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Short Communication

Anti-tumor Effect of All-*Trans* Retinoic Acid Loaded Polymeric Micelles in Solid Tumor Bearing Mice

Narin Chansri,[1] Shigeru Kawakami,[1] Masayuki Yokoyama,[2] Tatsuhiro Yamamoto,[2] Pensri Charoensit,[1] and Mitsuru Hashida[1,3]

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Purpose. All-*trans* retinoic acid (ATRA) polymeric micelles were developed for parenteral administration. The distribution characteristics and antitumor activities of ATRA polymeric micelles were evaluated after intravenous administration to mice bearing CT26 solid tumors.
Methods. ATRA incorporated in poly(ethylene glycol)-poly(benzyl aspartate) block copolymer was prepared by the evaporation method. The levels of [^{3}H]ATRA in blood and tissue including tumor were determined by measuring the radioactivity after injection into mice. The tumor volume and the survival of the mice were determined to assess the anticancer activity.
Results. The delivery of ATRA by polymeric micelles prolonged the blood circulation and enhanced the accumulation of ATRA in the tumor tissue compared with the administration of free ATRA. Tumor growth was significantly delayed and the survival time of mice was prolonged following the treatment by ATRA polymeric micelles demonstrating the improved anticancer activity of ATRA.
Conclusion. Polymeric micelles are a promising and effective carrier of ATRA in order to enhance tumor delivery and they have a promising potential application in the treatment of solid tumors.

KEY WORDS: all-*trans* retinoic acid; antitumor activity; biodistribution; drug targeting; nanomedicine; polymeric micelles.

INTRODUCTION

All-*trans* retinoic acid (ATRA) is an active metabolite of retinoids that has been shown to exert anti-cancer activities in a number of cancer cells and tissues. The pharmacology effects of ATRA are mainly mediated by nuclear retinoid receptor that is retinoic acid receptors, leading to growth inhibition, differentiation, and apoptosis of cancer cells (1–3). Recently, it has been extensively used for the treatment of acute promyelotic leukemia (APL) (4). However, a gradual decrease in the ATRA concentration in the blood circulation after prolonged treatment and highly variable bioavailability after oral administration were observed (5,6). Therefore, parenteral formulations that maintain the ATRA concentration in the blood could enhance its pharmacological effect.

Because of the low aqueous solubility of ATRA, drug delivery carriers such as liposomes (7), emulsions (8), and solid lipid nanoparticle (9) have been employed to improve its potency and duration of activity. In particular, clinical trials have demonstrated that liposomal ATRA offers potential pharmacological advantages over the oral administration of ATRA and appears to be an effective carrier of ATRA for APL therapy (6). Recently, we have demonstrated the inhibition of pulmonary and hepatic metastasis by ATRA incorporated in cationic liposomes (10,11) and O/W emulsions (12), respectively. However, sustained circulation of the carrier for ATRA is required for maintaining the required therapeutic level and reaching the target pathological sites in the body. Therefore, the delivery of ATRA by a micelle-forming agent is represents a novel parenteral delivery system for ATRA.

Polymeric micelles are a class of micelles that are formed from block copolymers typically consisting of hydrophilic and hydrophobic polymer chains (13). They are of particular interest because of their small particle size, their efficiency in entrapping a satisfactory amount of hydrophobic drug within the inner core, their stability in the circulation and their ability to gradually release the drug (14). Furthermore, the highly hydrated outer shells of the micelles prevent reticuloendothelial system (RES) uptake and inhibit intermicellar aggregation of their hydrophobic inner cores (15). The characteristics of these polymeric micelles could be advantage for passive delivery and extravasate the drug in the tumor site by an enhanced permeability and retention effect (EPR effect) (16). In this regard, polymeric micelles have been employed to enhance *in vivo* anticancer activity by solid tumor targeting of many anticancer drugs such as doxo-

[1] Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan.
[2] Kanagawa Academy of Science and Technology, KSP East 404, Sakado 3-2-1, Takatsu-ku, Kawasaki-shi, Kanagawa 213-0012, Japan.
[3] To whom correspondence should be addressed. (e-mail: hashidam@pharm.kyoto-u.ac.jp

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rubicin (17,18), camptothecin (19), paclitaxel (20), and cisplatin (21).

