Chronopharmacologic Cancer Treatment with an Angiogenic Vessel-Targeted Liposomal Drug

Kosuke SHIMIZU,a Yasuharu SAWAZAKI,a Toshiki TANAKA,b Tomohiro ASAI,a and Naoto OKU*,a

a Department of Medical Biochemistry, University of Shizuoka School of Pharmaceutical Sciences; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan; and b Department of Applied Chemistry, Faculty of Engineering, Nagoya Institute of Technology, Gokiso-cho, Nagoya 466–8555, Japan. Received June 5, 2007; accepted September 27, 2007

Antineovascular therapy (ANET), which eradicates angiogenic endothelial cells by specifically delivered anticancer drugs to tumor cells to obtain complete cutoff of blood supply, is an effective modality for cancer treatment. Since the expression of vascular endothelial growth factor (VEGF) in hypoxic tumor cells is known to fluctuate in a circadian oscillation, we investigated the chronopharmacologic treatment of tumors with ANET. Adriamycin-encapsulated liposomes modified with the Ala-Pro-Arg-Pro-Gly (APRPG) peptide (APRPG-LipADM) were prepared, after the APRPG peptide had been shown to have affinity to angiogenic sites. Colon 26 NL-17 tumor-bearing mice were injected three times with APRPG-LipADM at Zeitgeber time (ZT) 2, 8, 14, and 20 where ZT 0 was the time lights were turned on, and tumor growth was monitored. Tumor growth suppression changed with dosing time and was significantly (p<0.01) more potent at ZT 14 compared with ZT 20. The VEGF concentration in the plasma of the tumor-bearing mice was higher in the light phase compared with that in the dark phase, and this circadian oscillation was related to dosing time dependency with ANET. These results indicate that tumor growth suppression is correlated to some extent with the VEGF concentration in the plasma, and that chronopharmacologic treatment of cancer with ANET may enhance the therapeutic efficacy and reduce the side effects.

Key words vascular endothelial growth factor; tumor angiogenesis; antineovascular therapy; liposome; circadian rhythm; drug delivery system

Tumor cells demand oxygen and nutrients, and tumor angiogenesis is critical for the growth and maintenance of solid tumors.1-3 Therefore antiangiogenic inhibitors for cancer treatment have been developed.4,5 Angiogenesis is promoted by various proangiogenic factors such as vascular endothelial growth factor (VEGF) that stimulate endothelial cell proliferation and sprouting to form vascular construction.6 Antibodies against VEGF or VEGF receptor, kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1) are known to inhibit angiogenesis effectively and to suppress tumor growth. The humanized monoclonal anti-VEGF antibody bevacizumab (Avastin) has been used clinically.7,8 Since angiogenesis is critical for tumor growth, we focused on delivering anticancer drugs to the angiogenic endothelial cells and eradicating them to obtain complete cutoff of blood supply to tumor cells. Although this therapy potentially has side effects due to the usage of cytotoxic anticancer agents, these side effects might be decreased by drug delivery system (DDS) technology. For this purpose, we first isolated peptides specific for angiogenic vasculature by in vivo biopanning using a phage-displayed 15-mer-peptide library.9 As an epitope, we obtained the Ala-Pro-Arg-Pro-Gly (APRPG) peptide that specifically interacts with an angiogenic site. The APRPG peptide was then used for the modification of liposome, an active-targeting drug carrier. We previously observed that APRPG-modified liposome (APRPG-Lip) predominantly accumulated in tumor tissue and bound to VEGF-stimulated endothelial cells in vitro. When anticancer drugs such as adriamycin (ADM) are encapsulated in APRPG-Lip, it inhibits angiogenesis in dorsal air sac model mice, suppresses tumor growth with reduced side effects, and lengthens the survival time of tumor-bearing mice. ANET was effective in the drug-resistant tumor model.10 Confocal microscopic observation and histochemical staining demonstrated that APRPG is applicable to human cancer.11

Koyanagi and coworkers reported that the expression of VEGF in hypoxic tumor cells fluctuated in a circadian oscillation.12,13 The mRNA level of VEGF in implanted tumor cells was high at Zeitgeber time (ZT) 2 to 10, and low at ZT 14 to 18, and actual VEGF concentrations in plasma and tumor correspondingly oscillated. Furthermore, they observed higher tumor growth suppression by three different types of antiangiogenic agents, SU1498, TNP-470, and BB2516, at dosing time ZT 2 than at ZT 14 in sarcoma 180-bearing mice. After that elegant study, we attempted to investigate the chronopharmacologic treatment of tumors with ANET.

