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Research Paper

Tumor Endothelial Cell Targeted Cyclic RGD-modified Heparin Derivative: Inhibition of Angiogenesis and Tumor Growth

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Purpose. We prepared tumor endothelium targeted cRGD-modified heparin derivative (cRGD-HL) by coupling heparin-lithocholic acid (HL) with cRGDyK, and evaluated inhibition effects of cRGD-HL on angiogenesis and tumor growth.
Methods. To evaluate antiangiogenic activity of cRGD-HL, we performed tests on endothelial cell adhesion and migration to vitronectin, tube formation, binding affinity to purified $\alpha_v\beta_3$ integrin, and *in vivo* Matrigel plug assay. The antitumor activity of cRGD-HL was also evaluated by monitoring tumor growth and microvessel formation in squamous cell carcinoma (SCC7) tumor.
Results. The cRGD-HL significantly inhibited adhesion and migration of endothelial cells to vitronectin, and tubular structures of endothelial cells. Compared to cRGDyK and HL, cRGD-HL has high binding affinity to purified $\alpha_v\beta_3$ integrin. The enhanced antiangiogenic effect of cRGD-HL was confirmed in Matrigel assay by showing the significant inhibition of bFGF-driven angiogenesis and blood vessel formation. It was thought that potent antiangiogenic effect of cRGD-HL was probably due to the interference of $\alpha_v\beta_3$-mediated interaction, resulting in the enhanced antitumoral activity against SCC7 tumor.
Conclusion. These results demonstrated that cRGD-modified heparin derivative enhanced anti-angiotherapeutic effects against solid tumor, and therefore, it could be applied to treat various cancers and angiogenic diseases as a potent angiogenesis inhibitor.

KEY WORDS: angiogenesis; heparin derivative; lithocholic acid; RGD; SCC7.

INTRODUCTION

New blood vessel formation from preexisting vasculature (angiogenesis) is a fundamental process for tumor growth, metastasis and other angiogenic diseases (1–3). Angiogenic microvascular endothelial cells present a central challenge for therapeutic intervention because they are easily accessible (4,5). Because tumors cannot further grow without blood-supplied oxygen and nutrients, targeting of angiogenic endothelial cells could thus be a good strategy for tumor therapy. Therefore, many researchers have sought to develop antiangiogenic peptide drugs or angiogenesis inhibitors which target angiogenic endothelial cells for tumor therapy.

In the past several decades, researchers have demonstrated that dividing endothelial cells in the angiogenic vessels express several markers, whereas such would be either barely detectable or entirely absent in normal blood vessels (6–9). Of the markers, $\alpha_v\beta_3$ integrin is found to be over-expressed in the tumor vasculature, and thus drug targeting to $\alpha_v\beta_3$ integrin may provide the opportunity to destroy tumor vessels without affecting the microvessels of normal tissues. Targeting tumor vasculature by peptides containing RGD (Arg–Gly–Asp) sequence is a promising way for both tumor therapy and imaging because RGD sequence recognizes $\alpha_v\beta_3$ integrin over-expressed on tumor microvessels. For example, the RGD peptide analogues have been used in tumor imaging with fluorophores (10–12) or radionucleotides (13), and in tumor therapy with peptide conjugated chemotherapeutic drugs (14,15). To further improve therapeutic efficiency and tumor selectivity, various functionalized therapeutic systems such as gene delivery carriers (16–19), micelles (20) and protein carriers (21,22) have been developed to actively target tumors through

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; HL, heparin-lithocholic acid; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; SCC7, squamous cell carcinoma.

molecular recognition of $\alpha_v\beta_3$ integrin on angiogenic endothelial cells (23).

Recently, we have developed low anticoagulant bile acid acylated heparin derivatives (24–27) to overcome side effects such as hemorrhage, HIT (heparin-induced thrombocytopenia) (28) and osteoporosis (29) that are caused by the strong anticoagulant activity of heparin when heparin is used at high dosages and for extended periods of time. Other research groups have also developed chemically modified heparin derivatives including carboxyl-reduced heparin, *N*-*O*-desulphated/*N*-resulphated heparin, *N*-desulphated/*N*-reacetylated heparin (30), heparin-steroid conjugate (31), heparin-carrying polystyrene (32), and neoheparin (33). These chemically modified heparin derivatives have advantages that reduce the risk of hemorrhage, and prevent metastasis, angiogenesis, and tumor growth. Among them, bile acid-acylated heparin derivatives effectively prevented proliferation of tumor and endothelial cells (25), and further showed potent antiangiogenic effect *in vitro* and *in vivo* (26,27), thereby exerting similar or superior antitumor effect at much lower doses than heparin or other heparin derivatives. In addition, bile acid heparin derivatives are safe with respect to long-term treatment in a mouse model (25).

It has been reported that systemically injected heparin has high binding affinity, and also can be taken up by dividing vascular endothelial cells, which are abundant in tumors (34–36): this suggests that heparin or heparin derivatives might target susceptible dividing tumor endothelial cells. In the present study, to enhance targeting efficiency to dividing endothelial cells within tumor, we introduced a cyclic RGD peptide to heparin-lithocholic acid (HL) to produce RGD peptide conjugated HL derivative (cRGD-HL). Due to low anticoagulant activity, it is thought that cRGD-HL will minimize heparin's side effects. At the same time, by introducing cRGDyK peptides, cRGD-HL will enhance binding selectivity to dividing tumor endothelial cells, thereby exerting potent antiangiogenic and antitumoral effects. Herein, we evaluated the functionalized cRGD-HL as a potent angiogenesis inhibitor by performing tests on endothelial cell adhesion, migration, $\alpha_v\beta_3$ integrin binding affinity, Matrigel plug assays, and antitumoral effect.

MATERIALS AND METHODS

Chemicals

Unfractionated heparin (UFH, 167 IU/mg) of average molecular weight *approximately* 12,000 Da was purchased from Pharmacia Hepar Co. (Franklin, OH, USA). Anhydrous formamide, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *N*-(2-aminoethyl)-lithocholylamide (LCA-NH_2) was prepared as described in the previous report (24,27). RGD-bearing synthetic peptide (cRGDyK, cyclic Arg–Gly–Asp–D–Tyr–Lys) was the product of FutureChem Co. Ltd. (Seoul, Korea). Acetone was obtained from Merck (Darmstadt, Germany), and all reagents were of analytical grade and were used without further purification.

Preparation and Characterization of the Heparin-Lithocholic Acid

The HL conjugate was synthesized by coupling heparin with *N*-(2-aminoethyl)lithocholylamide (LCA-NH_2) as described in the previous report (24,27). In brief, heparin (100 mg) was dissolved in anhydrous formamide (5 ml) by gentle heating. EDAC (96 mg) was added to this heparin solution while mixing, followed by the addition of LCA-NH_2 (543 mg), which was dissolved in anhydrous DMF (10 ml). After 24 h, the mixture was precipitated in excess cold acetone, and the precipitate was carefully washed three times with cold acetone to remove excess LCA-NH_2. The dried HL conjugate was suspended in water, and then freeze-dried for 2 to 3 days. The anti-coagulant activity of the HL conjugate was evaluated using anti-FXa chromogenic assay (37).

Preparation of cRGDyK-Conjugated Heparin-Lithocholic Acid

The cRGD-HL conjugate was manufactured as follows (Fig. 1); HL (100 mg) was dissolved in anhydrous formamide (5 ml), followed by the addition of EDAC (9.2 mg). After 10 h, cRGDyK (35.6 mg) was added to the solution, which was further allowed to proceed for 12 h. Then, the mixture was precipitated in excess cold acetone, and the precipitate was carefully washed three times with DMSO and cold acetone to remove excess EDAC and cRGDyK, respectively. The dried cRGD-HL conjugate was suspended in water, and then freeze-dried for 2 to 3 days. The cRGD-HL was analyzed by ^{1}H NMR (JEOL JNM-LA 300 WB FT-NMR, Tokyo, Japan) to confirm the coupling of cRGDyK to HL. The contents of cRGDyK bound to the HL conjugate were determined using a Lambda Vis 7 spectrophotometer (Perkin-Elmer, CT). Lyophilized cRGD-HL (1 mg) was dissolved in 1 ml of formamide to obtain clear solutions, and cRGDyK contents were quantified by a UV-vis spectrometer.

Cell Adhesion and Migration Assays

HUVECs were isolated from human umbilical cord veins by collagenase treatment and used in passages 2–7 (38). The cells were grown in 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.

