Anti-neovascular therapy by liposomal DPP-CNDAC targeted to angiogenic vessels

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Abstract We previously reported that liposomalized 5′-*O*-dipalmitoylphosphatidyl 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (DPP-CNDAC), a hydrophobized derivative of the novel antitumor nucleoside CNDAC, is quite useful for cancer therapy. On the other hand, for anti-neovascular therapy, we recently isolated peptides homing to angiogenic vessels from a phage-displayed random peptide library, and observed that peptide-modified liposomal adriamycin strongly suppressed tumor growth, perhaps through damaging angiogenic endothelial cells. In the present study, we modified DPP-CNDAC-liposomes with one of the angiogenic homing peptides, APRPG, and examined their antitumor activity. Three doses of APRPG-modified DPP-CNDAC-liposomes (15 mg/kg as CNDAC) strongly inhibited tumor growth compared with the same number of doses of unmodified DPP-CNDAC-liposomes. The life span was increased 31.8%, with one completely cured mouse out of the six mice treated. Since the accumulation of liposomes in the tumor tissue was not so much different between APRPG-liposomes and non-modified liposomes, the enhanced therapeutic efficacy may be explained as the alteration of targets, i.e. APRPG-modified DPP-CNDAC-liposomes caused tumor growth suppression through damage of angiogenic endothelial cells. Anti-neovascular therapy promises no drug resistance, and should be effective against essentially any kind of solid tumor; and thus the present results demonstrate another benefit of the therapy, namely, high efficacy of cancer treatment. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Liposome; Anti-neovascular therapy; Angiogenesis; Drug delivery system; Active targeting; CNDAC

1. Introduction

Tumor angiogenesis affords new targets for cancer therapy, since inhibition of angiogenesis causes tumor dormancy and suppresses hematogenous metastases [1,2]. Besides anti-angiogenic therapy, anti-neovascular therapy, i.e. the indirect lethal damage to tumor cells through damage to neovessels, is also being explored. Since anticancer drugs cause damage to growing cells, neovascular endothelial cells as well as tumor cells are damaged by these drugs. In fact, cancer chemotherapy using anti-neovascular scheduling induced endothelial cell apoptosis prior to tumor cell damage and effectively eradicated the tumor [3].

Liposomes are thought to be an ideal drug carrier in targeted cancer chemotherapy. Liposomes have a tendency to passively accumulate in tumor tissues due to their enhanced permeability and retention effect [4,5]. In addition, modification of liposomes with specific ligands such as antibody, glycoconjugate, or oligopeptide enables active targeting of tumor tissues [6,7] Especially, small peptides having high affinity against certain antigens are suitable for modification of liposomes, since they are biocompatible, biodegradable, and less antigenic compared to antibody and other modifiers. Furthermore, liposomalization of oligopeptides requires only a very simple technique involving the use of a lipid derivative of an oligopeptide that is easily synthesized and readily incorporated into the liposomal bilayer [8,9]. Thus we planned to obtain oligopeptides specific for angiogenic sites, and to modify liposomes with such a peptide for the purpose of developing an effective drug carrier that would be applicable for anti-neovascular therapy.

For this purpose, we recently isolated peptides specific for tumor angiogenic vasculature by using a phage-displayed peptide library [10,11]. In vivo biopanning of phage-displayed peptide library in angiogenic model mice prepared by the dorsal air sac method [12] enabled us to isolate specific phage clones having the ability to bind only to angiogenic vessels, not to tumor cells. After determination of the epitope sequences of the obtained phages and modification of liposomes with these peptides, the APRPG-modified liposome was revealed to be favorable for the active targeting of the angiogenic endothelium. Furthermore, anti-neovascular therapy using this type of liposome as a carrier of adriamycin markedly suppressed tumor growth possibly through a cytotoxic effect of the drug against angiogenic endothelial cells [10], although an increase in the local concentration of adriamycin released from the liposomes may have partly contributed to this enhanced efficacy.