MATERIALS AND METHODS

Materials Stearoyl-APRPG derivative was synthesized as previously described.15 Distearoylphosphatidylcholine (DSPC) was a gift from Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Experimental Animals BALB/c male (3-week-old) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), housed under the standard laboratory conditions (23 °C and 55±5% humidity) and allowed access to tap water and food.
day 18, when the tumor volumes became about 0.15 cm$^3$, the mice were harvested in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma) in a CO$_2$ incubator. After harvesting of the cells with 0.02% EDTA in PBS, 1.0$\times$10$^6$ cells were carefully implanted subcutaneously into the posterior flank of 5-week-old male BALB/c mice. The mice were injected with Cont-LipADM or APRPG-LipADM intravenously via a tail vein (10 mg/kg as the dose of ADM) on day 18, when the tumor volumes became about 0.15 cm$^3$, with special care to minimize environmental changes. These liposomes were again administered on day 21 and 24 (30 mg/kg total ADM after three administrations, and about 0.045 mmol/kg liposomal dosage as DSPC in each injection). The dosing time of the sample on each day was determined to ZT 2 (10 mg/kg/d as ADM) through a tail vein at ZT 2 (10 mg/kg/d as ADM) through a tail vein at ZT 2, ZT 8, ZT 14, and ZT 20, respectively (Fig. 1a). The VEGF concentration in the plasma of C26 NL-17 tumor-bearing mice was determined using the ELISA (Mouse VEGF immunoassay, R&D Systems) according to the manufacturer’s instructions. A standard curve was obtained with mouse VEGF. Absorbance at 562 nm was measured with a microplate reader, and the amount of VEGF was expressed as picograms per milliliter.

**Preparation of Liposomes** Preparation of liposomes was performed as described previously.$^{11,12}$ In brief, DSPC and cholesterol with stearyl-APRPG (10 : 5 : 1 as a molar ratio), or DSPC and cholesterol without APRPG derivative (10 : 5 as a molar ratio) were dissolved in chloroform, 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 of citrate solution, pH 4.0, and frozen and thawed for three cycles using liquid nitrogen. Then, the liposomes were sized by extrusion three times through a polycarbonate membrane filter with 100-nm pores (Advantec, Tokyo, Japan). After adjusting the pH of the solution to pH 7.0 with sodium carbonate in HEPES buffer 20 mM, liposomes were incubated with ADM at 60 °C for 1 h, washed three times with PBS, and resuspended with glucose solution 0.3M. Particle size of the liposomes was about 150 nm as recorded on an ELS-800 electrophoretic light-scattering spectrophotometer (Otsuka Electronics Co., Ltd., Osaka, Japan). The concentration of ADM was determined based on absorbance at 484 nm, and the encapsulation efficiency of ADM was greater than 90% throughout the experiment.

**Therapeutic Experiment** C26 NL-17 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma) in a CO$_2$ incubator. After harvesting of the cells with 0.02% EDTA in PBS, 1.0$\times$10$^6$ cells were carefully implanted subcutaneously into the posterior flank of 5-week-old male BALB/c mice. The mice were intravenously administered glucose 0.3 M (©) or APRPG-LipADM intravenously via a tail vein (10 mg/kg as the dose of ADM) on days 34 and 35. As an indicator of side effects, we determined the body weight changes in the APRPG-LipADM groups and observed body weight loss in the ZT 2 and ZT 20 groups to some extent (Fig. 1b). The survival time was also monitored and the mean survival time for the control, ZT 2, ZT 8, ZT 14, and ZT 20 groups was 62.5, 77.4, 89.0, 77.8, and 73.2 d, respectively (Fig. 2). These data also indicate that APRPG-LipADM was less potent in the ZT 20 group, although it was also possible that the side effects shown as body weight loss in the ZT 20 group affected the life span.

