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Angiotensin II-induced hypertension enhanced therapeutic efficacy of liposomal doxorubicin in tumor-bearing mice

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ABSTRACT

In this study, we investigated whether the therapeutic efficacy of liposomal doxorubicin (DXR-SL) could be enhanced by angiotensin II (AT)-induced hypertension. AT-induced hypertension increased the volume of tumor blood flow in mice bearing a poorly vascularized Lewis lung carcinoma (LLC) tumor, but only slightly in mice bearing a well-vascularized colon carcinoma Colon 26 (C26) tumor. In therapeutic efficacy, AT-induced hypertension enhanced the antitumor activity of DXR-SL in mice bearing LLC and C26 tumors. Localization of DXR-SL after injection by AT-induced hypertension was observed outside tumor blood vessels in LLC and C26 tumors, but within them under the normotension. From these findings, AT-induced hypertension had potential to improve the delivery of DXR-SL to both well- and poorly vascularized solid tumors.

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1. Introduction

Cancer chemotherapeutic agents are in many cases administered systemically. The effectiveness of cancer therapy in solid tumors depends on adequate delivery of the therapeutic agent to tumor cells. Tumor blood supply plays an important role in the delivery of therapeutic agent to solid tumors, and tumor blood flow affects drug transport through the vascular space in a tumor. Many researchers have tried to modify tumor blood flow with pharmacological agents. Some catecholamines, including noradrenaline, adrenaline and isoproterenol, have been examined extensively, and are known to decrease tumor blood flow in general (Hori et al., 1993; Jirtle, 1988). Whereas a vasoactive peptide, angiotensin II (AT), has been shown to increase tumor blood flow (Burton et al., 1985; Trotter et al., 1991; Zlotecki et al., 1993; Tozer and Shaffi, 1993; Li et al., 1993; Suzuki et al., 1981).

AT, an octapeptide hormone, has direct vasoconstricting action, particularly in arterioles of normal tissues, and hence increases peripheral resistance with elevation of arterial blood pressure (Tozer and Shaffi, 1993). It is a potent arteriolar vasoconstrictor which acts by binding to specific receptors located on smooth muscle cells. It is generally supposed that the poorly differentiated blood vessels of tumors would be lacking in such structures. Therefore, the rationale for using AT is that vasoconstriction in most normal tissues would increase perfusion pressure to the tumor with little or no direct vasoconstriction of the blood vessels supplying the tumor. The consequence would be an increase in blood flow to the tumor.

Induced hypertension chemotherapy with intravenously infused AT achieved successful results experimentally and clinically (Fujii et al., 1991; Nagamitsu et al., 2009). AT-induced hypertension selectively enhanced the delivery of 5-fluorouracil (5-FU) in mice bearing mouse sarcoma 180 tumor (Okamoto et al., 1988), and also increased the antitumor effect by combination therapy with 5-FU, doxorubicin and mitomycin C in clinical trials for gastric cancer (Sato et al., 1995), advanced carcinoma of the stomach (Nakamura et al., 1992) and advanced gastrointestinal, pancreatic and hepatobiliary carcinoma (Hoshi and Sato, 1995). Based on these reports, it appears that AT-induced hypertension will yield better results for macromolecular drugs because these will not traverse freely through blood capillaries from the interstitial space of a tumor. The enhanced permeability and retention (EPR) effect of macromolecular agents such as polymers will be more prominent under AT-induced hypertension than under normontension. Indeed, it has already been reported that AT could improve the delivery of a macromolecular drug, SMANCS, which is a conjugate of the protein antitumor antibiotic neocarzinostatin with a hydrophobic copolymer of styrene/maleic acid, to the tumor (Maeda, 2001; Nagamitsu et al., 2009).

Liposomes have been extensively investigated as carriers for a variety of anticancer drugs. Due to the long circulation time of PEGylated liposomes and the leakiness of the microcirculation in solid tumors, the liposome accumulated preferentially in the tumors by the EPR effect. Similar to polymers, AT-induced hypertension is expected to improve liposome delivery into solid tumors by increasing tumor blood flow. To our knowledge, this is the first

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report to apply AT-induced hypertension to liposomal drug delivery. In this study, we investigated whether PEGylated liposomal doxorubicin (DXR-SL) could enhance therapeutic efficacy by AT-induced hypertension in mice bearing a poorly vascularized Lewis lung carcinoma (LLC) tumor and well-vascularized colon carcinoma Colon 26 (C26) tumor.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) and methoxy-poly (ethyleneglycol)-distearylphosphatidylethanolamine (PEG-DSPE, PEG mean molecular weight, 2000) were purchased from NOF Inc. (Tokyo, Japan). Cholesterol (Chol), doxorubicin hydrochloride (DXR) and AT were purchased from Wako Pure Chemical Industries Inc. (Osaka, Japan). All other chemicals were of the finest grade available.

