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RESEARCH PAPER

Inhibitory effect of non-anticoagulant heparin (S-NACH) on pancreatic cancer cell adhesion and metastasis in human umbilical cord vessel segment and in mouse model

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Abstract Metastasis is the most devastating aspect of cancer and it is the main cause of morbidity and mortality in cancer patients. Tumor cell adhesion to the vascular endothelial cell lining is an important step in metastatic progression and is prompted by platelets. Mucin 1 is overexpressed and aberrantly glycosylated in more than 60% of pancreatic ductal adeno-carcinomas, which mediate adhesion of pancreatic cancer cells to platelets via P-selectin. The anticoagulant low molecular weight heparins (LMWHs), which are commonly used in venous Thromboprophylaxis and treatment, appear to have an effect on cancer survival. The aim of this study is to investigate the effect of platelets on human pancreatic cancer MPanc96 cell adhesion to the endothelial cell vessel wall, and to examine the effect of heparin derivatives on MPanc96 adhesion using a novel, in vitro model of human umbilical cord vein. The modified heparin S-NACH (sulfated non-anticoagulant heparin), which is devoid of antithrombin (AT) binding and devoid of inhibition of systemic AT-dependent coagulation factors such as factor Xa and IIa, and the LMWH tinzaparin both potently reduced adhesion and invasion of fluorescence-labeled MPanc96 cancer cells to the endothelial layer of umbilical cord vein in a dose-dependent manner. S-NACH effectively inhibited P-selectin mediated MPanc96 cell adhesion, and inhibited cell adhesion and invasion similar to tinzaparin, indicating that systemic anticoagulation is not a necessary component for heparin attenuation of cancer cell adhesion, invasion, and metastasis. Also, S-NACH and tinzaparin versus unfractionated heparin, heparin derivatives enoxaparin, deltaparin, fraxiparin, and fondaparinux were evaluated for their effect on platelet-cancer cell adhesion. An in vivo anti-metastatic S-NACH-treated nude mouse model of MPanc96 pancreatic cancer cell metastasis demonstrated potent anti-metastasis efficacy as evidenced by IVIS imaging and histological staining.

Keywords Heparin · Low molecular weight heparin · Anticoagulant · Heparin derivatives · P-selectin glycoprotein ligand-1 · P-selectin · Platelet · Endothelial cells · Cancer cell adhesion · Invasion · Metastasis

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Introduction

Pancreatic adenocarcinoma is a highly lethal disease. It has the worst prognosis of any malignancy, and is the fourth most common cause of cancer death yearly in the United States [1]. One of the major hallmarks of pancreatic cancer is its extensive local tumor invasion and early systemic metastasis with few or no effective therapies [2]. Therefore, preventing its invasion and dissemination might improve the survival of pancreatic cancer patients.

Platelet interaction with cancer cells is thought to play a major role in cancer invasion and metastasis [3, 4]. Platelet

aggregation induced by tumor cells is important in hematogenous metastasis and also contributes to the prothrombotic state in cancer [5, 6]. Pancreatic cancer cell lines induce platelet aggregation in vitro via a thrombin-dependent mechanism [7]. Pancreatic cancer and many other malignant diseases are associated with the well-recognized complication of developing thromboembolic disease. The standard therapies include the use of heparin and low molecular weight heparin (LMWH), which serve as effective anticoagulants in the prevention and treatment of cancer-associated thrombosis, along with having potential anticancer effects and improving survival in cancer patients [8, 9].

The potential anti-metastatic activity of heparin and LMWH that is independent of its antithrombotic effect is supported by data from many in vitro and in vivo studies [10–21]. Several clinical studies as well as meta-analyses have found significant improvement in 3-month and 6-month survival in cancer patients who are treated with LMWH compared to cancer patients treated with unfractionated heparin [9, 21–26]. However, the risk of bleeding associated with heparin or LMWH anticoagulation therapy has limited their use in cancer patients. In that regard, non-anticoagulant heparins would be preferable for clinical use if proven equivalent in efficacy to LMWH since Sulfated Non-Anticoagulant Heparin (S-NACH) could be administered at high doses, without bleeding complications.

To study the complex polypharmacological properties of heparin and its derived non-anticoagulant heparins on cancer cell adhesion and invasion in a clinically relevant in vitro model system, we have modified our previously established in vitro perfusion system. In that system, human breast cancer cells (MDA-MB-231 and MCF7) were added to human blood and circulated into human umbilical cord at venous shear, and the effect of LMWH on adhesion and invasion to umbilical endothelium was determined [27]. To improve the information obtained from the vessel segment model, we cannulated the umbilical cord vein to prevent any physical damage to the endothelial layer of the vessel, used fluorescence-labeled cancer cells, and imaged attached cancer cells to vessel wall.