**Relationship between VEGF Concentration in Plasma and ANET Efficiency** Since the suppression of tumor growth by APRPG-LipADM was affected by dosing time, the VEGF concentration in the plasma of C26 NL-17 tumor-bearing mice was prepared as described above. On day 18 after tumor inoculation when the tumor volumes became about 0.15 cm$^3$, the mice (n=4) were killed under diethylether anesthesia and their blood was collected and centrifuged to obtain the plasma. The VEGF concentration in the plasma was determined using an ELISA (Mouse VEGF immunoassay, R&D Systems) according to the manufacturer’s instructions. A standard curve was obtained with mouse VEGF. Absorbance at 562 nm was measured with a microplate reader, and the amount of VEGF was expressed as picograms per milliliter.

**Statistical Analysis** Variance in a group was evaluated using the F-test, and differences were evaluated with Student’s t-test.

**RESULTS**

### Chronopharmacologic Treatment of Tumors with ANET

**TZ 2, 8, 14, and 20** corresponded to 10:00, 16:00, 22:00, and 04:00, respectively since the lights were automatically turned on at 08:00 and off at 20:00. As shown in Fig. 1a, tumor growth was significantly (p<0.01) suppressed in all APRPG-LipADM groups. Among them, APRPG-LipADM was less potent for tumor growth suppression in the ZT 20 group, and a significant difference in tumor volume was observed between the ZT 20 group and ZT 14 group on days 34 and 35. As an indicator of side effects, we determined the body weight changes in the APRPG-LipADM groups and observed body weight loss in the ZT 2 and ZT 20 groups to some extent (Fig. 1b). The survival time was also monitored and the mean survival time for the control, ZT 2, ZT 8, ZT 14, and ZT 20 groups was 62.5, 77.4, 89.0, 77.8, and 73.2 d, respectively (Fig. 2). These data also indicate that APRPG-LipADM was less potent in the ZT 20 group, although it was also possible that the side effects shown as body weight loss in the ZT 20 group affected the life span.

### C26 NL-17 Carcinoma-Bearing Mice

C26 NL-17 carcinoma cells were subcutaneously implanted into BALB/c mice. The mice were intravenously administered glucose 0.3 m (©) or APRPG-LipADM (10 mg/kg/d as ADM) through a tail vein at ZT 2 (©), ZT 8 (©), ZT 14 (©), or ZT 20 (©) on days 18, 21, and 24 after tumor implantation. Tumor volume (a) and body weight change (b) in each mouse were measured to evaluate therapeutic effects. Data in (a) are presented as the mean tumor volume and S.D., where S.D. bars are shown only for the last points (day 35) for the sake of graphic clarity. Significant differences between APRPG-LipADM treated groups are indicated; *p<0.05. Arrows indicate the days of treatment.
C26 NL-17 carcinoma cells were subcutaneously implanted into BALB/c mice. The mice were intravenously administered glucose 0.3 m (○) or APRPG-LipADM (10 mg/kg/d as ADM) through a tail vein at ZT 2 (●), ZT 8 (▲), ZT 14 (●), or ZT 20 (■) on days 18, 21, and 24 after tumor implantation. Arrows indicate the days of treatment.

Fig. 3. Chronopharmacological Relationship between VEGF Concentration in the Plasma and Therapeutic Effect

a) C26 NL-17 carcinoma cells were subcutaneously implanted into BALB/c mice, and the mice were killed on day 18 at ZT 2 (●), ZT 8 (▲), ZT 14 (●), or ZT 20 (■). Plasma VEGF concentration was determined as described in Materials and Methods. b) Tumor growth inhibition on day 35 was depicted based on Fig. 1 and arranged against ZT. Significant differences are indicated; *p<0.05.

bearing mice was determined at various ZTs. As shown in Fig. 3, the plasma VEGF concentration in the light phase was higher than that in the dark phase. This result is consistent with the previous study by Koyanagi and coworkers.14) Figure 3 also shows the tumor volume on day 35 after tumor implantation, depicted from the data shown in Fig. 1. Tumor growth suppression was roughly correlated with the VEGF concentration in the plasma, although the decrease in tumor growth suppression was retarded in comparison with the decline in plasma VEGF concentration: Tumor growth was highly suppressed in the ZT 14 group, although the VEGF concentration was low at ZT 14. These data suggest that ANET was effective when the growth of tumor angiogenic endothelial cells was enhanced by VEGF, and the growth was retarded for several hours after stimulation with VEGF.

