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Size Controlled Heparin Fragment–Deoxycholic Acid Conjugate Showed Anticancer Property by Inhibiting $VEGF_{165}$

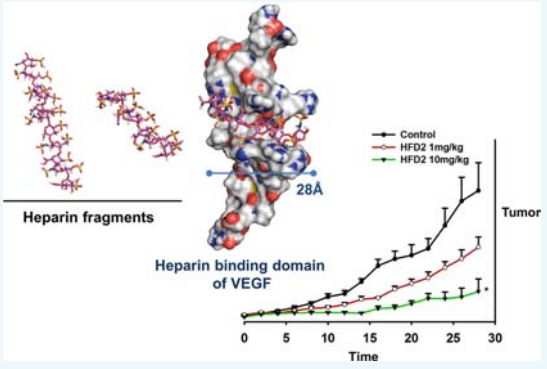
Jooho Park,[†] Jee-Heon Jeong,[‡] Taslim A. Al-Hilal,[†,§] Ji-young Kim,[†] and Youngro Byun*[,†,¶]

[†]College of Pharmacy and [¶]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea

[‡]College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Gyeongbuk South Korea

[§]Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 136-791, South Korea

ABSTRACT: Heparin is a highly sulfated, long, and linear polysaccharide, which can inhibit tumor growth by interacting with growth factors such as bFGF and VEGF. Several researchers have shown the anti-angiogenic effect of heparin and its conjugates in relation to growth factor inhibition. For drug development and inhibition of growth factors using heparin conjugates, the molecular size of heparin may be crucial considering the size of the heparin binding site of growth factors. In this study, we synthesized heparin fragments and deoxycholic acid conjugated heparin fragments (HFD) to search for the optimal size-controlled conjugate that will inhibit the angiogenic effect of $VEGF_{165}$. We have also shown that the HFDs could have an enhanced therapeutic effect in vitro and in vivo consequent to the molecular size control. HFDs have significant anti-angiogenic effects by blocking the angiogenic activity of $VEGF_{165}$ depending on its molecular size. Among them, HFD2 was a promising candidate for oral angiogenesis inhibitor. These results suggest that size-controlled synthesis is necessary for heparin-based drug development.



INTRODUCTION

Heparin is a highly sulfated, long, and linear polysaccharide which is widely used clinically as an anticoagulant.[1,2] Heparin can also bind to a variety of proteins including growth factors, growth factor receptors, and chemokines since heparin has a similar structure to heparan sulfate which is present on endothelial cell surface and in extracellular matrix (ECM).[3,4] Among growth factors, the members of the VEGF family are key regulators of normal and pathological angiogenesis.[5] In particular, $VEGF_{165}$ stands out, primarily having a heparin-binding domain consisting of arginine and lysine residues.[6]

Unfractionated heparin (UFH) is a polydispersed biomaterial with a molecular weight ranging from 5000–40 000 Da and an average molecular weight of 12 000 Da.[1] Low-molecular-weight heparin (LMWH) is generally prepared through the controlled chemical or enzymatic depolymerization of heparin having an average molecular weight of 4500–6000 Da.[1,7,8] Very-low-molecular-weight heparin (VLMWH), ultralow-molecular-weight heparin (ULMWH), or heparin fragments are usually described as a mixture having an average molecular weight of less than 4000 Da. Compared to UFH, smaller heparins have several benefits such as a higher bioavailability, longer half-life, wider therapeutic window, and lower bleeding risk.[9,10]

In the recent years, clinical application of heparin of lower molecular weights is preferred due to subsequent reduced adverse effects.[11,12] ULMWHs such as bemiparin and semuloparin showed similar or better efficacy than LMWHs, but with lower risk of bleeding and heparin-induced thrombocytopenia (HIT).[13,14] Smaller heparins also displayed anti-angiogenic effects potentially better than that of LMWH with a safety profile.[15,16] Therefore, they have been evaluated in clinical trials as adjuvants in patients with cancer because they are effective and suitable for long-term treatment.[12,17]

Many researchers have shown the anti-angiogenesis effect of heparin on the outgrowth of primary tumor.[18] Several studies also conducted on inhibition of tumor angiogenesis via the targeting of relevant angiogenic growth factors and showed therapeutic results using heparin fragment and heparin-based oligosaccharides.[4,19,20] Heparin-based materials competitively bind to heparin-binding growth factors, thereby inhibiting their interaction with heparan sulfate.[15,21] Structural studies about the heparin-binding site of growth factors provided important information for the optimal size requirement in heparin-based antitumor drug design.[19] Because the recognition of glycosaminoglycan by protein depends on molecular binding, the proper molecular size could be essential for the rational design of carbohydrate-derived drug.[22] The molecular size of unfractionated heparin is enormous, compared to the size of heparin binding domain of growth factors.