In our previous study, ATRA was successfully incorporated in poly(ethylene glycol)-poly(aspartate ester) (PEG-P(Asp)) block copolymer micelles in which 75% of the aspartic acid residues were esterified benzyl groups in order to increase the hydrophobicity of the inner core (22). Because ATRA is a hydrophobic drug, the interaction between ATRA and the hydrophobic inner core play a very important role in the stable incorporation. The ATRA polymeric micelles were effective in increasing the blood retention and lowering hepatic clearance compared with free ATRA and ATRA incorporated in liposomes and suggested the potential use of this system for the design of ATRA carriers in the treatment of solid tumors.

In the present study, the distribution and antitumor efficacy of ATRA incorporated in polymeric micelles was examined in mice bearing CT26 solid tumors. The distribution characteristics of ATRA delivered by polymeric micelles showed a prolonged blood retention and enhanced accumulation of ATRA at the tumor site. Finally, we evaluated the efficiency of ATRA incorporated in polymeric micelles for the treatment of solid tumors by measuring the tumor volume and survival of the mice.

MATERIALS AND METHODS

Chemical

ATRA was purchased from Wako Pure Chemicals Industry, Ltd. (Osaka, Japan). Clear-Sol I was obtained from Nacalai Tesque, Inc. (Kyoto, Japan), and Soluene 350 was purchased from Packard Co., Inc. (Groningen, The Netherlands). RPMI1640 medium was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD, USA). HCO-60 was obtained from Nikko Chemical Co. Ltd. (Japan). [^{3}H]ATRA was purchased from NEN Life Science Products, Inc. (Boston, USA). All other chemicals were of the highest purity available.

Synthesis of Block Copolymer

PEG-P(Asp) block copolymer was obtained by alkaline hydrolysis of poly(ethylene glycol)-poly(β-benzyl-L-aspatate) (PEG-PBLA) as reported previously (23). Briefly, the molecular weight of the poly(ethylene glycol) (PEG) chain was 5,000 and the average number of aspartic acid units was 27. Approximately 69% of the aspartic acid residues in the poly(aspartic acid) chain were converted to the β-amide form by alkaline hydrolysis during the synthesis of this block copolymer. A hydrophobic benzyl group was bound to 69% of the poly(aspartic acid) residues by an ester-forming reaction between benzyl bromide and PEG-P(Asp) as reported previously (24). Briefly, PEG-P(Asp) block copolymer was dissolved in *N,N*-dimethylformamide (DMF) and added to benzyl bromide along with a catalyst, 1, 8-diazabicyclo[5,4,0]7-undecene (DBU). The reaction mixture was stirred at 50°C for 15.5 h. Polymers were obtained by precipitation in an excess of diethyl ether and collected by filtration. The dried polymer was dissolved in dimethyl sulfoxide (DMSO), then 6 N HCl was added, followed by dialysis against distilled water and, finally, freeze-drying.

For determination of the polymer composition, such as the number of Asp units and the benzyl ester content, ^{1}H-NMR measurements were carried out on a 1% solution in 6D-DMSO containing 3% trifluoroacetic acid using a Varian Unity Inova NMR spectrometer at 400 MHz.