Also in the current study, we used this in vitro perfusion model on segments of human umbilical cord to examine the effect of platelets on pancreatic cancer cell adhesion to vascular endothelium, and to assess the role of S-NACH versus the anticoagulant LMWH tinzaparin on cancer-platelet endothelial adhesion and invasion. Additionally, the effect of other heparin derivatives (enoxaparin, deltaparin, fraxiparin, fondaparinux) on platelet-associated P-selectin mediated adhesion to pancreatic cancer cells was also studied.

Materials and methods

Reagents and tumor cell line

Cell culture reagents and Cell Tracker Green CMFDA were purchased from Invitrogen (Carlsbad, CA). Anti-mouse P-selectin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pancreatic cancer cell line MPanc96 was provided by Dr. T. Arumugam (MD Anderson Cancer Center, Houston, TX). Tinzaparin was obtained from Leo Pharma Inc. (Ballerup, Denmark) and S-NACH was synthesized at Rensselaer Polytechnic Institute. Both tinzaparin and S-NACH were solublized in PBS. After informal consent, fresh umbilical cord was collected in PBS along with cord blood immediately after a cesarean section delivery from different human subjects, at Albany Medical Center.

Preparation of cells for attachment study

MPanc96 cells (5×10^6) were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. In order to quantify tumor cell adhesion, pancreatic cancer cells were labeled with Cell Tracker Green CMFDA as per the product instructions, and collected in 50 ml of medium.

Human P-selectin mediated cancer cell adhesion

The ability of heparin and heparin derivatives to inhibit adhesion of LS180 cancer cells to immobilized P-selectin was examined as described previously [13]. Briefly, ELISA plates (Nunc, Rochester, NY), were coated overnight with soluble protein A, and then blocked with 1% bovine serum albumin (BSA) in Hebe's buffered salted solution (HBSS) for 30 min at RT. Mouse P- and L-selectin chimeras (400 ng/well in HBSS/BSA) were incubated for 3 h at RT. After 3 washes, calcein AM-labeled LS180 tumor cells (50,000 cells/well) were added to the plate in the presence or absence of serially diluted heparin derivatives at concentrations ranging from 0.24 to 250 μg/ml. Plates were incubated for 1 h at 4°C while rotating on an Orbital Rotor at 70 rpm. Wells were washed with HBSS/BSA and HBSS. Adherent cells were lysed with 1% Triton X-100 and quantified by measuring the fluorescence with an ELISA plate reader. The IC50 values were calculated from three independent experiments.

Perfusate

For the umbilical cord vein perfusion model, cord blood was collected in standard buffer containing sodium citrate (0.105 M = 3.2%) and used within 1–2 h. Platelet-rich

plasma was separated as described above. Just prior to the flow adhesion assay, an equal number of platelets ($\sim 10^9$) were added to 50 ml of medium containing labeled cancer cells. For inhibition studies, perfusates were pretreated for 30 min at 37°C with either S-NACH or the LMWH tinzaparin at different concentrations.

Flow adhesion assay

Cancer cell perfusion model using human umbilical cord vein

The human umbilical cord was cut into ~ 5 cm segments and cleaned with PBS. The cord vein was cannulated using an 18 g straight gavage needle in umbilical cord segment and attached to the BioRad Econo pump system. MPanc96 cells were perfused at a constant venous perfusion pressure of 40 mm Hg. Cancer cells were continuously mixed using a stir-bar at low speed and re-circulated through the vein. The system was maintained in a humidified atmosphere of 5% CO_2 and 95% air (Fig. 1). To prevent drying during the study, cord was covered with PBS.

Confocal microscopy and morphometric studies

Umbilical cord was removed from the perfusion system and the vein was cut longitudinally and placed on cover glass. Cancer cells attached to the wall of the vein were imaged using a Leica (LAS AF) Confocal microscope. Fluorescent signals (green) of the cancer cells were detected at excitation/emission 494 nm/518 nm wavelengths. Confocal images were obtained with the image plane parallel to the wall surface. All the cells attached to the length of vein were counted. The segment was then fixed in buffered formalin for histological studies.

