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Applicability of anti-neovascular therapy to drug-resistant tumor: Suppression of drug-resistant P388 tumor growth with neovessel-targeted liposomal adriamycin

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Abstract

Anti-neovascular therapy, one of the effective anti-angiogenic chemotherapy, damages new blood vessels by cytotoxic agents delivered to angiogenic endothelial cells and results in indirect eradication of tumor cells. We previously reported that liposomes-modified with a pentapeptide, Ala-Pro-Arg-Pro-Gly (APRPG-Lip) homing to angiogenic site, highly accumulated in tumor tissue, and APRPG-Lip encapsulating adriamycin (APRPG-LipADM) effectively suppressed tumor growth in tumor-bearing mice. In the present study, we examined the topological distribution of fluorescence-labeled APRPG-LipADM as well as TUNEL-stained cells in an actual tumor specimen obtained from Colon 26 NL-17 carcinoma-bearing mice. The fluorescence-labeled APRPG-Lip dominantly localized to vessel-like structure: A part of which was also stained with anti-CD31 antibody. Furthermore, TUNEL-stained cells were co-localized to the same structure. These data indicated that APRPG-LipADM bound to angiogenic endothelial cells and induced apoptosis of them. We also investigated the applicability of anti-neovascular therapy using APRPG-LipADM to ADM-resistant P388 solid tumor. As a result, APRPG-LipADM significantly suppressed tumor growth in mice bearing the ADM-resistant tumor. These data suggest that APRPG-LipADM is applicable to various kinds of tumor including drug-resistant

Abbreviations: ADM, adriamycin; DSPC, distearoylphosphatidylcholine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PEG, polyethylene glycol; VEGF, vascular endothelial growth factor

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tumor since it targets angiogenic endothelial cells instead of tumor cells, and eradicates tumor cells through damaging the neovessels.

Keywords: Liposome; Anti-neovascular therapy; Angiogenesis; Drug delivery system; Active targeting

1. Introduction

Tumor angiogenesis, construction of new blood vessels in tumor tissue, is critical for tumor growth, since the supply of oxygen and nutrients is essential for many tumors. Angiogenesis is also related to blood-borne metastasis to distal organs, since it is initiated through this angiogenic vasculature (Folkman, 1971). A number of previous studies on angiogenesis have elucidated the functions of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and the biological process of angiogenesis (Asahara et al., 1999; Ferrara, 2002). Based on these findings, cancer therapy targeted to angiogenesis has been considered and various inhibitors for angiogenic process have been developed (Kerbel and Folkman, 2002). These agents often prevent pro-angiogenic factors from binding to their receptors or inhibit signal transduction in angiogenesis. This therapy is generally called as anti-angiogenic therapy. For example, the treatment of antibody against VEGF receptor, KDR/flk-1 suppressed tumor growth by inhibition of angiogenesis (Brekken et al., 2000). We previously suggested that disruption of angiogenic vasculature by allowing cytotoxic agent to angiogenic endothelial cells could effectively inhibited tumor growth (Oku et al., 2002a). Since angiogenic endothelial cells have acquired enhanced growth ability, cytotoxic anti-cancer agents are able to damage angiogenic endothelial cells as well as tumor cells. This anti-neovascular therapy is expected to overcome several but critical problems in traditional cancer chemotherapy. For example, anti-neovascular therapy can reduce injected dose, since the angiogenic endothelial cells, which should be eradicated by this therapy, are fewer than the tumor cells in a tumor tissue. In addition, this therapy is promising to apply to various kinds of solid tumors including drug-resistant tumors since the nature of angiogenic vessels may be the same or quite similar among tumors despite the tumor cells acquire the drug-resistance (Browder et al., 2000).

