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「がん新生血管を標的としたAll in oneデバイスによる革新的siRNAデリバリーシステムとがん治療法の開発」に関する研究

平成１７～１９年度　総合研究報告書

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「がん新生血管を標的としたAll in oneデバイスによる革新的siRNAデリバリーシステムとがん治療法の開発」に関する研究

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研究要旨　がん新生血管を構成する血管内皮細胞内にsiRNAを選択的に導入し、細胞の恒常性の維持に深く関与しているRISC(RNA-induced silencing complex)の発現を抑制させ、結果的に細胞死（apoptosis）を誘導させることによりがん新生血管の破壊とそれに伴うがんの退縮を実現させうる革新的がん治療法の開発とそれを実現しうるデリバリーシステム（All in oneデバイス）の開発を実現させる事が本研究の目的である。これまでの検討から、①RISC構成成分の一つであるArgonaute2 (Ago2)をknockdownすることで細胞増殖抑制およびapoptosisが誘導されることを確認し、②がん新生血管に選択的にsiRNAを送達可能なキャリアー（リポソーム）を開発すると共に、siRNAを結合させた場合の方がむしろがん新生血管への親和性が高まることを見いだした。さらに、③細胞死を誘導するsiRNAを腫瘍内に直接投与することによって顕著に高い腫瘍増殖抑制効果が得られること、しかし一方で静脈内投与では有意な効果を得ることができないこと、を明らかにしてきた。残念ながら現状のシステム単独では当初の目的を果たすことはできなかったが、同様の現象が低分子や抗体などを用いた抗新生血管療法でも見られており、抗がん剤と併用投与することで高い抗腫瘍効果が得られるものと考えられる。以上の事から、今後本システムを発展させていくことで目的とした新規がん治療法の提案が十分果たされるものと考えている。

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A. 研究目的

RISC(RNA-induced silencing complex)は細胞の生存に深く関わっているとともに、普遍的に発現しているタンパク複合体である。RISCは、細胞内でsiRNAを取り込み、このsiRNAと相補的な配列を持つmRNAを酵素的に切断し、タンパクの発現を抑制する。しかし、RISCの存在量は限られており、①siRNAを細胞内に送達することでRISCを消費させる、②RISC構成タンパクに対するsiRNAを導入することによってRISCをknockdownする、ことにより細胞内RISC量を抑制し、細胞機能の破綻を導きapoptosisを誘導できるのではないかと考えた。がん新生血管を構成する血管内皮細胞内にsiRNAを選択的に導入し、細胞の恒常性の維持に深く関与しているRISCの発現を抑制させ、結果的に細胞死(apoptosis)を誘導させることによりがん新生血管の破壊とそれに伴うがんの退縮を実現させうる革新的がん治療法の開発とそれを実現しうるデリバリーシステム（All in oneデバイス）の開発を実現させる事が本研究の目的である。

B. 研究方法

（1）Knockdown効率の評価

HT-1080（ヒト繊維芽肉腫細胞）のwild typeおよびGFP安定発現株（GFP/HT-1080細胞）、ヒト臍帯静脈内皮細胞(HUVEC）を用いた。Knockdown効率はGFP由来の蛍光を指標に評価した。

（2）細胞増殖・毒性試験

MTT法およびLDH法によって評価した。

（3）細胞内動態解析

siRNAをFAMで蛍光標識したsiRNA/TFL-3複合体、あるいはリポソーム(TFL-3)をDilで蛍光標識した複合体を調製した。これら複合体を細胞に導入し、共焦点レーザー顕微鏡下観察した。

（4）Ago2-siRNA導入によるApoptosisの誘導

Caspase-3活性、およびPropidium iodideで核を染色すると同時に、annexin Vでphosphatidylserineを染色する事により、Ago2-siRNA導入後のapoptosis誘導状況を調べた。

（5）血管内皮細胞への標的化

DiDで修飾したsiRNA/TFLあるいはsiRNA/PCL複合体を調製し、HUVEC内に取り込まれたリポ

ソームを DiD の蛍光を指標として評価した。siRNA 導入量は、FAM 蛍光標識 siRNA の蛍光量を指標に評価した。

(6)血管内皮細胞への Ago2 knockdown の影響

HUVEC をモデルとし、Ago2 knockdown による血管類似構造体形成への影響を検討した。

(7)キャリアの新生血管集積性の検討

Dorsal air sac 法により人為的にマウス背部皮下に新生血管を誘導させ、これに蛍光ラベル siRNA あるいは蛍光ラベルリポソームを静脈内投与し、一定時間経過後動物を犠牲死させ、皮膚を採取し、皮内新生血管への集積を蛍光顕微鏡で評価した。

(8) siRNA/キャリア複合体の抗腫瘍効果の検討

siRNA/キャリア（リポソーム）複合体の in vivo 活性は、HT-1080 細胞移植ヌードマウスモデルを用い、腫瘍内への直接投与、あるいは静脈内への投与によって評価した。腫瘍内への直接投与の場合、細胞移植後 8 日目から投与を開始し、18 日目まで 2 日毎に計 6 回(総量 240μg)投与を行った。静脈内投与に関しては、細胞移植後 4 日後から 18 日目まで 2 日毎に計 8 回(総量 200μg)投与を行った。抗腫瘍活性は腫瘍体積の変化および生存日数を指標に評価した。

（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）細胞における Ago2 knockdown の影響

(Ⅰ)mRNA 量と細胞増殖への影響

Ago2-siRNA を Lipofectamine2000 により HT1080 細胞および HUVEC に導入し、Ago2-mRNA 量を PCR 法で測定した。Control として用いた luciferase に対する siRNA では Ago2-mRNA 発現量に影響はなく、両細胞共に Ago2-siRNA を用いた際に経時的な減少が確認された。

ついで、Ago2-siRNA が導入された際の細胞の増殖性の変化について検討した。導入した siRNA 量に依存した細胞増殖の抑制が観察された。また、Ago2 knockdown に対する感受性は、HUVEC の方が HT1080 細胞よりも高いことが分かった。

(Ⅱ)細胞増殖抑制のメカニズム

細胞増殖抑制効果がどのような機構によって誘導されているか、検討した。HT1080 細胞でも HUVEC でも、Ago2 knockdown によって持続的にアノイキスを介した apoptosis が誘導されている事を確認した。また、細胞周期解析の結果、細胞周期が G0/G1 期で停止しており、細胞分裂が顕著に抑制されている事が分かった。

(Ⅲ)血管類似構造体形成能への影響

次に血管類似構造体形成能力への影響について検討した。Ago2-siRNA で処置した細胞が有意に構造形成能力を失っていた。Ago2-siRNA 導入によって apoptosis まで至らなかった細胞群において、その血管類似構造体形成能が消失していることが明らかとなった。

（2）siRNA/TFL-3 複合体による細胞内 siRNA 導入機構の検討

siRNA/TFL-3 複合体形成時にエネルギー(攪拌、超音波など)を加える事で、高い遺伝子 knockdown 効果が得られた。これは、エネルギーを与えることによって複合体の粒子径が均一化され、各細胞に等しく siRNA を送達できたからであった。さらに、本来は膜融合によって細胞内に送達されていた siRNA が、エネルギーを与えた後には主として endocytosis によって送達されるようになっており、このような機構変化も knockdown 効果が向上した一因であることが示唆された。

（3）新生血管選択的キャリアの開発

Dorsal air sac model でマウス背部皮下に新生血管を誘導し、キャリアの移行性について検討した。種々の脂質組成に関して検討したところ、PEG を修飾し膜の流動性を高めたキャリアが高い親和性を示す事が分かった。さらに、興味深いことに、siRNA を結合させたものの方がより高い親和性を示すことが分かった。カチオニックリポソームは、正常血管内皮細胞との相互作用は弱いが、がん新生血管内皮細胞には比較的親和性が高い。新生血管内皮細胞は特徴的な proteoglycan などにより比較的高い負電荷を帯びており、この静電的相互作用がドライビングフォースになっているものと考えられる。一方、siRNA が結合したリポソームの表面電荷は負に傾き、静電的な相互作用は弱まるものと推測される。よって、siRNA に特徴的な結合機構が存在する可能性を考えている。本特徴を利用することで、より効率の良い siRNA デリバリーシステムの構築が可能になるものと期待される。

（4）siRNA/キャリア複合体による抗腫瘍効果

腫瘍内に直接投与する事により、in vitro で得られた細胞増殖抑制効果が in vivo で反映されるか

検討した。その結果、有意に高い腫瘍増殖抑制効果とともに生存日数の延長も観察された。よって、Ago2 を knockdown することによって in vivo でも高い効果が得られることが分かった。そこで、当初の目的である systemic injection（静脈内投与）による効果検討を行った。しかし、期待に反して、十分な効果を得ることができなかった。これは、①静脈内投与では直接投与ほど多量の siRNA を送達することができない、②マウスモデルにおける腫瘍内新生血管はマウス由来であり、ヒト Ago2 に対してデザインした siRNA が十分に機能しない、③他の新生血管阻害薬でも見られるように、血管新生抑制効果のみでは腫瘍体積の減少にはつながらない、などの理由によるものではないかと考えている。

D. 考察

本研究における目標は、①RISC 構成タンパクである Ago2 を選択的に knockdown することで細胞死を誘導すること、②siRNA をがん新生血管に選択的にデリバリーすることが可能なキャリアシステムを開発すること、③①②を組み合わせることで革新的ながん治療法を提案すること、であった。①に関しては、Ago2 を knockdown することで細胞増殖抑制およびアポトーシスが誘導されることを確認し、この siRNA に関して特許を出願した。②に関しては、がん新生血管に選択的に siRNA を送達可能なキャリアー（リポソーム）を開発すると共に、siRNA を結合させたときの方がむしろがん新生血管への親和性が高まることを見いだした。③に関しては、腫瘍内直接投与によって顕著に高い腫瘍増殖抑制効果が得られたが、静脈内投与では有意な効果を得ることができなかった。このような現象は、低分子や抗体などを用いた抗新生血管療法でも見られており、抗がん剤と併用投与することで高い抗腫瘍効果が得られるものと考えられる。以上の事から、当初の目標は９０％以上達成できたものと判断している。

siRNA は RISC に組み込まれ、配列特異的に mRNA を切断する。よって、その効果は available な RISC と mRNA の量に依存する。本検討では、RISC 自体を阻害することで細胞増殖抑制を誘導し、抗がん効果が得られることが明らかになっており、前述の課題を克服する革新的な成果である。

