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ORIGINAL ARTICLE

Investigation of anti-tumor effect of doxorubicin-loaded human serum albumin nanoparticles prepared by a desolvation technique

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Abstract

Purpose Nanoparticles of human serum albumin (HSA) prepared using a desolvation method possess sizes suitable for tumor accumulation. Here, we report on an investigation of the anti-tumor effects and biodistribution of doxorubicin–HSA nanoparticles in vitro and in vivo.

Methods The cytotoxicity of nanoparticles was evaluated in 2D and 3D colon 26 cell cultures. Furthermore, the biodistribution and the anti-tumor activity of nanoparticles in colon 26-bearing mice were investigated. Assessments on the effect on metastasis and the toxicity were also carried out.

Results Doxorubicin–HSA nanoparticles showed cytotoxicity in colon 26 cancer cell cultures, although the cytotoxicity was less in the case of nanoparticles than in free doxorubicin. In vivo anti-tumor activity was more pronounced in nanoparticles despite the fact that their accumulation in tumors was not superior to that of free doxorubicin, suggesting that factors other than accumulation contribute to the enhanced anti-tumor activity of these nanoparticles. The administration of nanoparticles also resulted in the suppression of metastasis.

Conclusions The prepared nanoparticles appear to be effective for cancer therapy although further studies will be needed to clarify the details of anti-tumor activity and the toxicity of these nanoparticles.

Keywords Human serum albumin · Nanoparticles · Desolvation · Doxorubicin

Introduction

The therapeutic use of nanoparticles as a drug carrier is an attractive approach especially for cancer therapy, since nanoparticles with a size below 200 nm are thought to accumulate in tumors due to the enhanced permeation and retention (EPR) effect [1–3]. The characteristics of these types of nanoparticles suggest that they could enhance therapeutic outcomes and reduce side-effects. Liposomes and polymeric micelles are well-known nanoparticulate carriers for anti-cancer drugs, and some of them are on the market or in the clinical trial stage of evaluation [4, 5]. Nanoparticles prepared using proteins such as albumin or gelatin has recently emerged as versatile carriers for anti-cancer drugs [6, 7]. Among these carriers, human serum albumin (HSA) is promising candidate for drug delivery because of its biodegradability and lack of toxicity and immunogenicity [6, 8]. A well-known example of this is albumin-bound paclitaxel nanoparticles (*nab*®-paclitaxel; Abraxane®) [9, 10]. In this case, in addition to the EPR effect, the accumulation of drugs in tumors via the active transport of HSA-bound drugs by an albumin receptor such as the 60-kDa glycoprotein (gp60) located on the endothelial cell surface is also thought to contribute to its efficacy [10].

As an alternate candidate, HSA nanoparticles prepared by a desolvation technique have been studied as carriers for anti-cancer drugs, since these nanoparticles have sizes suitable for EPR and the technique is simple [11–14]. Not only anti-cancer drugs (e.g., doxorubicin or paclitaxel) [14–16], but also other types of drugs (e.g., ganciclovir, atorvastatin, gabapentin, metformin, noscapine or piceatannol) [17–22]

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have been formulated based on this technique. Although numerous strategies have been evaluated for preparation [11–13, 23], in vivo studies regarding their efficacy using animals are limited. Furthermore, the efficacy of these nanoparticles in the context their pharmacokinetics have not been examined in detail.

In a previous study [14], we reported on the preparation of doxorubicin-loaded HSA nanoparticles with features amenable for the EPR effect (e.g., average size: 108 nm, slow release rate in plasma). However, information regarding their additional characteristics (e.g., anti-tumor effects or biodistribution and their relationship) is limited. In this study, we first investigated the efficacy of these particles in a colon carcinoma cell line, colon carcinoma 26. We also confirmed their therapeutic efficacy, toxicity and biodistribution in vivo using mice bearing colon 26 carcinoma tumors. Based on these results, we examined the issue of whether these nanoparticles would be suitably effective for treating cancer effectively.