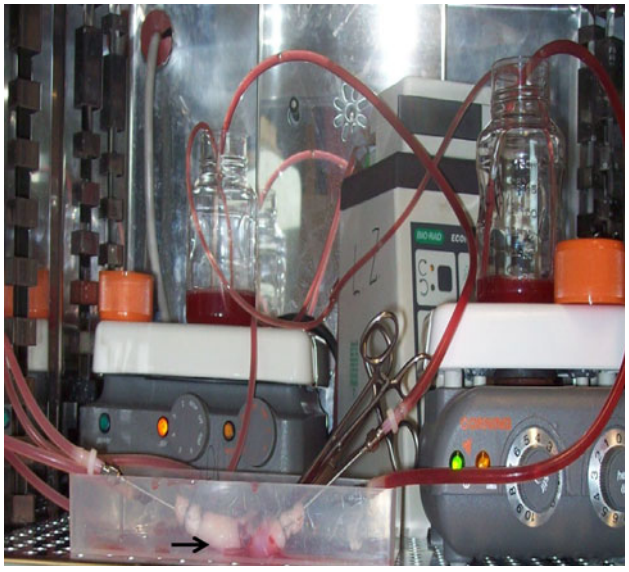

Fig. 1 Perfusion system showing the recirculation of pancreatic cells through the cannulated vein of human umbilical cord segment. Maintained in a humidified atmosphere with 5% CO_2 and 95% air; arrow points to cannulated umbilical cord vein

Protein studies in platelets and endothelial cells

Blood was collected from normal healthy donors and platelet-rich plasma was collected as described above. Platelets were separated by centrifuging at $15{,}000 \times g$ for 15 min and treated at different concentrations with either S-NACH or tinzaparin for 3 h and lysed in RIPA buffer. Lysates were run on SDS-PAGE, transferred to polyvinylidine membranes, and blotted with P-selectin antibody. Blots were detected using HRP-conjugated anti-mouse IgG.

MPanc96-luc metastasis study in nude mice

Animal studies

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

Cells

MPanc96-luc (0.5×10^6 cells/50 µl/animal).

Injection

Intravenous injection into the tail vein.

Treatment

S-NACH (20 mg/kg, subcutaneous) just prior to injection of MPanc96-luc cells versus vehicle (PBS) control group. S-NACH treatment (20 mg/kg, once a day, subcutaneous) was continued for 15 days.

In vivo imaging system (IVIS)

Imaging was performed at 15 min post-intravenous injection of cancer cells, 24 h after, 7 days after, and at the end of day 15 to monitor tumor invasion. Mice were

anaesthetized using isoflurane and post luciferin injection mice were 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 (ROI). Light emission between 5.5×10^6 and 7.0×10^{10} 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/second for each ROI. 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 on day 15.

Histology

After animals were sacrificed, primary tumors were excised and fixed in 4% buffered formaldehyde for 24 h, rinsed with phosphate buffer, dehydrated in a series of graded ethanol and embedded in paraffin. Sections of 5 μm thickness were cut and stained with haematoxylin and eosin (H.E.). To achieve a random distribution of each animal lung, the lungs were excised and fixed en block and cut into 1 mm thick slices. The slices were placed in warm agar and pressed down with a glass piston. After hardening of the agar these lung slices were processed paraffin-embedded as above. Ten slices from the 10th section out of the middle of each paraffin wax block were H.E. stained. Metastases were counted in each of the ten stained sections under a microscope (Zeiss, Axioplan 10× and 20×).

Statistical analyses

Experiments on the effects of shear rate on tumor cell adhesion were carried out with human umbilical cords and human blood. Experiments on the effect of heparin derivatives were performed as a series of three runs (control and test agent), each run being performed from human umbilical cord and blood from the same cord. For each assay, control perfusion was considered as 100% adhesion, and the results with either S-NACH or LMWH were expressed as percent yield of this control.

Results

Effect of heparin derivatives on cancer cell adhesion

The modified perfusion system (Fig. 1), which simulates physiological flow conditions in human vessel segments to test cancer cell adhesion to the endothelial layer, was used to determine the effect of S-NACH versus the LMWH tinzaparin. The adhesion of highly metastatic pancreatic cancer cells (MPanc96) to the vessel wall could be visualized within 2–3 h (Fig. 2a, b). The newly attached cancer cells were rounded and eventually, by 24 h, firmly adhered and spread out onto the endothelial layer (Fig. 2c). After 3 h there was no further increase in the number of attached cells (Fig. 2d), even after 24 h (not shown). Hence, the 3 h time point was used for investigating the effect of S-NACH and tinzaparin on the metastasis. Histopathology studies revealed that the cancer cells attached to the endothelial layer of the vessel (Fig. 3). Perfusion of cancer cells along plasma did not reveal any cancer cell adhesion to the vessel wall, even after 24 h of perfusion. S-NACH, with no anticoagulant effect (i.e., devoid of anti-Xa and anti-IIa activities), decreased tumor cell adhesion and invasion into vascular wall, similar to the potent anticoagulant LMWH tinzaparin.

