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The inhibition of the integrin VLA-4 in MV3 melanoma cell binding by non-anticoagulant heparin derivatives

Martin Schlesinger [a,*], Patrick Schmitz [a], Reiner Zeisig [b], Annamaria Naggi [c], Giangiacomo Torri [c], Benito Casu [c], Gerd Bendas [a]

[a] Department of Pharmacy, Rheinische Friedrich Wilhelms University Bonn, 53121 Bonn, Germany
[b] Max Delbruck Centre for Molecular Medicine, 13125 Berlin, Germany
[c] "G. Ronzoni" Institute for Chemical and Biochemical Research, 20133 Milano, Italy

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ABSTRACT

Introduction: The integrin VLA-4-mediated binding is important for the metastatic dissemination of melanoma cells. Recently we found that heparin possesses a binding capacity to VLA-4. This could contribute to the heparin function to attenuate metastasis in a selectin-dependent manner. Aiming to a purposive, anti-adhesive heparin application, structural requirements of heparin for VLA-4 recognition have to be elucidated.
Materials and methods: A series of non-anticoagulant heparin derivatives were investigated concerning their inhibitory capacities for VLA-4 mediated binding of human melanoma MV3 cells to VCAM-1 under physiological flow conditions *in vitro*. A surface acoustic wave biosensor was applied to detect kinetic constants of selected derivatives binding to both, VLA-4 or P- and L-selectin.
Results: Experimental metastasis of MV3 cells in mice confirmed the relevance of VLA-4 for metastatic dissemination. LMWHs (enoxaparin, tinzaparin) efficiently blocked VLA-4 cell binding, dominantly via the integrin`s α-chain. Desulfation at 2-O-position, N-acetylation or a size smaller than tetradecasaccharide disfavoured VLA-4 inhibition. Glycol-splitting of heparin and thus higher chain flexibility is a tolerable parameter. A derivative with 50% 6-O-desulfation appeared promising and exceeded tinzaparin in VLA-4 inhibition, both compounds displayed binding affinities to VLA-4 in the low micromolar range.
Conclusions: These findings provide structure-activity relationships for heparin VLA-4 binding, which partly differ from P- and L-selectin requirements. The data confirm that anti-coagulative and anti-adhesive function of heparin can be distinguished favouring applications of non-anticoagulant heparins in antimetastatic approaches without the risk of bleeding complications. The 50% 6-O-desulfated heparin-derivative appears promising to further evaluate the interference with selectin and VLA-4 binding functions *in vivo*.

Introduction

Tumor cell metastasis is the most fatal complication in cancer, causing the majority of cancer–related death. The course of hematogenous metastasis is complex and consists of multiple steps in which tumor cells invade into the blood circulation, interact with blood components, adhere to the endothelium at distant sites, extravasate and proliferate in the tissue [1,2]. Since adhesion molecules are crucially involved in this cascade, remarkable efforts on anti-adhesive strategies are being made as promising approaches in oncology [3].

The contribution of selectins to metastasis was confirmed in numerous preclinical studies [4–6]. At a very early stage of hematogenous tumor cell contacts, platelet P-selectin facilitates the formation of platelet-rich tumor cell emboli, which protect the cells from high shear stress and help to evade the innate immune system [7,8]. Leukocytes were recruited to the microemboli [9] and L-selectin helps to mediate endothelial contacts to endogenous L-selectin ligands [9,10].

In addition to the selectins and in dependence on the tumor cell entities, integrins contribute to the adhesive interactions between tumor cells and the endothelium. Recent focus lies on the integrin α4β1 (very late activation antigen-4, VLA-4) interacting with the vascular cell adhesion molecule-1 (VCAM-1). Malignant melanoma cells utilize VLA-4 to adhere to the endothelium [11], thereby promoting

Abbreviations: ECM, extracellular matrix; EDC, *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide; FCS, fetal calf serum; HRP, horseradish peroxidase; K_D, equilibrium dissociation constant; k_{off}, dissociation rate constant (off-rate); k_{on}, association rate constant (on-rate); LMWH, low molecular weight heparin; MS, monosaccharide; MW, molecular weight; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; SAW, Surface Acoustic Wave biosensor; SDS, sodium dodecylsulfate; TBS, tris-buffered saline; UFH, unfractionated heparin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

* Corresponding author at: Department of Pharmacy, Rheinische Friedrich-Wilhelms-Universität Bonn, An der Immenburg 4, 53121 Bonn, Germany. Tel.: +49 228 735238; fax: +49 228 734692.
E-mail address: mschlesi@gmx.de (M. Schlesinger).

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transmigration [12,13] and metastasis [14,15]. Although these finding suggest VLA-4 as target for antimetastatic approaches, and despite VLA-4 inhibition is a vital strategy to interfere with pathological inflammations [16], suchlike approaches have not been described in the cancer field so far. Recently we reported that VLA-4 can bind to heparin and LMWH, but not to the pentasaccharide fondaparinux [17]. These findings were stimulating since heparin is the topic of intensive research on tumor cell metastasis [4].

Commonly applied to cancer patients for prophylaxis and treatment of cancer-associated thrombosis [18,19], heparin was shown to prolong the survival of cancer patients in a number of prospective clinical trials [20]. Although several postulations on the molecular mechanisms exist that beside anticoagulation include (i) inhibition of P- and L-selectin, (ii) inhibition of angiogenesis via stimulation of tissue factor pathway inhibitor (TFPI)-release, (iii) modulation of growth factors and chemokines, and (iv) inhibition of heparanase activity [21–24], research in this field is further pursued. A recent study reported that heparin affects the adhesion and migratory activities of melanoma cells by interfering intracellularly with the signalling pathways, i.e. the focal adhesion kinase [25].

Depending on the tumor cell entities and the dominance of the VLA-4 pathway for dissemination, the inhibition of VLA-4 by heparin appears attractive as an extension to the well studied inhibition of selectin binding functions. Several aspects remain to be answered for that, mainly a further insight into the structural correlations of heparin binding to VLA-4. This could help to evaluate the prospects of a heparin application for anti-adhesive, antimetastatic approaches and maybe, a discrimination of anticoagulant activities.

In the present study we used the human melanoma cell line MV3, for which we demonstrate the importance of VLA-4 for metastatic dissemination in a mice model, to investigate structural parameters of heparin responsible for VLA-4 binding *in vitro*. Therefore, we applied different series of non-anticoagulant heparins and investigated the inhibitory effects on MV3 cell adhesion to VCAM-1 under flow conditions. In accordance to our recent study, which focused on the heparin size [17], the impact of site and degree of sulfation, of N-acetylation, and of chain flexibility for binding was studied here and complemented by the determination of heparin binding kinetics to VLA-4, using a Surface Acoustic Wave biosensor technology. Structural requirements were elucidated which are more restrictive than for P- and L-selectin binding. A derivative was found to exceed tinzaparin in VLA-4 inhibition representing a promising candidate for applying non-anticoagulant heparins for antimetastatic approaches.