The cell adhesion assay was carried out as follows. In brief, as a positive control, a flat-bottomed 96-well plate (Corning, NY, USA) was coated overnight at 4°C with vitronectin (5 μg/ml in PBS, pH 7.4) and then blocked for 1 h at room temperature with 2% BSA. HUVECs were suspended at a density of 3×10^5 cells per milliliter in the presence or absence of heparin, cRGDyK, HL, or cRGD-HL. As a negative control, a flat-bottomed 96-well plate was only coated overnight at 4°C with 2% BSA. The cell suspension (0.1 ml) was added onto the plates pre-coated with vitronectin

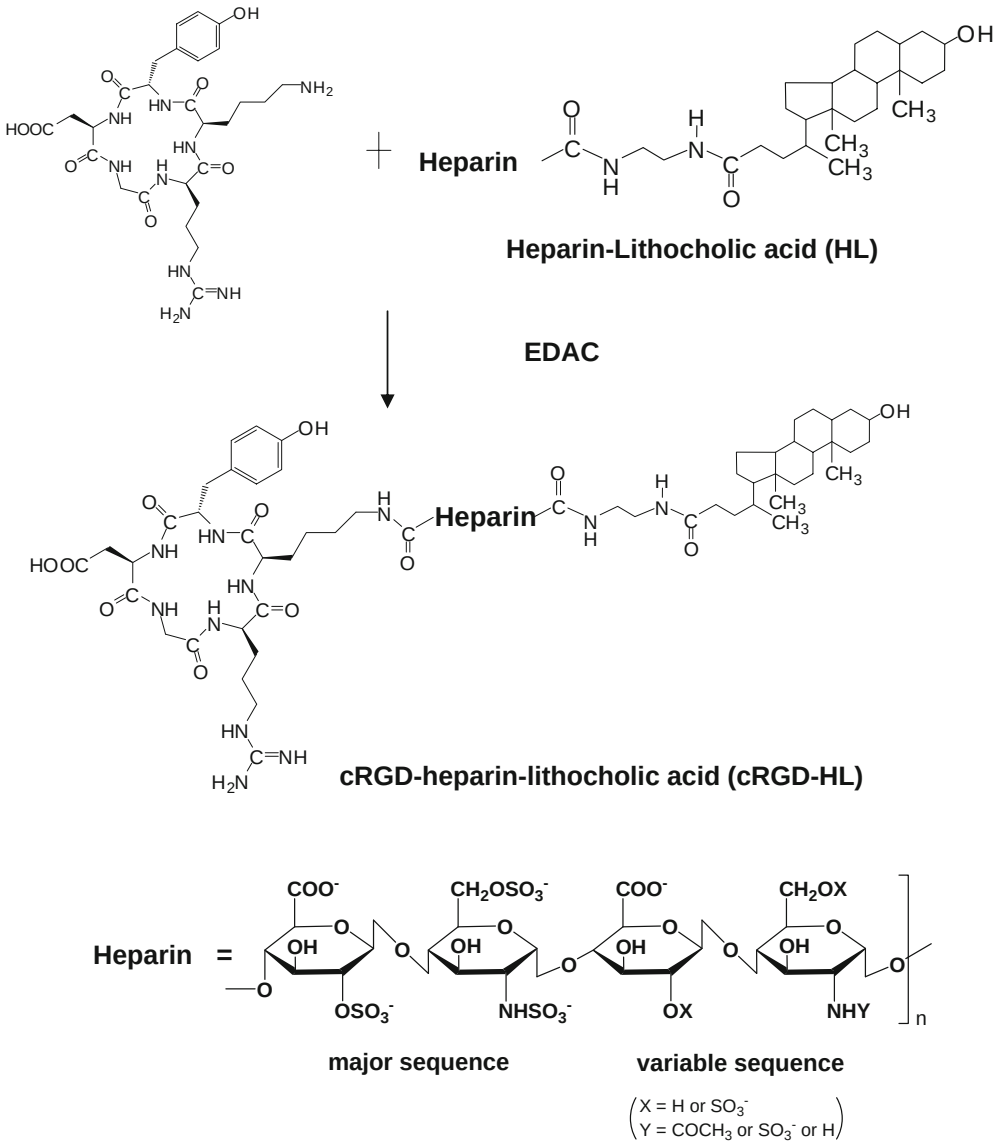


Fig. 1. Synthetic scheme for the preparation of cRGD-HL. The cRGD-HL was synthesized by reaction between the carboxylic groups of heparin-lithocholic acid (HL) with cRGDyK in the presence EDAC.

protein and incubated for 30 min at 37°C; unattached cells were removed by rinsing with PBS. Attached cells were then incubated for 1 h at 37°C in 50 mM citrate buffer (pH 5.0), containing 3.75 mM *p*-nitrophenyl-*N*-acetyl-D-glycosaminide and 0.25% Triton X-100. Enzyme activity was blocked by treating with 50 mM glycine buffer (pH 10.4), containing 5 mM EDTA. The amount of attached cells was estimated by measuring the absorbance at 405 nm using a microplate reader (Model 550, Bio-Rad Laboratories).

The cell migration assay was performed in transwell plates (8 μm in pore size, Corning, NY, USA). As a positive control, the undersurface of the membrane was coated overnight at 4°C with vitronectin (5 μg/ml in PBS, pH 7.4) and then blocked for 1 h at room temperature with 2% BSA. The HUVEC suspension (0.1 ml) was added to the upper compartment of the plate with or without heparin, cRGDyK, HL, or cRGD-HL. As a negative control, the undersurface of the membrane was only coated overnight at 4°C with 2% BSA. The cells were allowed to migrate for 8 h at 37°C, which was terminated by removing the cells from the upper compartment of the plate with a cotton swab. The migrated cells throughout the membrane were fixed with 8% glutaraldehyde and stained with crystal violet. Thereafter, the number of migrated cells was determined using an optical microscope (Nikon Diapot 300, Japan) under high power field (hpf) from ten representative areas per well.

Tubular Formation of Human Umbilical Endothelial Cells

In vitro endothelial tube formation was performed as described in the literature (26). 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 the medium at the density of 4×10^5 cells per milliliter, and 0.1 ml of the cell suspension was

added to each well coated with Matrigel in the presence of heparin, cRGDyK, HL, or cRGD-HL. Cells were incubated at 37°C, and then photographed. The branch points were counted and averaged.

Integrin $\alpha_v\beta_3$ Binding Assay

The standard assay was performed as described previously (11). Microtiter-2 96-well plates were coated with $\alpha_v\beta_3$ integrin (100 μl per well, 500 ng/ml) in coating buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM $MnCl_2$] for 16 h at 4°C, and the wells were blocked with 200 μl blocking buffer (coating buffer in the presence of 1% radioimmunoassay grade bovine serum albumin) for 2 h. The plate was washed twice with binding buffer (coating buffer in the presence of 0.1% bovine serum albumin) and then was incubated with ^{125}I-labeled echistatin (0.06 nM) in the presence of different concentrations of cRGDyK, HL, or cRGD-HL at room temperature for 3 h. After incubation, the plate was washed thrice with binding buffer, and the radioactivity was solubilized with 2 M boiling NaOH and was subjected to gamma-counting. Nonspecific binding of ^{125}I-labeled echistatin to $\alpha_v\beta_3$ was determined in the presence of 100 nM echistatin. The IC_{50} values were calculated by nonlinear regression analysis with the Sigma Plot 10.0 version program.

In Vivo Assessment of Angiogenesis Using Matrigel Plug Assay

To assess the antiangiogenic properties of cRGD-HL, Matrigel plug assays were performed as described in the literature (26). Briefly, 0.67 ml of liquid Matrigel–PBS mixture was injected subcutaneously into the backs of male C57BL/6 mice at 4°C; once inside the animals, the liquid Matrigel–PBS mixture was solidified. The Matrigel contained bFGF (R & D systems, Inc., McKinley, NE, USA) at the final concentration of 500 ng/ml to stimulate angiogenesis; heparin, cRGDyK, HL, or cRGD-HL were also loaded in the Matrigel, respectively. All of the treatment groups consisted of five mice. After 10 days, mice were sacrificed, Matrigel plugs were removed, and photographed. To evaluate the development of angiogenesis, the hemoglobin (Hb) content was measured using Drabkin's reagent kit (Sigma, St. Louis, MO, USA). The Matrigel plugs were homogenized in 50 μl of double-distilled water 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 cuvettes, and the absorbance was measured at 540 nm. Drabkin's solution was used as a blank. The absorption was proportional to the total hemoglobin concentration. Microvessels within Matrigel were also assessed by CD31 immunostaining on frozen sections with 8 μm. 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, USA) at 1:200 dilution, and with 4',6-diamidino-2-phenylindole (DAPI) counterstain. The images were taken with fluorescent microscope (CARL-ZEISS), and the number of blood vessels were counted and averaged under the five high-power fields.

Antitumor Effect of cRGD-HL and Immunohistochemistry

SCC7 cells were originally obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin at 37°C in a humidified 5% CO_2–95% O_2 atmosphere.

Male C3H/HeN mice (8-weeks old, Institute of Medical Science, Tokyo, Japan) were used for animal experiments. Subcutaneous tumors were established by inoculating 1×10^6 SCC7 cells into the backs of mice. The care and maintenance of animals were undertaken in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. Mice were allocated into six groups according to the treatments as follows: (1) normal saline (control group); (2) heparin 5 mg/kg; (3) cRGDyK 0.35 mg/kg; (4) HL 5 mg/kg; (5) cRGD-HL (0.5 mg/kg); (6) cRGD-HL (5 mg/kg). When tumors grew to approximately 50 to 100 mm^3, each animal received drugs in the indicated doses at every three day over a period of 12 days (a total of five injections) via lateral tail vein. The doses, dosing frequency and duration of therapy in this study were determined based on the previous study (27). Tumor volume was measured daily using a caliper. At 14th day, mice were sacrificed and tumor tissues in each group were isolated. The excised tumors were fixed with 4% (*v*/*v*) formaldehyde in PBS (pH 7.4), and sectioned into slices of

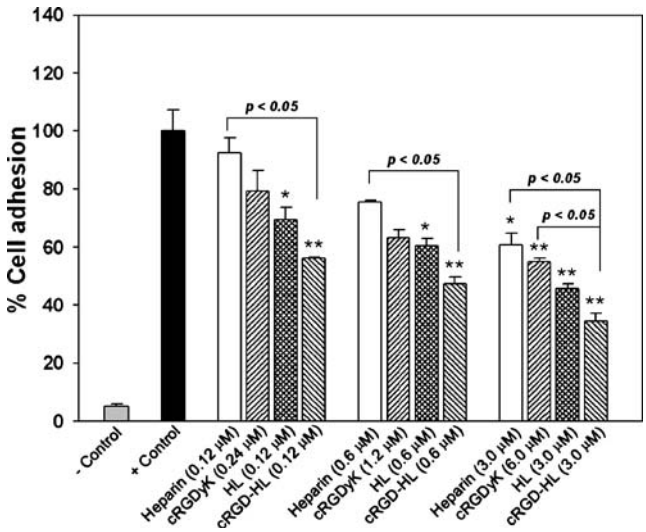


Fig. 2. Adhesion inhibition of HUVEC on vitronectin surface by heparin, cRGDyK, HL, and cRGD-HL. The cRGD-HL significantly decreased the adhesion of HUVECs to vitronectin surface compared to other agents. These experiments were repeated thrice. The *error bar* represents SE. *, $P<0.01$ *versus* + control, and **, $P<0.001$ *versus* + control. The *inset P* values in the figure indicate the significance between groups.

