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Research Paper

Antiangiogenic Effect of Bile Acid Acylated Heparin Derivative

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Purpose. Chemically modified heparin–DOCA was prepared and found to have markedly lower anticoagulant activity than heparin. In the present study, we elucidated the antiangiogenic and antitumoral activities of heparin–DOCA derivative.
Methods. To evaluate the antiangiogenic and antitumoral effects of heparin–DOCA, capillary-like tube formation assay, Matrigel plug assay *in vivo*, western blotting for FGFR phosphorylation, ERK and p38 MAPK activities, tumor growth of SCC *in vivo* and immunostaining of blood vessels in tumor tissues were performed.
Results. Heparin–DOCA inhibited capillary-like tubular structures of endothelial cells and bFGF-induced neovascularizations in Matrigel plug assays. Signaling experiments showed that heparin–DOCA significantly inhibited angiogenesis by suppressing the phosphorylation of FGFR and its downstream signal pathways (ERK and p38 MAPK activities). The antiangiogenic activity of this heparin derivative was found to be closely associated with antitumoral activity in a mouse model. In addition, histological evaluations supported the inhibitory effect of heparin–DOCA on blood vessel formation in tumor tissues.
Conclusion. Heparin–DOCA derivative exerted a significant antitumoral effect by inhibiting angiogenesis resulting from the disruption of FGF/FGFR and its downstream signal pathways, and could be applied to treat various angiogenic diseases.

KEY WORDS: antiangiogenesis; heparin; heparin–DOCA; HUVECs; SCC.

INTRODUCTION

Heparin is a highly sulfated natural polysaccharide, which is mainly composed of alternating units of sulfated glucuronic acid and glucosamine derivatives (1). In addition, it has a well known anticoagulant effect, and interferes with the activities of growth factors such as bFGF and VEGF, thus inhibiting angiogenesis and tumor progression (2,3). Heparin is also known to attenuate metastasis by blocking selectin-mediated intercellular interactions, for example, tumor-platelet and tumor-endothelial cells (4–7). Moreover, heparin inhibits or stimulates tumor growth and metastasis depending on cancer cell type and the animal model used (8). However, heparin can only be administered at relatively low concentrations because of its strong anticoagulant activity, which can induce hemorrhages as side effect (9).

To overcome the side effects induced by high dose or long-term heparin treatment, a number of heparin derivatives with non-anticoagulant activity have been developed, and evaluated their inhibitory effects on angiogenesis, tumor growth, and metastasis (10–15). Carboxyl-reduced heparin, (*N*-*O*-desulphated, *N*-resulphated) heparin, and (*N*-desulphated, *N*-reacetylated) heparin inhibit heparanase activity and significantly reduce lung metastasis by B16-BL6 melanoma cells (11). Moreover, heparin–steroid conjugates and non-anticoagulant heparin-carrying polystyrene (NAC-HCPS) inhibit angiogenesis and tumor growth (12,14). Neoheparin (carbodiimide-modified-heparin) can prevent cancer cell proliferation and induce apoptosis (15).

New chemically modified heparin derivative (heparin–DOCA) was prepared by covalently bonding the amine group of *N*-deoxycholylethylenediamine (DOCA-NH_2) to the carboxylic acids of heparin via amide formation, as we described previously (16,17). Because this heparin derivative has low anticoagulant activity, it can be used at higher concentrations than heparin. It has been reported that

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ABBREVIATIONS: aPTT, activated partial thromboplastin time; bFGF, basic fibroblast growth factor; DOCA-NH_2, *N*-deoxycholylethylenediamine; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; HSPGs, heparan sulfate proteoglycans; HUVECs, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; SCC, squamous cell carcinoma.

systemically administered heparin has high binding affinity, also it can be taken up by dividing vascular endothelial cells, which are abundant in tumors (18–20): this suggests that heparin or heparin derivatives might target susceptible dividing tumor endothelial cells. In the previous report, heparin–DOCA prevented the proliferation of endothelial cells and squamous cell carcinoma (SCC) cell. Heparin–DOCA had safety in mice model when it was repeatedly treated for long period, and it also exhibited a potential as a drug carrier for a variety of hydrophobic drugs due to its structural characteristics (17). It is considered that heparin–DOCA is more applicable for cancer therapy compared to other heparin derivatives. In this study, we evaluated heparin–DOCA as an angiogenesis inhibitor using *in vitro* and *in vivo* angiogenesis assays, and showed its antitumoral effect against squamous cell carcinoma (SCC) tumors.

MATERIALS AND METHODS

Chemicals

Unfractionated heparin (UFH, 167 U/mg) of average molecular weight *ca.* 12,000 Da, was purchased from Pharmacia Hepar Inc. (Franklin, OH, USA). Anhydrous dimethylformamide (DMF), anhydrous formamide, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were from Sigma Chemical Co. (St. Louis, MO, USA). Acetone was obtained from Merck (Darmstadt, Germany), and all reagents were of analytical grade and were used without further purification.

Synthesis and Characterization of Heparin–DOCA

Heparin–DOCA was synthesized by reacting heparin with the bile acid analogue, *N*-deoxycholylethylenediamine (DOCA-NH_2), as previously described (17). In brief, heparin (0.1 g) was dissolved in formamide (5 ml) by gentle heating. EDAC (0.096 g) was mixed with this heparin solution, and this was followed by DOCA-NH_2 (1.086 g) dissolved in anhydrous DMF (5 ml). This reaction was allowed to proceed at room temperature under a nitrogen atmosphere for 24 h. The mixture was then precipitated in excess cold acetone. The precipitate was carefully washed three times with cold acetone to remove excess DOCA-NH_2, and dried in vacuum. Dried heparin–DOCA was suspended in water and lyophilized to produce white powder.

