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がん微小環境制御を併用したナノドラッグによる難治性固形がん治療の実現

平成２１年度　総括・分担研究報告書

研究代表者　狩野　光伸

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目　　次

Ⅰ．総括研究報告
がん微小環境制御を併用したナノドラッグによる難治性固形がん治療の実現
------------------------------ 1
狩野　光伸

Ⅱ．分担研究報告
がん微小環境制御を併用したナノドラッグによる難治性固形がん治療の実現
（白金錯体制ガン剤DACHPtを内包した高分子ミセルの構築）
------------------------------ 6
西山　伸宏

Ⅲ．研究成果の刊行に関する一覧表 ------------------------------ 12

Ⅳ．研究成果の刊行物・別刷 ------------------------------ 14

別添3

厚生労働科学研究費補助金（医療機器開発推進研究事業）
総括研究報告書

がん微小環境制御を併用したナノドラッグによる難治性固形がん治療の実現

主任研究者　狩野光伸　東京大学大学院医学系研究科分子病理学　講師

研究要旨

ナノ粒子内包薬剤は副作用が少なく治療効果は高いことが期待されてきた。しかし難治性固形癌の治療ではあまり効果を示せていない。その原因として、腫瘍微小環境の性質も重要な一因を構成していると予想される。本研究は、この腫瘍微小環境制御の併用により、副作用の増悪を最小限にしながらナノDDSの薬効を増強することを目指す。本年度は、各種毒性解析を行った結果、TGF-β阻害剤の併用投与により有意な体重変化や各種異常所見の出現は認めなかった。また、適応患者の絞込みを行うべく、ヒト腫瘍病理標本に対する血管壁細胞被覆程度の解析を行った結果、膵癌、スキルス胃癌、悪性胸膜中皮腫については同様の血管構築を持ち、本研究による方法の適応対象となりうることが示唆された。

研究分担者

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A．研究目的

ナノ粒子内包薬剤は副作用が少なく治療効果は高いことが期待されてきた。しかし難治性固形癌の治療ではあまり効果を示せていない。その原因として、腫瘍微小環境の性質も重要な一因を構成していると予想される。本研究は、この腫瘍微小環境制御の併用により、副作用の増悪を最小限にしながらナノDDSの薬効を増強することを目指してきた。

我々は、これまでに膵癌・胃癌の動物モデルにおいて、TGF-β阻害剤を用いた血管新生制御の併用によってDDSの効果増大が見られることを、分担研究者らの合成した薬剤を用いて証明した。これに続いて、漏出性腫瘍血管を持つC26大腸癌モデルとそうでないBxPC3膵癌モデルを用いて、各種阻害剤の効果を比較した。この結果、C26腫瘍に対してはVEGF阻害が、BxPC3腫瘍ではTGF-β阻害剤がナノ粒子蓄積増強効果を示すことが明らかにされた。関連して組織学的には、血管の壁細胞による被覆が強いほど漏出性が低く、TGF-β阻害剤の効果が発揮されることが判明した。こ

れらの結果から、壁細胞被覆の強い血管を持つ腫瘍においては、本研究の戦略がふさわしいことが示唆された。またBxPC3モデルにおいて、分担研究者により開発された遺伝子発現ベクター内包およびMRI造影剤内包ナノ粒子に対して、TGF-β阻害併用時のみ腫瘍内への有意な蓄積が起こり、効果が観察されることを示した。

本年度は、本方法に関して、各種毒性解析を行うこと、また、適応患者の絞込みを行うべく、ヒト腫瘍病理標本に対する血管壁細胞被覆程度の解析を行うことを目的とした。

B．研究方法

1.毒性評価

ヒト膵癌由来BxPC3細胞株を移植したヌードマウスに対して、分担研究者によって供給されたDACHPt内包ナノDDSを投与し、TGF-β阻害剤1mg/kg併用投与の有無で、体重変化を投与開始後16日間追跡した。なお、DACHPt（ダハプラチン）は、白金系抗腫瘍剤であるオキザリプラチンの中間活性体である。

また、非担癌ヌードマウスに対して同ナノDDSを継続投与し、これに対して各回TGF-β阻害剤1mg/kg併用投与の有無で、2か月間継続観察し、体重変化及び各種異常所見の出現がないかを確認した。

さらに^{111}Inを内包したミセルを用いて、TGF-β阻害剤1mg/kg併用投与の有無で体内各臓器への蓄積が変化しないかを、ラジオグラフィーを用いて検討した。

2.組織学的評価

膵癌、胃癌（通常胃癌とスキルス胃癌双方）、大腸癌（組織型はmed, int, sciのうち最も症例数が多いintの症例）、卵巣癌、胸膜中皮腫について、各5症例以上の病理標本に対して、連続切片を作成し、通常のHE染色、血管内皮を染色するCD34の免疫染色、ペリサイトと考えられる血管外周に存在するsmooth muscle actin (SMA)の免疫染色を行った。

（倫理面への配慮）本研究では、あらかじめ研究機関の長等の承認、届出、確認等が必要な研究について、研究開始前に所定の手続を行った。具体的には、前記２のヒト病理検体を用いた研究内容は、平成20年1月東京大学医学部倫理委員会にて承認された「動物モデルにおいて腫瘍内での薬剤送達を規定したタンパク質の発現をヒト病理組織で確認する（1945）」に含まれる。また、前記１の動物を用いた実験は、東京大学医学部の定める規則に従い、動物実験の講習を修了し充分な知識と経験を有するものだけに従事させ行った。

これらから、倫理面の問題はないと判断している。

C．研究結果

1.毒性評価

BxPC3細胞担癌ヌードマウスにおいてDACHPt内包ナノDDSに対してTGF-β阻害剤1mg/kg併用投与の有無で、体重変化を投与開始後16日間追跡した。この結果、TGF-β阻害剤の有無で体重変化に有意な差は認められなかった。また、非担癌ヌードマウスにおいて同ナノDDSを継続投与し、これに対して各回TGF-β阻害剤1mg/kg併用投与の有無で、2か月間継続観察した結果でも、体重変化に有意な差は認めず、またTGF-β阻害剤の有無にかかわらず明らかな異常所見の出現は認められなかった。さらに^{111}Inを内包ミセル投与でTGF-β阻害剤1mg/kg併用投与の有無により^{111}Inの体内正常各臓器への蓄積が変化しないかを、ラジオグラフィーを用いて検討した結果、TGF-β阻害剤の有無で明らかな臓器分布の差は認められなかった。

