＜リポソーム化アデノシンの血行動態への影響＞

そこで、旧第一製薬との共同研究にて、リポソーム化アデノシンの開発をおこなった。水和法を用いて、アデノシンのリポソーム化に成功した（平均サイズ：126±12 nm、ζ電位：-2.3±0.4 mV）。最初に、ＲＩ標識したアデノシンを用いて、フリーアデノシンならびにリポソーム化アデノシンの血中滞留時間を比較検討した。フリーのアデノシンを静脈内投与したラットでは、投与10分後の血中アデノシン量は投与量の１％以下になっていた。一方、リポソーム化アデノシン投与ラットでは、投与後10分においては投与量の11%、投与30分後においても3.5%のアデノシンが存在していた。以上より、リポソーム化により、アデノシンは長時間（数時間）血中内に滞留しうることが明らかになった。通常、アデノシンは血管内皮細胞や血球成分に存在するアデノシンデアミナーゼにより分解されるため、血中での半減期は数秒であるが、リポソーム化によりアデノシンの分解が抑制するため、その血中滞留時間が増加したものと考えられる。

次に、フリーアデノシンならびにリポソーム化アデノシンの全身静脈内投与を行い、全身血圧ならびに脈拍数への影響を検討した。フリーアデノシンは用量依存的に全身血圧を低下させた（225ならびに450μg/kg/minの10分間静脈内投与にて、全身平均血圧はそれぞれ4.8%ならびに25.4%低下した）。一方、リポソーム化アデノシンは450μg/kg/minの投与量においても全身血圧の低下をきたさなかった。脈拍数に対しても同様の効果が認められた。すなわち、フリーアデノシンによる脈拍数数減少作用は用量依存性に認められたが、リポソーム化アデノシンでは上記投与量では脈拍数を低下させなかった。これらのことから、リポソーム化により、アデノシンによる血圧低下作用ならびに脈拍数減少作用は著明に軽減されることが明らかになった。次に、血行動態を変化させない最大用量のリポソーム化アデノシンによる心筋梗塞サイズ縮小効果を検討した。

＜リポソーム化アデノシンの心筋梗塞サイズ軽減効果＞

蛍光色素標識アデノシン投与実験と同様に、ラット心筋梗塞は左冠動脈を３０分間閉塞後、再灌流することにより作成した。再灌流開始５分前から開始後５分までの計１０分間、大腿静脈よりフリーアデノシン（450μg/kg/min）またはリポソーム化アデノシン（450μg/kg/min）を投与し、３時間後にTTC染色を用いて心筋梗塞サイズを評価した。興味深いことに、リポソーム化アデノシン投与群では、生理食塩水またはアデノシン単独投与群と比較して、心筋梗塞サイズが有意に縮小された。このリポソーム化アデノシンによる心筋梗塞サイズ抑制効果は非特異的アデノシン受容体拮抗薬(8-(p-sulfophenyl) theophylline)により抑制された。これらのことから、心筋梗塞部位に集積する

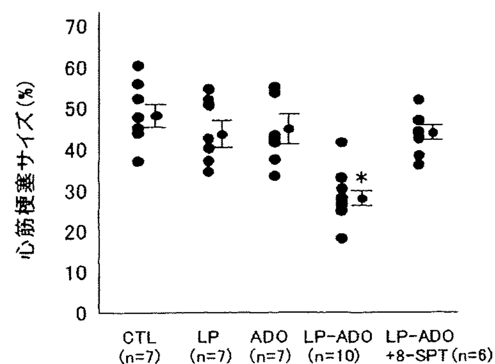

図3. リポソーム化アデノシンは心筋梗塞サイズ縮小効果を増強する(ラット)

リポソーム化アデノシンから漏出するアデノシンがアデノシン受容体を介して心筋保護効果を呈することが明らかになった（図3）。また、同様の検討を大型動物であるイヌを用いて行なった。心筋梗塞サイズは90分間虚血・6時間再灌流モデルにて検討した。リポソーム化アデノシン（450μg/kg/minの10分間静脈内投与）により、全身血圧・脈拍数に変化なく、心筋梗塞サイズが著明に抑制された（心筋梗塞領域／心筋危険領域：生理食塩水投与群 41%、リポソーム化アデノシン投与群 22%）。また、ラットでは見いだされなかった再灌流中の致死的不整脈である心室細動発生率も著明に抑制することが明らかになった（心室細動発生率：生理食塩水投与群60%、リポソーム化アデノシン投与群 17%）。以上より、アデノシンをリポソーム化することにより、アデノシンの副作用（血圧低下・徐脈作用）は軽減すると同時に、心筋梗塞サイズを縮小することが明らかになり、リポソーム化アデノシンは心筋梗塞治療薬として理想的な作用を有していることが明らかになった。心筋梗塞部位ではリポソームの集積により高濃度のアデノシンが存在するため心筋保護作用が生じる一方、末梢血中ではリポソーム化アデノシンの血中濃度は血行動態に変化させるほどに高くないと考えられる（図4）。

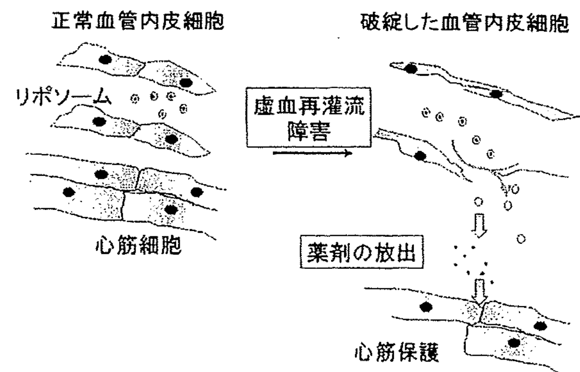

図4. 心筋虚血再灌流領域はリポソームの標的である

＜今後の課題＞

これまでの実験結果を踏まえ、リポソーム化アデノシンの心筋梗塞治療薬としての臨床応用をめざす。現在、提携企業にてfeasibility試験実施を予定している。同時に、抗不整脈薬、実験的に心臓保護作用が確認された薬剤、siRNAなどをリポソーム化することにより新たな心筋梗塞治療薬の開発をめざす。また、大阪大学微生物研究所との共同研究にて、心不全を標的としたＤＤＳの開発を進めていく予定である。心筋虚血領域へのリポソーム集積はpassive targetingにても十分に認められるが、active targetingの必要性や他の送達手段の有効性については今後の検討課題である。