Material and methods

Cell Culture

The human melanoma cell line MV3 was cultivated in RPMI 1640 medium containing 10% (v/v) FCS (fetal calf serum) in a humidified atmosphere with 5% CO_2 at 37 °C. For subcultivation, MV3 cells were detached at a confluency of about 90% with EDTA-solution (0.2 g/L EDTA × tetra sodium) for 5 min at 37 °C. All reagents were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Antibodies, adhesion molecules, siRNA

The humanized anti-VLA-4 mAb natalizumab was kindly provided by Biogen Idec GmbH (Ismaning, Germany). Anti human CD29 (integrin β1-chain) mAb, recombinant human P- and L-selectin Fc chimera, and recombinant human VCAM-1 Fc chimera were purchased from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). The FlexiTube siRNA mixture against the VLA-4 mRNA was obtained from Qiagen (Hilden, Germany). X-treme gene transfection reagent for insertion of siRNA into the cells was purchased from Roche Diagnostics (Mannheim, Germany).

Sulfated polysaccharides

LMWH enoxaparin (Clexane®) was from Sanofi-Aventis GmbH (Frankfurt/M, Germany). Tinzaparin was supplied by LEO Pharma (Ballerup, Denmark). The N-acetylated, 2-O- and 6-O-desulfated, and "glycol-split" heparin derivatives were prepared as previously described [26]. Chemically modified heparins have an average molecular weight in the order of 15 kD. All samples were recovered after freeze-drying and diluted to a concentration of 1 mg/mL. Hexa-, octa-, deca-, dodecasaccharide fractions of tinzaparin were isolated by size exclusion chromatography [27].

VLA-4 knockdown in MV3 cells

To decrease the number of VLA-4 receptors on the MV3 cell surface, siRNA was used for downregulation. At first, the suitable concentration of transfection reagent, siRNA concentration, and incubation time were determined by flow cytometry using the VLA-4 antibody natalizumab and a second FITC-labeled mAb. MV3 cells were cultivated in dishes and treated for 72 h with 0.5% (v/v) transfection reagent and with 10 nM siRNA. After this period, MV3 cells were detached with EDTA-solution (0.2 g/L EDTA tetrasodium) for 5 min at 37 °C, resuspended in FCS free medium, and used in the flow chamber assay.

Flow chamber assay

To determine the interaction of MV3 cells with the ligand VCAM-1 under physiological flow conditions, glass slides were coated with 0.2 μg VCAM-1 Fc chimera and incorporated into a parallel plate flow chamber as described in detail before [11,17]. The flow chamber was fixed on an inverted microscope, and PBS (pH 7.4) flow medium was driven at a shear rate of about 200 s^{-1}.

1×10^6 MV3 cells were suspended in FCS-free RPMI 1640 medium and treated with 1 mM Mn^{2+} for 5 min at 37 °C (stimulated cells) in comparison to unstimulated cells. For inhibition experiments, either 500 μg of a heparin derivative or 5 μg natalizumab were applied. For this purpose stimulated MV3 cells and the inhibitors were mixed to a total volume of 100 μL, incubated for 5 min at 37 °C by gently shaking. After injection into the flow chamber, cell adhesion was observed for a period of 5 s while video sequences were captured with the camera CSC-795 (Pacific Corporation, Tokyo, Japan). VLA-4 knockdown MV3 cells were compared with the inhibitor-free control experiments. Videos were analyzed with Imagoquant MultiTrack-AVI-2 software (Mediquant GmbH, Lützen, Germany).

Impact of heparin derivatives on P-selectin mediated platelet MV3 melanoma cell interaction

Platelets were isolated, labeled, and activated as previously described [11]. The influence of the heparin size on the P-selectin mediated interaction of human platelets with MV3 melanoma cells was determined on an octasaccharide unit derivative by counting the number of adherent platelets to a confluent layer of MV3 cells under gently shaking conditions.

SAW Biosensor measurements

Biosensor investigations were performed using an S-Sens™ K5 Surface Acoustic Wave biosensor supplied by SAW instruments GmbH, Bonn, Germany as described before [17].

Gold-coated sensors were incubated in a solution of 11-mercaptoundecanoic acid (10 mM) in ethanol abs. The carboxyl groups were activated with 200 mM *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) to bind 25 μg/mL P-selectin and L-selectin Fc chimera, respectively, forming carboxylamides. The excess of activated, unreacted NHS esters was

blocked by 1 M ethanolamine (pH 8.5). The amount of bound protein was determined using the protocol described in [28].

After immobilisation of P- and L-selectin, respectively, a baseline equilibration in PBS containing 1 mM Ca^{2+} and Mg^{2+} was performed for 15 min. The flow rate was adjusted to 40 µL/min to ensure sufficient time for heparin-selectin interaction. For binding experiments, different heparin derivatives were injected in dilution series between 10^{-10} M and 10^{-4} M and binding events were measured as phase shifts of the acoustic wave.

Preparation of membrane vesicles and immobilisation on SAW sensors

MV3 cells were harvested from 90% confluently grown cell dishes by washing them twice with PBS, and incubating them for 10 min in a 1% octyl glucoside solution in PBS by rocking gently on a horizontal shaker. Supernatants were collected and cell membrane vesicles were prepared according to the protocol described in detail in [29]. All steps were performed at 4 °C. Membrane vesicles were homogenized using a Potter-Elvejhem homogenizer. The protein yield was determined in an amido black protein assay [30]. VLA-4 was determined applying a SDS-PAGE under non-reducing conditions. Proteins from the gel were plotted by tank blot on a polyvinyldifluoride membrane at 100 V, 350 mA for 60 min. Unspecific binding sites on the membrane were blocked using a solution of skim milk protein (5%) in TBS with 0.2% Tween. VLA-4 was detected using natalizumab and anti-human IgG-HRP as secondary mAb. Chemiluminescence was detected after treating the membrane with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's protocol.

The immobilization of membrane vesicles on gold-coated SAW sensors was performed as described recently [17].

Determination of natalizumab and heparin kinetic binding constants to VLA-4

Natalizumab, LMWH or heparin derivatives, resp., were injected in dilution series between 10^{-9} M and 10^{-5} M to interact with the immobilized membrane vesicles detecting the resulting phase shifts. Kinetic binding constants were calculated from the slopes of the obtained curves applying a 1:1-binding model.

