Latent Heparanase Facilitates VLA-4–Mediated Melanoma Cell Binding and Emerges As a Relevant Target of Heparin in the Interference with Metastatic Progression

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Semin Thromb Hemost 2015;41:244–254.

Abstract

Heparanase is an endo-β-glucuronidase that enzymatically cleaves heparan sulfates (HS) and heparan sulfate proteoglycan (HSPG) structures. Heparanase expression levels by tumors were correlated with cell invasion, angiogenic activity, and poor prognosis. Heparanase can also possess pro-tumorigenic effects independent of its enzymatic activity. Using human melanoma MV3 cells, we demonstrate that latent heparanase activates in a tightly temporary-regulated manner the binding function of the integrin very late antigen-4 (VLA-4), an important component in the metastatic spread of melanoma cells. shRNA-mediated knockdown of syndecan-4 (SDC-4) indicated that this proteoglycan is the key element to convey heparanase binding via focal adhesion complex formation, detected by vinculin staining, to an upregulated VLA-4 binding function. This inside-out signaling pathway of VLA-4 involved activated FAK and Akt, but apparently not PKCα/δ. VLA-4, however, appears representative of other integrins which together impact the heparanase/integrin activation axis in tumorigenicity. Biosensor measurements provided an insight as to how heparin can interfere with this activation process. While low-molecular-weight heparin (LMWH) cannot replace heparanase bound to SDC-4, LMWH can compete with SDC-4 binding of heparanase. Since blockade of heparanase by LMWH has functional consequences for reduced VLA-4 binding, latent heparanase appears as a novel, so far unnoticed target of heparin, underlying its antimetastatic activity.

Keywords
- heparanase
- heparin
- melanoma
- cell adhesion
- migration
- syndecan-4
- VLA-4

Metastasis of tumors is the most fatal complication in cancer diseases and the major cause for the poor outcome of patients. Metastatic spread of tumors is a complex and versatile process, commonly described as the metastatic cascade. Tumor cells, which have been detached from the primary tumor, migrate through the extracellular matrix (ECM), often supported by the activity of matrix degrading enzymes. After entering the blood system, tumor cells undergo manifold contacts with soluble and cellular blood components, which help them to escape the immune surveillance, to adhere to

published online February 15, 2015

Issue Theme Anticoagulant Therapy: Present and Future; Guest Editor: Job Harenberg, MD.

DOI http://dx.doi.org/10.1055/s-0035-1544229.
ISSN 0094-6176.

the endothelium at distant sites, and subsequently extravasate and proliferate to form micrometastases.[1]

Cell adhesion molecules are critically involved in the metastatic process. Selectins, a family of carbohydrate-binding proteins, have attracted most attention in this context,[2] since P-selectin is a key component for rapid and massive coverage of tumor cells by platelets when entering the blood circulation.[3] This platelet clot protects the tumor cells from shear forces of blood circulation and provides a reservoir of abundant signaling molecules to mediate multiple steps in the formation of a metastatic niche.[4] L-selectin contributes dominantly to contact formation between tumor cell emboli and the endothelium and by recruiting leukocytes to sites of settlement.[5]

Integrins, a larger family of α/β heterodimeric cell adhesion receptors, have much less been considered in terms of their involvement in metastasis.[6] We have recently revisited the role of the integrin VLA-4 ($\alpha_4\beta_1$) in melanoma metastasis.[7] This integrin is strongly expressed by melanoma cells and few other tumor entities and its expression levels have been correlated with bad clinical prognosis.[8,9] Our data indicated that VLA-4 is of dominant importance in experimental metastasis of melanoma in mice through contact formation with the endothelial ligand VCAM-1.[7,10–12] Moreover, VLA-4 activity and thus metastasis were significantly attenuated by application of low-molecular-weight heparin (LMWH). Thus, blockade of VLA-4 added a novel target for understanding the antimetastatic activity of heparin, a relevant and still ongoing field of cancer research.

Heparin is the first-line therapy for the antithrombotic prophylaxis of cancer patients, which have commonly a higher risk of venous thromboembolic events. Several prospective clinical trials confirmed a positive effect of heparin treatment on cancer patients' survival,[13] especially for patients with an initial better prognosis. The molecular basis of this activity appears versatile and elicited multiple experimental approaches. Beside the antithrombotic efficiency of heparin, blockade of P- and L-selectin appears of dominant importance.[6] Heparin also interferes with the process of angiogenesis by triggering the release of tissue factor pathway inhibitor.[14]

In addition, heparin blocks heparanase, an endo-β-glucuronidase that cleaves HS and is thereby strongly implicated in tumor malignancy.[15] Heparanase activity of tumor cells provides them with the ability to degrade the ECM and thus facilitates tissue invasion and migratory activity. An impact on the bioavailability of heparin/HS-bound growth- and angiogenesis-promoting factors is also an accepted mode of action of this enzyme.[16,17] In fact, heparanase expression levels correlate with tumor vascularization, metastatic potential, and postoperative survival of cancer patients.[18] Several approaches in preclinical or clinical stages are ongoing to block heparanase using LMWH, heparin derivatives, or HS-like structures.[19–21]