Materials and methods

Materials

rHSA was donated by Nipro Corporation (Shiga, Japan) and, after receiving it, it was defatted using a charcoal treatment as described by Chen [24]. After dialysis against distilled water, the protein was freeze-dried and stored at − 20 °C until used. Doxorubicin (as hydrochloride) was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Glutaraldehyde and D-mannitol were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals obtained from commercial sources were of the highest grade.

Preparation of nanoparticles

Nanoparticles were prepared using a previously described desolvation technique [14]. Briefly, particles were generated by adding 3 mL of ethanol as a desolvating agent to 1 mL of a solution (pH 8.5) of HSA (20 mg) and doxorubicin (0.5 mg) with a syringe pump (flow rate 1 mL/min) under magnetic stirring (1500 rpm) at room temperature. The resulting particles were washed three times with 4-mL portions of ultrapure water. After washing, a sufficient amount of ultrapure water was added to give nanoparticles with an HSA concentration of 10 mg/mL. In addition, D-mannitol, an aggregation protectant, was dissolved in this sample at concentration of 3% (w/v) and the sample was then freeze-dried. Re-dispersed particles in ultrapure water showed mean particles size of 112 nm, a value comparable to the size of previously reported particles [14].

Cytotoxic effect of doxorubicin–HSA nanoparticles in 2D colon 26 cell cultures

Colon 26 carcinoma cells were maintained in RPMI-1640 (Wako Pure Chemical, Osaka, Japan) supplemented with 10% FBS containing 1% penicillin–streptomycin using a 5% CO_2/95% relative humidity incubator at 37 °C. To evaluate the cytotoxic effects of free doxorubicin and doxorubicin–HSA nanoparticles towards 2-dimensional (2D) colon 26 cell cultures, cells were seeded on a 96-well plate at a concentration of 1×10^4 cells per well and pre-incubated for 24 h. After the incubation, pre-determined amounts of doxorubicin or doxorubicin–HSA nanoparticles were added to give final drug concentrations of 1–10,000 ng/mL. In vitro cytotoxicity was determined using a cell counting kit-8 after a 72-h incubation and the 50% inhibitory concentration (IC_{50}) was calculated.

Cytotoxic effect of doxorubicin–HSA nanoparticles in 3D colon 26 cell cultures

Colon 26 cells were seeded on a 96-well spheroid microplate at a concentration of 2.5×10^3 cells per well and the plate was then centrifuged at 200 rpm for 5 min. After incubation for 3 days, pre-determined amounts of doxorubicin or doxorubicin–HSA nanoparticles were added to give final drug concentrations of 10–1000 ng/mL. The morphology of the spheroids was monitored using a BZ-8000 microscope (Keyence Corp., Osaka, Japan) 7 days after treatment. Cytotoxicity was also evaluated using an acid phosphatase assay (APH assay) kit and the IC_{50} was calculated.

Biodistribution of doxorubicin–HSA nanoparticles in colon 26-bearing mice

Five-week-old male Balb/c mice (Kyudo Co., Ltd, Saga, Japan) were housed in a room in which stable conditions of temperature and humidity were maintained with a standardized light/dark cycle. The animals were treated in accordance with the NIH guidelines. All animal experiments were reviewed and approved by the Animal Care and Use committee of Sojo University (Permit No. 2018-P-035). For tumor induction, mice were inoculated with colon 26 cells (1×10^6 cells) by subcutaneous injection into the dorsal skin. When tumors reached a diameter of approximately 10 mm, doxorubicin or doxorubicin–HSA nanoparticles were administered intravenously through tail vein. At the indicated time after the administration of doxorubicin or doxorubicin–HSA nanoparticles (dose: 5 mg/kg doxorubicin equivalent) into colon 26-bearing mice, the mice were killed, and tumors and organs were collected after perfusion with saline. Tissues were dissected,

weighed, PBS added (900 µL/100 mg tissue) for homogenization and 200 µL of the resulting homogenate was mixed with same amount of 2 M HCl followed by incubation at 50 °C for 90 min. After the addition of 700 µL of chloroform, the sample was incubated for 15 min, after which, 500 µL of a chloroform layer was added to the tube, and evaporated at 40 °C for 8 h. The residue was dissolved in acetonitrile/0.1 M acetate buffer, pH 5 (1:1), and the doxorubicin concentration was determined by HPLC, as described in a previous report [14].