Heparin–DOCA was analyzed using ^{1}H-NMR (JEOL JNM-LA 300 WB FT-NMR, Tokyo, Japan). The degree of substitution, defined as the number of DOCAs per heparin molecule, was determined using a titration method (21). The anticoagulant activity of heparin–DOCA was measured using established bioactivity assays, such as FXa chromogenic and aPTT assays (22).

Tubular Formation of Human Umbilical Endothelial Cells (HUVECs)

HUVECs were isolated from human umbilical cord veins by collagenase treatment and used in passages 2–7 (23). The cells were grown in the M199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF (Upstate Biotechnology, Lake Placid, NY, USA), and 5 U/ml heparin at 37°C under a humidified atmosphere of 5% CO_2 and 95% O_2.

In vitro endothelial tube formation was performed as described previously (24). Matrigel (100 μl, BD Bioscience, San Jose, CA, USA) was added to each well of a 96-well plate and allowed to polymerize for 30 min at 37°C. HUVECs were suspended in medium at a density of 4×10^5 cells/ml, and 0.1 ml of the cell suspension was added to each well coated with Matrigel, with/without varying levels of heparin or heparin–DOCA indicated in Fig. 2. Cells were incubated at 37°C, and then photographed.

In Vivo Assessment of Angiogenesis Using Matrigel Plug Assays

To assess the antiangiogenic properties of heparin–DOCA, Matrigel plug assays were performed as described previously (24). Briefly, 637.5 μl of liquid Matrigel–PBS mixture was injected subcutaneously into the backs of male C57BL/6 mice at 4°C; once inside the animals, it solidified. The Matrigel contained bFGF (R & D systems, Inc., McKinley, NE, USA) at a final concentration of 500 ng/ml to stimulate angiogenesis, and heparin or heparin–DOCA at a final concentration of 500 μg/ml. All treatment groups contained 5 to 6 mice. After 10 days, mice were sacrificed, Matrigel plugs were removed, and fixed in 4% paraformaldehyde. To evaluate angiogenesis, plugs were stained with H&E to observe blood vessels within Matrigel. The number of blood vessels in 5 to 6 high-power fields (×200) were counted and averaged. Microvessels were also assessed by CD31 immunostain on frozen sections of Matrigel. After rinsing and blocking, the sections were incubated with a rat anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, CA, USA) at 1:30 dilution, followed by Rhodamine-conjugated secondary goat anti-rat IgG (Santa Cruz, CA) at 1:200 dilution, and with 4′,6-diamidino-2-phenylindole (DAPI) counterstain. In addition, the hemoglobin (Hb) content within the Matrigel plugs was measured using the Drabkin's reagent kit (Sigma, St. Louis, MO, USA). The Matrigel plugs were homogenized in 50 μl of double-distilled H_2O using disposable pellet pestles for microtubes. Homogenates were incubated in 0.5 ml of Drabkin's solution for 15 min at room temperature. Samples were centrifuged to cuvetts, and the absorbance was measured at 540 nm. Drabkin's solution was used as a blank. The absorption is proportional to the total hemoglobin concentration.

Western Blot Analysis for FGFR, ERK and p38 MAPK Phosphorylation

Serum-starved HUVECs were treated with heparin–DOCA (250 μg/ml) at 37°C for 1 h and then stimulated with 500 ng/ml of bFGF for 10 min. Cells were lysed using modified radioimmunoprecipitation assay buffer [50 mM Tris–HCl (pH 7.4); 1% Triton X-100; 150 mM NaCl; 0.5 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM phenylmethylsulfonylfluoride (PMSF); 1 mM Na_3VO_4; 10% glycerol and 10 μg/ml of each of aprotinin, leupeptin, and pepstatin] on ice for 1 h. Lysates were clarified by centrifugation (12,000 ×*g* for 10 min at 4°C), and protein concentrations were determined using a Bio-Rad detergent-

compatible protein assay kit (Bio-Rad, Hercules, CA) using BSA as a standard. For detection of phosphorylated FGFR, supernatants were incubated for 2 h to overnight on ice with phosphorylated tyrosine (*pTyr*, Santa Cruz Biotechnology, CA, USA). Protein A-Sepharose was used to collect immunocomplexes and beads were washed with precipitation buffer. Immunoprecipitates were resuspended in 2× SDS sample buffer and lysates were separated by electrophoresis on SDS-polyacrylamide gels. Gels were transferred onto polyvinylidine difluoride membranes and blots were blocked with TBST (50 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20) containing blocking reagents for 1 h. The blots were then incubated with antibody against FGFR for 1 h at 4°C. For western blot analysis, protein lysates were analyzed by SDS-PAGE, and western blotted using anti-FGFR antibody (Santa Cruz Biotechnology). Proteins were visualized by enhanced chemiluminescence (Amersham Parmacia Biotech., Piscataway, NJ, USA) and exposed to film. For ERK and p38 MAPK phosphorylation, the blots were incubated with primary antibodies for 16 h at 4°C and washed in TBST. The visualized proteins were exposed to film. The following antibodies were used in this study: anti-phospho ERK, and anti-ERK antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho p38, and anti-p38 antibodies from Cell Signaling Technology (Beverly, MA, USA).

Inhibitory Effect of Heparin–DOCA on Tumor Growth in Mice

Male C3H/HeN mice (8-weeks old, Institute of Medical Science, Tokyo, Japan) weighing 23 to 26 g were given dorsal subcutaneous (s.c.) injections of 1×10^6 SCC cells. When tumors reached 50 to 70 mm^3, the mice were given i.v. injections of 100 μl of saline containing heparin (5 mg/kg/3 day) or heparin–DOCA (1 to 10 mg/kg/3 day) for 12 days. Tumors were measured daily with calipers. On the 14th day, mice were sacrificed and tumors were removed. Treatment groups contained 7 to 8 mice. Tumor tissues were isolated from three representative treated and untreated tumor-bearing mice. Microvessel detection and the determination of proliferating cell nuclear antigen (PCNA) maker expression in tumor tissues were carried out by immunohistochemistry using a specific anti-CD34 antibody and anti-PCNA antibody (Dako, CA, USA), respectively.