DISCUSSION

Tumor angiogenesis is critical for tumor growth and blood-borne metastasis. Therefore angiogenesis and angiogenic vessels have become a target for cancer treatment. Proangiogenic factors such as VEGF, angiopoietin, basic fibroblast growth factor, platelet-derived growth factor, and interleukin-8 may stimulate endothelial cells in a receptor-dependent manner and induce angiogenesis. The binding of VEGF to VEGF receptors initiates signal transduction in endothelial cells and these stimulated endothelial cells begin to proliferate and sprout to form neovessels. Therefore the inhibitors of VEGF binding or its signal transduction are thought to suppress tumor growth by blocking the neovessel formation that is critical for the supply of oxygen and nutrients to tumor cells. Traditional antiangiogenic therapy, however, is rather passive. Therefore we designed a modality for active degeneration of neovessel constructs, ANET.11,12) In this modality, we deliver cytotoxic agents to angiogenic endothelial cells, which directly eradicate angiogenic vasculature, since cytotoxic agents damage the growing endothelial cells as well as tumor cells.

A number of living organisms including humans show periodicity in biologic functions. Recent studies have revealed that the circadian rhythm is regulated by several specific clock gene produces.17—19) Circadian rhythm is known to affect various events in a body related to drug efficacy: The oscillation of sensitivity of target organs, drug metabolism, etc. changes the bioavailability and side effects of drugs. Therefore, in such cases, scheduling of dosing time enhances drug efficacy and reduces side effect.20—23) Anticancer drugs are also chronologically considered for scheduling to reduce side effects and enhance antitumor activity.24—28) These results indicate that the susceptibility of tumor cells to anticancer agents oscillates. Moreover, the efficacy of antiangiogenic therapy depends on dosing time,14,29) since the previous report suggested that VEGF production also fluctuates in a circadian fashion.

In the present study, we chronologically treated tumors with ANET, since the therapeutic efficacy of ANET is affected by dosing time. As shown in Fig. 1, the therapeutic efficacy of APRPG-LipADM was dependent on the dosing time; it was potent at ZT 2 to ZT 14 and weak at ZT 20. The plasma VEGF concentration also fluctuated in a circadian fashion, consistent with the data of Koyanagi et al.14) Considering these data, the oscillation of tumor growth inhibition by APRPG-LipADM may be explained as VEGF stimulates the growth of angiogenic endothelial cells, and the cells in growing phase are more susceptible to ADM. Thus the damaged endothelial cells caused regression of tumor growth. At ZT 14, the VEGF concentration in the plasma is low in the daily oscillation, although tumor regression due to treatment with APRPG-LipADM at ZT 14 was clear. We speculate that it took several hours from VEGF stimulation of angiogenic endothelial cells to the growth of the cells.

Alternatively, VEGF stimulation might augment the binding site of APRPG on the surface of angiogenic endothelial cells, enhancing the binding of APRPG-LipADM at ZT 2 to ZT 14. (The target molecule of APRPG, however, is not clear at present.) APRPG-Lip bound to human umbilical vein endothelial cells (HUVECs) when the cells had been stimulated with VEGF.31) If this was the case, APRPG-Lip did not bind sufficiently at ZT 20 when the APRPG-binding site was less present on the surface of the endothelial cells. In Fig. 1b, the
side effect was slightly more potent at ZT 20 and ZT 2 than at ZT 8 and ZT 14. Although we do not speculate that the unbound APRPG-LipADM caused greater side effects, since the accumulated amount of APRPG-Lip in tumors was less than 2% of the injected dose per gram of tumor tissue.11) To et al.30) examined the dosing time dependency of doxorubicin-induced cardiotoxicity and bone marrow toxicity after repeated administration in rats. They reported that the toxic effects were significantly high in the 9 HALO-treated group, corresponding to ZT 9 and that the reason was the increased AUC of the drug.30) The body weight change observed in Fig. 1b, however, is not explained by the results of To and coworkers, since the pharmacokinetics of free drug and liposomal drug are completely different.

In conclusion, chronopharmacological treatment of cancer with ANET promises enhanced therapeutic efficacy and reduced side effects. This dosing time dependency may be due, at least in part, by circadian oscillation of the plasma VEGF concentration.