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An apoptosis-homing peptide-conjugated low molecular weight heparin-taurocholate conjugate with antitumor properties

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ABSTRACT

Various angiogenesis inhibitors and apoptosis-targeting agents have been therapeutically applied in preclinical cancer models, some of which have been tested in clinical trials. In a previous study, we demonstrated that LHT7, a low molecular weight heparin (LMWH)-taurocholate conjugate, has strong antiangiogenic and tumor-suppressive activity and diminished anticoagulant properties. In this study, we developed LHT7-ApoPep-1, an apoptosis-homing peptide-conjugated variant of LHT7. LHT7-ApoPep-1 exhibited antiangiogenic activity in endothelial cell tube-formation assays and apoptotic cell-targeting ability in tumor cell binding assays; it also showed little toxicity toward healthy cells. Administration of LHT7-ApoPep-1 in mouse xenograft models of breast carcinoma delayed tumor growth compared to LHT7-only, and histological evaluations revealed decreased vessel formation and increased apoptotic area in tumor tissues. Moreover, an examination of LHT7-ApoPep-1-Cy7.5 localization within the body using *in vivo* live imaging showed accumulation at the tumor site of tumor-bearing mice, with a more prolonged circulation time and enhanced intensity compared to LHT7-Cy7.5. Inspection of the tumor microenvironment revealed that Cy5.5-labeled LHT7-ApoPep-1 was located on and near CD31-positive vessels in tumor tissue. We conclude that LHT7-ApoPep-1 has antiangiogenic and apoptosis-targeting properties and exerts antitumor effects by suppressing tumor vessel growth and homing to apoptotic cells within the tumor.

1. Introduction

Angiogenesis and apoptosis are prominent features of the environment of growing tumors [1]. Angiogenesis is a highly exquisite process that is critical to reproduction, wound healing, and progression of malignant tumors [2,3]. In the process of tumor angiogenesis, as well as during growth and/or tumor metastasis, tumors secrete multiple growth factors, some of which interact with heparan sulfate; these factors are potential targets for the development of angiogenesis inhibitors [4–7]. The anticoagulant properties of heparin, a plentiful sulfated polysaccharide, are well known, but heparin also has an important role as a growth factor-inhibitory agent [8–11]. In the past decade, it has been reported that heparin binds angiogenic growth factors and affects tumor progression through modulation of tumor vascularization and the spread of tumor cells [12–15].

Apoptosis is a fundamental process that occurs during the regulation of normal development and homeostasis of multicellular organisms. Intensive cancer treatments, such as chemotherapy and radiation, are accompanied by the iatrogenic induction of apoptosis, including in non-tumor cells such as vascular endothelial cells. Because of this, the concept of apoptosis-recognition has attracted considerable research attention, prompting the development of agents that home to apoptotic cells [16–18].

By exploiting two processes that are universal features of tumorigenesis—angiogenesis and apoptosis—it should be possible to develop therapeutic agents that provide synergistic antitumor effects [19,20]. We have demonstrated previously that the new

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angiogenesis inhibitor, LHT7, a conjugate of a low molecular weight heparin (LMWH) derivative and taurocholic acid, delays the growth of human MDA-MB-231 breast cancer tumors *in vivo* [21]. In the current study, we sought to design a new angiogenesis inhibitor that targets tumor tissue by introducing ApoPep-1 (tumor apoptotic and necrotic area binding peptide), an active-targeting moiety, to the previously developed LHT7. Here, we evaluated the antiangiogenic effects of LHT7-ApoPep-1 *in vitro* using endothelial cell tube-formation assays and assessed the apoptosis-homing and antitumor activity of LHT7-ApoPep-1 *in vivo* using mouse xenograft tumor models. Distribution of LHT7-ApoPep-1 in the apoptotic areas of tumor microenvironment was also analyzed by microscopically inspecting the excised tumor tissues from mice.

