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Original Article

Antitumor properties of a new non-anticoagulant heparin analog from the mollusk *Nodipecten nodosus*: Effect on P-selectin, heparanase, metastasis and cellular recruitment

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Abstract

Inflammation and cancer are related pathologies acting synergistically to promote tumor progression. In both, hematogenous metastasis and inflammation, P-selectin participates in interactions involving tumor cells, platelets, leukocytes and endothelium. Heparin has been shown to inhibit P-selectin and as a consequence it blunts metastasis and inflammation. Some heparin analogs obtained from marine invertebrates are P-selectin inhibitors and do not induce bleeding effects. The present work focuses on the P-selectin blocking activity of a unique heparan sulfate (HS) from the bivalve mollusk *Nodipecten nodosus*. Initially, we showed that the mollusk HS inhibited LS180 colon carcinoma cell adhesion to immobilized P-selectin in a dose-dependent manner. In addition, we demonstrated that this glycan attenuates leukocyte rolling on activated endothelium and inflammatory cell recruitment in thioglycollate-induced peritonitis in mice. Biochemical analysis indicated that the invertebrate glycan also inhibits heparanase, a key player in cell invasion and metastasis. Experimental metastasis of Lewis lung carcinoma cells was drastically attenuated by the mollusk HS through a mechanism involving inhibition of platelet–tumor-cell complex formation in blood vessels. These data suggest that the mollusk HS is a potential alternative to heparin for inhibiting P-selectin-mediated events such as metastasis and inflammatory cell recruitment.

Key words: anti-cancer activity, heparanase, mollusk heparan sulfate, P-selectin

Introduction

The relationship between cancer and inflammation has been increasingly reported during the last decade (Coussens and Werb 2002). However, the connection between these two pathologies was first suggested ~150 years ago (Balkwill and Mantovani 2001). Whereas acute inflammation is part of the organism defense response, chronic inflammation can lead to cancer. Indeed, patients with ulcerative colitis and Crohn's disease, have a higher risk to develop colorectal cancer (Itzkowitz and Yio 2004). On the other hand, during hematogenous metastasis, natural killer cells can attack tumor cells, decreasing the metastatic rate. Thus, the function of leukocytes in tumor biology is complex (Lanca and Silva-Santos 2012) and represents an attractive research area.

Hematogenous metastasis and inflammatory cell recruitment strongly depend on selectin function. P-selectin is a family member of glycan-recognizing adhesion molecules. In endothelial cells, P-selectin is stored in Weibel–Palade bodies, whereas in platelets it occurs in α-granules. P-selectin is readily exposed at the surface of platelets upon activation, mediating its interaction with leukocytes and endothelial cells (Kansas 1996). Tumor cells are characterized by aberrant glycosylation patterns (Altevogt et al. 1983; Kim et al. 1996), including the over-expression of highly branched or sialylated oligosaccharides, especially fucosylated glycans, such as sialyl-Lewis[X] and sialyl-Lewis[a]. These glycans are ligands for selectins and their presence is related to poor prognosis due to increased metastatic disease (Fukuoka et al. 1998; Tatsumi et al. 1998).

The metastatic process is comprised of several steps that include degradation of basement membrane, entry of cancer cell into the bloodstream, evasion of innate immune surveillance, adhesion to the vascular endothelium of secondary sites with subsequent extravasation and colonization. Once in the bloodstream, cancer cells are covered by platelets, in a P-selectin-mediated process, forming a natural barrier against immune system cells (Kim et al. 1998). On the other hand, leukocytes recruitment during inflammation is mediated by P- and L-selectins. These adhesion molecules are involved in the first steps of cellular recruitment by reducing the rolling velocity of leukocytes, contributing to their adhesion and arrest at sites of inflammation. In fact, P-selectin null mice exhibit reduced platelet aggregation, delayed leukocyte recruitment and attenuated metastasis, suggesting that P-selectin might be a common therapeutic target to treat cancer-related inflammation (Mayadas *et al.* 1993; Kim et al. 1998; Ludwig et al. 2007). The anticoagulant glycosaminoglycan heparin is a potent inhibitor of P- and L-selectin binding to their natural ligands (Nelson et al. 1993). Accordingly, heparin treatment has been shown to reduce leukocyte recruitment and metastasis through inhibition of P-selectin (Nelson et al. 1993; Borsig et al. 2001).

