Volumetric and Angiogenic Evaluation of Antitumor Effects with Acoustic Liposome and High-Frequency Ultrasound

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Abstract

Acoustic liposomes (AL) have their inherent echogenicity and can add functionality in serving as drug carriers with tissue specificity. Nonuniform vascular structures and vascular branches/bends are evaluated by imaging the intravascular movement locus of ALs with high-frequency ultrasound (HF-US) imaging. However, the evaluation of antitumor effects on angiogenesis by ALs and HF-US imaging has not been reported. Here, we show that the combination of ALs and an HF-US imaging system is capable of noninvasively evaluating antitumor volumetric and angiogenic effects in preclinical mouse models of various cancers. In this study, the antitumor effects of cisplatin on tumor growth and angiogenesis in mice bearing two different types of tumor cells were assessed. By tracking each AL flowing in the vessel and transferring the images to personal computers, microvessel structures were mapped and reconstructed using the color difference based on SD method. The antitumor effects were confirmed with an in vivo bioluminescence imaging system and immunohistochemical analysis. Our results show that cisplatin inhibits tumor growth by decreasing intratumoral vessel area but does not affect the angiogenesis ratio in the tumor. The vascular occupancy in the outer region of the tumor was larger than that in the inner region; however, both occupancies were similar to those of the control tumor. We propose that this method of mapping microvessels with ALs and an HF-US system can serve as a new molecular imaging method for the assessment of angiogenesis and can be applied to evaluate the antitumor effects by various therapeutic agents.

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Introduction

With the development of ultrasound contrast agents (UCA), contrast-enhanced ultrasound can image blood perfusion in organs and measure blood flow rate in the heart and other organs, thereby facilitating the detection of primary and metastatic cancer (1, 2). Acoustic liposomes (AL) are one kind of UCA and can add functionality along with echogenicity, serving as drug carriers with tissue specificity (3–6). When their diameter is reduced to 100 to 200 nm, the enhanced permeability and retention (EPR) effect (7) can be exploited, creating the possibility of using high-frequency ultrasound (HF-US) to evaluate vascular permeability, vascular number, and vascular occupancy by their extravascular US imaging properties. In addition, tumor-targeting chemotherapy can be achieved by collapsing extravascularized ALs with US, that is, sonoporation (4, 6, 8). However, the in vivo lifetime of ALs is relatively short compared with that of commercially available UCAs, thus the angiogenic evaluation of antitumor effects with ALs and HF-US imaging has not been reported. In this study, we evaluated tumor volume and vascular area in tumors with and without intratumoral injection of cisplatin (CDDP). Tumors after injected intravenously with ALs were imaged by a HF-US system. Images were evaluated using the color difference based on SD (CDSD) method (9). We found that CDDP induced cytoreductive effects but did not affect the angiogenesis ratio in the tumor. The vascular occupancy in the outer region of the tumor was larger than that in the inner region; however, both occupancies were similar to those of the control tumor (saline injection). The results indicate that CDDP may not inhibit angiogenesis specifically. We propose that this microvessel mapping system can serve as a new molecular imaging method for assessing the effects of therapy on angiogenesis.

Materials and Methods

In vivo studies were done in accordance with the ethical guidelines of Tohoku University.

Preparation of acoustic liposomes

The ALs were prepared as described previously (3, 10). The shell composition was 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC; NOF Co.) and N- (carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000-Ome; PEG molecular weight, 2,000; NOF Co.; 94:6 (mol/mol). The number of ALs in lipid solution

References


was calculated to be $3.3 \times 10^{12}$ bubbles/mL (6). The peak diameter of the number distribution was $0.20 \pm 0.08 \mu$m and the zeta potential was $-2.40 \pm 0.51$ mV. Approximately 20% of the ALs contained both liquid and gas, whereas approximately 80% contained liquid alone (i.e., nonacoustic; ref. 6).

