20101103IA

厚生労働科学研究費補助金

医療機器開発推進研究事業

「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

平成２２年度　総括研究報告書

研究代表者　石田　竜弘

平成２３（２０１１）年　５月

目　次

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研究代表者　石田　竜弘　徳島大学大学院ﾍﾙｽﾊﾞｲｵｻｲｴﾝｽ研究部・准教授

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。

申請者は２１～２２年度の研究において、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。TNF-α や TGF-β 阻害剤の併用によりナノキャリアの腫瘍への移行性が向上することが報告されているが、これらの薬剤は本邦では認可されておらず、現時点では臨床への応用は期待できない。一方、本検討で使用した S-1 は既に本邦において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化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。申請者は既に、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確認している。本研究では経口型抗がん剤(S-1)の繰り返し投与時の腫瘍内微小環境変化について検討を行い、siRNA・キャリア複合体の腫瘍移行性・拡散性の評価に基づく併用投与時の最適なregimenの確立を目的として研究を行う。

当該研究期間において、

1. S-1の低用量頻回投与によるsiRNA・リポソーム複合体の腫瘍内分布の変化
2. S-1 とsiRNA・リポソーム複合体の併用による抗腫瘍効果

について主として検討した。

B. 研究方法

(1) siRNA 搭載リポソームの腫瘍移行性・腫瘍内拡散性

最終的に臨床応用を目指すことから、ヒト大腸癌細胞をヌードモデルに移植し、これをモデルとした。siRNA 搭載リポソームの腫瘍内拡散性に関して、リポソームを蛍光ラベルし、投与後に腫瘍切片を作成し、これを蛍光顕微鏡で観察することで評価した。

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 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 *in vivo* の実験に用いた。

II. siRNA 搭載リポソームの調製（その１）

Cationic liposome (CL)は Bangham 法により調製した。脂質組成が DOPE:POPC:CHOL:DC-6-14= 3:2:3:2 (モル比) となるように脂質を試験管内で混合した。次に、減圧下溶媒（クロロホルム）を除去し、試験管内に脂質薄膜を形成させた。この脂質薄膜に内水相として 9% スクロース溶液 (pH 7.4) を加え、脂質薄膜を完全に水和させ、MLV (multilamellar vesicle) を得た。この MLV をエクストリュージョン法により整粒し、粒子径が約 110 nm となる LUV (large unilamellar vesicle) を

調製した。CL への PEG 修飾は、ポストインサーション法により行った。ポストインサーション法は、基本となるリポソームを調製後、9% スクロース溶液中に完全に溶解させた $mPEG_{2000}$-DSPE を脂質 (DOPE、POPC、DC-6-14) 総量に対してモル比で 5%となるように CL 溶液に添加し、振盪機付きインキュベータ中で 37℃、1 時間、軽く振盪させながら行った。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。

次いで、Core-siRNA を調製するために、siRNA 溶液と 0.2 mg/mL ヒアルロン酸(HA)溶液との混合溶液 (siRNA/HA= 1/1 (weight ratio)) と、0.2 mg/mL プロタミン(PRO)溶液を等量ずつ混合し、室温で 5 分間インキュベーションした。このとき、混合する (siRNA+HA) と PRO の重量比は、最適化検討の結果から、1.8 と固定した。次に Core-lipoplex を調製するために、PEG 修飾 CL 溶液と Core-siRNA 溶液を混合し、15 秒間攪拌した後、10 分間室温で放置した。このとき、混合する Lipid と siRNA のモル比は、最適化検討の結果から 2000 に固定し Core-lipoplex を調製した。

III. siRNA 搭載リポソームの調製（その２）

より汎用性を高めるため、オーソドックスな方法（PEG 修飾 cationic liposome と siRNA を混合するだけ）でも lipoplex を調製し、腫瘍への移行性について検討を行った。

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、II と同様に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。

IV. RI 標識・蛍光標識リポソームの調製

RI 標識リポソーム：脂質相マーカーとして ^{3}H-CHE を脂質(DOPE、POPC、DC-6-14)量 0.5 μmol あたり 720,000 dpm のカウントを持つように、脂質薄膜を形成させるときに添加した。DiD 修飾リポソーム：脂質薄膜を形成させる際、DiD を脂質 (DOPE、POPC、DC-6-14) 総量に対しモル比で 1% となるように添加した。

V. In vivo imaging system による siRNA 搭載リポソームの腫瘍移行性の定性的評価

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て、S-1 溶液を経口用ゾンデにより tegafur 量で 6.9 mg/kg となるように経口投与した。S-1 は投与開始から 7 日間、毎日投与を行った。最終 S-1 投与日に、DiD ラベル化 PEG 修飾 core-lipoplex を脂質 (DOPE、POPC、DC-6-14) 総量で 25 mg/kg となるように 200 μL ずつ尾静脈より投与した。その投与 6、12、24、48、96、144 時間後に Fluorescence Image Analyzer LAS-4000 IR multi color (Fujifilm)を用いて、イソフルランで麻酔をかけたマウスの全身像を撮影することにより腫瘍内への蓄積を評価した。コントロールとして、S-1 の metronomic 投与を施さなかったマウスを用いた。

VI. RI 用いた siRNA 搭載りポソームの腫瘍移行性の定量的評価

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て、V に示すように S-1 を投与し、最終 S-1 投与日に ^{3}H-CHE ラベル化 siRNA 搭載リポソームを脂質 (DOPE、POPC、DC-6-14) 総量で 25 mg/kg となるように 200 μL ずつ尾静脈より投与した。その 24 時間後に血液を心臓より 100 μL 採取し、また肺、肝臓、脾臓、腎臓、腫瘍も採取し、血液および各臓器中の放射活性を測定することで siRNA 搭載リポソームの腫瘍内移行性を評価した。

(2) 抗腫瘍効果の検討

I. PEG 修飾 siRNA 搭載リポソーム (Bcl-2, Argonaute2 (Ago2)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(siBcl2, siAgo2 もしくはコントロールとして siGFP 含有) を siRNA 量で 10-80 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(Bcl-2, Ago2 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 : DLD-1 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) siRNA 搭載リポソームの腫瘍移行性・腫瘍内拡散性

これまでの検討から、S-1 を metronomic 投与することによって腫瘍内の微小環境が変化し、全身投与した PEG 修飾 liposome の腫瘍蓄積量が有意に向上する (liposome に対する EPR 効果が増強される) ことが明らかになっている。また、平成２１年度の検討から、Core-siRNA を PEG 修飾リポソームで包埋した Core-lipoplex においても、PEG 修飾リポソームと同様に S-1 の metronomic 投与によって腫瘍内への移行性が亢進されることを確認した。

平成２２年度においては、汎用性を高める事を目的としてオーソドックスな方法により調製 (PEG 修飾 cationic liposome と siRNA を混合するだけ) し、この PEG 修飾 siRNA-lipoplex に対する S-1 併用による腫瘍内移行性の亢進に関して検討を行った。このような方法によって調製される lipoplex は比較的粒子径が大きく、静脈内投与されると mononuclear phagocyte system (MPS)に比較的取り込まれやすく血中滞留性が低いため、結果的に EPR 効果による腫瘍移行性が低い事が知られている。

In vivo imaging による評価の結果、S-1 非処置群 (Control) と比較し S-1 処置群では、腫瘍において蛍光が強く確認され、PEG 修飾 siRNA-lipoplex の腫瘍移行性が向上している事が確認できた。次いで RI を用いた定量的評価を行ったところ、Control と比較して S-1 処置群のほうが、siRNA-lipoplex の腫瘍蓄積量が有意に高いことが確認できた。また、この siRNA-lipoplex の蓄積量の増加は、他の臓器 (肺、肝臓、脾臓、腎臓) においては観察されなかった。以上より、PEG 修飾リポソームおよび PEG 修飾 core-lipoplex とは粒子径が全く異なる PEG 修飾 siRNA-lipoplex でも S-1 と併用することにより腫瘍移行性向上が認められ、さらにこの亢進効果は腫瘍部位のみにおいて生じることを確認することができた。

次いで、微視的に S-1 の低用量頻回投与が Peg 修飾 siRNA-lipoplex の腫瘍内分布に与える影響について検討を行った。蛍光顕微鏡による観察から、S-1 の処置日数依存的に、PEG 修飾 siRNA-lipoplex の腫瘍内分布が拡大することが明らかとなった。また、分布領域についても、Control では比較的腫瘍の端部分に蛍光色素の顕著な集積 (ホットスポット)が存在していたが、腫瘍の中心部ではあまり観察されなかった。しかし、S-1 処置により、PEG 修飾 siRNA-lipoplex が腫瘍中心付近にも分布していることが確認された。このことから、S-1 処置による変化は、腫瘍の端から内側にかけて生じているものと考えられる。循環血流は、腫瘍外部から腫瘍新生血管を通り腫瘍内へという方向に流れ込む。しかしながら、腫瘍内は間質圧が高く、また血流が不連続的であるため、S-1 を含有する血液が腫瘍深部まで到達するのは困難であることが予想される。腫瘍内微小環境の変化は S-1 が届く領域で変化が生じると考えられるため、分布の変化が起こる場所に偏りが見られるのだと推測される。

さらに、S-1 の投与日数が与える DiI 標識 PEG 修飾 siRNA-lipoplex の腫瘍移行に対する影響について検討を行った。S-1 投与 4 日目から PEG 修飾 siRNA-lipoplex の腫瘍内分布が増加する傾向があらわれはじめ、7 日間連続投与することで腫瘍内での分布が比較的均等に分布するようになることが分かった。このことから、S-1 投与によって腫瘍内微小環境が微粒子が蓄積しやすい状況になるためには、S-1 投与期間がある程度必要であることが明らかになった。

(2) 抗腫瘍効果の検討

I. siBcl2 と S-1 の併用

siBcl2含有PEG修飾core-lipoplex (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、siBcl2含有PEG修飾core-lipoplexを1日おきに計8回、siRNA量で10 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)と比較してsiBcl2含有PEG修飾core-lipoplexとS-1単独投与ではそれぞれ21.5%、31.4% (DLD-1接種後20日目)の腫瘍成長抑制効果を示したが、siBcl2含有PEG修飾core-lipoplexとS-1とを併用することによって62.8%と顕著に高い腫瘍成長抑制効果が得られた。この併用効果は、それぞれの薬剤単独時の効果を単純に足した効果 (52.9%) に比べて約10%も効果が増強しており、相乗的な効果であると言える。