In vivo anti-tumor activity of doxorubicin–HSA nanoparticles in colon 26-bearing mice

For tumor induction, mice were inoculated with colon 26 cells (1×10^6 cells) by subcutaneous injection into the dorsal skin. When the tumors reached a diameter of approximately 4–6 mm, doxorubicin or doxorubicin–HSA nanoparticles were administered intravenously through the tail vein (dose: 5 mg/kg doxorubicin equivalent). The tumor volume and body weight of the mice were measured during the study period. Tumor volume (V) was calculated as follows:

$$V = \frac{W^2 \times L}{2}, \quad (1)$$

where W and L are the width and length of tumor, respectively.

Effects of doxorubicin–HSA nanoparticles on lung metastasis which is known to occur in the colon 26 tumor model were also investigated according to the method of Suzuki et al. [25], with minor modifications. Briefly, after 28 days, the mice were killed and the lungs removed. The lungs were then rinsed in 0.9% NaCl solution and fixed for 24 h in acetone, to allow the numbers of macroscopic lung metastases to be determined.

Toxicity of doxorubicin–HSA nanoparticles in colon 26-bearing mice

Doxorubicin or doxorubicin–HSA nanoparticles (dose: 5 mg/kg doxorubicin equivalent) were administered into colon 26-bearing mice (see previous section). At 48 h after the administration, the mice were killed, blood samples were collected and hematology data evaluated (WBC; white blood cells and RBC; red blood cells) as well as data for liver, heart and kidney functions (*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CK* creatinine kinase, and *CRE* total creatinine levels).

Statistical data analysis

All data are expressed as the mean ± SD. Statistical analyses for multiple comparisons in the study were determined by the analysis of variance (two-way ANOVA). A probability value of $p < 0.05$ was considered to be significant.

Results

Cytotoxic effect of doxorubicin–HSA nanoparticles in colon 26 cell cultures

The cytotoxicity of nanoparticles was evaluated in 2D and 3D colon 26 cell cultures (Table 1 and Fig. 1). When nanoparticles were used, the IC_{50} value was significantly higher than that when free doxorubicin was used in 2D cell cultures (Table 1). The diameter of 3D spheroids at 7 days after the nanoparticle treatment was larger than those for the free doxorubicin treatment especially at higher doxorubicin concentrations (Fig. 1). Thus, the cytotoxicity of doxorubicin was decreased as the result of being incorporated in nanoparticles.

Biodistribution of doxorubicin–HSA nanoparticles

We further evaluated the biodistribution of doxorubicin in colon 26-bearing mice after the administration of free doxorubicin or doxorubicin–HSA nanoparticles (Fig. 2). In both administrations, a substantial fraction of doxorubicin was initially detected in the lungs, liver and spleen. The levels of doxorubicin in these organs after the administration of nanoparticles were higher than those after the administration of free doxorubicin, and were maintained for a longer period of time. Tumor accumulation that was detected when nanoparticles were administered was not superior to that when doxorubicin was administered alone. Furthermore, doxorubicin levels in the heart at 48 h and in the kidney at 96 h were higher in the case of nanoparticle administration compared to free doxorubicin administration. Doxorubicin levels in plasma were not significantly different for the administrations of nanoparticles and free doxorubicin.