Interestingly, heparanase can exert proadhesive effects on cells, which are apparently independent of its enzymatic activity. Initially found by elucidating the pH-dependency of heparanase enzymatic activity,[22] few studies reported that latent heparanase can induce spreading and adhesion of glioma, lymphoma, or T cells to ECM substrates.[15,23–25] Although the molecular mechanisms of these proadhesive events are not fully understood, β_1 integrins appear to play a role, indicated by the involvement of typical integrin-signaling molecules, such as Src, PKC, and PI3K[24,25] and the costimulatory effect of cellular HSPGs.[26] These findings appear to be related to the aforementioned VLA-4 activity in melanoma cells and its inhibition by heparin.[7] Clearly, a potential "autoactivation" of integrins by tumor cell heparanase could have strong implications for different steps of the metastatic cascade. Furthermore, if VLA-4 activity on melanoma would be stimulated by latent heparanase, the inhibitory effect of LMWH could also be attributed to blocking of latent heparanase, thus constituting a novel functional target. However, a heparanase/VLA-4 axis has not been described for solid tumors and interference with latent heparanase activity by heparin has not been regarded as a potential antimetastatic approach. Although a direct binding of heparin to VLA-4 has been reported,[27] an indirect inhibition of VLA-4 by affecting integrin signaling molecules is also likely for heparin as previously shown for the matricellular protein Cyr61 or fractalkine.[28,29]

Here, we provide evidence that latent heparanase activates VLA-4–mediated binding of MV3 melanoma cells via a SDC-4–mediated signaling pathway, a process that might have broader consequences on the metastatic cascade. We also introduce a mechanistic insight demonstrating how LMWH can interfere with this heparanase activity and show that latent heparanase is a relevant target for LMWH as a potential antimetastatic treatment option.

Materials and Methods

Chemicals and Proteins

Recombinant human (rh) proteins VCAM-1-Fc chimera, VLA-4 (His-tag), and SDC-4 (His-tag) and antibodies against human SDC-4, SDC-1, and integrin subunits α_1, α_4, α_5, α_V, β_1 and β_3 were purchased from R&D Systems (Wiesbaden, Germany). Fibronectin was from Roche Diagnostics GmbH (Mannheim, Germany), and bovine serum albumin (BSA), cyanuric chloride and the enzymes chondroitinase ABC, and heparinase III were from Sigma-Aldrich (Deisenhofen, Germany). PKCα inhibitor (Go6976) was obtained from Tocris (Wiesbaden, Germany). DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DGS-NTA(Ni) (1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) were purchased from Avanti Polar Lipids (Alabaster, AL). Tinzaparin was purchased from Leo Pharma GmbH (Neu Isenburg, Germany). Partially 6-O-desulfated (6O-DS-H; 56%), *N*-acetylated heparin (NAc-H; 100%), and glycol split heparin (RO-H, molecular weight of these derivatives are ~16,000 to 20,000 Da) were a kind gift by Dr. Annamaria Naggi, Ronzoni Institute of Chemical and Biochemical Research, Milano, Italy, and were prepared and analyzed as described.[30] Latent 65 kDa heparanase was purified from medium conditioned by HEK-293 cells overexpressing heparanase essentially as described.[31] All salts and buffers were of analytical grade.

Cell Culture

The human melanoma cell line MV3 was cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum, penicillin (0.1 U/mL), and streptomycin (0.1 mg/mL) (Sigma-Aldrich). For decreasing SDC-4 expression in MV3 cells, shRNA-technique was used for stable downregulation. MV3 cells were transfected by using a commercial shRNA-plasmid (Mission *sh*RNA, Sigma-Aldrich), which was recovered by Maxi-Prep (Sigma-Aldrich) of *Escherichia coli*. Lipid-based transfection of the cells was performed by using FuGene6 (Roche Applied Science, Roche Diagnostics GmbH, Mannheim, Germany); efficient DNA-lipid concentration has previously been tested by GFP-plasmid transfection. After transfection, cells stably expressing anti-SDC-4-shRNA were selected under permanent puromycin treatment. SDC-4 expression by MV3 cells was measured by flow cytometry (FACS Calibur, BD Heidelberg, Germany) using an anti-SDC-4 mAb (R&D Systems) and a secondary fluorescein isothiocyanate (FITC)-coupled antibody.

SDS-PAGE and Western Blotting

For protein separation, MV3 wild type (MV3wt), SDC-4 knockdown variants of MV3 cells (aSDC-4MV3kd), and cells of a non-target shRNA-plasmid (ntMV3) were rinsed with cold PBS, detached by 0.02% (w/v) EDTA and lysed by a commercial cell extraction buffer (Invitrogen GmbH, Karlsruhe, Germany) supplemented with phenylmethanesulfonyl fluoride and protease inhibitor cocktail according to manufacturer's instructions. Protein concentration was determined by BCA assay (Sigma-Aldrich). Equal amounts of proteins were separated by SDS-PAGE and transferred to polyvinyldifluoride membranes using a tank blot system at 100 V, 350 mA for 60 minutes. After blocking unspecific binding sites with a solution of 5% (w/v) skim milk protein in TBS with 0.2% (v/v) Tween 20, the membranes were incubated with primary antibody overnight. After three washes, antimouse IgG-HRP (Calbiochem, Merck Darstadt, Germany) was added as a secondary antibody. Chemiluminescence was detected after treating the membrane with Clarity Western ECL substrate chemiluminescence kit (Bio-Rad Lab GmbH, München, Germany) according to manufacturer's protocol.