In the previous studies, we developed orally active heparin conjugates which can enhance heparin absorption in the intestine using deoxycholic acid (DOCA).[23,24] In the oral

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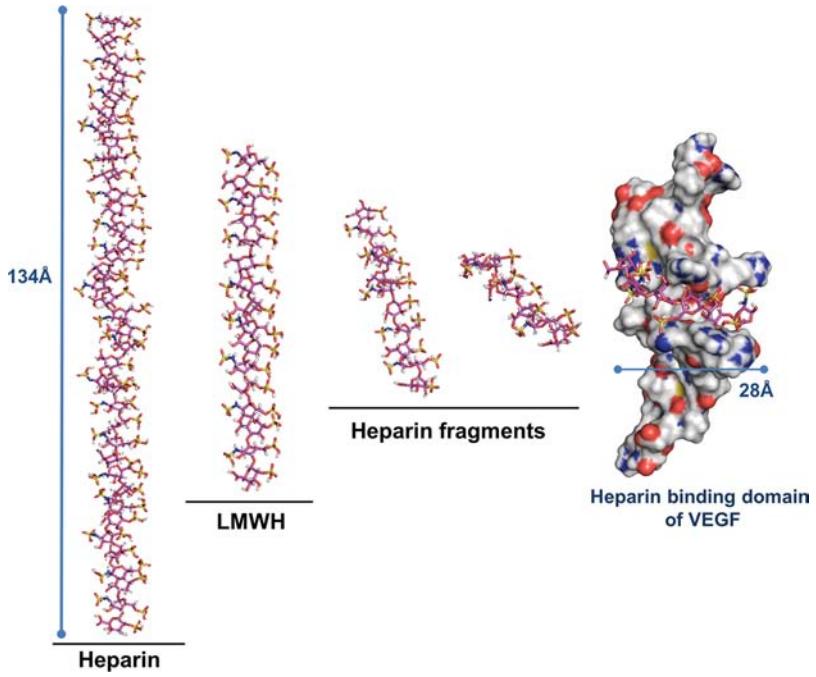

Figure 1. Molecular structures of unfractionated heparin (UFH), low-molecular-weight heparin (LMWH), heparin fragments with the heparin binding domain of vascular endothelial growth factor (VEGF).

Table 1. Characterization of Low-Molecular-Weight Heparin (LMWH) and Heparin Fragments

materials	average molecular weight[a] (Da)	average DP[b]	polydispersity index[c]	molecular lengths[d] (Å)	anti-Xa activity[e] (%)
LMWH	4765	18	1.65	64	100 ± 0.1
Heparin fragment 1	3289	12	1.68	47	92.5 ± 8.2
Heparin fragment 2	1835	7	1.22	25	72.9 ± 2.8
Heparin fragment 3	1443	5	1.04	19	39.6 ± 14.2

[a]Measured and calculated by gel permeation chromatography program. [b]Degree of polymerization (based on the average molecular weight). [c]Polydispersity (PD = M_w/M_n; M_w = weight-average molecular weight; M_n, number-average molecular weight). [d]Measured using PyMOL program. [e]Relative activity to LMWH, measured using Coatest anti-FXa chromogenic assay. Results are the mean ± standard deviation

delivery of heparin-like polysaccharides, bile acid conjugation to heparin proved to be effective for enhancing its absorption in the intestine while eliminating the anticoagulant activity of heparin.[25,26] The DOCA conjugation has been found to promote the intestinal absorption by enhancing the hydrophobic properties of LMWH and increase the membrane interaction of LMWH without any systemic and local toxicity.[27,28] The heparin conjugates also showed an enhanced anti-angiogenic effect and reduced primary tumor volume.[29−32] An orally available safe heparin conjugate is suitable for a long-term anticancer therapy and it has therapeutic potential to target the other heparin binding growth factors.

In the present study, we synthesized size-controlled heparin fragments in order to determine the size-optimized heparin-based conjugates ideal for inhibiting $VEGF_{165}$ induced angiogenic effects. Moreover, heparin fragments were conjugated with DOCA to minimize the anticoagulant effect as well as enhance oral absorption. We evaluated the anti-angiogenic therapeutic effect of DOCA-conjugated heparin fragments (HFD) using HUVECs and the tumor growth inhibition behavior in tumor animal model. We also proved that HFDs exhibit optimized therapeutic effects in relation to size control. This study could be discussed in terms of the controlled size requirements for heparin-based drug development considering the size of the target.

RESULTS

Synthesis and Characterization. The heparin fragments were synthesized from LMWH by depolymerization using nitrous acid. The molecular structures are shown in Figure 1 and the characterizations of these molecules are described in detail in Table 1. They have different average molecular weights according to the reaction ratio of $NaNO_2$. LHD, which is a LMWH and DOCA conjugate, was studied in our previous research.[30] DOCA conjugation was expected to enhance the bile acid transport mediated cellular uptake and eliminate the side effect of heparin for the oral anticancer therapy. The molecular coupling ratios of DOCA to the LMWH, heparin fragment 1, heparin fragment 2, and heparin fragment 3 were 4.0, 3.0, 1.9, and 1.3, respectively. Figure 2b shows the weight and molecular based coupling ratios of LHD and HFDs.

Anti-FXa and Cell Viability Assays. The anticoagulant activities of LMWH, heparin fragment, LHD, and HFD were evaluated by the anti-FXa chromogenic assay. Even though heparin fragment 1, heparin fragment 2, and heparin fragment 3 have decreased molecular sizes, they still exhibited anticoagulant effects of about 92.5%, 72.9%, and 40.6%, respectively (Table 1). However, their anti-FXa activities were significantly decreased after DOCA conjugation as shown in Figure 2. We then tested cell viability after synthesis of HFDs using the CCK-8 assay with HUVEC, SCC7, and MDA-MB-231 cell

