Abstract

Background: Some low molecular weight heparins (LMWHs) prolong survival of cancer patients and inhibit experimental metastasis. The underlying mechanisms are still not clear but it has been suggested that LMWHs (at least in part) limit metastasis by preventing cancer cell-induced destruction of the endothelial glycocalyx.

Methodology/Principal Findings: To prove or refute this hypothesis, we determined the net effects of the endothelial glycocalyx in cancer cell extravasation and we assessed the anti-metastatic effect of a clinically used LMWH in the presence and absence of an intact endothelial glycocalyx. We show that both exogenous enzymatic degradation as well as endogenous genetic modification of the endothelial glycocalyx decreased pulmonary tumor formation in a murine experimental metastasis model. Moreover, LMWH administration significantly reduced the number of pulmonary tumor foci and thus experimental metastasis both in the presence or absence of an intact endothelial glycocalyx.

Conclusions: In summary, this paper shows that the net effect of the endothelial glycocalyx enhances experimental metastasis and that a LMWH does not limit experimental metastasis by a process involving the endothelial glycocalyx.

Introduction

In experimental animal models and clinical studies it has been well established that some low molecular weight heparins (LMWH) inhibit experimental metastasis and prolong survival [1,2]. Although the underlying mechanisms are only partially understood, it has been suggested that the endothelial glycocalyx may play an important role in the lifelong prolonging effects of LMWH in patients.

The endothelial glycocalyx is a negatively charged, organized network of membrane-bound glycoproteins, proteoglycans and glycosaminoglycans that affects several biological processes with potential importance for cancer cell extravasation. First, the endothelial glycocalyx is essential for vascular barrier function. Its disruption by pro-inflammatory cytokines, including tumor necrosis factor (TNF-α) and glycocalyx-degrading enzymes such as heparanase and hyaluronidase, leads to increased vascular permeability [3-5]. Second, the glycocalyx has anticoagulant properties and thrombin generation is reduced by the glycocalyx because it stores various natural anticoagulant factors such as antithrombin, protein C and tissue factor pathway inhibitor [6]. Consequently, disruption of the endothelial glycocalyx instantly results in thrombin generation and platelet adhesion [7]. Third, through its diversity in biochemical make-up, the endothelial glycocalyx both prevents and facilitates cell adhesion to the endothelium. The size of the glycocalyx (predominantly its heparan sulphate proteoglycan and hyaluronate composition) exceeds the size of the adhesion molecules (syndecan-1, L- and P-selectin), thereby masking these proteins and preventing adhesion of among others leukocytes [8]. On the other hand, when glycocalyx bound components such as hyaluronic acid are released they may serve as ligands for the CD44 receptor expressed on many cells (including cancer cells). The glycocalyx thus plays an important role in cell adhesion to the vessel wall [9,10]. Fourth, the glycocalyx binds growth factors and extracellular matrix components via its proteoglycan syndecan-1. Moreover, syndecan-1 modulates fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) activity [11]. The glycocalyx is a sink of growth factors that in general are anti-apoptotic and of VEGF that can increase endothelial permeability [12]. Overall, the endothelial glycocalyx may thus be an important player in several biological processes with potential relevance for cancer cell
Results and Discussion

To assess the net effect of the endothelial glycocalyx on experimental metastasis, wild type mice were treated with hyaluronidase in order to remove hyaluronan and, in part, heparan sulphates from the endothelial glycocalyx. As it has previously been shown that one hour after hyaluronidase treatment vascular leakage is evident [4], B16F10 melanoma cells were injected intravenously 1h after intravenous hyaluronidase or saline administration. Experimental metastases in the lung were examined 14 days later. As shown in Figure 1, the number of pulmonary tumor foci was significantly reduced by approximately 30% after hyaluronidase treatment as compared to the saline injected control group. Enzymatic degradation of the glycocalyx (at least of its hyaluronan component) thus limits experimental metastasis. The relative importance of the particular pro- and anti-metastatic effects of the endothelial glycocalyx in vivo remains to be elucidated however.

Interestingly, cancer cells produce enzymes that are known to degrade the endothelial glycocalyx, such as heparanase and hyaluronidase [12–16]. These enzymes consequently influence vascular endothelial barrier integrity, adhesive properties of the endothelial lining, cytokine production and can liberate heparan sulfate-bound growth factors thereby inducing cancer cell extravasation. As heparin, LMWHs and heparin derivatives can abolish the activity or binding of heparanase [17,18] and hyaluronidase [19] by competing with heparan sulphates and hyaluronan [20–22], it has been hypothesized that LMWHs (at least in part) limit cancer progression by restoring cancer cell-induced glycocalyx damage thereby limiting cancer cell extravasation [23].