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Chapter 23

Angiogenic Vessel-Targeting DDS by Liposomalized Oligopeptides

Tomohiro Asai and Naoto Oku

Abstract

Liposomal oligopeptides are one of the promising nanocarriers to deliver a drug, DNA or siRNA to target tissues. In this chapter, we describe our methodology to develop liposomal oligopeptides targeting to tumor angiogenic vessels. At first, we introduce our strategies to identify objective peptides. We performed in vivo biopanning using a phage-displayed peptide library and identified Ala-Pro-Arg-Pro-Gly (APRPG) peptide as a ligand for angiogenic vessels. To modify APRPG peptide on the surface of PEGylated liposomes, we synthesized a novel lipid derivative of the peptide, distearoylphosphatidylethanolamine–polyethyleneglycol–APRPG (DSPE–PEG–APRPG). The lipid derivative of APRPG peptide is expected to be readily incorporated into liposomal membrane and enables to present the peptides on the surface of PEGylated liposomes.

We next describe how to evaluate the advantages of liposomal oligopeptides using specific examples; (1) Intratumoral distribution of APRPG–PEG-modified liposomes, (2) Therapeutic efficacy of adriamycin encapsulated in APRPG–PEG-modified liposomes, (3) Preparation of 5′-*O*-dipalmitoylphosphatidyl 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (DPP–CNDAC) liposomes modified with APRPG–PEG, and (4) Therapeutic experiment with APRPG–PEG-modified liposomal DPP–CNDAC.

Key words: Oligopeptides, Liposomes, Polyethyleneglycol, Angiogenic vessels, APRPG

1. Introduction

Active targeting to specific tissues such as tumors is achieved by modification of drug carriers with certain ligands (oligopeptides, proteins, antibodies, glycoconjugates, and so on). In this chapter, we describe the usefulness of liposomal oligopeptides for a drug delivery system. Liposomalization of oligopeptides requires only very simple technique by using lipid derivative of oligopeptides that is easily synthesized and readily incorporated into the liposomal membrane (1–4). In addition, oligopeptides can be bound

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to a polyethyleneglycol (PEG)–lipid conjugate such as, PEG–distearoylphosphatidylethanolamine (PEG–DSPE) and can be presented on the surface of liposomes (5–7). PEG-coating of liposomes has been used in a liposomal DDS, since PEGylated liposomes characteristically remain in the blood circulation longer than non-modified ones through avoidance of reticuloendothelial system (RES)-trapping of drug carriers (8). PEG-coating of the liposomal surface is known to form a fixed aqueous layer around the liposome due to the interaction between the PEG and water molecules. Thus, PEGylated liposomes prevent the binding of certain serum proteins and opsonins that are responsible for the RES-trapping (9). In case of PEGylated liposomes modified with oligopeptides, this long circulating characteristic in the bloodstream increases the opportunity for specific binding of ligand-modified liposomes to target tissues. Here, we present methodologies and results from our recent studies on liposomal oligopeptides. We have developed liposomal oligopeptides to construct angiogenic vessel-targeting DDS for cancer chemotherapy. Angiogenic vessel-targeting DDS has become a focus of interest, since angiogenic vessels have properties different from those of the preexisting systemic vessels (10) and certain drugs or drug carriers first meet angiogenic vessels before extravasation into tissues such as tumors. Angiogenic endothelial cells express specific address molecules that are not or little expressed on preexisting ones (10). Therefore, specific oligopeptides against these address molecules are applicable for active targeting to angiogenic vessels.

For constructing angiogenic vessel-targeting DDS, we firstly isolated a peptide that specifically bound to tumor angiogenic vessels from a phage-displayed peptide library. The epitope sequence of the peptide is determined to be Ala-Pro-Arg-Pro-Gly (APRPG) (2). Then, we synthesized stearoyl-APRPG for the modification of liposomes and demonstrated that APRPG is a useful probe for angiogenic vessel-targeting liposomal DDS (2). In fact, liposomes modified with stearoyl-APRPG highly accumulated in the tumor implanted in mice, and the liposomes encapsulating anticancer drugs strongly suppressed the tumor growth (2, 11, 12). Next, we endowed angiogenic vessel-targeted liposomes with long-circulating characteristic by PEGylation. This approach is expected to cause passive targeting of liposomes in tumor tissues in addition to active targeting of them by oligopeptides, since the angiogenic vessels are quite leaky and PEGylated liposomes as well as macromolecules easily accumulate in the interstitial tissues of tumors due to enhanced permeability and retention (EPR) effect (13). For this purpose, we designed a novel conjugate composed of APRPG peptide, PEG and hydrophobic anchor, namely DSPE, and examined the applicability of APRPG–PEG-modified liposomes for cancer treatment (5–7). As a result, it has been demonstrated that APRPG–PEG modification is superior to just APRPG-modification

for enhancing antitumor activity of liposomal doxorubicin (14). Furthermore, APRPG–PEG-modified liposomes could deliver an antiangiogenic agent to angiogenic vessels, resulting in suppression of angiogenesis and tumor growth (15).