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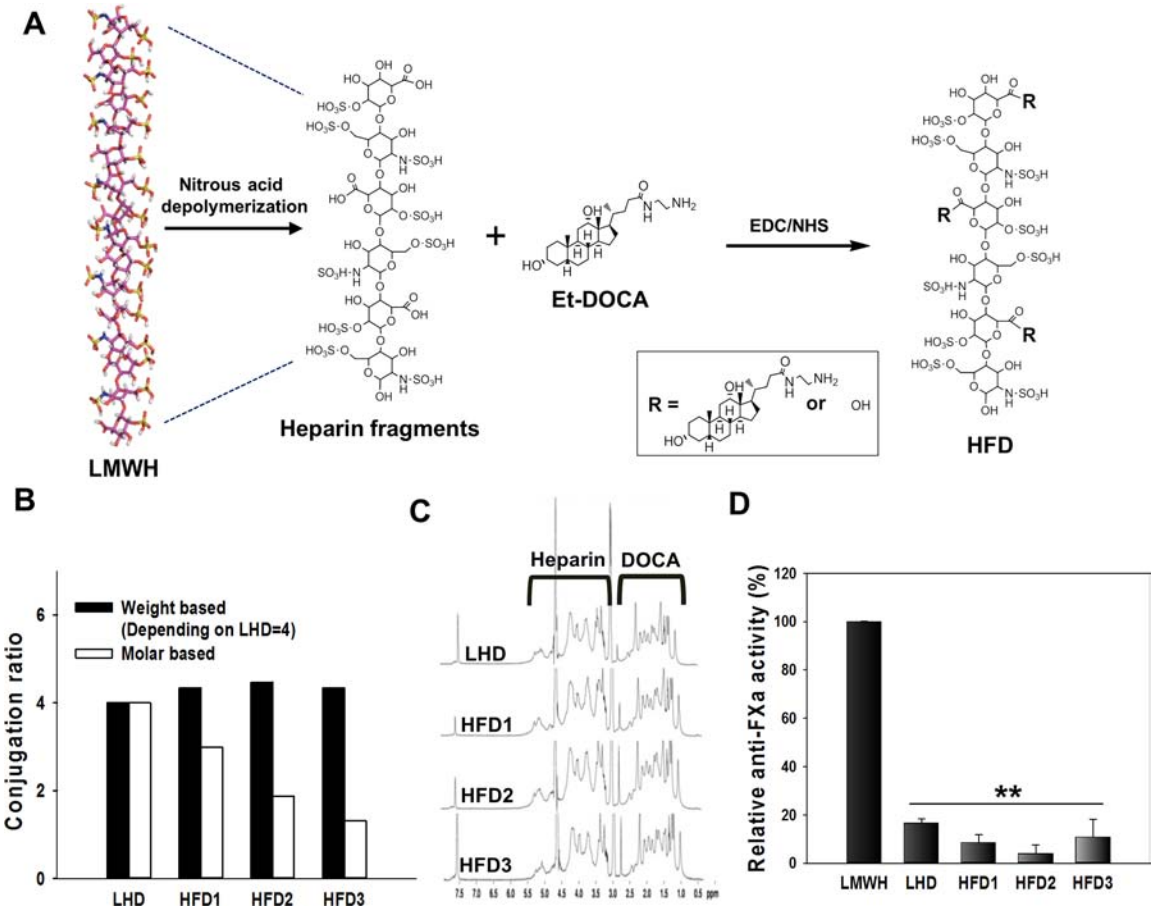

Figure 2. (A) Synthetic scheme for the preparation of heparin fragment-deoxycholic acid conjugate (HFD) to eliminate the anticoagulant activity of heparin fragment and enhance the oral uptake. After the size control of heparin using nitrous acid depolymerization, HFD was prepared by deoxycholic acid conjugation. (B) Conjugation ratio. (C) NMR peak data of LHD and HFDs. (D) Relative anticoagulant activities (Anti-FXa) of LHD and HFDs (n = 3). $^{**}p < 0.001$ vs control group.

lines. In various concentrations, LHD and HFDs did not influence cell viability (Figure 3).

In Vitro VEGF Inhibition Test. The inhibitory effects of LHD and HFDs on VEGF were evaluated using VEGF-accelerating angiogenesis conditions. In the case of $VEGF_{165}$ inducing HUVEC tubular formation assay, the tubular formation inhibitory effects of LHD, HFD1, HFD2, and HFD3 showed 71.7%, 53.5%, 46.8%, and 63%, respectively. Among them, HFD2 exhibited the maximum inhibition of tubule formation (Figure 4). However, at the same concentration (20 μg/mL), LHD did not exhibit significant effect. Using HUVEC, the effects of LHD and HFDs on cell proliferation were also evaluated. As shown in Figure 5A, LHD and HFDs both inhibited HUVEC cell proliferation at 100 μg/mL. They also inhibited the proliferation of HUVEC cells at 10 μg/mL. In VEGF-induced wound healing assay (Figure 5B), LHD and HFDs on scratch wound inhibited the migration of HUVECs after creating scratch wound and $VEGF_{165}$ treatment. Among HFDs, HFD2 strongly decreased the wound healing to 42.1% at 10 μg/mL and 25.1% at 100 μg/mL compared with 69.4% of untreated positive control group.

In Vivo Tumor Inhibition Effect of Orally Administered HFDs. Anticancer effects of HFDs were studied in a tumor graft model using SCC7 cells. After the oral administration of 10 mg/kg/day of HFD1, HFD2, and HFD3, all of them exhibited therapeutic potentials in SCC7 animal model. When 10 mg/kg/day of HFD1, HFD2, and HFD3 were treated, tumor growth rates were significantly lowered by 65%, 77%, and 58%, respectively, compared with the control group (Figure 6A). HFD treatment did not cause any loss of body weight (Figure 6B). HFD3, which has the lowest average molecular weight, showed less tumor inhibition effect in vivo compared with HFD2. Therefore, based on this experiment, HFD2 was determined to have the proper molecular size to inhibit cancer growth in the mouse model.