Heparanase Activity Assay

Preparation of $Na_2{}^{35}SO_4$-labeled ECM-coated 35-mm dishes and determination of heparanase activity were performed essentially as described in detail elsewhere.[32] Briefly, cells (2×10^6) were lysed by three cycles of freeze/thaw, and the resulting cell extracts were incubated (18 hours, 37°C) with ^{35}S-labeled ECM. The incubation medium (1 mL) 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 was counted in a β-scintillation counter. Degradation fragments of HS side chains produced by heparanase are eluted at $0.5 < K_{av} < 0.8$ (peak II, fractions 12–22). Nearly intact HSPGs released from the ECM are eluted just after the V_0 ($K_{av} < 0.2$, peak I, fractions 3–12).[30] These high-molecular-weight products are released by proteases that cleave the HSPG core protein.

Flow Cytometry

MV3 cells were detached with PBS containing 0.02% (w/v) EDTA. Where indicated, 1×10^5 cells were incubated at 37°C for different time periods with latent heparanase, heparins (50 µg/mL or 1 mM Mn^{2+}). The cells were centrifuged, washed with PBS, and incubated with 0.1 µM VCAM-1 (His-tag) for 30 minutes. After two washes, a secondary FITC-conjugated antibody targeted against histidine regions was added. VCAM-1 binding of MV3 cells was determined by flow cytometry.

Scratch Assay

2D-Migration was detected with a modified Scratch assay as described by Liang et al.[33] Briefly, wells of a 24-well plate were coated with fibronectin (10 µg/mL) for 2 hours at 37°C. Afterward, 1.5×10^5 cells were plated on the coated wells. After reaching 90% confluence, two scratches per well were performed using a pipette tip, detached cells were removed with the supernatant. The cells were incubated with starvation medium without FCS for 24 hours. Latent heparanase (2 µg/mL) was added and migration properties were detected by comparing the scratch areas at different time points, referring to zero time using a Nikon Eclipse Ti microscope (Nikon Corporation, Tokyo, Japan).

Surface Acoustic Wave Biosensor Measurements

Surface acoustic wave (SAW) measurements were performed to extract kinetic constants of latent heparanase binding to rhVLA-4, rhSDC-4, rhVCAM-1, LMWH, or heparin derivatives using a sam 5 blue sensor device (SAW Instruments GmbH, Bonn, Germany) as described earlier.[27] Binding events were measured as phase shifts of the acoustic wave.

Latent heparanase was immobilized on gold-coated sensors. Sensors were preincubated in ethanolic solution of 11-mercaptoundecanoic acid (10 mmol/L) forming a self-assembled monolayer. The terminal carboxyl groups were activated with a mixture of 200 mmol/L EDC and 50 mmol/L NHS and 5 µg latent heparanase or 2 µg rhVCAM-1 Fc chimer were covalently bound to the sensor surface. Excess of free NHS esters was blocked by 1 mol/L ethanolamine (pH 8.5). A constant buffer flow of 40 µL/min was applied and a baseline equilibration in PBS was performed for at least 15 minutes. Binding of heparins to latent heparanase was determined by injecting dilution series between 10^{-7} and 10^{-4} mol/L, and dilution series of latent heparanase binding to immobilized VCAM-1 were applied in the range of 10^{-12} to 10^{-6} mol/L.

For determination of latent heparanase binding to VLA-4 or SDC-4, sensors were preincubated in a chloroform solution of 10 mM hexadecanthiol. After drying the sensor, a model membrane consisting of 20 mol% DOGS-NTA and 80 mol% DPPC was formed by Langmuir-Blodgett technique and transferred to the sensors as described earlier.[28] rHis-tagged VLA-4 or SDC-4 were injected with 25 µg/mL at a flow rate of 20 µL/min to allow binding of the His-tag to the DGS-NTA lipid in the membrane. After indicated membrane binding of VLA-4 or SDC-4 (phase shift of ~10 degrees) binding of latent heparanase was performed using a dilution series from 10^{-12} to 10^{-6} mol/L.

Immunofluorescence

To determine the impact of heparanase on integrin signaling, MV3 cells were plated on rhVCAM-1 Fc chimera-coated coverslips. Cells adhered to the surface overnight and were incubated with 2 µg/mL latent heparanase or 1 mM Mn^{2+} for 10 minutes or were left untreated before fixation with 4% paraformaldehyde. Next, cells were permeabilized for 10 minutes with 0.5% Triton X-100 (Sigma-Aldrich), and incubated for 1 hour with a primary antibody against vinculin (SantaCruz Biotechnology Inc., Heidelberg, Germany). After three washes with 0.05% (v/v) Tween 20 in PBS, cells were treated with secondary antibody conjugated to FITC (R&D Systems). After three more washes, coverslips were mounted on glass slides using Fluoromount (Sigma-Aldrich) and imaged using a Nikon Eclipse Ti microscope.

Statistics

Data represent means ± standard deviations of at least three independent experiments. Comparisons were performed by the unpaired Student *t*-test.

Results

Knockdown of Syndecan-4 in MV3 Cells

Adhesive effects of latent heparanase have been demonstrated by increased spreading or ECM binding of glioma, lymphoma,[15,23] or T cells.[22,24] β_1 integrins have been postulated to be crucially involved in these binding interactions. To evaluate whether triggering of such integrin binding is relevant for metastatic steps of solid tumor cells, we selected the highly metastatic human melanoma cell line MV3. MV3 cells express among others the β_1 integrins VLA-5 and VLA-4. The latter has been shown to be critical for metastatic spread.[10–12,34,35] Integrin binding functions are partly supported by cell surface HSPGs. Especially the transmembrane HSPG SDC-4 has been related to integrin costimulation by its signaling pathway and its role in focal adhesion complex formation.[36,37] Consequently, SDC-4 appears as the most probable candidate to mediate effects of latent heparanase on integrins.