On the other hand, we developed angiogenic vessel-targeting liposomal 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC). CNDAC has a novel anticancer mechanism and induces DNA strand breaks after its incorporation into tumor cell DNA (16). We previously designed 5′-*O*-dipalmitoylphosphatidyl CNDAC (DPP–CNDAC) to incorporate it into the liposomal bilayer (17), since CNDAC itself is not suitable for the efficient encapsulation in liposomes (see Note 1). APRPG-modified liposomes containing DPP–CNDAC actually caused tumor growth suppression through damaging angiogenic endothelial cells (11). However, in vivo behavior of the 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 this case, the potential of APRPG-modification would be attenuated in the blood circulation. Therefore, we masked the CNDAC moiety on the liposomal surface with APRPG–PEG conjugate to erase this undesirable property of DPP–CNDAC in liposomalization (see Note 2). As a result, the improvement of the blood circulation afforded by the use of APRPG–PEG conjugate enhanced the accumulation of the liposomes in the tumor, enabled targeting to the angiogenic endothelial cells, and caused efficient damage to the tumor cells (18). Our studies suggest that PEG-shielding of the liposomal surface should be useful for designing active targeting DDS with oligopeptides as well as passive targeting.

2. Materials

2.1. Lipids, Cells, and other Materials

1. Distearoylphosphatidylcholine (DSPC), DSPE and cholesterol were the products of Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan).
2. A phage-displayed random peptide library expressing pentadecamer amino acid residues at the N terminus of pIII phage coat protein of M13 phage was kindly provided by Dr. Hideyuki Saya at Keio University.
3. 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 K.K., Tokyo, Japan).
4. A fluorescence dye for labeling liposomes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate

(DiI C18), was purchased from Molecular Probes Inc. (Eugene, OR, USA).

5. Other materials: Reduced Triton X-100 and fetal bovine serum were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Animals

1. Five-week-old BALB/c, C57BL/6, or BALB/c nu/nu male mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were cared according to the animal facility guideline of the University of Shizuoka.

3. Methods

3.1. Identification of Peptide

1. Angiogenic model mice: Angiogenic vessels were formed on murine dorsal skin for in vivo biopanning (19). Highly metastatic murine B16BL6 melanoma cells (1×10^7 cells/ring) were loaded into a Millipore chamber ring. The chamber rings were dorsally implanted into 5-week-old C57BL/6 male mice. Five days after the implantation, these mice bearing angiogenic vessels on the dorsal skin were used for in vivo biopanning.
2. In vivo biopanning was performed by a modified method as described by Paspualini et al. (20, 21). The phage-displayed peptide library (1×10^{13} cfu) was intravenously injected into angiogenic vessel-bearing mice. Four minutes after the injection, the phages that had accumulated in angiogenic vessels were recovered and titrated. The skin attached to the Millipore chamber ring where the angiogenic vessels had been formed was dissected, minced, and homogenized with ice-cold DMEM containing 1 mM phenyl methyl sulphonyl fluoride. This homogenate was washed three times ($30{,}000 \times g$ for 10 min) with ice-cold DMEM containing 1% BSA, and the accumulated phages were recovered by infecting *E. coli* K91KAN with them. A part of the phages in the homogenate was used for the titration of the accumulated phages, and the remaining phages were amplified in *E. coli* K91KAN and purified. Then, a second round of biopanning was performed similarly as per the first round. These biopanning steps were repeated for five cycles. At the fifth round of biopanning, the recovery rate of the phage (recovered phage titer to input phage titer) increased about thousand-fold over that of the first round, suggesting that selection of high-affinity phage clones capable of accumulating in the angiogenic site was successful (see Note 3).

3. The selected phages were cloned and the sequence of presented peptides was determined. For in vivo screening, 1.0×10^6 cells of B16BL6 were implanted subcutaneously into the posterior flank of 5-week-old C57BL/6 male mice. Each sample of phage clones (1.0×10^{11} cfu) was injected into tumor-bearing mice via a tail vein when the tumor size had become about 10 mm in diameter. Four minutes after the injection, the phages that had accumulated in the tumor were recovered and titrated. Similar experiment was performed in Meth A sarcoma-bearing mice. As a result, we demonstrated that PRPGAPLAGSWPGTS-presented phage clone highly accumulated in two types of murine tumor.

3.2. Characterization of Peptides

1. Pentadecamer peptides were synthesized by use of Rink amide resin (0.4–0.7 mmol/g) and a peptide synthesizer ACT357, resulting in an amide at the carboxyl terminus.
2. To confirm the capability of the synthetic peptides to accumulate in the tumor, we co-injected 0.25 μmol of synthetic peptide (PRPGAPLAGSWPGTS) and 5×10^8 cfu of corresponding phage clone into B16BL6 melanoma-bearing mice. Four minutes after injection, the titer of phages that had accumulated in the tumor was determined. Tumor accumulation of phage clone was inhibited in the presence of the corresponding synthetic peptide, although a random peptide, GLDLLGDVRIPVVRR, did not affect the phage accumulation.
3. To determine the epitope sequences of the peptides, we synthesized various short peptides based on original 15-mer sequence and examined the inhibitory effect of these peptides against tumor accumulation of the corresponding phage clone. Our results indicated that APRPG in original 15 mer sequences was essential for their affinity.

3.3. Synthesis of DSPE–PEG–APRPG

1. We designed the structure of DSPE–PEG–APRPG (1) as shown in Fig. 1. At first, we synthesized DSPE–PEG–SA (4) (Schemes 1 and 2) and APRPG respectively, and then condensed them to obtain DSPE–PEG–APRPG (1).