In vivo 応用を目的として、血中滞留性が高くがん新生血管へ高い親和性をもつ siRNA キャリアを開発した。細胞死を誘導する siRNA を搭載したキャリアを投与することで、がん新生血管の選択的な破壊とそれに伴うがん組織の退縮・血行性転移の抑制が実現されるものと期待される。本治療法の利点は、①化学療法とは異なり非選択的な毒性の発現が抑制でき、患者の quality of life(QOL)の向上が可能であること、②新生血管にはがん細胞のような多様性はなく全ての固形がんに適応可能であること、である。特にがん種を選ばない治療法の開発は、産業面ではデバイス開発の容易さ、治験の簡便さ、厚生労働行政面では認可手続きの簡略化にも通じる。

今後の課題としては、①Systemic injection でのデリバリー効率の改善、②種差の有無に関する検討と解決、および③治療法への展開、の３点であると考えている。

特に③に関しては、既存の新生血管阻害薬と同様に、抗がん剤と併用することによって高い抗腫瘍効果が得られる事が期待される。今後は、この可能性に関して検討を行う予定である。また、耐性を獲得したがんに対しても高い効果が得られる可能性があり、この点に関しても検討する。一方、安全性の観点からの情報は乏しく、繰り返し投与時の安全性の確認などを行う必要がある。

E. 結論

Ago2 knockdown による細胞増殖抑制と新生血管への選択的デリバリーシステムを融合させる事により、革新的がん治療法の開発が実現できる可能性が示された。

F. 研究発表

1. 論文発表

(1) Asai, T., Miyazawa, S., Maeda, N., Hatanaka, K., Katanasaka, Y., Shimizu, K., Shuto, S., Oku, N., Antineovascular therapy with angiogenic vessel-targeted polyethyleneglycol-shielded liposomal DPP-CNDAC. *Cancer Sci.*, **in press**

(2) Shiraga, E., Barichello, J.M., 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.*, **353**, 65-73 (2008)

(3) Asai, T., Suzuki, Y., Matsushita, S., Yonezawa, S., Yokota, J., Katanasaka, Y., Ishida, T., Dewa, T., Kiwada, H., Nango, M., Oku, N., Disappearance of the angiogenic potential of endothelial cells

caused by Argonaute2 knockdown. *Biochem. Biophys. Res. Commun.*, **368**, 243-248 (2008)

(4) Shimizu, K., Sawazaki, Y., Tanaka, T., Asai, T., Oku, N., Chronopharmacologic cancer treatment with an angiogenic vessel-targeted liposomal drug. *Biol. Pharm. Bull.*, **31**, 95-98 (2008)

(5) Hatakeyama, H., Akita, H., Ishida, E., Hashimoto, K., Kobayashi, H., Aoki, T., Yasuda, J., Obata, K., Kikuchi, K., Ishida, T., Kiwada, H., Harashima, H., Tumor targeting of doxorubicin by anti MT1-MMP antibody-modified PEG liposomes. *Int. J. Pharm.*, **342**, 194-200 (2007)

(6) Atobe K., Ishida T., Ishida, E., Hashimoto K., Kobayashi H., Yasuda J., Aoki, T., Obata K, Kikuchi H., Akita, H., Asai, T, Harashima, H., Oku, N., Kiwada, H., In vitro efficacy of a sterically stabilized immunoliposomes targeted to membrane type 1 matrix metalloproteinase (MT1-MMP). *Biol. Pharm. Bull.*, **30**, 972-978 (2007)

(7) Ishida, T., Wang X.Y., Shimizu, T., Nawata, K., Kiwada, H., PEGylated liposomes elicit an anti-PEG IgM response in a T-cells independent manner. *J. Control. Release*, **122**, 349-355 (2007)

(8) Katanasaka, Y., Asai, T., Naitou, H., Ohashi, N., Oku, N., Proteomic characterization of angiogenic endothelial cells stimulated with cancer cell-conditioned medium. *Biol. Pharm. Bull.*, **30**, 2300-2307 (2007)

(9) Isakari, Y., Harada, Y., Ishikawa, D., Matsumura-Takeda, K., Sogo, S., Ishida, T., Taki, T., Kiwada, H., Efficient gene expression in Megakaryocytic cell line using Nucleofection. *Int. J. Pharm.*, **338**, 157-164 (2007)

(10) Nguyen, L.T., Atobe, K., Barichello, J.M., Ishida, T., Kiwada, H., Complex formation with plasmid DNA increases the cytotoxicity of cationic liposomes. *Biol. Pharm. Bull.*, **30**, 751-757 (2007)

(11) Tagami, T., Barichello, J.M., Kikuchi, H., Ishida, T., Kiwada, H., The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex. *Int. J. Pharm.*, **333**, 62-69 (2007)

(12) Yonezawa, S., Asai, T., Oku, N., Effective tumor regression by anti-neovascular therapy in hypovascular orthotopic pancreatic tumor model. *J. Control. Release*, **118**, 303-309. (2007)

(13) Akita, N., Maruta, F., Seymour, L.W., Kerr, D.J., Parker, A.L., Asai, T., Oku, N., Nakayama, J., and Miyagawa, S., Identification of oligopeptides binding to peritoneal tumors of gastric cancer. *Cancer Sci.*, **97**, 1075-1081 (2006)

(14) Maeda, N., Miyazawa, S., Shimizu, K., Asai, T., Yonezawa, S., Kitazawa, S., Namba, Y., Tsukada, H., and Oku, N., Enhancement of anticancer activity in antineovascular therapy is based on the intratumoral distribution of the active targeting carrier for anticancer drugs. *Biol Pharm Bull.*, **29**, 1936-1940 (2006)

(15) Ishida, T., Li, W., Liu, Z., Kiwada, H., Stimulatory effect of polyethylene glycol (PEG) on gene expression in mouse liver following hydrodynamics-based transfection. *J. Gene Med.*, **8**, 324-334 (2006)

(16) Li, W., Ishida, T., Okada, Y., Oku, N., Kiwada, H., Increased gene expression by cationic liposomes (TFL-3) in lung metastases following intravenous injection. *Biol. Pharm. Bull.*, **21**, 701-706 (2005)

(17) Shimizu, K., Asai, T., Fuse, C., Sadzuka, Y., Sonobe, T., Ogino, K., Taki, T., Tanaka, T., Oku, N.: Applicability of anti-neovascular therapy to drug-resistant tumor: Suppression of drug-resistant P388 tumor growth with neovessel-targeted liposomal adriamycin. *Int. J. Pharm.*, **296**, 133-141 (2005)

(18) Ichikawa, K., Hikita, T., Maeda, N., Yonezawa, S., Takeuchi, Y., Asai, T., Namba, Y., Oku, N.: Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels. *Biochim. Biophys. Acta*, **1669**, 69-74 (2005)

(19) Shimizu, K., Asai, T. and Oku, N.: Antineovascular therapy, a novel antiangiogenic therapy. *Expert Opin. Ther. Targets.*, **9**, 63-76 (2005)

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

2. 学会発表

・招待講演・発表

(1) 石田竜弘、リポソームを基盤とした DDS の開

発研究～効果増強と安全性の観点から～、第三回 DDS 熊本シンポジウム（熊本）２００８年２月

(2) 石田竜弘、抗がん剤含有新生血管標的ナノキャリアの開発とその腫瘍休眠療法への展開、とくしま健康・医療クラスター創成フォーラム２００８（徳島）２００８年１月

(3) 石田竜弘、リポソームＤＤＳによる体内動態制御に基づく創薬・育薬の試み、Bio Japan2007, World Buisiness Forum, （横浜）２００７年９月

(4) 石田竜弘、ポリマー修飾ナノ微粒子の組織・細胞反応性とバイオ応用に関する研究、花王芸術・科学財団第９回助成研究発表会（東京）２００７年５月

(5) 石田竜弘、PEG 修飾リポソーム投与時に者忌諱される免疫反応に関する研究、奨励賞受賞講演、日本薬剤学会第２２年会（大宮）２００７年５月

(6) 石田竜弘、際田弘志、ポリエチレングリコール修飾リポソーム投与時に生ずる accelerated blood clearance (ABC)現象、シンポジウム S6、日本薬学会第１２７年回（富山）２００７年３月

(7) 石田竜弘、リポソーム DDS による薬物動態制御ー効果増強と安全性の観点からー、学長表彰受賞記念講演会（徳島大学）２００６年１２月

(8) 石田竜弘、がん新生血管を標的とした All in one デバイスによる革新的 siRNA デリバリーシステムとがん治療法の開発、総合科学技術会議・科学技術連携施策群・ナノバイオテクノロジー連携群　成果報告会「超早期診断と低侵襲医療の実現と一体化、生活の安全・安心を目指して」（東京）２００６年１２月

(9) 浅井知浩、石田竜弘、奥直人、際田弘志、がん新生血管を標的とした siRNA デリバリーの構築と Argonaute2 ノックダウンによる治療効果の検討、第２８回生体膜と薬物の相互作用シンポジウム（静岡）２００６年１１月

・一般講演

(1) 田上辰秋、廣瀬聖実、Barichello J.M., 石田竜弘、際田弘志、siRNA のリポフェクションが細胞内遺伝子発現および遺伝子サイレンシングに与える影響、日本薬学会第１２８年会（横浜）２００８年３月

(2) 廣瀬聖実、田上辰秋、Barichello J.M., 山崎尚志、浅井知浩、石田竜弘、奥直人、際田弘志、Argonaute2(Ago2)標的 siRNA 細胞内導入による細胞生存抑制効果に関わる遺伝子発現変化に関する検討、日本薬学会第１２８年会（横浜）２００８年３月

(3) Koide, H., Hatanaka, K., Tsuruta, A., Asai, T., Ishida, T., Kiwada, H., Oku, N., T cell-independent B cell response induces accelerated blood clearance phenomenon caused by a repeated injection o PEGylated liposomes. International Liposome Society 2007 Annual Meeting (London, UK) ２００７年１２月

(4) Barichello, J.M., 石田竜弘、城慎二、田上辰秋、廣瀬聖実、菊池寛、際田弘志、siRNA リポプレックス調製時における攪拌操作による遺伝子抑制効果促進メカニズムの検討：フローサイトメトリーによるリポプレックスー細胞相互作用およびリポプレックスの内在化に関する研究、第２９回　生体膜と薬物の相互作用シンポジウム（仙台）２００７年１１月

(5) 中村和也、鈴木卓也、嶋津史恵、田上辰秋、Barichello, J.M., 石田竜弘、際田弘志、siRNA カチオニックリポソーム複合体の体内動態と新生血管親和性との相関に関する検討、第４６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（高知）２００７年１１月

(6) Miyauchi, H., Ichikawa, K., Urakami, T., Yonezawa, S., Shimizu, K., Ishida, T., Kiwada, H., Asai, T., Oku, N., Antigen-conjugated liposomes enhanced hyposensitization immune therapy with extra-low doses. 34th Annual Meeting & Exposition of the Controlled Release Society (CA, USA) ２００７年７月