Anti-tumor activity of doxorubicin–HSA nanoparticles

The anti-tumor activities of doxorubicin–HSA nanoparticles and free doxorubicin were also compared in colon 26-bearing mice (Fig. 3). Both nanoparticles and free doxorubicin

Table 1 IC_{50} values for free doxorubicin and doxorubicin–HSA nanoparticles against 2D colon 26 cell lines

	IC_{50} (ng/mL)
Free doxorubicin	246 ± 140
Doxorubicin–HSA nanoparticles	1117 ± 514

Values are expressed as the mean ± SD ($n = 3$)

*$p < 0.05$ vs. free doxorubicin

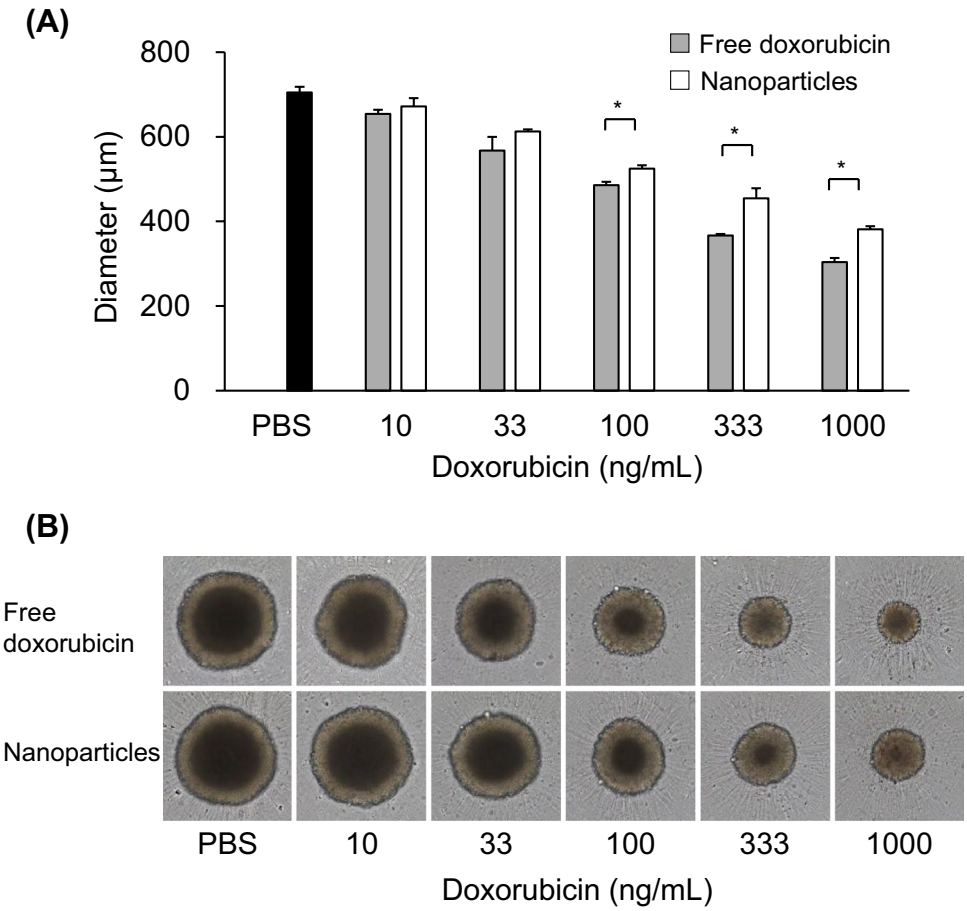

Fig. 1 Effects of doxorubicin and doxorubicin–HSA nanoparticles against 3D colon 26 spheroids at 7 days after treatment. Evaluations were carried out to monitor the diameters (**a**) measured from morphology of spheroids (**b**). Values are expressed as the mean ± SD ($n = 3$). *$p < 0.05$ vs. free doxorubicin