In the current manuscript, we aimed to assess whether the effect of a LMWH on experimental metastasis depends on restoration of the endothelial glycocalyx. To this end, we first determined the net effect of the endothelial glycocalyx in experimental metastasis. Next, we assessed the effect of a LMWH in the presence or absence of an intact endothelial glycocalyx to determine the contribution of the glycocalyx to the effect of this LMWH on the reduction of experimental metastasis.

Figure 1. Effect of hyaluronidase on the number of B16F10 pulmonary tumor foci. C57BI/6 mice were treated intravenously with 100U hyaluronidase 1h prior to the administration of 3.5×10⁵ B16F10 melanoma cells into the lateral tail vein. Mice were sacrificed 14 days after cancer cell injection and the number of tumor foci at the surface of the lungs was determined. Error bars represent means ± SEM (n = 8); *, p<0.05. doi:10.1371/journal.pone.0011200.g001

As already indicated, the glycocalyx is considered as an integrated and balanced carbohydrate layer in which both hyaluronan and heparan sulfate chains are key structural components. Importantly, our data show that targeting either hyaluronan (enzymatically by hyaluronidase treatment) or the heparan sulphate chains (genetic ablation of syndecan-1) of the glycocalyx leads to reduced experimental metastasis. As these two different interventions have a similar effect on experimental metastasis, our data imply that barrier protective-properties of the glycocalyx are less essential for metastasis than its functions in cancer cell adhesion or growth factor storage [28]. Future experiments are needed however to fully appreciate the role of specific components of the glycocalyx on metastasis and to elucidate the underlying mechanisms.

As mentioned before, some LMWHs protect against cancer progression in experimental animal models and clinical trials, including the B16F10 melanoma model of experimental metastasis. As suggested previously, these LMWHs may inhibit metastasis through competitive binding of heparanase or hyaluronidase thereby protecting the vascular endothelium and its barrier function from disruption caused by these enzymes. To assess whether the inhibitory effect of the administration of a LMWH on cancer progression are dependent on its protective effects on the glycocalyx, we compared the effect of enoxaparin administration on experimental metastasis in syndecan-1 deficient and wild type mice. As shown in Figure 2, enoxaparin injected intravenously at 30 min prior, and 6 and 12 h after cancer cell inoculation decreased the number of pulmonary tumor foci in wild type mice almost equally suggesting that systemic, heparinoid induced, anticoagulant effects of hyaluronidase may explain at least some of the reduction in lung metastasis. However, blocking the proteolytic activity of heparanase, heparinoid induced anticoagulant effects may also play a role in preventing growth factor mobilization and consequently cancer cell extravasation.
nously in a dose of 100 units per mouse 1 h prior to cancer cell inoculation [4]. LMWH (15 mg/kg; enoxaparin, Sanofi-Aventis, Paris, France) was injected 30 min prior to and 6, 12 and 24 h after cancer cell inoculation.

Cells and cell culture
Murine B16F10 melanoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin-streptomycin solution and 1% L-glutamine at 37°C as described before [29,30]. Single cell suspensions were prepared from 2 mM EDTA-treated monolayer's which were washed and diluted in phosphate-buffered saline (PBS) prior to counting and inoculation. Cells were stored on ice until administration.

Materials and Methods

Hyaluronidase and heparin
Bovine testicular hyaluronidase (type IV-S; Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% NaCl was administered intrave-

Statistical analysis
Statistical analysis was carried out in GraphPad Prism version 4.03. Data are expressed as means +/- SEM or medians with interquartile range. For normally distributed data, significance was assessed with the Student t-test. For not normally distributed data, non-parametric testing was performed using the Mann-Whitney test. Statistical significance was assumed when the p-value was <0.05.

Acknowledgments
We kindly acknowledge the carefully performed animal experiments by Joost Daalhuisen and Marieke ten Brink of the Center for Experimental and Molecular Medicine of the Academic Medical Center in Amsterdam. We gratefully acknowledge Dr. M Gotte (Medical Center, University of Munster, Münster, Germany) for the syndecan-1 deficient mice. We kindly acknowledge Dr Rops (Radboud University Medical Centre, Nijmegen, the Netherlands) for the breeding and genotyping of the syndecan-1 knock out mice.

Author Contributions
Conceived and designed the experiments: GvS CAS. Performed the experiments: GvS. Analyzed the data: GvS MN PK CAS. Contributed reagents/materials/analysis tools: JvdV. Wrote the paper: GvS MN PK CJFVN CAS.
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