**Tumor cell culture**

Murine colon carcinoma (Colon26) cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, on March 28, 2006. Murine mammary carcinoma (EMT6) cells were obtained from American Type Culture Collection on December 4, 2008. Colon26-Luc and EMT6-Luc cells stably expressing the firefly luciferase gene were prepared by Transfection of Colon26 and EMT6 cells with pEGFP (BD Bioscience) using Lipofection Transfer Reagent (Invitrogen), respectively (11). Colon26 and EMT6 cells were cultured in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% FBS and 1% penicillin/streptomycin. Colon26-Luc and EMT6-Luc cells were cultured in each medium added with Geneticin (G418 sulfate; Sigma-Aldrich; 1 mg/mL), respectively. Cells were incubated at 37°C in a mixture of 5% carbon dioxide and 95% air until 80% confluence was achieved. Cells were routinely verified by morphology and growth characteristics using Trypan blue. Cells were tested for Mycoplasma contamination on the day of the inoculation. Colon26-Luc, Colon26, Colon26-Luc, or EMT6-Luc cells were suspended in PBS without Mg$^2+$ and Ca$^2+$ (PBS). To form solid tumors, $1 \times 10^7$ cells/mL of 100 μL was injected intradermally into the right and left flanks of severe combined immunodeficient (SCID) mice (age 6–8 weeks, weight 20–25 g) or BALB/c mice (age 9 weeks, weight 25–30 g), in which the flanks to be injected were depilated with commercial hair removal cream. Colon26 cells were used for the BALB/c mice and Colon26-Luc and EMT6-Luc cells were used for the SCID mice. The day of inoculation was defined as day 0. Tumors were allowed to grow to a diameter of 10 to 15 mm, which was normally attained in 15 to 16 days.

**CDDP treatment**

For the treated groups, a total of 40 μL CDDP (molecular weight 300, 10 mg/20 mL, Nihon Kayaku Co.), and 60 μL PBS, that is, CDDP of 2 μg/g body weight, was injected into each solid tumor on days 4, 8, and 11 for the EMT6-Luc cells and on days 7, 12, and 15 for the Colon26-Luc cells. In the control group, 100 μL of PBS was injected intratumorally under the same conditions as for the treatment group (12). For the EMT6-Luc cells, tumor-bearing mice in the treatment group numbered $n = 21$, and in the control group, $n = 12$. For Colon26-Luc tumor-bearing mice in the treatment, $n = 9$, and in the control group, $n = 9$.

**Tumor growth assessment**

Two methods, an in vivo bioluminescence imaging system and the HF-US imaging system, were used for tumor growth assessment on day 4 ($n = 12$ for control, $n = 9$ for treatment), day 8 ($n = 9$ for control, $n = 9$ for treatment), day 11 ($n = 6$ for control, $n = 6$ for treatment), and day 15 ($n = 3$ for control, $n = 3$ for treatment) for the EMT6-Luc cells, and on day 4 ($n = 9$ for control, $n = 9$ for treatment), day 7 ($n = 9$ for control, $n = 9$ for treatment), day 12 ($n = 6$ for control, $n = 6$ for treatment) and day 15 ($n = 3$ for control, $n = 3$ for treatment) for the Colon26-Luc cells. With the bioluminescence imaging method, the luminescence intensities were assessed with the in vivo imaging system, IVIS Lumina (Caliper Life Science Inc.; refs. 6, 12). With the HF-US imaging method, B-mode images were acquired with the Vevo 770 system (VisualSonics Inc.) under room temperature of 22°C to 26°C. The scanner was equipped with a mechanical single element transducer (RMV-704, central frequency 40 MHz, axial resolution 40 μm, lateral resolution 80 μm, slice thickness resolution 40 μm, focal depth 6 mm, and length of field 1.5 mm). The transducer was fixed with a 3-dimensional (3D) stage control system (mark-204-MS; Sigma Koki). Mice were anesthetized with 2% isoflurane (Abbott Japan Co., Ltd.) and placed on a stage maintained at 35°C (TM150, VisualSonics Inc.). To maintain US transmission, US gel (Parker Laboratories Inc.) and placed on a stage maintained at 38°C (TM150, VisualSonics Inc.). To maintain US transmission, US gel (Parker Laboratories Inc.) was placed on the tumor formed in the flanks. In the tumor images, linear measurements were made across each axis to measure the length, width, and depth of the tumor. The center of the tumor was set at the focal length. 2D/3D images of the tumor were constructed by B-mode images. 2D B-mode images at the center of the longitudinal length were acquired at 30 frames/sec for 10 seconds, that is, the obtained cineloops consisted of 300 frames. The consecutive B-mode images acquired with a slice thickness of 100 μm, scanning across the tumor by the computer-controlled 3D motorized stage, were reconstructed into a 3D image, and the volume consisting of multiple polygons was calculated with the Vevo 770 software.