Incorporation of ATRA into Polymeric Micelles

Incorporation of ATRA into polymeric micelles was carried out by an evaporation method as previously described (22). Briefly, ATRA 1 mg and polymer 10 mg were dissolved in chloroform. After vacuum drying and desiccation, 3 ml of phosphate buffered saline (PBS) pH7.4 was added for hydration for 30 min at 25°C. The preparation was placed in the probe sonicator (200 W) for 3 min at 60°C. The obtained preparation was centrifuged at 1,500×*g* for 10 min before the supernatant was passed through a 0.450-μm filter. The maximum concentration of ATRA in the polymeric micelle preparation was about 0.066 mg/ml that quantified by the UV absorption at 340 nm after dissolving the preparation in a mixture of dimethylsulfoxide (DMSO) and water (DMSO:water=9:1 by volume). After the preparation was filtered, the concentration of ATRA in filtered preparation was adjusted to 0.060 mg/ml for *in vivo* experiments based on its UV absorption. The particle size of the polymeric micelles was measured by Zetasizer Nano Series instrument (Malvern Instruments, Ltd., Worcestershire, UK). To prepare [^{3}H]ATRA-labeled polymeric micelles, a trace amount of [^{3}H]ATRA (50 μCi) was dissolved in chloroform with ATRA and polymer and then treated exactly as described above. The filtered ATRA was adjusted to a concentration of 0.06 mg/ml based on the measured radioactivity.

Solubility of ATRA

The solubility of ATRA in serum was determined by mixing the excess amount of ATRA in serum and stirred overnight at 37°C. The undissolved ATRA was separated from the solution by filtration through 0.450-μm filter. The amount of ATRA dissolved in serum was determined by spectrophotometer at 340 nm (UV-visible Spectrophotometer, Shimazu Co., Ltd., Kyoto, Japan). The solubility of ATRA in serum was 1.426±0.009 mg/ml at 37°C (*n*=3).

In Vitro Cytotoxicity Experiment

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) assay was performed by the method described previously (10). The CT-26, mouse colon adenocarcinoma cells, was plated on a 96-well cluster dish at a density of 1×10^4 cells/0.28 cm^2. Twenty-four hours later, the medium containing various concentrations of unloaded polymeric micelles was added to the plates. After 48 h of incubation, the medium was removed and 5 mg/ml MTT solution was added to each well. Cells were incubated for 4 h at 37°C in 5% CO_2 and then 10% sodium dodecyl sulfate (SDS) solution was added followed by incubation overnight to dissolve formazan crystals. The absorbance was measured at wavelengths of 570 nm in a microplate photometer (Bio-

Rad Model 550, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

In Vivo Distribution Experiment

The *in Vivo* distribution study was performed by the method described previously (25). Briefly, five-week-old male ddY mice (23–25 g) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). [^{3}H]ATRA dispersed in serum or incorporated in polymeric micelles was injected into the tail vein of the mice. At each collection time point, blood was collected from the vena cava under anesthesia, and the mice were killed at the end of the experiment. Ten microliters of blood were incubated with 0.7 ml Soluene 350 overnight at 45°C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5N HCl, and 5.0 ml Clear-Sol I were added in this order. The samples were stored overnight and the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

Tumor Bearing Mouse Model

Four-week-old male CDF1 mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals. CT-26, mouse colon adenocarcinoma cells, were routinely grown in RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen Co., Carlsbad, CA, USA) in 5% CO_2 humidified air at 37°C. The cells were harvested from 2-day-old subconfluent cultures by trypsin and the cell concentration was adjusted to 10^6 cells/ml by Hank's balance salt solution (HBSS). Then, 0.1 ml of the cell suspension was inoculated subcutaneously in the lower back of each CDF1 mouse. A solid tumor was observed within 7 days after tumor inoculation.

Tissue Distribution in Tumor-Bearing Mice

The blood concentration, tissue distribution and tumor accumulation of [^{3}H]ATRA was examined in tumor-bearing mice on day 14 post-inoculation. After injection of [^{3}H]ATRA dispersed in serum or [^{3}H]ATRA polymeric micelles into the tail vein of the mice, blood, tumor and other tissues were collected and the radioactivity was determined as previously described in the *in vivo* distribution experiment.