S-NACH and tinzaparin were tested at 1, 5, 10, and 40 μg/ml concentrations on the adhesion of the MPanc96 cancer cells with human platelets through the umbilical cord segments. Reduction of metastasis by 50–80% was achieved by the inhibition of cancer cell adhesion to the human vessel segment (Fig. 4). The non-anticoagulant S-NACH attenuated the cancer adhesion by 60–75%, and not much variation in the dose–response was observed between 5, 10, and 40 μg/ml (Fig. 4a). Tinzaparin inhibited the adhesion of pancreatic cells in a dose-dependent manner (Fig. 4b).

Effect of heparin derivatives on P-selectin mediated cancer cell adhesion

The effect of unfractionated heparin, different LMWHs, and non-anticoagulant heparin derivatives at different concentrations on P-selectin mediated cancer cell adhesion was determined. S-NACH (average molecular weight = 4,000 Da) demonstrated greater potency in inhibiting P-selectin mediated cancer cell adhesion as compared to unfractionated heparin or the various LMWHs (Fig. 5). The efficacy of LMWHs in inhibiting P-selectin mediated cancer cell adhesion was proportional in efficacy to their average molecular weight, with potencies tinzaparin (6,500 Da) > deltaparin (5,500 Da) > enoxaparin (4,500 Da) > fraxiparin (3,800 Da), and no significant effect with fondaprinux (1,700 Da) (Fig. 5).

Western blot study

We evaluated the contribution of S-NACH and tinzaparin to the P-selectin mediated interaction of the metastatic process. To investigate whether the observed attenuation of metastasis was due to P-selectin inhibition, we tested the effect of these heparin derivatives on platelet P-selectin

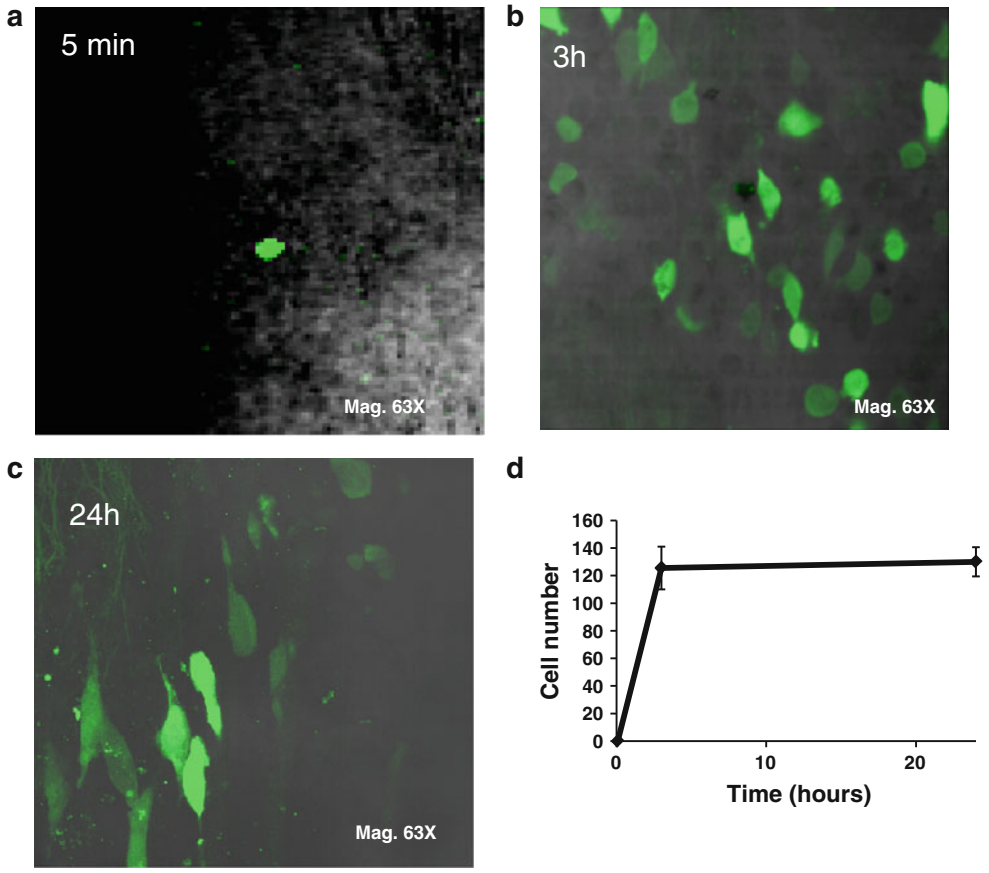

Fig. 2 Confocal images of attached green fluorescence-labeled MPanc96 pancreatic cancer cells on the endothelial layer of vein after perfusion with platelets: **a** after 5 min, showing few cells; **b** after 3 h, showing rounded cells; **c** after 24 h, showing elongated, well attached cells; **d** graph of number of attached MPanc96 cells during the time course of the experiment