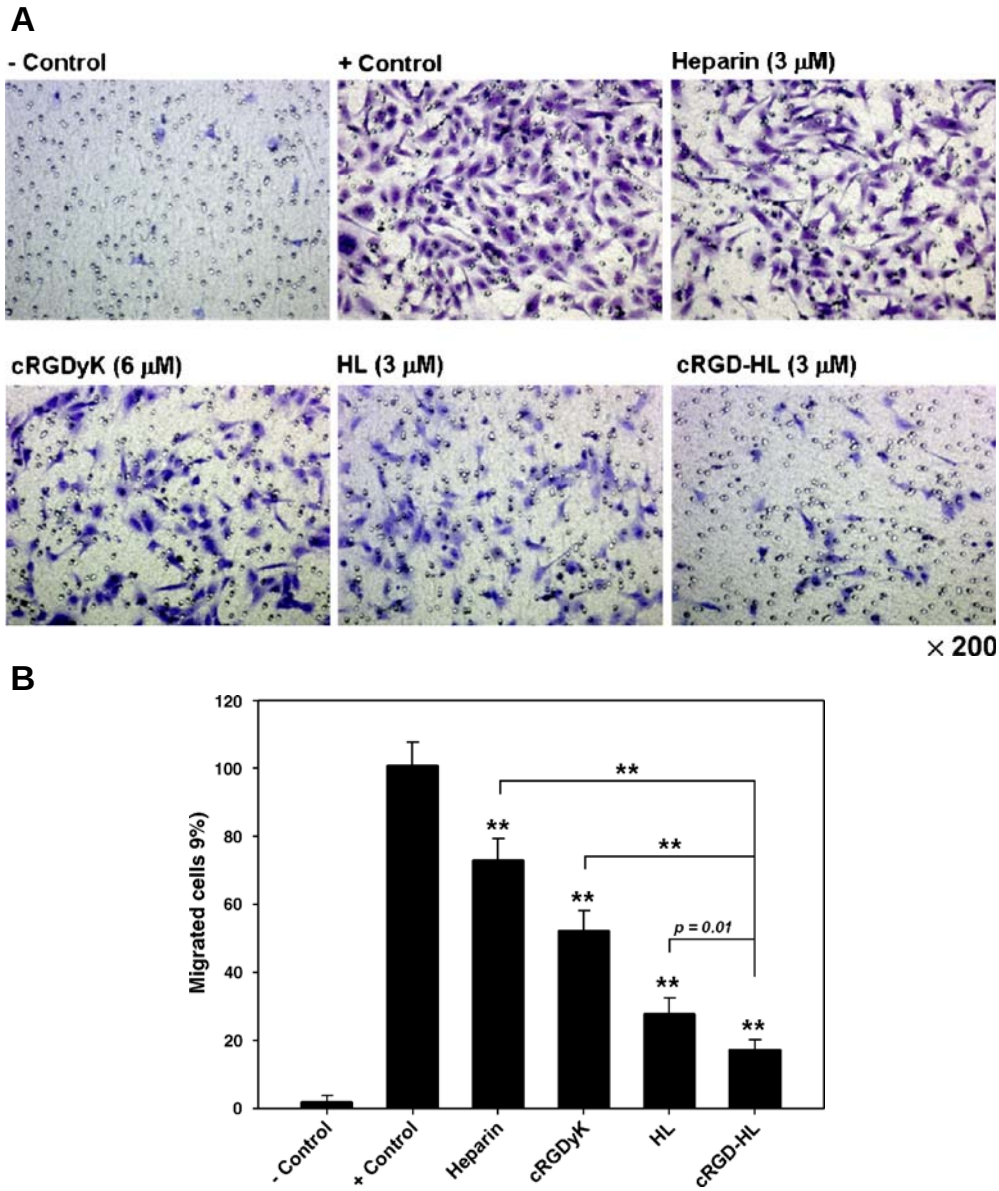


Fig. 3. Migration of HUVEC to vitronectin. **A** HUVECs migrated were observed using an optical microscope (magnification ×200). It can be seen that cRGD-HL significantly inhibits HUVEC migration compared to heparin, cRGDyK, and HL. **B** This observation was clearly supported by the quantitative measurement, obtained from the ten representative areas. The *error bar* represents SE (n=10). These experiments were repeated thrice. **, $P<0.001$ *versus* + control or between groups. The *inset P* values in the figure indicate the significance between groups.

8 μm thickness. Microvessels in tumor tissues were then stained by using a rat anti-mouse CD34 monoclonal antibody (Dako, CA, USA), and the number of microvessels were observed by an optical microscope (Nikon Diapot 300, Japan), and counted in the randomly selected fields (×200, eight fields).

Statistics

The statistical significance of the difference between experimental and control groups was determined using one-way ANOVA or post hoc test. *P* values considered significant are indicated in the figures by asterisks.

RESULTS

Characteristics of HL and cRGD-HL

HL was synthesized by covalently binding LCA-NH_2 to heparin in the presence of EDAC, and this binding was confirmed by ^{1}H NMR, in which the characteristic peaks of LCA appeared at 18-CH_3 (0.64 ppm), 19-CH_3 (0.91 ppm), 21-CH_3 (1.24 ppm) and new amide linkages between heparin and LCA, and the intrinsic sulfonamide of heparin showed at 8.04 ppm, respectively (27). The molecular weight of HL conjugate based on the degree of LCA substitution was 16,640 Da. This modification of carboxyl groups in heparin

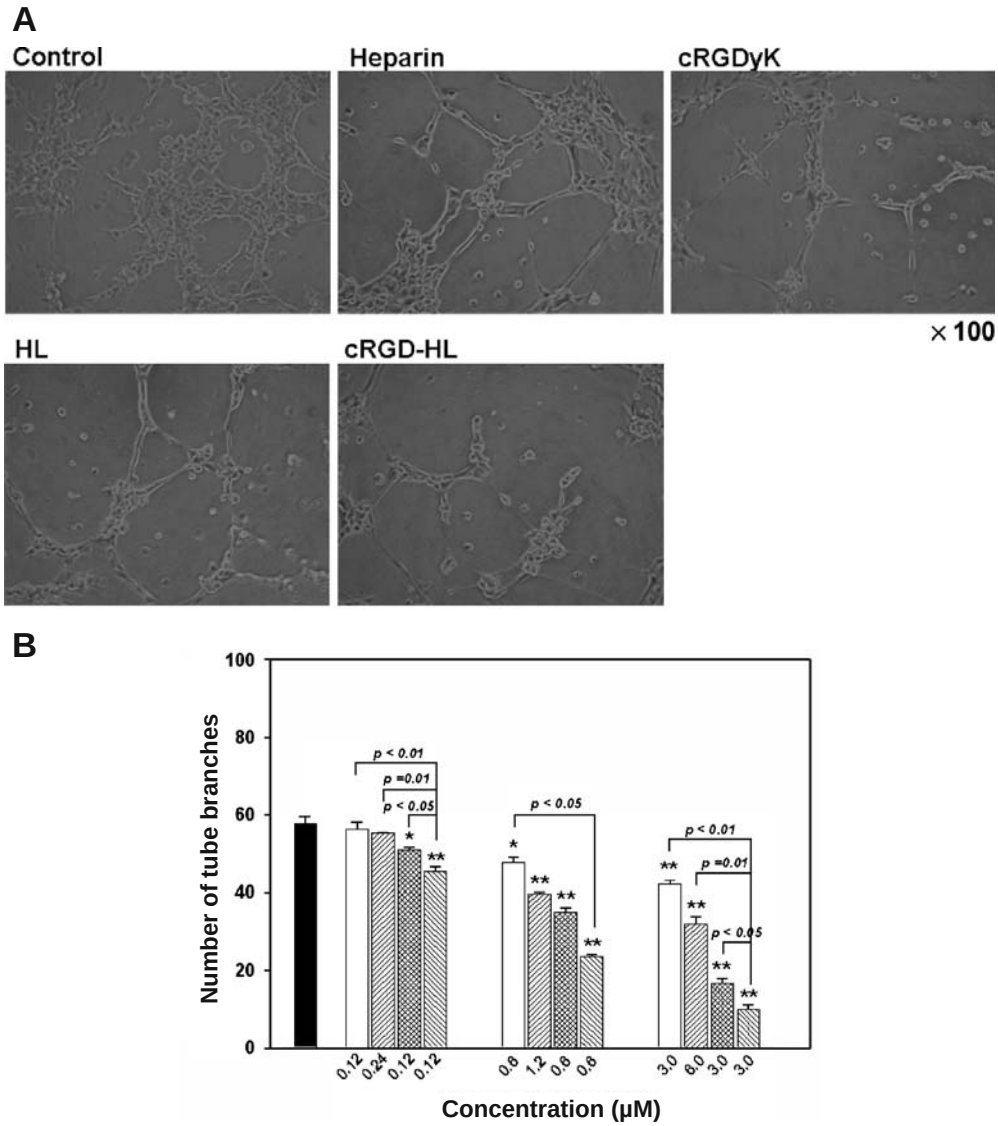


Fig. 4. Tube formation of HUVEC on Matrigel. **A** The representative photos for tube formation inhibition of HUVECs at the highest concentration (magnification ×100). Cells were seeded on Matrigel in the absence or presence of heparin (3 μM), cRGDyK (6 μM), HL (3 μM), or cRGD-HL (3 μM) and photographed after 7 h. B. Quantitative analysis of the tube formation assay at various concentrations. Groups: (■) control, (□) heparin, (▨) cRGDyK, (▩) HL, or (▧) cRGD-HL. These experiments were repeated thrice. The *error bar* represents SE. These experiments were repeated thrice. *, $P<0.02$ *versus* control, and **, $P<0.01$ *versus* control. The inset *P* values in the figure indicate the significance between groups.