2.組織学的評価

解析した各種腫瘍のうち、膵癌、スキルス胃癌、胸膜中皮腫については、腫瘍血管周囲にSMA陽性細胞がほぼ必ず存在し、ペリサイトによる被覆があることが確認された。一方で、スキルスではなく通常の胃癌、大腸癌、卵巣癌では、血管周囲にSMA陽性細胞はほぼ認められなかった。

D．考察

BxPC3細胞担癌ヌードマウス及び非担癌ヌードマウスを用い、DACHPtミセルを投与する、または、非担癌ヌードマウスを用い放射性ラベル物質を内包したミセルを用いた毒性評価の結果、本研究における手法、すなわちTGF-β阻害剤を併用したナノDDSでは、明らかな副作用の増悪は認められないことが強く示唆された。

また、組織学的評価の結果、膵癌、スキルス胃癌、胸膜中皮腫については、腫瘍血管周囲にSMA陽性細胞がほぼ必ず存在し、ペリサイトによる被覆がある血管パターン、一方で、通常胃癌、大腸癌、卵巣癌は、血管周囲にSMA陽性細胞はほぼ認められず、ペリサイトの被覆がほぼない血管パターンであることが判明した。昨年度のBxPC3膵癌モデルとC26大腸癌モデルを用いた比較で、前者はペリサイトの被覆がある血管パターン、後者はペリサイトの被覆がほぼない血管パターンであるとともに、前者はTGF-β阻害剤を併用することでナノDDSが初めて奏功し、後者ではナノDDS単独でも蓄積できることが判明している。この結果と合わせると、ヒトにおいても、膵癌、スキルス胃癌、胸膜中皮腫では、TGF-β阻害剤を併用することでナノDDSが初めて奏功する可能性が示唆された。

E．結論

本年度は、TGF-β阻害剤を併用したナノDDSの副作用が増悪はしないこと、またこの方法論のヒトでの適応疾患として、膵癌、スキルス胃癌、悪性中皮腫の可能性があることが示唆された。また分担研究者の西山により、DACHPtを含む中空ポリマーナノ粒子が新規に開発された。この粒子には蛍光物質やMRIなどに対して造影効果を持つ物質の内包も可能である。本研究終了後も、西山らと連携し、本方法を難治性がんの診断・治療法の実現へと展開していきたい。

F．健康危険情報

特記すべきことなし。

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別添4

厚生労働科学研究費補助金（医療機器開発推進研究事業）
分担研究報告書

がん微小環境制御を併用したナノドラッグによる難治性固形がん治療の実現
（白金錯体制ガン剤DACHPtを内包した高分子ミセルの構築）

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研究要旨
本研究では、難治ガンの標的治療を目的として、高分子ミセル型ナノキャリアの最適化と高機能化を目指している。本年度は、白金錯体制ガン剤であるDACHPtと同時に水溶性薬剤を送達できるナノキャリアとして高分子-金属錯体形成を駆動力とする中空ナノ粒子(プラチナソーム)の構築を行った。

A．研究目的

本研究では、高分子ミセル型ナノドラッグを研究代表者である狩野に供給し、新しい治療戦略に基づいて難治性固形ガンの標的治療を実現することを目指している。この目的において、前年度までに白金錯体制ガン剤であるdichloro (1,2- diaminocyclohexane) platinum (II)(DACHPt)(オキサリプラチンの中間活性体)を内包した高分子ミセルの調製を行い、そのin vivoにおける有効性を確認してきたが、本年度はDACHPtと同時に水溶性薬剤を送達できるナノキャリアとして高分子-金属錯体形成を駆動力とする中空ナノ粒子(プラチナソーム)の構築を行った。

B．研究方法

1. PEGasus-*b*-P(Glu)-Cholブロック共重合体の合成

PEGasus-NH_2(分子量20,000 (10,000×2))を開始剤としてγ-benzyl L-glutamate (BLG) N-カルボン酸無水物(BLG-NCA)を重合することにより、PEGasus-*b*-PBLGを合成した(PBLGの重合度:20)。次に、PEGasus-*b*-PBLGのN末端にcholesteryl chloroformateを反応させることによって、PEGasus-*b*-PBLG-Cholを合成した。合成したPEGasus-*b*-PBLG-Cholは0.5N NaOH中で脱保護することにより目的とするPEGasus-*b*-P(Glu)-Cholを合成した。

2. プラチナソームの調製

プラチナソームの調製はPEGasus-*b*-P(Glu)-CholとDACHPtを混合し、水中で120時間反応させることにより行った。その後、限外ろ過を行い、調製したサ

ンプルの動的光散乱(DLS)測定、透過型電子顕微鏡(TEM)観察を行った。

プラチナソームに内包させるモデル水溶性薬剤として蛍光(FITC, Alexa680)標識dextran(分子量: 10,000)の封入を検討した。薬剤の封入は、上記の方法によりプラチナソームを調製した。

3. プラチナソームの機能評価

プラチナソームの機能評価として、第一に、150mM NaCl含有リン酸緩衝液(pH7.4)中におけるPtおよび蛍光標識dextranのリリースを透析法により評価した。Pt量はICP-MSにより定量した。次に、プラチナソームの安定性をDLS測定により評価した。最後に、プラチナソームのin vitroにおける細胞毒性、担がんマウス(Colon-26細胞皮下移植モデル)における体内動態、蛍光標識dextranによる腫瘍のイメージング、制がん活性を評価した。