After the murine cancer experiment, we confirmed the anticancer therapeutic effect of HFD2 in a human breast carcinoma model. When the mice were treated with HFD2 orally, the tumor growth rates in HFD2 treated group were much lower than in the control group. Orally administered HFD2 at 10 mg/kg/day significantly decreased tumor volumes by 77%. In the examination of vascular structure at tumor tissues, tumor tissue sections were stained with anti-CD34 antibody and then visualized. Especially, treatment with HFD2 of 10 mg/kg/day decreased the number of CD34 positive blood vessels by 82% and inhibited cell proliferation compared to the control group in the PCNA-staining result. Therefore, we chose HFD2 as a bile acid conjugated and polysaccharide-based drug candidate with an optimized molecular size for tumor growth inhibitory effect.

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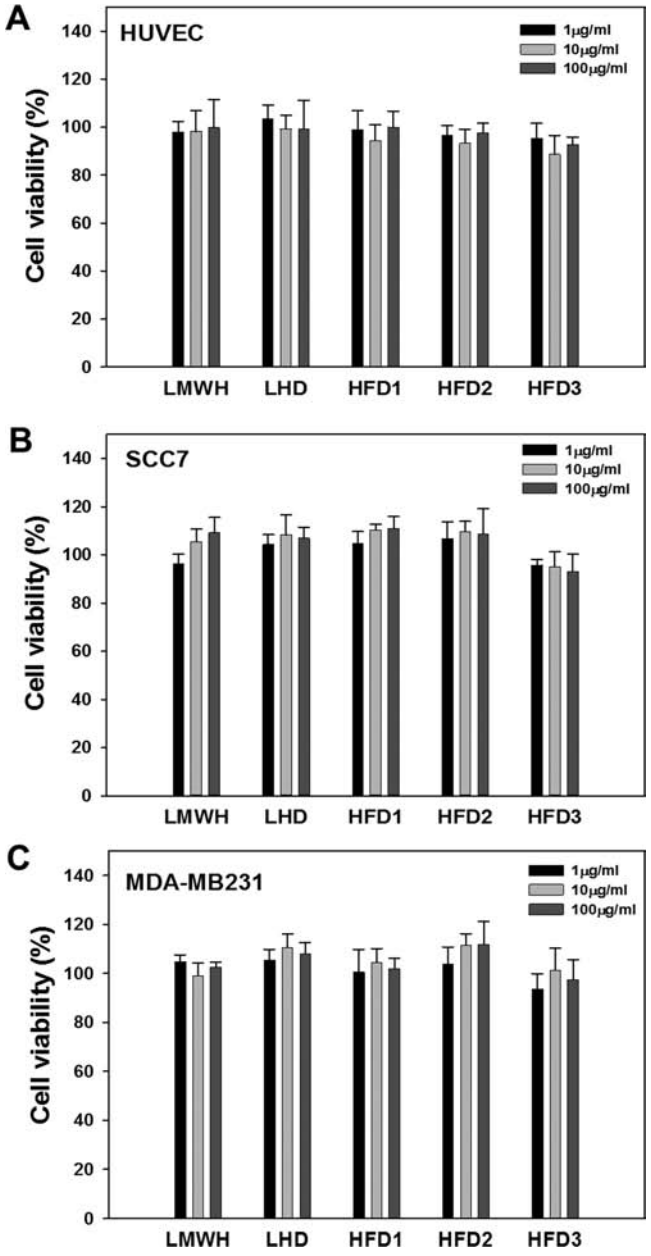

Figure 3. In vitro cytotoxicity assay of LMWH, LHD, and HFDs against (A) HUVEC, (B) SCC7, and (C) MDA-MB231, The percentage of live cells was calculated by dividing the number of live cells by the total number of live and dead cells compared to untreated control group. The data are plotted as mean ± SD (n = 6).

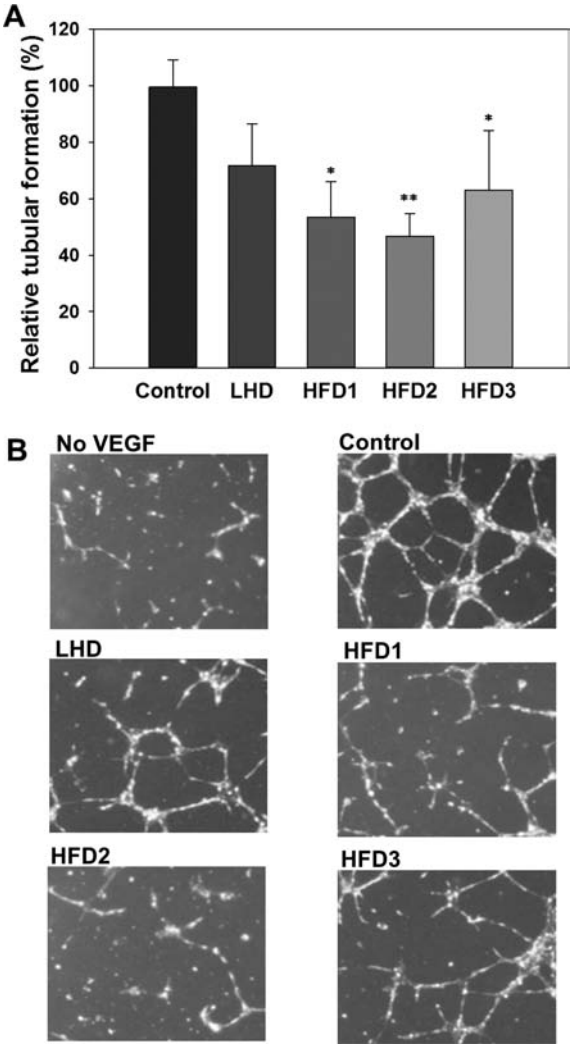

Figure 4. Anti-angiogenic effect of LHD and HFDs on endothelial tube formation with $VEGF_{165}$. (A) The degree of tube formation was quantified by counting the number of branch points. (B) Representative photomicrographs of HUVECs after treatment of LHD or HFDs. Each bar indicates the mean ± SD (n = 4). *p < 0.05 vs control group, **p < 0.001 vs control group.