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、siBcl2 含有PEG 修飾 core-lipoplex 単独投与群では 10.5%、S-1 単独投与群では 15.9%のアポトーシス誘導が見られたが、これら併用投与群では 29.6%と顕著に高いアポトーシス誘導が確認された。

II. siAgo2 と S-1 の併用

研究代表者は最近 RNAi において主たる役割を果たす Argonaute2 (Ago2) を siRNA によって knockdown する事によって細胞死が誘導される事を明らかにしている。そこで、DLD-1 細胞における siAgo2 による細胞成長抑制、そして siAgo2 を細胞に前処置した後に 5-FU を処置することで、細胞成長にどのような影響を生じるのか、検討を行った。結果として、5-FU を併用することで、siAgo2 単独の場合よりも高い細胞成長抑制率が得られることが分かった。この場合の IC50 の変化は、siRNA を前処置していない細胞(control)では 0.39μg/ml 、control 配列の siRNA(Luciferase 標的 siRNA)を前処置したものでは 0.43μg/ml、Ago2 標的 siRNA の前処置をしたものでは 0.19μg/ml であった。これらのことから、Ago2 標的 siRNA を前処置することで、5-FU に対する感受性が約 2.0 倍向上することを確認する事ができた。

前述のように in vitro において Ago2 遺伝子を knockdown することで、DLD-1 の 5-FU に対する感受性が上がり、高い細胞成長抑制効果を示すことを確認した。このことから、in vivo においても同様に高い抗腫瘍活性を示すことが考えられる。そこで、S-1 処置後に PEG 修飾 siRNA-lipoplex 投与を行い、抗腫瘍効果について検討を行った。その結果、siBcl-2 と同様に、併用投与を行った場合に極めて高い抗腫瘍効果が得られることを確認した。

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 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。

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。

我々が見出した 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次のように考察している。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いる。その結果として、全身投与した PEG 修飾 liposome の腫瘍蓄積量が増加しているのではないかと考えている。また、この現象は、粒子径が 400 nm 以上の PEG 修飾 siRNA-lipoplex でも見られており、ナノキャリアの粒子径に依存しない現象である可

能性が示唆されている。

この現象を利用して、Bcl-2 標的 siRNA (siBcl2) あるいは siAgo2 と経口型抗がん剤 S-1 を組み合わせた新規がん治療戦略を考えた。両 siRNA ともに標的遺伝子を knockdown することによって細胞死を誘導する事を確認しており、この siRNA 依存的な細胞死の誘導と S-1 の本体である 5-FU による細胞死誘導が相加・相乗的に作用して、結果として高い抗腫瘍効果が得られるのではないかと考えたのである。実際この併用療法が機能すれば、異なる 2 つの薬剤が互いに効果を向上し合うわけで『Double modulation therapy』と定義されるべきものである。検討の結果、顕著に高い、且つ相乗的な抗腫瘍効果が得られ、この併用療法の有用性を確認することができた。現在に至るまで、siRNA を effector (抗がん作用を示す薬剤)、もしくは modulator (effector の効果を変化させる作用を示す薬剤) としてのみ用いる研究が主流であった。しかし、組み合わせによっては siRNA に effector と modulator の両作用を発揮させることが可能であり、また、抗がん剤に関してもほとんどが effector として用いるのが通常であるが、組み合わせによっては modulator として機能させることが可能であることも明らかとなった。本検討で得られた知見は、今後、siRNA に代表される核酸医薬と化学療法とを組み合わせた新しいがん治療法の開発に向けた一歩になるものと期待している。

E. 結論

ナノキャリアを利用した抗がん剤の腫瘍へのデリバリー術はすでに確立されており、Doxil などの製剤が臨床で用いられている。抗がん剤の場合、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はキャリアからの放出性に乏しく、また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されなければ、その効果を発揮することはできない。したがって、siRNAが薬効を発現できる領域はナノキャリアの分布領域に依存する。そのため、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。S-1は大腸がん・胃がん・膵がん治療などで既に臨床応用されており、このS-1との併用によって、より腫瘍深部の細胞にsiRNAが導入され、薬効発現につながったことは、siRNAを用いたがん治療『Double modulation therapy』の実現に大きく貢献する新たな知見である。

今後、本併用療法の投与 regimen 最適化に寄与しうる経口抗がん剤投与時の腫瘍内微小環境変化についてさらに検討するとともに、本戦略の臨床応用を目指す目的で、5-FU 感受性を向上しうる因子群を標的とした siRNA をデザインし、その併用効果に関して検討を行う予定である。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

(1)Ishihara T., Maeda T., Sakamoto H., Takasaki N., Shigyo M., Ishida T., Kiwada H., Mizushima Y., Mizushima T., Evasion of the accelerated blood clearance phenomenon by coating of nanoparticles with various hydrophilic polymers. Biomacromolecules, 11, 2700-2706 (2010)

(2)Doi Y., Okada T., Matsumoto H., Ichihara M., Ishida T., Kiwada H., Combination therapy of metronomic S-1 dosing with oxaliplatin-containing PEG-coated liposome improves antitumor activity in a murine colorectal tumor model. Cancer Sci., 101, 2470-2475 (2010)

(3)Tagami, T., Nakamura, K., Shimizu, T., Yamazaki, N., Ishida, T., Kiwada, H., CpG motifs in pDNA-sequences increase anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes. J. Control. Release, 142, 160-166 (2010)

(4)Abu Lila, A., Doi, Y., Nakamura, K., Ishida, T., Kiwada, H., Sequential administration with oxaliplatin-containing PEG-coated cationic liposomes promotes a significant delivery of subsequent dose into murine solid tumor. J. Control. Release, 142, 167-173 (2010)

(5)Abu Lila, A., Ishida, T., Kiwada, H., Targeting anticancer drugs to tumor vasculature using cationic liposomes. Pharm. Res., 27, 1171-1183 (2010)

2. 学会発表

(1) Matsumoto, H., Doi, Y., Ishida, T., Kiwada, H., Tumor localization and therapeutic effect of PEG-coated liposomal anticancer agent: Tumor type-dependency. International Liposome Research Days & Lipids, Liposomes & Membrane Biophysics, Vancouver, Canada, Aug. (2010)

(2) Okada, T., Doi, Y., Abu Lila, A. Abu Lila., Ishida, T., Kiwada, H., Metronomic oral S-1, a fluoropyrimidine anticancer agent, dosing affects tumor accumulation of PEG-coated liposome. International Liposome Research Days & Lipids, Liposomes & Membrane Biophysics, Vancouver, Canada, Aug. (2010)

(3) Matsunaga, M., Nakamura, K., Doi, Y., Moriyoshi, N., Ishida, T., Kiwada, H., Development of new cancer treatment strategy that combines siRNA-lipoplex with oral tegafur anticancer drug S-1. International Liposome Research Days & Lipids, Liposomes & Membrane Biophysics, Vancouver, Canada, Aug. (2010)

(4) Iwaki, T., Tagami, T., Nakamura, K., Ishida, T., Kiwada H., The effect of PEGylation of siRNA-lipoplex on intracellular uptake quantity of siRNA and intracellular behavior of siRNA. International Liposome Research Days & Lipids, Liposomes & Membrane Biophysics, Vancouver, Canada, Aug. (2010)

(5) 石田竜弘、際田弘志、腫瘍内微小環境変化を利用したsiRNAデリバリーシステムの開発、第32回生体膜と薬物の相互作用シンポジウム（富山）、2010年11月

(6) 石田竜弘、腫瘍へのsiRNAデリバリーシステムの開発～効果と安全性の観点から～、第47回薬剤学懇談会研究討論会（高山）、2010年6月

(7) 石田竜弘、生体内動態検討を基盤としたリポソームDDSの開発、第26回日本ＤＤＳ学会（大阪）、2010年6月

(8) 石田竜弘、際田弘志、核酸デリバリーにおけるABC現象、遺伝子・デリバリー研究会第10シンポジウム（札幌）、2010年6月

(9) 長尾愛、市原理子、土井祐輔、石田竜弘、際田弘志、抗PEG IgM抗体誘導におけるS-1とl-OHP封入PEG修飾リポソーム併用条件が与える影響の検討、第49回日本薬学会・日本薬剤師会・日本病院薬剤師会 中国四国支部学術大会（鳥取）、2010年11月

(10) 北川瑞野、岩木雄大、田上辰秋、石田竜弘、際田弘志、細胞内導入後のsiRNA量の経時的変化に関する検討、第49回日本薬学会・日本薬剤師会・日本病院薬剤師会 中国四国支部学術大会（鳥取）、2010年11月

(11) 石田竜弘、田上辰秋、上原友美、森吉直人、岩木雄大、際田弘志、リポソームを用いた腫瘍へのsiRNAデリバリーシステムの開発、遺伝子・デリバリー研究会第１０回夏季セミナー（滋賀）、2010年9月

(12) 上原友美、田上辰秋、市原理子、石田竜弘、際田弘志、ナノ粒子ポリマー修飾剤の構造の違いが与える抗ポリマーIgMの分泌機構への影響に関する検討、ナノライフサイエンス・オープンセミナー2010（大阪）、2010年8月

(13) 岩木雄大、中村和也、田上辰秋、石田竜弘、際田弘志、siRNA-lipoplexへのPEG修飾がRNAi効果発現に与える影響、ナノライフサイエンス・オープンセミナー2010（大阪）、2010年8月

(14) 北川瑞野、岩木雄大、田上辰秋、石田竜弘、際田弘志、トランスフェクション後の細胞内siRNA量の経時変化に関する検討、ナノライフサイエンス・オープンセミナー2010（大阪）、2010年8月

(15) 長尾愛、市原理子、土井祐輔、石田竜弘、際田弘志、S-1とl-OHP封入PEG修飾リポソームの併用条件下における抗PEGIgM抗体分泌の検討、ナノライフサイエンス・オープンセ

ミナー2010（大阪）、2010年8月

(16) 森吉直人、中村和也、松永真理子、石田竜弘、際田弘志、テガフール製剤S-1とBcl-2標的siRNA封入liposomeの併用療法によるがん治療の有用性に関する検討、第26回日本ＤＤＳ学会（大阪）、2010年6月

