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Research Paper

Antiangiogenic Activity of Orally Absorbable Heparin Derivative in Different Types of Cancer Cells

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Purpose. Orally absorbable anticancer medications have great advantages for conventional cancer therapies to patients. Here we evaluated the potent anticancer effect of orally absorbable LHD, a chemical conjugate of low-molecular-weight heparin and deoxycholic acid, on tumor graft growth models.
Methods. We characterized the angiogenic factors, such as VEGF, heparanase, and MMPs, of murine squamous cell carcinoma (SCC7), melanoma (B16F10) or lung carcinoma (LLC1). Two weeks after oral administration of LHD into these cancer-cell-bearing mice, we evaluated the antiangiogenic activity of LHD.
Results. Although all cancer cells expressed the angiogenic factors, SCC7 cells had much higher angiogenic potential and grew rapidly after implantation into mice. When orally administered, LHD delayed tumor graft growth regardless of cancer types. Particularly, LHD powerfully diminished the SCC7-derived tumor growth. Also, the expression of angiogenic factors in all kinds of tumor tissues was decreased, thereby attenuating the neovascularization in tumor tissue.
Conclusion. Our study shows that LHD has potent anticancer and antiangiogenic effect on at least three kinds of tumor cells. LHD can be specifically used for preventing neovascularization in tumor tissue because it has therapeutical potential as an antiangiogenic drug and can be orally absorbed.

KEY WORDS: angiogenesis; anticancer effect; heparin derivative; oral absorption.

INTRODUCTION

It has been proposed that antiangiogenic cancer therapy designed to target tumor neovasculature might be more broadly effective and safer than either targeted or traditional therapies aimed at cancer cells, due to traditional impediments to successful cancer therapy, such as drug resistance and inadequate drug delivery (1–3). Recently, angiogenesis was validated as a target for conventional cancer therapies in a randomized trial using chemotherapy with an antibody to vascular endothelial growth factor (VEGF) (4,5). In addition, several studies have demonstrated that targeting the $\alpha_v\beta_3$ integrin overexpressed in the tumor neovasculature is a promising means of cancer therapy (6,7). However, in view of the complex angiogenesis process, targeting other angiogenic factors, such as fibroblast growth factor (FGF)-2, heparanase, and matrix metalloproteinases (MMPs), in addition to VEGF, might improve the efficacy of antiangiogenic and anticancer treatments (8,9).

Various independent studies have recently elucidated that the anticoagulant heparin's many more biologic effects may account for its therapeutic efficacy in tumor growth and metastasis (8,10–15). Compelling data also suggest that the critical anticancer effects of heparin are mediated primarily via interference with angiogenic factors and selectin binding (14–17). Interestingly, the diverse clinical efficacies of heparin have been shown without severe toxicity. Heparin-based therapeutic approaches, however, are limited because heparin is not absorbed in the intestine due to its high molecular weight, negatively charged structure and hydrophilic properties, and for these reasons, heparin should be given parenterally (18,19).

It is interesting to develop an orally active heparin because the long-term use of continuous parenteral heparin is not deemed practical. Various approach types, such as liposomes, enteric coatings and enhancers, have been studied for oral absorption of heparin (20–23): in our previous

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; BrdU 5-bromo-2′-deoxyuridine; DOCA, deoxycholic acid; LHD, LMWH-DOCA conjugate; LLC1, Lewis lung carcinoma; LMWH, low-molecular-weight-heparin; MMP, matrix metalloproteinases; SCC7, murine squamous cell carcinoma; VEGF, vascular endothelial growth factor.

reports, we developed an orally absorbable low-molecular-weight-heparin (LMWH)-deoxycholic acid conjugate, or LHD, by covalently bonding the amine group of *N*-deoxycholylethylenediamine (DOCA-NH_2) with the carboxylic acids of heparin via amide linkage (Fig. 1A) (24). We found that this new heparin derivative could be orally absorbable in the rodent and monkey with therapeutic efficacy (25,26). The oral absorption of heparin derivative was attributed to the conjugated DOCA molecule constituent, which could promote intestinal absorption by enhancing the hydrophobic properties of heparin and by increasing the interaction between heparin and the intestinal membrane (26,27). Interestingly, we demonstrated that chronic administration of the orally absorbable LHD can prevent cancer progression without adverse effects of heparin, suggesting the potential therapeutic efficacy of LHD as a new oral anticancer agent (28,29).

For conventional cancer therapy using LHD, it should have a universal remedy against various kinds of cancer cells as a new oral anticancer agent. Thus, we are wondering about the therapeutic effects of LHD on various kinds of cancer cells after oral administration of LHD. To this end, in this study we present *in vivo* data on the antiangiogenic effects of LHD on three kinds of cancers—squamous cell carcinoma, melanoma, and lung carcinoma— with respect to clinical application.

MATERIALS AND METHODS

Synthesis of Orally Absorbable Heparin Derivative (LHD)

LHD was prepared as described previously (26–29). In brief, it was synthesized by conjugating the amine group of DOCA-NH_2 with the carboxylic groups of LMWH (Fraxiparin®, average MW 4.5 kDa; GlaxoSmithKline, Brentford, Middlesex, UK). The initial stage of preparation involved activating the carboxylic group of DOCA, as follows: DOCA (196 mg) was mixed with dicyclohexylcarbodiimide (DCC; Sigma Chemical Co., St. Louis, MO) (165 mg) and hydroxysuccinimide (HOSu; Sigma) (92 mg) in 15 mL of dimethylformamide (DMF; Merck, Darmstadt, Germany), a molar feed ratio of 1:1.6:1.6, respectively. DCC and HOSu levels were slightly higher than that of DOCA in order to ensure complete DOCA activation. This mixture was reacted for 5 h at room temperature under vacuum condition. Unreacted DCC was precipitated by adding 1 mL of distilled water and then removed by filtration. The solution obtained was then added to 15 mL of distilled water in order to precipitate the activated DOCA. The obtained activated DOCA was then mixed with ethylenediamine (molar ration 3:1) in DMF and reacted for 5 h at room temperature to form deoxycholylethylamine. LMWH (100 mg) was then dissolved in 2 mL of formamide (Sigma) and 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC; Sigma) solution (11.49 mg) was added to activate the carboxylic acid of LMWH. Deoxycholylethylamine was then coupled with the activated carboxylic acid of LMWH (mole ratio 3:1) at 25°C for 12 h. The product DOCA-heparin conjugate, called to LHD, was then precipitated in acetone and then lyophilized with vacuum drying. The LHD was obtained as white powder. Finally, to bind the LHD with DMSO (Sigma), 5 mg of LHD was dissolved in 180 μL of distilled water and 20 μL of DMSO was added. The solution was then sonicated and freeze-dried to produce LHD.