DISCUSSION

Glycosaminoglycans are linear polysaccharides which have molecular masses of about 2×10^4 Da. They also hold a large number of water molecules in their molecular structure and occupy enormous hydrodynamic space.[22] Heparin and heparan sulfates, which are biologically important polysaccharides, have molecular weight greater than 10 kDa. Given the importance of protein−glycosaminoglycan interactions, small-sized polysaccharides and oligosaccharide fragments are important biomaterials for drug design. $VEGF_{165}$ is an important tumor-related growth factor in cancer research and its heparin-binding domain has been studied. The heparin-binding site on $VEGF_{165}$ consists of positively charged residues such as Arg123, Arg124, Arg145, Arg149, and Arg156.[6] The size of the heparin-binding site of $VEGF_{165}$ is similar to the heparin-derived hexasaccharide site observed in basic fibroblast growth factor (bFGF). Considering heparin binding sites on growth factors, we expected that the heparin-binding domain of $VEGF_{165}$ will be suitable for heparin and heparin derivatives of a smaller size.

The ability of heparin and LMWH to bind with $VEGF_{165}$ implies that they can act as anti-angiogenic agents to interfere with growth factor mediated angiogenesis processes. Molar based SPR study showed similar binding affinities of UFH, LMWH, and heparin fragment to $VEGF_{165}$ (data not shown). Therapeutic effects of heparin-based oligosaccharides also indicate that heparin needs a smaller molecular size ideal for optimum binding with $VEGF_{165}$. Considering the molecular weight of heparin, heparin fragments could be preferred because they have higher molar concentrations when they were treated with the same amount. However, the anticoagulant effect of heparin fragments still existed although the molecular sizes of heparin were decreased.

The purpose of this experiment was to determine the optimum molecular size of heparin derivative for an orally

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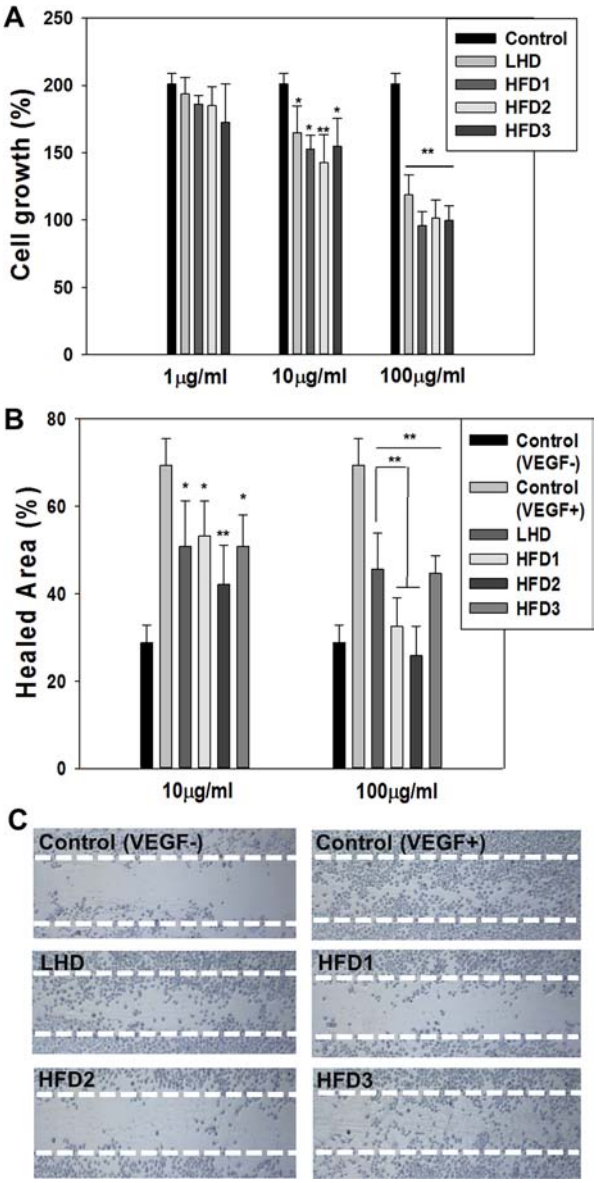

Figure 5. Effects of LHD and HFDs on VEGF-induced endothelial cell proliferation and wound healing assay. (A) Cell proliferation after 72 h incubation was evaluated using CCK assay. (B) Confluent layers of HUVECs were wounded and then, $VEGF_{165}$ (20 ng/mL) was added. The wound area was measured after 24 h. Each bar indicates the mean ± SD (n = 5). *p < 0.05 vs control group. **p < 0.001 vs control group.

available heparin based anticancer agent with minimized anticoagulant activity. We have already reported that DOCA conjugated heparin derivatives were orally available without anticoagulant activity. In the present experiment, to optimize the polysaccharide size, we prepared heparin fragments of different sizes by conjugating heparin fragments with DOCA molecules. They showed different molecular sizes but the conjugated DOCA amounts were maintained according to the NMR peak analysis. In this study, LHD and HFDs did not present any significant toxic effects on the cells and showed size-dependent anti-angiogenesis effects related to $VEGF_{165}$ inhibition. This means that the DOCA conjugated heparin fragments are not toxic and might not directly inhibit tumor cells by cytotoxic effect. HFDs inhibited the proliferation and migration of vascular endothelial cells at 100 μg/mL in vitro experiments. In particular, HFD2 showed improved effects in the $VEGF_{165}$-accelerated tubular formation assay by preventing microvessel formation of HUVECs more than other HFDs (Figure 4).