C．研究結果

1. プラチナソームの物性評価

PEGasus-*b*-P(Glu)-CholとDACHPtを水中で120時間反応させることよって120nmの粒径分布の狭い会合体の形成が確認された。DACHPt内包ミセルが35nmの粒径を有することを考えると、上記の会合体はミセル以外の形態を有するものと考えられたため、TEM観察を行った。その結果、中空ナノ粒子の形成が確認された。次に、FITC-dextran存在下でプラチナソームを調製し、封入の確認をゲルろ過クロマトグラフィーにより行った。その結果、プラチナソームが溶出する同じ時間にFITC-dextranも溶出することが確認され、水溶性のFITC-dextranがプラチナソームに封入されることが明らかになった。さらに、150mM NaCl含有リン酸緩衝液(pH7.4)中におけるプラチナソームからのDACHPtとAlexa680-dextranのリリースを評価した。その結果、DACHPtは、プラチナソームより持続的に放出(100時間後に約50%の放出)され、Alexa680-dextranは、最初の12時間は放出されないが、その後、徐放されることが明らかになった(100時間後に約30%の放出)。また、150mM NaCl含有リン酸緩衝液(pH7.4)中におけるプラチナソームの安定性に関しては、30時間後までに120nmから80-90nmに粒径が減少したが、その後はDACHPtのリリース量に拘わらず、80-90nmの一定の粒径が維持されることが確認され、プラチナソームは高い構造安定性を有することが示唆された。

2. プラチナソームの生物学的評価

プラチナソームの体内動態試験を実施したところ、静脈内投与24時間後において5%のPt、10%のFITC-dextranが血中に存在していることが明らかになった。ここで、DACHPtおよびFITC-dextranを単独で血中に投与した際は速やかに消失することが確認されている。一方、がんへの集積に関して

は、24時間後に10% dose/g組織のPt、20% dose/g組織のFITC-dextranががんに集積することが確認された。この値は、他の臓器への集積と比較しても有意に高く、プラチナソームのがんへの選択的な集積が示唆された。また、Alexa680-dextranを搭載したプラチナソームを担がんマウスに投与し、24時間後に生きたマウスの蛍光イメージングを行ったところ、がん組織のみがイメージングされることが確認された。さらに、担がんマウスを用いた制がん活性試験においては、oxaliplatinは、8mg/kgでは薬効を示さず、10mg/kgでは毒性死を示したが、プラチナソームは6m/kgで著明な制がん活性を示した。一方、体重変化による毒性試験では、プラチナソーム投与群で有意な体重の減少は認められなかった。

D．考察

本研究では、金属錯体形成を駆動力とする新しい超分子集合体としてプラチナソームを開発した。

調製したプラチナソームは、内水相に蛍光標識dextranを内包させることが可能であった。150mM NaCl含有リン酸緩衝液(pH7.4)中におけるミセルの安定性に関しては、PtのP(Glu)のカルボキシル基との配位子交換反応によってDACHPtが放出され、持続的な放出が確認されたが、プラチナソームの構造は長時間維持されることが示唆された。このような特性には、ブロック共重合体の末端に導入したCholesteryl基による高分子-金属錯体の構造安定化が寄与しているものと考えられる。一方、プラチナソームからDACHPtがリリースされることによって膜の透過性が増大し、12時間後からゆっくりとAlexa680-dextranがリリースされることが明らかになった。また、プラチナソームの生物学的評価に関しては、担がんマウスを用いた体内動態試験において、プラチナソームの長期血中滞留性、がん選択的集積性が確認され、制がん活性試験において著明な制がん活性が確認された。以上のようにプラチナソームは、ペプチドやタンパク質などの水溶性薬剤を搭載でき、キャリアが患部に到達する投与12時間後にそれらの溶性薬剤を放出することができる制御放出型のナノキャリアシステムとして今後のさらなる展開が期待できる。

E．結論

本年度は、DACHPtと同時に水溶性薬剤を送達できるナノキャリアとして高分子-金属錯体形成を駆動力とする中空ナノ粒子(プラチナソーム)の構築を行った。本システムは、低分子化合物、核酸医薬、生理活性ペプチドおよびタンパク質などの様々な水溶性薬剤を搭載することができ、複合デリバリーシステムとして非常に有望であると考えられる。また、プラチナソームには、MRI造影剤や蛍光分子などを搭載すること

が可能であり、患部のイメージングや研究ツールとしても非常に有用である。今後、狩野と連携として、本システムを難治性がんの診断・治療へと展開していきたいと考えている。

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GENE DELIVERY

Antiangiogenic gene therapy of experimental pancreatic tumor by sFlt-1 plasmid DNA carried by RGD-modified crosslinked polyplex micelles

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ABSTRACT

Disulfide crosslinked polyplex micelles with RGD peptides were formed through ion complexation of thiolated c(RGDfK)-poly(ethylene glycol)-*block*-poly(L-lysine) (c(RGDfK)-PEG-P(Lys-SH)) and plasmid DNA encoding sFlt-1 and tested for their therapeutic effect in BxPC3 pancreatic adenocarcinoma tumor bearing mice. These micelles, systemically injected, demonstrated significant inhibition of tumor growth up to day 18, as a result of the antiangiogenic effect that was confirmed by vascular density measurements. Significant therapeutic activity of the 15% crosslinked micelle (c(RGDfK)-PEG-P(Lys-SH15)) was achieved by combined effect of increased tumor accumulation, interaction with endothelial cells and enhanced intracellular uptake through receptor-mediated endocytosis. These results suggest that RGD targeted crosslinked polyplex micelles can be effective plasmid DNA carriers for antiangiogenic gene therapy.

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1. Introduction

Poly(ethylene glycol) (PEG)-polycation block copolymers have been widely investigated in the field of gene delivery as a potential non-viral vectors for systemic applications [1–7]. The complexes of plasmid DNA (pDNA) and block copolymers form self-assembling particles, termed polyplex micelles, with a core-shell structure. The outer hydrophilic shell layer, formed by PEG segment, increases micelle stability in serum, improves its pharmacokinetic properties, and reduces polymer toxicity [8–11]. Nevertheless, further stabilization and increased longevity in blood are required for polyplex micelles to achieve successful gene delivery *in vivo*.