**Evaluation of time-dependent changes in grayscale intensity in tumors**

Time-dependent changes in the grayscale intensity of mouse solid tumors were investigated. With the HF-US imaging method, B-mode images were acquired at the center of tumors using the HF-US imaging system with a 40 MHz transducer on day 8, as described in the previous section. ALs were used as an US contrast agent and were prepared 1 minute before each injection, as previously described. Colon26 tumor-bearing mice ($n = 4$) were given bolus intravenous injection of ALs (lipid concentration: 1 mg/mL) via the tail vein for a total volume of 100 μL. B-mode images were acquired from approximately 5 seconds before AL injection. The tumor grayscale intensities in each frame were calculated using the HF-US system software.

**Reconstruction of intratumoral microvasculatures using ALs**

The 2D images of intratumoral microvasculature were reconstructed in only one solid tumor per mouse on day 5 ($n = 9$ for control, $n = 9$ for treatment), day 9 ($n = 3$ for control, $n = 3$ for treatment), day 12 ($n = 6$ for control, $n = 6$ for treatment), and day 15 ($n = 3$ for control, $n = 3$ for treatment).
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Statistical analysis

All measurements are expressed as mean ± SEM. Any overall difference between the groups was determined by 1-way ANOVA. Simple comparisons of the independence of data of 2 groups were done using Student t test or Aspin–Welch t test. Normally distributed equal variables were compared by the Student t test, and normally distributed unequal variables were compared by Aspin-Welch t test. Nonnormally distributed variables were compared by the Mann–Whitney U test. The differences were considered significant at P < 0.05.

Immunohistochemical analysis

The solid tumors that were used for the vessel map construction as described above were then excised. The blocks used for immunohistochemical staining were fixed in 4% neutral buffered formalin (Wako Pure Chemical Industries) for 2 to 3 days at 4°C in 4% neutral buffered formalin (Wako Pure Chemical Industries), embedded with Paraplast (Sakura Finetek Japan Co., Ltd.), and sectioned into 3 to 3.5 μm sections, and deparaffinized with the Tissue clear (Sakura Finetek Japan Co., Ltd.). CD31-positive endothelial cells were stained with anti-mouse CD31 rabbit polyclonal antibody (Thermo Fisher Scientific K.K.), sectioned into 3 to 3.5 μm sections, and deparaffinized with the Tissue clear (Sakura Finetek Japan Co., Ltd.). A peroxidase stain with an anti-mouse CD31 rabbit polyclonal antibody was used as a primary antibody (dilution with 1% bovine serum albumin in PBS solution, 1:50; Abcam plc.). A peroxidase conjugated anti-rabbit antibody (Nichirei) was used as a secondary antibody. The sections were stained with 0.04% hydrogen peroxide and 0.2% 3′,3′-diaminobenzidine (Ventana Japan K.K). The hot spot method was used for calculating the vascular ratio (15, 16). First the tumor boundary was extracted under a microscope (BX51; Olympus) with a digital camera (DP72; Olympus) at a low magnification (×40 or ×100), as shown in Fig. 4A. Next, the boundary was reduced by half, and the inner and other regions were defined as Ac and Ao, respectively. Each region was divided into 4 subregions. In Ac region, the mean vascular density was calculated by dividing the vascular total area, consisting of vasculatures with a minor axis of 7 to 50 μm (×200), by the CD31-positive hotspot area (430 μm × 330 μm). Similarly, the mean vascular density in Ao was calculated. The vascular density measurement was done by 2 persons to reduce measurement error.
Results