Attempting to identify a functional link between latent heparanase and melanoma integrin activation, we performed a shRNA-mediated knockdown of SDC-4 in MV3 cells. The resulting aSDC-4MV3kd cells display downregulation of SDC-4 to 55% (►**Fig. 1A**), while SDC-1 and the various integrin subunits were not modified in their expression levels by the knockdown procedure (►**Fig. 1B**) compared with MV3wt cells. Furthermore, SDC-4 knockdown did not affect heparanase activity of the MV3 cells (►**Fig. 1C**). However, the uptake characteristics of heparanase, detected by blotting the intracellular heparanase, displayed a time lag in the knockdown cells indicating a role of SDC-4 for uptake and processing of heparanase (►**Fig. 1D**).

Another functional consequence of SDC-4 downregulation became evident by determining the 2D-migration of MV3wt and aSDC-4MV3kd cells on a fibronectin surface (►**Fig. 1E**). Although the differences in migratory capacity were not significantly different, aSDC-4MV3kd cells tend to exhibit a slower migration, especially at later time points of the experimental period. This can be related to the fact that SDC-4 has an intrinsic binding function to fibronectin[38] and a costimulating effect on VLA-5, thereby contributing to the migratory capacity of cells.[37] Consequently, deficiency in SDC-4 results in slightly slower migration.

This confirms that despite the incomplete downregulation, SDC-4 functionality is clearly affected and furthermore, this cell model appears appropriate to elucidate a potential latent heparanase/SDC-4 activation axis.

Latent Heparanase Facilitates VLA-4 Binding to VCAM-1

To investigate whether latent heparanase affects integrin functionality, we first focused on a 2D cell migration assay on fibronectin, as indicated earlier. For this purpose, we added recombinant latent heparanase (2 µg/mL) to both MV3wt and aSDC-4MV3kd cells and followed cell migration by a sequence of microscopic images within 4 hours. In general, there were neither significant effects of heparanase on cell motility nor significant differences between the two cell types (data not shown). A slight, but not significant, acceleration of the MV3wt cells, not the SDC-4 knockdown cells, was evident after 30 to 60 minutes of heparanase interaction (►**Fig. 1F**). However, this does not appear as explicit parameter for integrin activation by heparanase in light of the various influencing factors of this assay.

To focus more specifically on the heparanase/integrin axis, we selected VLA-4 as a highly expressed and relevant integrin for metastasis, detecting its selective binding to the cellular ligand VCAM-1. For this purpose, MV3wt and aSDC-4MV3kd cells were incubated for up to 1 hour with latent heparanase (2 µg/mL) and the resulting VCAM-1 binding was detected by flow cytometry (►**Fig. 2A**). While both cell types displayed an identical VCAM-1 binding capacity under normal untreated conditions, the binding intensities differ after incubation with latent heparanase. The highest affinity to the physiological binding partner was observed in MV3wt cells after 10 minutes preincubation with latent heparanase. A longer incubation of MV3wt cells with latent heparanase yielded no higher binding. The effect vanished after 15 minutes and the VCAM-1 attachment stayed on the level of untreated cells.

In contrast, throughout the whole experimental period, the aSDC-4MV3kd cells were not affected by latent heparanase for higher VCAM-1 binding, indicating SDC-4 as a key component to mediate the proadhesive effects of heparanase.

To examine if application of higher heparanase concentrations further increase VCAM-1 binding, we tested two additional concentrations (►**Fig. 2B**). Both were able to amplify the attachment, characterizing the process as not yet saturated.

We included manganese as a positive control to estimate the maximum stimulation range of VCAM-1 binding to MV3 cells, as manganese induces a conformational change of integrins leading to a high affinity state.[39] In this setting, MV3wt and aSDC-4MV3kd cells exhibited equal VCAM-1 binding capacity, excluding differences between the cell types in integrin stimulation. Furthermore, it became evident that