Statistics

The statistical significance of differences between the experimental and control groups was determined using one-way ANOVA. *P* values were considered significant and are indicated by asterisk.

RESULTS

Characteristics of Heparin–DOCA

The structure of heparin–DOCA is shown in Fig. 1. Heparin–DOCA was synthesized by covalently binding DOCA-NH_2 to heparin in the presence of EDAC, and this binding was confirmed by ^{1}HNMR, in which the characteristic peaks of DOCA appeared at 0.65–2.3 ppm. New amide linkages between heparin and DOCA and the intrinsic sulfonamide of heparin showed at 8.04 and 5.3 ppm, respectively. The molecular weight of heparin–DOCA based on the degree of DOCA substitution was 16,990 Da. This modification of carboxyl groups in heparin resulted in low anticoagulant activity *versus* the native material. As expected, the anticoagulant activities of

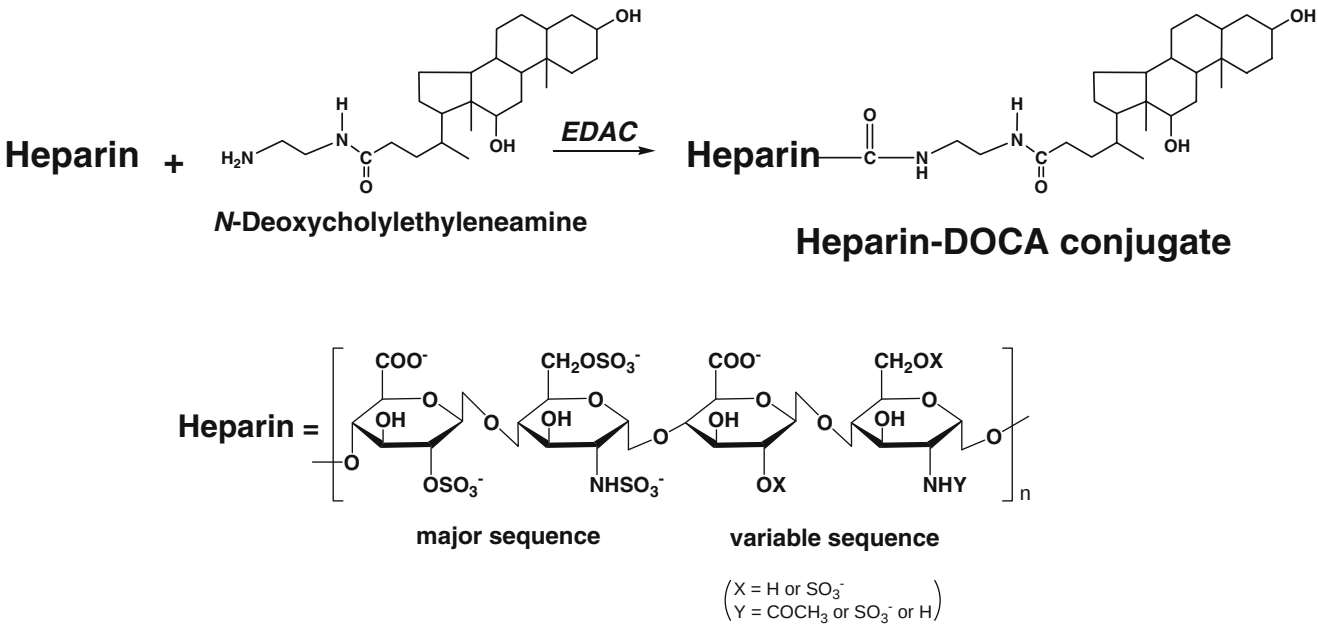


Fig. 1. Synthetic scheme for the preparation of heparin–DOCA. Heparin–DOCA was synthesized by reacting the carboxylic groups of heparin with *N*-deoxycholylethylenediamine (DOCA-NH_2) in the presence of EDAC.

heparin–DOCA as determined by FXa and aPTT assays were 23% and 27%, respectively, *versus* native heparin.

Inhibitory Effect of Heparin–DOCA on Angiogenesis *In Vitro* and *In Vivo*

The impact of heparin–DOCA on capillary-like tube formation by HUVECs was investigated. HUVECs attached to reconstituted extracellular matrix (Matrigel), and then formed capillary-like tubular structures. It is considered that these processes in the extracellular matrix are representative of the latter stages of angiogenesis during differentiation and that they are also involved in cell migration and invasion. HUVECs spread out and generated lateral processes 3 h after being placed onto Matrigel. The presence of heparin–DOCA affected the extent of tube formation by HUVECs (Fig. 2). At 7 h, cell alignments and tubular structure formations were observed in HUVECs treated with heparin–DOCA and the inhibitory effects of heparin–DOCA were evident. As the incubation period continued to 7 h, HUVECs treated with heparin–DOCA gradually lost intercellular contact. Additionally, in our previous study, the result of BrdU assay showed that heparin–DOCA prevented the proliferation of HUVEC by as much as 72.5% at 250 μg/ml, and the MTT assay indicated that cell viability was above 85% after the treatment of heparin–DOCA (17).