2.2. Cell culture

C26 and LLC cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were cultured in RPMI-1640 medium with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotecin B in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Blood pressure measurement

Female ddY mice (Sankyo Lab. Service Corp., Tokyo, Japan) were anesthetized with 1.5% isoflurane by inhaled air on a heated stage at 37 °C, and blood pressures were measured by the tail cuff method (BP-98A, Softron, Tokyo, Japan). The mouse body was kept fixed at 37 °C by keeping the mice on a heating stage throughout the experiment because anesthesia causes a decrease in body temperature, which may result in considerable deformation of the pharmacokinetic profile. AT was diluted in buffer containing 0.1 mg/mL gelatin and 0.2 mg/mL citric acid in saline, pH 5.0, and adjusted to 2.4 μg/mL. AT was continuously infused into the tail vein at a rate of 2 μg/kg/min with an infusion pump (Model TE-361; Terumo Co., Tokyo, Japan), and blood pressures (systolic blood pressure, SBP; mean blood pressure, MBP; diastolic blood pressure, DBP) were measured for 30 min.

2.4. Preparation of liposomes

Liposome was prepared from DSPC/Chol/PEG-DSPE at a molar ratio of 55/45/5 by a dry-film method, as previously reported (Yamada et al., 2008). Briefly, all lipids were dissolved in chloroform, which was removed by evaporation. The thin film was hydrated with 300 mM citrate buffer adjusted to pH 4.0 with NaOH at 60 °C by vortex mixing and sonication. Extraliposomal pH was adjusted to 7.4 with NaOH before DXR loading according to the pH gradient method. Particle sizes of the liposome were determined by the dynamic light scattering method (ELS-Z2; Otsuka Electronics, Osaka, Japan) at 25 °C after diluting the liposome suspension with water. DXR was added to the liposome at a DXR to total lipid weight ratio of 0.2:1.0, and incubated at 60 °C for 25 min for drug loading. Unencapsulated DXR was removed using a Sephadex G-50 column eluted with saline. DXR concentration was determined by measuring absorbance at 480 nm (V-1700 Pharmaspec; Shimadzu, Kyoto, Japan) after the addition of a large volume of methanol, and then the loading efficiency of DXR in liposomal DXR (DXR-SL) was calculated. The concentration of phospholipid (DSPC) was measured with the Phospholipids *C*-test Wako (Wako Pure Chemical Industries, Ltd.). For preparation of rhodamine-labeled PEGylated liposome (SL), *N*-(Lissamine Rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rhodamine-DHPE; Invitrogen, Carlsbad, CA, USA) was incorporated at 1 mol% into the total lipid.

2.5. Tumor distribution of Hochechest 33342

To generate Colon 26 and LLC tumors, 1×10^6 cells suspended in 100 μL RPMI-1640 medium were inoculated subcutaneously into the flank of female CDF1 and C57BL/6Cr mice, respectively (5 weeks of age; Sankyo Lab. Service Corp.). The tumor volume was calculated using the formula; tumor volume = $0.5 \times a \times b^2$, where a and b are the larger and smaller diameters, respectively. When the average volume of the tumors reached 100–200 mm^3, AT was continuously infused at a rate of 2 μg/kg/min and maintained for 5 min. For detection of tumor vessels with blood flow, fluorescent DNA-binding dye Hoechst 33342 was intravenously injected at 25 mg/kg under AT-induced hypertension and mice were sacrificed 1 min after the injection. The tumor was frozen on dry ice and sliced at 20 μm.

For detection of endothelial cells by immunostaining, antibody against CD31 (rat anti-mouse CD31 monoclonal antibody, clone MEC 13.3; BD Biosciences, CA, USA) was used to identify endothelial cells with Alexa 488-labeled goat anti-rat IgG (Invitrogen) as the secondary antibody. The localizations of CD31 and Hoechst 33342 were examined using an ECLIPSE TS100 microscope (Nikon, Tokyo, Japan) as previously reported (Minowa et al., 2009; Yamada et al., 2008).