In the previous study, we isolated peptides specific for tumor angiogenic vasculature by in vivo biopanning of a phage-displayed peptide library (Oku et al., 2002b). Obtained Ala-Pro-Arg-Pro-Ala (APRPG) peptide was used for an active targeting tool for angiogenic vessels. In fact, APRPG-modified liposomes (APRPG-Lip) highly accumulated in tumor tissue and adriamycin (ADM)-encapsulated APRPG-Lip (APRPG-LipADM) effectively suppressed tumor growth in Meth A sarcoma- and Colon 26 NL-17 carcinoma-bearing model mice. Furthermore, APRPG-Lip bound specifically to VEGF-stimulated human umbilical vein endothelial cells (HUVECs) compared with unmodified ones (Oku et al., 2002b). These findings suggested that APRPG-LipADM shows potent anti-tumor effect in order to eradicate angiogenic vasculature and, therefore, is expected to apply to drug-resistant tumor.

In the present study, to confirm that APRPG-LipADM really damages angiogenic endothelial cells, intratumoral distribution of APRPG-Lip and vessel damage in tumor tissue after the treatment of APRPG-LipADM were examined. Furthermore, to investigate whether APRPG-modified liposomal agent can be applied to drug-resistant cancer, we performed therapeutic experiment using ADM-resistant P388 solid tumor-bearing mice.

2. Materials and methods

2.1. Materials

Stearoyl-APRPG derivative was synthesized as previously described (Asai et al., 2002). Distearoylphosphatidylcholine (DSPC) was a gift from Nippon Fine Chemical Co. Ltd. (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Balb/c and DBA/2 male mice were purchased from Japan SLC, Inc.

2.2. Liposomal preparation

Liposomal preparation was performed as described previously (Oku et al., 2002b). In brief, DSPC, cholesterol and stearoyl-APRPG derivative (10/5/1 as a molar ratio) dissolved in chloroform were dried under reduced pressure and stored in vacuo for at least 1 h. Constituted lipid thin film was hydrated with 0.3 M citric acid solution (pH 4.0) to generate liposomal solution. This liposomal suspension (10 mM as DSPC) was frozen and thawed for three cycles with liquid nitrogen and sized to 100 nm by extruding through 100 nm-pore sized polycarbonate membrane filter (Advantec, Tokyo, Japan) attached to an extruder (Lipex, Vancouver, BC, Canada). ADM was encapsulated into APRPG-Lip as previously described (Oku et al., 2002b). Particle size of the liposomes was 154 ± 1 nm recorded on an ELS-800 electrophoretic light-scattering spectrophotometer (Otsuka Electronics Co., Ltd., Osaka, Japan), and the liposomes were stable during a 48-h incubation in the presence of 50% fetal bovine serum (FBS, Sigma Chemical Co.) at 37 °C (data not shown). Fluorescence-labeled APRPG-Lip was prepared as follows: Thin film composed of DSPC, cholesterol, and stearoly-APRPG derivative with a trace of $DiIC_{18}$ (Molecular Probes Inc., Eugene, OR, USA) was hydrated with 0.3 M glucose solution (DSPC/cholesterol/stearoyl APRPG derivative/$DiIC_{18}$ = 10/5/1/0.1 as a molar ratio, 10 mM as DSPC). Obtained liposomal suspension was extruded with a 100 nm-pored filter.

2.3. Preparation of tumor-bearing mice

Mouse Colon 26 NL-17 carcinoma (C26 NL-17) cells were grown in RPMI 1640 with 10% FBS at 37 °C in the presence of 5% CO_2. P388 and P388/ADM leukemia cells were grown in abdominal cavity of DBA/2 mouse. C26 NL-17, P388 or P388/ADM cells (1×10^6 cells per mouse) were subcutaneously implanted into left posterior flank of Balb/c male mice for C26 NL-17 or DBA/2 male mice for P388 and P388/ADM, respectively.