resulted in low anticoagulant activity (38% *versus* heparin by FXa assay). In general, cyclic RGD peptides display a higher binding affinity to activity compared to their linear counterparts, and this beneficial effect may be due to less conformational flexibility and more metabolical stability (39,40). Thus, cRGDyK peptide as a potent $\alpha_v\beta_3$ integrin antagonist was conjugated to HL. After coupling of cRGDyK to HL, the presence of cRGDyK to the HL conjugate was determined by ^{1}H NMR and UV-vis spectrometry because cRGDyK has an aromatic ring structure. The characteristic peaks of phenyl group in tyrosine residue of cRGDyK were shown at 7.67~7.93 ppm (data not shown), and two cRGDyK molecules were coupled to one HL molecule. After coupling of LCA-NH_2 and cRGDyK to heparin, 67% of carboxylic groups in heparin were consumed. FXa assay showed that the anticoagulant activity of cRGD-HL was accounted for by 27% intact heparin.

Cell Adhesion and Migration Inhibition

The potential antiangiogenic properties of heparin, cRGDyK, HL, and cRGD-HL were measured by evaluating the ability to inhibit endothelial cell adhesion and migration to extracellular matrix protein, vitronectin, which mediate endothelial cell adhesion by specific interaction with $\alpha_v\beta_3$ integrin (41). The presence of heparin, cRGDyK, HL and cRGD-HL effectively inhibited the HUVEC adhesion by increasing the concentrations (Fig. 2). At the highest concentration (3 μM), the HUVEC adhesion was decreased

by 39.1%, 45.0%, 54.2% and 65.6% by heparin, cRGDyK, HL, and cRGD-HL, respectively.

Similarly to the adhesion assay data, heparin, cRGDyK, HL and cRGD-HL inhibited the migration of HUVECs to the surface coated with vitronectin (Fig. 3A). The results were clearly supported by the quantitative measurement (Fig. 3B): the extent of cell migration was effectively reduced by 27.0% (heparin), 47.8% (cRGDyK), 72.1% (HL), and 82.8% (cRGD-HL), respectively. From these results, cRGD-HL showed significant anti-adhesion and anti-migration effects of HUVECs compared to other compounds.

Inhibition of Capillary-Like Tube Formation

The formation of capillary-like tube structures by HUVECs in ECM is the pivotal step in angiogenesis and is also involved in cell migration and invasion. The tube formation actively occurred in the control group. However, the presence of heparin, cRGDyK, HL and cRGD-HL affected the extent of tube formation caused by HUVECs (Fig. 4A). In particular, cRGD-HL exhibited the greatest inhibition effect against cell alignments and tubular structure formations. The number of tube branches in the control was reduced from 57.7±1.8 to 42.0±1.2 (heparin), 32.0±1.7 (cRGDyK), 16.7±1.2 (HL), and 10.0±1.0 (cRGD-HL), respectively. We found that cRGD-HL most profoundly exhibited capillary-like tube formation inhibition.

Receptor Binding Studies

To evaluate the $\alpha_v\beta_3$ integrin receptor binding characteristics of the cRGDyK, HL and cRGD-HL, we measured the IC_{50} values of cRGDyK, HL, and cRGDHL in competitive-type experiments. Binding of cold echistatin, cRGDyK, HL, and cRGD-HL were compared using ^{125}I-labeled echistatin in a concentration-dependent manner (Fig. 5). The modification of HL with cRGDyK somewhat increased its receptor binding affinity compared to that of HL and cRGDyK. The determined mean IC_{50} values for the $\alpha_v\beta_3$ integrin were 3.37 nM for cRGD-HL, 7.43 nM for HL, and 9.64 nM for cRGDyK, respectively. Therefore, cRGD-HL has higher binding affinity for purified $\alpha_v\beta_3$ integrin than HL and cRGDyK.

In Vivo Assessment of Angiogenesis Using The Matrigel Plug Assay

The Matrigel plug assay, which allows angiogenesis inhibitors to be examined, was used to assess the antiangiogenic properties of cRGD-HL in this model (Fig. 5). At 10 days, plugs treated with bFGF alone were dark red in color, whereas plugs treated concurrently with bFGF and heparin displayed red color, indicating the formation of new blood vessels and blood circulation in both plug types (Fig. 5A). Plugs with mixed cRGDyK, or HL, or cRGD-HL were pale in their color, implying a significant decrease in microvessel formation within Matrigel. The suppression of angiogenesis by cRGDyK, HL, and cRGD-HL was consistent with the decrease in the hemoglobin content within the Matrigel plugs. The hemoglobin contents were markedly reduced up to 32.0% by cRGDyK, to 66.0% by HL, and to 78.4% by cRGD-HL. The suppression of angiogenesis by cRGD-HL treatment was supported by CD31-positive microvessel immunostain (Fig. 5C). As shown in Fig. 5D, cRGD-HL significantly suppressed CD31-positive microvessels compared to heparin, cRGDyK, and HL. These results imply that cRGD-HL significantly inhibits bFGF-induced angiogenesis.

Inhibitory Effect of cRGD-HL on Tumor Growth in Mice

To evaluate whether cRGD-HL inhibits tumor growth *in vivo*, SCC7 was implanted into the backs of C3H/HeN male mice. Heparin produced 33.5% reduction in the tumor volume (not significant compared to the untreated controls). On the other hand, cRGDyK, HL, and cRGD-HL at 0.5 or 5 mg/kg also suppressed SCC7 tumor growth by as much as 46.9%, 52.5%, 59.5% and 74.1%, respectively, compared to the untreated control (Fig. 6A). This result showed a superior antitumor effect of cRGD-HL, compared to heparin and cRGDyK. CD-34 immunostaining data supported the superior antiangiogenic effect of cRGD-HL (Fig. 6B, C). The number of CD-34 positive microvessels was decreased up to 24.1% by heparin, 38.4% by cRGDyK, 46.8% by HL, 52.3% by cRGD-HL at 0.5 mg/kg, and 69.2% by cRGD-HL at 5 mg/kg. In contrast to other groups, mice treated with 5 mg/kg of cRGD-HL for every 3 days showed a profound decrease in the numbers of CD34-positive microvessels (Fig. 6C). These results demonstrate that cRGD-HL prevents tumor angiogenesis, and thereby suppresses tumor growth.

DISCUSSION

Targeting tumor vasculature is an exciting target for drug delivery in cancer therapy because dividing endothelial cells within tumor tissues are known to over-express specific target molecule such as $\alpha_v\beta_3$ integrin. Additionally, because

