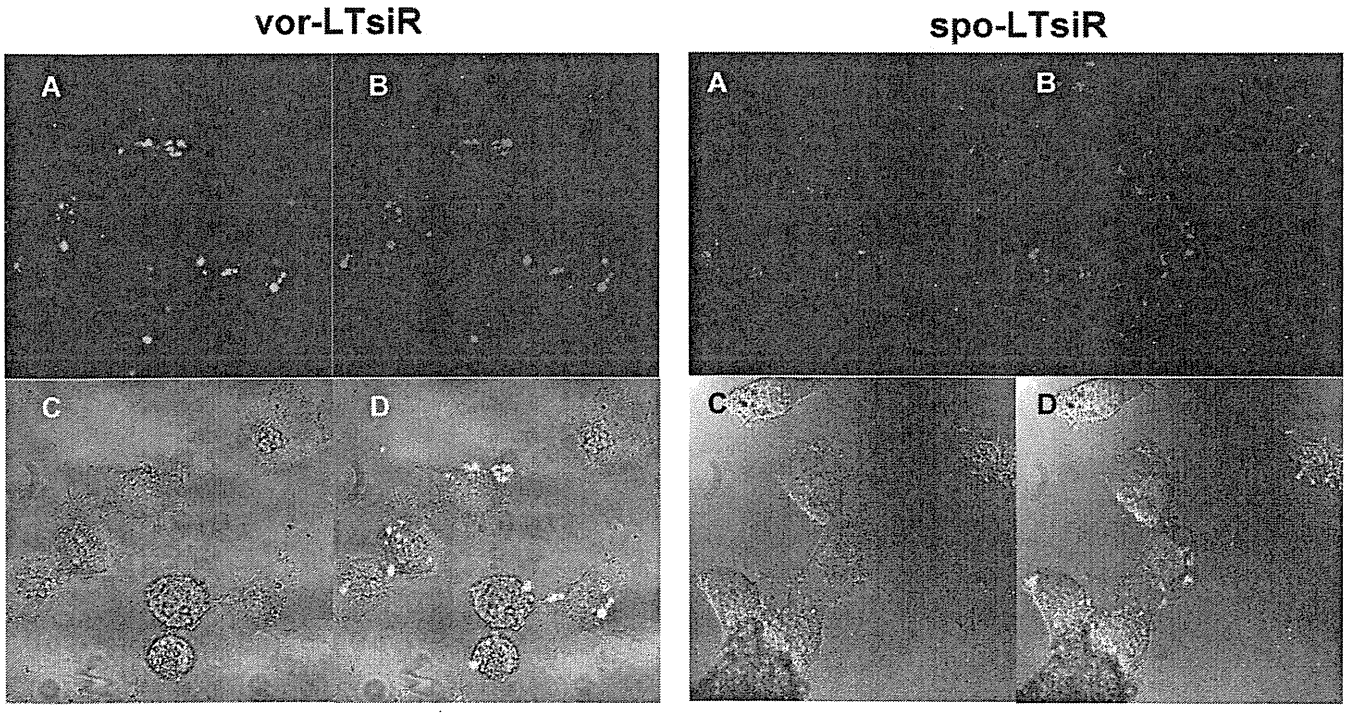

Fig. 3. Visualization of the intracellular vor-LTsiR (left) and spo-LTsiR (right) in the cell. Cationic liposome/siRNA amount was 9.6 μM/30.0 nM and corresponds to a charge ratio of 7.62. (A) siRNA (green), (B) LT (red), (C) phase-contrast image, and (D) merged image of A, B and C. Blue corresponds to the nucleus. (Magnification: ×630.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

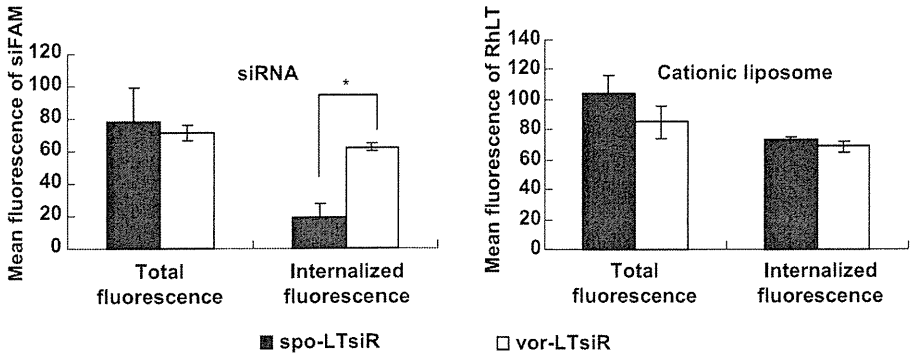

Fig. 4. Amount of siRNA associated with lipoplexes internalized by the cells. Total fluorescence represents the siRNA which was surface-bound and internalized in the cells. Cationic lipid/siRNA amount was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. Data represent the mean ± S.D. of three independent experiments performed in triplicate. *$P < 0.05$ was considered significant.