2.4. Intratumoral distribution of liposomes

$DiIC_{18}$-labeled APRPG-Lip or $DiIC_{18}$-labeled unmodified liposome (Cont-Lip) were administered via a tail vein of C26 NL-17-bearing mice when the tumor sizes had reached about 1 cm in diameter. Fifteen minutes or 2 h after injection of liposomes, mice were sacrificed under anesthesia with diethyl ether and tumors were dissected and kept in 20% formalin. Solid tumors were wrapped in O.C.T. compound (Sakura Finetechnochemical Co. Ltd., Tokyo, Japan) and frozen at −80 °C. Five-micrometer tumor sections were prepared by using cryostatic microtome (HM 505E, Microm, Walldorf, Germany). For endothelial cell staining, sections were washed and incubated with biotinylated anti-mouse CD31 antibody (PharMingen, San Diego, CA, USA) in wet chamber at room temperature, after the sections had been blocked with 1% BSA–PBS (−). After 1-h incubation, sections were washed and secondly stained with streptavidin–FITC conjugate (Molecular Probes Inc., Eugene, OR, USA) for 30 min. These sections were fluorescently observed by using microscopic LSM system (Carl Zeiss, Co. Ltd.).

2.5. Determination of apoptotic cells in tumor

ADM-encapsulated Cont-Lip (Cont-LipADM) or APRPG-LipADM (10 mg/kg as dose of ADM) were administered via a tail vain of C26 NL-17-bearing mice or P388/ADM-bearing mice when the tumor sizes had reached about 1 cm in diameter for C26 NL-17-bearing mice and at day 6 for P388/ADM-bearing mice, respectively. Two days after injection of liposomal ADM, each solid tumor was dissected from the mice and tumor sections were prepared as described in Section 2.4. Immunostaining of endothelial cells was performed as described in Section 2.4, except that streptavidin–Alexa 594 conjugate (Molecular Probes Inc., Eugene, OR, USA) was used as fluorescent dye instead of streptavidin–FITC conjugate. For visualizing apoptotic cells, TUNEL staining was performed by use of ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Intergen Co., Purchase, NY, USA) according to the recommended procedures of the Kit. In brief, tumor sections were washed and equilibrated for 15 min in wet chamber at room temperature, and sections were reacted with TdT enzyme for 1 h at 37 °C. Then, sections were stained with anti-digoxigenin–fluorescein antibody. These sections were observed with LSM system.

In some experiments, fixed tumor sections were stained with hematoxylin and eosin. In brief, sections were stained with Lillie-Mayer's hematoxylin (Muto

Pure Chemicals. Ltd., Tokyo, Japan) for 2 min and subsequently adjusted the color shade by 50 mM Tris-buffered saline, pH 7.5. Then, the sections were stained with eosin Y (Wako Chemical Co., Osaka, Japan) for 30 s and dehydrated with ethanol. The sections were mounted with DIATEX (AB Wilh. Becker, Stockholm, Sweden) and observed by light microscopy (Olympus, Tokyo, Japan).

2.6. Therapeutic experiment

Cont-LipADM or APRPG-LipADM were intravenously administered (10 mg/kg as dose of ADM) via a tail vein into P388- or P388/ADM-bearing mice at day 6 after tumor implantation. Tumor sizes were examined at selected days after the treatment and the tumor volume was calculated in an established formula 0.4 ($a \times b^2$), where 'a' was the largest and 'b' was the smallest diameter of the tumor. Body weight of each mouse was also monitored after injection of the formulations to evaluate the side effect.