2.6. In vivo therapeutic studies

When the average volume of the tumors reached 100–200 mm^3, DXR-SL was intravenously administered *via* lateral tail veins at a dose of 5 mg DXR/kg into mice bearing C26 and LLC tumors. For AT-induced hypertension, AT was continuously infused at a rate of 2 μg/kg/min immediately after injection of DXR-SL and maintained for 25 min. Tumor volume and body weights were measured for individual animals. Animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Hoshi University.

2.7. Biodistribution of DXR

When the average volume of the tumors reached 100–200 mm^3, DXR-SL was intravenously administered *via* lateral tail veins at a dose of 5 mg DXR/kg. For AT-induced hypertension, AT was continuously infused at a rate of 2 μg/kg/min to reach mean blood pressure of 100 mmHg immediately after the injection of DXR-SL and maintained at 100 mmHg for 25 min. For measurement of DXR concentration in tissues, tissues were excised 24 h after injection, and then homogenized in 0.1 M NH_4Cl/NH_3 buffer (pH 9.0). DXR was extracted with chloroform/methanol (2:1 v/v) and analyzed by HPLC, as previously described (Yamada et al., 2008).

2.8. Tumor accumulation

When the average volume of the tumors reached 100–200 mm^3, DXR-SL was intravenously administered *via* lateral tail veins at a dose of 5 mg DXR/kg and 24.4 mg total lipid/kg. For AT-induced hypertension, AT was continuously infused at a rate of 2 μg/kg/min immediately after the injection of DXR-SL and maintained for 25 min. For localization of DXR after administration of DXR-SL into the tumor by microscopy, Hoechst 33342 was intravenously injected to stain tumor blood vessels at 24 h after injection of DXR-SL and then mice were sacrificed.

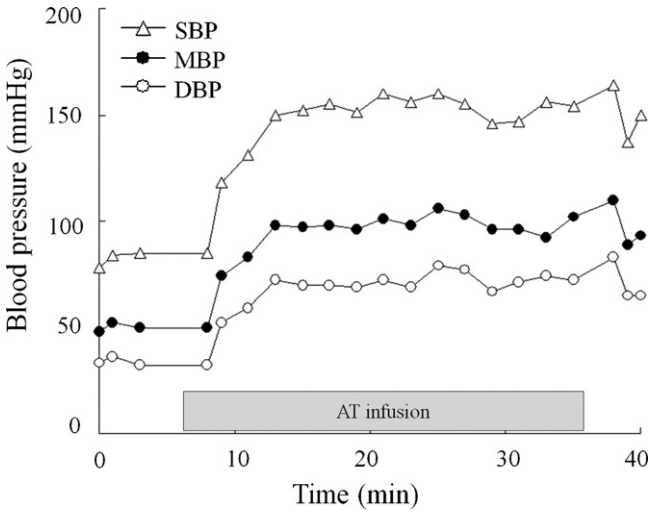

Fig. 1. Representative tracings of blood pressure by tail-cuff method under anesthesia in mice during infusion of AT for 30 min at a dose of 2 μg/kg/min. Infusion of AT was started at 5 min after tracking blood pressure and was maintained for 30 min. Systolic blood pressure, SBP; mean blood pressure, MBP; diastolic blood pressure, DBP.

For liposome localization after the administration of rhodamine-labeled SL, rhodamine-labeled SL was intravenously administered at a dose of 24.4 mg total lipid/kg. Immediately after injection of the liposome, AT-induced hypertension was performed as described above. Twenty-four hours after injection, Hoechst 33342 was intravenously injected for detection of tumor blood flow and then mice were sacrificed. The tumors were frozen on dry ice and sliced at 20 μm. The localizations of rhodamine-labeled SL, DXR and Hoechst 33342 were examined using an ECLIPSE TS100 microscope as described above.

2.9. Statistical analysis

Significant differences in the mean values were evaluated by Student's unpaired *t*-test. A *p* value of 0.05 or less was considered significant.

3. Results

3.1. Change of blood pressure with AT

We measured blood pressure changes with AT infusion at a dose of 2 μg/kg/min. Systolic blood pressure (SBP), mean blood pressure (MBP) and diastolic blood pressure (DBP) under anesthesia were approximately 80, 50 and 30 mmHg, respectively (Fig. 1). After infusion of AT, blood pressure was elevated to about 200% within 5 min (150, 100 and 70 mmHg in SBP, MBP and DBP, respectively), and was maintained at a high level until the end of AT infusion. Infusion of AT at doses of more than 2 μg/kg/min did not further increase blood pressure compared with that at a dose of 2 μg/kg/min. Therefore, a dose of 2 μg/kg/min AT was adopted in further experiments.