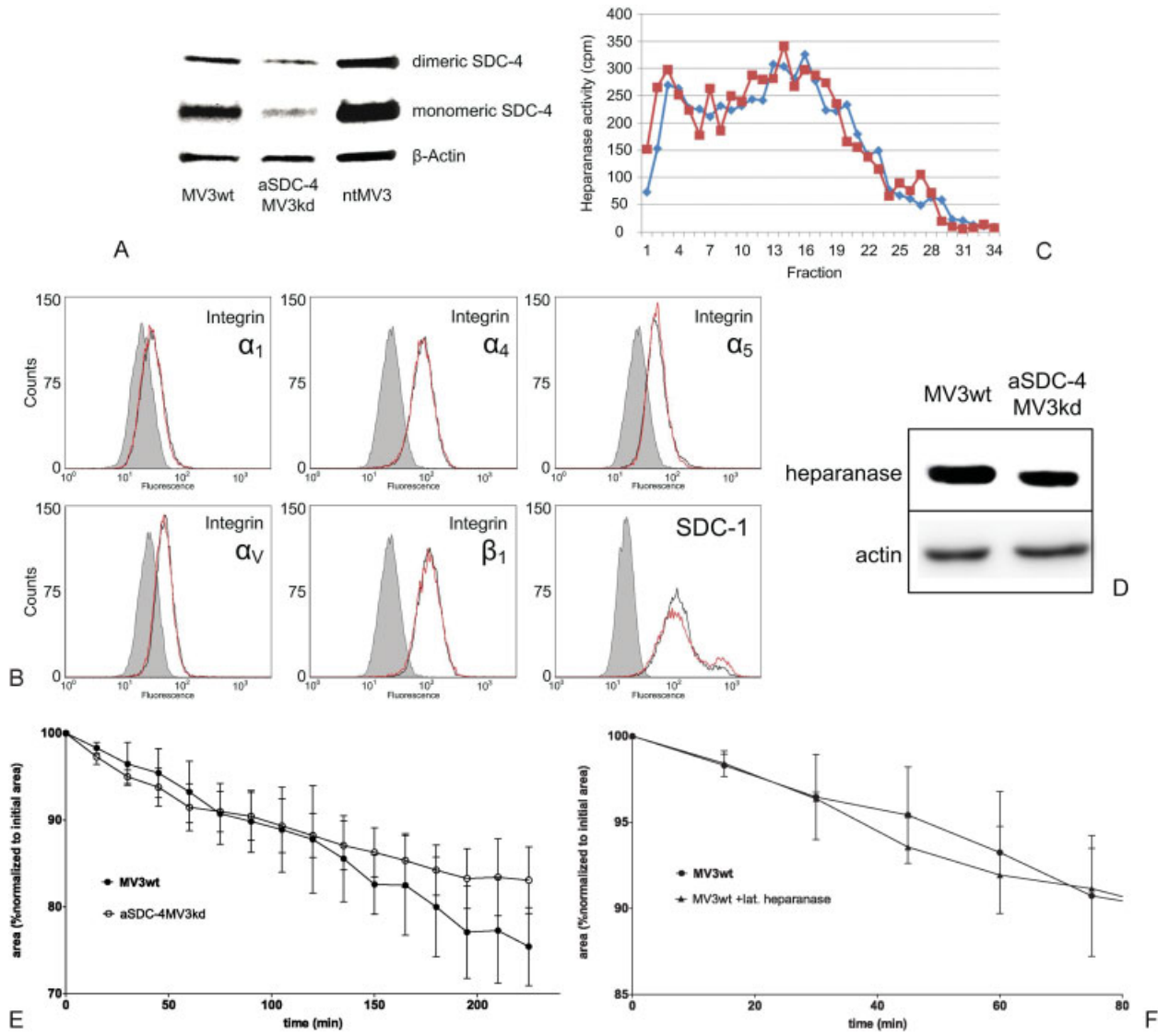

Fig. 1 shRNA-mediated knockdown of SDC-4 in MV3 human melanoma cells and consequences for cell function. (**A**) Protein characterization by Western blot of the aSDC-4MV3kd cells with respect to SDC-4 expression; SDC-4 monomer 32 kDa, SDC-4 dimer 68.2 kDa. Left: MV3wt, middle: aSDC-4MV3kd, right: ntMV3. (**B**) Flow cytometry data for comparison of the expression pattern of different integrin subunits and SDC-1 in MV3wt (black line) and aSDC-4MV3kd (red line) cells; the integrin subunit β_3 was not detectable. (**C**) Heparanase enzymatic activity. MV3wt (blue line) and aSDC-4MV3kd (red line) cells (2×10^6) were lysed by three cycles of freeze/thaw and the cell extracts were incubated (18 hour, 37°C) with ^{35} S-labeled ECM. The incubation medium was subjected to gel filtration on a Sepharose CL-6B column and the amount of sulfate labeled HS degradation fragments determined as described in section "Materials and Methods." (**D**) Cellular heparanase uptake. Heparanase (1 µg/mL) was added to MV3wt and aSDC-4MV3kd cells for 2 hours at 37°C. Cells were then washed twice with PBS and lysate samples were subjected to immunoblotting applying anti-heparanase antibodies. (**E**) 2D-migration of MV3wt and aSDC-4MV3kd cells on a fibronectin substrate, SDC-4 knockdown displayed a slower cell migration. (**F**) 2D-migration of MV3wt cells on a fibronectin substrate after adding 2 µg/mL of latent heparanase, which induces a temporal acceleration of migratory capacity of the MV3wt cells.

preincubation with the highest applied concentration of latent heparanase (10 µg/mL) reached the level of VCAM-1 binding similar to that observed upon artificial stimulation.

Altogether, the above results indicate that latent heparanase facilitates VLA-4 binding function via a SDC-4 dependent, timely regulated signaling process.

Insight into the Underlying Molecular Mechanisms of SDC-4 Involvement

To investigate the mechanism underlying latent heparanase stimulation of VLA-4 activity, first we simulated processes occurring at the cell membrane surface. For this purpose, we applied a model membrane biosensor approach and analyzed the binding affinity of heparanase to SDC-4. We also checked the potential interaction of heparanase with VCAM-1, VLA-4, or another integrin ($\alpha_V\beta_5$) as control. We performed a site-directed immobilization of SDC-4 (via His-tag) into a model membrane and added increasing concentrations of latent heparanase, as schematically presented in ►**Fig. 3A**. As expected, the results emphasized SDC-4 as important binding partner of latent heparanase at the cell surface with a remarkable affinity in the mean picomolar range (33.7 pmol/L; ►**Fig. 3B**). This high affinity binding is clearly related to the GAG side chains of SDC-4, as preincubation of