Experimental metastasis in vivo

All animal experiments were performed according to the German Animal Protection Law and with approval of the local responsible authorities. Female NMRI: nu/nu mice were obtained from Taconic Europe, Denmark. The animals were housed under pathogen-free conditions in individually ventilated cages (22 °C room temperature, 50 ± 10% relative humidity, 12 h light–dark rhythm). They received autoclaved food and bedding (Sniff, Soest, Germany) and acidified (pH 4.0) drinking water *ad libitum*. Experimental lung metastasis was induced in the control group by injecting 1×10^6 MV3 cells in 400 µl PBS into the tail vein. Animals were treated with the anti-VLA-4 mAb natalizumab (10 mg/kg) prior to tumor cell inoculation, or they received the antibody simultaneously with tumor cells after preincubation with 5 µg for 30 min.

Statistics

Data represent means ± standard deviations of at least three independent experiments. Comparisons were performed by the unpaired Student's *t*-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Relevance of VLA-4 in experimental metastasis of MV3 cells

The importance of adhesion receptors for mediating the hematogenous phase of tumor cell metastasis is well accepted. While the main focus lies on the role of selectins and their impact on formation of tumor cell microemboli with platelets and leukocytes, much less is known about the importance of integrins [12,14,31,32].

To evaluate the involvement of VLA-4 in MV3 hematogenous metastasis, an experimental metastasis study in mice was performed. The female nude mice in the control group were injected with 1×10^6 MV3 cells suspended in 0.4 mL PBS in the tail vein. The second group obtained 10 mg/kg natalizumab i.v. 30 min prior to the inoculation of MV3 cells. In a third group the same number of cells was administered, but cells were mixed prior injection with 5 µg natalizumab to obtain a total blockade of the melanoma VLA-4 function. At day 31, the experiment was terminated and the number and weight of pulmonary and extrapulmonary metastatic nodules was evaluated. In comparison to the control group, both natalizumab treatment regimes displayed a remarkable reduction and bisect the percentage number of metastatic foci (Fig. 1). This strong reduction is even evident considering size and weight of the foci using a scoring system (data not shown). The dosis of natalizumab (10 mg/kg; second group) appears sufficient for a VLA-4 blockade leading to a similar effect as in case of antibody-preincubated cells (third group). However, this clearly substantiates VLA-4 as a keyplayer in MV3 hematogenous metastasis and justifies the further use of this cell line for relevant inhibitory studies using heparins *in vitro*.

VLA-4 dependency of MV3 adhesion under in vitro conditions

In order to evaluate the capacity of heparins to inhibit the VLA-4 dependent cell adhesion of MV3 cells to immobilized VCAM-1 and to correlate the structural peculiarities with this activity, a dynamic *in vitro* cell binding assay was applied [17]. In addition to a recent study, where we initially described the inhibitory capacity of heparin [17], we further focused on the VLA-4 dependency of the MV3 cell adhesion assay. Therefore, we applied a siRNA approach to knockdown VLA-4 expression in the MV3 cells (MV3siRNA). Flow cytometry data (not shown) confirmed a nearly 60% reduction in VLA-4 at

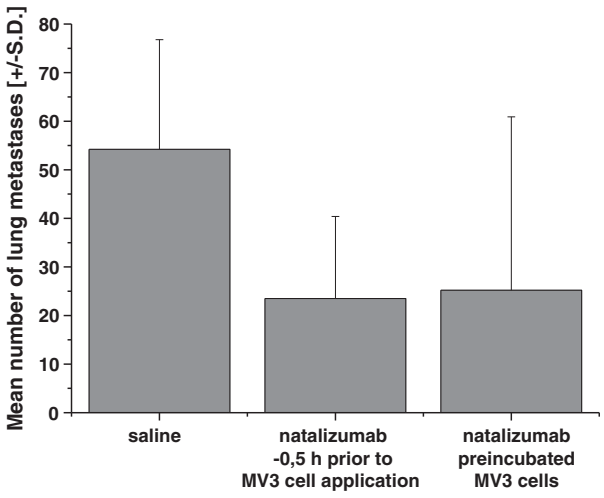

Fig. 1. Impact of VLA-4 on metastasis-like spread of MV3 cells in vivo. 1×10^6 MV3 melanoma cells were injected in tail veins of mice to simulate hematogenous metastasis. Natalizumab preincubated MV3 cells (5 µg) and mice treated with natalizumab (10 mg/kg) 30 min prior MV3 application revealed an attenuation of metastatic foci in the lungs. Data were presented as percentage ± SD of mean number of metastatic foci in lungs, taking saline treated mice (45 foci) as 100% (n = 6).

the cell surface of transfected cells. As illustrated in Fig. 2A, the MV3siRNA cells lost their ability to bind VCAM-1, the adhesion level is below that of the unstimulated cells. Both LMWHs, enoxaparin and tinzaparin reduced the adhesion of the stimulated wild type cells drastically and suggest excellent blocking capacities towards VLA-4. Enoxaparin displayed a slightly, but not significant higher efficiency and also reduced cell binding below the level of unstimulated cells.

There are no structural data available to explain the VLA-4 heparin interaction. To obtain a first insight how heparin interferes with the heterodimeric structure of VLA-4 in the binding process, MV3 cells were allowed to bind to both, immobilized antibodies against the α-4 or the β-1 chain and the interference of heparin with these bindings was evaluated (Fig. 2B). MV3 cells strictly adhered to both types of antibodies under dynamic conditions, but the binding towards the α-chain antibody was much stronger inhibited by heparin than binding to the β-chain. Although the antibody binding did not represent the conditions of ligand recognition, the data give a rough indication that heparin has a more dominant tendency to block VLA-4 via the α-chain.

Nevertheless, the questions arise whether and what types of structural modifications in the heparin molecule have an impact on VLA-4 binding capacity.