Cancer patients have high-risk of developing thromboembolic disease and therefore, heparin has been used as a prophylactic therapeutic agent. Retrospective analysis of patients under heparin therapy revealed a better prognosis of the malignant disease, which has not been associated with the anticoagulant effect of the drug (Kragh et al. 2005; Stevenson et al. 2007). Several studies aimed to investigate the anti-cancer effect of heparin revealed that it attenuates experimental metastasis in animals (Borsig et al. 2001), mainly by binding to P-selectin and decreasing the interaction between tumor cells and platelets. Additionally, heparin can also attenuate metastasis by inhibition of heparanase (Vlodavsky et al. 1994), the only known mammalian endoglycosidase that cleaves heparan sulfate (HS) and is over-expressed in essentially all human tumors. In fact, heparanase over-expression correlates with poor prognosis in a variety of cancers (Vlodavsky et al. 2012; Gomes et al. 2013).

We have previously shown that heparin analogues obtained from marine invertebrate bind to P-selectin, attenuating metastasis, inflammation and thrombosis (Borsig et al. 2007; Kozlowski et al. 2011). In a recent study, we described a unique HS isolated from the bivalve mollusk N. nodosus (Figure 1). This sulfated polysaccharide is formed by glucuronic acid and Glucosamine and can also contain a rare sulfation pattern on carbon 2 or 3 of the glucuronic acid units. We also described that this compound was able to inhibit thrombus growth without inducing bleeding effect (Gomes et al. 2010).

Although increasing evidence points to a beneficial therapeutic action of heparin in cancer patients, its bleeding effect still limits its use. Here, we addressed the ability of HS from N. nodosus to attenuate
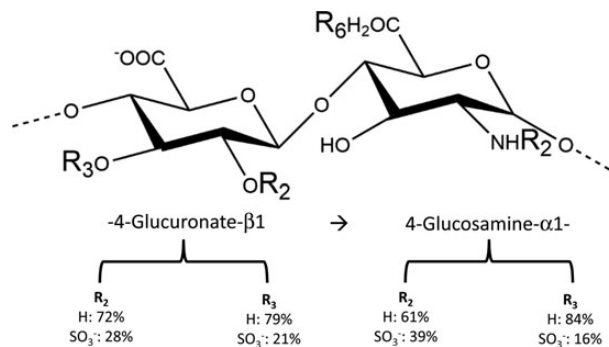

Fig. 1. *Nodipecten nodosus* HS structure. Major disaccharide unit of the *N. nodosus* HS.

leukocyte recruitment and cancer metastasis. Our results revealed that the mollusk HS effectively inhibits P-selectin activity, decreasing the binding of carcinoma cells to P-selectin *in vitro* and its interaction with platelets *in vivo*. The mollusk glycan also inhibits heparanase enzymatic activity *in vitro*. Moreover, we have demonstrated that this glycan attenuates hematogenous metastasis and polymorphonuclear cells recruitment *in vivo*.

Results

Nodipecten nodosus HS attenuates tumor cell binding to P-selectin

Because P-selectin is a crucial mediator of cell–cell interactions during inflammation and cancer, we evaluated whether HS from N. nodosus could inhibit P-selectin. For this purpose, we analyzed the ability of this compound to impair adhesion of LS180 cells to immobilized P-selectin. It is known from previous work that this colon cancer cell line expresses high content of selectin ligands (Mannori et al. 1995). *Nodipecten nodosus* HS decreased tumor cell binding to P-selectin in a dose-dependent manner, with an IC_{50} of 38.1 µg/mL. Heparin was more efficient in reducing tumor cell binding to P-selectin, showing an IC_{50} of 24.5 µg/mL (Figure 2). It is worth mentioning that, because heparin presents lower molecular weight (∼15 kDa) than N. nodosus HS (∼30 kDa), the molar concentration of heparin is higher than that of the mollusk HS. As expected, chondroitin sulfate did not inhibit P-selectin binding to tumor cells, even at high concentrations.

Nodipecten nodosus HS reduces leukocyte rolling and cell recruitment after inflammatory stimulus