We also confirmed the $VEGF_{165}$ inhibition effects of HFDs in vivo murine tumor graft model using SCC7 cells and human tumor graft model using MDA-MB231 cells. In our previous studies, we described the anti-angiogenesis effect of LHD and also reported the bile acid transporter mediated drug delivery system using DOCA. Here, we proved that the molecular size of LMWH could be controlled to the heparin binding site of VEGF and optimized for growth factor inhibition. In an animal study, HFDs were orally administered to SCC7 tumor bearing mouse at 10 mg/kg/day, HFD2 was determined as the therapeutic material with the proper molecular size to inhibit angiogenesis processes (Figure 6). Using MDA-MB231 human breast carcinoma, we also confirmed the tumor growth inhibitory effect of HFD2 as a drug candidate with optimized molecular size (Figure 7).

VEGF, bFGF, and other scatter factors have been reported to be dependent on the mechanics of heparin and heparan sulfate related to angiogenesis process.[33−35] The crystallographic structures of proteins in the presence of heparin have been determined by X-ray crystallography, providing the information about the proper molecular size of heparin for protein binding, basic fibroblast growth factor (Pentasaccharide), acidic fibroblast growth factor (Hexasaccharide), FGF1/FGFR2 (Decasaccharide), and hepatocyte growth factor (Pentasaccharide).[36−38] The crystallographic structure of $VEGF_{165}$ with heparin or heparin conjugates has not been confirmed, but on the basis of our results, the heparin binding to VEGF could be optimized by small heparin fragments or heparin fragments conjugates such as HFD2 (Hexasaccharide).

In conclusion, the present study showed that the HFDs could have anti-angiogenic effects by blocking the activity of $VEGF_{165}$ depending on its molecular size. For maximum angiogenesis inhibitory effect and low toxicity, HFD was synthesized using heparin fragments and DOCA. In several in vitro experiments, HFDs inhibited the angiogenic effect of $VEGF_{165}$ differently based on its average molecular weight. Among them, HFD2 could delay tumor growth significantly in both murine cancer and human breast carcinoma model. The results indicate that HFD2 could be an anticancer agent with an ideal molecular size.

EXPERIMENTAL PROCEDURES

Materials. Heparin, Deoxycholic acid (DOCA), *N*-hydroxylsuccinimide (NHS), 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDAC), ethylene-diamine, sodium nitrite ($NaNO_2$), and formamide were purchased from Sigma Chemical Co. (St. Louis, MO). LMWH (Nadroparin) was obtained from the Nanjing King-Friend Biochemical Pharmaceutical Company Ltd. (Nanjing, China). *N,N*-Dimethylformide (DMF) was purchased from Merck (Darmstadt, Germany). Coatest Anti-Factor Xa assay kit was purchased from Chromogenix (Milano, Italy). Growth factor reduced Matrigel was purchased from BD Bioscience. Endothelial basal medium (EBM) were obtained from Clonetics Corp. (San Diego, CA) and endothelial growth medium-2 (EGM-2) was from Lonza (Walkersville, ML).

Synthesis of Size-Controlled Heparins and Deoxycholic Acid Conjugated Heparins. Heparin fragments were prepared by controlled nitrous acid depolymerization of

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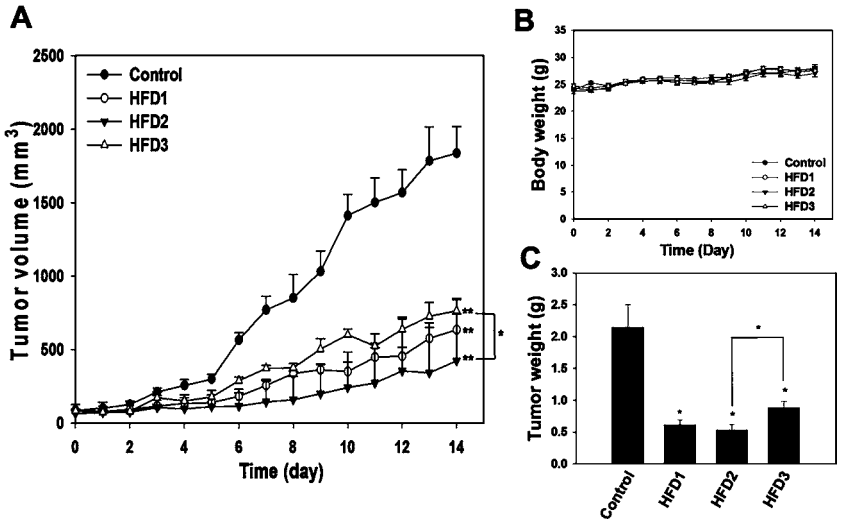

Figure 6. In vivo tumor growth inhibition test of HFDs in SCC7 inoculated mice. (A) Tumor volumes of control (●) or after oral administration of HFD1 (○), HFD2 (▼), and HFD3 (Δ) at a dose of 10 mg/kg/day. (B) Body weight changes. (C) Isolated tumor mass after 14 days (n = 7). Data are the mean ± standard error of mean. *p < 0.05 vs control group, **p < 0.001 vs control group.