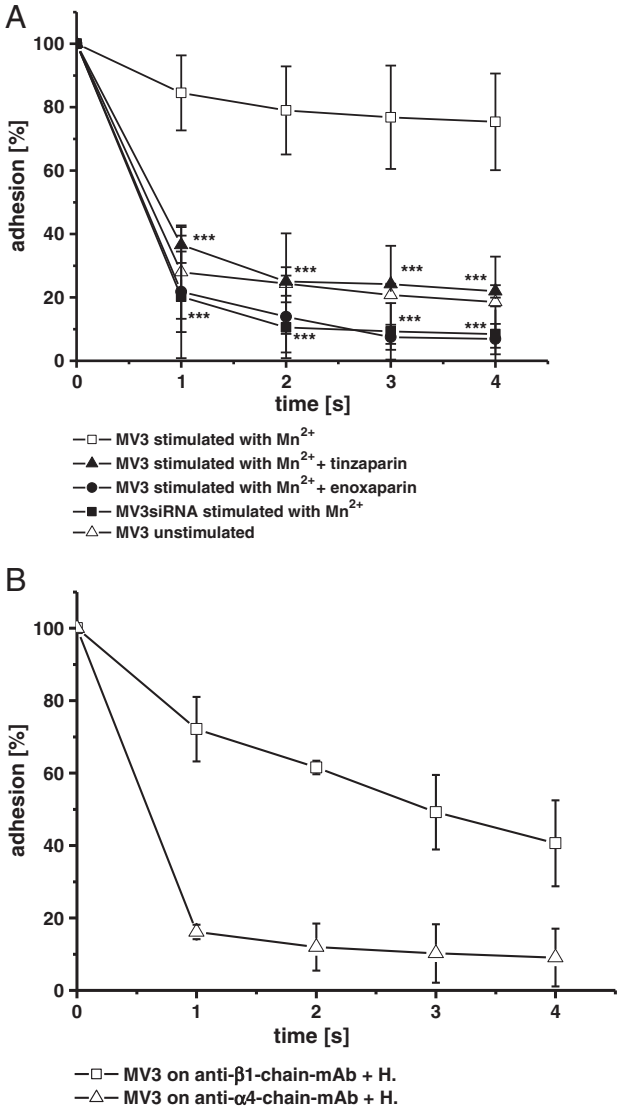

Fig. 2. Adhesion behaviour of VLA-4 knockdown MV3 melanoma cells and influence of LMWHs enoxaparin and tinzaparin on VLA-4 mediated adhesion to VCAM-1. Characterization of the heparin binding site. A) Adhesion of Mn^{2+}-activated MV3 cells (□) to immobilized VCAM-1 were determined in a microscopic flow chamber assay and compared to non activated cells (Δ). Binding of VLA-4 knockdown cells (MV3siRNA) (■) as well as enoxaparin (●) and tinzaparin treated cells (500 μg) (▲) is reduced to or below the level of non activated cells, respectively. B) Binding of MV3 cells to immobilized antibodies against the α4- (Δ) or the β1- (□) chain and the interference of unfractionated heparin with these bindings were ascertained. The interaction of MV3 cells with the antibody against the α4-chain is much more inhibited than the interaction with the β1-chain. These findings give a first hint for the raw localisation of the heparin binding site. Data are presented as mean values ± SD (n = 3; *p<0.05, **p<0.01, ***p<0.001 with respect to stimulated MV3 cells).

Structural requirements of heparin for VLA-4 binding

Impact of heparin size on inhibitory capacity

Recently, we evaluated UFH in this VLA-4 binding assay, which showed higher binding capacity than tinzaparin, while the pentasaccharide fondaparinux was without effect [17]. In addition, a fraction of tinzaparin consisting of 14–18 saccharide units could only slightly inhibit VLA-4 [17]. To investigate whether the size of about 16 saccharide units is the critical threshold cut across smaller and less efficient fractions, we examined tinzaparin fractions of 6, 8, 10, and 12 monosaccharide units. However, they failed to show any binding activity to VLA-4 (data not shown). This further emphasized the size of heparin as a critical parameter for VLA-4 inhibition.

Impact of heparin flexibility on inhibitory capacity

To get a deeper insight in the structural requirements for an optimal VLA-4 inhibition and further comparison to P-selectin inhibition, a number of non-anticoagulant heparins with various structural modifications were applied in the flow chamber assay. To consider the size dependency of heparin for VLA-4 binding, the derivatives were derived from UFH resulting in a mean molecular weight of 15 kD.

One can assume that the chain flexibility of heparin is an important parameter for a suitable binding to adhesion receptors at the cell surface such as was observed for P- and L-selectins with glycol-split heparins [27]. In order to consider this parameter, a glycol-split (RO-heparin) derivative was tested in which the C(2)-C(3) bonds of all non-sulfated uronic acid residues were cleaved, but the original chain length of heparin was maintained [26]. The expected increase in inhibition compared to the effect of tinzaparin held off (Fig. 3), but the RO-heparin displayed a significant inhibition slightly below the activity of tinzaparin. In opposite to the selectins, a flexible chain seems not to be required for an optimal VLA-4 binding.

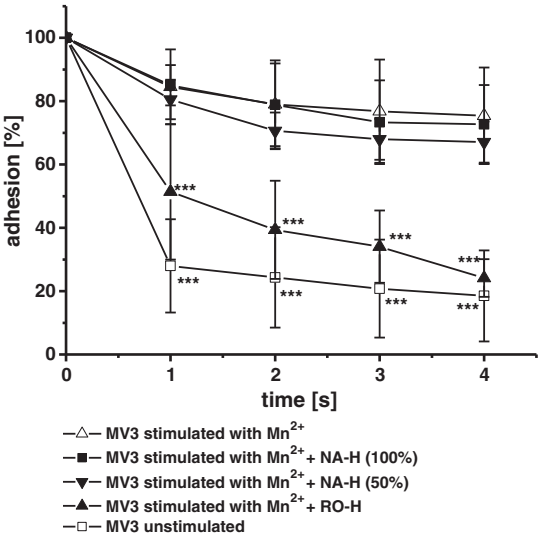

Fig. 3. Inhibitory activities of N-acetylated (NA-H) and "glycol-split" heparin (RO-H). Derivatives N-acetylated for 50% (▼) and 100% (■) (50 μg) exhibited no inhibitory capacity to the VLA-4 binding compared to stimulated MV3 cells (Δ). RO-heparin (▲) interfered with VLA-4 moderately. * This figure has already been published in an extended abstract presenting this part of the data [41].