While the involvement of inflammatory cells during several steps of cancer progression is well documented, little is known about the underlying mechanism. Because leukocyte rolling on activated endothelium during inflammatory response is mediated by P-selectin, we investigated how leukocyte rolling is affected by N. nodosus HS. Previous studies have shown that lipopolysaccharide (LPS) treatment induces P-selectin upregulation in endothelial cells (Gotsch et al. 1994). Based on that, we analyzed leukocyte rolling on LPS-activated endothelium by intravital microscopy, before or after N. nodosus HS administration. It was observed that treatment with the mollusk HS inhibited LPS-induced leukocyte rolling to the basal level (Figure 3A and Supplementary data). Because leukocyte rolling is the first step for cell recruitment, we further evaluated the effect N. nodosus HS on a thioglycollate-induced peritonitis model in mice. After 3 h of inflammatory stimulus the peritoneal lavage was harvested and differential
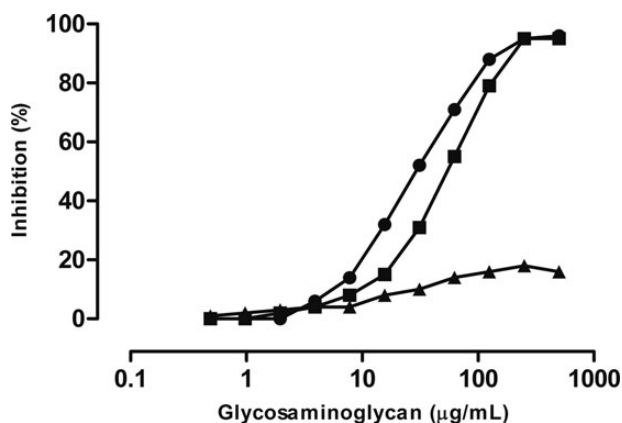
Fig. 2. *Nodipecten nodosus* HS attenuates P-selectin binding to LS180 tumor cells. Human colon carcinoma cells (LS180) adhesion to immobilized P-selectin chimera was measured in the presence of increasing concentrations of HS from *N. nodosus* (squares), unfractionated heparin (circles) or chondroitin sulfate (triangles). The curves are representative of three independent experiments. See the "Materials and methods" section for experimental details.

counting of leukocytes was performed. Figure 3B shows that thioglycollate promoted a significant increase in the recruitment of polymorphonuclear cells to the peritoneal cavity, compared with control. Whereas treatment with *N. nodosus* HS resulted in a 50% reduction in leukocyte recruitment mammalian heparin induced 60% of inhibition. This difference is not statistically significant. Interestingly, treatment of P-sel$^{-/-}$ mice with *N. nodosus* HS led to lower leukocyte recruitment in comparison with both HS-treated wild-type or untreated P-sel$^{-/-}$ mice (Figure 3C). These findings suggest an additional mechanism involved in the inhibition of leukocyte recruitment by *N. nodosus* HS besides P-selectin.

Nodipecten nodosus HS inhibits association between tumor cells and platelets in vivo

As mentioned earlier in the introduction, P-selectin-dependent leukocyte recruitment and intercellular interactions are crucial during inflammation as well as during tumor metastasis. P-selectin-mediated platelet association with tumor cells contributes to the success of metastasis. In previous studies, we showed that glycosaminoglycans from marine invertebrates could disrupt this association. In order to analyze the ability of HS from *N. nodusus* to inhibit tumor cell–platelet interactions *in vivo*, HS, heparin and chondroitin sulfate (8 mg/kg of each glycan) was injected via tail vein 5–10 min before injection of Lewis lung carcinoma (LLC) cells. The lungs were analyzed by immunofluorescence 30 min after tumor cell (calcein labeled) injection. While in the control group, ∼70% of tumor cells were associated with platelets (Figure 4), tumor cell–platelet complex was reduced to 30% after *N. nodosus* HS administration. Mammalian heparin attenuates tumor cell–platelet association to a similar extent as observed in mice treated with mollusk HS. Chondroitin sulfate did not inhibit tumor cell–platelet association.

### Mollusk HS inhibits heparanase and attenuates experimental metastasis

LLC cells express high amounts of heparanase (Takahashi et al. 2005), which plays a relevant role in tumor invasion and metastasis (Ilan et al. 2006). Because heparanase has been pointed as a molecular target of

heparin in cancer, we wondered whether *N. nodosus* HS inhibits heparanase enzymatic activity. For this purpose, we used a naturally produced sulfate-labeled extracellular matrix (ECM) as substrate and measured the release of HS degradation fragments upon incubation with heparanase (Vlodavsky et al. 1983; Vlodavsky 2001). As demonstrated in Figure 5, heparanase enzymatic activity was significantly inhibited by the mollusk HS. Because metastasis efficiency depends on P-selectin and heparanase, and heparin treatment is known to decrease tumor metastasis, we sought to assess the antimetastatic activity of *N. nodosus* HS (Kim et al. 1998; Borsig et al. 2001). In order to investigate that, we performed an experimental metastasis model in mice, using LLC cells that express P-selectin ligands and heparanase. This experiment involved administration of *N. nodosus* HS, mammalian heparin and chondroitin sulfate (8 mg/kg of each glycan) followed by injection (tail vein) of LLC carcinoma cells. Figure 6 shows the effect of the mollusk HS treatment on metastasis. Whereas the number of metastatic foci was high in control lungs, HS-treated animals presented just few metastatic foci. In other words, in control animals we observed around 10 foci per lung while in mollusk HS-treated animals an average of 1 focus per lung was detected. We also noticed that the tumor burden (size) in control lungs was markedly higher than in the treated animals. As expected, heparin attenuates experimental metastasis, however at the same potency observed in mice treated with mollusk HS. Injection of chondroitin sulfate did not affect metastasis. Overall, these results indicate that the mollusk HS may be an attractive therapeutic drug to block both P-selectin-mediated interactions and heparanase activity, blunting metastasis and inflammation without inducing bleeding effects.