(17) 上原友美、田上辰秋、石田竜弘、際田弘志、核酸デリバリーにおける修飾剤種が与えるポリマー特異性IgM分泌への影響、遺伝子・デリバリー研究会第10回シンポジウム（札幌）、2010年6月

(18) 岩木雄大、田上辰秋、中村和也、石田竜弘、際田弘志、Lipoplexのエンドソーム/ライソソームからの脱出メカニズムに関する検討、遺伝子・デリバリー研究会第10回シンポジウム（札幌）、2010年6月

(19) 松永真理子、中村和也、森吉直人、石田竜弘、際田弘志、腫瘍移行性向上を目指したsiRNA-lipoplexの改良と評価、遺伝子・デリバリー研究会第10回シンポジウム（札幌）、2010年6月

(20) Abu-Lila Amr、土井祐輔、中村和也、石田竜弘、際田弘志、Sequential administration with oxaliplatin-containing PEG-coated cationic liposomes promotes a significant delivery of subsequent dose into murine solid tumor、日本薬剤学会第25年会（徳島）、2010年5月

H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Doi Y., Okada T., Matsumoto H., Ichihara M., Ishida T., Kiwada H.	Combination therapy of metronomic S-1 dosing with oxaliplatin-containing PEG-coated liposome improves antitumor activity in a murine colorectal tumor model.	*Cancer Sci..*	101	2470-2475	2010
Tagami, T., Nakamura, K., Shimizu, T., Yamazaki, N., Ishida, T., Kiwada, H.	CpG motifs in pDNA-sequences increase anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes.	*J. Control. Release*	142	160-166	2010
Abu Lila, A., Doi, Y., Nakamura, K., Ishida, T., Kiwada, H.	Sequential administration with oxaliplatin-containing PEG-coated cationic liposomes promotes a significant delivery of subsequent dose into murine solid tumor.	*J. Control. Release*	142	167-173	2010
Ishihara T., Maeda T., Sakamoto H., Takasaki N., Shigyo M., Ishida T., Kiwada H., Mizushima Y., Mizushima T.	Evasion of the accelerated blood clearance phenomenon by coating of nanoparticles with various hydrophilic polymers.	*Biomacromolecules*	11	2700-2706	2010
Abu Lila, A., Ishida, T., Kiwada, H.	Targeting anticancer drugs to tumor vasculature using cationic liposomes.	*Pharm. Res.*	392	1171-1183	2010

Cancer Science
The official journal of the Japanese Cancer Association JCA

Combination therapy of metronomic S-1 dosing with oxaliplatin-containing polyethylene glycol-coated liposome improves antitumor activity in a murine colorectal tumor model

Yusuke Doi, Tomoko Okada, Haruna Matsumoto, Masako Ichihara, Tatsuhiro Ishida[1] and Hiroshi Kiwada

Department of Pharmacokinetics and Biopharmaceutics, Subdivision of Biopharmaceutical Science, Institute of Health Biosciences, The University of Tokushima, Tokushima, Japan

(Received May 23, 2010/Revised July 1, 2010/Accepted July 7, 2010/Accepted manuscript online July 13, 2010/Article first published online August 23, 2010)

Metronomic chemotherapy has been advocated recently as a novel chemotherapeutic regimen. Polyethylene glycol (PEG)-coated liposomes are well known to accumulate in solid tumors by virtue of the highly permeable angiogenic blood vessels characteristic for growing tumor tissue, the so-called "enhanced permeability and retention (EPR) effect". To expand the range of applications and investigate the clinical value of the combination strategy, the therapeutic benefit of metronomic S-1 dosing in combination with oxaliplatin (l-OHP)-containing PEG-coated liposomes was evaluated in a murine colon carcinoma-bearing mice model. S-1 is an oral fluoropyrimidine formulation and metronomic S-1 dosing is a promising alternative to infused 5-FU in colorectal cancer therapy. Therefore, the combination of S-1 with l-OHP may be an alternative to FOLFOX (infusional 5-FU/leucovorin (LV) in combination with l-OHP), which is a first-line therapeutic regimen of a colorectal carcinoma. The combination of oral metronomic S-1 dosing with intravenous administration of liposomal l-OHP formulation exerted excellent antitumor activity without severe overlapping side-effects, compared with either metronomic S-1 dosing, free l-OHP or liposomal l-OHP formulation alone or metronomic S-1 dosing plus free l-OHP. We confirmed that the synergistic antitumor effect is due to prolonged retention of l-OHP in the tumor on account of the PEG-coated liposomes, presumably via alteration of the tumor microenvironment caused by the metronomic S-1 treatment. The combination regimen proposed here may be a breakthrough in treatment of intractable solid tumors and an alternative to FOLFOX in advanced colorectal cancer therapy with acceptable tolerance and preservation of quality of life (QOL). (*Cancer Sci* 2010; 101: 2470–2475)

Oxaliplatin (l-OHP), an innovative third generation platinum compound, has powerful anti-neoplastic competence with no cross drug resistance with cisplatin and carboplatin[1,2] However, l-OHP shows relatively low anticancer effectivity when it is administered alone, because it shows poor accumulation in tumor tissues due to a high plasma protein binding ratio and high partitioning to erythrocytes, while in addition displaying peripheral neurotoxicity due to high protein binding in the tissue.[3] These features stand in the way of an effective continuous treatment with l-OHP. Oxaliplatin is frequently used for treatment of advanced colorectal cancer when combined with fluorouracil (5-FU) and leucovorin (LV) (FOLFOX).[4,5]

Chemotherapy using nanocarriers as a delivery system has been developed to improve the success of clinical treatment of solid tumors by achieving high accumulation of the chemotherapeutic agent in tumor tissues but with limited accumulation in healthy tissues. Polyethylene glycol (PEG)-modified (PEG-coated) liposomes show prolonged circulating times and thereby enhanced accumulation in solid tumors by virtue of the increased vascular permeability observed in tumor angiogenic blood vessels (the so-called ''enhanced permeability and retention (EPR) effect'').[6] Therefore, it is reasonable to assume that PEG-coated liposomes may improve the pharmacokinetic features of l-OHP and enhance its anticancer efficiency. We and other groups have shown therapeutic improvement of l-OHP by encapsulation in PEG-coated targeted liposomes.[7–9]

However, nanocarriers of various designs have repeatedly shown insufficient delivery of their payloads to solid tumors. One of the major limitations is insufficiency of the EPR effect due to a disordered intratumoral microenvironment represented for instance by hypovascularity. Recently, Kano *et al.*[10] reported that treatment with transforming growth factor-β type 1 receptor (TβR-1) inhibitor resulted in increased accumulation of nanocarriers accompanied by a pronounced antitumor response in a murine solid tumor model. ten Hagen and co-workers[11,12] have reported a similar observation with tumor necrosis factor α (TNF-α) in both rat and murine models. These approaches are regarded as a breakthrough that can overcome the insufficient EPR effect for nanocarriers in solid tumors. An obvious drawback of this approach is that TβR-1 inhibitor and TNF-α cannot be readily applied because these compounds have not yet been approved for clinical use worldwide.

Metronomic chemotherapy, which refers to the frequent administration of chemotherapeutics at doses significantly below the maximum tolerated dose (MTD) without prolonged drug-free breaks, is a novel approach to the control of advanced cancer.[13,14] The therapy shows a potent anti-angiogenetic effect by targeting genetically stable endothelial cells within the tumor vascular bed, rather than tumor cells with a high mutation rate. Drugs that can be administered orally, such as cyclophosphamide (CPA), capecitabine, UFT and S-1, would meet the requirements of prolonged daily administration schedules. Recently, we showed that metronomic CPA dosing augments intratumoral accumulation of co-administered doxorubicin (DXR)-containing PEG-coated liposomes and this combination exerted an excellent antitumor activity in a murine tumor model without overlapping severe side-effects.[15,16] This favorable therapeutic effect might be attributed to the following mechanism: metronomic dosing with CPA makes the newly forming tumor vessels leaky, and thereby enhances intratumoral accumulation of PEG-coated liposomes.

Oxaliplatin administered together with infusions of 5-FU and LV (FOLFOX) has become a standard treatment regimen for

[1]To whom correspondence should be addressed.
E-mail: ishida@ph.tokushima-u.ac.jp

doi: 10.1111/j.1349-7006.2010.01678.x

advanced colorectal cancer.[4,5] To extend our approach described above, in the present study we evaluated l-OHP/5-FU synergy by combination of metronomic S-1 dosing (orally, daily) with l-OHP-containing PEG-coated liposomes (intravenously, once a week) in a murine colorectal cancer model. S-1 consists of tegaful (a prodrug of 5-FU), 5-chloro-2, 4-dihydroxypyridine (CDHP: an inhibitor of 5-FU degradation) and potassium oxonate (Oxo: a reducer of gastrointestinal toxicity) at a molar ratio of 1:0.4:1. S-1 is one of the most frequently used drugs for oral administration in Japan and shows less toxic side-effects than 5-FU.[17–19] The biochemical modulation of S-1 leads to prolonged retention of 5-FU in the blood, which mimics the pharmacokinetic profile of infusional 5-FU. Daily oral administration with S-1 meets the concept of metronomic dosing and is assumed to enhance intratumoral accumulation of PEG-coated liposomes and l-OHP associated with the liposome.

Materials and Methods

Materials. Hydrogenated soy phosphatidylcholine (HSPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-n-(methoxy[polyethylene glycol]-2000) ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical (Osaka, Japan). S-1 and l-OHP were generously donated by Taiho Pharmaceutical (Tokyo, Japan). DiI (1, 1′-dioctadecyl-3, 3, 3′,3′-tetramethyl-indocarbocyanine perchlorate) and DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine perchlorate) were purchased from Invitrogen (Paisley, UK). ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) was purchased from Perkin Elmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

Preparation of l-OHP-containing PEG-coated liposomes. l-OHP-containing PEG-coated liposomes, composed of HSPC/CHOL/$mPEG_{2000}$-DSPE (2/1/0.2, molar ratio), were prepared using a reverse-phase evaporation method as described earlier.[9] Unencapsulated, free l-OHP was removed by dialysis by means of a dialysis cassette (Slyde-A-Lyzer, 10000MWCO; Pierce, Rockford, IL, USA) against 5% dextrose. Encapsulated l-OHP was quantified using an atomic absorption photometer (Z-5700; Hitachi, Tokyo, Japan). The phospholipid concentration was determined by colorimetric assay.[20] The particle size of the liposomes was 180 ± 52 nm, as determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, Mountain View, CA, USA). The encapsulation efficiency of l-OHP was calculated by dividing the drug to lipid ratio after the dialysis by the initial drug to lipid ratio and was approximately 20%. These values are three times higher than that reported recently by another group.