Disulfide crosslinks were previously introduced into the polyplex micelle core to stabilize its structure in the extracellular entity, while facilitating smooth release of the entrapped pDNA in the intracellular reductive environment [12,13]. Indeed, disulfide crosslinked polyplex micelles exhibited improved transfection of the reporter gene to cultured cells and mouse liver upon systemic administration [13]. In addition, cyclic RGD peptide ligands (c(RGDfK)) were recently installed onto the surface of the disulfide crosslinked polyplex micelles to achieve specific targeting to tumor neo-vasculature [14,15]. RGD (Arg–Gly–Asp) peptide is a recognition motif in multiple ligands of α_v integrin family [16]. Moreover, cyclic RGD peptides showed increased affinity to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors [17] which are overexpressed on tumor angiogenic endothelial cells [18]. Therefore, RGD peptide ligands have been intensively investigated as an active targeting strategy in antiangiogenic gene therapy for cancer [19–22]. Consequently, we hypothesized that polyplex micelles with cyclic RGD ligands and disulfide crosslinks may be a useful system for targeting angiogenic endothelial cells by systemic administration. RGD conjugated polyplex micelles showed remarkably increased transfection efficiency in cultured HeLa cells possessing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, as a result of increased cellular uptake and intracellular trafficking of micelles toward perinuclear region via caveolae-mediated endocytosis as was previously reported [14,15]. Caveolae- mediated endocytosis is a nondigestive internalization pathway, which does not result in pH decrease, thus avoiding pDNA degradation in acidic organelles in cell. This route might be especially essential for polylysine based pDNA carriers, which do not possess "proton buffering" ability to escape endosome.

Vascular endothelial growth factor (VEGF) is a major proangiogenic molecule, which stimulates angiogenesis via promoting endothelial proliferation, survival and migration [reviewed in [23,24]]. VEGF and VEGF receptors have been found to be up-regulated in

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GENE DELIVERY

various types of tumors and are usually associated with tumor progression and poor prognosis (reviewed in [25]). Inhibition of VEGF or its signaling pathway has been shown to suppress tumor angiogenesis and tumor growth [reviewed in [25–27]].

The soluble form of VEGF receptor-1 (soluble fms-like tyrosine kinase-1: sFlt-1) is a potent endogenous agent for antiangiogenic therapy. The sFlt-1 binds to VEGF with the same affinity and equivalent specificity as that of the original receptor, however inhibits its signal transduction [28–30]. Therefore, exogenous sFlt-1 is considered to be an effective therapeutic agent for antiangiogenic tumor therapy [20,21,31–35]. Recently, several reports were published on *in vivo* non-viral gene therapy with sFlt-1, carried by several types of polymers, for inhibition of tumor angiogenesis [21,35]. Kim WJ et al. reported effective tumor growth suppression in CT-26 colon adenocarcinoma bearing mice by systemic injection of polyethyleneimine based polyplexes, utilizing the RGD targeting approach [21].

In this study, thiolated PEG-poly(L-lysine) (PEG-PLys) block copolymer, combining long PEG chain with optimized crosslinking degree, was designed for construction of RGD-mediated gene delivery system. Here we report the therapeutic effect of sFlt-1 expressing pDNA complexed with 15% thiolated control poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-P(Lys-SH15)) and cyclic RGD conjugated (c(RGDfK)-PEG-P(Lys-SH15)) polymers, forming crosslinked polyplex micelles, after systemic administration to BxPC3 human pancreas adenocarcinoma tumor bearing mice. Note that BxPC3 xenografts are characterized by heterogeneous vascularity and stroma-rich histology [36], which limits access of therapeutic agents to tumor cells. Thus, the accessibility of endothelial cells by bloodstream, makes antiangiogenic approach an attractive strategy against pancreatic tumor.

2. Materials and methods

2.1. Materials

N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cyclo[RGDfK(CX-)] (c(RGDfK)) peptides (X = 6-aminocaproic acid: ε-Acp) was purchased from Peptide Institute (Osaka, Japan). The PEG-PLys block copolymer (PEG, 17,000 g/mol; polymerization degree of PLys segment, 73) was synthesized as previously reported [37]. Plasmid DNA coding for luciferase (Luc) under the control of CAG promoter was provided by RIKEN Gene Bank (Tsukuba, Japan), and a fragment cDNA of sFlt-1 was inserted into the pCAcc vector having CAG promoter. The pDNAs were amplified in competent DH5α *Escherichia coli* and purified by the HiSpeed Plasmid Maxi Kit purchased from QIAGEN Sciences Co., Inc. (Germantown, MD). Luc pDNA was labeled with Cy5 by the Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI) according to the manufacturer's protocol. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich Co (Madison, WI) and Dainippon Sumimoto Pharma Co., Ltd. (Osaka, Japan), respectively. Rat monoclonal antibody to CD31 (platelet endothelial cell adhesion molecule 1 (PECAM1)) was purchased from BD Pharmingen (Franklin Lakes, NJ), and Alexa Fluor 488-conjugated secondary antibody to rat IgG was from Invitrogen Molecular Probes (Eugene, OR).

2.2. Preparation of block copolymers

2.2.1. Synthesis of thiolated PEG-PLys (PEG-P(Lys-SH))

Pyridyldithiopropionyl (PDP) groups were introduced to the ε-amino groups of PLys side chain as reported previously [12]. Briefly, acetal-PEG-PLys (83 mg, 2.86 µmol) was dissolved in 10 mL *N*-methyl-2-pyrrolidone containing 5 wt.% LiCl and stirred with a heterobifunctional reagent, SPDP, (10 mg, 31 µmol) in the presence of *N,N*-diisopropylethylamine (10 mol excess against the SPDP reagent) for 3 h at room temperature. The mixture was then precipitated into 20 times excess volume of diethyl ether. The precipitated polymer was dissolved in 10 mM phosphate buffer (pH 7.0, 150 mM NaCl), dialyzed against the same buffer and then distilled water, and lyophilized to obtain PEG-P(Lys-PDP). The degree of PDP substitution for each polymer was determined from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2, $\delta = 3.5$ ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl group (C_5H_4N, $\delta = 7.2$–8.3 ppm) in the ^1H NMR spectrum (D_2O, 25 °C). Block copolymer with X % thiolation degree was abbreviated as B-SHX%.