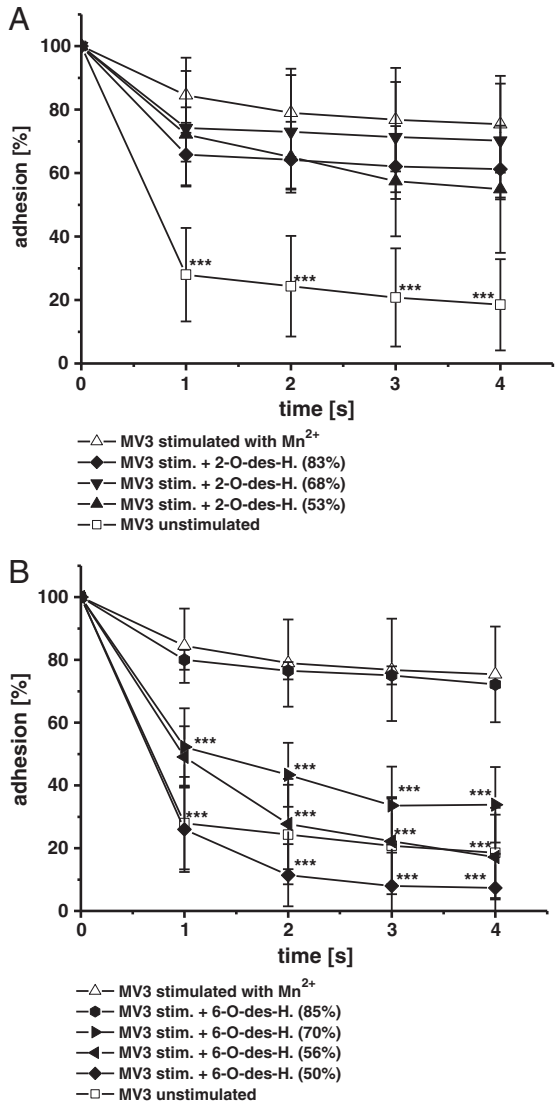

Fig. 4. Influence of heparin differing in degree and position of sulfation on the adhesion behaviour of MV3 cells to VCAM-1. A) 53% 2-O-desulfated derivatives (50 μg) showed only a slight reduction in adhesion (▲), whereas heparin desulfated to 68% (▼) and 83% (●) indicated hardly an inhibitory effect compared to Mn^{2+}-activated MV3 cells (△). B) However 6-O-desulfated derivatives (50 μg) reduced VLA-4 mediated binding under the level of unstimulated cells (□). Compounds desulfated to 50% (♦) exceeded tinzaparin and also enoxaparin in their inhibitory potencies. Only the compound desulfated to 85% (●) showed a decline in VLA-4 blocking capabilities and did not distinguish from stimulated MV3 cells (△).

Impact of N-acetylation on inhibitory capacity

Testing N-acetylated derivatives revealed that heparins with 50% as well as 100% N–acetylation are completely unable to inhibit the VLA-4 mediated binding (Fig. 3). There are no detectable differences in binding compared to stimulated MV3 cells. This is in contrast to what was observed for selectin interactions, since partial heparin N-acetylation displayed a remarkable increase in P-selectin inhibition [21,33].

Impact of 2-O and 6-O-desulfation on inhibitory capacity

Furthermore, samples with different degrees of 2-O-desulfated uronic acids were examined (Fig. 4A). Only the derivative with a desulfation degree of 53% could mediate a slight reduction in adhesion but this is not significantly different from stimulated MV3 cells. The samples with desulfation levels of 68% and 83% were unable to reduce the VLA-4 binding remarkably. One can conclude that sulfation in 2-O-position of uronic acids is a critical parameter for VLA-4 binding.

In contrast, a partial 6-O-desulfation of glucosamine residues appears to be tolerable, since a 50% desulfation reduced the VLA-4 mediated binding below the level of unstimulated MV3 cells (Fig. 4B). Only 10% of the MV3 cells still adhered to the VCAM-1 layer after 4 s of flow, which is comparable to the value found with enoxaparin and exceeds tinzaparin clearly. Further 6-O-desulfation reduced the inhibitory capacity. Heparin with 56% 6-O-desulfation diminished the inhibition to the level of unstimulated cells. A derivative with a 70% level of 6-O-desulfation revealed, although statistically significant, only a reduced inhibitory capacity, while further desulfation to 85% led to a loss in VLA-4 inhibition.

Detection of heparin binding kinetics to VLA-4 and selectins

The cell inhibition studies provide interesting insights into the structure-activity relationship of heparin for VLA-4 binding, which were furthermore confirmed using another VLA-4 positive melanoma cell line (B16.F10 murine origin; data not shown). However, these data do not allow an evaluation of the real binding intensities for an assay independent comparison, for instance with the therapeutically applied VLA-4 antibody natalizumab. Therefore, we analyzed a selection of derivatives with respect to their VLA-4 binding affinity using the SAW biosensor technique. For this, we used a membrane preparation of the MV3 cells, in which the presence of VLA-4 was detected by SDS-PAGE and Western blot. Membrane vesicles were immobilized at the sensor surface and binding to VLA-4 was analyzed, as described before [17]. The binding data in Table 1 confirm a high binding affinity of natalizumab in the nanomolar range.

The binding affinity of enoxaparin is in the low micromolar range, and slightly higher than the recently detected affinity of tinzaparin [17]. The small difference between the affinities of both LMWHs might reflect the slightly deviating activities in the cell binding assay in Fig. 2, but do not relate to their differences in their average molecular weight (tinzaparin 8.3 kD vs. enoxaparin 5.3 kD, resp.) [27]. The RO-heparin, which displayed compared to enoxaparin slightly weaker but comparable binding to tinzaparin has nearly identical kinetic binding parameters. The heparin with 50% 6-O-desulfation, the most effective non-anticoagulant VLA-4 inhibitor in this study, displayed surprisingly a somewhat lower affinity. In reference to the adhesion experiments (Fig. 4A), the partially (53%) 2-O-desulfated heparin is much clearly distinguished, and a VLA-4 binding

Table 1
Kinetic binding constants of natalizumab, and heparin derivatives binding to VLA-4 in an immobilized membrane preparation. Selected heparin derivatives, (RO-H), 53% 2-O-desulfated heparin (2-O-DS H.) and 50% 6-O-desulfated heparin (6-O DS H.) were investigated for their binding kinetics to immobilized MV3 membrane preparations containing VLA-4. The K_D values roughly corroborate to the inhibitory capacity results acquired in the flow chamber assay.

	K_D [M]	k_{on} [M^{-1} s^{-1}]	k_{off} [s^{-1}]
natalizumab	$3.51\times10^{-8}\pm0.84\times10^{-8}$	$1.71\times10^{5}\pm0.28\times10^{5}$	$5.36\times10^{-3}\pm1.75\times10^{-3}$
enoxaparin	$2.22\times10^{-6}\pm0.76\times10^{-6}$	$1.45\times10^{3}\pm0.30\times10^{3}$	$3.09\times10^{-3}\pm0.63\times10^{-3}$
tinzaparin	$4.61\times10^{-6}\pm2.68\times10^{-6}$	$1.27\times10^{3}\pm0.58\times10^{3}$	$5.13\times10^{-3}\pm1.56\times10^{-3}$
RO-H	$2.71\times10^{-6}\pm2.63\times10^{-6}$	$1.19\times10^{3}\pm0.25\times10^{3}$	$4.45\times10^{-3}\pm3.39\times10^{-3}$
6-O DS H. (50%)	$5.89\times10^{-6}\pm4.12\times10^{-6}$	$4.20\times10^{2}\pm0.60\times10^{2}$	$2.32\times10^{-3}\pm1.51\times10^{-3}$
2-O DS H. (53%)	$1.16\times10^{-5}\pm0.36\times10^{-5}$	$2.83\times10^{2}\pm1.02\times10^{2}$	$3.33\times10^{-3}\pm1.42\times10^{-3}$

affinity of the small size fractions (6 – 12 monosaccharide units) could not be detected. Although these data generally confirm the tendencies of structural modifications in the cell inhibition studies, the differences are quite less pronounced. This should be related to the peculiarities of the different assays used, comparing a functional inhibition of binding vs. kinetic evaluation of binding. However, the kinetic data, especially the association rates allow an interesting insight into the binding process. Charge density appears to be important for contact formation to VLA-4, since the two desulfated derivatives, 6-O and 2-O-desulfated heparin clearly show a slower association rate.