In the present study, we investigated the therapeutic efficacy of APRPG-modified liposomes containing a lipophilic anticancer drug, since such drugs would be expected to be deliverable to the target cells in a liposomal form. Therefore, the therapeutic efficacy directly reflects the destruction of the cells to which the liposomes gain access. For this purpose, a lipophilic derivative of a novel anticancer drug 5′-*O*-dipalmitoyl-

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phosphatidyl 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (DPP-CNDAC) was used. The mother compound 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) was originally synthesized by us as a novel antitumor nucleoside antimetabolite, which induces DNA strand breaks after its incorporation into tumor cell DNA [13,14]. Furthermore, 5′-phosphatidylation of the compound, i.e. DPP-CNDAC [15], and its liposomal formulation [16,17] indicated enhanced antitumor activity. Therefore, DPP-CNDAC was thought to be favorable for liposomalization and for anti-neovascular therapy using novel angiogenic vessel-homing peptides.

2. Materials and methods

2.1. Materials

Synthesis of CNDAC and DPP-CNDAC was performed as described previously [13,15]. A phosphatidyl group was introduced into CNDAC through transphosphatidylation from 1,2-dipalmitoyl-3-*sn*-glycerophosphocholine by using phospholipase D. APRPG peptide was synthesized by use of Rink amide resin (0.4–0.7 mmol/g) and a peptide synthesizer (ACT357; Advanced ChemTech, Louisville, KY, USA), resulting in an amide at the carboxy-terminus. A stearoyl moiety was introduced into the peptide by the DIPCI-HOBt coupling method. Distearoylphosphatidylcholine (DSPC) was kindly supplied by Dr. Yukihiro Namba of Nippon Fine Chemicals (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of liposomes

DPP-CNDAC, DSPC, and cholesterol with or without stearoyl-APRPG (10:10:5:2 or 10:10:5 molar ratio, respectively) dissolved in chloroform were dried under reduced pressure and stored in vacuo for at least 1 h. Liposomes were produced by hydration of the thin lipid film with phosphate-buffered glucose (pH 6.8) and freeze-thawed for three cycles by use of liquid nitrogen. Then the liposomes were sized by extrusion thrice through polycarbonate membrane filters with a 100-nm pore size (Nuclepore, Maidstone, UK). The liposomal solutions were centrifuged at $180\,000\times g$ for 20 min (Hitachi, CS120EX) to remove the untrapped DPP-CNDAC if present. Then the liposomes were resuspended in phosphate-buffered glucose. For the determination of trapping efficacy of 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 has been adjusted to 1.0. For a biodistribution study, a trace amount of [oleate-1-^{14}C]cholesteryl-ether (74 kBq/mouse, Amersham Pharmacia, Buckinghamshire, UK) was added to the initial chloroform solution.

2.3. Biodistribution of liposomes

C26 NL-17 carcinoma cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (Sigma). 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 (Japan SLC, Shizuoka, Japan). The biodistribution study was performed when the tumor size had become about 10 mm in diameter. Size-matched C26 NL-17 carcinoma-bearing mice were injected with the radiolabeled liposomes via a tail vein. Three hours after the injection, the mice were killed under diethyl ether anesthesia for the collection of the blood which was centrifuged ($600\times g$ for 5 min) to obtain plasma. 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 with a liquid scintillation counter (Aloka, LSC-3500). The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.4. Therapeutic experiment

Meth A sarcoma grown in the ascites of BALB/c mice under an appropriate schedule was diluted with saline to obtain a suspension of 5×10^6 cells/ml. Then 0.2 ml of the suspension was carefully injected subcutaneously into the posterior flank of 5-week-old BALB/c male mice (Japan SLC). Liposomes composed of DSPC, DPP-CNDAC, cholesterol, and stearoyl-APRPG (10:10:5:2 molar ratio; APRPG-LipCNDAC) or of DSPC, DPP-CNDAC, and cholesterol (10:10:5 molar ratio; LipCNDAC) were administered intravenously to Meth A sarcoma-bearing mice at day 5, 9, and 13 after tumor implantation. The amount of injected liposomes was 47.2 mg/kg/day as DPP-CNDAC (15 mg/kg as CNDAC moiety). The size of the tumor and body weight of each mouse were monitored daily thereafter. Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume and calculation was performed using the formula $0.4(a\times b^2)$, where a is the largest and b the smallest diameter. The tumor volume thus calculated correlated well with the actual tumor weight ($r=0.980$) [18].