Determination of the Plasma Concentrations of LHD in Mice

After fasting for 4 h, LHD (10 mg/kg) was administered to C57BL/6J and C3H/HeN mice (Samtako Bio Korea, Osan, South Korea) by oral gavage by carefully inserting the tubes into mice stomachs. The final volume administered was adjusted to 200 μL/mouse. Blood samples (450 μL) were collected from the retro-orbital plexus at 10, 20, 30, 60 and 120 min after oral administration of LHD using a capillary, directly mixed with 50 μl of sodium citrate (3.8% solution), centrifuged at 2500×*g*, and finally allowed to stand for 5 min at 4°C. After collecting blood samples at each time, 450 μl of saline solution (Choongwae Pharmaceutics, Co. Ltd., Seoul, Korea) was immediately injected via tail vein to compensate for the lost body fluid. The plasma concentration of orally absorbed LHD was measured using Coatest Factor Xa assay kit (Chromogenix, Milano, Italy).

All animal experimentations were reviewed and approved by our Institutional Animal Care and Use Committee, which is certified by the Institute of Laboratory Animal Resources at Seoul National University.

Characterization of Angiogenic Factors, VEGF, Heparanase, and MMP-2/9 of Different Cancer Cell Lines

Angiogenic factors of Head SCC7 (Murine Squamous Cell Carcinoma; Seoul National University Hospital, Seoul, Korea), B16F10 (Murine Melanoma; American Type Culture Collection, Manassa, VA) and LLC1 (Lewis Lung Carcinoma; ATCC) were characterized. Three kinds of cell lines were plated in the 100-mm culture dish (BD Falcon™; BD Bioscience, San Jose, CA) and then cultured in RPMI1640 (SCC7 and B16F10, Invitrogen, Carlsbad, CA) and DMEM (LLC1, Invitrogen) supplemented with 1.5 g/L sodium bicarbonate (Sigma), 4.5 g/L glucose (Sigma) and 10 mL of 100 mM sodium pyruvate (sigma), 10 mL antibiotic/antimyotic (Sigma) and 10% fetal bovine serum (Invitrogen). After the confluence of cells reached 70%, culture media were changed into serum-free media and incubated for 24 h. Upon completion of incubation, the serum-free media were harvested and centrifuged at 1,200 rpm for 3 min to obtain a clear supernatant.

The secreted amount of VEGF and the intracellular heparanase activity were measured using enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN for VEGF assay; Takara Bio Inc., Shiga, Japan for Heparanase assay). To evaluate the MMP-2/9 secreted from the cell lines, Gelatin Zymography was performed on 10% polyacrylamide gels containing 1 mg/mL gelatin under Laemmli conditions. Following electrophoresis (90 mV in stacking gel, 120 mV in resolving gel) combined with water cooling systems, the gels were washed tree times in 200 mL of 2.5% Triton X-100 (30 min each) and incubated in 100 mL of 50 mM Tris-HCl, 5 mM $CaCl_2$, 50 mM NaCl and 0.05% *v/v* Brij 35, pH 7.6 at 37°C for 24 h. The gels were stained with 0.25% Coomassie brilliant blue R-250 (50% methanol, 10% acetic acid) (30). Upon completion of

Zymography, the clear region in the gel was scanned by image densitometry (GS710, Bio-Rad, Hercules, CA).

BrdU Incorporation Assay

The anti-proliferative activity of LHD on cell lines was investigated using colorimetric 5-bromo-2′-deoxyuridine (BrdU) cell proliferation ELISA test (Roche Applied Science, Indianapolis, IN). The three kinds of cell lines—SCC7, B16F10 and LLC1—were plated in 96-well flat-bottomed culture plate at 5×10^3 cells/well and cultured overnight at 37°C and 5% CO_2 atmosphere. The cells were then treated with various dilutions (1~100 μg/mL) of LHD in culture media for 24 h. After 1-day culture, BrdU assays were performed according to the manufacturer's instructions.

Experimental *In Vivo* Tumor Graft Growth Assay

In these tumor graft animal models, SCC7-derived tumor grafts were established in C3H/HeN mice, and B16F10- or LLC1-derived tumor grafts were established in C57BL/6J mice (28,29). Briefly, cancer cells (1×10^6 cells/100 μL) were injected subcutaneously into the flanks of mice. On day 7, when each tumor became palpable (50–70 mm^3), mice were randomly allocated to two dosage groups. Mice in each group received a daily oral administration of LHD (10 mg/kg/day in 200 μL of filtered water) for 2 weeks, and mice were fasted for 4 h before administration. As a control, the dissolved water solution without LHD (0 mg/kg/day) was also orally administered once a day for 2 weeks. Tumor size was measured in two dimensions daily using a caliper, and the volume was calculated as $a^2\times b\times0.52$, where a is width and b is tumor length.