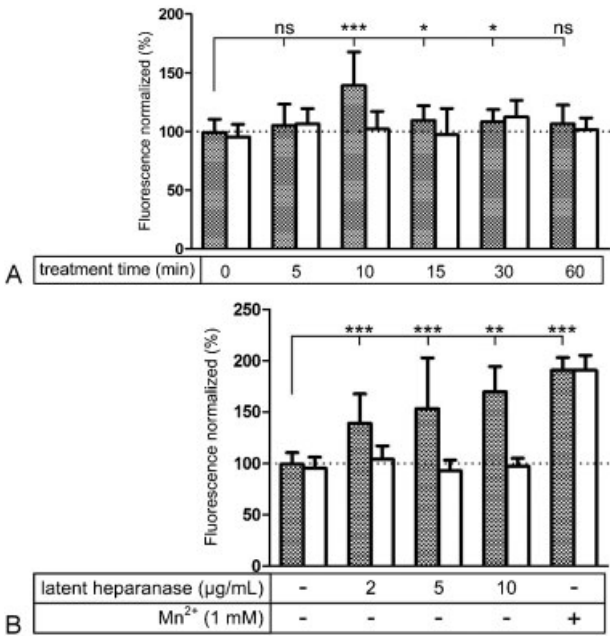

Fig. 2 The impact of latent heparanase on increased VLA-4 binding capacity. (**A**) MV3wt (black dotted) and aSDC-4MV3kd (white) cells were characterized by flow cytometry with respect to VCAM-1 binding capacity without activation, or after different incubation periods with 2 µg/mL latent heparanase. (**B**) Impact of different amounts of latent heparanase after 10 minutes to increase the binding capacity of VLA-4. Manganese of 1 mM was added as positive control to induce a conformational shift into high binding activity. Data from A and B confirm that downregulation of SDC-4 eliminates VLA-4 activation by heparanase.

SDC-4 with the enzymes heparinase III or chondroitinase ABC to cleave the GAG chains abolished the binding completely or attenuated the binding affinity by more than four orders of magnitude (data not shown).

In contrast, the binding capacity of latent heparanase to the integrin VLA-4 is much lower, as indicated in the second sensor shift of ►**Fig. 3B**, comparable to its binding to the GAG-cleaved SDC-4. This seems not to be a specific recognition of this integrin, as the integrin $\alpha_V\beta_5$ is also recognized by heparanase with a comparable affinity (not shown). Likewise, heparanase binding to VCAM-1 appears negligible.

These data confirm the assumption that SDC-4 is the preferred binding partner of latent heparanase, and thus VLA-4 activation occurs via a SDC-4–mediated signaling pathway.

SDC-4 shares similarities in the signaling pathways with integrins, although SDC-4 does not appear as an essential coreceptor to mediate VLA-4 binding.[37] In agreement with studies on the adhesive effects of latent heparanase in glioma cells,[23] MV3wt cells displayed a strong activation of both Akt and FAK as indicated by the appearance of their phosphorylated forms in response to incubation with latent heparanase (►**Fig. 3C**). This strongly indicates that VLA-4 is activated by triggering focal adhesion complex formation. In contrast, upregulation and activation of FAK was not detectable in SDC-4 knockdown cells. However, as these cells showed a higher level of activated Akt than MV3wt cells, the impact of heparanase on pFAK is less informative. Integrin-linked kinase (ILK) expression was not affected by heparanase leading to the assumption that VLA-4 or integrins in general are short term activated by latent heparanase via the focal adhesion kinase and not via the ILK pathway.

Dimerization of SDC-4 upon extracellular stimuli is known to induce intracellular downstream signals by their association with phosphatidylinositol 4,5-bisphosphate (PIP_2), which promotes the activity of PKCα. In a counteracting process, PKCδ phosphorylates SDC-4 and markedly decreases the affinity of SDC-4 to PIP_2 and thus activation of PKCα. We also investigated the expression and activation of PKCα and PKCδ. However, although we could find, as expected, a lower activation of PKCα in the aSDC-4MV3kd cells, an impact of latent heparanase on deregulation of PKC expression and phosphorylation was not evident (►**Fig. 3C**). Furthermore, as a functional confirmation, we could not detect any effect of adding a PKCα inhibitor on VLA-4 binding to VCAM-1 (data not shown). This obvious independence of VLA-4 activation on PKC signaling function is in accordance with former findings,[37,40] referring to a direct binding capability of paxillin to VLA-4 to form a complex, for example, with vinculin, talin and actin. Consequently, vinculin as a linking protein between integrins and the cytoskeleton is an indicator to detect adhesion complex formation. Therefore, vinculin was immunostained in cells that were seeded on a VCAM-1 surface to detect microscopically the appearance of local vinculin spots. Untreated MV3wt and aSDC-4MV3kd cells revealed a scattered vinculin pattern, which implies a diffuse distribution of integrins on the cell surface and only minor focal adhesion complexes. However, after incubation with latent heparanase, MV3wt cells showed clustered vinculin spots, which were quantified in representative cellular areas (►**Fig. 3D**). In contrast, SDC-4 knockdown cells did not display this vinculin redistribution. Interestingly, Mn^{2+} treatment of both cell types, which was shown to strongly increase VLA-4 binding affinity (►**Fig. 2B**), had no effect on vinculin clustering. These findings affirm the specific activation of VLA-4 and cytoskeleton reorganization by latent heparanase via SDC-4 and underline the inside-out activation of VLA-4.