Animal and tumor cell. Male BALB/c mice, 5 weeks old, were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

The Colon 26 (C26) murine colorectal carcinoma cell line was purchased from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). To develop tumor-bearing mice, C26 cells (2×10^6) were inoculated subcutaneously into the back of BALB/c mice.

Combination therapy with S-1 and l-OHP formulation. Treatments began when the tumor volumes reached a volume of 40–60 mm^3. The day treatment began was defined as day 0. The dosing schedule of each chemotherapeutic treatment was as follows:

1 Metronomic S-1 dosing: S-1 (6.9 mg tegafur/kg/dose) was administered orally every day from day 0 to day 21.
2 Free or liposomal l-OHP dosing: Free or liposomal l-OHP (4.2 mg/kg/dose) was intravenously administered at day 0, 7 and 14.
3 Combination dosing (S-1 plus free or liposomal l-OHP): S-1 (6.9 mg tegafur/kg per dose) was orally administered daily from day 0 to day 21. Either free or liposomal l-OHP (4.2 mg/kg per dose) was intravenously administered at day 0, 7 and 14.

Tumors were measured externally every third day. Tumor volume was approximated by using formula A below. The antitumor activity was determined by evaluating the change of the relative tumor volume (formula B) and the tumor growth inhibition rate was calculated through formula C.

$$\text{Tumor volume [TV, (mm}^3\text{)]} = 0.5 \times \text{Length} \times \text{Width}^2 \quad \text{(A)}$$

$$\text{Relative tumor volume (RTV)} = \text{Tumor volume on day n/Tumor volume on day 0} \quad \text{(B)}$$

$$\text{Tumor growth inhibtion rate [TGI, (\%)]} = [1 - (\text{mean RTV of treated group})/(\text{mean RTV of control group})] \times 100 \quad \text{(C)}.$$

Effect of S-1 dosing on blood clearance and tumor accumulation of l-OHP encapsulated in PEG-coated liposomes. Treatment with S-1 (6.9 mg tegafur/kg, daily, 7 days) was started when the tumor volumes reached a volume of 40–60 mm^3. To evaluate blood clearance and tumor accumulation of l-OHP encapsulated in PEG-coated liposomes, the liposomal l-OHP formulation (4.2 mg l-OHP/kg) was intravenously injected right after the final S-1 administration. At 6, 12, 24, 36, 48, 72 and 120 h post-injection of l-OHP formulation, plasma was collected, and then the mice were killed to remove the tumor. Tumors were harvested and weighed. Thereafter, concentrated nitric acid was added to the tumor or plasma (100 μL), which was then digested in a microwave oven (600 W for 25 min at 50°C ETHOS TC; Milestone general, Kanagawa, Japan). The content of platinum (Pt) in the plasma and tumor was measured using an ICP-MS (Agilent 7500 series; YOKOKAWA analytical systems, Tokyo, Japan). Europium was added to the assay mixture and calibration standards, respectively. The l-OHP concentrations were calculated from ion counts Pt using the calibration method with internal standard correction. The l-OHP concentration in the tumor was expressed as μg l-OHP per g tissue. Pharmacokinetic parameters were calculated on the basis of l-OHP concentration using poly-exponential curve fitting and the least-squares parameter estimation program SAAM II (SAAM Institute, Seattle, WA, USA).

Effect of S-1 dosing on biodistribution of PEG-coated liposomes. Treatment with S-1 (6.9 mg tegafur/kg, daily, 7 days) was started when the tumor volumes had reached a volume of 40–60 mm^3. To assess the biodistribution of PEG-coated liposomes, ^{3}H-CHE-labeled liposomes (25 mg total lipid/kg) were intravenously injected right after the final S-1 administration. At 24 h after liposome injection, samples (tumor, blood [100 μL], heart, lung, liver, spleen, kidney) were collected. Tissue samples were washed and weighed after removing excess fluid. Radioactivity in the samples was assayed as described previously.[22]

Effect of S-1 dosing on tumor accumulation and distribution of PEG-coated liposomes. Treatment with S-1 (6.9 mg tegafur/kg, daily, 7 days) was started when the tumor volumes had reached a volume of 40–60 mm^3. In order to assess the effect of S-1 dosing on intratumoral accumulation of PEG-coated liposomes, DiI- or DiD-labeled PEG-coated liposomes (25 mg phospholipids/kg) were intravenously injected right after the final S-1 administration. At defined time points (6, 12 and 24 h) after injection, fluorescence imaging was performed with Fluorescence Image Analyzer LAS-4000 IR (Fujifilm, Tokyo, Japan). The fluorescence images were acquired with a 1/100 s exposure

time. For the intratumoral liposome distribution study, at 24 h post-injection, the tumors were harvested and snap-frozen in Optical Cutting Compound (OCT) compound (Sakura Fintechnical, Tokyo, Japan) with dry-iced acetone. Sections of frozen samples (5 μm thick) were directly observed using a fluorescence microscope (Axiovert 200 M; Zeiss, Oberkohen, Germany). Three tumors per group were studied. Thirty images from 10 randomly selected sections per tumor (three images from one section) were analyzed using AxioVision software (Zeiss).

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

Results

Tumor growth suppressive effect of metronomic S-1 dosing plus l-OHP-containing PEG-coated liposomes. As shown in Figure 1, free l-OHP showed relatively low antitumor activity compared with other treatments and the TGI was only 19.0%. Metronomic S-1 dosing showed a higher tumor growth suppressive effect than free l-OHP (36.5%, TGI). Liposomal l-OHP showed even a much higher tumor suppressive effect (52.9%, TGI) compared with free l-OHP, and the therapeutic efficiency was very similar to that of conventional combination therapy (metronomic S-1 dosing plus free l-OHP) (57.7%, TGI). Metronomic S-1 dosing plus liposomal l-OHP showed by far the strongest tumor growth suppressive effect (87.0%, TGI) of all treatments. This result indicates that metronomic S-1 dosing combined with l-OHP-containing PEG-coated liposomes produces a superior tumor growth suppressive effect in a murine colorectal tumor model.

To assess toxicity of each mono- or combination therapy, change of bodyweight and blood cells (white cells, red cells and platelets) were determined. Only combination chemotherapy of metronomic S-1 dosing plus l-OHP formulations showed a slight suppression of bodyweight increase. However, there was no significant difference between S-1 plus free l-OHP and S-1 plus liposomal l-OHP (data not shown). In addition there were no significant changes in blood cell counts between each mono- and combination therapy (data not shown).

Effect of metronomic S-1 dosing on clearance and tumor accumulation of l-OHP associated with PEG-coated liposomes. The plasma clearance of l-OHP in S-1 treated mice was very similar to that in control mice; the $t_{1/2}$ in S-1 treated mice amounting to 18.5 h and the $t_{1/2}$ in control mice to 18.1 h. In the tumor without S-1 treatment, l-OHP concentration reached the maximum level (approximately 1500 ng/g tissue) at 24 h after injection, and then precipitously decreased (Fig. 2). In the S-1-treated tumor, l-OHP concentration reached the maximum level, similar to the control, at 24 h after injection, being retained at this level until 48 h and then gradually decreasing (Fig. 2). The area under the concentration–time curve (AUC) of l-OHP in the S-1-treated tumor was approximately 1.4-fold higher than that in the control tumor; 131.6 (μg/g tissue h) in S-1-treated tumor *vs* 96.2 (μg/g tissue h) in the control. These results indicate that the S-1 treatment prolonged the retention of l-OHP within the tumor tissue.

Effect of metronomic S-1 dosing on biodistribution and tumor accumulation of PEG-coated liposomes. To gain more insight into the underlying mechanism of the improved tumor suppressive effect (Fig. 1) and in the prolonged l-OHP retention within the tumor (Fig. 2), we investigated the effect of metronomic S-1 dosing on the biodistribution and tumor accumulation of co-administered PEG-coated liposomes. The effect of daily metronomic S-1 dosing (for 7 days) on the biodistribution of PEG-coated liposomes was investigated with a radio-labeled liposome. The S-1 treatment yielded significant enhancement of accumulation of PEG-coated liposomes (1.3-fold) in tumor at 24 h following administration (Fig. 3). Interestingly, in the S-1-treated mice, there appeared to be a discrepancy between the tumor accumulation of l-OHP (Fig. 2) and liposomes at 24 h following administration. Although the mechanism is uncertain,

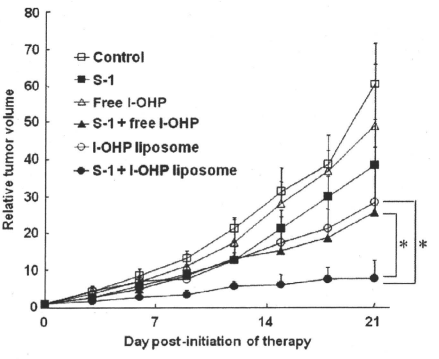

Fig. 1. Antitumor effect of mono- or combination chemotherapy in colorectal tumor-bearing mice. Control (non-treated, □); S-1 dosing (daily, ■); free l-OHP (weekly, △); S-1 dosing (daily) plus free oxaliplatin (l-OHP) (weekly) (▲); l-OHP-containing polyethylene glycol (PEG)-coated liposomes (weekly, ○); S-1 dosing (daily) plus l-OHP-containing PEG-coated liposomes (weekly) (●). Data represent mean ± SD ($n = 5$). *$P < 0.05$.