2. Materials and methods

2.1. Preparation and characterization of an apoptosis-targeting, peptide-conjugated LHT7

Peptide-conjugated LHT7 was prepared by first dissolving 50 mg of end-amidated LMWH (Celsus, Cincinnati, OH) and 6.67 mg of sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC; Pierce, Rockford, IL) in 5 ml formamide and 3 ml *N,N*-dimethylformamide (DMF), respectively. The two solutions were mixed and stirred overnight, after which a solution containing 25 mg of ApoPep-1, prepared in 1 ml formamide–water cosolvent (1:1), was added and allowed to react for 5 h. The product was purified by dialysis (molecular weight cut-off [MWCO], 2000 Da) in water, and ApoPep-1-conjugated LMWH was acquired by lyophilization. The successful introduction of ApoPep-1 to end-amidated LMWH was confirmed using Fourier transform-nuclear magnetic resonance spectroscopy (Avance 500 MHz FT-NMR; Bruker, Billerica, MA) [22,23]. Next, 43.1 mg of heparin–SMCC–ApoPep-1 was mixed with 20.4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in an ice batch for 5 min and the pH was adjusted to pH 5–6. To this solution, 11.8 mg of *N*-hydroxysuccinimide (HOSu) was then added and the resulting solution was agitated for 30 min at room temperature. In the final step, 66.04 mg of ethylene amine derivative of taurocholic acid (Et-TCA) was added in water and allowed to react overnight. The product, LHT7-ApoPep-1, was dialyzed with membrane (2000 Da MWCO) to remove EDC, HOSu and unreacted Et-TCA, and was lyophilized by vacuum drying (yield = 60%).

2.2. Endothelial capillary-like tube-formation assay

Human umbilical vein endothelial cells (HUVECs; Clonetics, San Diego, CA) were seeded onto 96-well plates (4×10^4 cells/well) coated with 50 μl/well of growth factor-reduced Matrigel (BD Bioscience, San Jose, CA) that had been allowed to polymerize at room temperature for 1 h. The plates were examined for tube-formation at different incubation times (3, 7 and 12 h) after seeding. Each experiment was performed in triplicate and was repeated several times to assure repeatability. Capillary-like tube-formation was observed with an inverted phase-contrast microscope, and the number of tubes was quantified.

2.3. Immunofluorescence of peptide binding

MDA-MB-231 breast cancer cells and H460 lung cancer cells were cultured in RPMI-1640 medium. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin). Apoptosis was induced by incubating with 50 μM etoposide (H460 cells) or 2.5 nM TRAIL ligand (MDA-MB-231 cells) for the indicated durations. For immunofluorescence analyses, cells were treated with 1% bovine serum albumin (BSA) at 37 °C for 30 min to block nonspecific binding and then with 250 μM Cy5.5-conjugated LHT7-ApoPep-1 at 4 °C for 1 h. Apoptotic cells were then labeled with Alexa-594-annexin V (Invitrogen, Carlsbad, CA) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 15 min at room temperature. After fixation, cells were incubated with a 4′,6′-diamidino-2-phenylindole (DAPI)-containing mounting solution (Sigma–Aldrich, St Louis, MO) to stain nuclei before observing under a fluorescence microscope (Zeiss, Oberkochen, Germany).

2.4. Antitumor efficacy of ApoPep-1-LHT7 in vivo

In vivo studies used the same mouse models of human tumor xenografts (5-wk-old female athymic nude mice) and treatment strategies as those described in our previously reported LHT7 experiments [21]. Briefly, MDA-MB-231 human breast cancer cells (1.0×10^7) were injected subcutaneously into the back of a mouse. After the tumors had grown to approximately 50–80 mm^3, ApoPep-1, LHT7-ApoPep-1 or LHT7 were injected via the tail vein. Mice were divided into six groups ($n = 5$/group) receiving different intravenous (i.v.) injections, as follows: (1) saline (control group); (2) ApoPep-1; (3) LHT7, 5 mg/kg; (4) LHT7-ApoPep-1, 0.2 mg/kg; (5) LHT7-ApoPep-1, 1 mg/kg; and (6) LHT7-ApoPep-1, 5 mg/kg. Each sample was injected daily for 14 days. The body weights of mice were recorded, and tumor volumes were calculated as $a \times b \times b \times 0.54$, where "a" is the largest axis and "b" is the smallest axis.