3.2. Hoechst dye perfusion in C26 and LLC tumors

Here, we used two types of mice bearing C26 and LLC tumors for comparison of therapeutic efficacy for the different vascu-

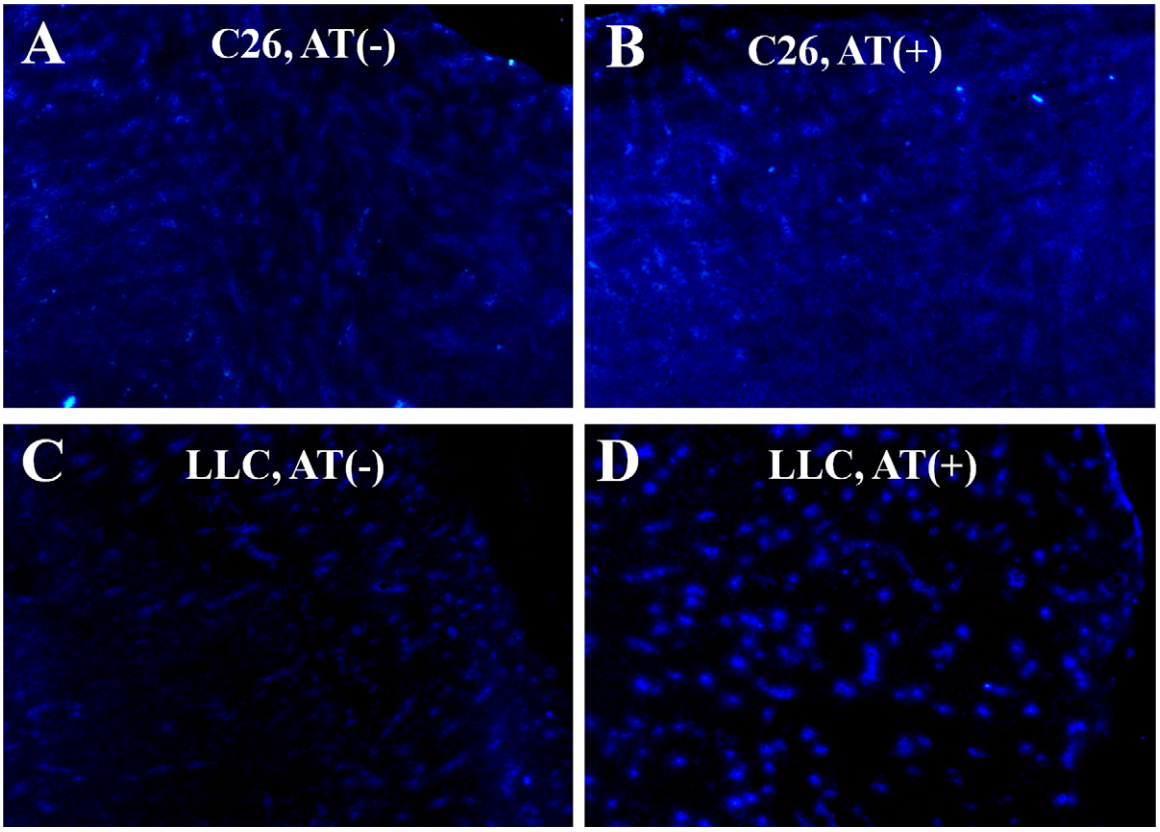

Fig. 2. Increased amount of fluorescent DNA-binding dye Hoechst 33342 in C26 (A and B) and LLC tumors (C and D) by AT-induced hypertension. C26 (A and B) and LLC tumors (C and D) were harvested 1 min after infusion of saline as a control (A and C) or AT (B and D) for 5 min, followed by injection of Hoechst 33342. Blue indicates tumor vessels with blood flow.