(7) Barichello, J.M., 城慎二、田上辰秋、菊池寛、石田竜弘、際田弘志、Dose the application of vortex-mixing during complex formation minimize the electrostatic association of cationic liposomes and siRNA? 第１６回ＤＤＳカンファランス（静岡）２００７年７月

(8) 城慎二、Barichello, J.M., 田上辰秋、跡部一孝、小林英夫、橋本浩一、菊池寛、石田竜弘、際田弘志、siRNA リポプレックス(LP)の新規調製法がもたらすノックダウン効率向上のメカニズムに関する検討、第２３回日本ＤＤＳ学会（熊本）２００７年６月

(9) 鈴木卓也、跡部一孝、嶋津史恵、田上辰秋、Barichello J. M., 浅井知浩、奥直人、石田竜弘、際田弘志、マウス皮下新生血管誘導モデルを用いたカチオニックリポソームの新生血管親和性の検討、第２３回日本ＤＤＳ学会（熊本）２００７年６月

(10) 松下小緒里、鈴木佑子、浅井知浩、石田竜弘、際田弘志、前田典之、奥　直人：腫瘍新生血管を標的とした siRNA デリバリーの構築とArgonaute2-siRNA による抗血管新生効果、第7 回遺伝子・デリバリー研究会シンポジウム（東京）２００７年５月

(11) Ishida, T., Tagami, T., Barichello, J.M., Hirose, K., Yamazaki, N., Asai, T., Oku, N., Kiwada, H., Argonaute2 (Ago2) gene silencing by liposomal transfection with siRNA for Ago2 induces apoptosis on HT1080 cells and HUVECs. American Society of Gene Therapy 10th Annual Meeting（WA）US, ２００７年５月

(12) Barichello, J.M., Ishida, T., Soares, L.A.L., Kizuki, S., Tagami, T., Hirose, K., Kobayashi, H., Hashimoto, K., Kikuchi, H., Kiwada, H., Agitation during siRNA-cationic liposomes (CL) complex formation is a key step to improve in vitro RNA interference by lipofection. American Society of Gene Therapy 10th Annual Meeting（WA）US, ２００７年５月

(13) Asai, T., Suzuki, Y., Matsushita, S., Yokota, J., Ishida, T., Kiwada, H., Oku, N., Development of Antineovascular RNAi Cancer Therapy Targeting to Argonaute2. American Society of Gene Therapy 10th Annual Meeting（WA）US, ２００７年５月

(14) 廣瀬聖実、田上辰秋、鈴木卓也、Barichello, J.M., 山崎尚志、浅井知浩、石田竜弘、奥直人、際田弘志、Argonaute2 (Ago2)標的 siRNA 細胞内導入による細胞増殖抑制に関する検討、日本薬剤学会第２２年会（大宮）２００７年５月

(15) 田上辰秋、廣瀬聖実、Barichello, J.M., 山崎尚志、浅井知浩、奥直人、石田竜弘、際田弘志、Argonaute2 の遺伝子サイレンシングがヒト血管内皮細胞(HUVEC)のチューブ形成能力に与える影響に関する検討、日本薬学会第１２７年会（富山）２００７年３月

(16) 米澤　正、鈴木佑子、浅井知浩、石田竜弘、際田浩志、出羽毅久、南後　守、奥　直人：標的化ポリカチオンリポソームによる siRNA の導入・がん治療を目的として、日本薬学会127 年会（富山）２００７年３月

(17) 鈴木佑子、横田洵一、松下小緒里、浅井知浩、石田竜弘、際田弘志、出羽毅久、南後　守、奥　直人、Argonaute2 を標的とした RNAi による腫瘍新生血管障害療法の開発、第 16 回アンチセンスシンポジウム（京都）２００６年１１月

(18) Jose Mario Barichello, 石田竜弘、城慎二、田上辰秋、廣瀬聖実、小林英夫、橋本浩一、菊池寛、際田弘志、The mechanism of gene silencing of agitation during siRNA-liposome complex formation: physicochemical and in vitro cell uptake study. 第２８回生体膜と薬物の相互作用シンポジウム（静岡）２００６年１１月

(19) 廣瀬聖実、田上辰秋、Barichello, J.M., 山崎尚志、石田竜弘、際田弘志、Anti-angiogenic therapy を目指した Argonaute 2(Ago2)標的 siRNA による細胞死誘導に関する検討、第４４回日本癌治療学会（東京）２００６年１０月

(20) 城慎二、Jose Mario Barichello、田上辰秋、小林英夫、橋本浩一、菊池寛、際田弘志、石田竜弘、siRNA リポプレックス調製時における vortexing と knockdown 効率との相関に関する研究：物性面からの検討、第４５回日本薬学会・日本病院薬剤師会中国四国支部学術大会（広島）２００６年１０月

(21) 鈴木卓也、跡部一孝、嶋津史恵、田上辰秋、Jose Mario Barichello、際田弘志、石田竜弘、マウス皮下新生血管誘導モデルを用いたカチオニックリポソームの新生血管親和性の検討、第４５回日本薬学会・日本病院薬剤師会中国四国支部学術大会（広島）２００６年１０月

(22) Jose Mario Barichello, 石田竜弘、田上辰秋、廣瀬聖実、小林英夫、橋本浩一、菊池寛、際田弘志、Effective exogenous and GFP stably expressed genes silencing in vitro using agitation during siRNA-liposome complex formation、第７回　長井長義記念シンポジウム（徳島）、２００６年９月

(23) Asai, T., Angiogenic Vessels-Targeted Liposomal DDS For Cancer Treatment. Drug Delivery & Licensing（Orchard, Singapore）２００６年７月

(24) Suzuki, Y., Yokota, J., Asai, T., Ishida, T., Kiwada, T., Dewa, T., Nango, M., Oku, N., development of Polycation Liposomes for Delivering Short Interfering RNA. 33rd Annual Meeting & Exposition of the Controlled Release Society (Vienna) ２００６年７月

(25) 鈴木佑子、横田洵一、浅井知浩、石田竜弘、際田弘志、出羽毅久、南後　守、奥　直人、腫瘍新生血管を標的としたsiRNAデリバリーシステムとがん治療法の開発、第22回日本DDS学会（東京）２００６年７月

(26) Barichello. J.M., Ishida, T., Tagami, T., Hirose, K., Soares, L., Kobayashi, H., Kikuchi, H., Kiwada, H., Selective and efficient delivery of siRNA in vitro by means of TFL-3 liposomes. 10th Liposome Research Days Conference, Chapel Hill (NC), USA, ２００６年５月

(27) Tagami, T., Barichello, J., Hirose, K., Yamazaki, N., Asai, T., Oku, N., Kiwada, H., Ishida, T., Liposomal transfection of siRNA targeting for Argonautes induces apoptosis on HT1080 and HUVEC cells. 10th Liposome Research Days Conference, Chapel Hill (NC), USA, ２００６年５月

(28) Suzuki, Y., Yokota, J., Asai, T., Ishida, T., Kiwada, H., Dewa, T., Nango, M., Oku, N., Investigation of Polycation Liposomes for Short Interfering RNA Delivery. 10th Liposome Research Days Conference, Chapel Hill (NC), USA, ２００６年５月

(29) 横田洵一、鈴木佑子、浅井知浩、石田竜弘、際田弘志、出羽毅久、南後　守、奥　直人、ポリカチオンリポソームを用いた新規siRNAデリバリーシステムの構築、第１２６年会日本薬学会（仙台）２００６年３月

(30) 田上辰秋、廣瀬聖実、バリケロ　ジョゼマリオ、山崎尚志、石田竜弘、際田弘志：Argonaute2を標的としたsiRNAによるapoptosisの誘導に関する検討、日本薬剤学会21年会（金沢）２００６年３月

(31) バリケロ　ジョゼマリオ、石田竜弘、Soares Luis A.L., 田上辰秋、廣瀬聖実、小林英夫、菊池寛、際田弘志：Setting TFL-3 formulation parameters for siRNA use in functional genomics studies、日本薬剤学会21年会（金沢）２００６年３月

(32) 菅直恵、金平実、石田竜弘、伊藤智佳、久保和弘、菊池寛、際田弘志：ドキソルビシン封入ペプチドスペーサー含有PEG修飾リポソームの有用性に関する検討、日本薬剤学会21年会（金沢）２００６年３月

(33) 西村武広、菅直恵、石田竜弘、橋本浩一、菊池寛、際田弘志：肺転移性メラノーマ(B16BL6)への癌抑制遺伝子PTEN導入によるdoxorubicinの感受性増強に関する検討、日本薬学会第126年会（仙台）２００６年３月

(34) 三原陽、田上辰秋、Jose M. Barichello、石田竜弘、橋本浩一、小林浩一、菊池寛、際田弘志：新規カチオニックリポソームによる遺伝子導入効率に関するスクリーニング的検討、日本薬学会第126年会（仙台）２００６年３月

(35) 沢崎靖治、浅井知浩、奥　直人：腫瘍新生血管傷害療法の時間薬理学的検討 日本薬学会東海支部例会（名古屋）２００５年１２月

(36) 嶋津史恵、跡部一孝、白髪恵美、石田竜弘、際田弘志：Metronomic Chemotherapyにおける血管新生阻害に関する検討　第44回日本薬学会中四国支部大会（松山）２００５年１１月

(37) 白髪恵美、西村武広、石田竜弘、際田弘志：シクロフォスファミドを用いたMetronomic Chemotherapyにおけるドキソルビシン封入リポソームの併用効果の検討　第44回日本薬学会中四国支部大会（松山）２００５年１１月

(38) 西村武広、小林友貴、石田竜弘、際田弘志：Studying the mechanism overcoming P-gp mediated MDR cells by doxorubicin-containing transferring-modified liposomes. 第27回生体膜と薬物の相互作用シンポジウム（京都）２００５年１１月

(39) 宮澤壮一郎、前田典之、清水広介、浅井知浩、塚田秀夫、難波幸弘、奥　直人：腫瘍新生血管標的化血中滞留性リポソームの体内動態PET解析及びがん治療 第1回放射薬学研究発表会（東京）２００５年８月

(40) 宮澤壮一郎、前田典之、清水広介、浅井知浩、難波幸弘、周東　智、松田　彰、奥　直人：腫瘍新生血管標的化血中滞留性リポソームによるがん治療 第21回日本DDS学会（長崎）２００５年７月