The results of the present study clearly showed that the preparation procedure affects not only the physicochemical property of siRNA lipoplex but also their gene knockdown efficiency.

The interaction of particles with cells is significantly influenced by particle size (Rejman et al., 2004; Khalil et al., 2006b; Gratton et al., 2008) and surface charge (Lima et al., 2001). The surface charges of both lipoplexes were positive at the siRNA concentration (N/P ratio of 7.62 (9.6 μM/30 nM; $L^+/siRNA^-$)) where the most distinct difference in the RNAi effect was observed. The size of both lipoplexes did not vary significantly when prepared in 9% sucrose, a medium free of counter ions. However, when prepared in Opti-MEM, the medium most routinely used for lipofection, the lipoplexes displayed a heterogeneous size distribution (Table 1) due to the effect of counter ions that stimulate the aggregative properties of lipoplexes (Spagnou et al., 2004). The mean particle size of spo-LTsiR was at least 3-fold larger than that of vor-LTsiR. In addition, the lipoplexes prepared by different procedures displayed a very heterogeneous association of siRNA to distinct lipoplex size population. In vor-LTsiR, approximately 80% of the siRNA molecules attached to the lipoplex smaller than 200 nm, whereas in spo-LTsiR,

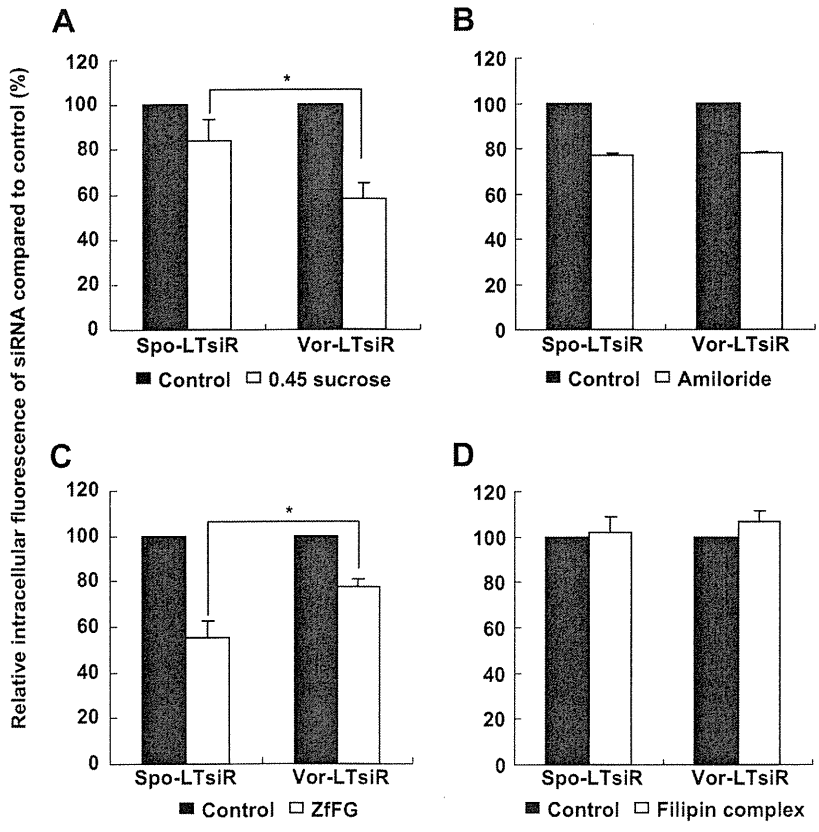

Fig. 5. Uptake mechanism of vor-LTsiR (left) and spo-LTsiR (right) by the cells. siRNA lipoplexes were incubated with the cells, which were treated with various uptake inhibitors. The fluorescence intensity relating to siRNA in the cells was determined by flow cytometry. Data represent the mean ± S.D. of three independent experiments performed in triplicate. *$P < 0.05$ was considered significant. Inhibition of (A) clathrin-mediated endocytosis, (B) macropinocytosis, (C) membrane fusion, and (D) caveolae-mediated endocytosis.