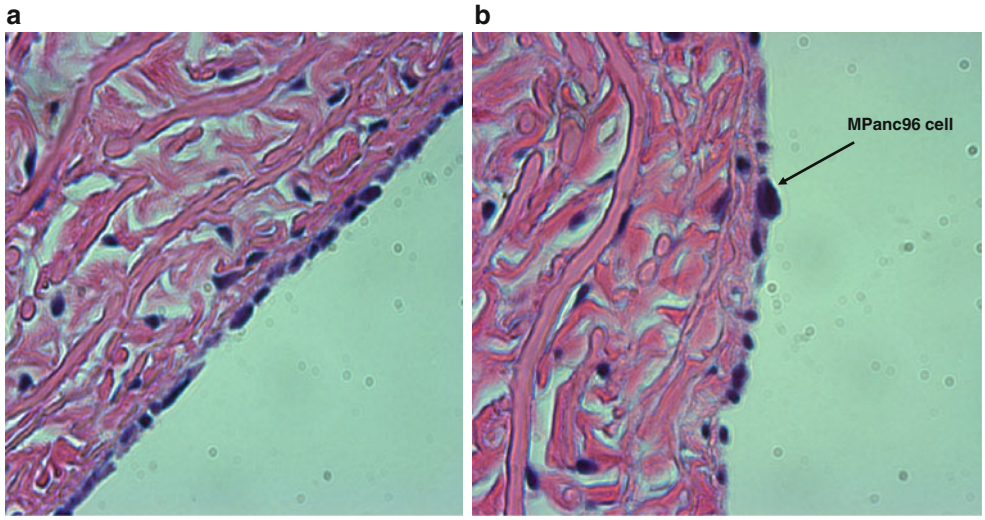

Fig. 3 Representative H.E. stain of the vein after perfusing with MPanc96 cells for 3 h. **a** Control vein with normal endothelium; **b** endothelium showing the attachment of cancer cell

proteins. To further evaluate the role of P-selectin that was expressed on platelets in the process of cancer cell attachment to the endothelial layer of vessel, human platelets were treated with S-NACH or tinzaparin at different concentrations. Both of the heparin derivatives significantly decreased P-selectin expression; notably,

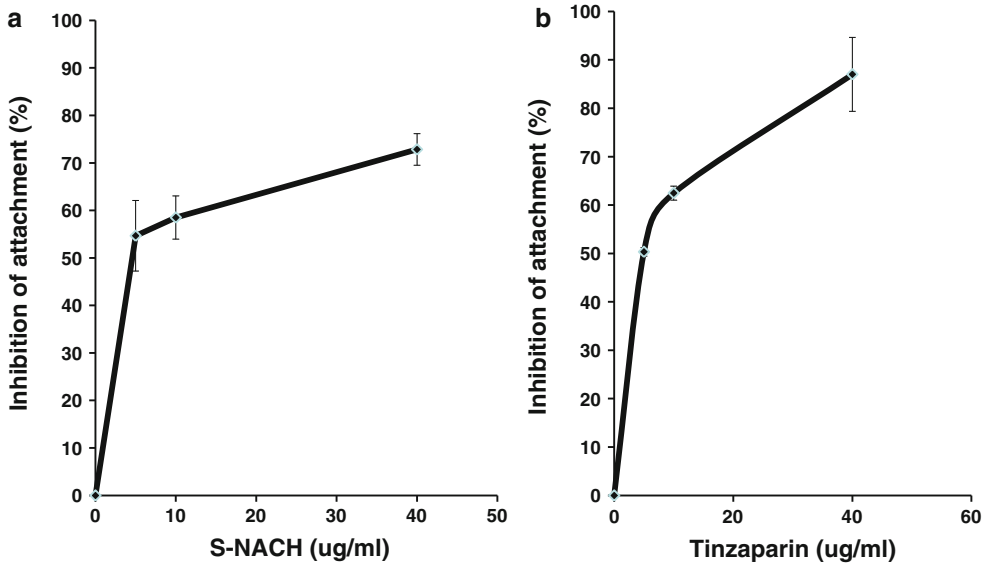

Fig. 4 Inhibitory effect of S-NACH and tinzaparin (0, 5, 10 and 40 μg/ml) on MPanc96 cell adhesion to the endothelial layer of umbilical cord vein after perfusion along with platelets for 3 h. **a** Treatment with S-NACH; **b** treatment with tinzaparin. Data represent mean ± SD, n = 5

