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Suppression of pancreatic cancer by sulfated non-anticoagulant low molecular weight heparin

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ABSTRACT

Sulfated non-anticoagulant heparins (S-NACHs) might be preferred for potential clinical use in cancer patients without affecting hemostasis as compared to low molecular weight heparins (LMWHs). We investigated anti-tumor effects, anti-angiogenesis effects, and mechanisms of S-NACH in a mouse model of pancreatic cancer as compared to the LMWH tinzaparin. S-NACH or tinzaparin with or without gemcitabine were administered, and tumor luminescent signal intensity, tumor weight, and histopathology were assessed at the termination of the study. S-NACH and LMWH efficiently inhibited tumor growth and metastasis, without any observed bleeding events with S-NACH as compared to tinzaparin. S-NACH distinctly increased tumor necrosis and enhanced gemcitabine response in the mouse pancreatic cancer models. These data suggest the potential implication of S-NACH as a neoadjuvant in pancreatic cancer.

Introduction

Pancreatic cancer, which is usually diagnosed at an advanced stage, carries the highest fatality rate among all human cancers [1,2]. Reasons for low survival include aggressive tumor, high metastatic potential, and late presentation at the time of diagnosis. Despite the introduction of gemcitabine and attempts at developing combination chemotherapy regimens, pancreatic cancer remains a highly aggressive and chemo-resistant tumor [1], and there is need for improved methods to treat this deadly disease. Heparin and low molecular weight heparins (LMWHs) are used in pancreatic and other cancer patients mainly to prevent or treat deep vein thrombosis [3]. In addition to antithrombotic effects, LMWHs release tissue factor pathway inhibitor proteins [4] and nitric oxide [5], attenuate TNF-alpha induced inflammation [6], and inhibit heparinases [7] and selectin [8], supporting their potential anti-cancer and anti-inflammatory role. However, a direct anti-cancer effect for heparins or LMWHs in cancer patients without thrombosis still remains to be demonstrated clinically.

LMWHs have been shown to illicit significant anti-tumor responses in a variety of cancers in both animal and some clinical studies [9–16], suggesting the potential for increasing patient survival. However, it has been difficult to draw definitive conclusions about survival benefits because the studies often involved populations that were heterogeneous in terms of histology type and stage of tumor. Major bleeding problems associated with systemic effects of LMWH on thrombin and factor Xa [17,18] are the limiting factor in continuation of treatments or dose escalation in clinical trials. The anti-thrombin-binding sequence accounts for most of the systemic anticoagulant activity of clinically used heparins and LMWH, and it is localized in only one third of the large molecule [19]. It has been shown that the anti-metastatic efficacy is not primarily based on its anticoagulant activity [20],

Abbreviations: CAM, chick chorioallantoic membrane; LMWH, low molecular weight heparin; S-NACH, sulfated non-anticoagulant heparin; THBS1, Thrombospondin-1; XIAP, X-linked inhibitor of apoptosis protein.

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and animal studies using non-anticoagulant species of heparin indicate that it is possible to separate the anti-metastatic and anticoagulant activities of heparin [21,22].

We have formulated LMWH that has no effect on the systemic coagulation factors yet releases tissue factor pathway inhibitor protein, a key endogenous inhibitor of the TF/VIIa complex from the endothelium [22]. We have also demonstrated the efficacy of a sulfated non-anticoagulant form of LMWH, S-NACH, as an anti-metastatic agent in a B16 melanoma mouse model, without any significant impact on coagulation [22,23]. The additional finding that S-NACH exhibits anti-angiogenesis activity suggests another mechanism that could contribute to its role in tumor suppression [22].

In this study, we investigated a possible role of S-NACH on tumor growth in an orthotopic pancreatic cancer mouse model, comparing the results with a standard LMWH, tinzaparin, which is used clinically. Our data show that S-NACH has direct anti-cancer effects, with comparable effects on the inhibition of pancreatic cancer proliferation and angiogenesis in the mouse pancreatic tumor model and the chick chorioallantoic membrane (CAM) model, but without any effects on hemostasis. We used *in vivo* and *ex vivo* bioluminescent imaging, correlating tumor signal intensity with viability, and histopathological data to identify a profile of activity and possible mechanism for S-NACH anti-tumor efficacy in this pancreatic cancer model.

Materials and methods

Cancer cell lines and reagents

Human pancreatic cancer cell lines, MPanc96 and SUIT2 expressing firefly luciferase, were provided by Dr. Arumugam (MD Anderson Cancer Center, Houston, TX). Cell culture reagents and hemoglobin standard, Drabkin's reagent, and other common reagents were purchased from Sigma (St. Louis, MO). D-Luciferin potassium salt was purchased from Caliper Life Sciences (Hopkinton, MA), and gemcitabine was purchased from Thermo Fisher Scientific (Waltham, MA). Matrigel was purchased from BD Bioscience (San Jose, CA). Tinzaparin was obtained from Leo Pharma Inc. (Ballerup, Denmark), and S-NACH was synthesized at Rensselaer Polytechnic Institute (Rensselaer, NY). Anti-pSer15-p53 (9286) was obtained from Cell Signaling (Danvers, MA), Anti-XIAP (sc-55550), Anti-THBS1 (sc-65612), and Anti-p21 (sc-6246) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Cells and cell culture

Mpanc96-luc and SUIT2-luc cells were grown in DMEM supplemented with 5% fetal bovine serum, 1% penicillin, and 1% streptomycin. Cells were cultured at 37 °C to sub-confluence and treated with 0.25% (w/v) trypsin/EDTA to affect cell release from culture flask. After washing cells with culture medium, cells were suspended in DMEM (free of phenol red and fetal bovine serum) and counted.