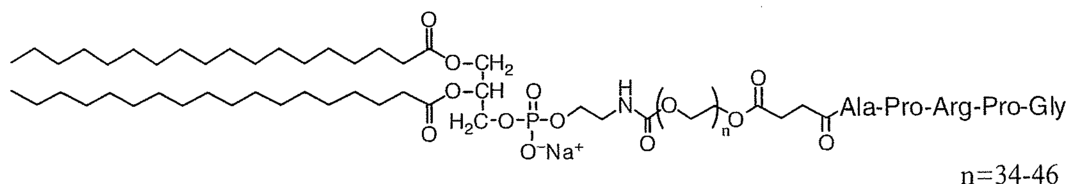
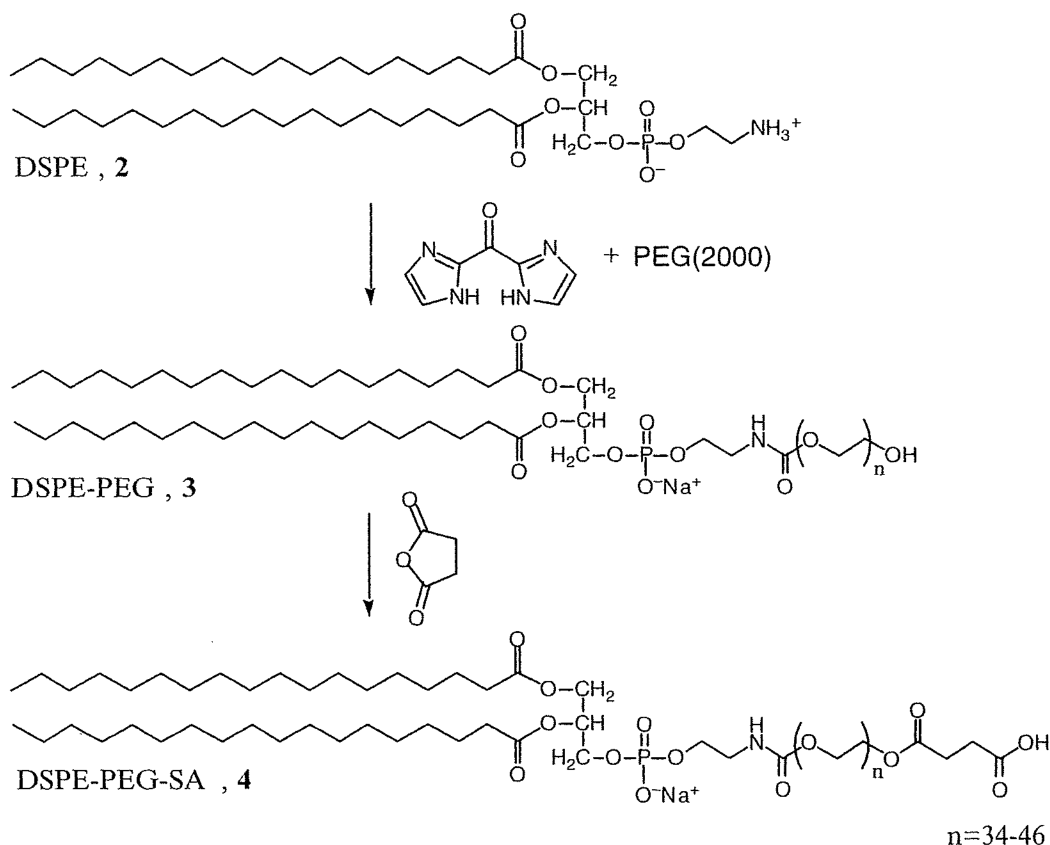
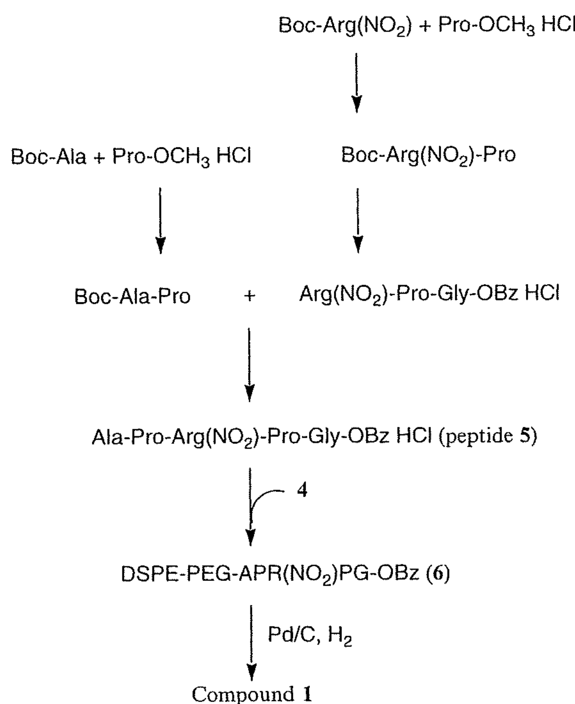


Fig. 1. Structure of DSPE–PEG–APRPG (1). Reproduced with permission from (5)



Scheme 1. Pathway for synthesis of DSPE–PEG–SA. Reproduced with permission from (5)

2. To synthesize DSPE–PEG–SA, DSPE (2) 15.0 g and carbonyl diimidazole (CDI) 3.9 g were dissolved in 70 mL of toluene. Reaction was performed at 100 °C for 1 h after addition of triethyl amine 2.0 g. Then, PEG (average molecular weight; 2,000) 40.0 g dissolved in toluene was added dropwise to the solution. The solvent was evaporated in vacuo followed by the reaction, and the product was dissolved in acetone 500 mL and insoluble materials were filtrated and the solvent was evaporated. The reaction mixture was exchanged into Na^+ salt with ion exchange resin. Purification by column chromatography on silica gave 11.4 g of the desired product (3) in a 26% yield. In order to use the PEG-end of obtained DSPE–PEG as a carboxylic group (referred to as (4)); it was allowed to react with succinic anhydride 2.1 g in the presence of pyridine 1.7 g in 100 mL of toluene. After powdering with ether, the yield of 4 was 80%.
3. Preparation of APRPG peptide moiety was carried out by the liquid-phase method as shown in Scheme 2. *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.1 equiv. based on peptide) and 1-hydroxybenzotriazol (HOBt, 1.1 equiv. based on peptide)



Scheme 2. Pathway for synthesis of DSPE–PEG–APRPG. Reproduced with permission from (5)

were used for peptide coupling in DMF. HCl in 1,4-dioxane was used for deprotection of the Boc group of N-terminal and NaOH was used for deprotection of methyl ester group of C-terminal in water and methanol. In order to avoid racemization, segment condensation was proceeded between Boc-Ala-Pro and Arg(NO_2)-Pro-Gly-OBz to yield 78% of Boc-Ala-Pro-Arg(NO_2)-Pro-Gly-OBz. Next, the Boc protecting group was deprotected by HCl in 1,4-dioxane to obtain peptide (5).