Immunohistochemistry

Animals were sacrificed after 2-week oral administration of LHD for the harvest of tumor tissues. For immunohistochemistry, tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Paraffin sections were dewaxed in xylene and hydrated in a graded series (100 to 70%) of ethanol solutions. Paraffin sections were pretreated with 2% hydrogen peroxide, blocked in Universal blocking reagent (BioGenex, San Ramon, CA) and incubated with specified primary antibodies overnight at 4°C. The antibodies used were rat anti-CD34 (Abcam, Cambridge, UK) against microvessel, mouse anti-heparanase (InSight Biopharmaceuticals Ltd., Rehovot, Israel), mouse anti-MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-MMP-9 (Santa Cruz Biotechnology). Slides were incubated in secondary antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). Immunostained sections were counterstained with hematoxylin, dehydrated with a graded series of ethanol, cleaned with xylene, mounted and visualized by light microscopy. In all experiments, the secondary antibody alone served as a negative control. To count the anti-CD34 positive microvessel in the tissue, the sections were evaluated by blindly counting microvessels in randomly selected fields (× 400, 5 fields) under a light microscope. Microvessel counts per field were expressed as mean vessel numbers in these areas.

Statistics

Data were expressed as means ± SEM. The paired *t*-test and one way ANOVA were used to compare groups. $P<0.05$ was considered significant.

RESULTS

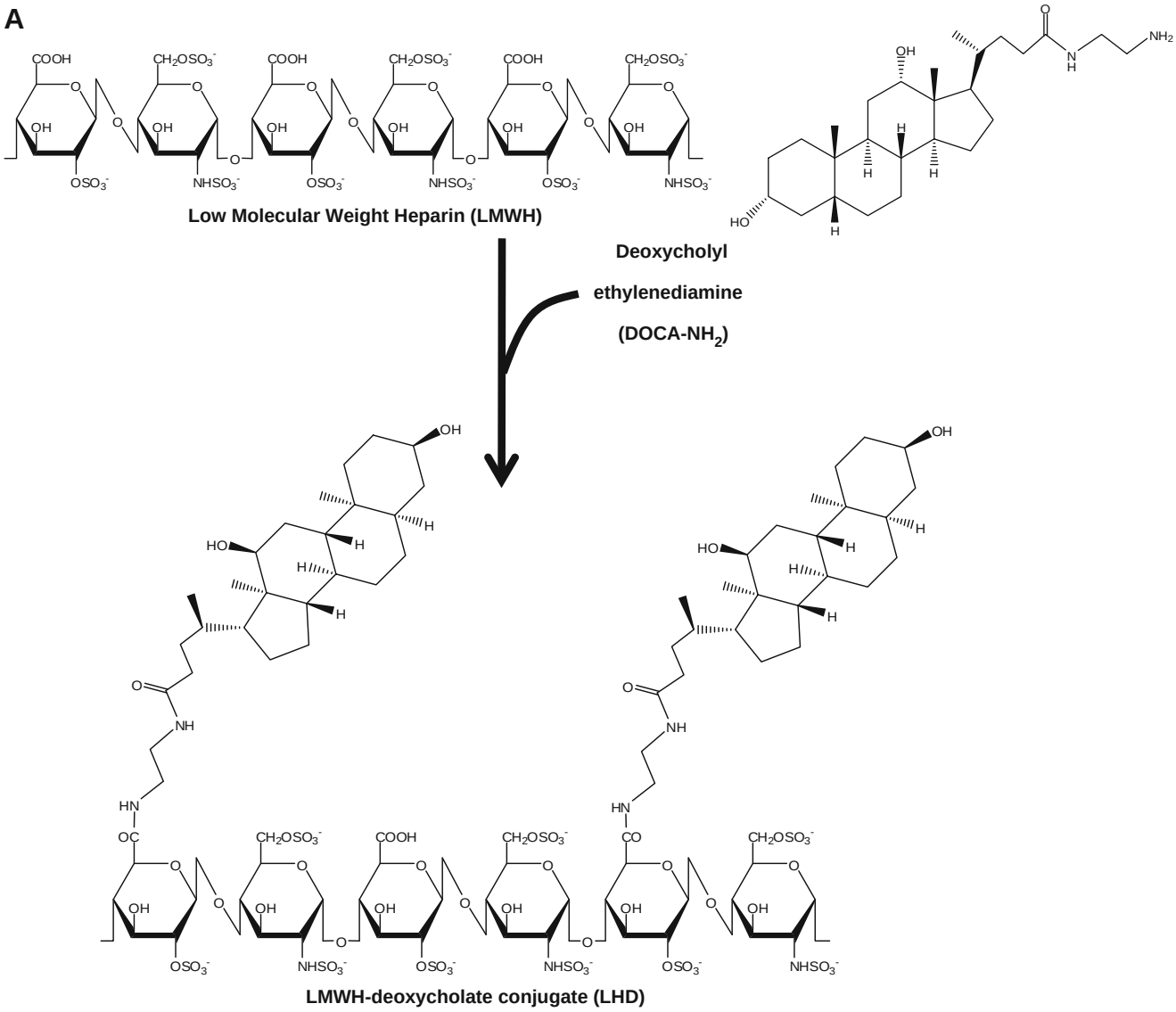
Characterization and Oral Absorption of LHD in Mice

Hydrophilic LMWH and hydrophobic DOCA were conjugated via amide bonds formed by coupling carboxylic groups of heparin with amine groups of *N*-deoxycholylethylenediamine. When the conjugation ratio of DOCA to LMWH was analyzed, two molecules of DOCA were found to be conjugated with one molecule of LMWH (Fig. 1A). LHD formed self-assembled nanoparticles in water because of the conjugated hydrophobic DOCA molecules. However, after physical complexation with DMSO molecules, a well-known amphiphilic solubilizer, LHD was completely dissolved in aqueous solution without forming any particles (31). In addition, the anticoagulant activity of LMWH was 97 IU/mg, whereas that of LHD was about 86 IU/mg. To evaluate the oral absorption of LHD, the plasma concentration of LMWH derivatives was measured by using anti-FXa chromogenic assay after oral administration into mice (Fig. 1B). LMWH was hardly absorbed in the intestine; however, 10 mg/kg of LHD was orally absorbed, and its maximum concentration (C_{max}) in the plasma to C3H/HeN and C57BL/6J mice was 6.0±0.5 and 6.5±0.4 μg/mL, respectively, and at this concentration level, neither side effects nor bleeding was induced. In the previous work, we showed the efficacy of oral absorption of LMWH derivatives regardless of species, such as mouse, rat, and monkey (25).