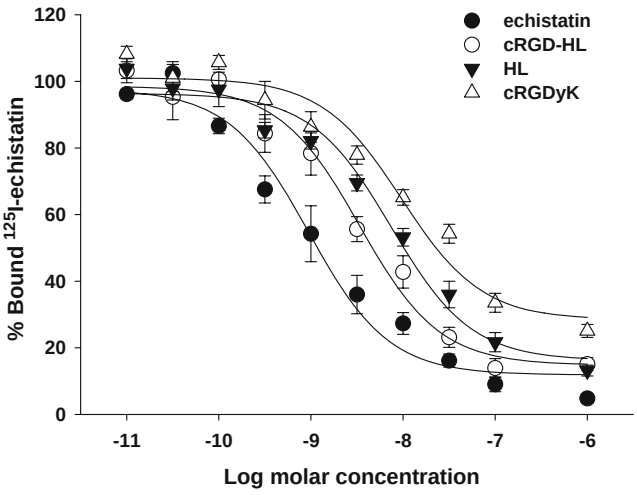
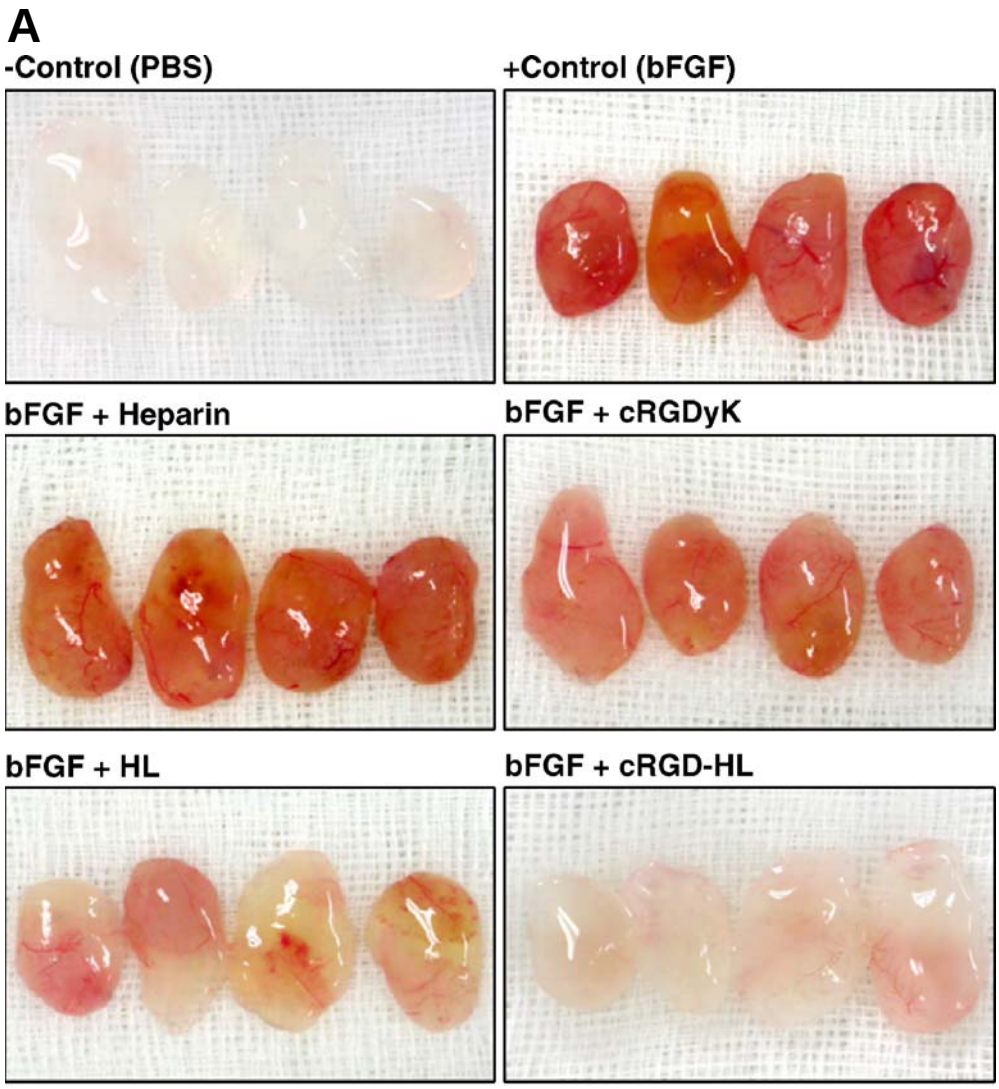
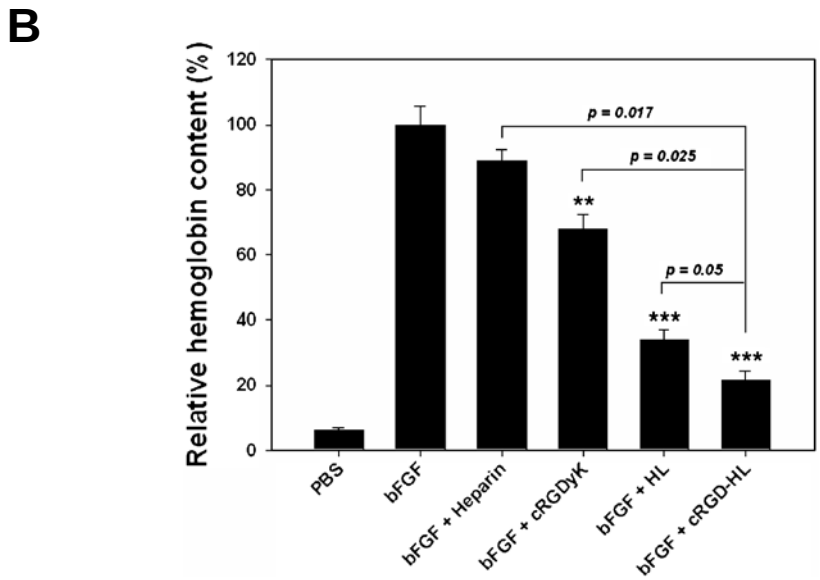


Fig. 5. Competition of specific binding of 125I-labeled echistatin with unlabeled echistatin (*filled circles*), cRGDyK (*empty triangles*), HL (*filled triangles*),or cRGD-HL (*empty circles*) to purified $\alpha_v\beta_3$ integrin as determined with the solid-phase receptor assay. The experiments were done in triplicate.


A
-Control (PBS)
+Control (bFGF)
bFGF + Heparin
bFGF + cRGDyK
bFGF + HL
bFGF + cRGD-HL


B
Relative hemoglobin content (%)
120
100
80
60
40
20
0
p = 0.017
p = 0.025
p = 0.05
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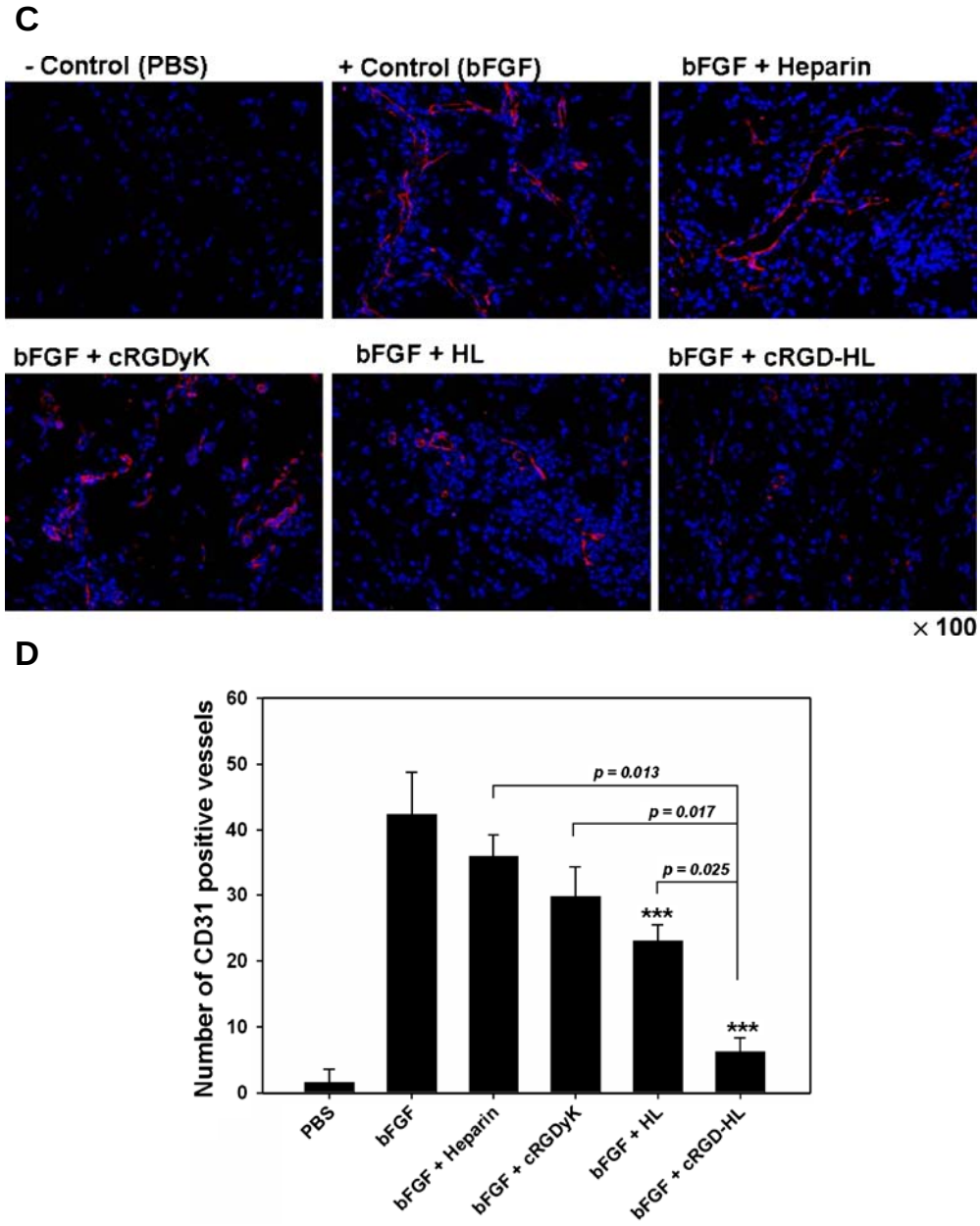
***
***
PBS
bFGF
bFGF + Heparin
bFGF + cRGDyK
bFGF + HL
bFGF + cRGD-HL

Fig. 6. (continued)

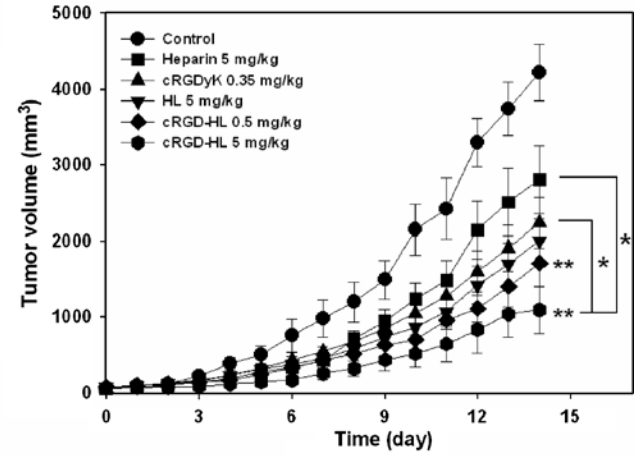


◀ **Fig. 6.** Inhibition of the bFGF-induced angiogenesis in Matrigel plugs. **A** Matrigel plugs containing PBS alone (− control), bFGF alone (+ control), bFGF + heparin (3 μM), bFGF + cRGDyK (6 μM), bFGF + HL (3 μM), or bFGF + cRGD-HL (3 μM) were photographed. **B** Hemoglobin contents within Matrigel were determined by the Drabkin's method. The data are presented as means±SE (n=8). **C** Microvessel assessment of Matrigel plugs containing bFGF and heparin, cRGDyK, HL, or cRGD-HL. The plugs were sectioned and immunostained with anti-CD31 antibody (*red*) and DAPI (*blue*; magnification ×100). **D** The number of blood vessels in five high power fields was counted and averaged (CD31 immunostain). These experiments were repeated thrice. **, P=0.002 + control (bFGF alone), and ***, P<0.001 *versus* + control. The *inset P* values in the figure indicate the significance between groups.