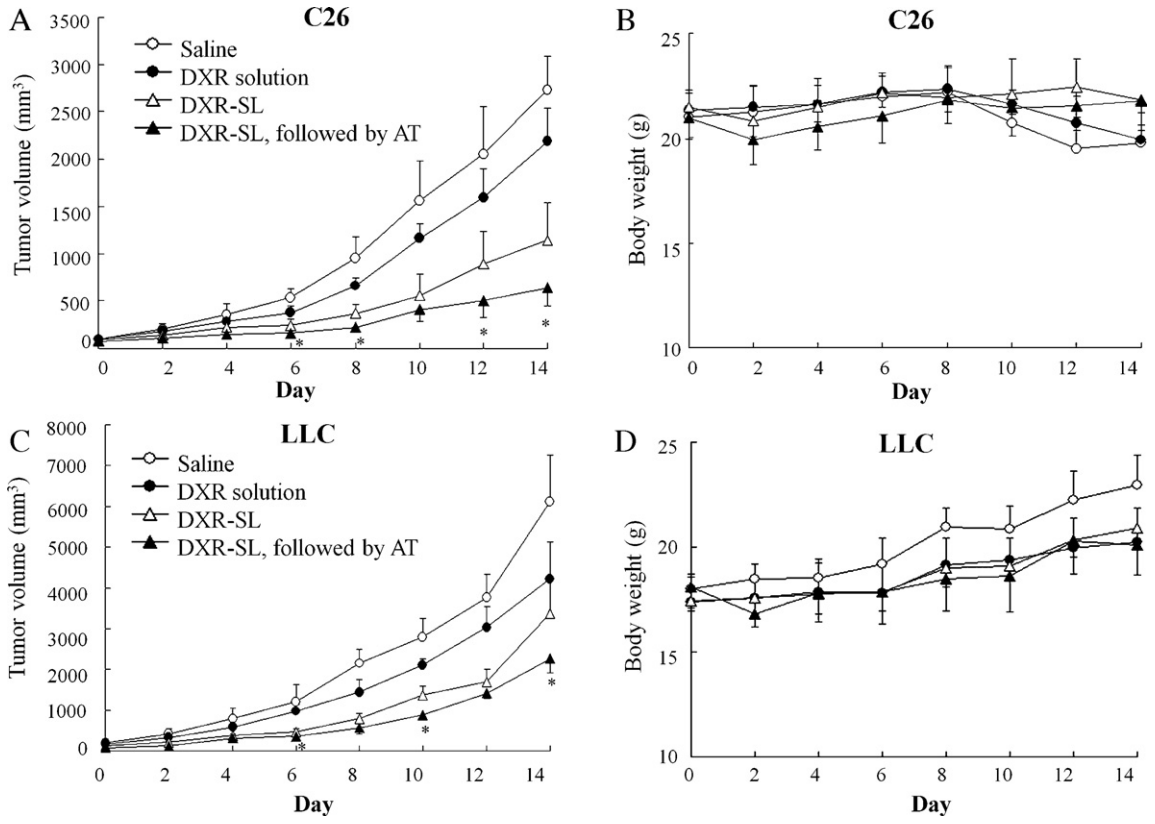

Fig. 3. *In vivo* anti-tumor effects of DXR-SL (5 mg DXR/kg) by AT-induced hypertension for C26 (A and B) and LLC (C and D) tumor-bearing mice. Each value represents the mean ± S.D. (n = 6 in A and B, n = 4 in C and D). Tumor size (A and C) and body weight change (B and D) were measured after single administration of DXR-SL. $^{*}p < 0.05$; compared with mice injected with DXR-SL under normotension.

larity of tumor blood vessels. It has been reported that C26 and LLC tumors had well- and poorly vascularized blood vessels, respectively (Ogawara et al., 2008), and LLC tumor had severely hypoxic regions throughout the tumor volume due to poor vascularity (Ziemer et al., 2005). To confirm the increase of tumor blood flow under AT-induced hypertension, Hoechest 33342 was intravenously injected under hypertension in mice bearing C26 and LLC tumors (Fig. 2). In C26 tumors, the fluorescence of Hoechst 33342 was slightly increased in the tumor by infusion of AT (Fig. 2A and B), whereas in LLC tumor, higher fluorescence was detected in tumors by infusion, indicating that AT-induced hypertension could improve blood flow in even to the poorly vascularized tumors (Fig. 2C and D). We confirmed that the localization of Hoechst 33342 signals corresponded to those of CD31 immunostaining as a marker of endothelial cells (Supplemental Fig. S1).