4. Peptide (5) was condensed with (4) (0.93 equiv. based on (5)) in $CHCl_3$ by DCC (1 equiv. based on (5)) and HOBt (1 equiv. based on (5)). The progress of the reaction was monitored by TLC. The reaction was almost complete overnight without any serious side reactions. It was purified by column chromatography on silica. The yield was 83% based on (4). Deprotections of NO_2 group of arginine side chain and benzyl ester group of glycine C-terminal were carried out by 10% palladium–carbon catalytic reduction under hydrogen atmosphere in methanol. It was purified by column chromatography on silica and ion exchange resin. This compound of single spot on TLC was in a 43% yield. This compound (DSPE–PEG–APRPG (1)) was positive for Sakaguchi reagent, while negative for UV lamp on TLC. These showed that NO_2 protecting group and benzyl ester protecting group were deprotected simultaneously (Fig. 1).

3.4. Preparation of PEGylated Liposomal Oligopeptides

1. DSPC and cholesterol with DSPE–PEG or DSPE-PEG–APRPG (10:5:1 as a molar ratio; PEG-lip or APRPG–PEG-lip, respectively) were dissolved in chloroform or chloroform/methanol, dried under reduced pressure, and stored in vacuo for at least 1 h. Liposomes were prepared by hydration of the thin lipid film with 0.3 M glucose, and frozen and thawed for three cycles using liquid nitrogen. Then liposomes were sized by extruding three times through a polycarbonate membrane filter with 100-nm pores (Nucleopore, Maidstone, UK).
2. For an observation of intratumor distribution of liposomes, DiI C18 of the quantity equivalent to 1 mol% of DSPC was added to the liposome. DiI C18-labeled PEG-lip and PEG–APRPG-lip were composed of DSPC, cholesterol, DSPE–PEG and DSPE–PEG–APRPG and DiI C18 (10:5:1:0.1 as molar ratio).
3. For therapeutic experiment, adriamycin (ADM)-encapsulated liposomes were prepared by modification of the remote-loading method as described previously (22). The concentration of ADM was determined by 484 nm absorbance.
4. Particle size and ζ-potential of liposomes diluted with PBS were measured by the use of a Zetasizer Nano ZS (MALVERN, Worcestershire UK, USA).

3.5. Intratumoral Distribution of Liposomal Oligopeptides

DiI C18-labeled liposomes were administered via tail vein of orthotopic tumor model mice (7) on the day 3, 9 and 18 after tumor implantation. Two hours after injection of liposomes, mice were sacrificed and the tumor was dissected. Then, these sections were fluorescently observed by using a microscopic LSM system (Carl Zeiss, Co., Ltd.) (Fig. 2).

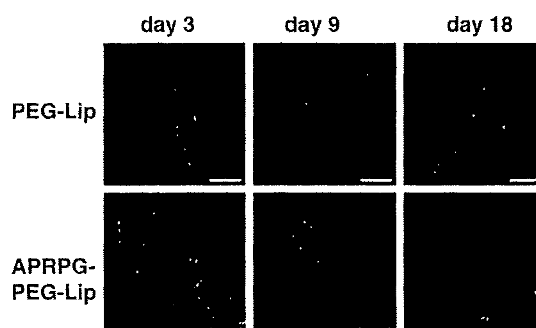


Fig. 2. Intratumoral distribution of DiIC18-labeled liposomes in the orthotopic pancreatic tumors. Mice with orthotopic pancreatic tumor were injected with PEG-Lip or APRPG-PEG-Lip labeled with DiI C18 via a tail vein at the day 3, 9, and 18 after tumor implantation. At 2 h after injection of fluorescence-labeled liposomes, frozen-sections of each tumor were prepared. Green portions indicate CD31-positive regions, red portions liposomal distribution, and yellow portions show the localization of liposomes at the site of vascular endothelial cells. Scale bar represents 100 μm. Reproduced with permission from (7)

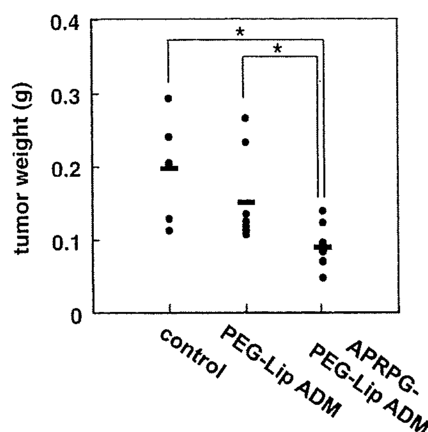


Fig. 3. Therapeutic effect of APRPG–PEG-modified liposome encapsulating ADM on mice with orthotopic pancreatic tumor. Mice with orthotopic pancreatic tumor were injected i.v. with 0.3 M Glucose (control), PEG–LipADM or APRPG–PEG–LipADM for four times at the day 3, 6, 9 and 12 after tumor implantation ($n = 6–8$). Injected dose of liposomal ADM were adjusted to 10 mg/kg as ADM dose in each time. The weight of the tumors was measured at the day 15. Significant differences are shown with asterisks: *$P < 0.05$. Reproduced with permission from (7)

3.6. Therapeutic Efficacy of Adriamycin Encapsulated in Liposomal Oligopeptides

Liposomes encapsulating ADM or 0.3 M glucose solution were administered intravenously into SUIT-2-bearing mice at day 3, 6, 9 and 12 after the implantation of tumor cells. The injected dose of ADM in each administration was 10 mg/kg. The weight of tumor was observed at day 15 (Fig. 3).