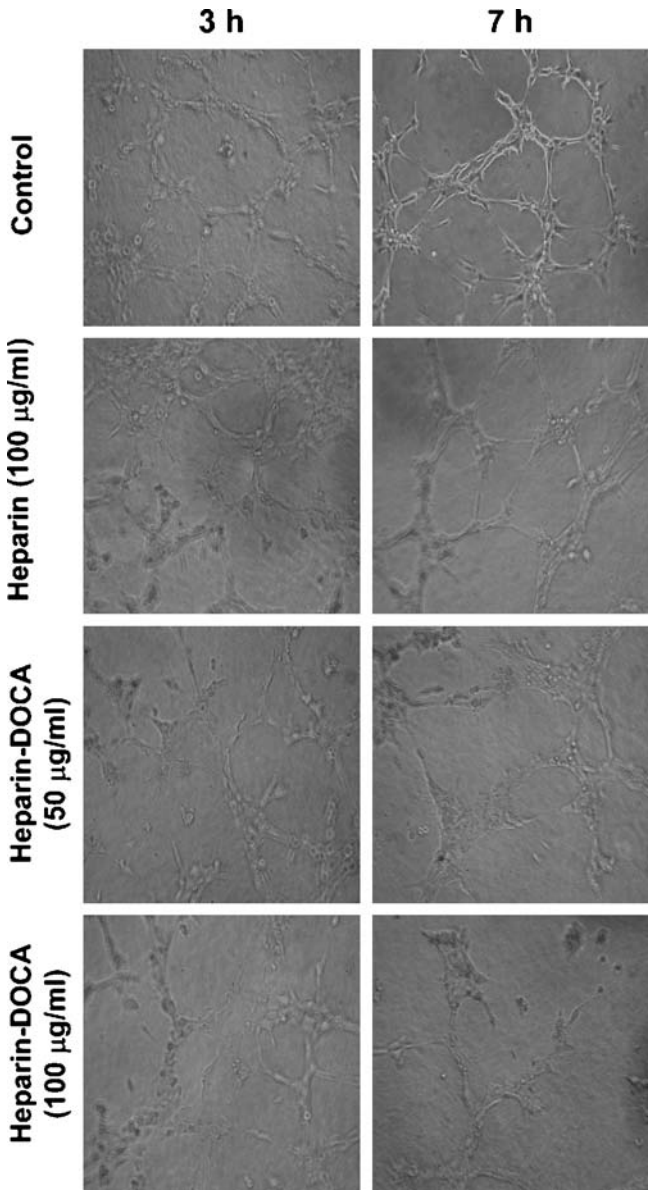


Fig. 2. Heparin–DOCA inhibits human umbilical vein endothelial cell (HUVEC) tube formation. HUVECs were preincubated for 30 min with the indicated concentrations of heparin or heparin–DOCA in Matrigel-coated plates at a density of 4×10^4 cells/well.

Matrigel plug assays allow the angiogenic effects of agents to be examined, and thus, we tested the ability of heparin–DOCA to inhibit bFGF-induced angiogenesis in this model (Fig. 3). When Matrigel plugs were removed from mice at 10 days, plugs treated with bFGF alone were dark-red in color, whereas plugs treated with heparin and bFGF appeared red, indicating the formation of new blood vessels and blood circulation due to new vasculatures in both plus types. In contrast, plugs with mixed heparin–DOCA were pale in their color, implying a significant decrease in blood vessel formation. The number of vessels induced by bFGF alone was nearly 19 vessels per high power field, whereas heparin–DOCA markedly inhibited the number of blood vessels by approximately 85%. The suppression of angiogenesis by heparin–DOCA treatment was consistent with the decrease in the CD31-positive microvessels. In addition, the fact that the hemoglobin content within the Matrigel plugs was reduced up to 82% by heparin–DOCA indicates that heparin–DOCA effectively inhibited angiogenesis within the Matrigel plugs. These results imply that heparin–DOCA inhibits bFGF-induced angiogenesis *in vivo*.

Phosphorylation of FGFR, ERK and p38 MAPK

To determine how heparin–DOCA inhibits angiogenesis by endothelial cells, we investigated the effect of heparin–DOCA on the signal pathways (i.e., phosphorylation of FGFR, ERK and p38 MAPK) induced by bFGF. HUVECs were incubated with/without bFGF in the presence or absence of heparin–DOCA, and FGFR, ERK and p38 MAPK phosphorylation were analyzed. The phosphorylated FGFR, ERK, and p38 MAPK were markedly activated by adding bFGF alone, implying that bFGF caused the increased cell proliferation. But, the addition of heparin–DOCA at 250 μg/ml profoundly inhibited the bFGF-induced phosphorylation of FGFR, ERK and p38 MAPK (Fig. 4). These results suggest that heparin–DOCA directly binds to bFGF and inhibits the interactions between bFGF and FGF receptors, hence inhibiting FGFR phosphorylation as well as ERK and p38 MAPK activation, which may affect cell proliferation, angiogenesis, and tumor progression.

Inhibitory Effect of Heparin–DOCA on Tumor Growth in Mice

To evaluate whether heparin–DOCA inhibits tumor growth *in vivo*, SCC was implanted into the backs of C3H/HeN male mice. Heparin–DOCA inhibited tumor growth and was superior to that of heparin alone (Fig. 5). Heparin produced a 34.4% reduction in tumor volume. On the other