(41) 前田典之、宮沢壮一郎、清水広介、浅井知浩、北沢貞哉、難波幸弘、奥 直人：新生血管標的化血中滞留リポソームによる腫瘍新生血管傷

害療法 日本薬学会第125年会（東京）、２００５年３月

(42)沢崎靖治、浅井知浩、奥 直人：時間薬理学に基づいた抗腫瘍新生血管療法 日本薬学会第125年会（東京）、２００５年３月

G. 知的財産権の出願・登録状況

1. 特許取得

石田竜弘、際田弘志、細胞死を誘導する２本鎖RNA 特願 2006-023224

2. 実用新案登録

なし

3. その他

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
Asai, T., Miyazawa, S., Maeda, N., Hatanaka, K., Katanasaka, Y., Shimizu, K., Shuto, S., Oku, N.	Antineovascular therapy with angiogenic vessel-targeted polyethyleneglycol-shielded liposomal DPP-CNDAC.	*Cancer Sci.*			in press
Asai, T., Suzuki, Y., Matsushita, S., Yonezawa, S., Yokota, J., Katanasaka, Y., Ishida, T., Dewa, T., Kiwada, H., Nango, M., Oku, N.	Disappearance of the angiogenic potential of endothelial cells caused by Argonaute2 knockdown.	*Biochem. Biophys. Res. Commun.*	368	243-248	2008
Shimizu, K., Sawazaki, Y., Tanaka, T., Asai, T., Oku, N.	Chronopharmacologic cancer treatment with an angiogenic vessel-targeted liposomal drug.	*Biol. Pharm. Bull.*	31	95-98	2008
Shiraga, E., Barichello, J.M., 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.*	353	65-73	2008
Hatakeyama, H., Akita, H., Ishida, E., Hashimoto, K., Kobayashi, H., Aoki, T., Yasuda, J., Obata, K., Kikuchi, K., Ishida, T., Kiwada, H., Harashima, H.	Tumor targeting of doxorubicin by anti MT1-MMP antibody-modified PEG liposomes.	*Int. J. Pharm.*	342	194-200	2007
Atobe K., Ishida T., Ishida, E., Hashimoto K., Kobayashi H., Yasuda J., Aoki, T., Obata K, Kikuchi H., Akita, H., Asai, T, Harashima, H., Oku, N., Kiwada, H.	In vitro efficacy of a sterically stabilized immunoliposomes targeted to membrane type 1 matrix metalloproteinase (MT1-MMP).	*Biol. Pharm. Bull.*	30	972-978	2007
Katanasaka, Y., Asai, T., Naitou, H., Ohashi, N., Oku, N.	Proteomic characterization of angiogenic endothelial cells stimulated with cancer cell-conditioned medium.	*Biol. Pharm. Bull.*	30	2300-2307	2007
Ishida, T., Wang X.Y., Shimizu, T., Nawata, K., Kiwada, H.	PEGylated liposomes elicit an anti-PEG IgM response in a T-cells independent manner.	*J. Control. Release*	122	349-355	2007

Isakari, Y., Harada, Y., Ishikawa, D., Matsumura-Takeda, K., Sogo, S., Ishida, T., Taki, T., Kiwada, H.	Efficient gene expression in Megakaryocytic cell line using Nucleofection.	*Int. J. Pharm.*	338,	157-164	2007
Nguyen, L.T., Atobe, K., Barichello, J.M., Ishida, T., Kiwada, H.	Complex formation with plasmid DNA increases the cytotoxicity of cationic liposomes.	*Biol. Pharm. Bull.*	30	751-757	2007
Tagami, T., Barichello, J.M., Kikuchi, H., Ishida, T., Kiwada, H.	The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex.	*Int. J. Pharm.*	333	62-69	2007
Maeda, N., Miyazawa, S., Shimizu, K., Asai, T., Yonezawa, S., Kitazawa, S., Namba, Y., Tsukada, H., and Oku, N.	Enhancement of anticancer activity in antineovascular therapy is based on the intratumoral distribution of the active targeting carrier for anticancer drugs.	*Biol Pharm Bull.*	29	1936-1940	2006
Akita, N., Maruta, F., Seymour, L.W., Kerr, D.J., Parker, A.L., Asai, T., Oku, N., Nakayama, J., and Miyagawa, S.	Identification of oligopeptides binding to peritoneal tumors of gastric cancer.	*Cancer Sci.*	97	1075-1081	2006
Yonezawa, S., Asai, T., Oku, N.	Effective tumor regression by anti-neovascular therapy in hypovascular orthotopic pancreatic tumor model.	*J. Control. Release*	118	303-309	2006
Li, W., Ishida, T., Okada, Y., Oku, N., Kiwada, H.	Increased gene expression by cationic liposomes (TFL-3) in lung metastases following intravenous injection.	Biol. Pharm. Bull.	21	701-706	2005
Nguyen, L.T., Ishida, T., Kiwada, H.	Gene expression in primary cultured mouse hepatocytes with a cationic liposomal vector, TFL-3: Comparison with rat hepatocytes.	*Biol. Pharm. Bull.*	28	1472-1475	2005
Ishida, T., Li, W., Liu, Z., Kiwada, H.	Stimulatory effect of polyethylene glycol (PEG) on gene expression in mouse liver following hydrodynamics-based transfection.	*J. Gene Med.*	8	324-334	2005
Shimizu, K., Asai, T., Fuse, C., Sadzuka, Y., Sonobe, T., Ogino, K., Taki, T., Tanaka, T., Oku, N.	Applicability of anti-neovascular therapy to drug-resistant tumor: Suppression of drug-resistant P388 tumor growth with neovessel-targeted liposomal adriamycin.	*Int. J. Pharm.*	296	133-141	2005
Ichikawa, K., Hikita, T., Maeda, N., Yonezawa, S., Takeuchi, Y., Asai, T., Namba, Y., Oku, N.	Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels.	*Biochim. Biophys. Acta*	1669	69-74	2005

Shimizu, K., Asai, T., Oku, N.	Antineovascular therapy, a novel antiangiogenic therapy.	*Expert Opin. Ther. Targets.*	9	63-76	2005

Antineovascular therapy with angiogenic vessel-targeted polyethyleneglycol-shielded liposomal DPP–CNDAC

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Causing damage to angiogenic vessels is a promising approach for cancer chemotherapy. The present study is a codification of a designed liposomal drug delivery system (DDS) for antineovascular therapy (ANET) with 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC). The authors have previously reported that liposomalized 5′-*O*-dipalmitoylphosphatidyl CNDAC (DPP–CNDAC), a phospholipid derivative of the novel antitumor nucleoside CNDAC, is quite useful for ANET. DPP–CNDAC liposomes modified with APRPG, a peptide having affinity toward angiogenic vessels, efficiently suppressed tumor growth by damaging angiogenic endothelial cells. In the present study, the authors masked the hydrophilic moiety of DPP–CNDAC, namely, CNDAC, on the liposomal surface with APRPG–polyethyleneglycol (PEG) conjugate to improve the availability of DPP–CNDAC liposomes. The use of the APRPG–PEG conjugate attenuated the negative ζ-potential of the DPP–CNDAC liposomes and reduced the agglutinability of them in the presence of serum. These effects improved the blood level of DPP–CNDAC liposomes in colon 26 NL-17 tumor-bearing BALB/c male mice, resulting in enhanced accumulation of them in the tumor. Laser scanning microscopic observations indicated that APRPG–PEG-modified DPP–CNDAC liposomes (LipCNDAC/APRPG–PEG) colocalized with angiogenic vessels and strongly induced apoptosis of tumor cells, whereas PEG-modified DPP–CNDAC liposomes (LipCNDAC/PEG) did not. In fact, LipCNDAC/APRPG–PEG suppressed the tumor growth more strongly compared to LipCNDAC/PEG and increased significantly the life span of the mice. The present study is a good example of an effective liposomal DDS for ANET that is characterized by: (i) phospholipid derivatization of a certain anticancer drug to suit the liposomal formulation; (ii) PEG-shielding for masking undesirable properties of the drug on the liposomal surface; and (iii) active targeting to angiogenic endothelial cells using a specific probe. (*Cancer Sci* 2008)

Because the inhibition of angiogenesis suppresses tumor growth and hematogenous metastases, antiangiogenic therapies have been widely investigated.[1–3] These therapies are also expected to be effective toward a broad spectrum of cancers, including drug-resistant cancers. Besides antiangiogenic therapy, ANET, namely, the causing of indirect lethal damage to tumor cells by the complete cut-off of the supply of oxygen and nutrients through damaging neovessels, is being developed.[4,5] For ANET, the authors previously isolated from a phage-displayed peptide library a peptide that specifically binds to the tumor angiogenic vasculature. The epitope sequence of the peptide was determined to be APRPG.[6,7] The authors demonstrated that APRPG is a useful probe for the active targeting of angiogenic vessels, although the target molecule of APRPG is still unknown. In contrast, PEG-coating of liposomes has been used in a liposomal DDS. It is known that PEG-modified liposomes characteristically remain in the circulation longer than non-modified ones through avoidance of RES-trapping of drug carriers.[8–10] PEG modification of the liposomal surface is known to form a fixed aqueous layer around the liposome due to the interaction between the PEG-polymer and water molecules, and thus prevents the binding of certain serum proteins and opsonins that are responsible for RES-trapping.[11] The feature of long circulation causes liposomal accumulation in tumor tissues, because the angiogenic vasculature in tumor tissues is quite leaky and macromolecules easily accumulate in the interstitial tissues of the tumor due to the EPR effect.[12,13] In the case of ANET, this long time in circulation increases the opportunity for specific binding of ligand-modified liposomes to angiogenic vessels. For this purpose, the authors previously designed a compound composed of APRPG, PEG, and DSPE.[14,15] It has been demonstrated that APRPG–PEG modification is superior to just APRPG modification for enhancing the antitumor activity of liposomal doxorubicin.[16]

In the present study, the authors used CNDAC as a chemotherapeutic agent. CNDAC had been originally synthesized as a novel antitumor nucleoside. anti-metabolite by Matsuda *et al.*, who showed that CNDAC has a novel anticancer mechanism and induces DNA strand breaks after its incorporation into tumor cell DNA.[17] The results of phase I clinical studies of its N4-palmitoyl derivative (CS-682) in patients with malignant solid tumors were reported recently.[18,19] The authors previously designed DPP–CNDAC for liposomalization,[20] because liposomal drugs show improved biodistribution and bioavailability in tumor-bearing animals. In fact, liposomal DPP–CNDAC showed enhanced activities for reducing tumor growth and increasing the life span of mice than conventional liposomes or soluble CNDAC.[21,22] As the next step, DPP–CNDAC liposomes were modified with APRPG for the purpose of ANET. APRPG-modification of DPP–CNDAC liposomes actually caused effective tumor growth suppression, possibly through damaging angiogenic endothelial cells.[23] These results also indicated that the therapeutic efficacy should reflect the damage to the angiogenic endothelial cells to which the liposomes gain access, because lipophilic drugs should be delivered to the cells in a liposomal form. However, the *in vivo* behavior of APRPG-modified DPP–CNDAC

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Abbreviations: ANET, antineovascular therapy; APRPG, Ala-Pro-Arg-Pro-Gly; BSA, bovine serum albumin; CNDAC, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DDS, drug delivery system; $DiIC_{18}$, 1,1′-dioctadecyl-3,3,3′,3′,-tetramethylindo-carbocyanine perchlorate; DPPC, dipalmitoylphosphatidylcholine; DPP–CNDAC, 5′-*O*-dipalmitoylphosphatidyl–CNDAC; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; EPR, enhanced permeability and retention; FBS, fetal bovine serum; FITC, fluoroscein-5-isothiocyanate, LipCNDAC, DPP–CNDAC liposomes; LipCNDAC/APRPG–PEG, APRPG–PEG-modified DPP–CNDAC liposomes; LipCNDAC/PEG, PEG-modified DPP–CNDAC liposomes; PBS, phosphate-buffered saline; PEG, polyethyleneglycol; RES, reticuloendothelial system; TUNEL, terminal dUTP nick end labeling.