Is Latent Heparanase a Target for Heparin with Functional Consequences for Cell Adhesion?

Enzymatically active heparanase has been intensively investigated as target to establish potent inhibitors to attenuate tumor progression and metastasis. Heparin or heparin-derived structures have been described as potential inhibitors.[30] Latent heparanase binds heparin as well,[25] but detailed studies on structural correlations have not been described.

In general, to pre-estimate the perspectives of inhibiting latent heparanase by heparin, blocking the GAG binding function in a competitive way appears to be the essential step. Binding and binding competition seem to be adequately simulated using the biosensor approach as indicated earlier to provide a simplified scenario of the cell surface and the interplay of all three components: latent heparanase, heparin, and SDC-4.

To provide first information on the binding affinity of heparin to heparanase, latent heparanase was immobilized on a sensor chip surface and dilution series of different heparins were applied. Tinzaparin, a commercial LMWH,

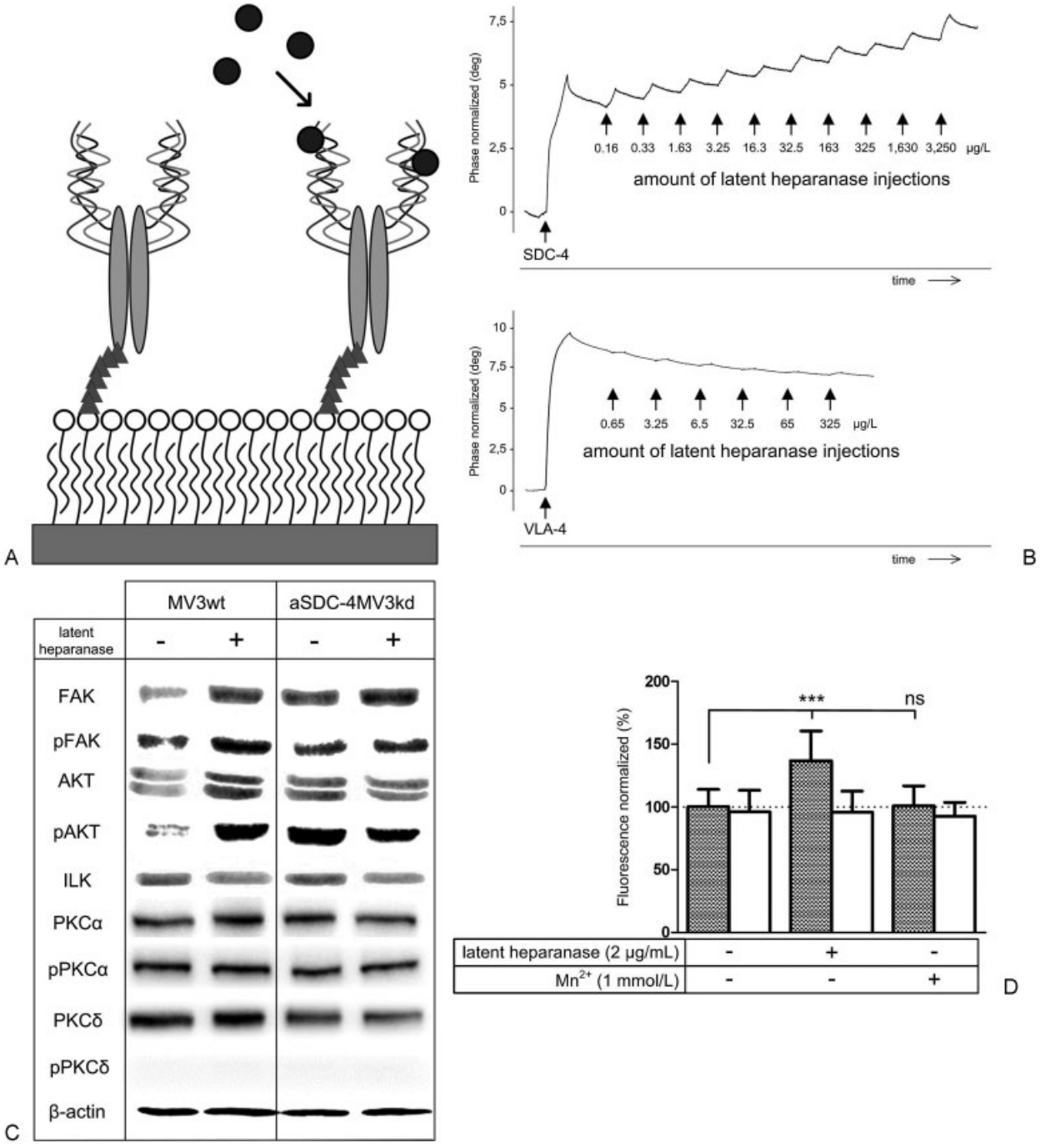

Fig. 3 Insight into binding processes of latent heparanase at the cell surface and induced signaling pathways. (**A**) A SAW biosensor has been applied to simulate the binding of heparanase at the cell surface, schematically depicted in A showing a sensor-immobilized model membrane with SDC-4 immobilization via His-tag binding (gray triangles) to a membrane chelator. (**B**) Phase shift graph representing the binding of heparanase to SDC-4 (upper panel) compared with binding of heparanase to immobilized VLA-4. It is evident that SDC-4 is the favored target for heparanase high affinity binding at the cell surface. (**C**) Western blot of selected signaling molecules in MV3wt and aSDC-4MV3kd cells, either untreated or after adding latent heparanase (2 µg/mL) for 10 minutes. (**D**) Vinculin staining revealed a significant higher clustering of activated VLA-4 per cell area in MV3wt than in aSDC-4MV3kd cells after incubation with latent heparanase (2 µg/mL, 10 minutes).