Anti-Tumor Efficacy in Tumor-Bearing Mice

The anti-tumor efficacy of ATRA was evaluated in CDF1 mice bearing CT26 cells. We and other group demonstrated that intravenous administration of ATRA incorporated in cationic liposome, O/W emulsion, sterylglucoside liposome at dose of ATRA 0.585 or 0.600 mg/kg exhibited anti-tumor in mice model (11,12,26). Therefore, ATRA dispersed in 5% HCO-60 solution or ATRA incorporated in polymeric micelles were repeatedly injected through the tail vein at an ATRA dose of 0.600 mg/kg/day every other day from day 3 to 11 (5 doses administered) after tumor inoculation (seven mice per group). In the control group, saline (10 ml/kg/day) was administered instead of ATRA. The survival of the mice was recorded every day up to 60 days after tumor inoculation. At the same time, tumor volume and body weight were measured for individual animals as long as more than three mice survived. The tumor volume was determined by measuring the tumor diameter with calipers and calculated as follows:

$$\text{Volume} = \pi/6 \times LW^2$$

Where L is the long diameter and W is the short diameter

Statistical Analysis

Statistical comparisons were performed using Student's t test for two groups. Statistical analysis of tumor volume and survival curves was carried out using Dunnett's test and the log-rank test respectively. $P<0.05$ was considered significant.

RESULTS

Characteristics of ATRA Incorporated in Polymeric Micelles

The block copolymer was successfully synthesized from PEG-P(Asp), and about 69% of the aspartic residues were esterified with benzyl groups as reported previously (23). ATRA was incorporated into the polymeric micelles by the evaporation method, and yielded a clear solution after being passed through 0.450 μm filter. The mean particle size (volume) of the micelles after incorporation of ATRA was 36.3±0.624 (73.7±2.54%) and 290±51.9 (25.6±3.50%) (n=3), respectively. The percentage recovery of ATRA in the polymeric micelle solution detected by UV absorption at 340 nm was 14.0±2.0% (n=3) when compared with the initial amount of ATRA. As previously reported the incorporation ratio of ATRA in polymeric micelles was 96.4±0.17% suggesting ATRA almost completely incorporated in micelles (22). The particle size and concentration of ATRA in the polymeric micelle solution remained constant for at least 1 month when stored at 4°C and protected from light under nitrogen gas.

Cytotoxic Effect of Polymeric Micelles

The cytotoxicity of polymeric micelles was evaluated in CT-26 cells and no significant cytotoxicity was observed at the concentration of 2–40 μg/ml of polymeric micelles, suggesting polymeric micelles itself is low cytotoxic (data not shown).

Distribution Characteristics of [^{3}H]ATRA in Mice

Figure 1 shows the blood concentration profiles of [^{3}H]ATRA dispersed in serum (representing the inherent distribution of ATRA) and [^{3}H]ATRA incorporated in polymeric micelles after intravenous injection into mice. The blood concentration of [^{3}H]ATRA in serum was significantly lower than that of [^{3}H]ATRA in polymeric micelles over 4 h suggesting that polymeric micelles with benzyl groups can enhance the blood retention of ATRA by their encapsulation efficacy.