Evaluation of CDDP-induced cytoreductive effect with the HF-US imaging system and the bioluminescence imaging system

The CDDP-induced cytoreductive effects were assessed by using the HF-US imaging system and bioluminescence imaging system up to 15 days postimplantation of tumor cells (Fig. 1). We measured the minimum volume \( V_{\text{min}} = 3.3 \text{ mm}^3 \) (using volume = \( \pi D_e^3/6 \), in which \( D_e \) = equivalent diameter of a sphere with the same volume, and \( D_e = 1.8 \text{ mm} \)) to the maximum volume \( V_{\text{max}} = 153 \text{ mm}^3 \) (\( D_e = 6.6 \text{ mm} \)) for the EMT6-Luc tumor (Fig. 1B), whereas for the Colon26-Luc tumor, \( V_{\text{min}} \) ranged from 4.8 mm\(^3\) (\( D_e = 2.1 \text{ mm} \)) to \( V_{\text{max}} = 320 \text{ mm}^3 \) (\( D_e = 8.5 \text{ mm} \); Fig. 1C). As shown in Fig. 1A and B, the EMT6-Luc tumor volumes (Fig. 1B, open circle) and the luciferase activities (white bar) increased in the control group over time; however, they were inhibited in the CDDP treatment group (solid circle, volume; gray bar, luciferase activity). On days 11 and 15, the mean volumes of the treatment groups were significantly lower than those of the control groups (\( P < 0.01 \), Aspin–Welch \( t \) test). Also, on days 8, 11, and 15, the mean luciferase activities of the treatment groups were significantly lower than those of the control groups (\( P < 0.01 \), Aspin–Welch \( t \) test). For the Colon26-Luc tumors (Fig. 1C), the tumor growth was suppressed by the treatments. However, the cytoreductive effects were lower than in the EMT6-Luc tumor; that is, for the EMT6-Luc tumor, the mean volume and the luciferase activity of the treatment group on day 15 decreased by approximately 1/10th compared with that of the control group. However, in the Colon26-Luc tumors, by day 15, the mean volume and the luciferase activity of the CDDP treatment group decreased by only 60% and 70% of those in the control group, respectively.

Time–intensity relation of ALs in tumors and constructed vascular images by ALs, HF-US image system, and the CDSD method

To determine the optimal diagnostic window allowed to capture vascular imaging in the presence of ALs, we investigated the time–intensity relation of B-mode images of tumors injected with ALs. Figure 2A shows a representative time–intensity relation in tumors of Colon26 on day 8 in the absence of CDDP. Immediately after the AL tail vein injection, the mean grayscale value increased dramatically and then decreased gradually to the background level within 6 minutes. An optimal diagnostic window for detection of ALs was established between 20 and 90 sec postinjection. B, representative 2D images of the Colon26-Luc tumor on day 8 without CDDP treatment (control). (a) and (b) are B-mode images of tumor before and after AL injection, respectively. (c) and (d) are the extracted 2D vascular structures in tumor before and after AL injection, respectively. The image was constructed from the B-mode image using the CDSD method. The margins of the tumor areas are delineated by red lines and scale bars indicate 1 mm.

Figure 2. Construction of intratumoral vessels with the combination of ALs, the HF-US imaging system, and the CDSD method. A, representative time-dependent changes in grayscale intensity in tumors of Colon26 without CDDP treatment. After the injection of ALs, significantly higher grayscale intensity was observed and maintained for approximately 6 minutes. An optimal diagnostic window for detection of ALs was established between 20 and 90 sec postinjection. B, representative 2D images of the Colon26-Luc tumor on day 8 without CDDP treatment (control). (a) and (b) are B-mode images of tumor before and after AL injection, respectively. (c) and (d) are the extracted 2D vascular structures in tumor before and after AL injection, respectively. The image was constructed from the B-mode image using the CDSD method. The margins of the tumor areas are delineated by red lines and scale bars indicate 1 mm.