2.7. 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. Intratumoral distribution of APRPG-Lip

Previous study showed that APRPG-Lip highly accumulated in tumor tissue in vivo and bound to VEGF-stimulated human endothelial cells in vitro (Oku et al., 2002b), although the actual binding of APRPG-Lip to tumor angiogenic vasculature has not been determined. Therefore, we examined the intratumoral distribution of APRPG-Lip by confocal laser scanning microscopy. Fluorescently labeled APRPG-Lip or Cont-Lip was administered intravenously into C26 NL-17-bearing mice and allowed to circulate for 15 min or 2 h. The tumor was dissected, devoted to immunofluorescence staining of endothelial cells, and examined liposomal distribution. Fluorescence micrographs 2 h after injection of the liposomes are shown in Fig. 1. As shown in the figure, fluorescence of APRPG-Lip was dominantly observed as vessel like structure and some of fluorescent dots were co-localized with CD31 staining (Fig. 1c and d). In contrast, fluorescence of Cont-Lip was observed in all over the tumor tissue without co-localization with CD31 (Fig. 1b). Similar results were also observed 15 min after injection (data not shown). These data suggest that APRPG-Lip bound to tumor angiogenic endothelial cells in an actual tumor tissue.

3.2. Apoptosis of angiogenic endothelial cells by APRPG-LipADM

Since APRPG-Lip seemed specifically bound to angiogenic endothelial cells in tumor tissue, it is possible that APRPG-LipADM damages the cells. Therefore, we determined the apoptotic cells in tumor tissue after treatment of APRPG-LipADM by using double immunostaining method, namely, CD31 staining for observing endothelial cells and TUNEL staining for observing apoptotic cells. Cont-LipADM

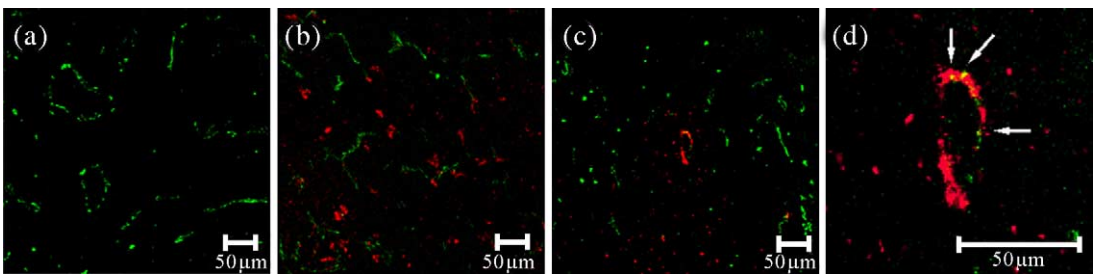

Fig. 1. Binding of APRPG-Lip to tumor angiogenic endothelial cells. C26 NL-17-bearing mice were intravenously administered with glucose (a), DiI-labeled Cont-Lip (b) or DiI-labeled APRPG-Lip (c) on day 17 after tumor implantation. Two hours later, each tumor was dissected, prepared for frozen-section and devoted to CD31-immunostaining to stain endothelial cells (green). Panel (d) shows the magnified image of the area of interest in panel (c). Liposomal fluorescence is shown in red. White arrows show the co-localization of liposomes with the marker of endothelial cells.

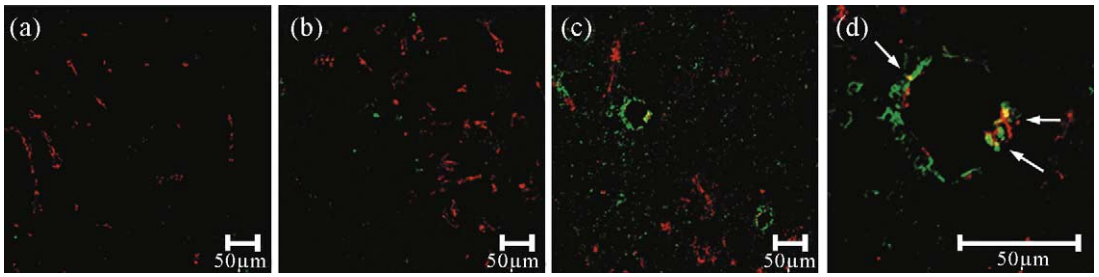

Fig. 2. Double immunostaining of endothelial cells and apoptotic cells on C26 NL-17 tumor following the treatment of APRPG-LipADM. C26 NL-17-bearing mice were administered with glucose (a), Cont-LipADM (b) or APRPG-LipADM (c) (10 mg/kg as dose of ADM) on day 19 after tumor implantation. Two days later, each tumor was dissected and prepared for frozen-section. Microvessels were stained with red (CD31-staining) and apoptotic cells were stained with green (TUNEL method). Panel (d) shows the magnified image of the area of interest in panel (c). White arrows showed apoptotic endothelial cells (yellow).