3.3. Antitumor effect on C26 and LLC tumors

In a preliminary study, we injected DXR solution at a dose of 5 mg/kg into mice bearing a C26 tumor under AT-induced hypertension and maintained high blood pressure for 25 min, as previously reported (Okamoto et al., 1988). However, the accumulation and distribution of DXR in tumor could not be increased by infusion of AT (Supplemental Fig. S2A and B); therefore, we decided to use liposomal DXR (DXR-SL) and modified the schedule of drug administration under AT-induced hypertension; DXR-SL administration, followed by AT-induced hypertension, because DXR-SL can circulate longer in blood compared with free DXR. After injection of DXR-SL, we immediately infused AT for 25 min, and investigated whether AT-induced hypertension could increase antitumor activity by DXR-SL in mice bearing C26 and LLC tumors (Fig. 3A and C). In mice bearing C26 and LLC tumors, a single administration of DXR-SL, followed by hypertension, significantly enhanced the suppression of tumor growth compared with that in normotension (Fig. 3A and C). The percentage of tumor growth inhibition (T/C%), which represents the mean difference (%) in tumor size for treated tumors (T) compared with control tumors (C), was calculated from relative tumor volume at day 14 in C26 and LLC tumors. The calculated T/C treated with DXR-SL and DXR-SL, followed by AT-induced hypertension, was 42 and 23% in C26 tumor and 55 and 37% in LLC tumor, respectively. Moreover, no tendency of weight loss was seen after intravenous injection of DXR-SL, followed by AT-induced hypertension (Fig. 3B and D).

3.4. Localization of DXR and liposome in tumor

Next, we investigated whether the accumulation of DXR-SL in tumor was increased by AT-induced hypertension. Surprisingly, the accumulation of DXR by DXR-SL in C26 and LLC tumors was not affected by AT-induced hypertension (Supplemental Fig. S3A and B). In normal organs of mice bearing C26 and LLC tumors, DXR distribution by DXR-SL, followed by hypertension, was the same as under normotension (Supplemental Fig. S3A and B). Therefore, to confirm the distributions of DXR and liposome in tumors after intravenous injection of DXR-SL, we administrated DXR-SL or rhodamine-labeled SL in mice bearing C26 and LLC tumors. Under normotension, the fluorescence of DXR and rhodamine-labeled SL was observed within area stained with Hoechst 33342 in C26 (Figs. 4A and 5A) and LLC tumors (Figs. 4C and 5C). In contrast, by AT-induced hypertension, the fluorescence of both DXR and rhodamine-labeled SL was strongly observed outside the