Characterization of Cancer Cell Lines

To establish the tumor growth animal models, we used three kinds of cancer cell lines: SCC7, B16F10, and LLC1. Before the evaluation of anticancer effect of LHD on the animal models, we first characterized the cancer cell lines. Among the three tumor types, SCC7 cells had significantly higher expression of the angiogenic factors, such as VEGF, heparanase and MMPs, than the other two cell lines, B16F10 and LLC1. The amount of VEGF from SCC7, B16F10 and LLC1 was 565.2±28.6, 198.0±10.5 and 181.2±9.6 pg/mL, respectively, and the intracellular heparanase activity was 133.4±11.2, 70.7±10.9 and 64.0±9.2 μU/mL, respectively (Fig. 2A–B). Notably, the MMP-2/9 enzymes were highly expressed in SCC7 cells, whereas they were very rarely expressed in B16F10 and LLC1 cells (Fig. 2C). The anti-proliferative effect of LHD was evaluated using BrdU incorporation assay (Fig. 2D). Although the proliferation of all cancer cells was inhibited by treating with LHD, SCC7 was most significantly inhibited by the treatment. When treated with 10 μg/mL of LHD, an amount that is similar to the plasma concentration of LHD in mice after the oral administration of 10 mg/kg of LHD, the relative cell proliferation rate of SCC7, B16F10 and LLC1 was 40.7±3.9, 61.9±5.0 and 67.8±2.0%, respectively.

A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Deoxycholyl
ethylenediamine
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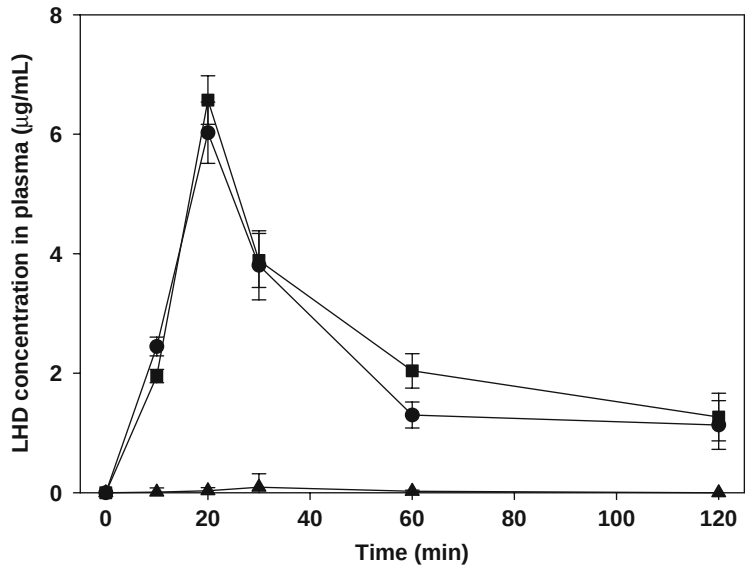
LMWH-deoxycholate conjugate (LHD)

B


LHD concentration in plasma (μg/mL)
Time (min)
0
2
4
6
8
0
20
40
60
80
100
120

◀ **Fig. 1.** **A** The structure of chemical conjugate LDH of low-molecular-weight-heparin (LMWH) and deoxycholic acid (DOCA). An orally absorbable LHD was prepared by covalently bonding the amine group of *N*-deoxycholyl-ethylenediamine to the carboxylic acids of LMWH via amide linkage. **B** Concentration profiles of orally administered LMWH derivatives in mice plasma after single administration. 10 mg/kg of LHD was orally administered to C3H/HeN (●) and C57BL/6J mice (■). Separately, 10 mg/kg of LMWH was orally administered to C3H/HeN (▲). Data were expressed as means ± SEM (n=7).

Inhibition of Tumor Growth by Orally Absorbable LHD

To provide evidence that orally absorbable LHD could inhibit three kinds of tumor growth, the antitumoral effect of LHD was investigated in an *in vivo* tumor graft model using SCC7, B16F10 or LLC1. Cancer cells were subcutaneously implanted into the flanks of mice, and subsequent tumor growth was monitored for 14 days of oral administration of 10 mg/kg/day of LHD or water alone as a control (Fig. 3A–C). Compared