or APRPG-LipADM was intravenously administered into C26 NL-17-bearing mice, and tumor was dissected, sectioned at 5 μm, and stained at 48 h after the administration. Each derived section was fluorescently observed by use of confocal laser scanning microscopy. As a result, the apoptotic cells located widespread in the tumor and some of them located on the vessel like structure after the treatment of APRPG-LipADM (Fig. 2c and d). Furthermore, CD31 staining was observed on the same vessel like structure. These data strongly suggested that APRPG-LipADM induced apoptosis of both angiogenic endothelial cells and surrounding tumor cells in the solid tumor. In contrast, apoptotic cells located apart from each other, and none of them was co-localized with CD31 staining after the treatment of Cont-LipADM (Fig. 2b).

3.3. Therapeutic experiment using APRPG-LipADM against ADM-resistant tumor

Next, we performed therapeutic experiment using APRPG-LipADM against ADM-resistant tumor. Free ADM, Cont-LipADM or APRPG-LipADM was administered into drug-sensitive P388- or P388/ADM-bearing mice and the tumor regression was evaluated. When we examined the anti-tumor effect against ADM-sensitive P388-bearing mice, free ADM and Cont-LipADM as well as APRPG-LipADM suppressed tumor growth (Fig. 3a). In contrast, treatment of free ADM or Cont-LipADM rarely showed the suppression of tumor growth of P388/ADM-bearing mice under the present experimental condition (Fig. 3b). On the contrary, APRPG-LipADM significantly suppressed tumor growth of P388/ADM solid tumor under the present experimental condition that free ADM or Cont-LipADM did not show the therapeutic effect (Fig. 3b). These data suggest that APRPG-LipADM is superior to free ADM or Cont-LipADM for the treatment of ADM-resistant tumor. Moreover, APRPG-LipADM, as well as Cont-LipADM, did not show the body weight loss, an indicator for side effects, unlike free ADM administration (data not shown).

To confirm the possibility that APRPG-LipADM suppressed tumor growth through damaging angiogenic endothelial cells of ADM-resistant tumor, we performed double immunostaining of apoptotic cells and endothelial cells on tumor section treated with Cont-LipADM or APRPG-LipADM. As shown in Fig. 4, a few apoptotic cells apart from each other was observed after the treatment of Cont-LipADM, and lining apoptotic cells co-localized with CD31 staining was observed after the treatment with APRPG-LipADM. In the case of APRPG-LipADM treatment, many apoptotic cells were also observed around the lining apoptotic cells. These data suggest that APRPG-LipADM induced apoptosis of endothelial cells which caused surrounding tumor cell apoptosis due to cut off of oxygen and nutrients through damaging the neovessels. Hematoxylin and eosin stained tumor sections also indicate that the number of vessel like structures was reduced after the treatment with APRPG-LipADM (Fig. 4, upper right). These data suggest that APRPG-LipADM suppressed drug-resistant tumor growth through the eradication of angiogenic endothelial cells and subsequent disruption of angiogenic vasculature.