Discussion

Cell interactions among leukocytes, platelets and endothelium are mediated by selectins and contribute to the pathophysiology of inflammation and metastasis. Several studies have shown that heparin can block these interactions and therefore attenuate hematogenous metastasis and inflammatory cell recruitment. In 2010, we described a unique HS obtained from the bivalve mollusk *N. nodosus*, which presents 2 or 3 O-sulfation on glucuronic acid (Figure 1). This compound is absent in the adductor muscle, and extracted from organs commonly discarded during preparation for commercialization. The mollusk glycan significantly reduces experimental thrombus growth *in vivo* without inducing any bleeding effect (Gomes et al. 2010). Therefore, we decided to investigate if this glycan could inhibit P-selectin. In order to evaluate this inhibitory potential we performed *in vitro* and *in vivo* experiments and showed that the mollusk HS inhibits P-selectin interaction with colon carcinoma cell line (LS180), decreases cell rolling and inflammatory cell recruitment. Additionally it attenuates platelets–tumor cell association and heparanase enzymatic activity, thereby blunting metastasis.

Leukocyte function in tumor biology has deserved attention in cancer research (Mantovani et al. 2008; Alderton and Bordon 2012). It has been proposed that, in some types of cancer, the inflammatory cascade is already activated before the tumor initiates (Itzkowitz and Yio 2004). Conversely, in other situations the malignant microenvironment promotes inflammatory