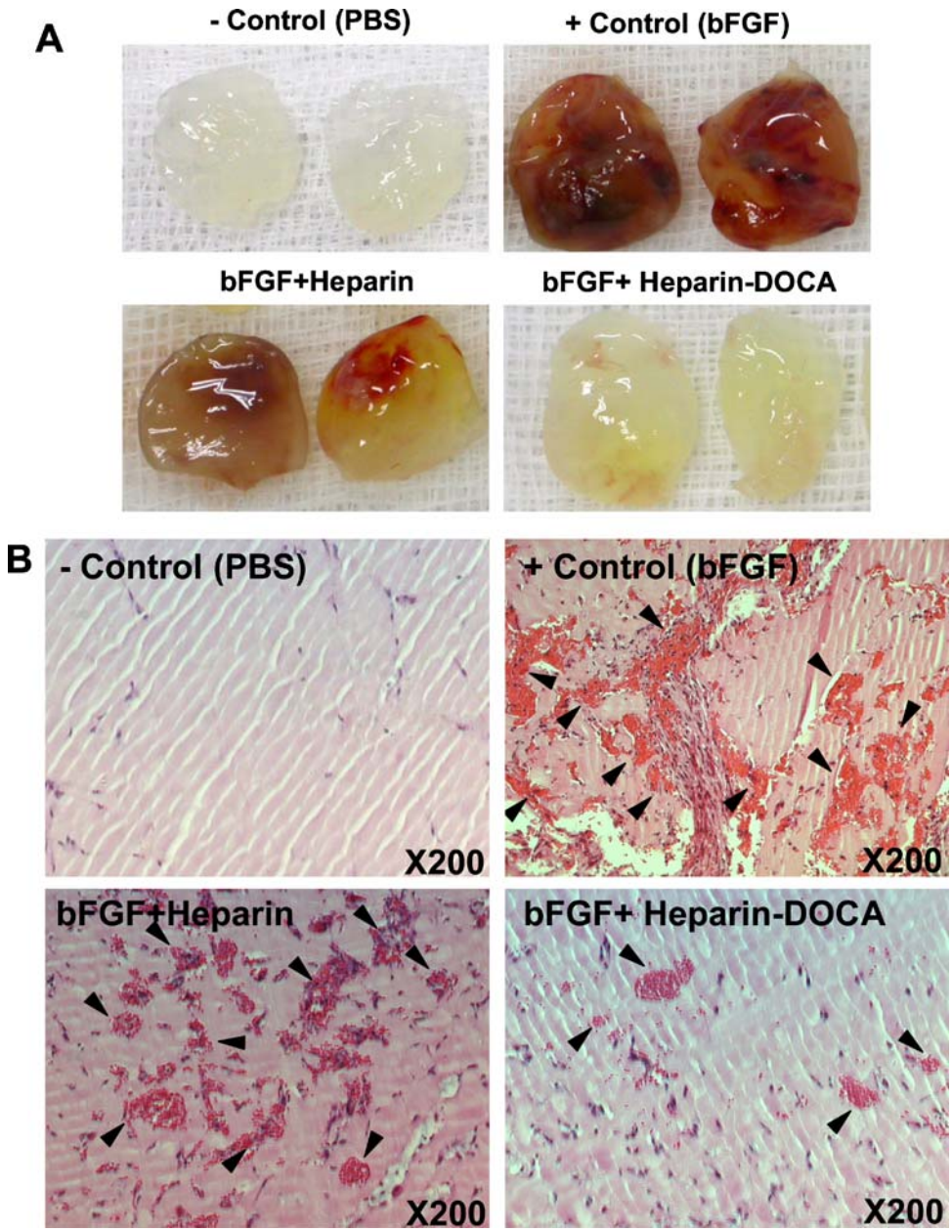
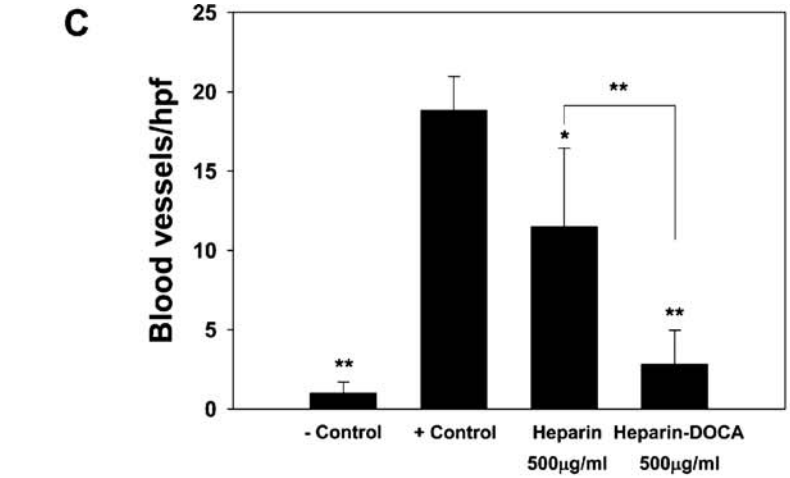
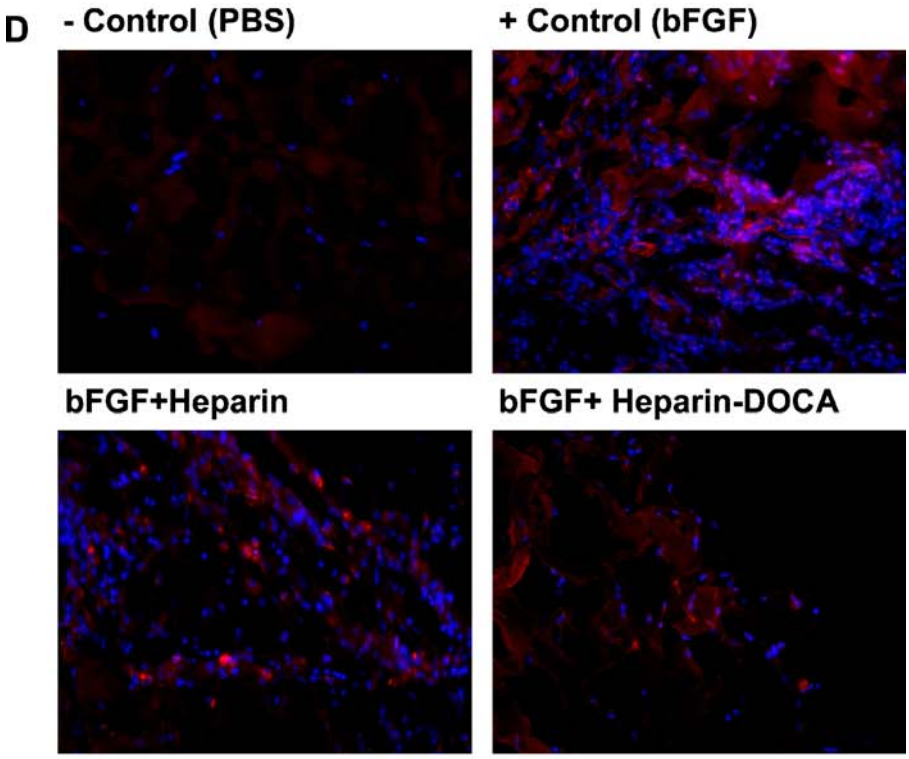
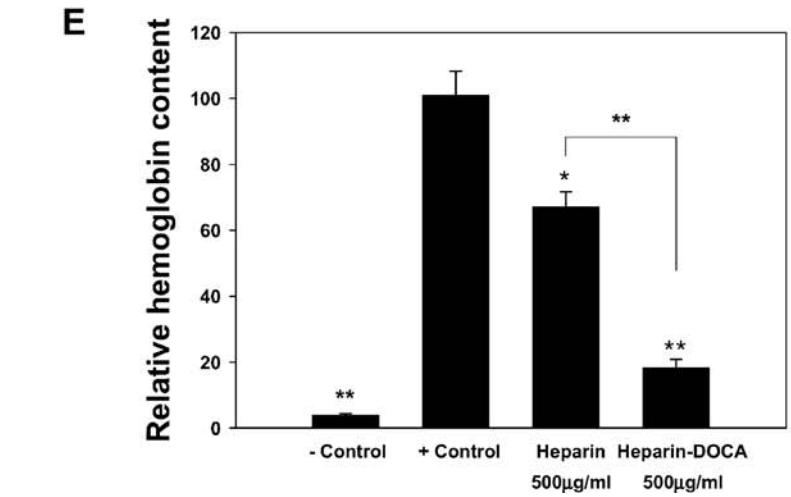