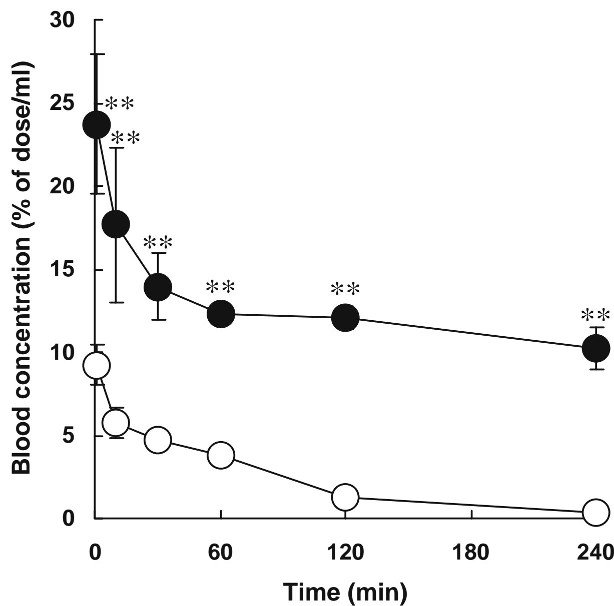

Fig. 1. Blood concentration of [^{3}H]ATRA (*open circle*) and [^{3}H]ATRA incorporated in polymeric micelles (*filled circle*) following intravenous administration to normal mice. Mice were intravenously administered with [^{3}H]ATRA dispersed in serum or [^{3}H]ATRA incorporated in polymeric micelles at 0.600 mg/kg dose of ATRA. At indicated time point, blood was collected and level of radioactivity was measured. Each value represents the mean ± SD of three experiments. Statistically significant differences compared with [^{3}H]ATRA (*double asterisk*, $P<0.01$).

Distribution of [^{3}H]ATRA in Tumor-Bearing Mice

The blood concentration and tumor accumulation of [^{3}H]ATRA dispersed in serum or [^{3}H]ATRA incorporated in polymeric micelles was evaluated in mice bearing CT26 solid tumors. The distribution of [^{3}H]ATRA was determined between 4 and 48 h after a single intravenous injection of 0.600 mg/kg ATRA. The blood concentration of ATRA in polymeric micelles was higher than that of ATRA dispersed in serum at every time point. Following a single injection of ATRA polymeric micelles, the ATRA level was prolonged to almost 48 h while ATRA dispersed in serum was rapidly eliminated and hardly any remained 24 h after administration (Fig. 2a). This sustained blood concentration of ATRA produced by polymeric micelles resulted in a higher accumulation of ATRA in the tumor tissue compared with ATRA dispersed in serum (Fig. 2b). Moreover, the liver accumulation of ATRA delivered by polymeric micelles was