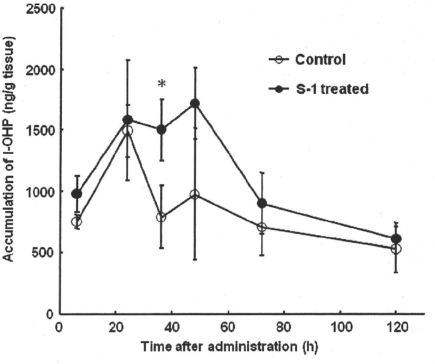

Fig. 2. Effect of S-1 dosing on tumor accumulation of oxaliplatin (l-OHP) delivered by polyethylene glycol (PEG)-coated liposomes. Oxaliplatin-containing PEG-coated liposomes were intravenously administered into tumor-bearing mice that were pre-treated with or without S-1 dosing for 7 days. At various time points, tumor tissue was collected and then l-OHP in the tissue was determined. Data represent mean ± SD ($n = 3$). *$P < 0.05$ *vs* control.

doi: 10.1111/j.1349-7006.2010.01678.x

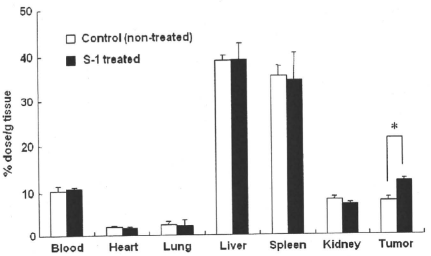

Fig. 3. Effect of S-1 dosing on biodistribution of polyethylene glycol (PEG)-coated liposomes. Biodistribution of PEG-coated liposomes was determined at 24 h following intravenous injection in tumor-bearing mice pretreated with or without S-1 dosing for 7 days. Data represent mean ± SD (n = 3). *P < 0.05.

l-OHP that leaked from the liposome and then bound to plasma proteins and partitioned to erythrocytes might affect the platinum concentration in the tumor. In addition, the treatment did not affect accumulation of PEG-coated liposomes in the major organs (Fig. 3). This observation indicates that the S-1 treatment does not affect the biodistribution of PEG-coated liposomes and the permeability of blood vessels towards the liposomes already existing in normal tissues.

In addition, *in vivo* imaging studies indicated a similar tendency of intratumor accumulation of PEG-coated liposomes as a function of time following injection (Fig. 4). Both the control and S-1-treated mice showed time-dependent augmentation of PEG-coated liposome accumulation. These findings indicate that PEG-coated liposomes accumulated in tumor tissue due to the EPR effect, and S-1 treatment facilitated the EPR effect towards PEG-coated liposomes, resulting in further accumulation of PEG-coated liposomes in solid tumor.

To investigate the intratumoral distribution of PEG-coated liposomes, a histological analysis was carried out. Fluorescence associated with PEG-coated liposomes was observed in the section of both control and S-1-treated tumor (Fig. 5A). The number and size of fluorescence spots in the section of S-1-treated tumor were substantially larger than those in the section of the control tumor, indicating that the S-1 treatment enhanced liposome distribution in tumor tissue. The area density of fluorescence in the tumor section indicated that the sections of S-1 treated tumor contain a much larger amount of PEG-coated liposomes than the section of control tumor (Fig. 5B).

Discussion

In the foregoing section we showed that the combination of oral metronomic S-1 dosing with oxaliplatin (l-OHP)-containing PEG-coated liposomes exerts improved antitumor activity in a murine colorectal tumor model without causing severe side-effects, as compared with conventional combination therapy (metronomic S-1 dosing plus free l-OHP) (Fig. 1). This improvement resulted from enhanced accumulation of l-OHP-containing PEG-coated liposomes and prolonged retention of l-OHP in the tumor induced by metronomic S-1 treatment (Figs 2–5). The FOLFOX regimen (5-FU/LV plus l-OHP) has frequently been used for treatment of advanced colorectal cancer in the clinic.[4,5] However, extended periods of infusional 5-FU (≈48 h) have the disadvantage of increased inconvenience and morbidity of patients related to the use of a portable infusion pump and a central venous catheter. Daily oral administration of

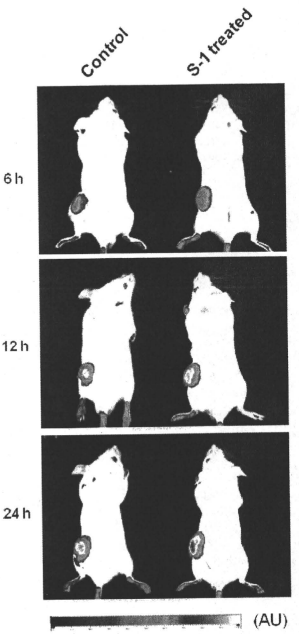

Fig. 4. *In vivo* optical imaging of tumor accumulation of polyethylene glycol (PEG)-coated liposomes. Tumor-bearing mice, pretreated with S-1 dosing for 7 days, received an intravenous injection of DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine perchlorate)-labeled PEG-coated liposomes. At 6, 12 and 24 h post-injection, *in vivo* optical images were recorded. AU, arbitrary unit.

S-1 can mimic the pharmacokinetic profile of infusional 5-FU and overcome the problems related to infusional 5-FU treatment. In fact, Yamada *et al.*[23] recently reported in a Phase I/II trial that the combination of S-1 with free l-OHP (SOX) is a preferable alternative to the FOLFOX regimen in metastatic colorectal cancer. In contrast to cisplatin, l-OHP has no renal toxicity, only mild hematological and gastrointestinal toxicity, while neurotoxicity is the dose-limiting toxicity.[24,25] The selective delivery of l-OHP to tumors by PEG-coated liposomes raises the possibility of reducing the side-effects of l-OHP in the FOLFOX and SOX regimens. Accordingly, the proposed combination regimen (i.e. addition of S-1 dosing to l-OHP-containing PEG-coated liposomes) may be an alternative to FOLFOX and SOX in advanced colorectal cancer therapy.

Accumulation of nanocarriers into solid tumor after systemic administration is thought to involve the following three processes: (i) distribution through the vascular compartment; (ii) transport across the angiogenic vascular wall (extravasation from neo-vasculature); and (iii) diffusion within the tumor interstitium.[26] It is generally believed that the major target of metronomic chemotherapy is endothelial cells of the growing

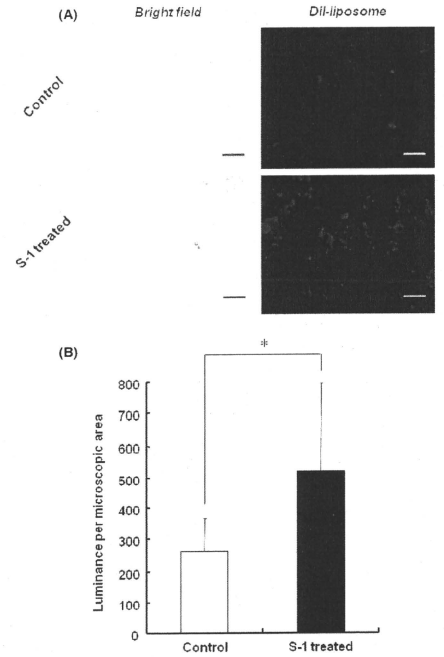

Fig. 5. Effect of S-1 dosing on intratumoral distribution of polyethylene glycol (PEG)-coated liposomes. Tumor-bearing mice, pretreated with S-1 dosing for 7 days, received DiI (1, 1′-dioctadecyl-3, 3, 3′,3′-tetramethyl-indocarbocyanine perchlorate)-labeled PEG-coated liposomes. At 24 h post-injection, the section of tumor was examined with fluorescence microscopy. (A) Intratumoral distribution of PEG-coated liposomes. Red spots represent liposomal distribution. Bar, 100 μm. Original magnification, ×200. (B) Mean fluorescence intensity per microscopic area. Data represent mean ± SD. $*P < 0.05$.

vasculature in the solid tumor. Ooyama *et al.*[27] recently demonstrated that metronomic S-1 dosing damages endothelial cells of tumor vasculature. Loss of the endothelial lining of vessels may make tumor vasculature much leakier. On the basis of our current and previous results,[16] we conclude that the therapy enhances the EPR effect towards PEG-coated liposomes in the following manner: the therapy causes blood vessels in the tumor to become more leaky, resulting in enhanced extravasation of PEG-coated liposomes from the vasculature into the interstitial space of the tumor (Figs 3,4). Moreover, metronomic therapy with S-1 may also exert a cytotoxic effect on viable tumor cells and stromal cells and thus bring about a decrease in the number of both cell types and, consequently, a decrease in the tumor interstitial pressure and enlargement of tumor interstitial space, which, in turn, will allow deeper penetration of the extravasated PEG-coated liposomes (Fig. 5). A similar observation was recently reported by Nagano *et al.*;[28] paclitaxel-induced tumor cell death enhanced the penetration and distribution of virus vector and microspheres in tumor tissue.

In addition to intratumoral accumulation of PEG-coated liposomes, the liposome distribution to major organs was investigated. Metronomic S-1 dosing did not affect accumulation of the liposomes in major organs and blood clearance of the liposomes (Fig. 3). This finding suggests that S-1 treatment does not affect normal vasculature pre-existing in normal tissues, but only the vasculature in tumors, although the mechanism by which S-1 changes only tumor vascular permeability remains unclear. This clearly relates to a safety issue in the proposed combination therapy. In addition, it appears that S-1 treatment does not affect the essential phagocytic uptake activity of hepatic and splenic macrophages, because the treatment did not affect blood clearance of PEG-coated liposomes. Daemen *et al.*[29] have previously reported that injection of DXR-loaded PEG-coated liposomes has a toxic effect on liver macrophages, both in terms of specific phagocytic activity and cell numbers. It is known that defects in the phagocytic uptake mechanism of macrophages can enhance metastatic growth, as reported in numerous animal studies.[30] The cytotoxic effect of l-OHP-containing PEG-coated liposomes on macrophages has not been elucidated yet. Hence, further experiments are in progress to ascertain the alteration of the tumor microenvironment (such as vascular permeability and compressive mechanical force of growing tumor cells) induced by metronomic S-1 dosing and cumulative toxicity of combination therapy of S-1 with l-OHP-containing PEG-coated liposomes.