3.7. Preparation of DPP–CNDAC Liposomes Modified with Oligopeptides

1. Synthesis of CNDAC and DPP–CNDAC was performed as described previously (16, 17). Briefly, a phosphatidyl group was introduced into CNDAC through transphosphatidylation from 1,2-dipalmitoyl-3-sn-glycerophosphocholine by using phospholipase D.
2. Liposomes were prepared as follows: DPP–CNDAC, DSPC, 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, 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 by use of liquid nitrogen. Then the liposomes were sized by extrusion thrice through polycarbonate membrane filters with 100-nm-diameter pores. The liposomal solutions were centrifuged at 180,000 $\times g$ 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.

3. For the determination of 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, and the amount of DPP–CNDAC was optically determined at 280 nm after the pH of the solution had been adjusted to 1.0. As a result, the encapuslation percent was almost 100%.

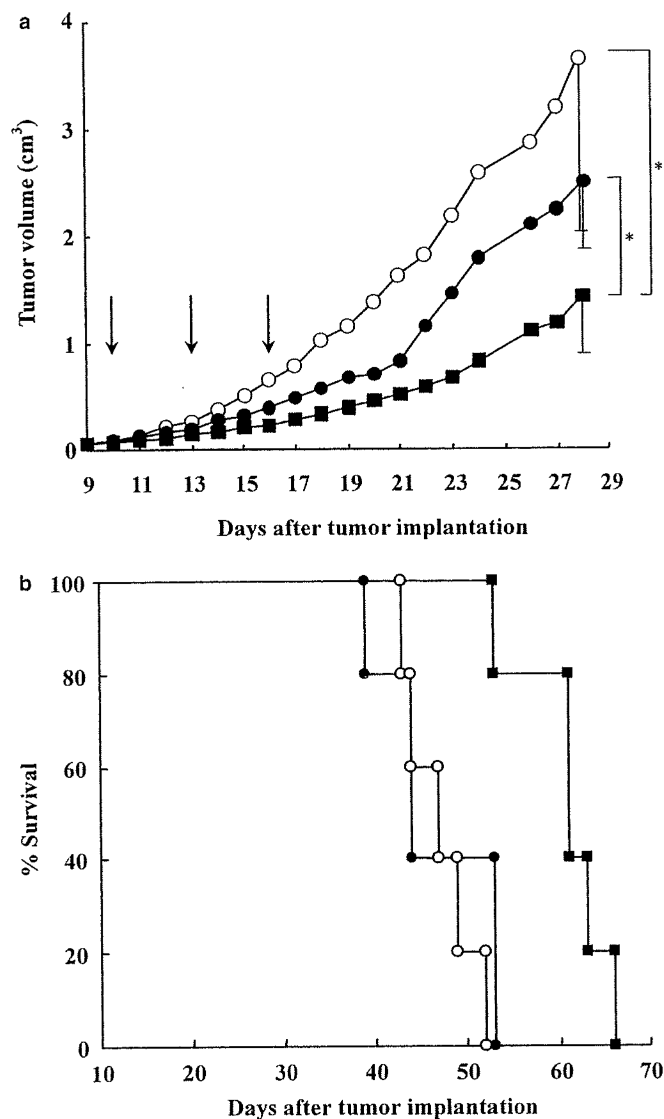


Fig. 4. Therapeutic efficacy of LipCNDAC/APRPG–PEG in tumor-bearing mice. Five-week-old Balb/c male mice (5 or 6 per group) were implanted s.c. with Colon 26 NL-17 carcinoma cells into their left posterior flank. They were injected i.v. with control liposomes (open circle), LipCNDAC/PEG (closed circle) or LipCNDAC/APRPG–PEG (*closed square*) at 15 mg/kg as CNDAC on days 10, 13, and 16 (*arrows*) after tumor implantation. The tumor volume (**a**) and survival time of mice (**b**) were monitored to evaluate the therapeutic efficacy of DPP–CNDAC liposomes. Significant differences from the control liposome-treated group are indicated (*$P<0.05$). Reproduced with permission from (18)

4. 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.
5. Particle size and ζ-potential of liposomes diluted with PBS were measured by use of a Zetasizer Nano ZS. They were 121 ± 4 nm and −29.2 mV for LipCNDAC, 122 ± 6 nm and −6.1 mv for LipCNDAC/PEG, and 102 ± 2 nm and −3.6 mV for LipCNDAC/APRPG–PEG, respectively.

3.8. Therapeutic experiment with APRPG–PEG-Modified Liposomal DPP–CNDAC

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 approxiamtely 0.1 cm^3. The size of the tumor and the body weight of each mouse were monitored daily thereafter (Fig. 4a). Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume. Calculation of the tumor volume was performed by 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 ($r = 0.980$) (22). The life spans of tumor-bearing mice were also monitored (Fig. 4b).

4. Notes

1. In general, encapsulation efficiency of drugs into liposomes is dependent on the logP value (octanol/water partition coefficient) of them, when they cannot be liposomalized by special techniques such as a remote-loading method (22). In many cases, it is difficult to encapsulate drugs into liposomes with high encapsulation efficiency since logP value of drugs is not always suit for liposomalization. In addition, it is also difficult to guarantee the quality and the stability of liposomal drugs in such difficult cases. CNDAC is also difficult to be liposomalized with high encapsulation efficiency by a general hydration method. Therefore, phospholipid derivatization of certain drugs to suit liposomal formulations is the useful methodology to develop liposomal drugs.
2. PEG-shielding of the liposomal surface should be useful for designing active targeting DDS with oligopeptides as well as passive targeting. An important aspect of PEGylation is that it serves for not only RES-avoiding but also for the construction of a practical liposomal oligopeptides. In fact, the biodistribution of APRPG-modified DPP–CNDAC liposomes without

PEG was strongly affected by the presence of cyano group of DPP–CNDAC on the liposomal surface. It induced aggregation of the liposomes, resulting in reduced blood circulation of the liposomes. However, the fixed aqueous layer formed by PEG can mask the undesirable surface properties of liposomes, which prevent attenuation of the effect of oligopeptides. The technology used in this study is also applicable to liposomalization of other compounds, DNA or siRNA etc.