Tumor growth in the CAM cancer implant model

The CAM cancer implant model has been described previously [24] and was used here to study the effect of heparin derivatives (S-NACH versus tinzaparin) along with gemcitabine at 1.0 μg/CAM on tumor angiogenesis and tumor growth. Briefly, pancreatic cancer cells in exponential growth phase were harvested as described above. One $\times 10^6$ cells in 30 μl of medium were mixed with an equal volume of Matrigel and implanted in the chorioallantoic membrane of 7-day old chick eggs. The effect of these treatments was determined after 8 days of implantation. Results are presented as a mean tumor weight (g) per treatment group and tumor hemoglobin (mg/dl) ± SEM, $n = 10$ per group.

Animal studies

Immune-deficient female NCr nude homozygous mice aged 5–6 weeks and weighing 18–20 g were purchased from Harlan Laboratories (Indianapolis, IN). All animal studies were conducted at the animal facility of the Veteran Affairs Medical Center, Albany, NY, in accordance with the institutional guidelines for humane animal treatment and according to the current NIH guidelines. Mice were maintained under specific pathogen-free conditions and housed under controlled conditions of temperature (20–24 °C), humidity (60–70%), and 12 h light/dark cycle with ad libitum access to water and food. Mice were allowed to acclimatize for 5 d prior to the start of study.

Pancreatic tumor orthotopic implant and treatments

MPanc96-luc and SUIT2-luc cells were harvested as described above and were orthotopically implanted (2×10^5 cells in 50 μl PBS per mouse) in the pancreas of anesthetized athymic nude mice. Just before treatment initiation, animals ($n = 5$–10 per group) were randomized by tumor mass detected by an *in vivo* imaging system (IVIS, described below). Initially heparin derivatives S-NACH and tinzaparin at 10 mg/kg were administered subcutaneously (s.c.) daily. In the subsequent studies S-NACH at 20 mg/kg and tinzaparin at 5 mg/kg were administered s.c. daily. Gemcitabine at 100 mg/kg was injected intraperitoneally twice a week alone or in combination with either S-NACH or tinzaparin. Treatment protocols for this study are summarized in Table 1. All mice used for treatment response evaluations were euthanized after 28 days.

In vivo imaging system (IVIS)

Imaging was performed once per week to monitor tumor growth. Mice bearing MPanc96-luc or SUIT2-luc tumors were anaesthetized using isoflurane, injected s.c. with 50 μl D-luciferin (30 mg/ml), then imaged. Photographic and luminescence images were taken at constant exposure time. Xenogen IVIS® Living Image software version 3.2 was used to quantify non-saturated bioluminescence in regions of interest. Light emission between 5.5×10^6 and 7.0×10^{10} photons was assumed to be indicative of viable luciferase-labeled tumor cells while emissions below this range were considered as background. Bioluminescence was quantified as photons/s for each region of interest. *In vivo* tumor kinetic growth and metastasis were monitored by signal intensity. *Ex vivo* imaging was performed to confirm the signal intensity in the tumors after the termination the study.

Histopathology

All specimens were analyzed by histology for routine analysis. Specimens were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Then, after fixation, the specimens were transferred into the embedding chambers to hold the specimens in position until the paraffin became solid to prevent further rotation. Four μm serial sections were cut, and then stained using haematoxylin and eosin. Sections were evaluated for various pathologic parameters using a light microscope (Leica, Buffalo Grove, IL).

Studies of cultured pancreatic cell growth in vitro

Cells were cultured as previously described for other cell lines [25], except that the medium contained 10% fetal bovine serum throughout the course of each study. Media were replenished daily, including the addition of tinzaparin or S-NACH at 40 μg.

Immunoblotting

Extracts of cytosolic proteins were obtained from control and treated cells, after which the total protein content was quantitated and proteins resolved on discontinuous PAGE. Proteins were then electro-blotted to nitrocellulose membranes (Millipore, Bedford, MA) as previously described [26]. The membranes were treated with 5% milk in tris-buffered saline containing 0.1% Tween™ and incubated overnight with one of the following: monoclonal anti-pSer15-p53 (hyperphosphorylated p53), anti-XIAP, anti-THBS1, and anti-p21. Primary antibody incubation was followed by treatment with the secondary rabbit anti-mouse IgG antibody. Immunoblots of β-actin were also prepared to control for equalization of proteins. Results presented reflect 3 western blot experiments, and data are represented as mean ± SEM, $n = 3$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

Statistical analysis

Analysis of the *in vivo* study results was by one-way ANOVA using StatView software (Adept Scientific, Acton, MA). The mean ± SEM from each experimental group was compared with its respective control, and statistical significance was defined as $P < 0.05$. For the *in vitro* studies, the unpaired *t*-test was used for analysis.

Results

Treatment effects on tumor angiogenesis and tumor growth in the CAM model

Either S-NACH or tinzaparin at 1 μg/CAM significantly ($^{*}P < 0.01$) inhibited pancreatic cancer cell (MPanc96-luc) medi-

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