Fig. 3. Inhibition of the bFGF-driven angiogenesis in Matrigel plugs by heparin–DOCA. Matrigel (0.67 ml) containing heparin or heparin–DOCA was injected s.c. into mice. Matrigel plugs contained bFGF (500 ng/ml) with/without heparin (500 μg/ml) or heparin–DOCA (500 μg/ml). After 10 days, mice were sacrificed and Matrigel plugs were excised. (A) Matrigel plugs containing PBS alone (− control), bFGF alone (+ control), bFGF + heparin, or bFGF + heparin–DOCA were photographed. (B) Sections of H&E stained Matrigel plugs were examined by light microscopy. *Arrowheads* indicate blood vessels. (C) The numbers of blood vessels in 5 to 6 high power fields was counted and averaged (H&E stain). (D) Microvessel assessment of Matrigel plugs containing bFGF and heparin or heparin–DOCA. The plugs were sectioned and immunostained with anti-CD31 antibody (*red*) and DAPI (*blue*). (E) Hemoglobin contents within Matrigel were determined by the Drabkin's method. *, $P = 0.002$; **, $P < 0.001$ *versus* + control (bFGF alone), and **, $P < 0.001$ (heparin–DOCA *versus* heparin). The data are presented as means ± SE.

hand, heparin–DOCA at 1, 5 or 10 mg/kg significantly suppressed SCC tumor growth by as much as 49.9%, 62.6% and 78.6%, respectively, *versus* untreated controls. Additionally, in our previous study, the result of BrdU assay showed that heparin–DOCA prevented the proliferation of SCC (17). Indeed, immunostaining data supported the superior antiangiogenic effect of heparin–DOCA (Fig. 6). In contrast to untreated controls and heparin treated mice, heparin–DOCA treated mice showed a profound decrease in the number of CD34-positive microvessels (Fig. 6). These results demonstrate that heparin–DOCA prevents tumor angiogenesis, and thereby suppresses tumor cell proliferation and tumor growth.







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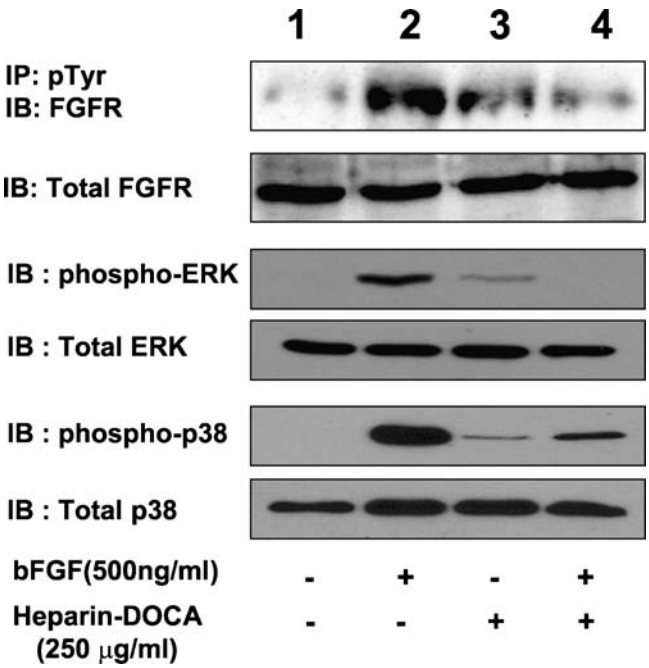

Fig. 4. Effect of heparin–DOCA on bFGF-mediated FGF receptor phosphorylation and ERK and p38 MAPK phosphorylation. Serum-starved HUVECs were preincubated with/without heparin–DOCA (250 μg/ml) and then stimulated for 10 minutes with bFGF (500 ng/ml), and lysates were analyzed by Western blot using antibodies against the indicated protein (*p*, antibody specific for phosphorylated protein).

DISCUSSION

We prepared and characterized heparin–DOCA, comprised of covalently bound heparin as a hydrophilic segment, and DOCA as a hydrophobic segment, as described previously (16,17). The modification of carboxylic groups in heparin resulted a significant decrease in its anticoagulant activity. However, this modification may retain its affinity for growth factors and other proteins, because heparin–DOCA showed high negative charge (nearly −56 mV) in zeta potential measurement (17).