2.5. Statistical analysis

Variance in a group was evaluated by the *F*-test, and differences in mean tumor volume were evaluated by Student's *t*-test.

3. Results

3.1. Biodistribution of APRPG-modified DPP-CNDAC liposomes

Previously, we demonstrated that APRPG-modified liposomes accumulated more in tumor tissues than peptide-unmodified ones [10]. However, since DPP-CNDAC was incorporated into the lipid bilayer of the liposomes, the CNDAC moiety on the liposomal surface may affect the targeting character of APRPG-liposomes. Therefore, firstly the effects of DPP-CNDAC incorporation on the biodistribution of APRPG-modified liposomes were examined. As shown in Fig. 1, the distribution of APRPG-LipCNDAC was not so very different from that of LipCNDAC, although APRPG-LipCNDAC accumulated in the tumor tissue a little more than LipCNDAC. Therefore, DPP-CNDAC may affect the

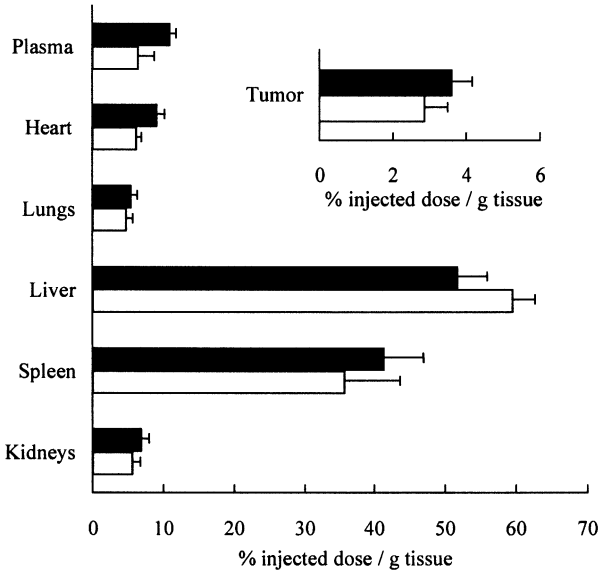

Fig. 1. Biodistribution of APRPG-LipCNDAC. Liposomes were prepared and labeled as described in Section 2. Size-matched C26 NL-17 carcinoma-bearing mice were injected with LipCNDAC (open bar) or APRPG-LipCNDAC (closed bar) via a tail vein. Three hours after the injection, the mice were killed under diethyl ether anesthesia, and the organs were removed. The radioactivity in each organ was determined in a liquid scintillation counter. Data are presented as the percentage of the injected dose per g tissue and S.D. in each tissue (in the case of plasma, value was per ml instead of per gram). The radioactivities of cholesteryl [^{14}C]oleate are shown.

in vivo behavior of APRPG-modified liposomes and reduce their targeting character. However, this characteristic was not fully abolished by the incorporation of this amount of DPP-CNDAC into the liposomes.

3.2. Therapeutic efficacy of APRPG-modified DPP-CNDAC liposome

Next, the antitumor activities of LipCNDAC and APRPG-LipCNDAC were examined. In this experiment, Meth A sarcoma-bearing mice were used instead of C26 NL-17 carcinoma-bearing ones, since Meth A sarcoma cells form a round tumor after subcutaneous injection, thus affording a more accurate determination of tumor volume.