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liposomes was affected by the presence of the cyano group of DPP–CNDAC on the liposomal surface. It induced aggregation of liposomes, resulting in reduced blood circulation of liposomes.

In the present study, the CNDAC on the liposomal surface was masked with APRPG–PEG conjugate to erase this undesirable property of DPP–CNDAC in liposomalization. The authors integrated their previous observations to formulate angiogenic vessel-targeted long-circulating DPP–CNDAC liposomes and applied them to ANET.

Materials and Methods

Materials. Synthesis of CNDAC and DPP–CNDAC was performed as described previously.[17,20] A phosphatidyl group was introduced into CNDAC through transphosphatidylation from 1,2-dipalmitoyl-3-sn-glycerophosphocholine using phospholipase D. Preparation of DSPE–PEG and DSPE–PEG–APRPG was performed as described previously.[14] DSPC and cholesterol were obtained from Nippon Fine Chemical Co., Ltd (Takasago, Hyogo, Japan). Colon 26 NL-17 colon carcinoma cells were established by Dr Yamori (Japanese Foundation for Cancer Research, Tokyo, Japan) and kindly provided by Dr Nakajima (Johnson & Johnson KK, Tokyo, Japan).

Animals. Five-week-old BALB/c male mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

Preparation of liposomes. DPP–CNDAC, DSPC, and cholesterol with DSPE–PEG (LipCNDAC/PEG) or DSPE–PEG–APRPG (LipCNDAC/APRPG–PEG) (10:10:5:2 as a molar ratio), or DPP–CNDAC, DSPC, and cholesterol without PEG-conjugate (LipCNDAC, 10:10:5 as a molar ratio) were dissolved in chloroform/methanol, dried under reduced pressure, and stored *in vacuo* for at least 1 h. Liposomes were produced by hydration of a thin lipid film with 10 mM phosphate-buffered 0.3 M sucrose (pH 6.8), and frozen and thawed for three cycles using liquid nitrogen. Then the liposomes were sized by extrusion thrice through polycarbonate membrane filters with 100-nm-diameter pores (Nucleopore, Maidstone, UK). The liposomal solutions were centrifuged at 180 000*g* for 20 min (CS120EX, Hitachi, Japan) to remove the untrapped DPP–CNDAC if present. Then the liposomes were resuspended in 10 mM phosphate-buffered 0.3 M sucrose. To determine the efficacy of trapping DPP–CNDAC in the liposomes, an aliquot of the liposomal solution was solubilized by the addition of reduced Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA), and the amount of DPP–CNDAC was optically determined at 280 nm after the pH of the solution had been adjusted to 1.0. For a biodistribution study, a trace amount of [1α, 2α(n)-^{3}H] cholesterol oleoyl ether (74 kBq/mouse, Amersham Pharmacia, Buckinghamshire, England) was added to the initial organic solution. To examine the intratumoral localization of liposomes in tumor syngrafts, $DiIC_{18}$ (Molecular Probes Inc., Eugene, OR, USA) was added to the initial organic solution (DPP–CNDAC:DSPC: cholesterol:$DiIC_{18}$:DSPE–PEG or DSPE–PEG–APRPG = 10:10:5:0.1:2; DPP–CNDAC : DSPC : cholesterol : $DiIC_{18}$ = 10:10:5:0.1, as a molar ratio). For the therapeutic study, control liposomes composed of DPPC, DSPC, and cholesterol (10:10:5 as a molar ratio) were prepared similarly as for the other liposomes.

Characterization of liposomes. Particle size and ζ-potential of liposomes diluted with PBS(–) were measured using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Aggregation testing was performed as follows: The liposomal solution was incubated in PBS(–) or in the presence of 50% FBS (Sigma-Aldrich) at 37°C for 1 h. The turbidity of the liposomal solution was determined at 450 nm, and relative turbidity compared with that in 0.3 M sucrose was then calculated.

Biodistribution of liposomes. Colon 26 NL-17 cells were cultured in DME/F12 medium (Nissui, Tokyo, Japan) supplemented with 10% FBS (Sigma-Aldrich). After harvesting of the cells, 1.0×10^6 cells were carefully injected subcutaneously into the posterior flank of 5-week-old BALB/c male mice. The biodistribution study was performed when the tumor size had become approximately 10 mm in diameter. Size-matched colon 26 NL-17 carcinoma-bearing mice were injected with the radiolabeled liposomes containing [1α, 2α (n)-^{3}H] cholesterol oleoyl ether via a tail vein. One hour after the injection, the mice were killed under diethyl ether anesthesia for the collection of blood. The plasma was obtained by centrifugation (600*g* for 5 min). After the mice had been bled from the carotid artery, the heart, lung, liver, spleen, kidney, and tumor were removed, washed with saline, and weighed. The radioactivity in each organ was determined using a liquid scintillation counter (LSC-3100, Aloka, Tokyo, Japan). Distribution data were presented as percentage dose per gram of tissue or per 0.1 mL plasma. The total amount in plasma was calculated based on the average body weight of the mice, where the average plasma volume was assumed to be 4.27% of the body weight based on the data on total blood volume.

Histochemical analysis of liposomal distribution in tumor syngrafts. $DiIC_{18}$-labeled liposomes were administered via a tail vein of colon 26 NL-17 carcinoma-bearing mice when the tumor sizes had reached approximately 10 mm in diameter. Two hours after the injection of liposomes, the mice were bled from the carotid artery under diethyl ether anesthesia, and the tumors were dissected. Histochemical analysis was performed according to the method described previously.[16] In brief, solid tumors were embedded in optimal cutting temperature compound (Sakura Finetechnochemical Co. Ltd, Tokyo, Japan) and frozen at –80°C. Five-micrometer tumor sections were prepared using a cryostat microtome (HM 505E, Microm, Walldorf, Germany), mounted on MAS-coated slides (Matsunami Glass Ind., Ltd, Japan), and air-dried for 1 h. The tissue sections were then fixed with acetone, and washed twice with PBS(–). After the sections had been blocked with 1% BSA in PBS(–), they were incubated with biotinylated antimouse CD31 rat monoclonal antibody (Becton Dickinson Laboratory, Franklin Lakes, NJ, USA) for 18 h at 4°C and then visualized after incubation with streptavidin–FITC conjugates (Molecular Probes Inc., Eugene, OR, USA) for 30 min at room temperature in a humid chamber. These sections were observed for fluorescence using an LSM microscope system (Carl Zeiss, Co. Ltd, Jena, Germany).

Determination of apoptotic cells in tumors. LipCNDAC/PEG or LipCNDAC/APRPG–PEG was administered intravenously into colon 26 NL-17 tumor-bearing mice when the tumor size had reached approximately 10 mm in diameter. Twelve hours after injection of the liposomes, the tumors were dissected from the mice; and tumor sections were then prepared. Next, immunostaining of endothelial cells was performed as described above, except that streptavidin–Alexa 594 conjugate (Molecular Probes Inc.) was used as fluorescent dye instead of streptavidin–FITC conjugate. For visualizing apoptotic cells, TUNEL staining was performed using an ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Intergen Co., Purchase, NY, USA) according to the recommended procedures supplied in the kit. In brief, tumor sections were washed and equilibrated for 15 min in a humid chamber at room temperature, and then reacted with TdT enzyme for 1 h at 37°C. Thereafter, they were stained with antidigoxigenin–fluorescein antibody, after which the sections were observed using the LSM system. Apoptotic signals (green signals) were analyzed using Image J software (NIH).

Therapeutic experiment. LipCNDAC/PEG, LipCNDAC/APRPG–PEG or control liposomes were administered intravenously into colon 26 NL-17 tumor-bearing mice. The injected dose for each administration was 15 mg/kg as CNDAC moiety. The treatment was started when the tumor volume became approx. 0.1 cm^3. The size of the tumor and the body weight of each mouse were

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monitored daily thereafter. Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume. Calculation of the tumor volume was performed using the formula 0.4 ($a \times b^2$), where a is the largest and b is the smallest diameter. The calculated tumor volume correlated well with the actual tumor weight ($r = 0.980$).[24] The life spans of tumor-bearing mice were also monitored.

Statistical analysis. Variance in a group was evaluated using the F-test; and differences in mean tumor volume using Student's t-test.

Results

Characterization of DPP–CNDAC liposomes. The efficiency of entrapment of DPP–CNDAC into liposomes was approximately 100% in all experiments (data not shown). Because DPP–CNDAC was easily incorporated into the lipid bilayer of liposomes as a lipid component, the CNDAC moieties of DPP–CNDAC were speculated to be exposed on the liposomal surface. In fact, the ζ-potential of LipCNDAC was negative due to the presence of the cyano group in CNDAC (Table 1). In contrast, PEG- or APRPG–PEG-modification reduced the negativity of the ζ-potential of DPP–CNDAC liposomes, suggesting that PEG shielded CNDAC moieties on the liposomal surface by forming a fixed aqueous layer (Table 1). As shown in Fig. 1, the agglutinability of both LipCNDAC/PEG and LipCNDAC/APRPG–PEG in the presence of serum was considerably low compared with that of LipCNDAC.

Biodistribution study. The biodistribution of these three types of liposomes was determined in colon 26 NL-17 carcinoma-bearing mice. At 1 h after administration of these liposomes, the plasma concentrations of LipCNDAC/PEG and LipCNDAC/APRPG–PEG were significantly higher than that of LipCNDAC (Fig. 2). These data suggest that the use of PEG or APRPG–PEG reduced the aggregation of these liposomes in the blood circulation, which prevented recognition of them by RES and endowed them with long circulation. In addition, APRPG–PEG modification significantly improved the blood circulation of DPP–CNDAC liposomes at 3 or 24 h administration of these liposomes (data not shown). Therefore, LipCNDAC/PEG and LipCNDAC/APRPG–PEG showed high accumulation in the tumors compared with LipCNDAC, possibly through the EPR effect. Particularly in LipCNDAC/APRPG–PEG, this characteristic of long circulation would increase the opportunity for specific binding of these liposomes to angiogenic vessels.