In this study, heparin–DOCA inhibited the organization of endothelial cell tubular structure by preventing intercellular attachments. In addition, heparin–DOCA inhibited bFGF-induced angiogenesis by 85% in Matrigel plug assays *in vivo*. These findings indicate that the inhibition of bFGF-induced angiogenesis by heparin–DOCA may efficiently inhibit tumor angiogenesis (25). Immunostaining data supported the notion that the antiangiogenic effect of heparin–DOCA leads to significant tumor angiogenesis suppression *in vivo*. Therefore, the antitumoral effect of heparin–DOCA is due to its inhibition of tumor angiogenesis, which may be relevant to the biology of malignancy and thus important therapeutically.

Heparin as FGF antagonist inhibits angiogenesis and tumor progression by interfering the activities of growth factors (i.e., bFGF and VEGF) (2,3). Heparin–protein interactions such as the FGF1–heparin or FGF1–FGFR–heparin complex were well studied (26,27). Sulfate groups or carboxyl groups of heparin interact with basic and polar residues (i.e., Asn, Lys, Arg, Gln, etc.) of proteins due to their electrostatic natures. Furthermore, more specific nonionic interactions, such as hydrogen bonding and van der Waals packing are also important in heparin–protein (i.e., FGF or FGFR) interactions. The 6-*O*-sulphate groups of GlcN in heparin are involved in hydrogen bonding to Lys, His and in van der Waals contact with Thr and Val residues (27). Also, FGF or FGFRs can interact with large molecules through hydrophobic interactions because it contains hydrophobic residues (i.e., Tyr, Phe, Leu, Pro, Val, etc.). Indeed, suramin (angiosuppressive agent) blocked the receptor binding of bFGF through hydrophobic interaction between the naphtyl or phenyl rings of suramin and Pro, Cys, Gly, Arg, Gln, or Lys residues of bFGF, as well as ionic, hydrogen bonding interactions (28).

This study shows that heparin–DOCA significantly inhibits bFGF-induced angiogenesis by suppressing the phosphorylation of FGFR, ERK and p38 MAPK (Fig. 4). It is thought that heparin–DOCA interacts with bFGF and changes the conformation and biological activity of bFGF, thereby effectively disrupting the import of HSPG/bFGF/FGFR ternary complex on the surface of endothelial cells (13). Because heparin–DOCA derivative has a high negative charge and hydrophobic moiety (DOCA), it can strongly interact with various proteins through electrostatic as well as hydrophobic interactions. Therefore, heparin–DOCA may interact with bFGF more tightly compared to heparin. In terms of conformation, heparin–DOCA adopts geometries unfavorable for forming ternary complexes with bFGF and FGFR, thereby suppressing FGFR, ERK and p38 MAPK phosphorylation, which regulate cell survival, and finally inhibiting the proliferation of endothelial cells and tumor growth. On the other hand, it is possible that interaction between heparin–DOCA firstly interacts with FGFR instead of FGF. But, heparin has 200 times higher binding affinity for FGF compared to that of FGFR (29), and thus the interaction between heparin–DOCA and FGFR may be negligible. Therefore, we consider that the antiangiogenic

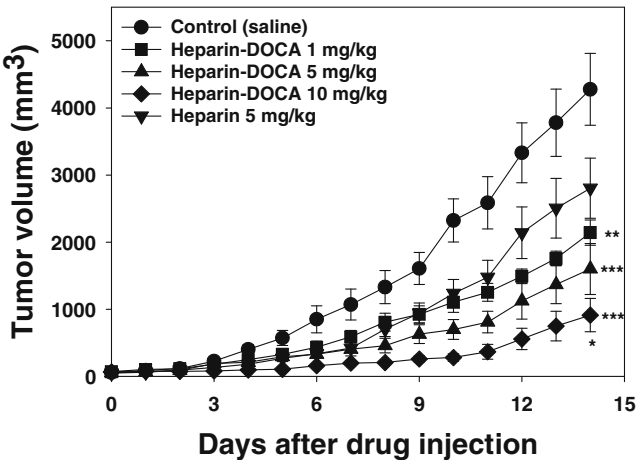

Fig. 5. Antitumoral effects of heparin–DOCA against solid SCC tumors. Mice were given injected s.c. with SCC, and when tumor volumes were 50 to 70 mm^3, the mice were given injected i.v. with normal saline (●), heparin (▼ 5 mg/kg/3 day) and heparin–DOCA (■; 1 mg/kg/3 day, ▲; 5 mg/kg/3 day, ◆; 10 mg/kg/3 day). Each group contained 7 to 8 mice. The sizes of tumors were measured daily. Data are presented as means ± SE. (**$P = 0.012$ and ***, $P < 0.001$ *versus* control; *, $P = 0.039$ heparin *versus* heparin–DOCA at 10 mg/kg).