cells recruitment, which in turn can induce tumor growth (Pollard 2004; Sica et al. 2006) or lysis of tumor cells. Inflammatory leukocyte rolling on activated endothelium is a P-selectin mediated event. Applying intravital microscopy, we showed that mollusk HS decreases leukocyte rolling after endothelial cell activation (Figure 3A). We speculate that as a
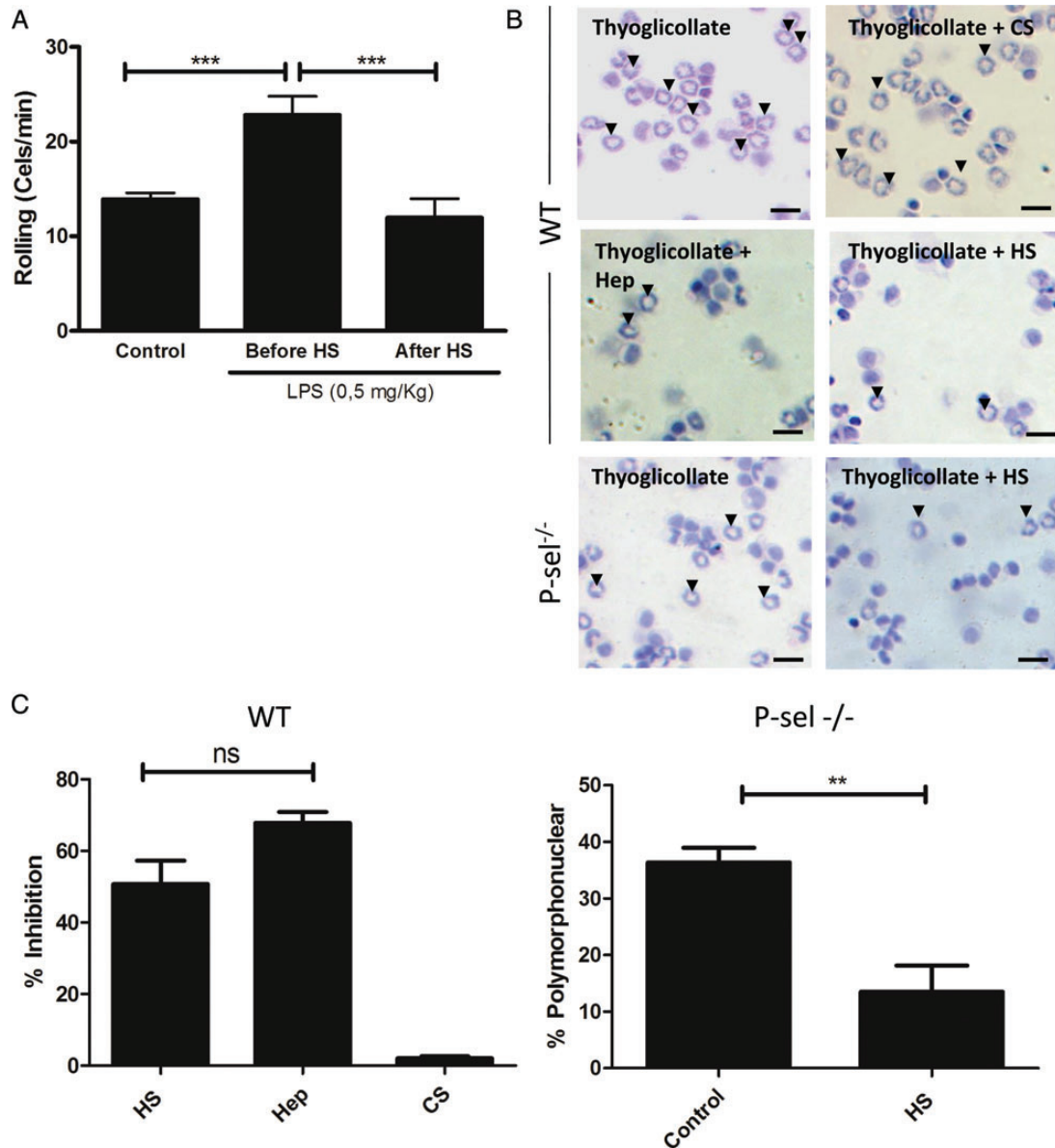
Fig. 3. *Nodipecten nodosus* HS inhibitis leukocyte rolling and polymorphonuclear cell recruitment. (**A**) Leukocyte rolling (cells/min) along the endothelium of postcapillary venules in distal ileum was measured by use of intravital microscopy. Four hours before counting, rats were intravenously injected with LPS (0.5 mg/kg) or saline. In rats treated with LPS the leukocyte rolling was measured before and after intravenous administration of HS from *N. nodusus* (2 mg/kg). (**B**) Wild-type (WT) mice were injected intraperitoneally with thioglycollate, followed by intravenous injection of mollusk HS, unfractionated heparin, chondroitin sulfate (20 mg/kg of each glycan) or PBS 5 min later. Peritoneal lavage was harvested 3 h later. Cells were stained with hematoxylin and eosin and differentially counted for mononuclear and polymorphonuclear cells. (**C**) Counting of polymorphonuclear cells in WT or P-sel$^{-/-}$ mice injected intraperitoneally with thioglycollate, followed by injection of mollusk heparan sulfate. Scale bar represents 20 mm. The statistical significance was determined by one-way analysis of variance (ANOVA) or *t*-test (***$P < 0.05$).