2.5. Histological analysis

The localization of LHT7 in the tumor environment was analyzed by first injecting animals (i.v.) with LHT7-Cy5.5. After 6 h, mice were sacrificed, and perfused and fixed with 4% paraformaldehyde; thereafter, 10-μm-thick sections were prepared. Microvessels were assessed by immunostaining for CD31 (rat anti-mouse monoclonal antibody, MEC-13.3, 1:20; BD Pharmingen, San Diego, CA), and apoptotic cell were assessed by terminal deoxy-nucleotidyl transferase-mediated nick-end labeling (TUNEL) assays. After washing with phosphate-buffered saline (PBS), sections were incubated with fluorescein isothiocyanate (FITC)- or Alexa-594-conjugated secondary antibody (diluted 1:200 in 1% BSA) for 60 min at room temperature. Sections were then washed with PBS, incubated with DAPI (to stain nuclei), slide-mounted, and sealed. Samples were visualized under a fluorescence microscope. Localization of LHT7 in tumor tissue was assessed by injecting animals (i.v.) with LHT7-Cy5.5 2 h prior to sacrificing, fixing, and sectioning as described above. Microvessels were assessed by immunostaining for CD31 (MEC-13.3, 1:20; BD Pharmingen), and apoptosis was detected using TUNEL assays. Samples were visualized by confocal microscopy (Carl Zeiss, Inc., Jena, Germany).

2.6. Fluorescence imaging of the in vivo bio-distribution of Cy7.5-tagged LHT7-ApoPep-1 and LHT7

Female athymic nude mice bearing a subcutaneous MDA-MB-231 tumor (approximately 50–80 mm^3) were injected (i.v.) with 5 mg/kg LHT7-Cy7.5 or LHT7-ApoPep-1-Cy75. *In vivo* fluorescence imaging was performed by scanning the mouse at the indicated times using an Optix eXplore molecular imaging system (ART, Montreal, Canada) and Cy7.5 fluorophore excitation ($\lambda_{excitation}$ = 778 nm) and emission ($\lambda_{emission}$ = 805 nm) filtersets [24]. The shown fluorescent images of organs from mice were obtained 6 h post-injection.

2.7. Statistical analysis

The statistical significance of differences between experimental and control groups was analyzed using one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$; significant differences are denoted by asterisks in the figures.

3. Results

3.1. Characteristics of LHT7-ApoPep-1

The structure of LHT7-ApoPep-1 is shown in Fig. 1A. In the synthesis of LHT7-ApoPep-1, ApoPep-1 was conjugated to end-amidated LMWH using SMCC as an amide-to-sulfhydryl cross-linker. The fidelity of ApoPep-1-conjugated LMWH synthesis was confirmed by 500 MHz FT-NMR, which revealed characteristic peaks at 1.30–1.45 ppm (Fig. 1B). Sodium taurocholate (TCA) was conjugated to the LMWH moiety of ApoPep-1-LMWH to produce ApoPep-1-LHT7.

3.2. Inhibition of endothelial tube-formation by LHT7-ApoPep-1

Initially, to demonstrate that the effect of LHT7-ApoPep-1 on endothelial tube-formation was similar to that of LHT7, we used an *in vitro* endothelial cell tube-formation assay on Matrigel. Endothelial cells were treated with the indicated concentrations of LHT7-ApoPep-1 or cultured with endothelial cell culture medium (EGM) alone (control) and then incubated on Matrigel for 16–18 h at 37 °C. Endothelial cells cultured with EGM showed formation of tube vessels after 1 h of incubation and tube vessel formation with capillary loops after 3 h. Treatment of endothelial cells with LHT7-ApoPep-1 produced an inhibitory effect on tubular formation of HUVECs similar that of LHT7 treatment (Fig. 2A). Quantification of tube-formation by counting the number of capillary tube-like structures confirmed that LHT7-ApoPep-1 significantly inhibited tube-formation by HUVECs *in vitro* (Fig. 2B).