Aiming to define anti-adhesive heparin derivatives including VLA-4 and selectins, we also detected the binding ability of the most efficient derivative (50% 6-O-desulfated heparin) towards P- and L-selectin in comparison to tinzaparin (Table 2). Therefore, P- or L-selectin Fc chimera were immobilized on the sensor surface and the dynamic binding characteristics were calculated from the resulting phase shifts. Both heparins show identical binding characteristics and affinities to P- and L-selectin in the low micromolar range, which are also in agreement to previous studies on other LMWHs [34]. Since the chain length of heparin appeared as critical parameter to distinguish selectin and VLA-4 binding ability, we also included the small size tinzaparin fractions into the P- and L-selectin binding experiments. In contrast to the inability to interact with VLA-4, all three derivatives displayed remarkable binding affinities to both, P- and L-selectin with intensities comparable to tinzaparin. To confirm that the high binding affinities of the small size derivative are relevant for functional interference with P-selectin binding, the octasaccharide unit heparin fraction was applied in a microscopic assay to inhibit the P-selectin mediated platelet interaction with MV3 cells. Data in Fig. 5 suggest a significant inhibitory capacity of the derivative indicating that a full P-selectin binding ability is maintained despite of the size reduction.

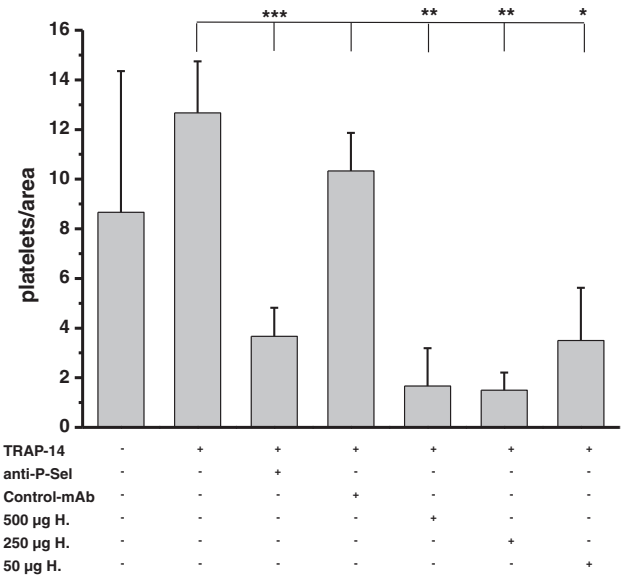

Fig. 5. P-selectin dependent interaction of MV3 cells with platelets and the interference by an octasaccharide unit heparin fraction. TRAP-14 activated platelets showed an increased interaction with MV3 cells compared to non-activated platelets. This interaction could be abrogated by addition of a P-selectin binding mAb (2.5 µg) or different amounts of octasaccharide heparin fraction (50 – 500 µg). A control mAb (2.5 µg) had no influence on the MV3 cell platelet interaction.

Discussion

Melanoma cells display a high tendency for metastatic spread in the body. One can assume that the high expression levels of the integrin VLA-4 at melanoma cells partly contribute to this malignancy by driving the contact formation with the endothelium at distant sites, binding to the ligand VCAM-1. Nevertheless, only very few studies exist, which describe the involvement of VLA-4 for the melanoma cell metastasis or the interference with this binding pathway [12,14]. Recently we reported that the VLA-4 binding of murine or human melanoma cells to VCAM-1 can be inhibited by heparin [17]. Among the different molecular mechanisms and explanations for the antimetastatic activities of heparin, the inhibition of P- and L-selectin appears as primary target covering tumor cells of multiple entities. Therefore, searching for the structural requirements of heparin to bind VLA-4 and comparing these to the selectin binding prerequisites could lead to the identification of favoured anti-adhesive, chemically modified heparins with distinction from other activities, i.e. anticoagulant properties.

However, VLA-4 is a vital receptor on multiple leukocyte populations and involved in immune response in course of leukocyte transmigration. Therefore, the systemic application of VLA-4 inhibitors could also induce partly immuno-suppressive effects, as aimed in anti-inflammatory approaches i.e. for the treatment of multiple sclerosis using the antibody natalizumab. On the other side, the involvement of leukocytes, i.e. monocytes in tumor cell microemboli formation and their contributions to signalling forcing tumor cell settlement and transmigration would also be interfered [35]. Consequently, blocking VLA-4 next to selectins appears as an attractive target.

Our initial experimental metastasis study in mice indicates a nearly identical reduction of metastatic foci in both, the systemically VLA-4 blocked mice and the natalizumab preincubated tumor cells, which suggest that VLA-4 function on the tumor cells is dominant for the

Table 2
Kinetic constants of tinzaparin, 6-O-desulfated heparin and size fractionated heparin derivatives binding to immobilized P- or L-selectin, detected by using SAW biosensor technology. The 6-O-desulfated heparin derivative possesses identical ability to bind P- and L-selectin compared to tinzaparin. Surprisingly, size fractionation of heparin up to 6 monosaccharide units does not affect the P- and L-selectin binding.

Heparin / derivative	K_D [M]	k_{on} [M^{-1} s^{-1}]	k_{off} [s^{-1}]
P-selectin			
tinzaparin	$5.74\times10^{-6}\pm0.03\times10^{-6}$	$4.74\times10^{2}\pm0.56\times10^{2}$	$2.73\times10^{-3}\pm0.33\times10^{-3}$
50% 6-O DS H.	$6.16\times10^{-6}\pm1.35\times10^{-6}$	$2.48\times10^{2}\pm0.91\times10^{2}$	$1.47\times10^{-3}\pm0.23\times10^{-3}$
6 saccharide units	$4.65\times10^{-6}\pm2.88\times10^{-6}$	$2.74\times10^{2}\pm1.65\times10^{2}$	$1.41\times10^{-3}\pm1.05\times10^{-3}$
8 saccharide units	$2.17\times10^{-6}\pm0.26\times10^{-6}$	$2.21\times10^{2}\pm1.67\times10^{2}$	$5.09\times10^{-4}\pm4.44\times10^{-4}$
12 saccharide units	$3.88\times10^{-6}\pm2.75\times10^{-6}$	$0.64\times10^{2}\pm0.31\times10^{2}$	$1.99\times10^{-4}\pm0.51\times10^{-5}$
L-selectin			
tinzaparin	$3.61\times10^{-6}\pm0.70\times10^{-6}$	$3.94\times10^{2}\pm1.04\times10^{2}$	$1.46\times10^{-3}\pm0.65\times10^{-3}$
50% 6-O DS H.	$3.11\times10^{-6}\pm1.36\times10^{-6}$	$6.80\times10^{2}\pm1.57\times10^{2}$	$1.67\times10^{-3}\pm0.29\times106^{-3}$
6 saccharide units	$4.29\times10^{-6}\pm2.10\times10^{-6}$	$1.74\times10^{2}\pm0.66\times10^{2}$	$6.96\times10^{-4}\pm3.68\times10^{-4}$
8 saccharide units	$7.16\times10^{-6}\pm5.54\times10^{-6}$	$4.29\times10^{2}\pm3.47\times10^{2}$	$2.22\times10^{-3}\pm1.14\times10^{-3}$
12 saccharide units	$5.56\times10^{-7}\pm2.77\times10^{-7}$	$4.24\times10^{3}\pm0.75\times10^{3}$	$2.37\times10^{-3}\pm1.41\times10^{-3}$