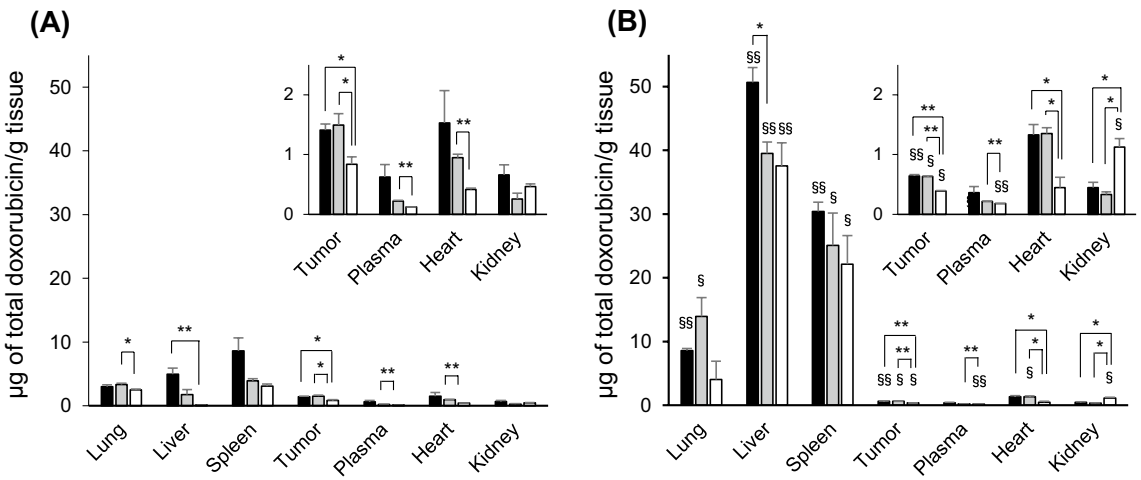

Fig. 2 Biodistribution of doxorubicin for the administration of free doxorubicin (**a**) and doxorubicin–HSA nanoparticles (**b**) to colon 26-bearing mice. Black, gray and white columns are data at 24, 48 and 96 h after administration, respectively. Values are expressed as the mean ± SE ($n = 3$). *$p < 0.05$, **$p < 0.01$ between times. $^{§}p < 0.05$, $^{§§}p < 0.01$ vs. free doxorubicin

were found to suppress tumor growth, and suppression by the nanoparticles was more pronounced than that by free doxorubicin. In both cases, no decreases in body weights were observed. Furthermore, the extent of reduction of metastasis in the lungs by nanoparticles was also remarkable when compared with control and free doxorubicin (Fig. 4).