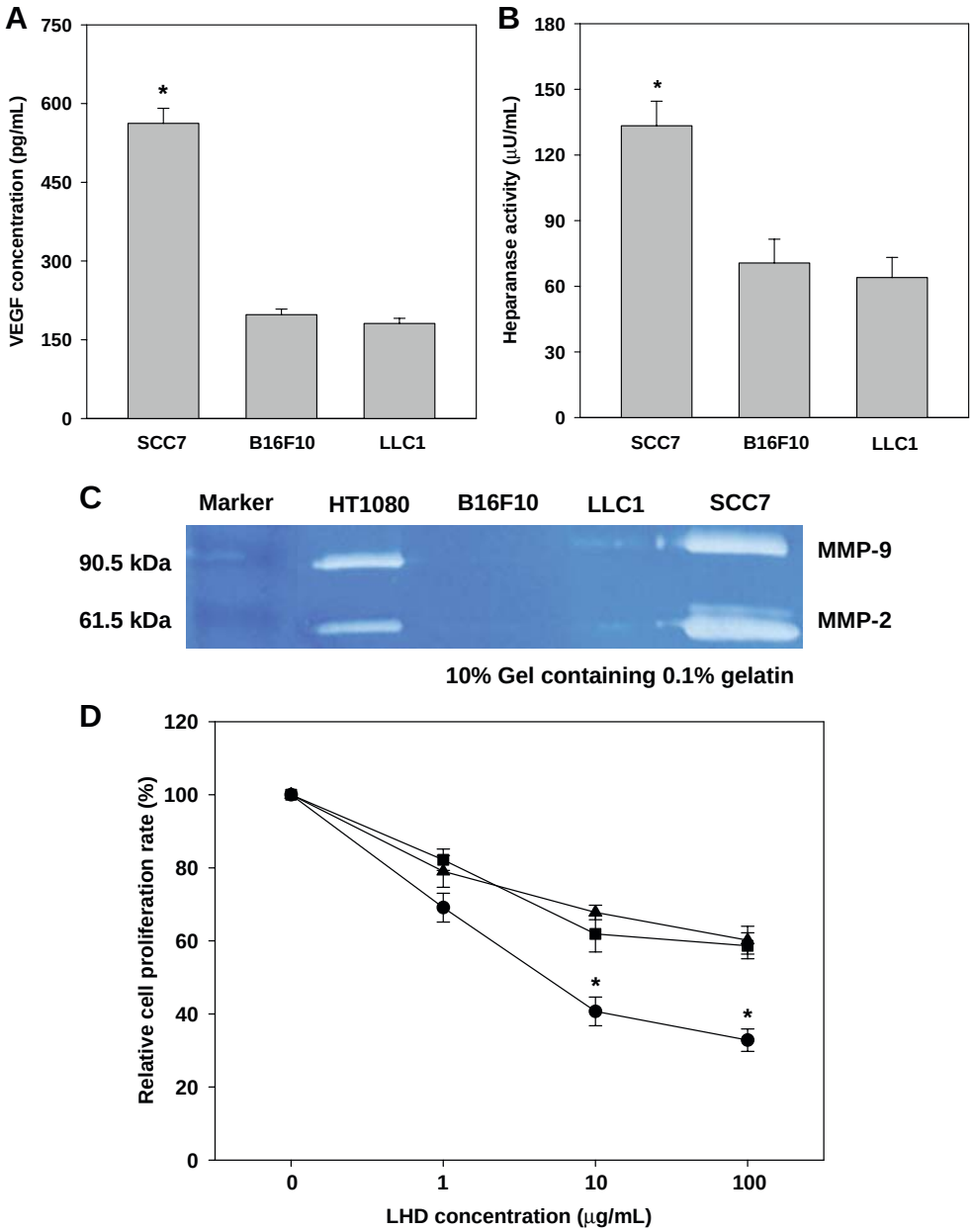


Fig. 2. **A** The amount of VEGF secreted from the SCC7, B16F10 and LLC1. Data are expressed as means ± SEM (n=5). *P<0.001. **B** Intracellular heparanase activity of SCC7, B16F10 and LLC1. Data are expressed as means ± SEM (n=5). *P<0.001. **C** Gelatin Zymography to demonstrate the enzyme activity of MMP-2/9 secreted form SCC7, B16F10 and LLC1. For the control, HT1080 cell lines were used. **D** BrdU incorporation assay to evaluate the relative cell proliferation rate (%) of SCC7 (●), B16F10 (■) and LLC1 (▲) according to the treatment of different concentrations of LHD for 24 h. Data are expressed as means ± SEM (n=7). *P<0.001.