Histochemical analysis of the tumor. Colon 26 NL-17-bearing mice were given a single i.v. dose of LipCNDAC/PEG or LipCNDAC/APRPG–PEG. As shown in Fig. 3a–c, when the fluorescently labeled LipCNDAC/PEG was injected, the liposomal distribution (red fluorescence) was observed to be separate from endothelial cells (green fluorescence). In contrast, LipCNDAC/APRPG–PEG was colocalized with endothelial cells (Fig. 3d–f). These data suggest that LipCNDAC/APRPG–PEG became selectively localized on angiogenic endothelial cells. Cellular

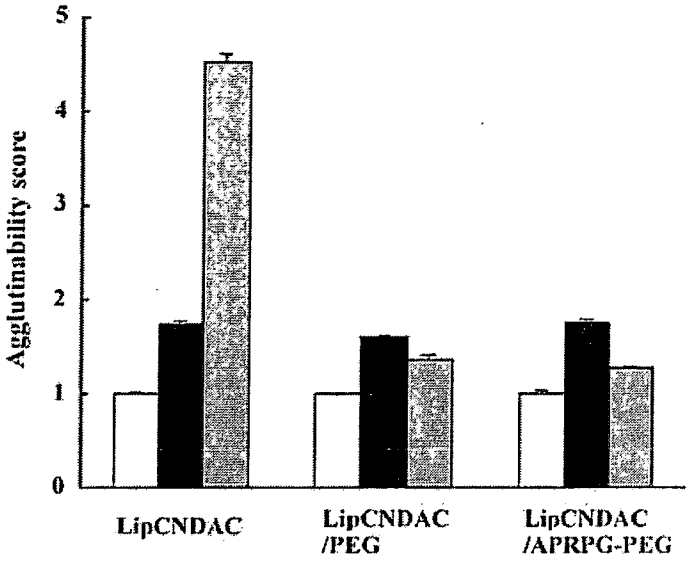

Fig. 1. Aggregation of DPP–CNDAC liposomes in the presence of serum. LipCNDAC, LipCNDAC/PEG or LipCNDAC/APRPG–PEG was incubated in (□) 10 mM phosphate-buffered 0.3 M sucrose, (■) PBS(–) or (▨) 50% FBS at 37°C for 1 h. The turbidity of the solutions was measured by absorption at 450 nm. The turbidity of the PBS or the FBS group relative to that of the sucrose group is shown, along with SD bars. Similar results were obtained in separate experiments. APRPG, Ala-Pro-Arg-Pro-Gly; CNDAC, 2′-C-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DPP, 5′-*O*-dipalmitoylphosphatidyl; FBS, fetal bovine serum; LipCNDAC, DPP–CNDAC liposomes; PBS, phosphate-buffered saline; PEG, polyethyleneglycol.

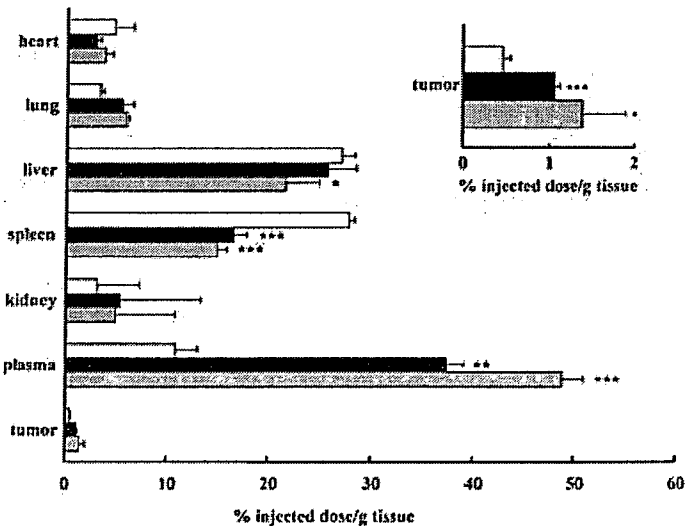

Fig. 2. Biodistribution of DPP–CNDAC liposomes in tumor-bearing mice. Liposomes were prepared and radiolabeled as described in Materials and Methods. Size-matched colon 26 NL-17 carcinoma-bearing mice ($n = 4$) were injected with (□) LipCNDAC, (■) LipCNDAC/PEG or (▨) LipCNDAC/APRPG–PEG, at 15 mg/kg as CNDAC, into a tail vein. One hour after injection, these mice were killed under diethyl ether anesthesia and each organ was dissected. The radioactivity in the plasma and each organ was determined in a liquid scintillation counter. Data are presented as the amount incorporated as a percentage of the injected dose per gram of tissue and SD in each tissue. (In the case of plasma, the value was per 0.1 mL instead of per gram). Significant differences from LipCNDAC are indicated (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). APRPG, Ala-Pro-Arg-Pro-Gly; CNDAC, 2′-C-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DPP, 5′-*O*-dipalmitoylphosphatidyl; LipCNDAC, DPP–CNDAC liposomes; PEG, polyethyleneglycol.

Table 1. Size and ζ-potential of DPP–CNDAC liposomes

	Size ± SD (nm)	ζ-potential (mV)
LipCNDAC	120.8 ± 3.5	–29.2
LipCNDAC/PEG	121.5 ± 5.7	–6.1
LipCNDAC/APRPG–PEG	102.4 ± 2.2	–3.6

Particle size and ζ-potential of DPP–CNDAC liposomes diluted with phosphate-buffered saline(–) were measured using a Zetasizer Nano ZS. APRPG, Ala-Pro-Arg-Pro-Gly; CNDAC, 2′-C-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DPP, 5′-*O*-dipalmitoylphosphatidyl; LipCNDAC, DPP–CNDAC liposomes; LipCNDAC/APRPG–PEG, APRPG–PEG-modified DPP–CNDAC liposomes; LipCNDAC/PEG, PEG-modified DPP–CNDAC liposomes; PEG, polyethyleneglycol.

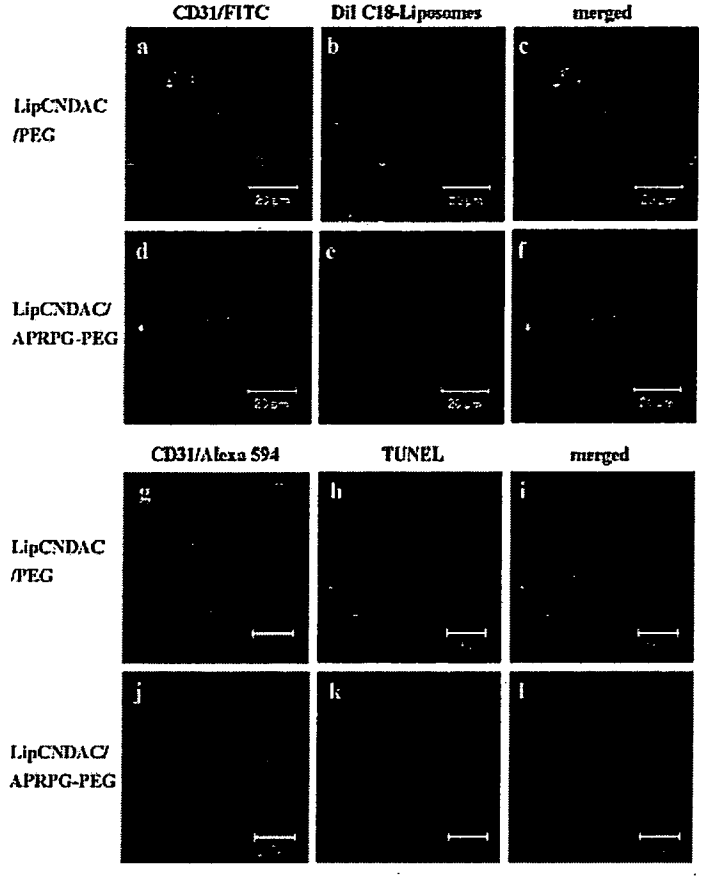

Fig. 3. Histochemical analysis of the tumor after injection of DPP–CNDAC liposomes. Size-matched colon 26 NL-17 carcinoma-bearing mice were injected i.v. with (a–c) LipCNDAC/PEG or (d–f) LipCNDAC/APRPG–PEG, at 15 mg/kg as CNDAC, on day 12 after tumor implantation. Two hours after the injection, each tumor was dissected and frozen sections were prepared. (a,d) Immunofluorescence staining for CD31 with FITC (green), and (b,e) liposomes labeled with $DiIC_{18}$ (red) are shown. (c,f) The merged images. The yellow portions indicate localization of liposomes at the site of vascular endothelial cells. In the experiment assessing apoptosis, colon 26 NL-17 carcinoma-bearing mice were similarly treated with (g–i) LipCNDAC/PEG or (j–l) LipCNDAC/APRPG–PEG. At 12 h after the injection, each tumor was dissected and frozen-sections were prepared. (g,j) The results of immunofluorescence staining for CD31 with Alexa 594 (red) and (h,k) TUNEL staining of apoptotic cells (green) are shown. (i,l) The merged images. APRPG, Ala-Pro-Arg-Pro-Gly; CNDAC, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DPP, 5′-*O*-dipalmitoylphosphatidyl; LipCNDAC, DPP–CNDAC liposomes; FITC, fluoroscein-5-isothiocyanate; PEG, polyethyleneglycol; TUNEL, terminal dUTP nick end labeling.