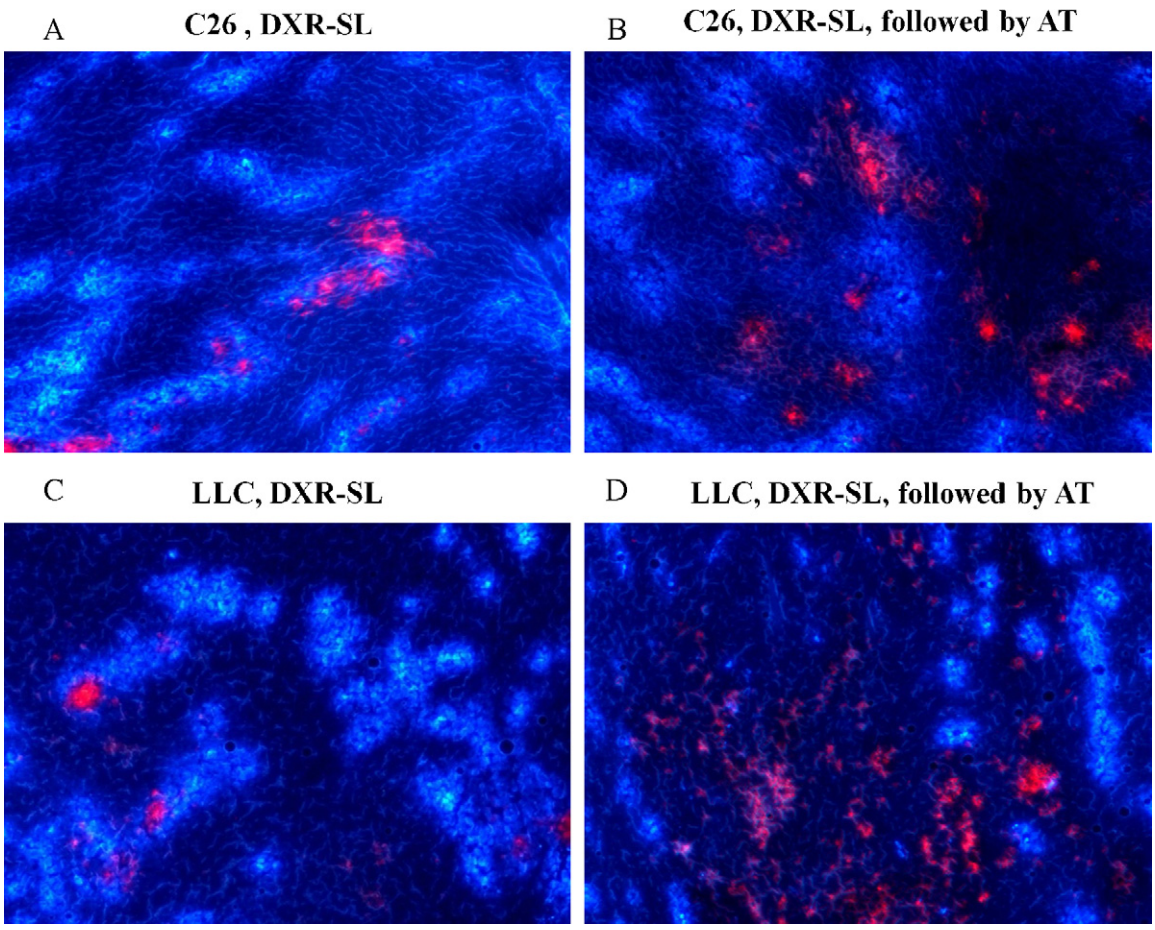

Fig. 4. Tumor accumulation of DXR 24 h after intravenous administration of DXR-SL (5 mg DXR/kg) into C26 (A and B) and LLC tumor-bearing mice (C and D) under normotension (A and C) or followed by AT-induced hypertension (B and D). Hoechst 33342 was injected into the tail vein 1 min before sacrifice. Localization of DXR is shown in red and tumor blood flow in blue. Magnification, 100×.

area stained with Hoechst 33342 in C26 (Figs. 4B and 5B) and LLC tumors (Figs. 4D and 5D). These findings suggested that AT-induced hypertension affected the distribution of DXR-SL in the tumors.

4. Discussion

Here, we demonstrated that AT-induced hypertension increased therapeutic efficacy by improving liposomal delivery within tumors. C26 and LLC tumors were used as models of well- and poorly vascularized tumors, respectively, according to previous reports (Ogawara et al., 2008; Ziemer et al., 2005; Li et al., 1993). It has been reported that liposomal DXR did not result in efficient DXR delivery to LLC tumors under normotensive state (Parr et al., 1997). Since the delivery of DXR-SL is thought to exploit well-vascularized tumors with hyperpermeable vasculature, AT-induced hypertension is expected to improve the accumulation of DXR-SL by increasing tumor blood flow in poorly vascularized tumors, such as LLC tumors; however, the accumulation of DXR-SL in C26 and LLC tumors was not affected (Supplemental Fig. S3). In contrast to drug accumulation, significantly higher antitumor effects by DXR-SL, followed by AT-induced hypertension *in vivo*, were observed in both mice bearing C26 and LLC tumors. In the administration of DXR-SL or rhodamine-labeled liposome, followed by AT-induced hypertension, the localizations of DXR and liposome were strongly observed outside the area stained with Hoechst 33342 in C26 (Figs. 4B and 5B) and LLC tumors (Figs. 4D and 5D), indicating that AT-induced hypertension might increase antitumor activity by pushing DXR-SL out into the tumor vasculature. As a result, AT-induced hypertension could enhance antitumor activity in mice bearing both well- and poorly vascularized solid tumors.

AT-induced hypertension chemotherapy is most benefit for macromolecular drugs (Li et al., 1993), but not much for low molecular drug (Supplemental Fig. S2). AT could improve the delivery of a macromolecular drug, SMANCS, to the tumor (Maeda, 2001; Nagamitsu et al., 2009). Nagamitsu et al. (2009) reported that AT-induced hypertension clinically improved the antitumor effect of SMANCS with lipiodol in various difficult-to-treat solid tumors including metastatic liver cancer, cholangiocarcinoma, massive renal cell carcinoma, pancreatic and other abdominal solid cancers. It has been suggested that the major barrier to the delivery of macromolecules to solid tumors was elevated interstitial fluid pressure in the tumor (Jain, 1990). AT-induced hypertension may be effective for overcoming such a barrier of intratumoral fluid pressure by increasing blood pressure and flow in a tumor. Accordingly, AT-induced hypertension could improve the therapeutic efficacy of liposomal anticancer drugs. Clinical studies using SMANCS with AT-induced hypertension in Japan have shown a definite regression of advanced tumors; in addition, toxicities were easily manageable and almost all patients had a good quality of life (Nagamitsu et al., 2009). The use of AT-induced transient hypertension is a new treatment option for tumors with nanoparticles, such as liposomes and micelles.