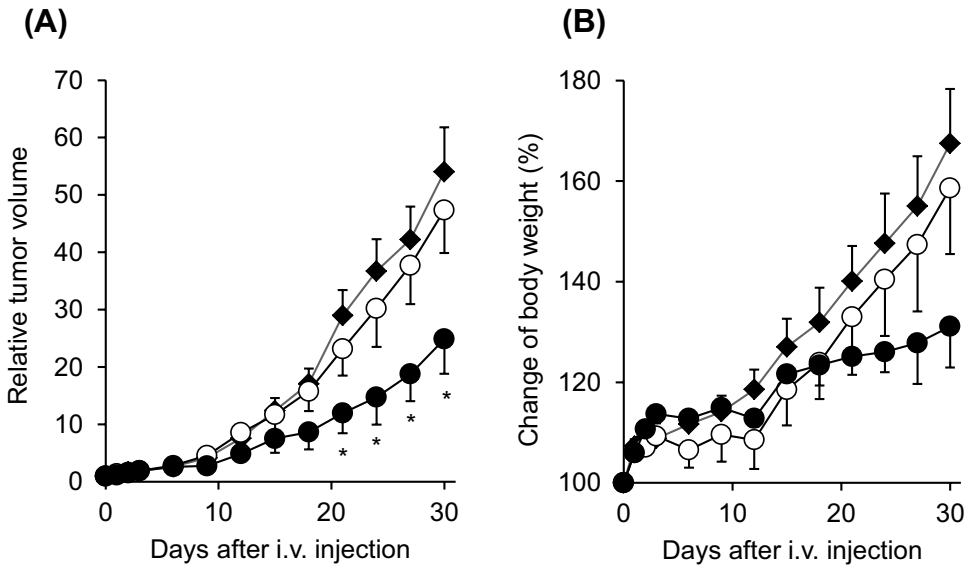

Fig. 3 Anti-tumor effects of doxorubicin–HSA nanoparticles against colon 26 tumor (**a**) and their effects on the body weight of tumor-bearing mice (**b**). Closed diamonds, open and closed circles are data for the administration of saline, free doxorubicin (5 mg/kg) and nanoparticles (5 mg/kg doxorubicin equivalent), respectively. Values are expressed as the mean ± SE ($n = 3$). *$p < 0.05$ vs. saline administration

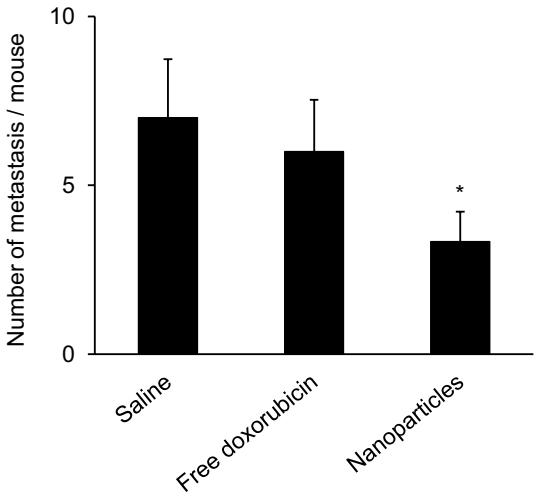

Fig. 4 Effects of free doxorubicin (5 mg/kg) and nanoparticles (5 mg/kg doxorubicin equivalent) against lung metastasis in colon 26 tumor-bearing mice. Values are expressed as the mean ± SD ($n = 3$). *$p < 0.05$ vs. saline and free doxorubicin

Toxicity of doxorubicin–HSA nanoparticles

Hematology data and data for liver, heart and kidney functions at 48 h after administration were also evaluated (Table 2). No apparent changes in the WBC and RBC counts were found after the administration of nanoparticles compared to those for the control and free doxorubicin. AST levels were increased slightly when nanoparticles were administered, while ALT was marginally increased when free doxorubicin was administered. CK levels were increased in the case of the administration of both free doxorubicin and nanoparticles. CRE was slightly increased in the case of the administration of free doxorubicin.

Discussion

HSA has recently been recognized as a versatile carrier that enhances the pharmacokinetic profiles of drugs or for drug targeting [26, 27], since its properties include a long half-life (about 19 days) and accumulation in tumor tissue. Among the products using HSA as a drug carrier, nanoparticles prepared by a simple desolvation technique is one of

Table 2 Effects of free doxorubicin and doxorubicin–HSA nanoparticles on functional parameters associated with hematology, liver, heart and kidney

	WBC (10^2/μL)	RBC (10^4/μL)	AST (IU/L)	ALT (IU/L)	CK (IU/L)	CRE (mg/dL)
Saline	61.3 ± 4.8	891.0 ± 10.6	59.3 ± 4.4	31.7 ± 1.8	78.7 ± 7.0	0.08 ± 0.01
Free doxorubicin	70.0 ± 1.5	849.7 ± 6.5	65.3 ± 4.1	43.0 ± 0.6	163.3 ± 50.8	0.13 ± 0.01*
Nanoparticles	82.3 ± 15.0	864.3 ± 20.3	81.0 ± 8.5	37.0 ± 7.9	175.7 ± 52.3	0.10 ± 0.01

Values are expressed as the mean ± SE ($n = 3$)

*$p < 0.05$ vs. saline

the promising candidates for the delivery of several types of drugs [14–22]. Using this technique, we previously prepared doxorubicin–HSA nanoparticles with characteristics that were suitable for drug delivery to target tumors (mean particle size: 108 nm, slow drug release rate in plasma) [14]. Researchers have now prepared other similar doxorubicin–HSA nanoparticles and confirmed their in vivo efficacy on glioblastoma [28] or liver cancer [16]. In the present study, we investigated the efficacy of such nanoparticles on colon carcinomas, and their biodistribution and toxicity.