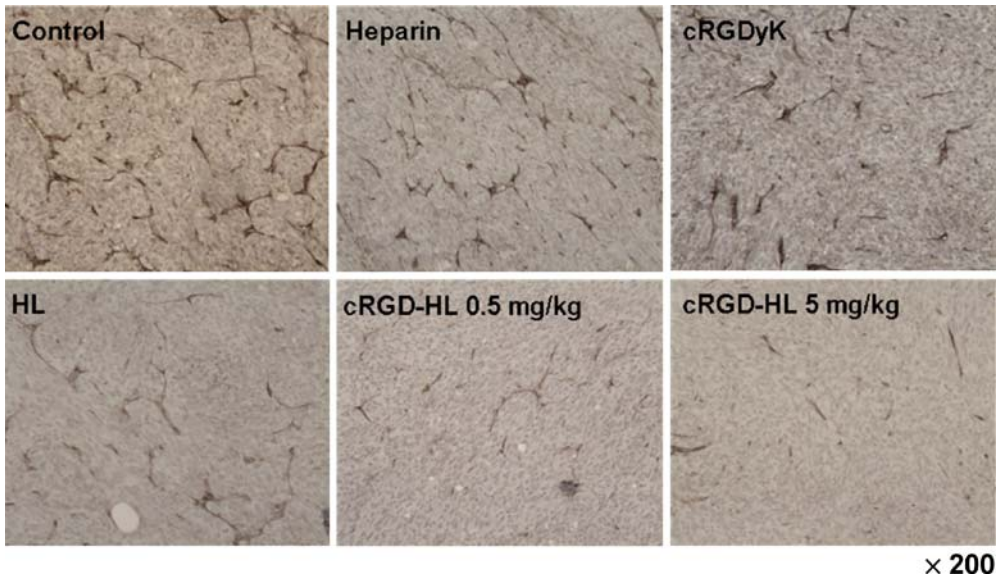
endothelial cells are genetically stable and therefore unlikely to mutate into drug-resistant variants, the targeting tumor endothelium is ideal for cancer treatment (42,43). In the present study, a new functionalized bile acid-acylated heparin derivative was developed by introducing the cRGDyK peptide to the heparin-lithocholic acid to actively target tumor endothelial cells, and to improve therapeutic efficiency.

In antiangiogenic therapies, $\alpha_v\beta_3$ integrin is one of the most important targets in antiangiotherapy. It is known that $\alpha_v\beta_3$ integrin expressed on endothelial cells modulates cell adhesion and migration by binding to RGD-containing components of the interstitial matrix such as vitronectin, fibronectin, and thrombospondin (44). In this study, heparin, cRGDyK, HL and cRGD-HL inhibited endothelial cell adhesion and migration to vitronectin coated surfaces. Among them, cRGD-HL showed superior anti-adhesion

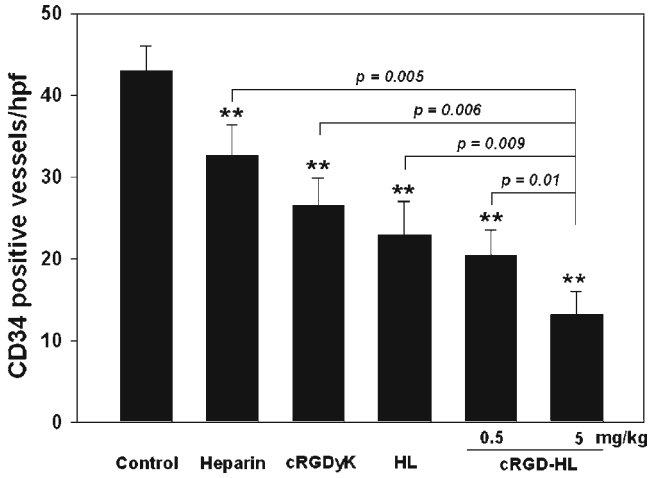
A


Control
Heparin 5 mg/kg
cRGDyK 0.35 mg/kg
HL 5 mg/kg
cRGD-HL 0.5 mg/kg
cRGD-HL 5 mg/kg
Tumor volume (mm3)
Time (day)
0
1000
2000
3000
4000
5000
0
3
6
9
12
15
**
*
*
**

B


Control
Heparin
cRGDyK
HL
cRGD-HL 0.5 mg/kg
cRGD-HL 5 mg/kg
× 200

C


CD34 positive vessels/hpf
0
10
20
30
40
50
p = 0.005
p = 0.006
p = 0.009
p = 0.01
**
**
**
**
**
Control
Heparin
cRGDyK
HL
0.5
5
mg/kg
cRGD-HL

◀ **Fig. 7.** Tumor growth inhibition and CD-34 microvessel immunostain. **A** Mice received s.c. injection with SCC7, and when tumor volumes were 50 to 100 mm^3, the mice received i.v. injection with normal saline (*filled circles*), heparin (*filled squares*; 5 mg/kg/3 day), cRGDyK (*filled upright triangles*; 0.35 mg/kg per 3 day), HL (*filled inverted triangles*; 5 mg/kg/3 day), cRGD-HL (*filled diamonds*; 0.5 mg/kg per 3 day, *filled hexagon*; 5 mg/kg per 3 day). Each group contained six to seven mice. The size of tumors was measured daily. Data are presented as means± SE. *, $P<0.05$ *versus* cRGD-HL at 5 mg/kg per 3 day, and **, and $P<0.001$ *versus* control (saline alone), respectively. These experiments were repeated thrice. **B** CD-34 immunostain of endothelial cells and representative vessels in sections (dark brown color; magnification ×200). **C** Blood vessel quantification in tumor tissues. The numbers of CD-34 positive blood vessels in eight high power fields (×200) were counted and averaged. *Bars*, means±SE. (**, $P<0.001$ *versus* control saline alone). The *inset P* values in the figure indicate the significance between groups.

and anti-migration effects. The cRGDyK peptide can directly interact with $\alpha_v\beta_3$ integrin expressed on endothelial cells, and prevent adhesion and migration of endothelial cells to vitronectin because of higher binding selectivity for $\alpha_v\beta_3$ integrin in both cell culture and living subjects (11,12). On the other hand, heparin and HL have negative charge and they interact with numerous extracellular proteins (e.g. laminin, collagen, fibronectin, and vitronectin) via electrostatic interaction, and thereby also suppress the interactions between endothelial cells to extracellular proteins. However, HL is more effective than heparin in inhibition effects. HL (−50 mV) has a much lower negative charge than heparin (−75 to −100 mV), and it also has a hydrophobic moiety as a LCA analog. It is postulated that HL might strongly interact with the ECM proteins via electrostatic and hydrophobic interactions (26,45), leading to more effective anti-adhesion and anti-migration effects. The RGD peptide conjugated to HL (cRGD-HL) profoundly improved its inhibitory effect on adhesion and migration of endothelial cells to vitronectin compared to other compounds. The potent inhibitory effects might be attributed that cRGD-HL contains a RGD peptide as its $\alpha_v\beta_3$ integrin targeting moiety, and has negative charge as well as a hydrophobic moiety. On the other hand, it is previously reported that among α_v integrins, only the $\alpha_v\beta_3$ integrin is found in focal contacts and leads to spreading and migration of (endothelial cells) vitronectin (46). In this regard, it is considered that the anti-adhesion and anti-migration of cRGD-HL is due to the interference of $\alpha_v\beta_3$ integrin-mediated interactions between endothelial cells and vitronectin. Furthermore, the result that cRGD-HL has higher binding affinity for the purified $\alpha_v\beta_3$ integrin was confirmed by the receptor binding studies compared to HL and cRGDyK (Fig. 5). This result suggests that the RGD peptide in cRGD-HL retains the $\alpha_v\beta_3$ integrin activity, and cRGD-HL is more active than HL. Therefore, we believe that the potent antiangiogenic effect of cRGD-HL in adhesion, migration and tube formation assays is probably due to the interference of the $\alpha_v\beta_3$ integrin-mediated interactions between endothelial cells and ECM protein.

In this study, we also found out that SCC7 cells hardly interact with $\alpha_v\beta_3$ monoclonal antibody in FACS analysis (data not shown), and SCC7 tumor cells *in vivo* did not express $\alpha_v\beta_3$ integrin (47), indicating that SCC7 cells are $\alpha_v\beta_3$-negative cells. However, we observed that a lot of microvessels were newly developed within murine SCC7 tumor tissues, as confirmed in CD31 immunostain (Fig. 7B), and endothelial cells in angiogenic microvessels of SCC7 tumor highly expressed $\alpha_v\beta_3$ integrin (47). Compared to HL without RGD peptide, cRGD-HL (RGD-modified heparin derivative) might more effectively interact with $\alpha_v\beta_3$-positive endothelial cells, leading to substantially enhanced antiangiogenic and antitumor effect on SCC7 tumors. Indeed, the cRGD-HL treatment could significantly decrease the microvessel density within SCC7 tumor tissues (Fig. 7B, C), suggesting that the use of cRGD-HL might be more effective in decreasing the growth of highly vascularized tumors.