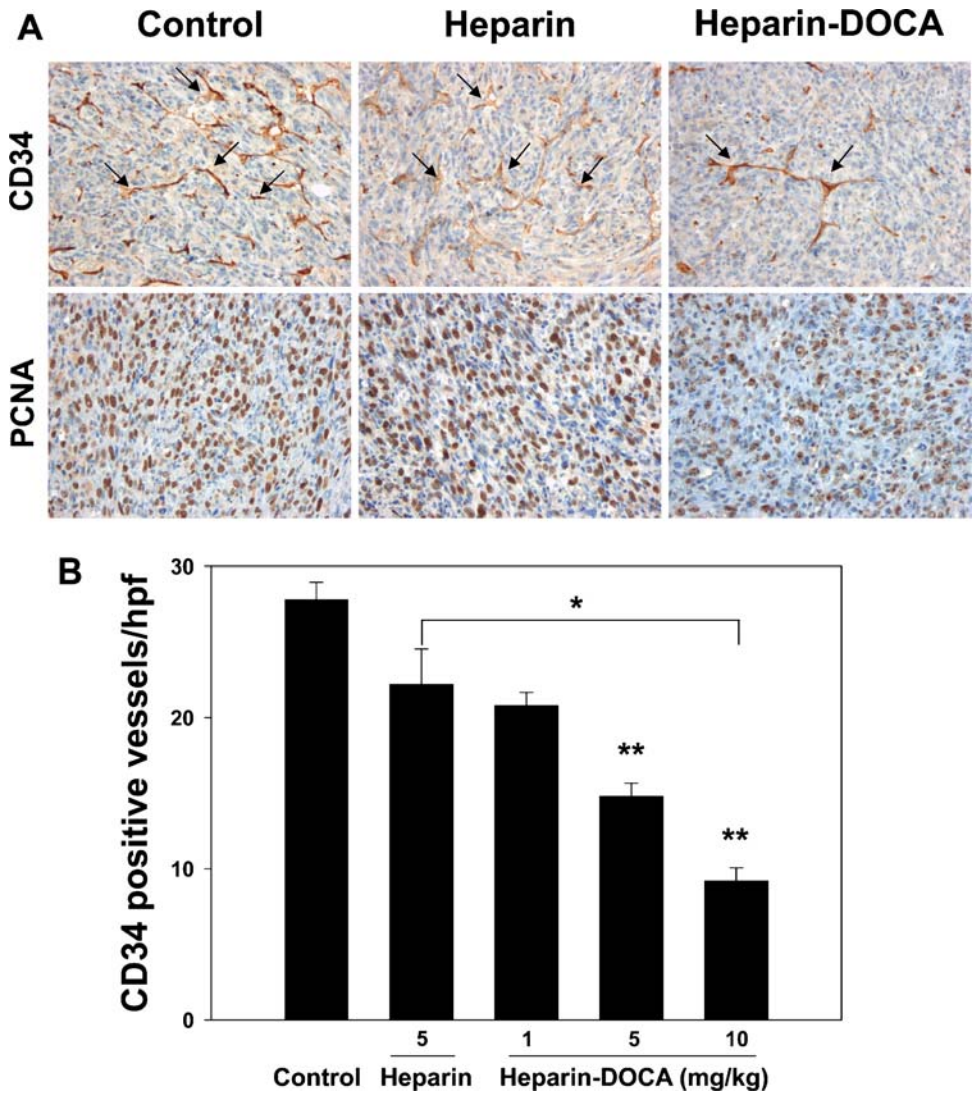

Fig. 6. Immunohistochemistry and blood vessel quantification in SCC tumor tissue. Tumor tissues from animals treated with heparin or heparin–DOCA (5 mg/kg/3 day) were isolated on day 14. (A) CD-34 immunostaining (×200) of endothelial cells and representative vessels in sections (*indicated arrow*), and PCNA immunostaining to detect cell proliferation (×200, proliferating cells: *brown color*). (B) blood vessel quantification in tumor tissues. The numbers of CD34-positive blood vessels in 5 high power fields (×200) were counted and averaged. *Bars*, means ± SE. (**, $P < 0.05$ *versus* control; *, $P < 0.05$ heparin *versus* heparin–DOCA at 10 mg/kg).

effect of heparin– DOCA is responsible for the inhibition of bFGF activity, followed by the suppression of phosphorylation of FGFR.

It has been reported that endothelial cells are freely accessible to blood and preferentially accumulate heparin after i.v. injection (18,30). The systemically administered heparin/heparin derivatives target endothelial cells in tumors because dividing endothelial cells in tumors take up 10 times as much heparin *in vitro* than non-dividing cells (31). In spite of chemical modification, heparin–DOCA may retain the ability to interact with endothelial cells because its negative charge interacts with proteins on the surfaces of endothelial cells. Furthermore, it is possible that heparin–DOCA is localized to blood vessels in tumors due to the high binding affinities to dividing endothelial cells, which may influence endothelial cell proliferation and angiogenesis in tumor tissues.

In order to minimize incidences of side effects (i.e., bleeding) at high doses of heparin, heparin derivatives with low anticoagulant activities have been suggested as promising approaches. Three representative products are heparin–steroid conjugates, 2,3-*O*-desulphated heparin, and NAC-HCPS (Non-anticoagulant heparin-carrying polystyrene), and they have all shown antiangiogenic and antitumor effects in tumors (10,12,14). These heparin derivatives are needed at high amounts of dosages (heparin–cortisol conjugate: 1 to 7 mg/mouse/day; 2,3-*O*-desulphated heparin: 1.5 mg/mouse/day; NAC-HCPS: 2 mg/mouse/day) to achieve their antitumoral effects. Heparin-DOCA exhibits similar antitumor effects against SCC tumors at much lower concentration (0.25 mg/mouse/3 day) compared to other heparin derivatives, even though it is impossible to directly compare their efficacies due to different tumor types and administration routes. In

the previous study, we reported that Heparin-DOCA is safe because they did not induce unexpected side effects and body weight changes when they were periodically administered at various doses (1, 10 and 20 mg/kg/3 day) for nearly one month (17). Moreover, Heparin-DOCA with a high negative charge and a hydrophobic segment may strongly interact with various proteins, blocking more efficiently biological activities of proteins compared to other heparin derivatives. Furthermore, Heparin-DOCA with anti-angiogenic effect can be used as drug carriers that include a number of anti-cancer drugs or angiogenesis inhibitors based on its amphiphilicity, and may improve therapeutic effects against various tumors. Therefore, we find that Heparin-DOCA is more applicable and has high potential for cancer therapy.

CONCLUSION

We report here that heparin–DOCA inhibits the formation of capillary-like vascular structures, retards vascularization within the Matrigel plug implanted into mice, and prevents the proliferation of endothelial cell and tumor cells, thereby suppressing angiogenesis and tumor growth. Moreover, this effect was attributed to its ability to block the biological effects of bFGF, which lead to the suppression of FGFR phosphorylation and its downstream signaling pathways. Thus, heparin–DOCA has potential for the treatment of human diseases related with angiogenesis and may also be used as a drug carrier because of its amphiphilic properties.

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