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Angiogenic evaluation by ALs, the HF-US image system, and the CDSD method

We quantified the CDDP-induced longitudinal angiogenesis suppression using the combination of ALs, the HF-US imaging system, and the CDSD method. In the EMT6-Luc tumor (Fig. 3A), the tumor area (Cont. TA) and vessel area (Cont. VA) increased in the control group over 15 days, but the tumor area (CDDP TA) and vessel area (CDDP VA) decreased in the CDDP treatment group. Similarly, in the Colon26-Luc tumor (Fig. 3B), the growth of the tumor area (CDDP TA) and vessel
area (CDDP VA) were restricted with CDDP treatments, in which TA is the internal area that is defined by the circumference of the solid tumor in B-mode image and VA is the area occupied by vessel in the TA extracted by the CDSD method. Therefore, both TA and VA have unit. The extent of suppression in the EMT6-Luc tumor was larger than in the Colon26-Luc tumor. Significant differences between the control and treatment group for the EMT6-Luc tumors were observed on days 12 and 15 (P < 0.05, Student t test), whereas they were detected on day 13 for Colon26-Luc tumors (P < 0.05, Student t test). Figure 3C shows the relation between the normalized vessel area in the CDDP-treated tumor and the number of days after inoculation, in which (normalized vessel area) = [(CDDP VA)/(CDDP TA)]/[(Cont. VA)/(Cont. TA)]. In both tumors, there was no significant difference in the normalized vessel ratio over the experiment period (1-way ANOVA).

Although CDDP contributed to inhibition of tumor growth, these results indicate that the intratumoral vessel ratios in both tumors were constant; that is, the ratio is independent of the tumor size.

Evaluation of the vessel ratio per inner or outer regions of the Colon26-Luc tumor, extracted from the 2D vascular image constructed using AL, the HF-US imaging system, and the CDSD method, and immunohistochemical staining

Next, we investigated the change of the vessel ratio in the inner and outer regions of the Colon26-Luc tumor. Figure 4A shows an image that was binarized to enhance the vascular structure based on Fig. 2Bd. The tumor area was divided into inner and outer areas. Similarly, the immunohistochemical slides were divided into inner and outer areas. The endothelial-specific antigen CD31-positive cells in the inner and outer areas are shown in Fig. 4C and D, respectively.
Figure 5A shows the vascular ratio in the inner and outer regions with and without CDDP treatments on days 8, 13, and 16, obtained by 2D US images. The vessel ratios of the outer region were about double those of the inner region on all days in both the control and treatment groups. Figure 5B shows the vascular ratio obtained by immunohistochemical analysis. Similarly, the vessel ratio of the outer region was about double that of the inner region on all days, although the inner vessel ratios on day 13 were decreased compared with the ratios on day 8. The vascular ratio obtained by AL, the HF-US imaging system, and the CDSD method (Fig. 5A) was about 10 times higher than the ratio obtained by immunohistochemical analysis (Fig. 5B).