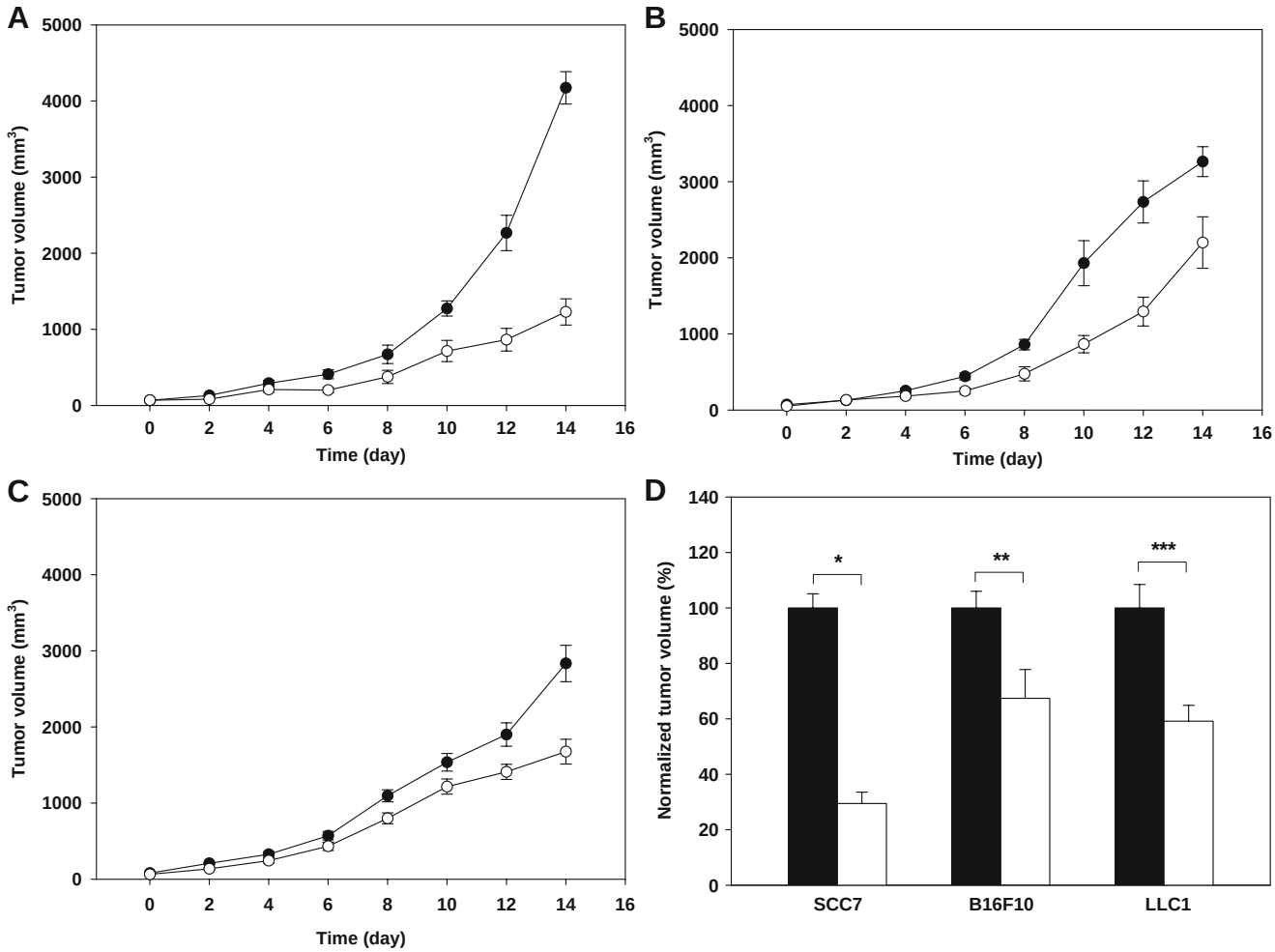


Fig. 3. *In vivo* tumor graft growth profiles of SCC7 (**A**), B16F10 (**B**), and LLC1 (**C**) subcutaneously implanted in the flank of mice during daily oral administration of the dissolved water alone (0 mg/kg/day) (●) or 10 mg/kg/day of LDH (○) for 14 days. Tumor size was measured in two dimensions daily using a caliper, and volumes were calculated. Data are expressed as means ± SEM (n=7). **D** Normalized tumor graft growth rate (%) of SCC7, B16F10 and LLC1 during daily oral administration of the dissolved water alone (■) or 10 mg/kg/day of LDH (□) for 14 days. Data were expressed as means ± SEM (n=7). *P<0.001. **P=0.019. ***P=0.002.

to the control group, the orally administered LHD significantly delayed tumor growth of all cancer cells. At day 14, the tumor volumes of the group of control or LHD for SCC7, B16F10 and LLC1 were 4173.1±211.9 or 1228.6±172.4, 3264.0±196.1 or 2200.1±338.3, and 2833.8±239.5 or 1675.9±162.5 mm^3, respectively. Interestingly, the inhibition rate of SCC having high expression of angiogenic factors was higher than that of B16F10 or LLC1 when the tumor growth rate was normalized (Fig. 3D). The normalized rate of tumor growth of SCC7, B16F10 and LLC1 at day 14 was 29.4±4.1, 67.4±10.4 and 59.1±5.7%, respectively.

Immunohistochemical Evaluation

To determine whether this anticancer effect of LHD on *in vivo* tumor growth was correlated with angiogenesis, tumor tissue sections were stained with anti-CD34, anti-heparanase, and anti-MMP-2/9 antibodies and visualized under a microscope. These experiments demonstrated that the orally absorbed LHD reduced the density of anti-CD34-positive microvessels (Fig. 4A). In addition, when the anti-CD34-positive microvessels of the group of control or treating LHD for SCC7, B16F10 and LLC1 were counted, the microvessel numbers were 18.6±1.5 or 6.2±1.5, 7.2±1.2 or 3.8±0.4 and 10.6±1.1 or 7.8±0.6, respectively (Fig. 4B). On the other hand, the orally treated LHD also inhibited the amount of the anti-heparanase-positive cells (Fig. 5A). In the case of anti-MMPs immunostain of SCC7-derived tumor tissue which has only higher expression of MMPs enzymes among three cancer cells, the orally treated LHD reduced the amount of the anti-MMP-2/9-positive cells (Fig. 5B).

DISCUSSION

Apart from the well-recognized anticoagulant activity of heparin, mechanisms by which heparin potentially affects tumor development and/or metastasis have been described by various researchers (8,10–17). In our previous studies, we had demonstrated that the chemically modified heparin, or LHD, could inhibit the processes of tumor progression, including growth, proliferation and metastasis, when LHD is orally administered. Briefly, as shown by our previous works on

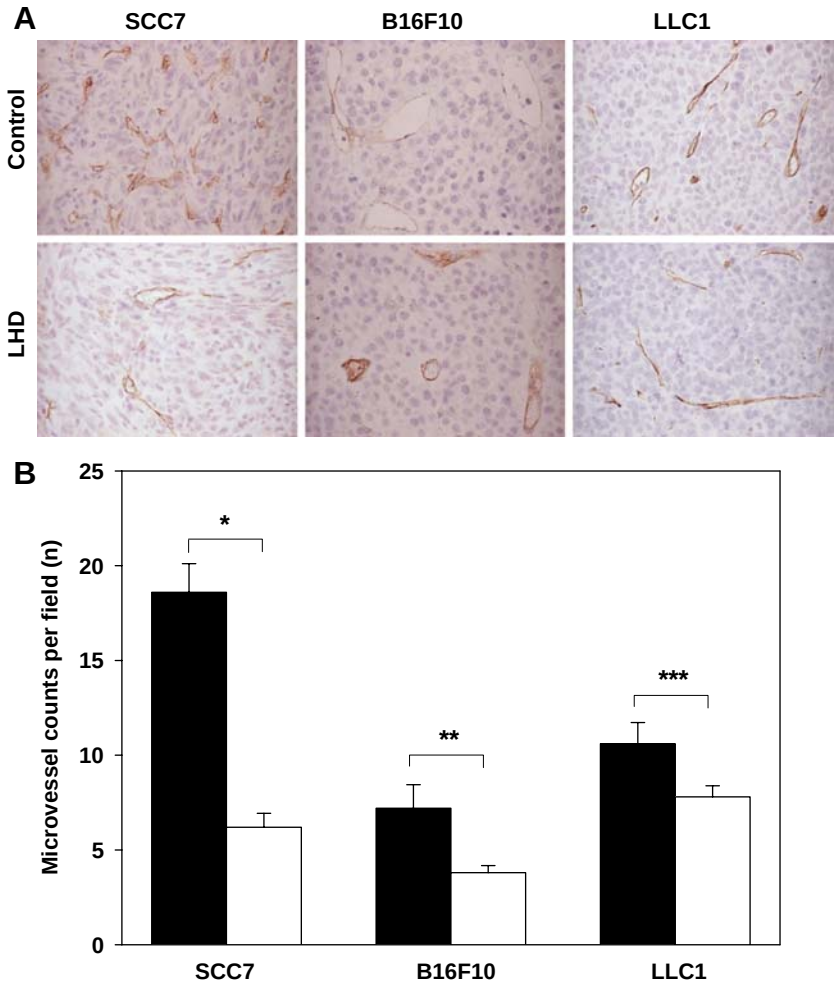


Fig. 4. **A** Immunohistochemistry to stain the newly formed microvessels using anti-CD34 antibody in SCC-7-, B16F10- and LLC1-derived tumor tissues (brown color). **B** Anti-CD34-positive microvessel counts on the tumor tissue sections after daily oral administration of the dissolved water alone (■) or 10 mg/kg/day of LDH (□) for 14 days. Data are expressed as means ± SEM (n=5). *P<0.001. **P=0.031. ***P=0.058. Magnification: ×400.