2.2.2. Synthesis of c(RGDfK)-PEG-P(Lys-SH)

Acetal-PEG-P(Lys-PDP) (30 mg, 1 µmol) was dissolved in 10 mM Tris–HCl buffer solution (pH 7.4) (3 mL) with 10 eq. of dithiothreitol (DTT). After 30 min incubation at room temperature, the polymer solution was dialyzed against 0.2 M AcOH buffer (pH 4.0). c[RGDfK(CX-)] (8 mg, 6.5 mmol) in AcOH buffer (3 mL) was then added to the polymer solution. After stirring for 5 days, DTT (6.67 mg, 43.9 µmol) was added and stirred at room temperature for 3 h. The reacted polymer was purified by dialysis sequentially against 10 mM phosphate buffer pH 7.0 with 150 mM NaCl and distilled water, and lyophilized to obtain c(RGDfK)-PEG-P(Lys-SH) [14].

2.3. Preparation of polyplex micelles

The above polymers were dissolved in 10 mM Tris–HCl buffer (pH 7.4) containing 10% volume of 100 mM DTT. After 30 min at ambient temperature, twice-excess volume of pDNA solution (50 µg/mL) in the same buffer was added to the polymer solution to form a polyplex micelle at *N/P* ratio of 2. The *N/P* ratio was defined as the residual molar ratio of amino groups of thiolated PEG-PLys to phosphate groups of pDNA. After an overnight incubation at ambient temperature, the polyplex micelle solutions were dialyzed against 10 mM Tris–HCl (pH 7.4) containing 0.5% dimethylsulfoxide (DMSO) at 37 °C for 24 h, followed by additional 2 days dialysis for the DMSO removal. During these dialysis processes, thiol groups of the polymers in the micelles were oxidized to form disulfide crosslinks. The concentration of pDNA in each micelle solution was determined by absorbance at 260 nm. Polyplex micelles with and without cyclic RGD peptide ligands were abbreviated as RGD(+) and RGD(−), respectively.

2.4. Quantitative determination of transfection efficiency by real time reverse transcription-polymerase chain reaction (RT-PCR) for sFlt-1

HeLa cells, expressing the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, were seeded on 24-well culture plates (10000 cells/well) and incubated for 24 h in 500 µL of DMEM medium containing 10% FBS. Micelle solutions were then added at a concentration equivalent to 1 µg of pDNA per well and the cells were incubated for 48 h. Following this incubation period, total RNA was extracted from the cells and transcripted to cDNA. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using the following human specific primers: 5′-CCACTCCCTTGAACACGAG-3′ and 3′-CGCCTTACG-GAAGCTCTCT-5′. Amplification conditions were as recommended by the manufacturer (QIAGEN Sciences Co., Inc.). Unknown and standard samples were run in triplicate. Concentrations of unknown samples were interpolated from a standard curve, established by simultaneous amplification of sFlt-1 plasmid standards.

2.5. In vivo studies

2.5.1. Mice

Five-week-old female Balb/c nude mice were purchased from Charles River Laboratories (Tokyo, Japan). Mice were maintained on ad libitum rodent feed and water. The experimental animals were allowed to acclimate for at least 1 week before tumor implantation. All studies were performed in accordance to the Guide for the Care

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and Use of Laboratory Animals as stated by the National Institutes of Health.

2.5.2. Tumor implantation

BxPC3 cell line (ATCC, Manassas, VA), derived from human pancreatic tumor was inoculated to nude mice subcutaneously to develop xenografts (100 μl of 5×10^7 cells/mL PBS suspension). Tumors were allowed to grow for 3 weeks till their size reached approximately 120–160 mm^3.

2.5.3. Blood circulation

Polyplex micelles loading Cy5-labeled pDNA (100 μg pDNA/mL, 200 μL) were intravenously injected to the mice via the tail vein at a dose of 20 μg pDNA/mouse. Blood was collected from the postcaval vein under anesthesia 15 min after injection and centrifuged to obtain blood plasma. Two microliters of 10X trypsin-EDTA were added to 20 μL of the plasma and incubated overnight at 37 °C to release pDNA from the micelle by digesting PLys segment of the block copolymer. The fluorescence intensity of the sample solution was measured at $\lambda=670$ nm by spectrofluorometer (ND-3300, Nano Drop, Wilmington, DE), and percent of pDNA dosage in the blood was calculated according to the following equation:

$$\%\ \text{injected pDNA in the blood} = (F_{670(\text{sample})} / F_{670(\text{control})}) \times 100 \quad (1)$$

where the $F_{670(\text{control})}$ represents the fluorescence intensity of micelle solution mixed with blood sample (time 0).

2.5.4. In vivo tumor growth inhibition

Polyplex micelles, loading pDNA equivalent to 20 μg and dissolved in 10 mM Hepes buffer (pH 7.4) with 150 mM NaCl, were administered intravenously on days 0, 4, and 8. Tumor size was measured every 2 days by a digital vernier caliper across its longest (a) and shortest diameters (b) and its volume (V) was calculated according to the formula $V=0.5ab^2$. Tumor progression was evaluated in terms of relative tumor volume (to day 0) over a period of 18 days.

2.5.5. Quantification of microvessel density

At the end of *in vivo* tumor growth studies, xenografted tumors were excised and frozen in tissue-Tek-OCT. The frozen tumors were cut into 10 μm thick slices with a cryostat maintained at −23 °C. Vascular endothelial cells were immunostained by incubation of the cryosections with anti-CD31 antibody followed by incubation with Alexa Fluor 488-conjugated secondary antibody. The tumor cryosections were observed by a confocal laser scanning microscope (CLSM), LSM 510 (Carl Zeiss, Oberlochen, Germany). Microvessel density was quantified by counting the percentage area of CD31 positive pixels per image with at least 21 images per sample (i.e., three animals per sample x 7 cryosections per tumor).