Anticancer chemotherapy using nanocarriers has shown marked therapeutic effects in many tumor models; however, nanocarriers do not always accumulate effectively in solid tumors, probably due to barriers generated by the tumor microenvironment. Recently, a number of approaches have been introduced that render chemotherapeutics associated with a nanocarrier more efficient. Iyer *et al.*[31] demonstrated that hypertension induced by infusion of angiotensin-II (AT-II) increased the blood flow volume and generated a pressure gradient between the intra- and extravascular space in tumor tissue, resulting in increased extravasation from the tumor vessel of an anticancer agent associated with a nanocarrier. They assumed that the selective accumulation in a solid tumor can be attributed to the absence of a vascular smooth-muscle layer in tumor vasculature. This approach also did not increase the amount of nanocarrier accumulating in healthy organs because of vasoconstriction and tighter endothelial gap junctions of the vasculature. Kano *et al.*[10] demonstrated that low-dose treatment with transforming growth factor-β type 1 receptor inhibitor resulted in further enhanced accumulation of PEG-coated liposomes and micelles to a solid tumor. Seynhaeve *et al.*[12] showed similar results with low-dose TNF-α administration. Our approach also has a potential to achieve enhanced accumulation of PEG-coated liposomes in solid tumors. Hence, the approach that actively causes alteration of the tumor microenvironment by treatment with vaso-active agents or anticancer agents may become a breakthrough in improved delivery of anticancer agents associated with nanocarriers.

Acknowledgments

We thank Dr G.L. Scherphof for his helpful advice on writing the English manuscript. This study was supported in part by a Grant-in-Aid for Young Scientists (A) (21689002), the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Disclosure Statement

No potential conflicts of interest are disclosed.

doi: 10.1111/j.1349-7006.2010.01678.x

References

1 Kidani Y, Noji M, Tashiro T. Antitumor activity of platinum(II) complexes of 1,2-diamino-cyclohexane isomers. *Gann* 1980; **71**: 637–43.
2 Mathe G, Kidani Y, Noji M, Maral R, Bourut C, Chenu E. Antitumor activity of l-OHP in mice. *Cancer Lett* 1985; **27**: 135–43.
3 Pendyala L, Creaven PJ. In vitro cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. *Cancer Res* 1993; **53**: 5970–6.
4 Saltz LB, Cox JV, Blanke C *et al.* Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 2000; **343**: 905–14.
5 Goldberg RM, Sargent DJ, Morton RF *et al.* A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol* 2004; **22**: 23–30.
6 Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986; **46**: 6387–92.
7 Suzuki R, Takizawa T, Kuwata Y *et al.* Effective anti-tumor activity of oxaliplatin encapsulated in transferrin-PEG-liposome. *Int J Pharm* 2008; **346**: 143–50.
8 Abu Lila AS, Kizuki S, Doi Y, Suzuki T, Ishida T, Kiwada H. Oxaliplatin encapsulated in PEG-coated cationic liposomes induces significant tumor growth suppression via a dual-targeting approach in a murine solid tumor model. *J Control Release* 2009; **137**: 8–14.
9 Abu-Lila AS, Suzuki T, Doi Y, Ishida T, Kiwada H. Oxaliplatin targeting to angiogenic vessels by PEGylated cationic liposomes suppresses the angiogenesis in a dorsal air sac mouse model. *J Control Release* 2009; **134**: 18–25.
10 Kano MR, Bae Y, Iwata C *et al.* Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-beta signaling. *Proc Natl Acad Sci USA* 2007; **104**: 3460–5.
11 ten Hagen TL, van Der Veen AH, Nooijen PT, van Tiel ST, Seynhaeve AL, Eggermont AM. Low-dose tumor necrosis factor-alpha augments antitumor activity of stealth liposomal doxorubicin (DOXIL) in soft tissue sarcoma-bearing rats. *Int J Cancer* 2000; **87**: 829–37.
12 Seynhaeve AL, Hoving S, Schipper D *et al.* Tumor necrosis factor alpha mediates homogeneous distribution of liposomes in murine melanoma that contributes to a better tumor response. *Cancer Res* 2007; **67**: 9455–62.
13 Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 2004; **4**: 423–36.
14 Laquente B, Vinals F, Germa JR. Metronomic chemotherapy: an antiangiogenic scheduling. *Clin Transl Oncol* 2007; **9**: 93–8.
15 Shiraga E, Barichello JM, Ishida T, Kiwada H. A metronomic schedule of cyclophosphamide combined with PEGylated liposomal doxorubicin has a highly antitumor effect in an experimental pulmonary metastatic mouse model. *Int J Pharm* 2008; **353**: 65–73.
16 Ishida T, Shiraga E, Kiwada H. Synergistic antitumor activity of metronomic dosing of cyclophosphamide in combination with doxorubicin-containing PEGylated liposomes in a murine solid tumor model. *J Control Release* 2009; **134**: 194–200.
17 Shirasaka T, Nakano K, Takechi T *et al.* Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxypyridine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. *Cancer Res* 1996; **56**: 2602–6.
18 Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T. Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur-0.4 M gimestat-1 M otastat potassium) in advanced gastric cancer patients. *Eur J Cancer* 1998; **34**: 1715–20.
19 Sakuramoto S, Sasako M, Yamaguchi T *et al.* Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357**: 1810–20.
20 Bartlett GR. Colorimetric assay methods for free and phosphorylated glyceric acids. *J Biol Chem* 1959; **234**: 469–71.
21 Cabral H, Nishiyama N, Kataoka K. Optimization of (1,2-diamino-cyclohexane)platinum (II)- loaded polymeric micelles directed to improved tumor targeting and enhanced antitumor activity. *J Control Release* 2007; **121**: 146–55.
22 Harashima H, Yamane C, Kume Y, Kiwada H. Kinetic analysis of AUC-dependent saturable clearance of liposomes: mathematical description of AUC dependency. *J Pharmacokinet Biopharm* 1993; **21**: 299–08.
23 Yamada Y, Tahara M, Miya T *et al.* Phase I/II study of oxaliplatin with oral S-1 as first-line therapy for patients with metastatic colorectal cancer. *Br J Cancer* 2008; **98**: 1034–8.
24 Grothey A. Oxaliplatin-safety profile: neurotoxicity. *Semin Oncol* 2003; **30**: 5–13.
25 Pietrangeli A, Leandri M, Terzoli E, Jandolo B, Garufi C. Persistence of high-dose oxaliplatin-induced neuropathy at long-term follow-up. *Eur Neurol* 2006; **56**: 13–6.
26 Lu D, Wientjes MG, Lu Z, Au JL. Tumor priming enhances delivery and efficacy of nanomedicines. *J Pharmacol Exp Ther* 2007; **322**: 80–8.
27 Ooyama A, Oka T, Zhao HY, Yamamoto M, Akiyama S, Fukushima M. Anti-angiogenic effect of 5-Fluorouracil-based drugs against human colon cancer xenografts. *Cancer Lett* 2008; **267**: 26–36.
28 Nagano S, Perentes JY, Jain RK, Boucher Y. Cancer cell death enhances the penetration and efficacy of oncolytic herpes simplex virus in tumors. *Cancer Res* 2008; **68**: 3795–802.
29 Daemen T, Regts J, Meesters M, ten Kate MT, Bakker-Woudenberg IA, Scherphof GL. Toxicity of doxorubicin entrapped within long-circulating liposomes. *J Control Release* 1997; **44**: 1–9.
30 Phillips NC. Kupffer cells and liver metastasis. Optimization and limitation of activation of tumoricidal activity. *Cancer Metastasis Rev* 1989; **8**: 231–52.
31 Iyer AK, Khaled G, Fang J, Maeda H. Exploiting the enhanced permeability and retention effect for tumor targeting. *Drug Discov Today* 2006; **11**: 812–8.

Journal of Controlled Release 142 (2010) 160–166

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Journal of Controlled Release

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CpG motifs in pDNA-sequences increase anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes

Tatsuaki Tagami [a], Kazuya Nakamura [a], Taro Shimizu [a], Naoshi Yamazaki [b], Tatsuhiro Ishida [a], Hiroshi Kiwada [a,*]

[a] Department of Pharmacokinetics and Biopharmaceutics, Subdivision of Biopharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan

[b] Department of Medicinal Biochemistry, Subdivision of Biopharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan

ARTICLE INFO

Article history:
Received 6 July 2009
Accepted 14 October 2009
Available online 20 October 2009

Keywords:
Accelerated blood clearance (ABC) phenomenon
Polyethylene glycol (PEG)
Anti-PEG IgM
PEG-coated pDNA-lipoplex
CpG motifs

ABSTRACT

Gene therapy is largely dependent on the development of efficient delivery vehicles. To prolong their circulating time, PEGylation of the surface of a delivery vehicle is frequently applied. However, we have reported previously that anti-PEG IgM produced by intravenous injection of PEG-coated liposome is responsible for enhanced clearance of second dose PEG-coated liposomes, which is known as the "accelerated blood clearance (ABC) phenomenon." A similar phenomenon has been observed with PEG-coated pDNA-lipoplexes (PDCLs) upon their repeated injection. But the effect of the sequence of pDNA in PDCLs on inducing the ABC phenomenon has not been thoroughly investigated. Here, we focus on CpG motifs in pDNA, which are known to have a potent immune-stimulatory activity. PDCLs with non-CpG pDNA (PNDCL) diminished the anti-PEG IgM response, resulting in significant accumulation of a second dose in tumor tissue, comparable to that of a single injection, but not in enhanced accumulation in liver. In addition, PDCL induced proliferation of IgM^+ splenic cells including B cells. These results suggest that the CpG motif is a major cause of the induction of the ABC phenomenon when PDCLs are repeatedly injected. Immunogenicity is a relevant point of concern for non-viral delivery systems. Our results indicate that the use of non-CpG pDNA may allow meaningful repeated dosing of pDNA formulations without the induction of a strong immune reaction and thus may have important implications for therapeutic use of liposomal formulations of nucleic acids.

1. Introduction

Gene therapy has been proposed as a promising strategy for treating genetic and acquired diseases, but convincing therapeutic results have been limited thus far, predominantly because of a lack of sufficiently efficient and safe delivery system [1,2]. Although viral vectors are highly efficient, there are justified concerns about their safety related to immunogenicity and random incorporation of the delivered gene into the host genome. On the other hand, non-viral vectors, despite their relatively low transfer efficiency, are attractive alternatives to viral vectors because of their safety, versatility and ease of preparation and scaling-up.