Discussion

To our knowledge, this is the first article on the assessment of effects on tumor volume and angiogenesis by CDDP by using ALs, a HF-US system, and the CDSD method. Because the in vivo half-life of ALs with a mean diameter of 200 nm is less than 90 seconds (Fig. 2), enough amount of ALs may not leak out to the vascular space by the EPR effect within the short limited period time and thus ALs may not be used as pure EPR tracers. In case of mice bearing sarcoma 180 tumor, within 10 minutes radioiodinated N-(2-hydroxypropyl) methacrylamide copolymers of MW ranging from 4.5 k to 800 k were accumulated effectively in the tumor (17). Therefore, development of long-circulating liposomes with a lifetime more than at least 10 minutes may be desired to reveal the EPR effect (3). Previously, angiogenesis has been evaluated by US backscatter images (18, 19). The simplest model of acoustic scattering from small particles is described by Rayleigh’s model (20). When the particle diameter, d, is much smaller than the wavelength of the sound, the scattering cross section, σ_s, increases by frequency, f, to the 4th power, and by d to the 6th power. However, Rayleigh’s model is not suitable for calculating the scatter of sound from a single bubble because it ignores resonance. Following a scattering model based on a linear bubble equation, σ_s increases by f^4 for frequencies well below resonance, whereas σ_S is independent of the frequency, for frequency above resonance. For diameters smaller than the resonance diameter, σ_S increases to the 6th power of the diameter, σ_S ∝ d^6. For diameters larger than the resonance diameter, σ_S ∝ d^2 (21, 22). Assuming that ALs are free bubbles and their mean diameter is 200 nm, the natural frequency was calculated to be 27 MHz (23). This frequency is on the same order as that used in the present experiment, that is, a central frequency of 40 MHz with an axial resolution of 40 μm. The calculated scattering cross section is 0.03 μm^2, which is emitted by a single bubble with a diameter of 200 nm. Because the bubble size is within the
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spatial resolution of the US system, the single bubble cannot be visualized. Thus, the intratumoral vascular images observed in the experiment might be the result of scattering of AL aggregation, ALs with larger sizes that cannot be eliminated from AL production process, or the oscillation of ALs at around 27 MHz.

The main function of CDDP is to damage DNA and induce apoptosis (24, 25). Although CDDP is not thought to inhibit angiogenesis specifically, some researchers have reported that CDDP provided antiangiogenic effects against several human and mouse cancer cells, including Colon26 carcinoma (26, 27). In addition, the larger vessels in the outer area of tumor are less affected by the antiangiogenic therapy, but small vessels perfused in the tumor are more sensitive to the therapy (1, 28, 29). It is believed that this effect depends on the accessibility of drugs to the endothelial cells in each vessel. In other words, most large vessels were stabilized by smooth muscle actin-positive cells, but immature small vessels in the tumor lacked coverage by smooth muscle cells and pericytes (29, 30). In the present experiment, both the tumor volume and the luciferase activities of murine mammary carcinoma EMT6-Luc tumor and murine colon carcinoma Colon26-Luc tumor were suppressed by about 1/10th and two-thirds of each control group by intratumoral injection of CDDP 3 times, respectively (Fig. 1). Because the vessel ratios of the inner and outer regions have similar values to those of the controls, CDDP may not inhibit angiogenesis specifically (Fig. 5). In the rapid growth phase of solid tumors, enhanced permeability of the neovascularity and limited lymphatic drainage was induced, resulting in the accumulation of fluid in the central tumor region, followed by an increase in the interstitial fluid pressure. The increased pressure constricts the vessels and decreases blood flow in the central regions (30, 31), leading to a decrease in the vessel ratio in the inner region rather than in the outer region (Fig. 5). The vessel ratio obtained by using ALs, the HF-US imaging system and the CDSD method (Fig. 5A) is 10 times higher than that of the value obtained by immunohistochemical analysis (32). Conventional noncontrast-enhanced Doppler US has been applied successfully to monitor antiangiogenic therapies; however, it is relatively insensitive to slow blood flow velocities and capillary blood flow in small nonstabilized vessels (28), which mostly respond to antiangiogenic treatments (33, 34). Recently, high-frequency volumetric power Doppler ultrasound (HF-VPDU; ref. 31) and contrast-enhanced HF-VPDU (1) have been investigated to assess antiangiogenic therapy effects. Ultrasound microbubble contrast agents that have been used in clinic are pure intravascular probes (35). They have the potential to target disease-related cellular and molecular processes within the vascular compartment (35). Although ALs are one of several kinds of UCAs, they can encapsulate gas and liquid, thereby being used as drug carriers as well as contrast agents. In addition, ALs can potentially be used to evaluate EPR effects and can be used for molecular delivery of agents under US guidance (6).

In conclusion, the use of ALs and HF-US in combination with the CDSD method has the potential to accurately and noninvasively assess the efficacy of therapies designed to target tumor growth and angiogenesis. In the future, the present method may be used as a new molecular imaging method for assessing angiogenesis, and it can be applied to evaluate antitumor effects by various therapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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