3. We indentified oligopeptides specifically bound to tumor angiogenic vessels from a phage-displayed peptide library and applied to a liposomal DDS. The advantage of *in vivo* biopanning the library is that the selected phages have the ability to bind only to angiogenic vessels, not to other tissues. In fact, the amino acid sequences of the phage clones thus obtained were different from any reported sequences. The selected phage clones had high affinity to murine angiogenic vessels.
4. One of the important things to develop liposomal oligopeptides for clinical use, we should investigate whether the peptides selected in the murine model have affinity for angiogenic endothelium in human tumors. In our studies, we demonstrated that our peptides have affinity for human angiogenic endothelium by histochemical staining of the peptides in human cancer samples (2).

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1章

重症心不全を理解する

—代償から非代償のメカニズム—

1 心臓力学とはなにか？

1 心臓の働き

心臓はポンプであり，その役割は，全身からの血液を右室にためて，肺に送り，肺からの血液を左室から全身に送ることである．心臓はそのポンプの原動力となる心筋細胞，その心筋への酸素・栄養分を供給する冠血管系細胞，心臓を統合的に動かす刺激伝導系細胞・神経系細胞，これらの支持母体である線維芽系細胞から成り立つ．これらの協調的・統合的作用により，胸腔のほぼ中央に位置する心臓は，毎分 60 ～ 80 回の割合で収縮と弛緩を繰り返しながら，毎分約 6*l* の血液を全身へ送り出している．心臓は 1 年に 4000 万回以上も収縮・弛緩を繰り返し，その間 200 万 *l* もの血液を駆出することにより，全身の血液循環を維持する．心臓の駆動力を発生させる源になっているのが心筋であり，収縮・弛緩を繰り返すことによりポンプとしての役割をはたしている．その収縮と弛緩を直接受けもっているのが心筋細胞である．

2 心筋細胞とは

心筋細胞は心臓の主要な構成要素となっており，周期的に収縮と弛緩を繰り返す．電子顕微鏡下の心筋細胞は横紋を有しており骨格筋細胞に形態は似ているが，骨格筋細胞と異なり介在板 intercalated disk を有している．介在板により心筋細胞が接合されることにより，隣同士の心筋細胞の原形質がつながっていないのにもかかわらず，骨格筋と異なり刺激によりあたかも一つの合胞体のようにふるまうことが可能となっている．

筋鞘 sarcolemma とよばれる細胞膜によって包まれた心筋線維は，細胞長軸に走行する多数の筋原線維により構成されている．さらに筋原線維は，ミオシン重合体からなる太いフィラメントとアクチン線維とトロポニンなどのアクチン結合蛋白からなる細いフィラメントにより構成されている．電子顕微鏡下には屈折率の違いにより，横紋が存在する．フィラメント密度が高いために暗く見える A 帯とフィラメント密度が低いために明るく見える I 帯が存在する．明るい I 帯の中央に一筋の暗いバンドが見え Z 帯とよばれる．この隣り合った 2 つの Z 帯で仕切られた間を筋節 sarcomere とよぶ．暗い A 帯のなかにやや明るく見える H 帯が存在し，H 帯の中央に一筋のバンドが存在し，M 帯とよばれる．

これらは，図 1-1 の模式図に示したようにフィラメントの重なり具合により明るさが異なっていることがわかる．ミオシン重合体により構成されている太いフィラメントが A 帯を作る．アクチンおよびアクチン結合蛋白（トロポニン，トロポミオシンなど）により構成される細いフィラメントからなる部分が明るい I 帯を作る．また筋弛緩時に細いフィラメントと重ならない太いフィラメントの部分が H 帯として見える．Z 帯は，筋原線維を横切っていて，アクチンフィラメントに連結している膜である．A 帯の横断面から，図 1-1 に示すように 1 本の太い

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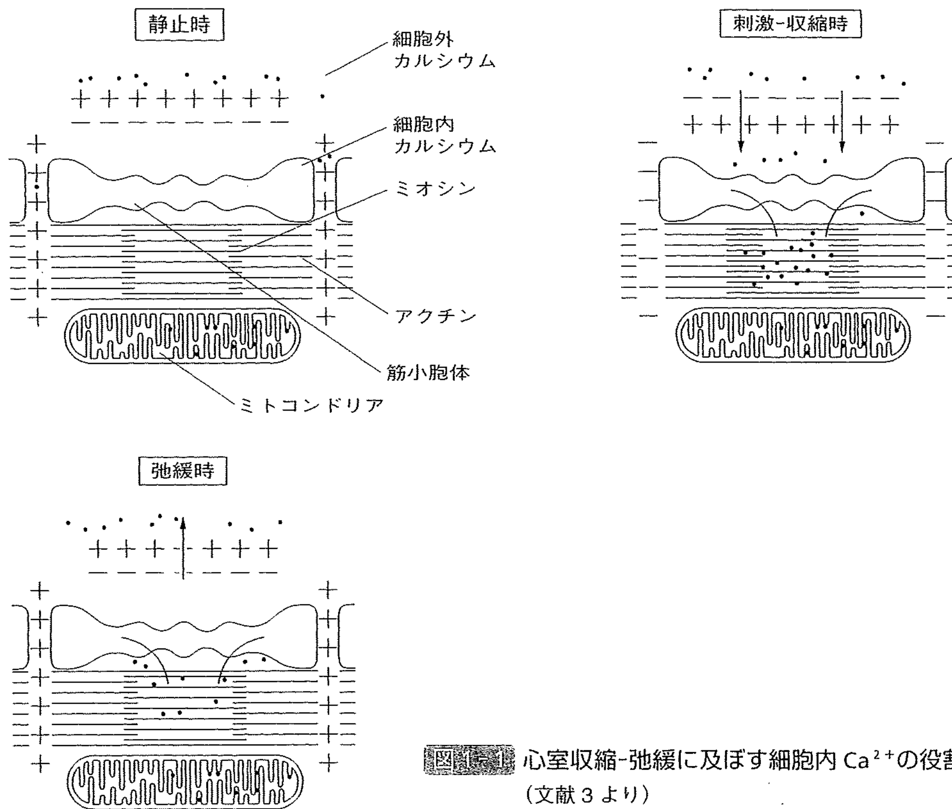