Cationic liposomes present one of the most useful non-viral vector systems [3]. pDNA/cationic liposome complexes (pDNA-lipoplexes) can bind efficiently to the cell surface via charge–charge interactions, followed by internalization into the cells and subsequent gene expression *in vitro*. However, therapeutic success of pDNA-lipoplexes in the clinical situation, requiring for example systemic delivery, is jeopardized due to short blood circulation times. Surface modification of pDNA-lipoplexes with polyethylene glycol (PEG)-conjugated lipid (PEGylation) is frequently applied to prolong circulation time of lipoplexes [4]. It is believed that the PEG on the liposomal surface attracts a water shell, resulting in the reduced adsorption of opsonins and thus the recognition of the lipoplexes by the cells of the mononuclear phagocyte system (MPS) [5,6]. The PEG-coated pDNA-lipoplexes (PDCLs) thus obtained possess long circulation properties, and therefore can accumulate efficiently in solid tumors [7] because of the enhanced vascular permeability and retention effect in growing tumors [8].

However, we and others have reported that an intravenous injection of PEG-coated liposomes causes a second dose of material, injected a few days later, to lack the long-circulating characteristics of the first dose and to extensively accumulate in the liver [9,10]. This phenomenon is known as the "accelerated blood clearance (ABC) phenomenon." Based on earlier results [11,12], we have proposed the following tentative mechanism of this phenomenon: anti-PEG IgM,

* Corresponding author. Tel.: +81 88 633 7259; fax: +81 88 633 7260.
E-mail address: hkiwada@ph.tokushima-u.ac.jp (H. Kiwada).

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doi:10.1016/j.jconrel.2009.10.017

produced in the spleen in response to a first dose, selectively binds to the PEG of the second dose of liposomes injected several days later and subsequently activates the complement system. This, in turn, leads to opsonization of the second dose of liposomes by C3 fragments and, as a consequence, to enhanced uptake of the liposomes by the Kupffer cells in the liver.

The ABC phenomenon involving anti-PEG IgM production is an important factor to be considered in designing an efficient delivery system of genes or nucleic acids. Judge et al. [7] have recently reported that PEG-coated lipid nano-particles encapsulating pDNA greatly enhance anti-PEG IgM production when compared with PEG-coated nano-particles without encapsulated pDNA, and that, as a consequence, gene expression relating to pDNA in tumor tissue was strongly diminished following its second injection. However, the mechanism underlying the enhancing effect of pDNA on anti-PEG IgM response and the accelerated blood clearance of the second dose of PEG-coated lipid nano-particles encapsulating pDNA has not been elucidated yet. Here, we focus on CpG motifs in the pDNA-sequence, which can activate the toll-like receptor 9 (TLR9) signaling pathway in immune competent cells and induce the production of a variety of inflammatory cytokines and interferons [13]. In addition, CpG motifs have a strong adjuvant effect which contributes to the increased immunogenicity [14]. In view of these considerations it would not be surprising if the presence of CpG motifs in pDNA formulations would act as a potent stimulator of the IgM response. In the present study, we investigated the effect of CpG motifs in pDNA-sequence on anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes and the contribution of splenic B cells to the anti-PEG IgM responses caused by PEG-coated pDNA-lipoplexes.

2. Materials and methods

2.1. Materials

2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and dioleoylphosphatidylethanolamine (DOPE) 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 (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Life Science (MA, USA). All other reagents were of analytical grade.

2.2. Preparation of pDNA

pEGFP-N1 which contains CpG motifs (4733 bp, 321 CpG points (13.56%)) was purchased from Clontech (CA, USA). pCpG-mcs (3066 bp) which lacks CpG motifs was purchased from Invivogen (CA, USA). pEGFP-N1 and pCpG-mcs were amplified in E. coli strain DH5α and GT115, isolated by using EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). The level of endotoxin in the DNA preparation was always <0.1 endotoxin unit/μg DNA as measured by the Limulus amoebocyte lysate assay (BioWhittaker, MO, USA).

2.3. Animals and cells

Male Std-ddY mice aged 4–5 weeks (20–25 g), male BALB/cCr Slc mice aged 4–5 weeks (20–25 g) and male BALB/c Slc-nu/nu mice aged 5–6 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.

Mouse sarcoma, sarcoma 180 (S-180) cells were injected intraperitoneally and maintained in the peritoneum of ddY mice. Mice were sacrificed at 2 weeks after intraperitoneal inoculation of S-180 cells and the ascitic fluid containing S-180 cells was collected. Cells were washed 3 times by phosphate buffered saline (PBS), and the prepared cell suspension was used for subsequent experiments.

2.4. Preparation of cationic liposomes

Cationic liposomes were composed of DC-6-14:POPC:CHOL:DOPE (10:30:30:30, molar ratio). Liposomes were prepared as previously described [15]. Briefly, the lipids were dissolved in chloroform, and after evaporation of the organic solvent, the resulting lipid film was hydrated in 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion thorough polycarbonate membrane filters (Nucleopore, CA, USA) with consecutive pore sizes of 400, 200 and 100 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 for cationic liposomes were 93.4 nm and +20.4 mV ($n=3$), respectively. The lipid concentration of the liposomes was determined by using a Cholesterol E-test Wako kit (Wako Pure Chemical, Osaka, Japan) and approximately 25 mM.

2.5. Preparation of PEG-coated pDNA-lipoplexes (PDCLs) and PEG-coated "empty" cationic liposomes (PCL)

For the formulation of pDNA-lipoplexes, pDNA (10 μg) and cationic liposomes (1 μmol, phospholipids) were mixed at 3.82 (+/−) charge ratio and incubated for 20 min at room temperature. For PEGylation, a post-insertion technique was employed [16,39]. Briefly, $mPEG_{2000}$-DSPE (5 mol% of total lipid) in 9% sucrose solution was added into either pDNA-lipoplex or cationic liposome solution. The mixture was vortexing and gently shaking for 1 h at 37 °C. Under the condition, almost 100% of $mPEG_{2000}$-DSPE added could be incorporated into preformed pDNA-lipoplexes [16]. The mean diameter was 315.3 nm for PEG-coated pDNA-lipoplexes (PDCLs), 309.9 nm for PEG-coated non-CpG pDNA-lipoplexes (PNDCL) and 108.1 nm for PEG-coated cationic liposomes (PCL) ($n=3$). The mean zeta potential was +14.5 mV for PDCLs, +13.1 mV for PNDCL and +20.5 mV for PCL ($n=3$). To determine the biodistribution of PEG-coated lipoplexes, cationic liposomes were labeled with a trace amount of ^{3}H-CHE (40 μCi/μmol of phospholipids) as a non-exchangeable lipid phase marker.

2.6. Biodistribution of single and second dose in tumor-bearing mice

S-180 cells (2×10^6 cells) were implanted subcutaneously in the dorsal skin of ddY mice. On day 2 after tumor inoculation, pretreatment was given. For the pretreatment, either PDCL, PNDCL (5 μmol phospholipids and 50 μg pDNA/mouse, approximately 125 μmol phospholipids and 1.25 mg pDNA per kg of body weight, respectively) or saline was intravenously administered into mice via the tail vein. On day 7 after tumor inoculation (5 days after the pretreatment) when the tumor had reached a diameter of 4–5 mm but no necrotic areas were apparent, radio (^{3}H-CHE)-labeled test dose (PDCL or PNDCL, 5 μmol phospholipids and 50 μg pDNA/mouse, respectively) was intravenously administered into the treated mice via the tail vein. At 24 h after the injection, the mice were sacrificed. Blood samples were withdrawn by heart puncture, and then tumor tissue as well as normal tissues including liver, kidney and lung were collected from the mice and weighed after withdrawing the blood samples. Radioactivities in blood and tissues were assayed as described previously [17].

2.7. Detection of anti-PEG IgM

A simple ELISA procedure as described previously [15] was employed to detect anti-PEG IgM in the serum. Briefly, 10 nmol of

$mPEG_{2000}$-DSPE in 50 μl ethanol was added to 96-well plates. Lipid-coated plates were allowed to air dry completely for 2 h. The plates were then blocked for 1 h with Tris-buffered saline containing 1% BSA and were subsequently washed three times. Diluted serum samples (1:100) (100 μl) were then applied in the wells, incubated for 1 h and washed. 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. 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 2 N 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. In vivo B-cell proliferation (BrdU incorporation assay)

To assess *in vivo* B-cell proliferation, mice were given drinking water containing BrdU (Sigma) at 0.8 mg/ml, which was made fresh and changed daily. One day after the start of BrdU administration, either PDCL, PNDCL (5 μmol phospholipids and 50 μg pDNA/mouse) or PCL (5 μmol phospholipids/mouse) was intravenously administered via the tail vein. Two days later, the spleen was removed. Spleen single-cell suspensions were prepared as described previously [15]. Briefly, spleen slices were pressed through a Cell Strainer (100 μm, Becton Dickinson, NJ, USA) and cells were subsequently washed by PBS (pH 7.4, Nissui Pharmaceutical, Tokyo, Japan). Red blood cells were lysed by treatment with 5 mL of ammonium chloride lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ 0.1 mM Na_4EDTA, pH 7.2) for 5 min on ice and subsequently washed.

IgM-expressing splenic B cells were stained with FITC-conjugated anti-mouse IgM (Goat anti-mouse IgM-FITC conjugate; American Qualex Antibodies, CA, USA) for 1 h at room temperature and subsequently washed. For staining BrdU incorporation, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2% Triton-X for 15 min on ice and treated with 1 U of DNase/ml for 1 h at 37 °C and subsequently washed for each procedure. Cells were then incubated with Phycoerythrin (PE)-conjugated anti-mouse BrdU (Goat anti-mouse BrdU-PE conjugate; Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature and subsequently washed. Cells were analyzed by using a flow cytometer, Guava EasyCyte Mini (Guava Technologies, CA, USA).