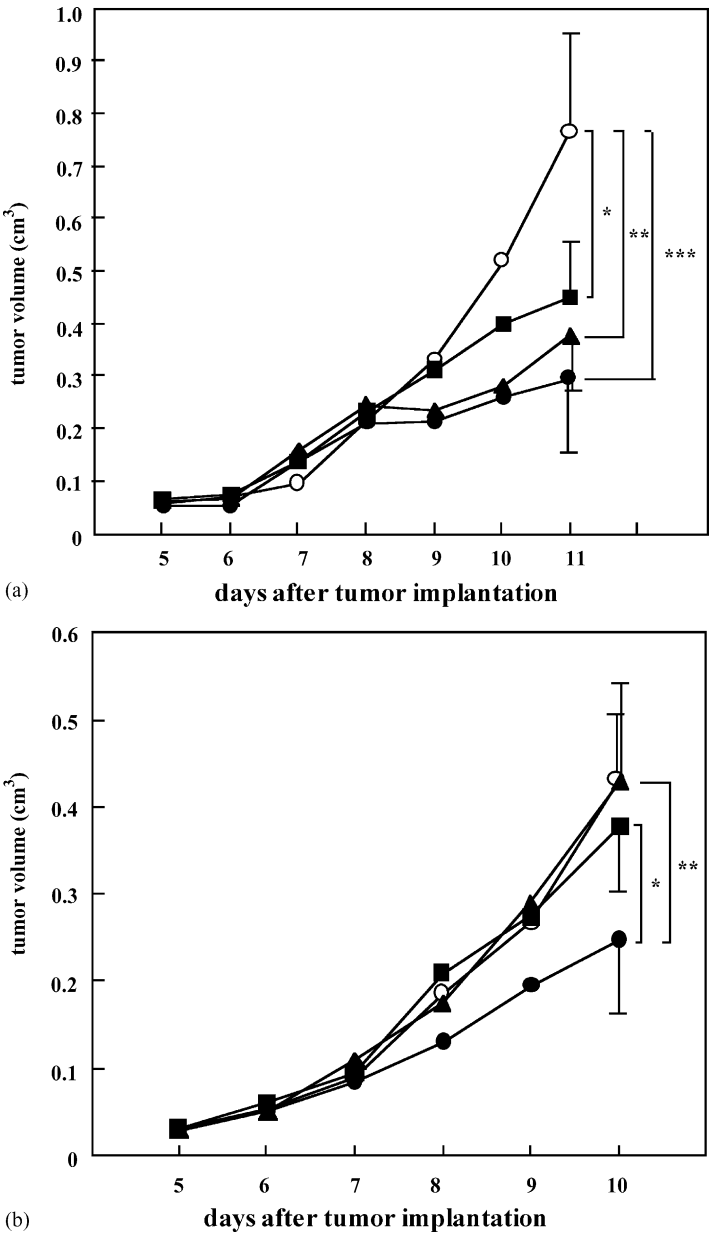

Fig. 3. Suppression of tumor growth by APRPG-LipADM in ADM-resistant tumor-bearing mice. The mice bearing P388 (a) or P388/ADM (b) ($n = 5$) were intravenously administered with glucose (○), free ADM (■), Cont-LipADM (▲), or APRPG-LipADM (●) (10 mg/kg as dose of ADM) at day 6 after tumor implantation. Evaluation of tumor regression was described in Section 2. Data points represent the mean ± S.D.; and S.D. bars are shown only for the data points of day 11 for (a) and day 10 for (b), respectively, for the sake of graphic clarity. Significant difference from control (a) or from APRPG-LipADM treatment (b) is shown ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