tubular formation of HUVECs (human umbilical vein endothelial cells), LHD is effective at preventing microvessel formation and, thus, by extrapolation, angiogenesis (28). Also, it could inhibit formation of microvessel as indicated by the results of CAM (chicken chorioallantoic membrane) and *in vivo* Matrigel plug assays using bFGF growth factor (28). In addition, the metastasis steps were interrupted by LHD at subsequent steps, namely the establishment and subsequent growth (i.e., colonization) of the metastasis at the lung metastasis animal model (29). The suppression of the interactions was due to the competitive inhibition by LHD of the P- or E-selectin-mediated interactions. Furthermore, this LHD significantly inhibits bFGF-induced angiogenesis by suppressing the phosphorylation of FGF receptor, ERK and p38 MAPK, indicating that it interacts with bFGF via ionic interaction and changes the conformation and biological activity bFGF, thereby effectively disrupting the import of endothelial cells (32).

In the present study, we used several different experimental tumor graft models to show that LHD has potent anticancer and antiangiogenic activity in different cancer cell types. The effects of heparin on cancer have been studied in several animal models (33), but only a few studies have reported that heparin treatment induced a significant reduction of tumor growth. The reason for this may be that the proliferation and angiogenic activities of cancer cells are significantly different for various kinds of cancer cell types. In this study, we show that different types of cancer cells have different tumor growth rates by showing that the expression and secretion of angiogenic factors of cancer cells of SCC7, B16F10 and LLC1 were significantly different. It is possible that the effect of heparin is affected by different methods of tumor induction and the interval between the start of heparin treatment and tumor induction for tumor growth inhibition. More importantly, the potential factors influencing the effect of heparin are the dose and type of heparin used and the duration of treatment. According to several studies, high doses of heparin (100 U three times for 15 days) seem to be effective, although results have not been always consistent (34–36). Low doses of heparin or less than 15 days of administration did not affect tumor growth. Moreover, a very high dose of heparin (200 U) given only three times a week

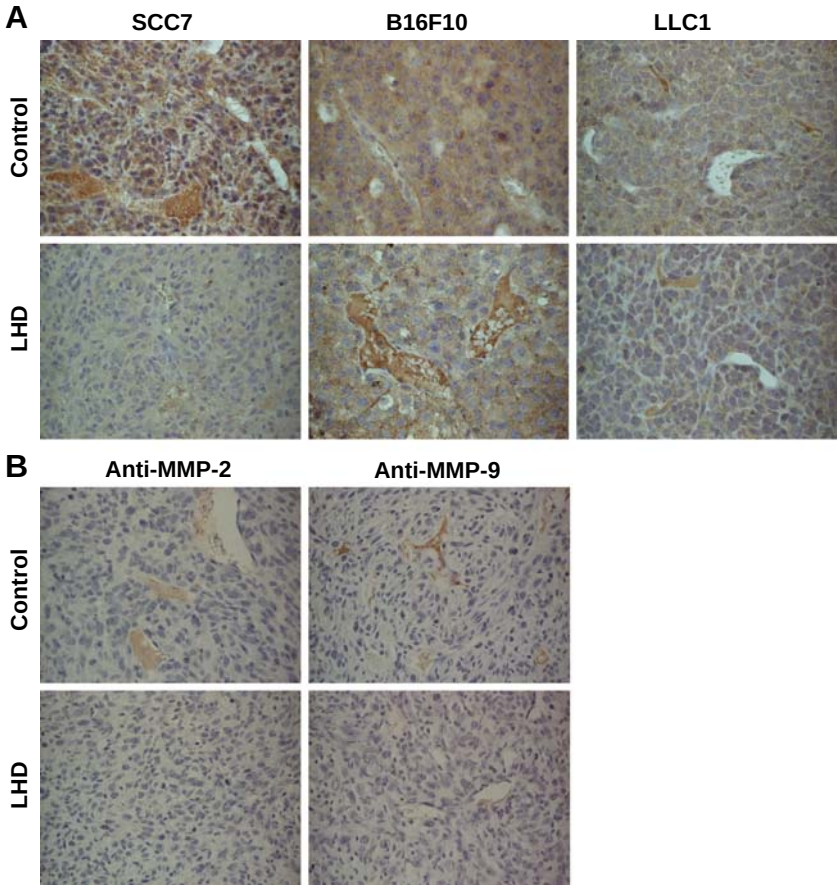


Fig. 5. **A** Immunohistochemistry to stain the expressed heparanase using anti-heparanase antibody in SCC-7-, B16F10- and LLC1-derived tumor tissues (brown color). **B** Immunohistochemistry to stain the expressed MMP-2 using anti-MMP-2 antibody and the expressed MMP-9 using anti-MMP-9 antibody in SCC7-derived tumor tissue (brown color). Magnification: ×400.

was not apparently capable of affecting the primary tumor growth (37). Therefore, these studies indicate that long-term heparin administration at high concentration levels is necessary to have an effect on the primary tumor growth. The development of orally absorbable heparin is attractive in this regard, where the main advantage of orally active heparin is that cancer progression, such as metastasis and tumor growth, can be prevented and/or delayed by chronic administration of orally active heparin.