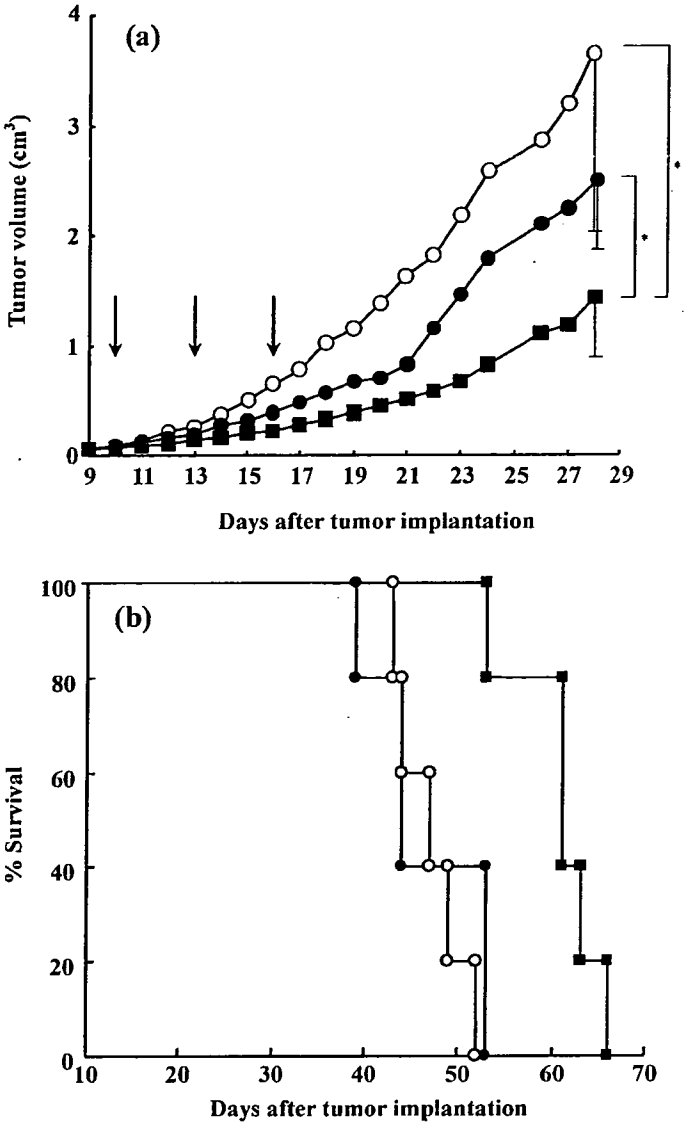

Fig. 4. Therapeutic efficacy of LipCNDAC/APRPG–PEG in tumor-bearing mice. Five-week-old BALB/c male mice (5 or 6 per group) were implanted s.c. with colon 26 NL-17 carcinoma cells into their left posterior flank. They were injected i.v. with (○) control liposomes, (●) LipCNDAC/PEG or (■) LipCNDAC/APRPG–PEG at 15 mg/kg as CNDAC on (→) days 10, 13, and 16 after tumor implantation. (a) The tumor volume and (b) survival time of mice were monitored to evaluate the therapeutic efficacy of DPP–CNDAC liposomes. Significant differences from the control liposome-treated group are indicated (*$P < 0.05$). APRPG, Ala-Pro-Arg-Pro-Gly; CNDAC, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine; DPP, 5′-*O*-dipalmitoylphosphatidyl; LipCNDAC, DPP–CNDAC liposomes; PEG, polyethyleneglycol.

apoptosis in the tumor tissues was evaluated 12 h after administration of the liposomes (Fig. 3g–l). The signals of apoptotic cells were approximately 4.6-fold greater for LipCNDAC/APRPG–PEG than for LipCNDAC/PEG. CD31-staining did not show any vessel-like structure in the tumor of either liposome-treated group, suggesting that LipCNDAC/PEG also damaged angiogenic endothelial cells to some degree. These results indicate that LipCNDAC/APRPG–PEG had preferentially damaged angiogenic endothelial cells that induced effective apoptosis of tumor cells surrounding the damaged vessels.

Therapeutic efficacy of LipCNDAC/APRPG–PEG in tumor-bearing mice. LipCNDAC/APRPG–PEG suppressed tumor growth more efficiently than LipCNDAC/PEG: Significant differences in the tumor volume of the LipCNDAC/APRPG–PEG-treated group from that of the LipCNDAC/PEG-treated group were observed from day 22–28, although the SD data are shown only for day 28 (Fig. 4a). In addition, the tumor volume of the LipCNDAC/APRPG–PEG-treated group was significantly different from that of the control liposome-treated group from day 20–28. The body-weight change, an indicator of side-effects, was not observed in either the LipCNDAC/PEG- or LipCNDAC/APRPG–PEG-treated groups (data not shown). Corresponding to the tumor growth suppression, treatment with LipCNDAC/APRPG–PEG elongated the survival time of the mice: The mean survival times of the control liposomes, LipCNDAC/PEG-, and LipCNDAC/APRPG–PEG-treated groups were 47.6 ± 3.7, 46.6 ± 6.2, and 60.8 ± 4.8 days, respectively (Fig. 4b). The survival time of the LipCNDAC/APRPG–PEG-treated group was significantly longer than that for the mice treated with the control liposomes ($P < 0.001$) or LipCNDAC/PEG ($P < 0.01$).

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Discussion

The use of a DDS for targeting tumors is a promising strategy particularly for drugs with severe side-effects such as those used in cancer chemotherapy. CNDAC was developed as an effective anticancer drug,[17] but has severe side-effects like other anticancer drugs. To design a targeting DDS, the authors previously derivatized CNDAC as a phospholipid mimetic[20] because it was readily incorporated into liposomes, the most widely used drug carrier for a DDS. This mimetic, DPP–CNDAC, was well suited to liposomalization for cancer treatment.[21,22]

For the active targeting strategy for delivery of anticancer drugs, angiogenic vessels were selected as a target organ and a novel type of antiangiogenic therapy, antineovascular therapy (ANET), was examined. Vascular targeting has become an interesting issue in DDS, because anticancer drugs or their carriers first meet angiogenic vessels before extravasation into the tumor tissue. The authors previously applied APRPG-modified liposomes for antineovascular therapy using DPP–CNDAC. Because lipophilic drugs should be delivered to the cells in a liposomal form, the therapeutic efficacy should reflect the damage to the cells to which the liposomes gain access rather than a change in the local concentration of the agent in the tumor tissue. If the therapeutic efficacy of APRPG-modified DPP–CNDAC liposomes is superior to that of non-modified liposomal DPP–CNDAC, such a result would suggest that the destruction of angiogenic endothelial cells is superior to the direct destruction of tumor cells for effective tumor treatment. The authors' previous results indicate that the delivery of DPP–CNDAC to angiogenic endothelial cells is, in fact, useful for the suppression of tumor growth.[23]

PEG-shielding of the liposomal surface should be useful for designing active targeting DDS as well as passive targeting. In the present study, the significant efficacy of APRPG–PEG-modified DPP–CNDAC liposomes for tumor growth suppression was shown. An important aspect of the present study is that PEGylation served for not only RES-avoidance but also construction of a practical liposomal formulation using a lipid-derivatized drug. When the liposomal surface is modified with anticancer drugs such as DPP–CNDAC, the fixed aqueous layer formed by PEG can mask the undesirable properties of such liposomes for DDS. Thus, the RES avoidance afforded by the use of PEG enhanced the accumulation of the liposomes in the tumor tissue, enabled targeting of angiogenic endothelial cells, and caused efficient damage to tumor cells. Therefore, APRPG–PEG-modified liposomal DPP–CNDAC caused efficient tumor growth suppression without severe side-effects.

The present study is a good example of liposomalization if the property of the objective compound is not suitable to liposomalize, and the technology used is applicable to other agents. The present study indicates the importance of designing drug, carrier, and therapeutic strategy in the development of DDS pharmaceutics.

Acknowledgments

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References

1 Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003; **3**: 643–51.
2 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
3 Kerbel RS. Antiangiogenic drugs and current strategies for the treatment of lung cancer. *Semin Oncol* 2004; **31**: 54–60.
4 Shimizu K, Asai T, Oku N. Antineovascular therapy, a novel antiangiogenic approach. *Exp Opin Ther Targets* 2005; **9**: 63–76.
5 Asai T, Oku N. Liposomalized oligopeptides in cancer therapy. *Meth Enzymol* 2005; **391**: 163–76.
6 Oku N, Asai T, Watanabe K *et al.* Antineovascular therapy using novel peptides homing to angiogenic vessels. *Oncogene* 2002; **21**: 2662–9.
7 Asai T, Nagatsuka M, Kuromi K *et al.* Suppression of tumor growth by novel peptides homing to tumor-derived new blood vessels. *FEBS Lett* 2002; **510**: 206–10.
8 Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett* 1990; **268**: 235–7.
9 Lasic DD. Doxorubicin in sterically stabilized liposomes. *Nature* 1996; **380**: 561–2.
10 Sakakibara T, Chen FA, Kida H *et al.* Doxorubicin encapsulated in sterically stabilized liposomes is superior to free drug or drug-containing conventional liposomes at suppressing growth and metastases of human lung tumor xenografts. *Cancer Res* 1996; **56**: 3743–6.
11 Sadzuka Y, Nakade A, Hirama R *et al.* Effects of mixed polyethyleneglycol modification on fixed aqueous layer thickness and antitumor activity of doxorubicin containing liposome. *Int J Pharm* 2002; **238**: 171–80.
12 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.
13 Muggia F. Doxorubicin-polymer conjugates. Further demonstration of the concept of enhanced permeability and retention. *Clin Cancer Res* 1999; **5**: 7–8.
14 Maeda N, Takeuchi Y, Takada M, Namba Y, Oku N. Synthesis of angiogenesis-targeted peptides and hydrophobized polyethylene glycol conjugate. *Bioorg Med Chem Lett* 2004; **14**: 1015–17.
15 Maeda N, Takeuchi Y, Takada M, Sadzuka Y, Namba Y, Oku N. Antineovascular therapy by use of tumor neovasculature-targeted long-circulating liposome. *J Control Release* 2004; **100**: 41–52.
16 Maeda N, Miyazawa S, Shimizu K *et al.* Enhancement of anticancer activity in antineovascular therapy is based on the intratumoral distribution of the active targeting carrier for anticancer drugs. *Biol Pharm Bull* 2006; **29**: 1936–40.
17 Matsuda A, Nakajima Y, Azuma A, Tanaka M, Sasaki T. Nucleosides and nucleotides. 100. 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*furanosyl-cytosine (CNDAC): design of a potential mechanism-based DNA-strand-breaking antineoplastic nucleoside. *J Med Chem* 1991; **34**: 2917–19.
18 Delaunoit T, Burch PA, Reid JM *et al.* A phase I clinical and pharmacokinetic study of CS-682 administered orally in advanced malignant solid tumors. *Invest New Drugs* 2006; **24**: 327–33.
19 Gilbert J, Carducci MA, Baker SD, Dees EC, Donehower R. A Phase I study of the oral antimetabolite, CS-682, administered once daily 5 days per week in patients with refractory solid tumor malignancies. *Invest New Drugs* 2006; **24**: 499–508.
20 Shuto S, Awano H, Shimazaki N, Hanaoka K, Matsuda A. Nucleosides and nucleotides. 150. Enzymatic synthesis of 5′-phosphatidyl derivatives of 1-(2′-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl) cytosine (CNDAC) and their notable antitumor effects in mice. *Bioorg Med Chem Lett* 1996; **6**: 1021–4.
21 Asai T, Kurohane K, Shuto S *et al.* Antitumor activity of 5′-*O*-dipalmitoylphosphatidyl 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine is enhanced by long-circulating liposomalization. *Biol Pharm Bull* 1998; **21**: 766–71.
22 Asai T, Shuto S, Matsuda A, Kakiuchi T, Ohba H, Tsukada H *et al.* Targeting and anti-tumor efficacy of liposomal 5′-*O*-dipalmitoylphosphatidyl 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine in mice lung bearing B16BL6 melanoma. *Cancer Lett* 2001; **162**: 49–56.
23 Asai T, Shimizu K, Kondo M *et al.* Antineovascular therapy by liposomal DPP–CNDAC targeted to angiogenic vessels. *FEBS Lett* 2002; **520**: 167–70.
24 Oku N, Doi K, Namba Y, Okada S. Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice. *Int J Cancer* 1994; **58**: 415–19.