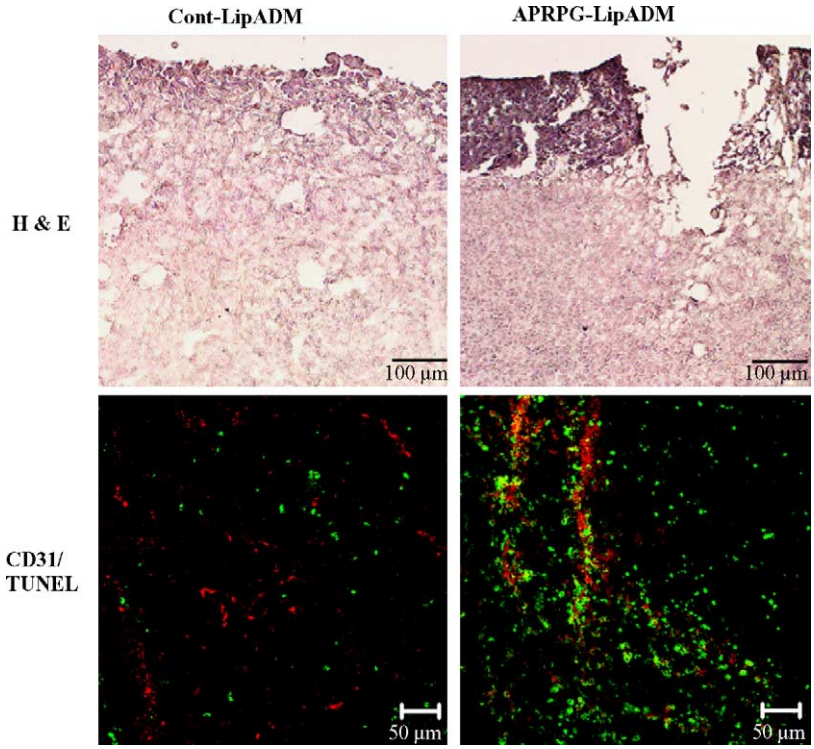

Fig. 4. Histological and immunochemical analysis of ADM-resistant tumor treated with APRPG-LipADM. Tumor sections were prepared as described in Section 2. Upper panels show the micrographs of tumor section stained with hematoxylin–eosin. In histological analysis, edges of tumor sections were observed. Lower panels show the fluorescence micrographs of tumor section immunostained with CD-31 (red) and TUNEL-stained (green). Yellow shows the apoptotic endothelial cells.

4. Discussion

Cytotoxic agents against rapidly growing cells are commonly used for cancer chemotherapy. These agents effectively eradicate cancerous cells, but also damage rapidly dividing normal cells such as bone marrow cells and intestinal lining cells. This is the most serious problem as severe side effects in traditional cancer chemotherapy. Additionally, tumor cells tend to acquire the drug resistance because of their gene instability (Bailar and Gornik, 1997), and therefore a certain cytotoxic agent at selected dose cannot affect on tumor cells. These problems further burden the patients on therapeutic treatment. For these reasons, the development of strategies for cancer chemotherapy such as usage of DDS technology has been required.

Since Folkman et al. advocates the necessity of angiogenesis in tumor progression (Folkman, 1971), a novel strategy for cancer therapy, which is called anti-angiogenic therapy, has been focused and various kinds of angiogenic inhibitor has been developed (Kerbel and Folkman, 2002). There may be some advantages of anti-angiogenic therapy over traditional cancer therapy: Anti-angiogenic therapy can reduce the side effects due to appreciable specificity to angiogenic endothelial cells (Brooks et al., 1994, 1996) and due to reduction of injected dose since angiogenic endothelial cells account for low population compared with tumor cells in tumor tissue. Anti-angiogenic therapy can get rid of concern about acquirement of drug-resistance because this therapy targets normal endothelial cells. Anti-angiogenic therapy may be applicable to most kinds of tumor including drug-resistant tumor because they often induce similar angiogenesis despite of them for their maintenance and development.

Recently, we developed a novel anti-angiogenic therapy, anti-neovascular therapy (Oku et al., 2002a). The concept of this therapy is different from conven-

tional anti-angiogenic therapy in that anti-neovascular therapy does not inhibit a part of angiogenic process but directly eradicate angiogenic endothelial cells by using DDS drugs against neovessels (Shimizu and Oku, 2004). As a result, this therapy strongly promises disruption of angiogenic vasculature following effective suppression of tumor growth with little side effects. To develop an angiogenic vasculature-targeting carrier for cytotoxic agents, we firstly determined pentapeptide sequence (APRPG), which had high affinity to angiogenic site. Cytotoxic agents-encapsulating liposomes modified with the peptide showed enhanced anti-tumor effect (Oku et al., 2002b; Asai et al., 2002).