2.5.6. Micelle accumulation in tumor tissue

Polyplex micelles loading Cy5-labeled pDNA were intravenously injected at a dose of 20 μg pDNA/mouse. Mice were sacrificed after 24 h and the excised tumors were fixed in formalin for 1 h, followed by 1 h incubation periods with 10, 15 and 20% sucrose/PBS solutions at room temperature. The tumors were frozen in tissue-Tek-OCT and cryosections were prepared for CLSM visualizations as described in the previous section. The nuclei were stained with Hoechst 33342 (Dojindo Lab., Kumamoto, Japan). The CLSM observations were performed at the excitation wavelengths of 488 nm (Ar laser) for the Alexa Fluor 488, 633 nm (He-Ne laser) for Cy5, and 710 nm (MaiTai laser, two photon excitation) for Hoechst 33342, respectively. The percentage of pDNA positive pixels per image was counted to quantify the micelle accumulation inside the tumor tissue.

2.6. Data analysis

The experimental data was analyzed by Student's *t*-test. $P<0.05$ was considered as significant.

3. Results

Thiolated acetal-PEG-PLys block copolymers, composed of 17 kDa M.W. PEG and 73 lysine units, were prepared as described elsewhere [12,14,37]. SPDP was used as a thiolating reagent and conjugated to the ε-amino group of lysine unit. Conjugation of c(RGDfK) peptide ligands into the PEG terminus of acetal-PEG-P(Lys-PDP) was achieved through the formation of a thiazolidine ring between the *N*-terminal cysteine and the aldehyde group converted from the acetal group [14,15]. The targetable polyplex micelles were prepared through ion complexation of the above polymers with pDNA at $N/P=2$ (Fig. 1), and analyzed for their size and ζ-potential by DLS and laser-doppler electrophorsis, respectively. The cumulant diameters of the B-SHX% micelles were approximately 104 ± 18 nm, with a moderate polydispersity index of 0.2. The ζ-potentials were found to be approximately 0.5 mV, as a result of the PEG palisade formation surrounding the polyplex core [8,14].

Following *in vitro* transfection in HeLa cells, the mRNA expressions of sFlt-1 were quantitatively analyzed by real time RT-PCR. From this analysis, presented in Fig. 2, it is clear that the cells were successfully transfected by the polyplex micelles. The highest transfection efficiency was achieved by RGD(+) B-SH15% crosslinked (15(+)) micelle. Worth noting, detectable protein level of sFlt-1 by ELISA, specific to human VEGF-R1/sFlt-1 (R&D Systems), could be achieved for this formulation only (1.2 ± 0.05 ng/mL) (data not shown). Other micelles, probably, resulted in sFlt-1 levels which are beyond the sensitivity of this assay (<13 pg/ml). The increased transfection efficiency of the 15(+) micelle results from the combination of crosslinked core and receptor targeting ligand, consistent with our previous studies [15].

The blood circulation experiments were carried out in BxPC3 tumor bearing mice upon intravenous injections of the Cy5-labeled pDNA (20 μg pDNA/ mouse). Blood was collected from the postcaval vein 15 min after administration and analyzed for its fluorescence intensity. Disulfide crosslinks prolonged blood circulation time, while the RGD conjugation resulted in significantly lower blood circulation period of polyplex micelles, as shown in Fig. 3. In the case of crosslinked system, 28% and 21% of injected pDNA were observed in plasma for RGD(−) and RGD(+) micelles, respectively. Significantly lower recovered doses of pDNA, 11 % and 7 % for RGD(−) and RGD(+) micelles, respectively, were found for non crosslinked system. We further evaluated micelle accumulation in tumor by iv administration of RGD-conjugated or non-conjugated 15% crosslinked micelles prepared with Cy5-labeled pDNA at a dose of 20 μg pDNA/mouse. Both micelles were found to be localized in the tumor blood vessels, 24 h after administration, as was indicated by colocalization of the Cy5-labeled pDNA (red) and the CD31 positive endothelial cells (green) (Fig. 4A). However, quantitative analysis of the pDNA positive area per image revealed significantly higher accumulation of the RGD-conjugated micelle than non-conjugated micelle inside the tumor tissue (Fig. 4B): 3.08 % and 2.44 % of red pixels per image for RGD(+) and RGD(−) micelle, respectively ($P<0.05$).

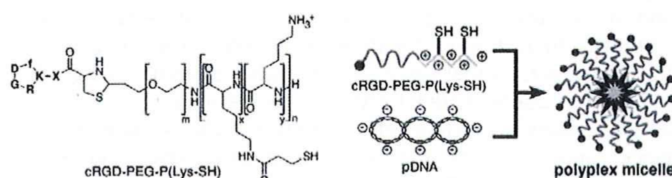

Fig. 1. Structure of cRGD-PEG-P(Lys-SH) and its polyplex micelle.

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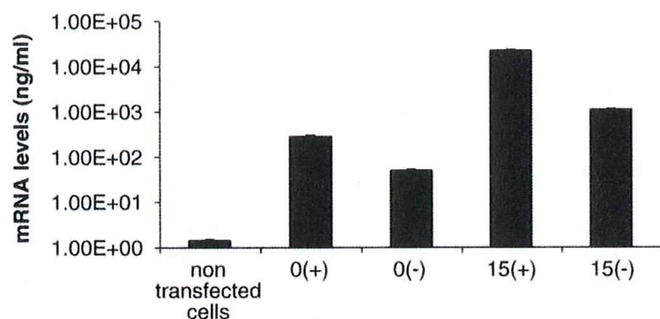

Fig. 2. *In vitro* transfection efficiency of sFlt-1 plasmid DNA in HeLa cells. The cells were transfected with RGD(+) and RGD(−) non crosslinked micelles (0(+) and 0(−)) and RGD(+) and RGD(−) 15% crosslinked micelles (15(+) and 15(−)), respectively. Non transfected cells were used as control. Each well was transfected with 1 μg of pDNA for 48 h and analyzed for sFlt-1 mRNA levels by real time RT-PCR.