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Disappearance of the angiogenic potential of endothelial cells caused by Argonaute2 knockdown

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Abstract

Argonaute2 (Ago2), a component protein of RNA-induced silencing complex, plays a central role in RNA interference. We focused on the involvement of Ago2 in angiogenesis. Human umbilical vein endothelial cells (HUVECs) stimulated with several growth factors such as vascular endothelial growth factor were used for angiogenesis assays. We applied polycation liposomes for transfection of small interfering RNA (siRNA) to determine the biological effects of siRNA for Ago2 (siAgo2) on HUVECs. The proliferation study indicated that siAgo2 significantly suppressed the growth of HUVECs compared with control siRNA. TUNEL staining showed a certain population of HUVECs treated with siAgo2 underwent apoptosis. Furthermore, the treatment with siAgo2 suppressed the tube formation of HUVECs and significantly reduced the length of the tubes. These present data demonstrate that siAgo2 inhibited indispensable events of angiogenesis *in vitro*. This is the first report suggesting that Ago2 is required for angiogenesis.

Keywords: Argonaute2; Angiogenesis; siRNA; Polycation liposomes

Double-stranded RNA (dsRNA) provokes sequence-specific gene silencing, commonly called RNA interference (RNAi) [1,2]. RNAi is a type of post-transcriptional gene silencing, and gene therapy using small interfering RNA (siRNA) is expected to be a novel treatment strategy [3]. For inducing RNAi, siRNA needs to be incorporated into an RNA-induced silencing complex (RISC). MicroRNAs (miRNAs), which are endogenous small non-coding RNAs that negatively regulate gene expression, are also incorporated into RISC for the cleavage or translational inhibition of the target mRNA [4]. Argonaute2 (Ago2) is a component protein of RISC and plays a central role in RNAi [5]. When a guide (antisense) strand of siRNAs or miRNA binds to its target mRNA, Ago2 expresses enzymatic activity to cleave the mRNA [6]. Ago2 is distinct from other Argonaute family members in the point that only Ago2-containing RISC is able to catalyze cleavage [5–7]. Ago2 is thus considered to be an indispensable protein for inducing RNAi. In addition, Ago2 cleaves the passenger strand of siRNAs, which facilitates the assembly of siRNAs into RISC [4,8]. On the other hand, Ago2-deficient mice show several developmental abnormalities such as a cardiac failure and a defect of neural tube closure [6]. Since these animals show an embryonic-lethal phenotype, Ago2 is essential for embryonic development.

Pathological angiogenesis is involved in diseases such as cancer [9]. Understanding of the mechanisms of angiogenesis

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leads to various antiangiogenic therapeutic modalities [10]. For instance, Avastin, a neutralizing antibody for vascular endothelial growth factor, is already used in clinical cancer chemotherapy [11]. While the participation of many proteins such as cytokines and signaling molecules in angiogenesis is well known, certain kinds of miRNAs have recently been shown to participate in angiogenesis [12]. Also, Dicer, a protein that cleaves long dsRNAs, is required for embryonic angiogenesis during mouse development [13]. Taken together, available information suggests that a miRNA system might be closely related to the regulation of angiogenesis. However, the involvement of Ago2 in angiogenesis is not known at all.

The present study is mainly focused on the possible involvement of Ago2, in addition to Dicer, in the regulation of angiogenesis. Our next concern is the application of Ago2 knockdown to antiangiogenic therapy. For establishment of RNAi therapy, a siRNA delivery system is quite important as well as a therapeutic target [14]. Polycation liposomes (PCLs), one of the non-viral types of vectors, possess the advantages of both cationic liposomes and polycations for gene delivery [15]. PCLs are simply prepared by modification of the liposomal surface with cetylated PEI (cetyl-PEI). Our previous study demonstrated that PCLs show various advantageous properties such as high transfection efficiency of plasmid DNA, low cytotoxicity, and applicability for *in vivo* use [15]. In this study, we optimized the formulation of PCLs for siRNA transfection and used such liposomes for analyzing the biological effects of Ago2 knockdown on angiogenesis.

Materials and methods

Preparation of siRNA/PCL complexes. Cetyl-PEI was synthesized as described previously [15]. Cholesterol was kindly provided by NFC Co. (Takasago, Hyogo, Japan). Dioleoylphosphatidylethanolamine (DOPE) was purchased from NOF Co. (Tokyo, Japan).

Cetyl-PEI, DOPE, and cholesterol (0.05:1:0.5 or 0 as a molar ratio) were dissolved in *tert*-butyl alcohol and freeze–dried. PCLs were produced by hydration of the lipid mixture with DEPC-treated RNase-free water. PCLs were sized by extruding them 10 times through a polycarbonate membrane filter having 100-nm pores. PCLs and siRNA solution were diluted with serum-free medium corresponding to the respective cell lines used. Then, PCLs and siRNA were mixed gently and incubated for 15 min at room temperature to form siRNA/PCLs complexes. The ratio of the nitrogen moiety of PCLs to the phosphate one of siRNA (*N/P* ratio) was varied from 18 to 30 for formulation screening. The particle size and ζ-potential of siRNA/PCLs complexes diluted with DEPC-treated water were measured by using a Zetasizer Nano ZS (Malvern, Worcs, UK). All siRNAs used in this study were purchased from Hokkaido System Science Co. (Hokkaido, Japan).

Cell cultures. HT1080 human fibrosarcoma cells (HT1080 cells) were cultured in DME/Ham F12 medium containing 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin (MP Biomedicals, Irvine, CA), and 100 μg/ml streptomycin (MP Biomedicals). HT1080 cells constitutively expressing EGFP (EGFP/HT1080 cells) had been previously established [16] and were cultured in the above medium supplemented with 100 μg/ml geneticin (Sigma–Aldrich). Human umbilical vein endothelial cells (HUVECs, Cambrex Bio Science Walkersville, Walkersville, MD) were cultured on gelatin-coated dishes containing endothelial growth medium-2 (EGM-2; Cambrex Bio Science Walkersville).

PCL-mediated transfection with siRNA. Cells were seeded and precultured overnight. The medium was then changed to fresh medium containing FBS but no antibiotics. Prepared siRNA/PCLs complexes (*N/P* ratio: 24 equiv) were added to the medium at a final concentration of 40 nM (as siRNA). After 4-h incubation, the siRNA/PCLs complexes were removed. These cells were subsequently incubated at 37 °C for additional periods of time as described for each experimental procedure.

Determination of the amount of siRNA taken into cells. The amounts of siRNA taken into cells were determined fluorometrically by using siRNA for Argonaute2 (siAgo2) labeled with 6-fluorescein-6-carboxamido hexanoate (FAM) at the 3′-terminal of its antisense strand. The nucleotide sequences of siAgo2 with a 2-nucleotide overhang (underline) were 5′-GC ACGGAAGUCCAUCUGAAUU-3′ (sense) and 5′-UUCAGAUGGAC UUCCGUGCUU-3′ (antisense). These sequences of siAgo2 correspond to the nucleotide region 1425–1443 and have been validated by G. Meister et al. [7].

HT1080 cells (3×10^4 cells/well) or HUVECs (5×10^4 cells/well) were seeded onto 24-well plates. After the cells had been cultured overnight, FAM-labeled siAgo2 (final concentration: 40 nM) in complex with PCLs was added to the cultures, which were then incubated for 4 h. The transfected cells were lysed with 2% reduced Triton X-100 containing protease inhibitors (2 mM PMSF, 200 μM leupeptin, 50 μg/mL aprotinin, and 100 μM pepstatin A). The fluorescent intensities of FAM were measured by using a spectrophotofluorometer (Wallac ARVO™ SX 1420 Multilabel Counter, Perkin-Elmer Life Sciences, Boston, MA) and corrected for protein amounts by using a BCA Protein Assay Reagent Kit (PIERCE Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Evaluation of the RNAi efficiencies obtained with PCLs. The nucleotide sequences of the siRNA for EGFP (siEGFP) with a 2-nucleotide overhang (underline) were 5′-GGCUACGUCCAGGAGCGCACC-3′ (sense) and 5′-UGCGCUCCUGGACGUAGCCUU-3′ (antisense). The sequences of siEGFP correspond to the nucleotide region 118–141.

EGFP/HT1080 cells were seeded onto 24-well plates at the density of 6×10^4 cells/well and transfected with siEGFP complexed with PCLs. After these cells had been transfected, the cells were lysed at 48 h after culture. The fluorescence intensity of EGFP was measured with a spectrophotofluorometer and corrected for protein amounts. EGFP/HT-1080 cells were also transfected with siEGFP mixed with Lipofectamine™ 2000 (LFA2K, Invitrogen, Rockville, MD) according to the manufacturer's instructions. The transfection time schedule was similar to that used for the PCLs.

Cytotoxicity assay. EGFP/HT-1080 cells were seeded onto 96-well plates at the density of 1.2×10^4 cells/well and transfected with siEGFP. After 24-h incubation, Tetracolor ONE™ (Seikagaku, Tokyo, Japan) was added to each well in accordance with the manufacturer's instructions. The amount of formazan formed in 3 h was measured on a microplate reader (MTP-120, Corona Electric, Ibaraki, Japan) at a test wavelength of 492 nm and a reference wavelength of 630 nm.

RT-PCR. Total RNA was isolated by using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) at 8, 16 or 24 h post-transfection with siRNA. Complementary DNA was generated from total RNA samples (5 μg) by use of a Ready-To-Go T-primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ). The PCR conditions were as follow: for Ago2, 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 67 °C for 30 s, and 72 °C for 30 s and then 72 °C for 15 min; for β-actin and EGFP, 94 °C for 2 min followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and then 72 °C for 10 min; and for GAPDH, 95 °C for 5 min followed by 20 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min and then 72 °C for 10 min. The primers for Ago2 were 5′-TGAACAACATC CTGCTGCCCCAGGGC-3′ (sense) and 5′-TCATGTTCGATGCTGGC TGTCACGGAAGGG-3′ (antisense); for β-actin, 5′-TGACGGGGTCAC CCACACTGTGCCCATCTA-3′ (sense) and 5′-CTAGAAGCATTTGC GGTGGACGATGGAGGG-3′ (antisense), for EGFP, 5′-TACGGCAA GCTGACCCTGAAGTTC-3′ (sense) and 5′-CGTCCTTGAAGAAGAT GGTGCG-3′ (antisense); and for GAPDH, 5′-TGTTGCCATCAATGA CCCCTTC-3′ (sense) and 5′-AGCATCGCCCCACTTGATTTTG-3′ (antisense). The PCR products were applied onto 2.0% (β-actin, EGFP,