We hypothesized that APRPG-LipADM caused tumor regression through damaging the neovessels. In the present study, we firstly investigated the intratumoral distribution of APRPG-LipADM and topological distribution of apoptotic cells after the treatment with APRPG-LipADM. The specific binding of APRPG-Lip to angiogenic endothelial cells in tumor tissue was observed (Fig. 1c and d), suggesting that APRPG-Lip actively targets and binds to angiogenic endothelial cells in tumor tissue after intravenous administration. Moreover, the data of Fig. 2 suggested that APRPG-LipADM induced apoptosis of endothelial cells as well as tumor cells in tumor tissue. In contrast, Cont-LipADM seemed to damage only tumor cells. We speculate that APRPG-LipADM directly eradicate angiogenic endothelial cells. Although it is still ambiguous whether tumor cells are directly damaged by APRPG-LipADM or their apoptosis is caused by cut off of oxygen and nutrients through damaging the endothelial cells. Even if, both mechanisms might be worked, we speculate that the latter contribute more than the former, since angiogenic vessels might be damaged by APRPG-LipADM, and since the population of apoptotic cells in tumor tissues is greater for APRPG-LipADM-treated group than for Cont-LipADM-treated one.

If the majority of tumor cells are damaged indirectly through the damage of neovessels after treatment with APRPG-LipADM, this formulation of ADM may also cause damage of ADM-resistant tumor. Therefore, we challenged to apply the APRPG-LipADM against ADM-resistant tumor. As a result, APRPG-LipADM actually suppressed tumor growth (Fig. 3). Since a single dose of treatment of free ADM or Cont-LipADM was not toxic to ADM-resistant tumor cells whereas they suppressed tumor growth of ADM-sensitive tumor cells, APRPG-LipADM may damage growing angiogenic endothelial cells, which causes the suppression for tumor growth indirectly. In fact, we observed the apoptotic cells in angiogenic vessels and in surrounding tumor tissue of ADM-resistant tumor after the treatment of APRPG-LipADM, (Fig. 4). These results indicate that APRPG-LipADM actively disrupts angiogenic vasculature and subsequently suppresses the ADM-resistant tumor growth. The present study strongly suggests that APRPG-LipADM targets to angiogenic vasculature and has potent anti-tumor effect against various kinds of tumor including drug-resistant tumor. Furthermore, reduction of severe side effects is expected by use of APRPG-LipADM due to targeting effects. The modification of liposomes with hexapeptide, GPLPLR, targeted to membrane-type 1 matrix metalloproteinase (MT1-MMP), which is expressed on the surface of angiogenic endothelial cells, also enhanced anti-tumor activity of encapsulated hydrophobic anti-cancer drug (Kondo et al., 2004).

Recently, we observed that APRPG-polyethyleneglycol (PEG) modified liposomes (APRPG-PEG-Lip) showed long-circulating character and accumulated in tumor tissue of tumor-bearing mice (Maeda et al., 2004a). Administration of these liposomes encapsulating ADM into tumor-bearing mice caused strong suppression of tumor growth without remarkable side effects (Maeda et al., 2004b). Availability of anti-neovascular therapy by using PEG-coated angiogenic-vasculature targeting liposomal agent was also reported by several research groups: One used NGR peptide as a targeting probe (Pastorino et al., 2003), and another used RGD peptide (Schiffelers et al., 2003). These accumulating data from ours and other research groups indicate the usefulness of anti-neovascular therapy using DDS drugs in the treatment of cancer, and the present study additionally confirms the availability of the therapy for drug-resistance-acquired cancer.

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