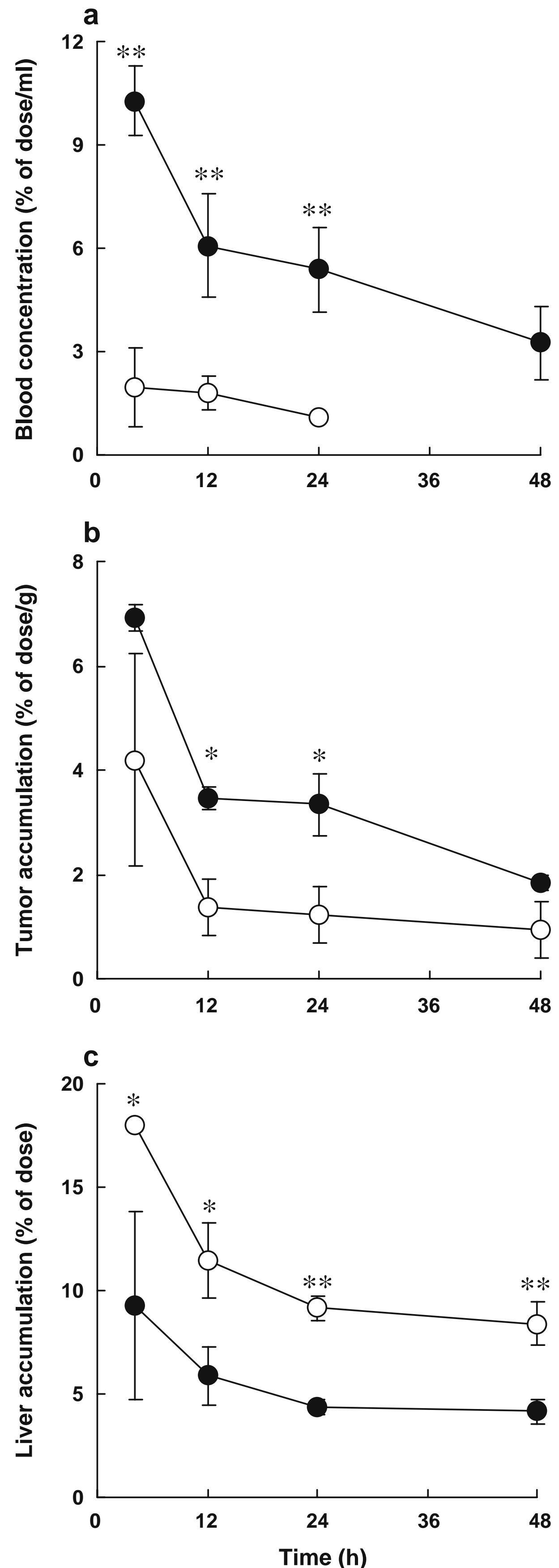

Fig. 2. Blood concentration (**a**), tumor accumulation (**b**), and liver accumulation (**c**) of [^{3}H]ATRA (*open circle*) and [^{3}H]ATRA incorporated in polymeric micelles (*filled circle*) following intravenous administration to tumor-bearing mice. Mice were intravenously administered with [^{3}H]ATRA dispersed in serum or [^{3}H]ATRA incorporated in polymeric micelles at 0.600 mg/kg dose of ATRA. At indicated time point, blood, tumor, and liver were collected and their radioactivity levels were measured. Each value represents the mean±SD of three experiments. Statistically significant differences compared with [^{3}H]ATRA (*double asterisk*, $P<0.01$; *single asterisk*, $P<0.05$).

lower than that of ATRA dispersed in serum (Fig. 2c) suggesting that polymeric micelles could reduce uptake by the liver, lead to retention in the blood and accumulate in the liver tissue. The accumulations of ATRA in lung, spleen, and kidney were very low and almost below the limit of detection within 4 h of injection (data not shown).

Anti-Tumor Effect of ATRA Incorporated in Polymeric Micelles in Tumor-Bearing Mice

The anti-tumor effect of ATRA was evaluated in mice bearing CT26 solid tumors by intravenous administration of saline (control), ATRA in HCO-60 micelles and ATRA in polymeric micelles at an ATRA dosage of 0.600 mg/kg/day or injection of 10 ml/kg/day every other day from day 3 to day 11. The body weight of mice ranged from 19 to 26 g regardless of the treatment group (data not shown). The results demonstrate that only ATRA in polymeric micelles delayed the growth of tumor lesions when compared with the controls treated with saline, while ATRA in HCO-60 micelles with the same administered dose of ATRA did not have any effect on tumor growth (Fig. 3). It was found that the tumor volume correlated with the survival of the mice and the mice died when the tumor reached around 4 cm^3. Following treatment with ATRA polymeric micelles, the survival time of tumor bearing mice was increased (median survival time=50 days) when compared with the control mice treated with saline (median survival time=29 days) and ATRA in HCO-60 micelles (median survival time=37 days; Fig. 4).