metastatic spread. However, further experiments are needed to elucidate potential immuno-suppression or effects of reduced leukocyte function, but the role of VLA-4 as promising target for reducing the metastatic spread can be confirmed.

The VLA-4 cell binding function *in vitro* (Fig. 2A) and the non-complete VLA-4 knockdown in MV3siRNA cells indicate either that not all the VLA-4 molecules are involved in the cell binding process, or that a certain threshold level of functional VLA-4 molecules is essential to mediate cell binding under the applied flow conditions. Compared to the MV3siRNA cells and their 60% reduction in VLA-4, one can speculate that the applied amounts of enoxaparin and tinzaparin to the wild type MV3 cells are able to block a similar fraction of the VLA-4 molecules.

A recent study reported on the role of non-anticoagulant heparins as inhibitors of P- and L-selectin or heparanase in course of metastasis [21]. The present findings add the knowledge referring to VLA-4 inhibition of these or similar non-anticoagulant derivatives and open the way for detailed discussions on anti-adhesive heparins. Partial heparin N-acetylation caused a remarkable increase in P-selectin inhibition, L-selectin inhibition was reduced [21], but VLA-4 recognition is totally abolished. 2-O-desulfation appears critical for both, L- and P-selectin binding [21] as well as VLA-4 recognition. However, other studies on the impact of heparin desulfation identified the 2-O position as more tolerable for P- and L-selectin binding [33,36-38].

Size fractionation of heparin appears as the most critical parameter to differentiate selectin and VLA-4 binding capacity. We could confirm by both adhesion studies and kinetic affinity data that fractions smaller than tetradecasaccharide are not recognized by VLA-4. Recently it was reported on the antimigratory and antimetastatic effects of heparin fractions from 4 to 22 monosaccharide units and profound interference of a tetrasaccharide derivative with melanoma metastasis was found [39]. However, the authors did not refer to integrin or selectin activities.

Size dependency of heparin binding was also described for the selectins. Koenig et al. [40] found a decrease in both, P- and L-selectin binding with reducing the heparin size and the critical threshold for high binding activity was the dodecasaccaride size [41]. However, this is partly in contrast to our biosensor affinity studies, in which the small size fractions displayed remarkable affinities to P- and L-selectin in the low micromolar range, comparable to tinzaparin and other LMWHs [34]. Probably, the differences might be explained by the experimental settings. While Koenig et al. analyzed the binding strength as an inhibitory capacity in cell binding of an ELISA-like assay, we directly analyzed the heparin binding to the receptors. In order to investigate whether the small size fractions in our study are able to inhibit the P-selectin binding function, the P-selectin dependent interaction of MV3 cells with activated platelets and the interference of the octasaccharide unit thereof was analyzed. The data in Fig. 5 confirm that this small size fraction maintained the full P-selectin inhibition and possessed a comparable inhibitory capacity to the blocking antibody. Nevertheless, other factors such as different starting heparins or fractionation procedures might be a reason for the differences of our data and the findings reported by Koenig et al. [40].

However, a partial heparin 6-O-desulfation to nearly 50% resulted in the most effective VLA-4 blockade and also retained the selectin binding affinities (Table 2). Therefore, this derivative appears as the most promising anti-adhesive heparin candidate without anticoagulant activities of the present study. High P-selectin binding but a somewhat lower L-selectin inhibition has also been reported recently for this modified heparin [21]. A similar study reported on the strong impact of 6-O-desulfation of LMWH on reducing the inhibitory capacity to P-selectin/PSGL-1 interaction [33], while other studies described the total loss of P- and L-selectin binding capacity for 6-O-desulfated heparin [36,37]. However, the degree of desulfation was not reported in the latter two studies and might explain the findings. Referring to the fact, that the presence of sulfate groups at either, the 2-O of IdoA or at 6-O of GlcN was found to be sufficient for effective inhibition of heparanase [26], the 50% 6-O-desulfation of heparin appears to be sufficient for antimetastatic approaches interfering with adhesion receptors and heparanase.

In conclusions, this study justifies the integrin VLA-4 as an important target of heparin, which besides P- and L-selectin or heparanase appears attractive to be considered for antimetastatic approaches using heparin or chemically modified heparin derivatives. The structure-function elucidation presented here, and independently confirmed using the murine melanoma cells B16.F10 (not shown) favour a 50% 6-O-desulfated heparin derivative as the most promising candidate to meet the requirements for VLA-4, P- and L-selectin recognition as well as heparanase inhibition and to distinguish these activities from the anticoagulant properties of LMWH. These findings are essential if heparin will be applied in antimetastatic approaches while reducing the risk of bleeding complications.

Further in vivo studies are needed to elucidate the antimetastatic potential of 6-O-desulfated heparin derivatives in comparison to the non-modified LMWH to figure out, whether further, yet non-considered activities of heparin contribute to the antimetastatic potential.

Conflict of interest statement

The authors state that they do not have any conflict of interest with respect to the content of the manuscript.