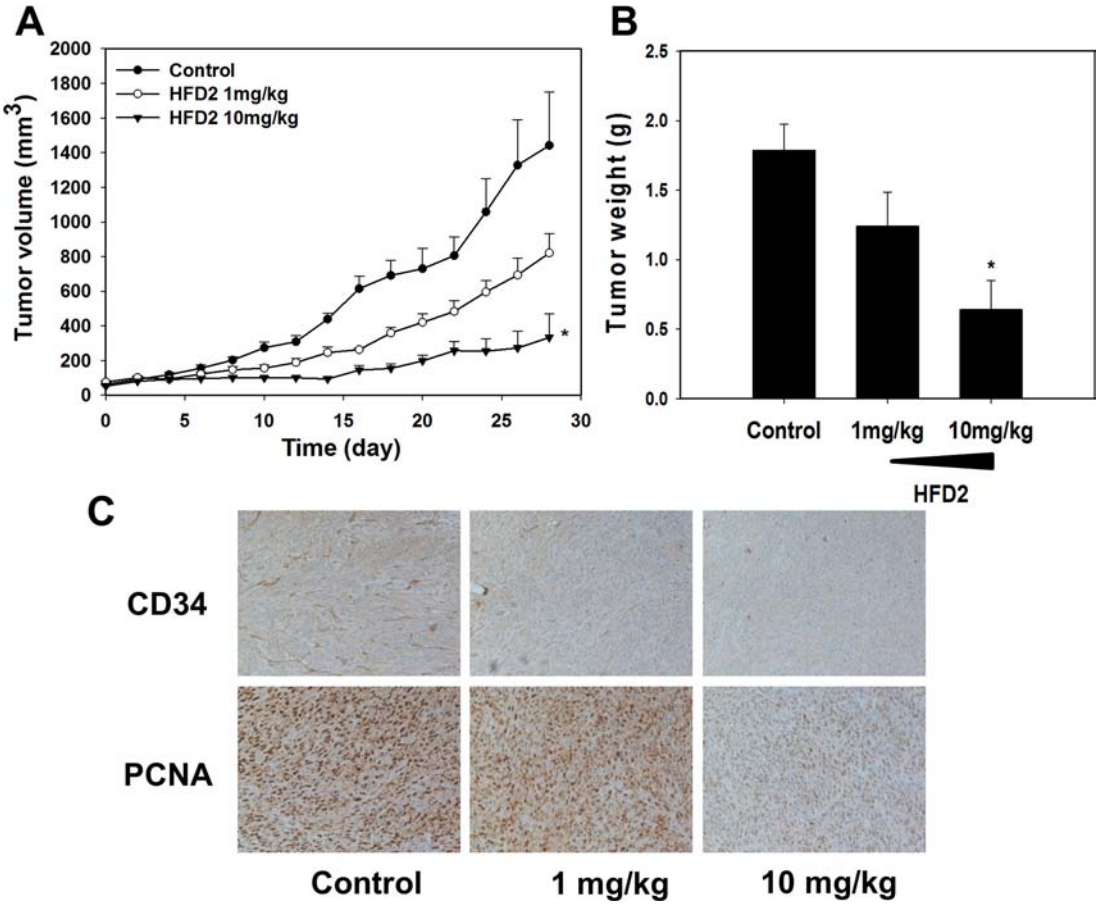

Figure 7. Anticancer effect of HFD2 in MDA-MB231 human breast carcinoma xenograft model (n = 7). (A) Tumor growth curves for control (●), HFD2 (○, 1 mg/kg/day; ▼, 10 mg/kg/day). (B) Isolated tumor weight. (C) Immunohistochemistry with CD34, PCNA of tumor tissue (200×). Administration of HFD2 (1 mg/kg/day and 10 mg/kg/day) decreased the number of CD34 positive blood vessels 69.4% and 82%, respectively. The error bar represents SE *p < 0.05 vs Control group.

LMWH. Briefly, LMWH (1 g) was dissolved in 20 mL of water and its pH was adjusted to 2 with 1 N HCl. $NaNO_2$ was added, and the reaction mixture was incubated at 4 °C and stirred for 30 min. The molar ratios between LMWH and $NaNO_2$ were 1:1, 1:2.5, and 1:4. The pH of the solution was adjusted to 6 with NaOH, and then the solution was precipitated in ethanol. Both the precipitant and 100 mg of $NaBH_4$ were dissolved in 20 mL of water, followed by incubation for 1 h. Heparin fragments were recovered by precipitation in ethanol. HFDs were synthesized following the previous procedure.[39] Briefly,

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heparin fragments and Et-DOCA (*N*-deoxycholylethylenediamine) were reacted using EDAC and NHS in formamide/DMF at room temperature for 12 h. Then the products were purified by methanol precipitation, followed by ethanol. The purity of products was checked by TLC using methanol solvent.