Although the effect of heparin on cancer progression remains an issue for debate, various studies have suggested that heparin is an attractive candidate for an anti-cancer therapy, especially for the fact that heparin is an efficient inhibitor of angiogenesis. In spite of these attributes of heparin, heparin-based therapeutic approaches are limited because its high molecular weight and negatively charged hydrophilic structure require heparin to be given parenterally only, and not orally. In our previous study, we first developed an amphiphilic heparin derivative by coupling hydrophobic DOCA molecules to hydrophilic LMWH, with the goal of enhancing its intestinal absorption (24). The chemically modified heparin derivative, LHD, had the increased liphophilicity due to the hydrophobic DOCA, thereby increasing the passive penetration through the mucous layer in the intestine wall. In addition, the chemically conjugated DOCA can interact with bile acid transporters that are highly expressed in the ileum, thereby enabling the transporter-mediated active penetration (25–27). In our previous study, we experimentally showed the direct interaction between LHD and ileal brush border membrane (BBM) surface by using surface Plasmon resonance technique (27). Also, from the mechanism studies of LHD absorption using Caco-2 cell monolayers for mimicking the intestine, we demonstrated that LHD highly permeated by passive diffusion through the transcellular route, and its permeation was partially affected by bile acid transporters (26). In general, bile acid transporter, localized in the distal ileum, is believed to comprise the major channel for the re-entry of bile acids into the portal blood in humans and other mammals (38,39).

In the current study, LHD could effectively prevent the growth and angiogenesis in three kinds of tumor tissue after oral administration, although their reduction rates by orally treated LHD were different. Interestingly, the orally administered LHD showed relatively more powerful anticancer activities on the SCC7-derived tumor having higher anti-angiogenic activities. This is because the SCC7 cells express and secrete greater amounts of VEGF, heparanase and MMP-2/9 to rapidly grow the tumor tissue. Therefore, it is possible that the treated LHD can interact more readily with the angiogenic factors secreted from SCC7 than from B16F10

and LLC1 cancer cells. The reduction rate of tumor growth by the treated LHD on SCC7, B16F10 and LLC1 was about 70%, 33% and 40%, respectively, although their tumor volumes were factually similar on day 14 after daily oral administration of LHD as shown in Fig. 3. In addition, the immunohistochemical analysis showed that the treated LHD attenuated the anti-CD34-positive microvessels, anti-heparanase-positive cells, and anti-MMP-2/9-positive cells. The formation of microvessels in SCC7, B16F10 and LLC1-derived tumor tissue was significantly inhibited by orally absorbed LHD. In the cases of anti-heparanase and anti-MMP-2/9 immunostains, the intensity of each immunostain was blurrily reduced when compared with control group. It is hard to quantify the effect of LHD against heparanase and MMP-2/9 because they were deeply or blurrily stained in the broad field of sectioned tissue. Furthermore, the microvessel numbers in tumor tissues were consistent with the inhibition rate of tumor growth.

To establish the *in vivo* tumor xenografts animal models, we used male C3H/HeN for SCC7 and C57BL/6J mice for B16F10 and LLC1 cancer cells. Therefore, it is possible that bioavailability of LHD in these mice can be different, thereby affecting the different tumor growth inhibition. Fortunately, after oral administration of LHD in both mice, the concentration profiles and C_{max} values of orally absorbed LHD were similar, indicating that the different antiangiogenic effect of LHD in these three kinds of cancer cells was not attributed from different oral absorption of LHD in the same genus of mice. However, when LHD was orally administered to mice, rats and monkeys, its oral bioavailability was different (25).

In our previous study, we found that the orally absorbed LHD could inhibit *in vitro* tubular formation of Human umbilical vein endothelial cells (HUVECs) (28). Also, we demonstrated anti-metastatic effect of the orally absorbable LHD in the experimentally induced lung metastasis by A549 human lung carcinoma cells (29), and the antiangiogenic effect of intravenously injected heparin-taurocholate conjugate (conjugation of taurocholate instead of DOCA) in MDA-MB231 human breast cancer cell xenografts model (40). These findings suggested that the heparin derivatives can inhibit the growth of human cancer cells for the purpose of human cancer therapeutics even though we did not directly show the result of orally absorbable LHD using one mouse xenografts model of human cancer. On the other hand, in our previous studies, we demonstrated the anticancer and anti-angiogenic effect of heparin derivatives after intravenous injection to confirm their similar activities (41). The intravenously injected heparin derivatives could significantly inhibit the growth of SCC7-derived xenografts in male C3H/HeN mice. Furthermore, the intravenously injected heparin derivatives could attenuate the formation of tumor nodules in lung tissue on experimentally induced metastasis model (29,42). Therefore, the heparin derivatives could have the same activity on tumor growth and metastasis model. Collectively, these findings suggest that the orally absorbable LHD could have potent anticancer and anti-angiogenic activities regardless of cancer cell types. In addition, the treated LHD could specifically and strongly affect cancer cells that have higher expression of angiogenic factors.

As an anticoagulant, heparin has the possibility of causing osteopenia, bleeding and heparin-induced thrombocytopenia (HIT), which restricts the application of heparin in treating cancer patients (43–45). Peak levels of heparin in plasma at the upper limit of the recommended therapeutic range (0.6–1.0 IU/ml) may be associated with an increased risk for bleeding (43). In the current study, when orally administered at 10 mg/kg/day as an optimal dose, peak levels of orally absorbed LHD in plasma were below the toxic concentration as shown in Fig. 1B. Therefore, we expect that LHD could exhibit anticancer activity and antiangiogenic activity without causing any bleeding problems. On the other hand, in our previous study, we demonstrated that when the high dose of LHD (500 mg/kg) was orally administered in monkeys for 2 weeks, there were no significant adverse effects of LHD, although its peak concentration was increased up to 2.2 IU/ml at day 14 (46). However, since the target plasma level of LHD for attenuating adverse effects is below 1.0 IU/ml, 10 mg/kg of LHD could be very safely used.

In summary, our findings demonstrate that orally absorbable LHD have anticancer and antiangiogenic effects on three kinds of cancer cells, indicating that LHD could be used as a potent anticancer medication. Although the present study shows the possibility of LHD as a first-line anticancer medication, we think that LHD could be more suitable for chronic therapy in treating primary cancer progression and preventing secondary cancer recurrence because LHD has therapeutical potential as an anti-angiogeneic and anti-metastatic drug, has lower side effects, and can be orally absorbed.

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