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. Anti-PEG IgM production induced by a single injection of either PCL, PDCL or PNDCL

We determined the effect of the presence of pDNA in the PEG-coated lipoplex on anti-PEG IgM production. Anti-PEG IgM production was assessed on day 5 after a single injection of either PEG-coated "empty" cationic liposome (PCL) or PEG-coated pDNA-lipoplexes (PDCLs), by which time the ABC phenomenon is markedly manifest [18]. We confirmed that a low-dose single injection of PCL caused a significant induction of anti-PEG IgM production (Fig. 1). Consistent with our earlier observations [19], the level of induction was reversely related to the dose of PCL: the higher dose the lower the induction. PDCLs also induced substantial production of anti-PEG IgM at low dose, but here no decline in response was observed upon increasing

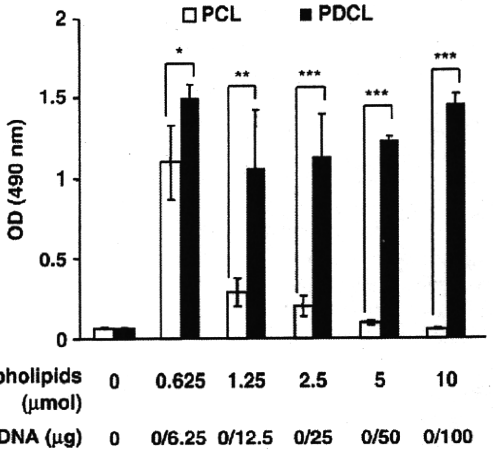

Fig. 1. Anti-PEG IgM production induced by a single injection of either PCL or PDCL. PCLs or PDCLs were intravenously injected at the indicated doses. Five days later, blood was withdrawn from each treated mouse and serum was collected. The sera collected from the naïve mice were used as controls (dose 0). PCL did not contain pDNA. Anti-PEG IgM was detected with ELISA as described in Materials and methods. Each value represents the mean ± SD ($n=4$). *$p<0.05$, **$p<0.01$, ***$p<0.005$.

the dose. Apparently, pDNA in the lipoplexes strongly stimulated the immune system to produce anti-PEG IgM.

Fig. 2 presents the results of experiments in which we studied the effect of CpG motifs in the pDNA entrapped in the PCL, on anti-PEG IgM production. On day 5 after a single injection of either PDCL or PEG-coated non-CpG pDNA-lipoplex (PNDCL), anti-PEG IgM in serum was determined. Clearly, PNDCL with non-CpG pDNA caused a much lower the anti-PEG IgM response than PDCL with CpG-containing pDNA. It is well known that CpG motifs in pDNA are a potent immune stimulator [14]. Hence, it is likely that the CpG motifs in pDNA were a major cause of the enhanced anti-PEG IgM production at the higher dose of PDCLs, as presented in Fig. 1.

3.2. Effect of prior dosing on biodistribution of test doses of PDCL or PNDCL

The biodistribution of radio-labeled test doses of PDCL or PNDCL was investigated with or without pre-dosing 24 h after injection. As shown in Fig. 3, without pre-dosing there were no significant differences between PDCL and PNDCL in the fractions of injected

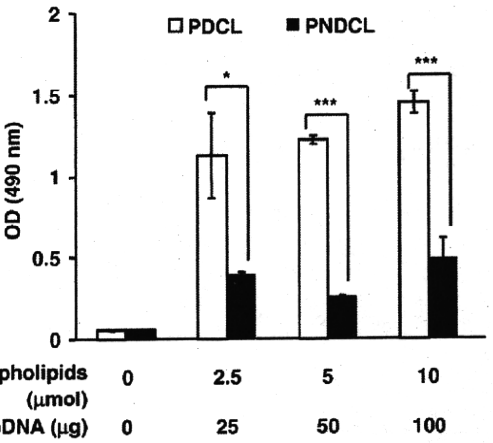

Fig. 2. Effect of CpG motifs in pDNA on anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes. Experimental conditions were identical to those in Fig. 1 except that PDCLs or PNDCLs were injected.

dose remaining in blood and accumulating in all organs including tumor. This indicates that the CpG motif in pDNA by itself does not affect the biodistribution of PEG-coated lipoplexes in mice.

After pre-dosing, however, significant differences were observed between biodistribution of the second test doses of the two types of particle. Again, tumor-bearing mice received a radio-labeled test dose of either PDCL or PNDCL, but now after first having received a pre-dose of the same particles 5 days earlier. Test-dose radioactivity in blood, major organs and implanted tumor was determined 24 h after injection. After pre-dosing, blood clearance and biodistribution of the second PDCL dose were markedly altered (Fig. 3). The amount remaining in blood was less than half of that found without pre-dosing, while accumulation of PDCLs in liver and spleen was significantly enhanced and that in tumor substantially reduced. A prior injection of PNDCL, on the other hand, did not at all affect the biodistribution of a second test PNDCL dose. These results suggest that the CpG motifs in the pDNA in PDCLs, are the major cause of the enhanced blood clearance of test-dose particles, resulting in substantially reduced accumulation in tumor tissue.

3.3. Anti-PEG IgM production in T-cell-deficient (nude) mice

We recently reported that an intravenous injection of PEG-coated "empty" liposomes or PEG-coated siRNA-lipoplex caused anti-PEG IgM production in a T-cell-independent manner [15,18]. To study the contribution of T cells to anti-PEG IgM production, T-cell-deficient (nude) mice received either PCL, PDCL or PNDCL. As shown in Fig. 4, anti-PEG IgM production was detected in all pretreated nude mice. This indicates that T cells do not play an important role in the anti-PEG IgM production. Interestingly, anti-PEG IgM production induced by PCL (both doses) or PNDCL (only higher dose) was significantly enhanced in nude mice compared to in naïve mice. It would appear that T cells in naïve mice rather attenuate the production of anti-PEG IgM in a dose-dependent manner.

3.4. Proliferation of IgM-expressing splenic B cells in vivo

In our earlier studies, we showed that spleen plays an important role in the production of anti-PEG IgM [11,18]. Because it is well known that B cells secrete IgM while extensively proliferating in spleen [20–22], we assumed that CpG motifs in pDNA might act as "B-cell mitogen," resulting in enhanced proliferation of splenic B cells.

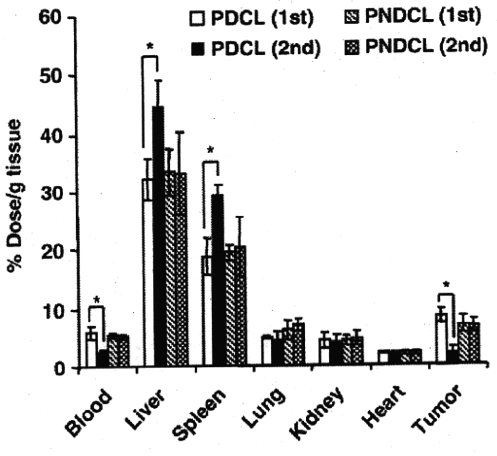

Fig. 3. Effect of prior dose on biodistribution of test doses of PDCL or PNDCL. At day 5 after the pretreatment with either PDCLs or PNDCLs (5 μmol phospholipids and 50 μg pDNA/mouse), ^{3}H-CHE labeled PDCLs or PNDCLs (5 μmol phospholipids and 50 μg pDNA/mouse) were intravenously injected. At 24 h after injection, ^{3}H activity in each tissue and blood was determined as described in Materials and methods. "1st" means without pretreatment. "2nd" means with pretreatment. Each value represents the mean ± SD ($n = 4$). * $p < 0.05$.

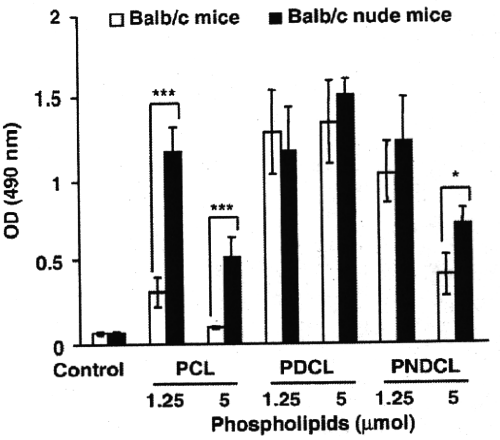

Fig. 4. Anti-PEG IgM production in T-cell-deficient (nude) mice. Either PCL, PDCL or PNDCL was intravenously injected into (A) BALB/c mice or (B) BALB/c nude mice. Five days later, blood was withdrawn from each mice and serum was collected. The sera collected from the naïve (non-treated) mice were used as controls. Anti-PEG IgM was detected with ELISA as described in Materials and methods. Each value represents the mean ± SD ($n = 4$). *$p < 0.05$, ***$p < 0.005$.

Therefore we determined BrdU incorporation and IgM expression in splenic cells by means of double immunofluorescent staining and flow-cytometric analysis at day 2 after a single injection of either PCL, PDCL or PNDCL (5 μmol phospholipids/mouse) (Fig. 5). The treatments with PCL, PDCL and PNDCL induced BrdU incorporation by IgM^{+} splenic cells, as compared to saline treatment. As expected, among these treatments, PDCLs showed the highest BrdU incorporation by IgM^{+} splenic cells, indicating that PDCLs did trigger proliferation of IgM-expressing splenic B cells. It appeared that CpG motifs in pDNA of PDCLs play a role as "B-cell mitogen," resulting in proliferation of splenic IgM-expressing cells, presumably splenic B cells.

4. Discussion

The use of non-viral vectors for gene therapy has many advantages over viral vectors such as safety, productivity and simplicity [1,2]. However, for *in vivo* use, repeated injections of such vectors will be required to compensate for their low transfection efficiency relative to viral vectors [23,24]. PEG-coated non-viral vectors have substantial advantages in prolonging blood circulation of drugs, including nucleic acids such as pDNA, oligodeoxynucleotide (ODN) or small interference RNA (siRNA). However, we have reported earlier that an intravenous injection of PEG-coated "empty" liposome causes a second dose of PEG-coated liposome, injected a few days later, to lose its long-circulating characteristics and to accumulate extensively in the liver [10] as a result of induction of anti-PEG IgM production by the first dose of PEG-coated "empty" liposomes [12,15]. This phenomenon is known as the "ABC phenomenon" [9]. Judge et al. recently reported that PEG-coated lipid nano-particles encapsulating pDNA cause an acute loss of prolonged blood circulation of a second dose due to enhanced anti-PEG IgM production [7]. However, the influence of the sequence of the encapsulated pDNA in the PEG-coated lipoplexes, namely the occurrence of CpG motifs, on the anti-PEG IgM response has not been thoroughly investigated yet.

In the present study, we showed that encapsulation of pDNA containing CpG motifs in PEG-coated cationic liposomes further facilitated the induction of anti-PEG IgM production (Fig. 1) while the use of non-CpG pDNA instead of pDNA with CpG motifs rather diminished the anti-PEG IgM production (Fig. 2). These findings clearly indicate that CpG motifs in pDNA play a key role in the induction of anti-PEG IgM production by intravenous injection of PEG-coated pDNA-lipoplexes and that the encapsulation of immune-