The therapeutic effect of polyplex micelles following intravenous administration of the sFlt-1 expressing pDNA was evaluated by tumor growth inhibition study in BxPC3 tumor bearing mice. When tumors reached the volume of 120–160 mm^3, animals were injected with three doses of polyplex micelles containing either sFlt-1 or Luc expressing plasmid (20 μg pDNA/dose) on days 0, 4 and 8. The results of these studies, in terms of relative tumor volumes (Fig. 5), indicate the ability of RGD(+) and RGD(−) crosslinked polyplex micelles as vehicles for therapeutic gene delivery in BxPC3 tumor bearing mice. In the case of animals treated with 15(+) micelles, the tumor progression was significantly inhibited from day 6, compared to control mice. By the end of the experiment, the mean tumor volume in this group was 1.67 ± 0.18 of initial tumor volume. In the group of animals treated with pDNA encapsulated in RGD(−) micelles, significant inhibition of tumor progression was observed only from day 12, and the mean tumor volume reached 1.93 ± 0.52 of initial tumor volume by the end of the experiment. On the other hand, tumors grew much faster in the control groups, and reached 2.58 ± 0.5 of initial tumor volume.

Intravenous administration of crosslinked polyplex micelles containing sFlt-1 pDNA to BxPC3 tumor bearing mice resulted in significant reduction in the tumor neo-vasculature, as shown by CD31 immunostaining of the tumor cryosections. Representative images are shown in Fig. 6A. Increased density of blood vessels throughout the tissue was observed in control tumors. In contrast, very few blood vessels could be observed in the sFlt-1 treated groups. The quantitative results of microvessel density in tumor tissue cryosections were obtained by counting the area of stained blood vessels (green pixels) per image (Fig. 6B). Systemic administration of sFlt-1 expressing pDNA in the RGD(+) micelles resulted in the lowest average microvessel density of only 8.6% per image, whereas the RGD (−) micelle carrying pDNA led to 12.3% vessels per image. The control group had an average microvessel area of 23.7% per image, significantly higher as compared to the treated groups.

4. Discussion

In this study, we demonstrate that crosslinked polyplex micelles formed by electrostatic interaction of thiolated PEG-PLys block copolymers, modified on their surface with cRGD peptide ligand, and sFlt-1 pDNA are effective for *in vivo* tumor regression upon systemic administration. The thiolated PEG-PLys block copolymer, in this study, was further optimized by higher molecular weight PEG (17,000 Da) against 12,000 Da M.W. PEG used so far [2,3,8,12–15], to achieve enhanced shielding effect and thus higher stability in blood. Block copolymer with 15% thiolation degree, which showed the highest transfection efficiency *in vitro* and *in vivo* (data not shown), was selected for construction of RGD-mediated gene delivery vector.

The results of sFlt-1 transfection in Hela cells show higher mRNA expression levels in the cells transfected by RGD(+) crosslinked micelle relative to either RGD(−) or non crosslinked micelles (Fig. 2). This result is consistent with our previous studies, indicating the greater stability of crosslinked micelles in the medium and specific affinity of RGD ligand to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors expressed in HeLa cells [14,15]. Micelle internalization to the cell via integrin-mediated endocytosis contributes to the accelerated accumulation of pDNA in the perinuclear region through the change in its intracellular trafficking from clathrin-mediated to caveolae-mediated endocytosis, resulting in enhancement of gene expression [15].

When administrated intravenously into BxPC3 tumor bearing mice, blood levels of Cy5-labeled pDNA were significantly lower for the RGD(+) micelle compared to the RGD(−) micelle. This observation might be partly explained by enhanced accumulation of pDNA in tumor site when carried by RGD(+) micelle over RGD(−) (Fig. 4B) and other organs as well. These observations are in good agreement with other works using cyclic RGD-modified particles, which reported significantly lower blood circulation times [38–40] while higher accumulation in tumor tissue [21,38–41], liver [21,38–42] and spleen [28–31] compared to the control. Moreover, CLSM observations demonstrated colocalization of both micelles with tumor endothelial cells, confirming their potential as effective antiangiogenic gene delivery vehicles (Fig. 4A).

In vivo tumor growth assay revealed significant ($P < 0.05$) tumor growth inhibition when the sFlt-1 pDNA was administrated by crosslinked micelles as compared to control groups. Compared to RGD(−), the RGD(+) micelle was more effective in suppressing tumor growth. The significant difference in relative tumor volumes between RGD(+) injected and control groups was observed from day 6 till the end of the experiment. In comparison, significant difference between RGD(−) injected and control groups was observed only from day 12. In addition, relative tumor volumes in the RGD(+) injected group were lower than those in the RGD(−). These findings may be explained by greater tumor accumulation and higher transfection efficiency of RGD-modified micelle, resulted from more effective intracellular plasmid delivery through specific receptor binding and endocytosis. The lack of significant difference in relative tumor volumes between the RGD(+) and RGD(−) injected groups might be due to the lower circulation time in blood of the RGD(+) micelle and its enhanced accumulation in organs such as liver and spleen. Accumulation in liver [21,38–42] and spleen [39–42] was shown for various cyclic RGD-modified vectors and was, in general, attributed to their accelerated clearance through the phagocytosis by macrophages located on reticuloendothelial system (RES) [39–41].

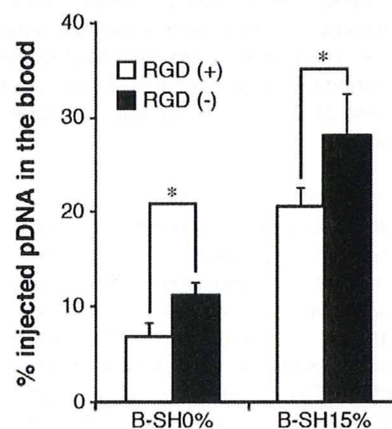

Fig. 3. Blood circulation of plasmid DNA carried by RGD (+/−) polyplex micelles. Micelles loading Cy5-labeled pDNA were intravenously administrated to the tumor bearing mice (20 μg pDNA/mouse). Blood was collected 15 min after administration and analyzed for its fluorescence intensity. $N = 3$, Mean ± s.d. $^*P < 0.05$ compared to RGD(−).