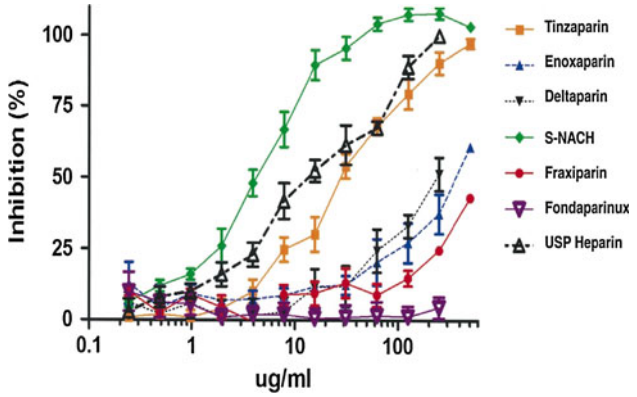

Fig. 5 Effect of S-NACH, unfractionated Heparin, various LMWHs (tinzaparin, deltaparin, enoxaparin, and fraxiparin), and the pentasaccharide fondaparinux on platelet-cancer cell adhesion. Cell adhesion was on immobilized chimeric P-selectin protein. Data represent mean ± SD, n = 5

S-NACH attenuated P-selectin protein expression in a concentration-dependent manner (Fig. 6).

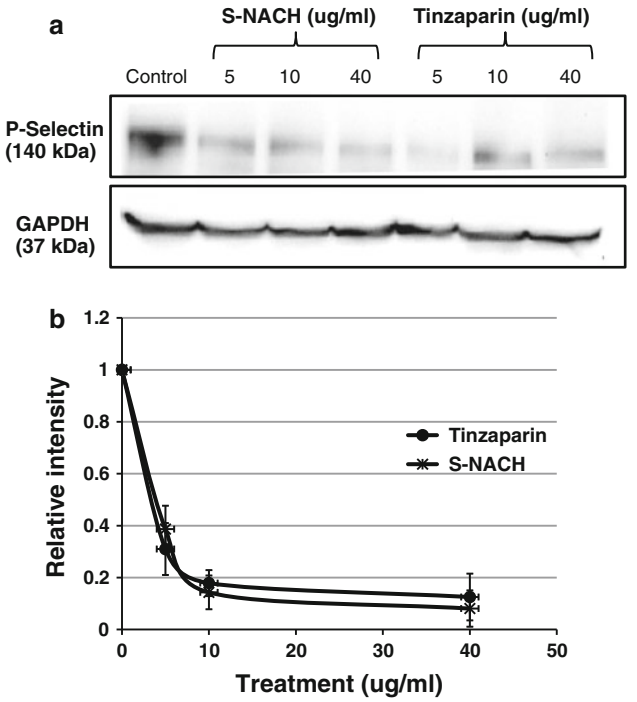

Fig. 6 Inhibition of platelet-associated P-selectin protein expression with S-NACH and tinzaparin (0, 5, 10, and 40 μg/ml) after 3 h treatment. **a** Western blots, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and S-NACH; **b** relative intensity of proteins

Effect of S-NACH on pancreatic cancer metastasis

In vivo anti-metastatic efficacy of S-NACH in a nude mouse model of MPanc96 pancreatic cancer cell lung metastasis demonstrated potent anti-metastasis efficacy as evidenced by IVIS image analysis. The anti-metastasis efficacy was demonstrated at 24 h post-administration of S-NACH as shown in the representative images (Fig. 7).

Furthermore, at the end of the experiment (15 days), IVIS imaging of isolated lungs demonstrated significant suppression ($P < 0.01$) of MPanc96 pancreatic cancer cell accumulation into the lung by S-NACH as compared to vehicle control (Fig. 8a). Histological H.E. staining of isolated and fixed lung tissues confirmed the effective suppression ($P < 0.01$) of MPanc96 lung metastasis (Fig. 8b).

