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

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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.

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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