As shown in Fig. 2, APRPG-LipCNDAC showed the most potent suppression of tumor growth (significantly different from the control, $P < 0.001$, and from the LipCNDAC treatment, $P < 0.05$, for the data points of day 19). Concerning the side effects, both liposomal formulations of DPP-CNDAC induced a slight and transient reduction in the body weight that was reversed within a few days (data not shown). The survival time of these mice is shown in Fig. 3. The mean survival time of control, LipCNDAC-treated, and APRPG-LipCNDAC-treated groups was 30.5, 32.2, and 40.2 days, respectively; thus the increase in survival was 5.5% for the LipCNDAC-treated group and 31.8% for the APRPG-LipCNDAC-treated group. Furthermore, one out of the six mice was completely cured by the treatment with APRPG-LipCNDAC. These data indicate that APRPG modification enhanced the antitumor activity of the DPP-CNDAC liposomes. Since the drug distribution to the tumor was not so very different between the two types of liposomes, the effect of APRPG modification might not have been due to an increased amount of DPP-CNDAC in the tumor tissue but rather to a topological change in the delivered DPP-CNDAC, i.e. APRPG-LipCNDAC may have delivered more DPP-CNDAC to angiogenic endothelial cells, the damage to which would be more serious for the survival of the tumor cells.

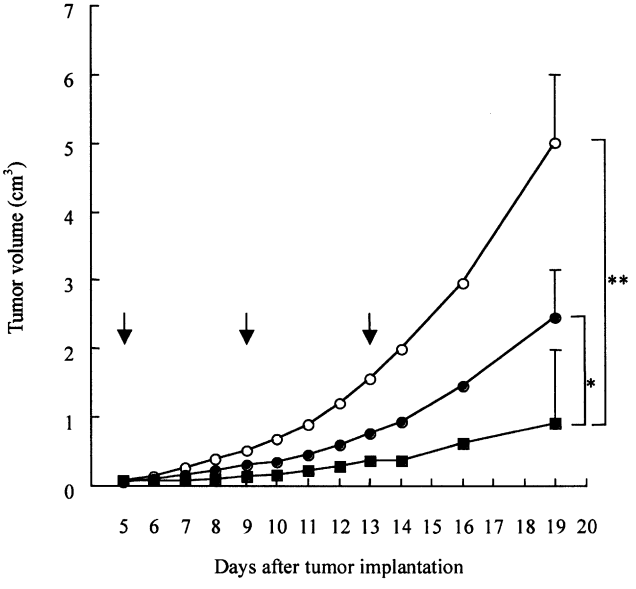

Fig. 2. Suppression of tumor growth by APRPG-LipCNDAC in Meth A sarcoma-bearing mice. Meth A sarcoma-bearing BALB/c mice ($n = 6$) were injected i.v. with 0.3 M phosphate-buffered glucose (open circle), LipCNDAC (closed circle), or APRPG-LipCNDAC (closed square). The weight of each mouse, an indicator of side effects, and size of the tumor were monitored every day after initiation of administration. Data are presented as the mean tumor volume and S.D. The S.D. bars are shown only for the last points for the sake of graphic clarity. Arrows show the days of treatment. Significant differences are indicated (*$P < 0.05$; **$P < 0.001$).

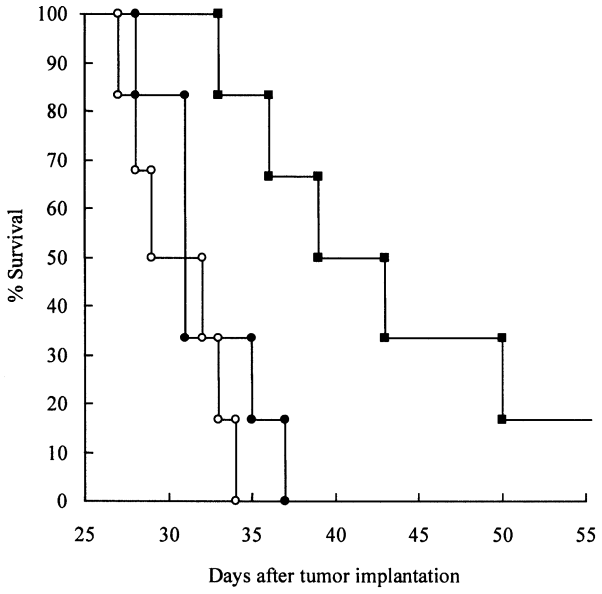

Fig. 3. Elongation of survival time of tumor-bearing mice treated with APRPG-LipCNDAC. Mice ($n = 6$) were implanted subcutaneously with Meth A sarcoma into the left posterior flank. At 6, 10, and 14 days after tumor implantation, they were injected intravenously with 0.3 M glucose (open circle), LipCNDAC (closed circle), or APRPG-LipCNDAC (closed square).