In the previous studies, we have shown that bile acid-acylated heparin derivatives have angiogenesis inhibition property and significant antitumor efficacy against SCC7 (squamous cell carcinoma) and KB (human nasopharyngeal carcinoma) tumors in mouse models (26,27). We reported that bile acid-acylated heparin derivatives may interact with bFGF, leading to decrease the biological activity of bFGF. Subsequently, they suppressed FGFR phosphorylation and its down stream (ERK and p38 MAPK activity) to inhibit the proliferation of endothelial cells, angiogenesis and tumor growth (26). This study exhibited that the conjugation of cRGDyK to HL substantially enhanced angiogenesis inhibition and antitumor efficacies compared to bile acid-heparin derivatives. Taking these results into consideration, therefore, we suggested that the preexisting antiangiogenic characteristics (inhibition of bFGF activity) of HL were maintained, and simultaneously, and the introduction of cRGDyK to HL enhanced the newly additional anti-adhesion and anti-migration effect (inhibition of the $\alpha_v\beta_3$ integrin-mediated cell to cell or cell to matrix interactions). Indeed, cRGD-HL significantly decreased the tube formation and migration of endothelial cells *in vitro*, bFGF-induced angiogenesis as well as microvessel density in *in vivo* Matrigel plug and in tumor tissues compared to other compounds tested in this study, and thereby enhancing antitumor effects. Also, it is possible that cRGD-HL can be internalized into cells through receptors similar to RGD-modified albumin (21,22) or folic acid-HL (FHL) (27), and then may affect cell proliferation inhibition.

The systemically administered heparin/heparin derivatives with negative charge interacts with proteins on the surfaces of endothelial cells, leading to localization of angiogenic blood vessels in tumors by binding affinities to dividing endothelial cells (34), which may influence endothelial cell proliferation and angiogenesis in tumor tissues. In addition, it may be possible that macromolecule drugs such as antiangiogenic heparin derivative might be localized to tumor sites by enhanced permeability and retention (EPR) effects (48), and thereby showing the anti-tumor effects (25). In the present study, we demonstrated that the therapeutic efficacy of HL as an angiogenesis inhibitor could be improved by coupling cRGDyK to the HL derivative. In addition, its antiangiogenic and antitumoral effects are markedly upgraded when compared to cRGDyK, heparin–deoxycholic acid and HL derivatives. It is thought that bile acid-acylated heparin derivatives can circulate in the blood stream for longer periods than small molecule drugs can

(25), and this prolonged circulation is thought to increase the possibility of cRGD-HL to access target sites and enhance therapeutic effects compared to cRGDyK peptide. Also, cRGD-HL probably has much higher binding affinity toward $\alpha_v\beta_3$ integrin expressed on dividing endothelial cells than HL due to the presence of a RGD peptide, resulting in the augmented antiangiogenic and antitumoral effects in both *in vitro* and *in vivo* experiments at the same dose as HL. These potent antiangiogenic and antitumoral effects of cRGD-HL are due to the antiangiogenic effect of HL as well as the added RGD peptide activity.

Recently, the multivalent RGD-human serum albumin (RGD-HSA) derivatives have been developed by Temming *et al.* (21,22). They coupled c(RGDfK) peptide to HSA and further conjugated monomethyl-auristatin-E (MMAE) as a potent antimiotic agent. These multivalent RGD-HSA derivatives with high binding affinity and specificity for the $\alpha_v\beta_3$ integrin delivered therapeutic drugs to kill the target cells at low nM concentrations *in vitro* and displayed excellent tumor homing properties. In this study, we have shown that cRGD-HL has higher binding affinity to purified $\alpha_v\beta_3$ integrin compared to cRGDyK peptide, and effectively inhibited proliferation of endothelial cells and angiogenesis *in vitro* and *in vivo*, and thereby showing significant tumor growth regression.

Low molecular weight heparin (LMWH) with reduced toxicity has potential antitumor effects by modulating growth factor activity. In the case of High molecular weight heparin (HMWH), it may induce side effects due to strong anticoagulant effects. However, HMWH can more strongly bind to various proteins, and thus inhibit activities of proteins including growth factors. The toxicity problem of HMWH can be overcome by chemical modification because the chemically modified heparin derivatives could reduce risk of hemorrhage, but they prevent metastasis, angiogenesis, and tumor growth (25,26). In our group, we have developed various heparin derivatives including heparin–deoxycholic acid with high or low molecular weight and folic acid-conjugated HL. Compared to heparin–deoxycholic acid (HD) in the previous studies, the antiangiogenic effect of cRGD-HL is more potent. Angiogenesis inhibition effect of cRGD-HL has increased nearly ten times compare to that of high molecular weight HD derivative. Recently, we developed functionalized heparin derivatives such as FHL and glucosylated heparin (GH). FHL was designed to target folate-receptor (FR) over-expressed tumor cells. This FHL is easily internalized into FR over-expressing KB cells to induce apoptosis of tumor cells *in vitro* and *in vivo*. On the other hand, malignant tumor cells display abnormally enhanced glucose uptake and over-expressed glucose transporters (GLUTs) compared to normal cells. Thus, GH was developed to block glucose transporters (GLUTs) expressed on tumor cells, and resulted in tumor regression (49). Based on these facts, three functionalized heparin derivatives such as cRGD-HL, FHL and GH showed good antitumor effects *in vivo*. We thought that cRGD-HL might be more widely available than FHL or GH. cRGD-HL can be applied for the treatment of $\alpha_v\beta_3$ integrin-expressing endothelial cells and tumors, whereas FHL or GH is limited for the treatment of FR- or GLUTs-over-expressing tumor cells. We believe that the functionalized cRGD-HL as a potent angiogenesis inhibitor can offer many opportunities in targeted drug delivery to cancerous tumors and improve the therapeutic outcome in the treatment of human diseases related with angiogenesis.

CONCLUSION

In this study, we designed the functionalized bile acid-acylated heparin derivative by chemically conjugating cyclic RGDyK peptide with heparin-lithocholic acid to actively target angiogenic endothelial cells within tumor and to profoundly improve antiangiogenic and antitumor effects. The functionalized cRGD-HL significantly inhibited endothelial cell adhesion, migration to vitronectin, capillary-like tubular structures of endothelial cells. In particular, cRGD-HL had higher binding affinity to purified $\alpha_v\beta_3$ integrin than cRGDyK peptide and HL, resulting in the effective anti-angiotherapeutic effects. We suggest that the enhanced therapeutic effects are probably due to the combination the preexisting antiangiogenic characteristics (inhibition of bFGF activity) of heparin derivative and the additional antiangiogenic effect (inhibition of the $\alpha_v\beta_3$ integrin-mediated interactions) of the functionalized heparin derivative. Therefore, the use of functionalized heparin derivative as a potent angiogenesis inhibitor can offer affirmative outcome in human cancer diseases related with angiogenesis.

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REFERENCES

1. J. Folkman. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **285**:1182–1186 (1971).
2. J. Folkman. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* **82**:4–6 (1990) doi:10.1093/jnci/82.1.4.
3. J. Folkman. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**:27–31 (1995) doi:10.1038/nm0195-27.
4. A. W. Griffioen, and G. Molema. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases and chronic inflammation. *Pharmacol. Rev.* **53**:237–268 (2000).
5. P. Carmeliet, and R. K. Jain. Angiogenesis in cancer and other diseases. *Nature*. **407**:249–257 (2000) doi:10.1038/35025220.
6. D. Srivastava, P. Cserjesi, and E. N. Olson. A subclass of bHLH proteins required for cardiac morphogenesis. *Science*. **270**:1995–1999 (1995) doi:10.1126/science.270.5244.1995.
7. H. P. Hammes, M. Brownlee, A. Jonczyk, A. Sutter, and K. T. Preissner. Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. *Nat. Med.* **2**:529–533 (1996) doi:10.1038/nm0596-529.
8. R. O. Schlingemann, F. J. Rietveld, R. M. de Waal, S. Ferrone, and D. J. Ruiter. Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *Am J Pathol*. **136**:1393–1405 (1990).
9. M. A. Burg, R. Pasqualini, W. Arap, E. Ruoslahti, and W. B. Stallcup. NG2 proteoglycan-binding peptides target tumor neovasculature. *Cancer Res.* **59**:2869–2874 (1999).
10. S. Zitxmann, V. Ehemann, and M. Schwab. Arginine–glycine–aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells *in vivo*. *Cancer Res.* **62**:5139–5143 (2000).
11. X. Chen, P. S. Conti, and R. A. Moats. *In vivo* near-infrared fluorescence imaging of integrin $\alpha_v\beta_3$ in brain tumor xenografts. *Cancer Res.* **64**:8009–8014 (2004) doi:10.1158/0008-5472.CAN-04-1956.