consequence of this inhibition, polymorphonuclear cells recruitment to the peritoneal cavity was attenuated (Figure 3B). We also observed a decrease in leukocyte recruitment in P-selectin deficient mice (Figure 3C). Because some glycosaminoglycans also bind to L-selectin, inhibition of leukocyte recruitment through L-selectin interaction could also be expected. Therefore, we suggest that HS from *N. nodosus* might also inhibit L-selectin.

Another set of P-selectin-mediated interactions occurs during dissemination of metastatic cancer cells. Along this process, into the bloodstream, tumor cells are often covered with platelets in a P-selectin-dependent manner. This interaction confers tumor cells with physical shielding mediated by platelets, avoiding natural killer cell-mediated tumor cell lysis. We found that the mollusk glycan significantly inhibited tumor-cell platelet association, as early as 30 min after tumor cells injection (Figure 5). We suggest that without this interaction tumor cells would be more vulnerable to immune surveillance and, as a result, the metastasis rate would decrease. Using LLC cells, which are carcinoma cells that express selectin ligands at the cell surface (Brown et al. 2006), we demonstrated that the mollusk glycan drastically attenuates seeding of tumor cells to the lungs. An elegant
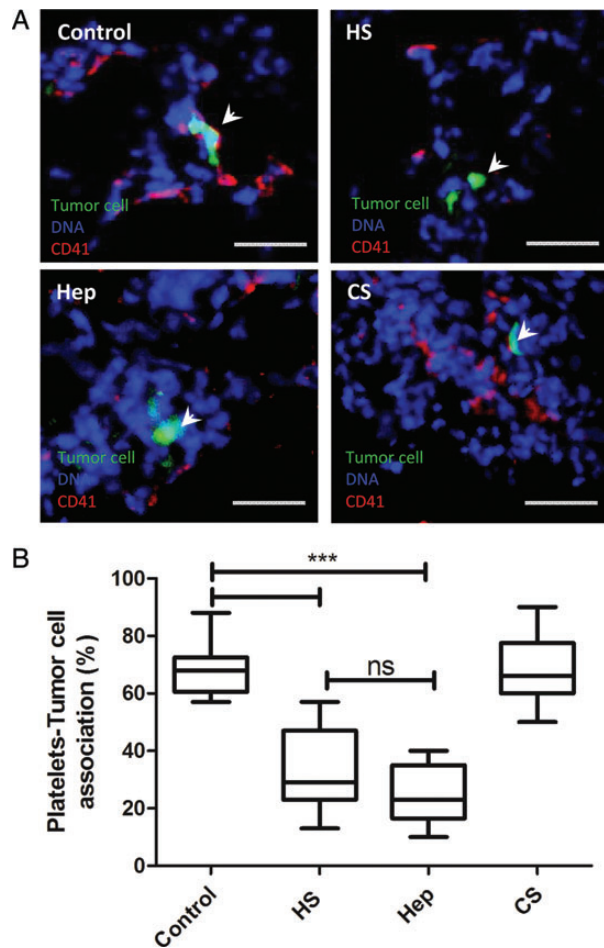
Fig. 4. *Nodipecten nodosus* HS inhibits platelet adhesion to tumor cells *in vivo*. LLC cells labeled with calcein AM were intravenously injected via tail vein. (**A**) Representative images of platelet–tumor cell association in the lungs of mice injected with *N. nodosus* HS, unfractionated heparin, chondroitin sulfate (8 mg/kg of each glycan) or PBS and euthanized 30 min later. Scale bars represent 20 μm; (**B**) Numbers of platelet–tumor cell aggregates are presented as percentages (in columns) of all counted tumor cells. The statistical significance of tumor cell–platelet association was determined by ANOVA (***$P < 0.05$).
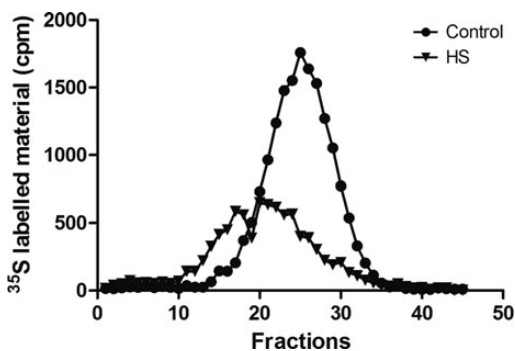
Fig. 5. *Nodipecten nodosus* HS is a heparanase inhibitor. The ability of the mollusk HS to inhibit recombinant heparanase enzymatic activity was determined as described in the "Materials and methods" section. Data are representative of three independent experiments.

work of Labelle et al. (2011) showed that apart of protection from leukocytes, platelets is also able to induce epithelial-mesenchymal transition in tumor cells via transforming growth factor beta (TGF-β) mediated-mechanism and thereby promotes metastasis. Similarly, through inhibition of platelet–tumor cell interaction the mollusk glycan may exert antimetastatic effect by suppressing the epithelial-mesenchymal transition induced by platelets.

When tumor cells exit the bloodstream they need to degrade the sub-endothelial basement membrane, which is rich in HS, in order to colonize a secondary site (Liotta et al. 1980). Previous studies have shown that heparin-mimicking compounds attenuate metastasis by acting as heparanase inhibitors (Borsig et al. 2011). Therefore, we suggest that the heparanase inhibitory activity of the mollusk glycan contributes to the antimetastatic effect observed in our experiments.

Overall, the present work identifies a new P-selectin inhibitor that does not induce hemorrhagic effects observed in mammalian heparin. We also showed that this compound attenuates thrombosis, inflammation and metastasis. Since cancer disease is usually associated with thrombotic events we suggest that the mollusk HS is an attractive candidate to be a therapeutic drug for cancer-associated thrombosis and cancer-related inflammation.

Materials and methods

Cell lines and reagents

Human colon carcinoma cells (LS180; ATCC, Manassas, VA) were grown in minimum essential medium-α (MEM-α) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Mouse LLC cells were grown in Dulbecco's modified Eagle's medium (Vitrocell) supplemented with 10% FBS. All reagents were from Sigma (St. Louis, MO), unless otherwise stated. Heparin (Liquemine) was obtained from Roche Pharma (Reinach, Switzerland).