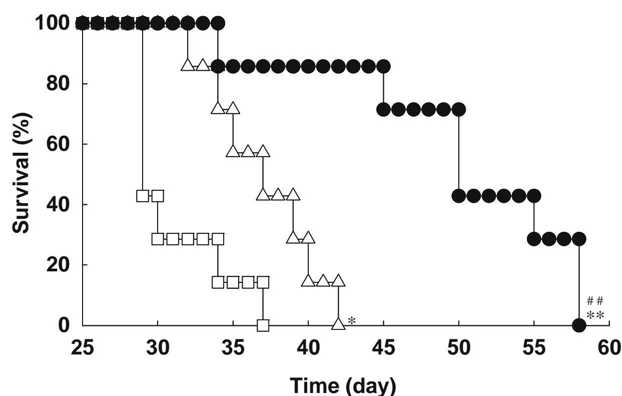

Fig. 4. Survival time of tumor-bearing mice after intravenous administration of saline (*open square*), ATRA in HCO-60 micelles (*open triangle*), and ATRA incorporated in polymeric micelles (*filled circle*) at 3, 5, 7, 9, and 11 days (total five doses administered) after tumor inoculation. Survival of mice was observed daily for 58 days and the percentage survival of each group (seven mice per group) is represented. Statistically significant differences compared with the administration of saline (*double asterisk*, $P<0.01$; *single asterisk*, $P<0.05$) and the administration of ATRA in HCO-60 micelles (*double pound sign*, $P<0.01$).

DISCUSSION

Polymeric micelles have attracted particular interest as a carrier for anti-tumor drugs, including ATRA, by enhancing drug retention in tumors by an EPR effect. Recently, Zuccari *et al.* (27) developed ATRA polymeric micelles by forming a complex of between ATRA and the modified polyvinylalcohol (PVA) substituted with oleylamine and showed enhanced cytotoxicity of the drug towards neuroblastoma cells compared with pure ATRA. Moreover, Jeong *et al.* developed a poly(ε-caprolactone)/poly(ethylene glycol) diblock copolymer for ATRA incorporation. They examined the ATRA incorporation into polymeric micelles and ATRA release and demonstrated that the ATRA incorporation efficacy is increased by the molecular weight of hydrophobic poly(ε-caprolactone) in the poly(ε-caprolactone)/poly(ethylene glycol) diblock copolymer (28). Although these studies showed the advantages of polymeric micelles as ATRA carriers, the *in vitro* studies were not enough to provide any information about their therapeutic effectiveness. In this regard, the present study was performed to evaluate the *in vivo* efficiency of ATRA incorporated in polymeric micelles for anticancer therapy.

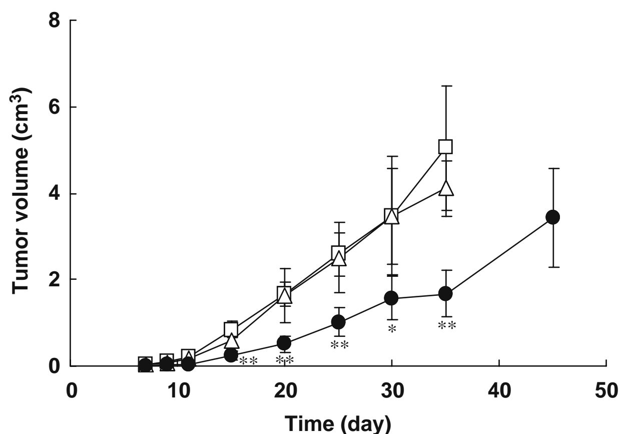

Fig. 3. The tumor volume of tumor-bearing mice after intravenous administration of saline (*open square*), ATRA in HCO-60 micelles (*open triangle*), and ATRA incorporated in polymeric micelles (*filled circle*) at 3, 5, 7, 9, and 11 days (total five doses administered) after tumor inoculation. Each value represents the mean±SD of seven mice. Statistically significant differences compared with the administration of saline (*double asterisk*, $P<0.01$; *single asterisk*, $P<0.05$).

Since ATRA is a highly lipophilic drug (log PC_{oct}=6.6) (29), it was stably incorporated in the lipophilic inner core of the polymeric micelles and was protected from elimination by a hydrophilic outer shell. Moreover, the particle size of the prepared ATRA polymeric micelles around 160 nm provides a system to reduce elimination by the RES, prolong the retention in blood vessels, and allow passage through the leaky vasculature into the interstitial space of the tumor tissue. Therefore, the anticancer activity of ATRA would be enhanced by the incorporation in polymeric micelles.