The doxorubicin–HSA nanoparticles that were prepared herein showed cytotoxicity against 2D and 3D colon 26 cell cultures (Table 1 and Fig. 1), but the degree of cytotoxicity was weaker than that of free doxorubicin. Similar findings were observed in MCF7 and HepG2 2D cell cultures [14]. Thus, nanoparticulation using a desolvation technique was found to attenuate the cytotoxicity of doxorubicin against cancer cells. In our previous report, fluorescent confocal laser microscopy was used to examine the intracellular uptake of doxorubicin–HSA nanoparticles in MCF7 2D cell cultures [14]. Signals corresponding to doxorubicin were co-localized in non-nuclear regions inside the cell, and the doxorubicin fluorescence intensity was significantly increased at the cell nucleus and decreased in nanoparticles with passing time. These findings suggest that the cytotoxicity of doxorubicin–HSA nanoparticles against cancer cells was accompanied by the intracellular uptake of nanoparticles followed by the release of doxorubicin from the nanoparticles and its subsequent accumulation in the nucleus. Unlike 2D cultures in the form of monolayers, 3D cultures such as multicellular tumor spheroids are known to possess an established extracellular matrix which is similar to the microenvironment in hypovascular or avascular regions of solid tumors [29, 30]. Therefore, tumor spheroids are recently recognized as a more useful and valid model for evaluating the efficacy of chemotherapy than when 2D cultures are used [31–34]. The cytotoxicity of doxorubicin–HSA nanoparticles against 3D colon 26 cells may more closely reflect the in vivo efficacy of nanoparticles.

Although the sizes of the doxorubicin–HSA nanoparticles prepared in the present study were small (112 nm) and optimum for accumulation by tumors through the EPR effect, the tumor accumulation of these nanoparticles was not superior to that for free doxorubicin. Instead, a substantial fraction of doxorubicin was detected in the lungs, liver and spleen after the administration of nanoparticles (Fig. 2). Nevertheless, the nanoparticles showed surprising level of in vivo anti-tumor activity compared to free doxorubicin (Fig. 3). Polyak et al., using technetium (Tc-99m)-labeled-doxorubicin-loaded albumin nanoparticles (diameter 176 nm), reported that 73% of the radioactivity was detected in the liver at 5 min after their administration to healthy rats and more than 50% of the radioactivity remained in the body, even after 22 h [35]. Miao et al. indicated that the in vivo anti-tumor effect of doxorubicin–albumin nanoparticles (diameter 170 nm) against mice that had been inoculated with liver cancer cells were superior to that of doxorubicin alone [16]. Thus, as of this writing, the anti-tumor and biodistribution profiles of doxorubicin-loaded albumin nanoparticles produced by desolvation have been separately investigated in vivo. Since the in vivo anti-tumor effects of the doxorubicin–HSA nanoparticles prepared in present study cannot be completely explained by their ability to allow doxorubicin to accumulate in tumors, other factors that enhance the anti-tumor activity also need to be considered.