図1-1 心室収縮-弛緩に及ぼす細胞内 Ca^{2+}の役割
（文献3より）

フィラメントの周りに規則正しく6角形に配列した6本の細いフィラメントにより囲まれている．太いフィラメントは数百のミオシン分子よりなっており，ミオシン分子は2つの重鎖 heavy chain と4つの軽鎖 light chain から構成されている．C末端が大きな球状を呈しており，アクチン結合部位とATP分解酵素のある部位が存在する．細いフィラメントは球状のアクチン蛋白が連なった2本の鎖から構成されており，2本の鎖の間の溝に長いフィラメント状のトロポミオシン分子が埋まっている．トロポニンT，トロポニンC，トロポニンIの3つのサブユニットからなる小球状のトロポニン分子は，図に示すようにトロポミオシン分子に沿って一定の間隔にて並んでいる．ミオシン分子頭部とアクチン分子が連結しており，アクチンフィラメントがミオシンフィラメント上を滑走することにより収縮が起こる．

3 心筋収縮の生化学と生理学

a）心筋の収縮特性とは

心筋の収縮は，骨格筋と同様に，ATPをエネルギー源とした，収縮蛋白ミオシン連結橋 myosin cross-bridge のアクチンフィラメント actin filament への周期的結合・解離反応により惹起されると考えられている[1]．その力学特性は筋線維の走行が骨格筋と類似した乳頭筋 papillary muscle あるいは肉柱 trabecula を用いて，筋長-張力関係や張力-速度関係を求めること

により解析されてきた[2]．この2つの関係により心筋の動態は記述されうることが明らかになっている．この2つの座標軸は，心筋の異なった収縮力の評価指標と考えられており，その考えは臨床にも生かされている．

b）心筋収縮とCaイオン—生化学的観点

さて，心筋の収縮は，交互に並んだアクチンとミオシンの2種の収縮蛋白がATPをエネルギー源として架橋を形成し，滑り込み（スライディング）により生じることが知られている．このスライディング理論は骨格筋においてHodgkin-Huxleyの理論としてよく知られており，これを心筋に応用して心筋収縮機構を体系づけたのがSonnenblickであった．心筋長がのびるとアクチン-ミオシンの重なりが増加するため，心筋張力が増加する．この架橋形成はCaイオンがトロポニンCに結合することにより，促進される．つまり，心筋収縮弛緩は細胞内Caイオンが制御していることがわかる（図1-1）[3]．最近の私どもの研究によると，Caイオンは，心臓特異的ミオシン軽鎖キナーゼのリン酸化を促進して心機能を修飾していることも示唆されている[4]．

心筋収縮と細胞内Caイオンはいかに関与するのか．通常静止時には，心筋細胞内は細胞外に比べて−90mV電位が低く保たれている．心筋細胞膜に対して電気的興奮が生じるとNa^{+}チャンネルを介してNa^{+}の細胞内への流入が起こり，心筋細胞が脱分極した状態となる．かかる電気的興奮は，通常の心臓では刺激伝導系を介して行われる．すると，筋原線維の折れ込みであるT管系に存在する膜電位依存性のL型Ca^{2+}チャンネルを介して細胞外から微量のCa^{2+}が流入する（図1-2）．細胞内のCa^{2+}濃度が上昇すると，細胞内小器官でCa^{2+}の所蔵庫である筋小胞体

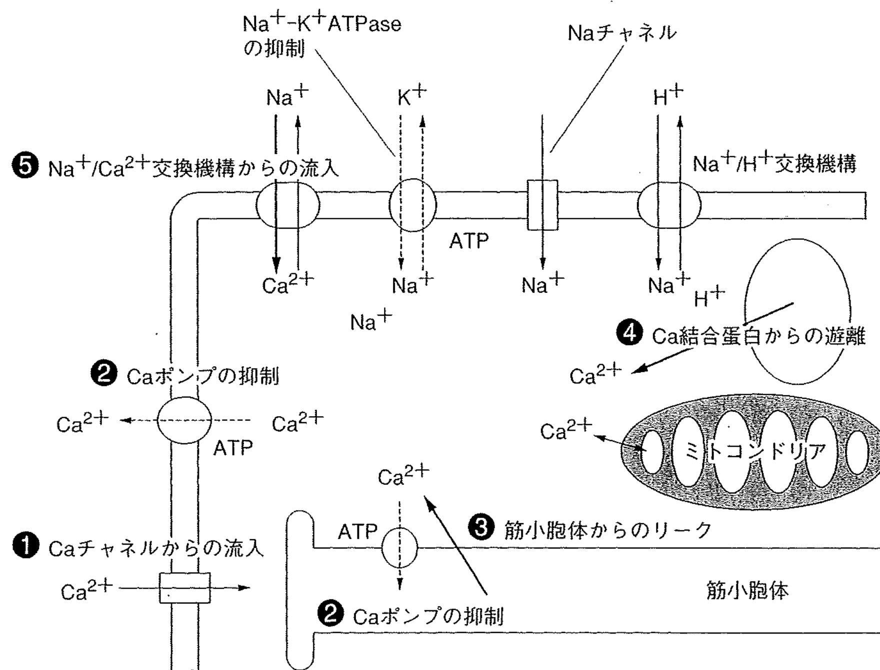

図1-2 心筋細胞内Ca^{2+}ハンドリング

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