### Isolation of HS from *N. nodosus*

Adult specimens of the bivalve mollusk *N. nodosus* (Linnaeus 1778) were collected from Baia da Ilha Grande, Angra dos Reis, Rio de Janeiro, Brazil. The polysaccharides were extracted by protease digestion and purified as described previously (Gomes et al. 2010).

Inhibition of tumor cell binding to immobilized P-selectin

The ability of glycosaminoglycans to inhibit the adhesion of calcein acetoxymethyl (AM)-labeled LS180 cells to immobilized P-selectin chimeras was investigated as described previously (Hostettler et al. 2007). The glycans were tested in triplicate wells at each concentration.

Intravital microscopy

Intravital microscopy was performed according to (Fortes et al. 1991) with slight modifications. Leukocyte rolling in the mesenteric venules was analyzed after 4 h of LPS (Sigma; 0.5 mg/kg) or phosphate-buffered saline (PBS) intravenous injection. Adult male and female Wistar rats (250 g body weight) were anesthetized with an intramuscular injection of 100 mg kg^{-1} of ketamine (Cristália, São Paulo, Brazil) and 16 mg kg^{-1} of xylazine (Bayer AS, São Paulo, Brazil). An abdominal midline incision (~1.5 cm) was performed and the mesentery was exposed for analysis. After positioning under the microscope, a 30 min equilibration period preceded quantitative measurements. The microscope used was a Zeiss Axio ImagerA1. Leukocyte rolling was counted for 10 min. In the group that received
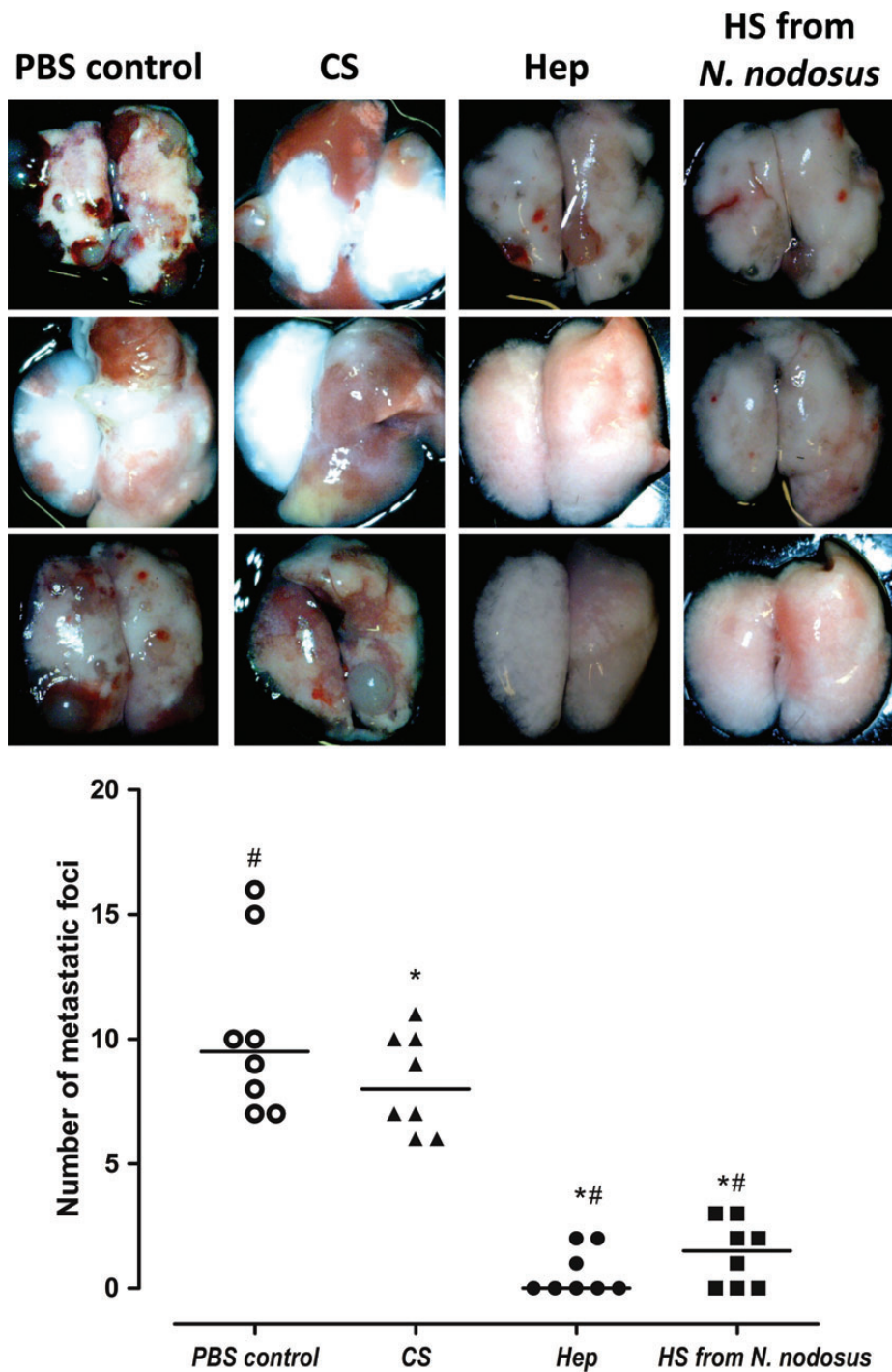
Fig. 6. Experimental lung metastasis of LLC carcinoma cells is attenuated by mollusk HS treatment. Mice were intravenously injected with of PBS, mollusk HS, heparin or chondroitin sulfate (8 mg/kg of each glycan) 10 min before injection of 10^6 LLC cells and terminated 21 days later. Metastatic foci in the lungs were evaluated macroscopically. (**A**) Representative images of lungs harvested from mice injected with mollusk HS versus control. (**B**) Counting of metastatic foci. The statistical significance was determined by ANOVA.