Ponta et al. investigated the biodistribution and anti-tumor activity of doxorubicin-loaded block copolymer micelles (size 54–117 nm) in lung cancer A549-bearing mice [36]. They reported that micelles that released doxorubicin slowly showed the most effective anti-tumor activity. In their study, no significant differences in tumor accumulation were observed for free doxorubicin and doxorubicin-loaded micelles. Based on these results, they proposed an intriguing mechanism in which micelles fail to deliver the drug into the core of the tumor, while doxorubicin that accumulates in peripheral areas of tumors is released, resulting in the doxorubicin concentration in tumors to be maintained above the therapeutic levels. Thus, a continuous supply of nanoparticles and/or doxorubicin from reservoir organs such as the liver may also contribute to the in vivo anti-tumor activity of the nanoparticles prepared in this study although their accumulation in tumors was not superior to that for free doxorubicin within 96 h after administration. Soma et al. proposed an alternate mechanism for the anti-tumor effect of doxorubicin-loaded nanoparticles [37]. They proposed that macrophages, which entrap nanoparticles may release cytotoxic factors like nitric oxide, superoxide, hydrogen peroxide and/or TNF-α in response to macrophage activation, thus resulting in the development of tumoricidal effects. The findings reported herein serve to confirm that the doxorubicin-free HSA nanoparticles have no cytotoxic effects in colon 26 cell cultures. Miao et al. also reported that doxorubicin-free albumin nanoparticles prepared by desolvation do not show in vivo tumor inhibition [16]. However, we did not confirm the in vivo tumor inhibition of doxorubicin-free HSA nanoparticles in present study. Therefore, additional studies will be needed to confirm whether such indirect effects of doxorubicin–HSA nanoparticles could contribute to the anti-tumor activity in the case of the nanoparticles prepared in this study.

Metastasis and toxicity are major causes of therapeutic failure in cancer chemotherapy. Doxorubicin–HSA nanoparticles were found to suppress, not only the growth of colon

26 cells implanted in the dorsal skin of mice, but also metastasis to the lungs (Fig. 4). In addition to the direct effects of nanoparticles on the growth of metastatic lung cancer, inhibiting the growth of the primary tumor by nanoparticles is also considered to be a cause for the suppression of lung metastasis as suggested in other studies [38, 39]. After the administration of doxorubicin–HSA nanoparticles, changes in ALT and CRE, indicative of hepatic and renal injuries were not indicated in spite of significant accumulation in the liver and kidney (Table 2). Furthermore, changes in parameters regarding hematotoxicity (WBC and RBC) were also not observed as the result of the administration of nanoparticles. It is well known that cardiotoxicity such as cardiomyopathy and congestive heart failure is dose-limiting for doxorubicin [40]. A number of nanoparticles such as liposomes have been shown to decrease this toxicity [41]. We monitored CK and AST levels as markers of cardiotoxicity. Increases in CK levels as the result of the administration of free doxorubicin and nanoparticles and an increase in AST levels as the result of the administration of nanoparticles suggested that nanoparticles may not possess suppressive effects on the cardiotoxicity of doxorubicin. Using healthy rats, Pereverzeva et al. suggested that doxorubicin–HSA nanoparticles (diameter: 404 nm) which were prepared by a desolvation method reduces the cardio- and testicular toxicities observed in the use of free doxorubicin [42]. They monitored electrocardiographic parameters to evaluate cardiotoxicity at 15 or 30 days after administration (our monitoring: 48 h after administration). Thus, the doxorubicin–HSA nanoparticles prepared in the present study can be considered to be effective in cancer therapy. However, further detailed studies to clarify the mechanism of their anti-tumor effects and their long-term toxicity should be conducted. Based on such accumulated data, modification of nanoparticle formulations such as pegylation [43] for achieving more effective chemotherapy will be possible in the future.

Conclusions

The findings presented herein indicate that doxorubicin–HSA nanoparticles prepared by a desolvation method possess anti-tumor activity in vitro and in vivo. In vivo anti-tumor activity was more pronounced in nanoparticles, despite the fact that the tumor accumulation of nanoparticles was not superior to that of free doxorubicin, suggesting that factors other than accumulation are responsible for enhancing the anti-tumor activity of nanoparticles. The nanoparticles also appeared to suppress metastasis. Thus, the nanoparticles prepared in this study are considered to be promising candidates for use in cancer therapy although further studies will need to be carried out to clarify the details of the anti-tumor activity and toxicity of such nanoparticles. Such detailed studies will provide useful information for developing more effective and safer nanoparticle formulation using HSA for chemotherapy.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving animal experiments were in accordance with the ethical standards of Animal Care and Use committee of Sojo University. This article does not contain any studies with human participants.

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