Characterization of Size-Controlled Heparins and Deoxycholic Acid Conjugated Heparins. The average molecular weight and polydispersion were measured by gel permeation chromatography (GPC) and mass spectrometer. The lyophilized samples were dissolved in 1 mL of distilled water and further analyzed by GPC using TSK-2000 column at a flow rate of 1 mL/min. The eluted materials were monitored by the refractive index (RI) detector and the eluent was 0.05 M Na_2SO_4 buffer. The dextran standard curve was used for the molecular weight calculation. The synthesized materials were characterized using proton nuclear magnetic resonance (^{1}H NMR, JEOL JNM-LA NMR, Japan). ^{1}H NMR spectra were recorded at 500 MHz using D_2O and DMSO-d_6 as solvent. The chemical conjugation ratio of heparin and deoxycholic acid was determined by analyzing the NMR peaks at 1−1.2 and 5.0−5.5 ppm using mixed LMWH and DOCA solution with different weight ratios.

Anticoagulant Activity Test. Heparin and heparin conjugates (100 μL) was mixed with 100 μL of antithrombin III (ATIII) solution and incubated at 37 °C for 3 min and 100 μL of Factor Xa (FXa) was added to the solution. The substrate (200 μL) was then added and incubated at 37 °C for 3 min. Anti-FXa activity was calculated from the absorbance at 405 nm by UV/vis spectrometer (n = 3).

Cell Viability Test. The cytotoxic effect of DOCA conjugated heparin was evaluated by using SCC7 and MDA-MB231 cancer cells, respectively. The cells were seeded into 96-well plate (1×10^4 cells/well) in 100 μL of supplemented DMEM. When the plate was confluent with cells, LHD and HFDs were added to reach a total medium volume of 200 μL. The samples were incubated at 37 °C for 24 h. To determine the number of live cells, 10 μL of CCK-8 solution was added to each well and incubated for 1 h. Absorbance was quantified at 450 nm using a microplate fluorescence reader. Cell viability was expressed as a ratio of absorbance by the untreated control group (n = 6).

Endothelial Tubular Formation Assay. HUVECs were cultured in EGM-MV2 medium for 6 days. We prepared a 96-well plate in incubator and a growth factor free Matrigel in ice bath. 100 μL of Matrigel was added to the 96-well plate and incubated for 30 min at 37 °C. Then, HUVECs (4×10^4 cells/well) were placed on the Matrigel and cultured in 50 μL of EBM medium containing $VEGF_{165}$ (40 ng/mL, Peprotech). Then, 50 μL of LHD or HFDs (40 μg/mL) was added. After 6 h of incubation in 5% CO_2 at 37 °C, the vessel formation was observed through a reverse phase-contrast photomicroscope and branch points were counted (n = 4).

Cell Proliferation Assay. The antiproliferation effect of LMWH, LHD, and HFDs was assayed with HUVECs with VEGF. Cells were seeded at a density of 1×10^4 cells per well in 100 μL of M199 medium (1% FBS). The samples were incubated with VEGF (10 ng/mL) at 37 °C for 72 h and the medium was changed every day. To determine the final cell growth, 10 μL of CCK-8 solution was added. Absorbance was quantified at 450 nm using a microplate fluorescence reader after 2 h. Cell growth was expressed as ratios of absorbance by the $VEGF_{165}$ untreated control group (n = 5).

Wound Healing Assay. After HUVECs were harvested by trypsin-EDTA, the cells were seeded in a 24-well plate in EGM-MV2 medium. After the cells reached confluence, HUVECs were wounded with a pipet chip by creating a wound in the center of well and incubated further in M199 medium (FBS 1%) for 2 h. LHD and HFDs were added into each well with different concentrations and incubated with $VEGF_{165}$ (10 ng/mL). The migration of the cells was stopped 24 h later by exchanging the M199 medium with 4% paraformaldehyde solution. After the cells were fixed for 20 min at 4 °C, they were washed with normal saline, and stained with 0.001% toluidine blue (Sigma-Aldrich Corp.) for 60 min (n = 5).

Tumor Growth Inhibition by HFDs (SCC7). SCC7 (murine squamous cell carcinoma) was cultured in high glucose DMEM medium containing 10% fetal bovine serum and antibiotics. After SCC7 cells were harvested, 10-week-old male C3H/HeN mice (Orient Bio, Korea) were injected with cells (1×10^6 cells/60 μL) subcutaneously into the flanks of mice. On day 6, mice in each group received 10 mg/kg daily of HFD1, HFD2, or HFD3, with 2 mg/kg of poloxamer and labrasol as solubilizer. Mice were fasted for 4 h before oral administration. The size of tumor and body weight was daily measured. Tumor volumes were calculated as $a^2 \times b \times 0.52$ (a = width, and b = length) (n = 7). All procedures for animal experiments were approved by the Committee on the Use and Care on Animals according to the regulations of the Institutional Animal Ethics Committee of the Seoul National University animal care facility.

Human Tumor Growth Inhibition by HFD2. We investigated the therapeutic effect of HFD2 on growth of human breast cancer (MDA-MB231) in 10-week-old male athymic nude mice (n = 7). A subcutaneous tumor was established by inoculating MDA-MB231 cells (1×10^7 cell/100 μL) into the back of a mouse. After sacrifice of the mice, tumor tissues were histologically evaluated by staining with anti-CD34 antibodies and proliferating cell nuclear antigen (PCNA). The number of microvessels was measured by counting in four areas at 200×.

Statistical Analysis. We analyzed the results by comparing the means of groups in in vitro and in vivo experiments using one-way analyses of variance (ANOVA) followed by Bonferroni's post hoc tests using Sigmaplot 12. P-values less than 0.05 were considered to be statistically significant.

AUTHOR INFORMATION

Corresponding Author

*E-mail: yrbyun@snu.ac.kr.

Notes

The authors declare no competing financial interest.

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