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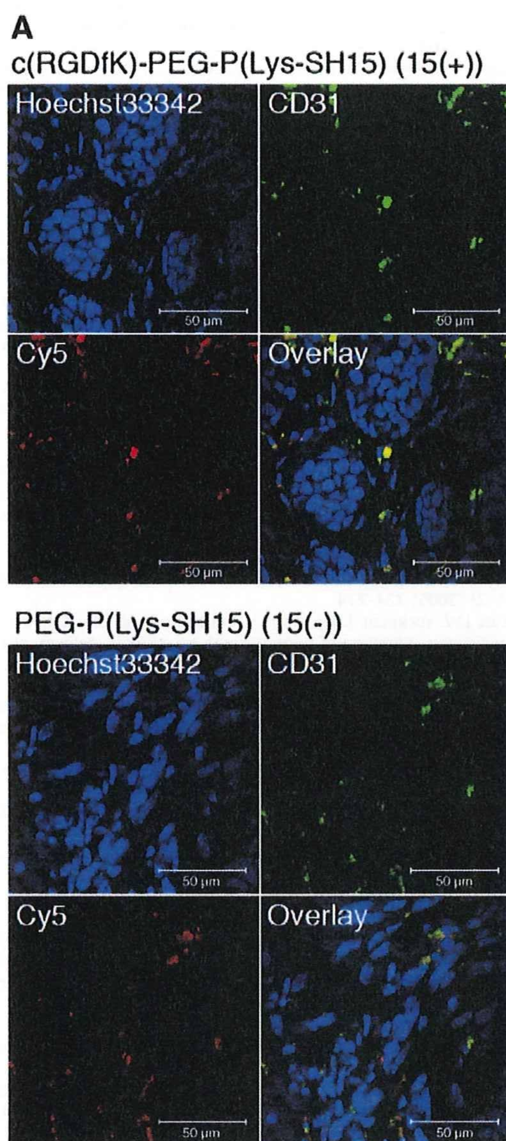
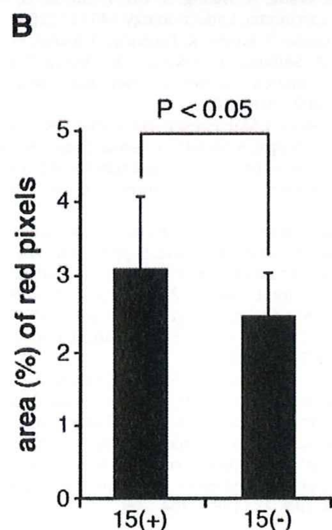
The antiangiogenic effect of expressed sFlt-1 was confirmed by CD31 immunostaining of the tumor cryosections and quantification of microvessel density. From these studies, it is clear that sFlt-1 was able to significantly suppress tumor neo-vasculature formation when the





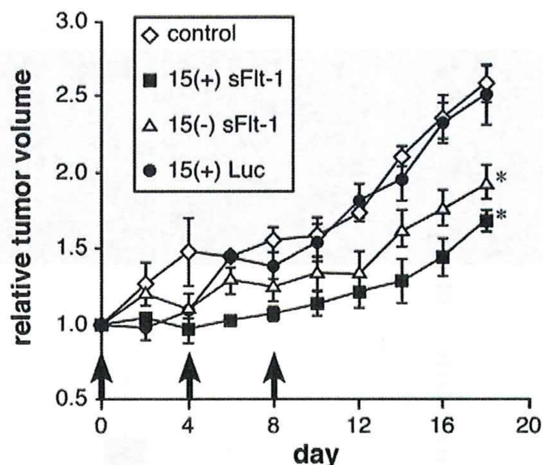


Fig. 5. *In vivo* tumor growth inhibition. RGD (+) and RGD (−) 15% crosslinked polyplex micelles loading plasmid DNA coding either sFlt-1 or Luc were administrated intravenously to BxPC3 tumor bearing mice at a pDNA dose of 20 μg on days 0, 4 and 8, as indicated by arrows. Control animals were injected with either Hepes buffer or 15 (+) micelle loading Luc expressing pDNA. Tumor volumes were measured every 2 days up to day 18 and normalized to the initial tumor volume (day 0). Results are presented in terms of relative tumor volumes, mean ± s.d., $N=6$. $^{*}P<0.05$ compared to control group.

pDNA was delivered in RGD(+) and RGD(−) crosslinked micelles. The most pronounced effect on microvessel density was observed with the plasmid administrated in RGD(+) micelles. This is probably due to the combined effect of tumor accumulation and increased transfection efficiency of the RGD-conjugated 15% crosslinked polyplex micelle.

5. Conclusion

Our data contributes to the list of successful non-viral systems for antiangiogenic cancer gene therapy utilizing sFlt-1 pDNA as VEGF sequester [21,35] and RGD targeting of tumor endothelial cells [19,21]. Worth noting, the antiangiogenic gene therapy by sFlt-1 pDNA, delivered by non-viral vector with cRGD ligand, appears to be a promising strategy to treat an intractable pancreatic tumor.

The significant inhibitory effect of tumor growth shown in this study, confirms the potential of c(RGDfK)-PEG-P(Lys-SH15) and PEG-P(Lys-SH15) polyplex micelles as effective systemic gene delivery systems to the neo-vasculature of solid tumors. Both of these formulations showed accumulation and interaction with tumor endothelial cells. The therapeutic activity of c(RGDfK)-PEG-P(Lys-SH15) was pronounced by combined effect of increased tumor accumulation and enhanced intracellular delivery. Based on these studies, c(RGDfK)-PEG-P(Lys-SH15) can be employed as an effective platform for systemic administration of therapeutic plasmid DNA for antiangiogenic therapy.

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Fig. 4. Micelle localization in tumor tissue. (A) Tumor endothelium and pDNA localization. Immunostaining of CD31 (green) revealed colocalization of Cy5-labeled pDNA (red) with tumor vasculature for both RGD-conjugated (15(+)) and non-conjugated (15(−)) micelles, 24 h after administration. The cell nuclei were stained with Hoechst 33342 (blue). (B) Quantitative analysis of Cy5-labeled pDNA (red pixels). The results represent percentage areas of pDNA-positive pixels per image. Seven images were taken from each tumor tissue, from 3 mice, mean ± s.d. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)