12. Z. Cheng, W. Yun, X. Zhengming, S. S. Gambhir, and X. Chen. Near-infrared fluorescent RGD peptides for optical imaging of integrin $\alpha_v\beta_3$ expression in living mice. *Bioconj. Chem.* **16**:1433–1441 (2005) doi:10.1021/bc0501698.
13. B. Haubner, H. J. Wester, W. A. Weber, and M. Schwaiger. Radiotracer-based strategies to image angiogenesis. *Q. J. Nucl. Med.* **47**:189–199 (2003).
14. W. Arap, R. Pasqualini, and E. Ruoslahti. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science*. **279**:377–380 (1998) doi:10.1126/science.279.5349.377.
15. H. M. Ellerby, W. Arap, L. M. Ellerby, R. Kain, R. Andrusiak, G. D. Rio, S. Krajewski, C. R. Lombardo, R. Rao, E. Ruoslahti, D. E. Bredesen, and R. Pasqualini. Anti-cancer activity of targeted pro-apoptotic peptides. *Nat. Med.* **5**:1032–1038 (1995).
16. W. Suh, S. O. Han, L. Yu, and S. W. Kim. An angiogenic, endothelial-cell-targeted polymeric gene carrier. *Mol. Ther.* **6**:664–672 (2002) doi:10.1016/S1525-0016(02)90721-5.
17. W. J. Kim, J. W. Yockman, M. Lee, J. H. Jeong, Y. H. Kim, and S. W. Kim. Soluble Flt-1 gene delivery using PEI-g-PEG-RGD conjugate for anti-angiogenesis. *J. Control. Release*. **106**:224–234 (2005) doi:10.1016/j.jconrel.2005.04.016.
18. W. J. Kim, J. W. Yockman, J. H. Jeong, L. V. Christensen, M. Lee, Y. H. Kim, and S. W. Kim. Anti-angiogenic inhibition of tumor growth by systemic delivery of PEI-g-PEG-RGD/pCMV-sFlt-1 complexes in tumor-bearing mice. *J. Control. Release*. **114**:381–388 (2006) doi:10.1016/j.jconrel.2006.05.029.
19. J. D. Hood, M. Bednarski, R. Frausto, S. Guccione, R. A. Reisfeld, R. Xiang, and D. A. Cheresh. Tumor regression by targeted gene delivery to the neovasculature. *Science*. **296**:2404–2407 (2002) doi:10.1126/science.1070200.
20. N. Nasongkla, E. Bey, J. Ren, H. Ai, C. Khemtong, J. S. Guthi, S. F. Chin, A. D. Sherry, D. A. Boothman, and J. Gao. Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. *Nano Lett.* **6**:2427–2430 (2006) doi:10.1021/nl061412u.
21. K. Temming, D. L. Meyer, R. Zabinski, E. C. Dijkers, K. Poelstra, G. Molema, and R. J. Kok. Evaluation of RGD-targeted albumin carriers for specific delivery of auristatin E to tumor blood vessels. *Bioconjug. Chem.* **17**:1385–1394 (2006) doi:10.1021/bc060087z.
22. K. Temming, D. L. Meyer, R. Zabinski, P. D. Senter, K. Poelstra, G. Molema, and R. J. Kok. Improved efficacy of alphavbeta3-targeted albumin conjugates by conjugation of a novel auristatin derivative. *Mol. Pharm.* **4**:686–694 (2007) doi:10.1021/mp0700312.
23. J. A. Varner, and D. A. Cheresh. Integrins and cancer. *Curr. Opin. Cell Biol.* **8**:724–730 (1996) doi:10.1016/S0955-0674(96)80115-3.
24. K. Park, K. Kim, I. C. Kwon, S. K. Kim, S. Lee, D. Y. Lee, and Y. Byun. Preparation and characterization of self-assembled nanoparticles of heparin-deoxycholic acid conjugates. *Langmuir*. **20**:11726–11731 (2004) doi:10.1021/la048646i.
25. K. Park, G. Y. Lee, Y. S. Kim, M. Yu, R. W. Park, I. S. Kim, S. Y. Kim, and Y. Byun. Heparin-deoxycholic acid chemical conjugate as an anticancer drug carrier and its antitumor activity. *J. Control. Release*. **114**:300–306 (2006) doi:10.1016/j.jconrel. 2006.05.017.
26. K. Park, Y. S. Kim, G. Y. Lee, J. O. Nam, S. K. Lee, R. W. Park, S. Y. Kim, I. S. Kim, and Y. Byun. Antiangiogenic effect of bile acid acylated heparin derivative. *Pharm. Res.* **24**:176–185 (2007) doi:10.1007/s11095-006-9139-6.
27. M. K. Yu, D. Y. Lee, Y. S. Kim, K. Park, S. A. Park, D. H. Son, G. Y. Lee, J. H. Nam, S. Y. Kim, I. S. Kim, R. W. Park, and Y. Byun. Antiangiogenic and apoptotic properties of a novel amphiphilic folate-heparin-lithocholate derivative having cellular internality for cancer therapy. *Pharm. Res.* **24**:705–714 (2007) doi:10.1007/s11095-006-9190-3.
28. M. N. Levin, J. Hirsh, and J. G. Kelton. Heparin-induced bleeding. In D. A. Lane, and E. Lindhal (eds.), In heparin: chemical and biological properties, clinical applications, CRC Press, Boca Raton, 1989.
29. J. D. Douketis, J. S. Ginsberg, R. F. Burrows, E. K. Duku, C. E. Webber, and P. Brill-Edwards. The effects of long-term heparin therapy during pregnancy on bone density. A prospective matched cohort study. *Thromb. Haemost.* **75**:254–257 (1996) doi:10.1159/000134495.
30. T. Irimura, M. Nakajima, and G. L. Nicolson. Chemically modified heparins as inhibitors of heparan sulfate specific endo-beta-glucuronidase (heparanase) of metastatic melanoma cells. *Biochemistry*. **25**:5322–5328 (1986) doi:10.1021/bi00366a050.
31. P. E. Thorpe, E. J. Derbyshire, S. P. Andrade, N. Press, P. P. Knowles, S. King, G. J. Watson, Y. C. Yang, and M. Rao-Bette. Heparin-steroid conjugates: new angiogenesis inhibitors with antitumor activity in mice. *Cancer Res.* **53**:3000–3007 (1993).
32. K. Ono, M. Ishihara, K. Ishikawa, Y. Ozeki, H. Deguchi, M. Sato, H. Hashimoto, Y. Saito, H. Yura, A. Kurita, and T. Maehara. Periodate-treated, non-anticoagulant heparin-carrying polystyrene (NAC-HCPS) affects angiogenesis and inhibits subcutaneous induced tumour growth and metastasis to the lung. *Br. J. Cancer*. **86**:1803–1812 (2002) doi:10.1038/sj.bjc.6600307.
33. C. Y. Pumphrey, A. M. Theus, S. Li, R. S. Parrish, and R. D. Sanderson. Neoglycans, carbodiimide-modified glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis. *Cancer Res.* **62**:3722–3728 (2002).
34. B. Gimelius, C. Busch, and M. Hook. Binding of heparin on the surface of cultured human endothelial cells. *Thromb. Res.* **12**:773–782 (1978) doi:10.1016/0049-3848(78)90271-2.
35. L. M. Hiebert, and L. B. Jaques. The observation of heparin on endothelium after injection. *Thromb. Res.* **8**:195–204 (1976) doi:10.1016/0049-3848(76)90262-0.
36. N. Sakamoto, and N. G. Tanaka. Mechanism of the synergistic effect of heparin and cortisone against angiogenesis and tumor growth. *Cancer J.* **2**:9–16 (1988).
37. Y. Lee, H. T. Moon, and Y. Byun. Preparation of slightly hydrophobic heparin derivatives which can be used for solvent casting in polymeric formulation. *Thromb. Res.* **92**:149–156 (1998) doi:10.1016/S0049-3848(98)00124-8.
38. D. A. Jaffe, R. L. Nachman, C. G. Becker, and C. R. Minick. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52**:2745–2756 (1973) doi:10.1172/JCI107470.
39. M. Aumailley, M. Gurrath, G. Muller, J. Calvete, R. Timpl, and H. Kessler. Arg–Gly–Asp, constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1. *FEBS Lett.* **291**:50–54 (1991) doi:10.1016/0014-5793(91)81101-D.
40. S. J. Bogdanowich-Knipp, S. Chakrabarti, T. D. Williams, R. K. Dillman, and T. J. Siahaan. Solution stability of linear *vs*. cyclic RGD peptides. *J. Pept. Res.* **53**:530–541 (1999).
41. K. M. Yamada. Adhesive recognition sequences. *J. Biol. Chem.* **266**:12809–12812 (1991).
42. J. Denekamp. Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *Br. J. Radiol.* **66**:181–196 (1993).
43. F. J. Burrows, and P. E. Thorpe. Vascular targeting: a new approach to the therapy of solid tumors. *Pharmacol. Ther.* **64**:155–174 (1994) doi:10.1016/0163-7258(94)90037-X.
44. J. P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, and M. A. Arnaout. Crystal structure of the extracellular segment of integrin alpha v beta3 in complex with an Arg-Gly-Asp ligand. *Science*. **296**:151–155 (2002) doi:10.1126/science.1069040.
45. P. A. Raj, E. Marcus, and R. Rein. Conformational requirements of suramin to target angiogenic growth factors. *Angiogenesis*. **2**:183–199 (1998) doi:10.1023/A:1009244623717.
46. D. I. Leavesley, G. D. Ferguson, E. A. Wayner, and D. A. Cheresh. Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J. Cell Biol.* **117**:1101–1107 (1991) doi:10.1083/jcb.117.5.1101.
47. F. Hunter, J. Xie, C. Trimble, M. Bur, and K. C. Li. Rhodamine-RCA *in vivo* labeling guided laser capture microdissection of cancer functional angiogenic vessels in a murine squamous cell carcinoma mouse model. *Mol. Cancer*. **5**:5 (2006) doi:10.1186/1476-4598-5-5.
48. Y. Matsumura, and H. Maeda. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and antitumor agent smancs. *Cancer Res.* **46**:6387–6392 (1986).
49. G. Y. Lee, S. K. Kim, and Y. Byun. Glucosylated heparin derivatives as non-toxic anti-cancer drugs. *J. Control. Release*. **123**:46–55 (2007) doi:10.1016/j.jconrel.2007.07.017.