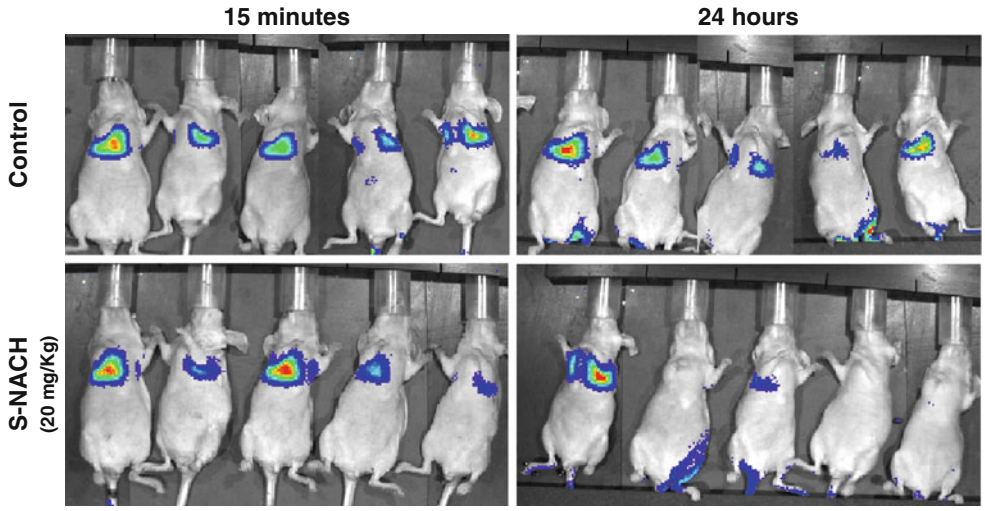

Fig. 7 IVIS imaging of nude mice control (vehicle), S-NACH (treated), and then all mice intravenously injected with MPanc96 cells. Images were taken at 15 min post-MPanc96 cells administration and at 24 h post-MPanc96 cells administration

Discussion

The results rule out any contribution of antithrombin III-mediated activities (anti-Xa and anti-IIa) to the anti-metastatic action of S-NACH as reported from our laboratory and in animal models as reported by other groups [24, 28, 29]. There have been a variety of in vitro perfusion models and techniques developed to study the hemostatic system and its interaction with human vessel walls [30]. Gomes et al. [30] studied the adhesion of MDA-MB-231 breast adenocarcinoma cells in whole blood to human umbilical vein endothelial cell extracellular matrix in vitro under physiological shear conditions, and showed that cooperation between platelet and tumor cell was needed to promote cancer cells.

The present perfusion model is an improved in vitro perfusion system developed by our group using umbilical cord vessel segment [27]. Current screening procedures usually involve highly artificial, expensive, time consuming, and technically complex in vitro models for metastasis. Use of intact vessel segment with cannulation truly represents an in vivo human blood vessel. Fluorescence-labeled human cancer cells permit us to study the kinetics of cancer cell adhesion and invasion into the vessel. The present model offered more physiologically relevant data than the available in vitro techniques, and more readily generated data. A number of experimental variables could be eliminated through our model before animal experiments were performed, consequently reducing the costs incurred with animal use. In summary, a significant volume of data could be generated at low cost, permitting animal models to be used more selectively.

Although anti-angiogenic drugs are expected to restrict the growth of secondary tumors, substances that directly interfere with tumor cell invasion and the subsequent spread of tumor cells to distant sites might have clinical implications. Furthermore, studies from our laboratory showed either LMWH or S-NACH to be effective inhibitors of tumor angiogenesis and tumor growth [8, 10, 24].

Based on the present data and other studies [8, 10, 24, 30], platelets play a key role in the adhesion of cancer cells to the endothelial layer, and as plasma alone show no enhanced cancer cell attachment. Data in the present study show that pancreatic cancer cell adhesion to endothelial cells is accelerated in the presence of human platelets relative to its absence. The effect of the heparin derivatives (LMWH or S-NACH) is due to the inhibition of mainly pancreatic cancer cell-platelet adhesion and also pancreatic cancer cell to endothelial cell adhesion that is mediated by selectins. Since selectins are known to initiate the first steps of cell–cell interactions, the inhibition of selectin-mediated interactions probably belongs to one of the earliest actions associated with metastasis [13, 26]. Heparin acts as a ligand for P-selectin glycoprotein ligand-1 (PSGL-1) and blocks binding to its carbohydrate ligands [10–14]. In addition, it has been reported that the absence of P-selectin in animal studies leads to decreased platelet–tumor cell interactions, resulting in attenuation of metastasis [26]. Despite the different nature of LMWHs, targeted inhibition of P-selectin with S-NACH or tinzaparin markedly reduced metastasis, which is in agreement with previous observations obtained in P-selectin deficient mice [10, 14, 26]. This opened up the possibility that heparin derivatives may inhibit interactions of selectins with endogenous ligands, e.g., P-selectin binding to PSGL-1. Additionally, pancreatic ductal adenocarcinoma mice lacking mucin 1 have a profound defect in tumor growth and metastasis [31]. These data suggest that blocking at the P-selectin or mucin