4. Discussion

If a drug could be delivered to only the desired site in the correct concentration at the right time, we could expect a more beneficial therapeutic effect of the drug, especially for drugs having severe side effects such as anticancer drugs. Liposomes have been used to prolong the circulation time of such drugs, to deliver them to the target, namely, tumor tissue, and to reduce side effects [19]. Among active targeting strategies, vascular targeting has become an issue of recent interest, since anticancer drugs or drug-carrying liposomes first meet angiogenic vessels before extravasation into the tumor tissue. Since these vessels have properties different from those of the preexisting systemic vasculature [20,21], it is possible to develop a probe specific for the neovasculature. Furthermore, anti-neovascular therapy, i.e. the therapy for indirect tumor cell killing through cutting off the supply of oxygen and nutrients to tumor cells by direct destruction of angiogenic endothelial cells, should be achievable by vascular targeting.

For the purpose of vascular targeting, we recently isolated peptide probes specific for neovasculature by using a phage-displayed random peptide library [10,11]. Thus obtained WRP-containing peptides showed anti-angiogenic activity. Another peptide, a pentadecapeptide containing an APRPG sequence, did not show such anti-angiogenic activity; however, it strongly bound to angiogenic endothelial cells as determined by histochemical analysis using human islet cell tu-

mor of the pancreas and glioblastoma specimens. The histochemical analysis also indicated that this pentadecapeptide did not interact with tumor cells. Furthermore, confocal microscopic observation indicated that APRPG-modified liposomes specifically bound to vascular endothelial growth factor-stimulated human umbilical vein endothelial cells, for the binding was cancelled by the addition of APRPG peptide [10]. In fact, APRPG-modified liposomes showed about three-fold higher accumulation in tumor tissues of tumor-bearing mice than non-modified ones, and APRPG-modified liposomes entrapping adriamycin strongly suppressed the tumor growth of tumor-bearing mice [10]. This enhanced antitumor activity of these liposomes may be explained by the efficient destruction of neovascular endothelial cells through active targeting of the drug to the cells, although an increase in the local concentration of adriamycin would also partly contribute to the enhancement.

In the present study, we applied these APRPG-modified liposomes for anti-neovascular therapy by using a lipophilic derivative of an anticancer drug, DPP-CNDAC. Since 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-liposomal DPP-CNDAC 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, and vice versa. Our present results indicate that the delivery of DPP-CNDAC to angiogenic endothelial cells is, in fact, far superior for the suppression of tumor growth.

A previous study demonstrated that DPP-CNDAC incorporated into long-circulating liposomes showed enhanced activities for reducing tumor growth and increasing the lifetime of the mice compared to conventional liposomes or soluble CNDAC [16]. In that study, the long-circulating property was achieved by modification of the liposomes with palmityl-D-glucuronide, and the enhanced therapeutic efficacy might have been due to the passive targeting effect of long-circulating liposomes. In other words, the topical concentration of DPP-CNDAC in the tumor was increased in the form of liposomes without the change in target cells. The tumor model and doses of DPP-CNDAC were the same between the former and present experiments, although the treatment schedule was not completely the same. The enhanced therapeutic efficacy, however, was much higher in the present study than in the former one. This difference can be explained by the fact that the liposomal long-circulating character results in a quantitative difference in DPP-CNDAC in the tumor tissue, whereas liposomal APRPG modification causes a qualitative difference, namely, tumor growth suppression through damaging angiogenic endothelial cells. In fact, the bulk accumulation of liposomes in the tumor tissue was not so very different between the APRPG-liposomes and non-modified ones (Fig. 1).

Taken together, the available data indicate that anti-neovascular therapy is an efficient modality for cancer treatment and that APRPG modification is useful for anti-neovascular therapy. The APRPG-liposome is able to deliver not only anticancer agents for anti-neovascular therapy, but also anti-angiogenic agents for tumor dormancy therapy. Direct conjugation of the peptide with anticancer agents or angiogenesis inhibitors would also be a hopeful approach.

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