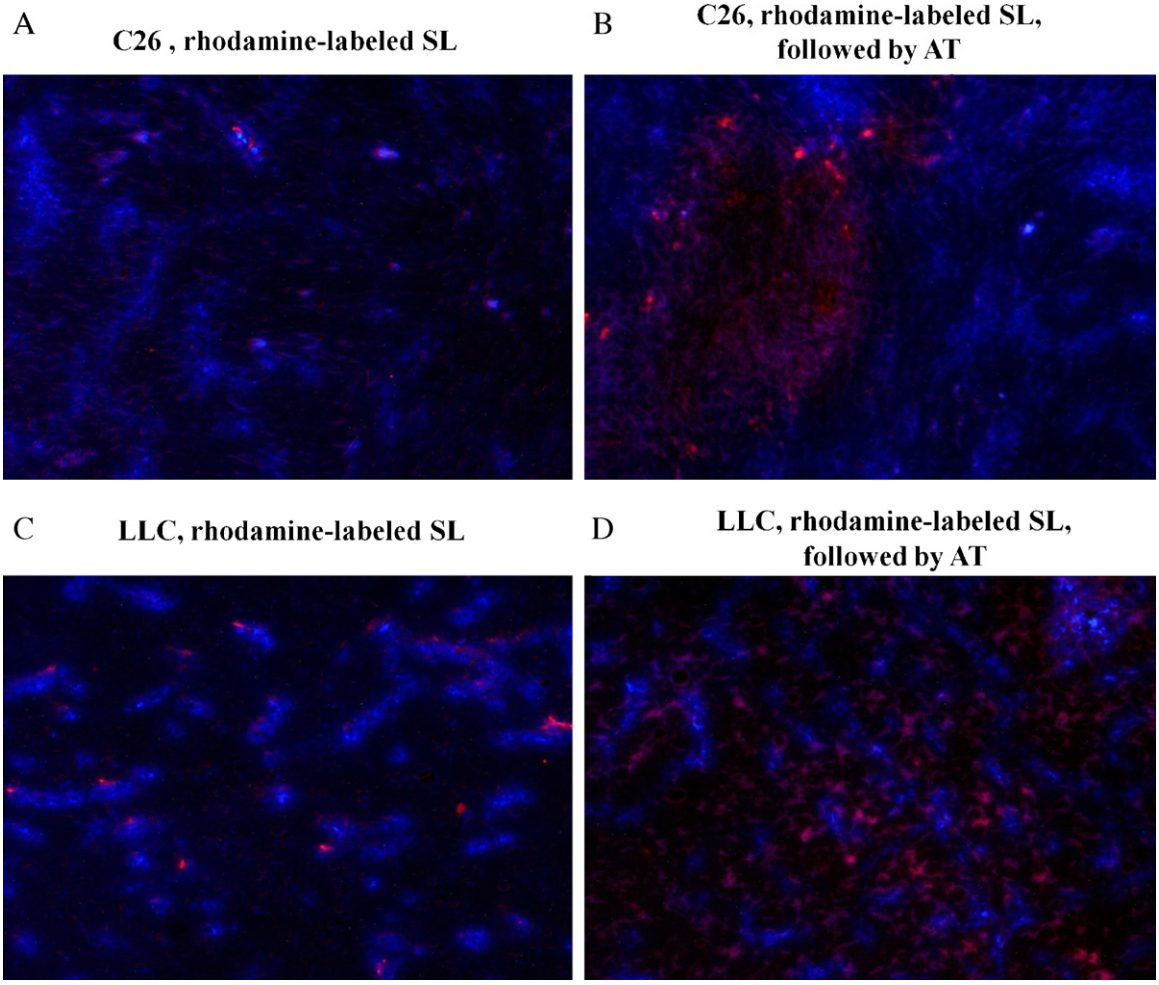

Fig. 5. Tumor accumulation of rhodamine-labeled liposomes 24 h after intravenous administration into C26 (A and B) and LLC (C and D) tumor-bearing mice under normotension (A and C) or followed by AT-induced hypertension (B and D). Hoechst 33342 was injected into the tail vein 1 min before sacrifice. Localization of rhodamine-labeled liposomes is shown in red and tumor blood flow in blue. Magnification, 100×.

5. Conclusions

In this study, we demonstrated that DXR-SL could enhance therapeutic efficacy by AT-induced hypertension. AT-induced hypertension improved the distribution of DXR-SL in tumors, and enhanced antitumor activity by DXR-SL in mice bearing well- and poorly vascularized solid tumors. This strategy involves the use of AT-induced transient elevation of blood pressure to enhance the delivery of liposomal DXR into solid tumor tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.10.009.

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