The biodistribution of the prepared [^{3}H]ATRA polymeric micelles and [^{3}H]ATRA dispersed in serum (30,31) which represents the inherent distribution of ATRA after intravenous administration into normal mice was examined in order to compare the distribution characteristics of ATRA. As shown in Fig. 1, [^{3}H]ATRA dispersed in serum was rapidly eliminated from the blood. In contrast, [^{3}H]ATRA was significantly retained in the blood circulation when ATRA was incorporated in polymeric micelles. These results lead us to believe that when ATRA was incorporated in polymeric micelles with 69% benzyl ester groups this resulted in sustained blood concentrations of ATRA.

Since the retention in the blood circulation would benefit the passive diffusion of the molecules allowing them to accumulate in the tumor tissue by the EPR effect (16), the distribution of [^{3}H]ATRA was also evaluated in mice bearing CT26 solid tumors. The distribution of [^{3}H]ATRA in tumor-bearing mice corresponded to that in normal mice in which the blood concentration and tumor accumulation of ATRA after administration with polymeric micelles was sustained whereas the intratumor concentration of [^{3}H]ATRA dispersed in serum fell rapidly in parallel with the blood concentration (Fig. 2a and b). In addition, the lower liver accumulation of ATRA polymeric micelles (Fig. 2c) suggested a reduction of ATRA taken up by the hepatocytes or RES that benefited the retention of ATRA in the blood. Our results in prolonging the blood concentration and enhancing tumor accumulation suggested that the EPR effect plays a role in this tumor accumulation.

To investigate whether polymeric micelle-enhanced ATRA accumulated in solid tumors can improve the anticancer activity of ATRA, the therapeutic efficiency of ATRA incorporated in polymeric micelles was evaluated in tumor-bearing mice. The delayed tumor growth produced by ATRA incorporated in polymeric micelles (Fig. 3) demonstrated that ATRA incorporated in polymeric micelles exhibited superior activity against tumors compared with the administration of saline or ATRA dispersed in HCO-60 micelles. The increased survival time of tumor-bearing mice treated with ATRA in polymeric micelles compared with other treatment groups (Fig. 4) confirmed the potential therapeutic efficacy of ATRA incorporated in polymeric micelles. Altogether, these results confirmed the therapeutic efficiency of the micellar structure of PEG-P(Asp) block copolymer with 69% benzyl ester groups as an effective delivery system of ATRA to solid tumors to exert its anti-cancer activity.

One of the most interesting features of polymeric micelles is the small particle size that blocks uptake by the RES and extravasation in the tumor tissue. However, the particle size of the partial polymeric micelles in the present study was larger than previously reported (22). This may be due to the increased concentration of the prepared ATRA polymeric micelles (from 1.667 to 3.333 mg/ml) that might induce temporary aggregation of the particles during storage. However, they were small enough to escape RES scavenging and enhanced the permeability and retention at the solid tumor site since their particle size remained below 200 nm (32).

ATRA has been widely used as a chemopreventive agent, which exerts strong anti-tumor activity by suppressing tumor growth (33,34). However, like many other anticancer drugs, sophisticated delivery and targeting is required for successful *in vivo* application. Apart from the benefit with regard to the EPR effect, ATRA polymeric micelles may be able to improve the outcome of APL disease since the high plasma concentration would alleviate the progressive decline in the plasma drug concentration after prolonged oral ATRA administration. Although further study is required, ATRA incorporated in polymeric micelles could be useful for cancer differentiation therapy, including the treatment of APL in the future.

In conclusion, we have examined the biodistribution characteristics of ATRA incorporated in polymeric micelles composed of PEG-P(Asp) with 69% esterification after intravenous administration. We have demonstrated that polymeric micelles prolonged the blood retention of ATRA, produced passive accumulation in the tumor site and resulted in superior therapeutic benefits of ATRA in mice with solid CT26 tumors.

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