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References

[1] Sleeman J, Steeg PS. Cancer metastasis as a therapeutic target. Eur J Cancer 2010;46:1177–80.
[2] Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 2006;12:895–904.
[3] Schmidmaier R, Baumann P. ANTI-ADHESION evolves to a promising therapeutic concept in oncology. Curr Med Chem 2008;15:978–90.
[4] Borsig L. Antimetastatic activities of heparins and modified heparins. Experimental evidence. Thromb Res 2010;125(Suppl 2):66–71.
[5] Läubli H, Borsig L. Selectins promote tumor metastasis. Semin Cancer Biol Jun 2010;20:169–77.
[6] Ludwig RJ, Boehme B, Podda M, Henschler R, Jager E, Tandi C, et al. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res 2004;64:2743–50.
[7] Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A 2001;98:3352–7.
[8] Nieswandt B, Hafner M, Echtenacher B, Männel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res 1999;59:1295–300.
[9] Läubli H, Stevenson JL, Varki A, Varki NM, Borsig L. L-selectin facilitation of metastasis involves temporal induction of Fut7-dependent ligands at sites of tumor cell arrest. Cancer Res 2006;66:1536–42.
[10] Krause T, Turner GA. Are selectins involved in metastasis? Clin Exp Metastasis 1999;17:183–92.
[11] Fritzsche J, Simonis D, Bendas G. Melanoma cell adhesion can be blocked by heparin in vitro: suggestion of VLA-4 as a novel target for antimetastatic approaches. Thromb Haemost 2008;100:1166–75.
[12] Okahara H, Yagita H, Miyake K, Okumura K. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. Cancer Res 1994;54:3233–6.
[13] Liang S, Dong C. Integrin VLA-4 enhances sialyl-Lewisx/a-negative melanoma adhesion to and extravasation through the endothelium under low flow conditions. Am J Physiol Cell Physiol 2008;295:701–7.
[14] Garofalo A, Chirivi RG, Foglieni C, Pigott R, Mortarini R, Martin-Padura I, et al. Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastases. Cancer Res 1995;55:414–9.
[15] Schadendorf D, Heidel J, Gawlik C, Suter L, Czarnetzki BM. Association with clinical outcome of expression of VLA-4 in primary cutaneous malignant melanoma as well as P-selectin and E-selectin on intratumoral vessels. J Natl Cancer Inst 1995;87:366–71.

[16] von Andrian UH, Engelhardt B. Alpha4 integrins as therapeutic targets in autoimmune disease. N Engl J Med 2003;348:68–72.
[17] Schlesinger M, Simonis D, Schmitz P, Fritzsche J, Bendas G. Binding between heparin and the integrin VLA-4. Thromb Haemost 2009;102:816–22.
[18] Akl EA, Terrenato I, Barba M, Sperati F, Muti P, Schünemann HJ. Extended perioperative thromboprophylaxis in patients with cancer. A systematic review. Thromb Haemost 2008;100:1176–80.
[19] Akl EA, Gunukula S, Barba M, Yosuico VED, van Doormaal FF, Kuipers S, et al. Parenteral anticoagulation in patients with cancer who have no therapeutic or prophylactic indication for anticoagulation. Cochrane Database Syst Rev 2011;1:CD006652.
[20] Engman CA, Zacharski LR. Low molecular weight heparins as extended prophylaxis against recurrent thrombosis in cancer patients. J Natl Compr Canc Netw 2008;6:637–45.
[21] Hostettler N, Naggi A, Torri G, Ishai-Michaeli R, Casu B, Vlodavsky I, et al. P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. FASEB J 2007;21:3562–72.
[22] Mousa SA, Mohamed S. Inhibition of endothelial cell tube formation by the low molecular weight heparin, tinzaparin, is mediated by tissue factor pathway inhibitor. Thromb Haemost 2004;92:627–33.
[23] Jayson GC, Gallagher JT. Heparin oligosaccharides: inhibitors of the biological activity of bFGF on Caco-2 cells. Br J Cancer 1997;75:9–16.
[24] Vlodavsky I, Abboud-Jarrous G, Elkin M, Naggi A, Casu B, Sasisekharan R, et al. The impact of heparanese and heparin on cancer metastasis and angiogenesis. Pathophysiol Haemost Thromb 2006;35:116–27.
[25] Chalkiadaki G, Nikitovic D, Berdiaki A, Katonis P, Karamanos NK, Tzanakakis GN. Heparin plays a key regulatory role via a p53/FAK-dependent signaling in melanoma cell adhesion and migration. IUBMB Life 2011;63:109–19.
[26] Naggi A, Casu B, Perez M, Torri G, Cassinelli G, Penco S, et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. J Biol Chem 2005;280:12103–13.
[27] Bisio A, Vecchietti D, Citterio L, Guerrini M, Raman R, Bertini S, et al. Structural features of low-molecular-weight heparins affecting their affinity to antithrombin. Thromb Haemost 2009;102:865–73.
[28] Perpeet M, Glass S, Gronewold TM, Malave A, Stoyanov I, Tewes M, et al. SAWSensor System for Marker-Free Molecular Interaction Analysis. Anal Lett 2006;39:1747–57.
[29] Lever JE. Active amino acid transport in plasma membrane vesicles from Simian virus 40-transformed mouse fibroblasts. Characteristics of electrochemical Na + gradient-stimulated uptake. J Biol Chem 1977;252:1990–7.
[30] Kaplan RS, Pedersen PL. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. Anal Biochem 1985;150:97–104.
[31] Varki A, Varki NM, Borsig L. Molecular basis of metastasis. N Engl J Med 2009;360:1678–9 1679–80.
[32] Klemke M, Weschenfelder T, Konstandin MH, Samstag Y. High affinity interaction of integrin alpha4beta1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. J Cell Physiol 2007;212:368–74.
[33] Roy S, Lai H, Zouaoui R, Duffner J, Zhou H, Jayaraman LP, et al. Bioactivity screening of partially desulfated low-molecular-weight heparins: a structure/activity relationship study. Glycobiology 2011;21:1194–205.
[34] Simonis D, Fritzsche J, Alban S, Bendas G. Kinetic analysis of heparin and glucan sulfates binding to P-selectin and its impact on the general understanding of selectin inhibition. Biochemistry 2007;46:6156–64.
[35] Qian B-Z, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141:39–51.
[36] Wang L, Brown JR, Varki A, Esko JD. Heparin's anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins. J Clin Invest 2002;110:127–36.
[37] Wei M, Tai G, Gao Y, Li N, Huang B, Zhou Y, et al. Modified heparin inhibits P-selectin-mediated cell adhesion of human colon carcinoma cells to immobilized platelets under dynamic flow conditions. J Biol Chem 2004;279:29202–10.
[38] Li Y, Luo J-Y, Cui H-F, Zheng L, Du S. Study on anti-metastasis of heparin derivatives as ligand antagonist of p-selectin. Biomed Pharmacother 2010;64:654–8.
[39] Kenessey I, Simon E, Futosi K, Bereczky B, Kiss A, Erdödi F, et al. Antimigratory and antimetastatic effect of heparin-derived 4–18 unit oligosaccharides in a preclinical human melanoma metastasis model. Thromb Haemost 2009;102:1265–73.
[40] Koenig A, Norgard-Sumnicht K, Linhardt R, Varki A. Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. J Clin Invest 1998;101:877–89.
[41] Schlesinger M, Naggi A, Torri G, Zeisig R, Alexander M, Schmitz P, et al. Blocking of integrin-mediated human MV3 melanoma cell binding by commercial and modified heparins. Int J Clin Pharmacol Ther 2010;48:448–50.