LPS, leukocyte rolling was evaluated before and after circulation of *N. nodosus* HS for 5 min (2 mg/kg—intravenous injection). Videos are available at Supplementary data. All animal work was performed according to institutional guidelines for the use and care of experimental animal, approved by the protocol number IBqM 014. Representative videos of experimental groups are available as Supplementary data.

Thioglycollate-induced peritoneal inflammation

Mice were injected intraperitoneally with 4% thioglycollate (1 mL). After 5 min, *N. nodosus* HS was administrated via tail vein injection. Mice were sacrificed after 3 h and peritoneal lavage was collected using 4 mL ice-cold PBS, containing 3 mM Ethylenediamine tetraacetic acid (EDTA) to prevent clotting. The peritoneal fluid, 200 μL, was analyzed

after cytospin preparation by hematoxylin and eosin staining. Then, differential counting was performed to evaluate the amount of polymorphonuclear cells present in the peritoneal cavity. P-sel$^{-/-}$ and wild-type mice were used in this experiment.

Tumor cell–platelets association in vivo

The formation of tumor cell–platelet complex was performed as described before (Borsig et al. 2001). Briefly, LLC cells were harvested with 2 mM EDTA in PBS, labeled with calcein AM, and injected intravenously into mice with or without previous intravenous application of 200 µg of *N. nodosus* HS. After 30 min, the lungs were harvested for analysis. Lung sections were stained with goat anti-integrin αIIb (CD41) (Santa Cruz biotechnology-sc6602), followed by anti-goat Cy3-conjugated antibody (Sigma), and analyzed by immunofluorescence microscopy. The extent of platelet association with tumor cells was quantified by evaluating calcein-labeled cells present in 40 fields of lung sections.

Heparanase enzymatic activity assay

Preparation of sulfate-labeled extracellular matrix (ECM)-coated dishes and determination of heparanase enzymatic activity were performed as previously described (Vlodavsky et al. 1983; Vlodavsky 2001). Briefly, sulfate-labeled ECM coating the surface of 35-mm culture dishes was incubated (4 h, 37°C, pH 6.0) with constitutively active recombinant human heparanase (120 ng/mL) in the absence or presence of 5 µg/mL of mollusk HS, as described [18]. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 mL) were eluted with PBS and their radioactivity counted in a β-scintillation counter. HS degradation fragments were eluted at 0.5 < K_{av} < 0.8 (peak II, fractions 10–25).

Experimental metastasis model

Mice (8–10 weeks old) were intravenously injected with 10^6 LLC cells via the tail vein. *Nodipecten nodosus* HS, heparin or chondroitin (8 mg/kg of each glycan) administration was performed 10 min prior to cell injection. Mice were sacrificed after 21 days. The lungs were macroscopically evaluated for the number of metastatic foci.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding

Conflict of interest

None declared.

Abbreviations

AM, acetoxymethyl; ANOVA, analysis of variance; ECM, extracellular matrix; EDTA, Ethylenediamine tetraacetic acid; FBS, fetal bovine serum; HS, heparan sulfate; Hep, unfractionated heparin; LLC, Lewis lung carcinoma; LPS, lipopolysaccharide; MEM-α, minimum essential medium-α; *N. nodosus*, *Nodipecten nodosus*; P-sel$^{-/-}$, P-selectin-null mice; PBS, phosphate-buffered saline; TGF-β, transforming growth factor beta; WT, wild-type.

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