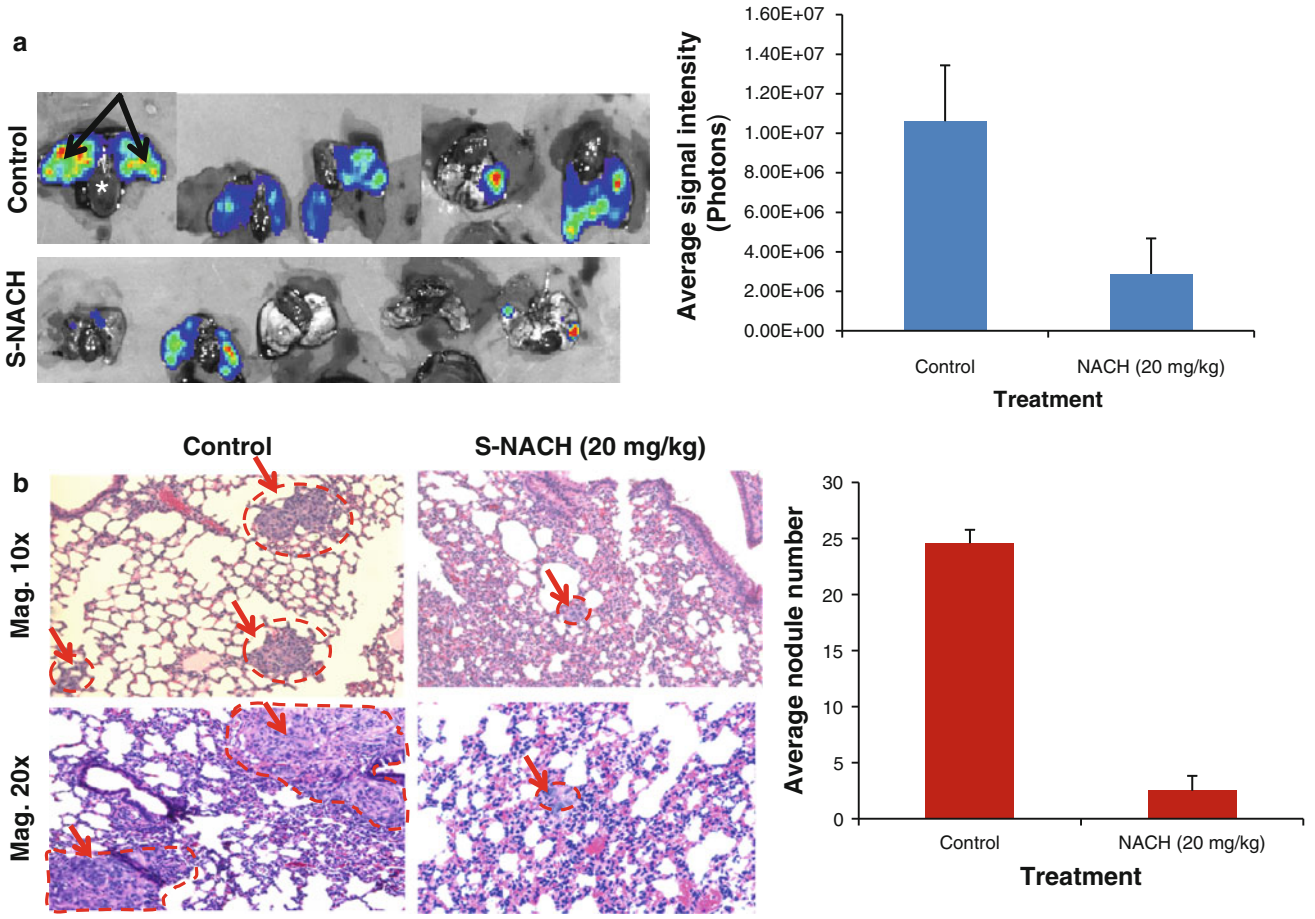

Fig. 8 **a** IVIS imaging of isolated lung tissues obtained from control and S-NACH treated animals, and quantitation of signal intensity of control versus S-NACH treated animals (data represent mean ± SD, $n = 5$). *Arrows* indicate tumor nodules. **b** H.E. staining of formalin fixed 5 μm lung tissues obtained from control and S-NACH treated animals and the measurement of tumor nodules in control versus S-NACH. *Arrows* indicate tumor nodules

1 levels could lead to diminished tumor metastasis. Furthermore, in our study S-NACH demonstrated potent inhibition of pancreatic cancer adhesion (isolated perfused vessel segment model), invasion, and metastasis (experimental metastasis mouse model) in addition to its inhibitory effects of tumor growth and tumor angiogenesis, which is not the case with other non-anticoagulant heparins [32].

Conclusion

Using a simple in vitro assay mimicking the human vessel under physiological shear conditions showed the inhibitory effect of LMWHs and S-NACH on pancreatic cell adhesion to endothelium in a dose-dependent manner. The results also suggest that S-NACH and tinzaparin inhibited the pancreatic cancer cell metastasis through the suppression of platelets' P-selectin, of the platelets, one of the underlying mechanisms in metastasis. Furthermore, potent in vivo anti-metastatic efficacy of S-NACH was demonstrated by IVIS imaging and histological staining in an experimental lung metastasis model of pancreatic cancer. Since there are no viable treatments currently available for metastasis of pancreatic cancer, S-NACH might be an effective and safe therapy in the prevention and treatment of cancer metastasis.

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