displayed an affinity in the mean micromolar range (►**Table 1**). Semisynthetic heparin derivatives with structural modifications, such as 6-O desulfation (6O-DS-H), glycol split (RO-H), or *N*-acetylation (NAc-H), were also included and all exhibited higher affinities than tinzaparin. The superior activity of RO-H followed by NAc-H and 6O-DS-H is roughly in line with former data on blocking the enzymatic activity of heparanase with heparin derivatives.[30] Nevertheless, the binding affinity of LMWH or heparin derivatives is by far lower than that of SDC-4 binding to latent heparanase (►**Fig. 3B**), so that a replacement of heparanase from SDC-4 by heparin is unlikely.

In a following experiment, we simulated this situation to check whether latent heparanase bound to SDC-4 could be

Table 1 Kinetic binding constants of tinzaparin, RO-H, NAc-H, and 6O-DS-H derivatives binding to immobilized latent heparanase

	K_D (μmol/L)	k_{on} (L/mol/s)	K_{off} (s)
Tinzaparin	32.5 ± 4.5	$2.6 \times 10^2 \pm 0.7 \times 10^2$	$8.46 \times 10^{-3} \pm 2.89 \times 10^{-3}$
6O-DS-H	10.7 ± 1.7	$15.2 \times 10^2 \pm 2.8 \times 10^2$	$16.04 \times 10^{-3} \pm 2.24 \times 10^{-3}$
RO-H	4.3 ± 0.6	$19.6 \times 10^2 \pm 2.1 \times 10^2$	$8.37 \times 10^{-3} \pm 1.28 \times 10^{-3}$
NAc-H	8.2 ± 1.1	$6.7 \times 10^2 \pm 1.4 \times 10^2$	$5.45 \times 10^{-3} \pm 1.09 \times 10^{-3}$

detached by increasing amounts of tinzaparin. This was not achieved and on the contrary, tinzaparin attached additionally to the immobilized proteins (►**Fig. 4A**). We hypothesize that heparanase acts in this experiment as a linker between SDC-4 and tinzaparin, as heparanase binding to the GAG side chains of SDC-4 likely not completely saturated the binding domains.

In contrast, preincubation of heparanase with tinzaparin and thus a simultaneous contacting of both components to SDC-4 prevented the binding of latent heparanase to SDC-4 at the simulated cell surface (►**Fig. 4B**), when exceeding a critical tinzaparin concentration. One can conclude that heparin interferes with the proadhesive effects of heparanase by masking the released soluble form of the latent enzyme, but not by replacing it from the cell surface HSPGs.

Next we figured out whether an interference with latent heparanase by heparin has functional consequences on cell adhesion. However, solely inhibiting heparanase is difficult because heparin can also directly affect VLA-4 binding function. We have previously demonstrated that LMWH or heparin derivatives attenuate VLA-4–mediated melanoma cell binding to endothelial cells or directly to VCAM-1.[34] Nevertheless, certain structural variations, such as *N*-acetylation of heparin, were shown not to be tolerated for direct VLA-4 blocking. Consequently, NAc-H appears as a suitable heparin to provide an insight into the separate activities when compared with tinzaparin, which possesses dual inhibitory function to both VLA-4 and heparanase.

The detection of VCAM-1 binding capacity of MV3wt or aSDC-4MV3kd cells after treatment with heparanase (2 μg/mL, 10 minutes) with or without simultaneous incubation with heparin (►**Fig. 4C**) displayed an insightful picture. While heparanase stimulates the binding of MV3wt, but not the knockdown cells, tinzaparin diminished the VCAM-1 binding capacities of both cell types in absence of heparanase to the same level below that of untreated cells, indicating a VLA-4 blockade. Since simultaneously given tinzaparin and heparanase reduced VCAM-1 binding to identical levels as in absence of heparanase, blockade of heparanase by tinzaparin appears as vital, but not discretely detectable contribution for inhibitory capacity.

In contrast, NAc-H did not affect the VCAM-1 binding of both cell types in absence of heparanase, indicating inability to interfere with VLA-4. However, simultaneous NAc-H and heparanase application prevented the rise in VLA-4 binding in MV3wt cells and thus the proadhesive effect of latent heparanase, but left the SDC-4 knockdown cells unaffected. These findings emphasize that heparanase inhibition by heparin is relevant for reducing cell binding, albeit this activity appears to be covered by other inhibitory effects of heparin.

Discussion

The enzymatic activity of heparanase, secreted by tumor cells into the microenvironment, is traditionally related to its ability to degrade matrix HSPG substrates and thus provide the cells an invasive and metastatic phenotype. Consequently, therapeutic approaches to interfere with heparanase enzymatic activity are a promising field of research in preclinical and clinical cancer studies to provide novel treatment options in the battle against cancer.[21] Notably, heparanase can also mediate pro-tumorigenic effects apparently independent of the well-established enzymatic cleavage of HSPG structures.

The enzymatic function of heparanase is controlled by the cell environment, that is, the pH, with a maximum activity at slightly acidic conditions between pH 6.2 and 6.8 found in tumor tissues.[41] At neutral and higher pH, heparanase may function primarily as an adhesion and signaling molecule.[22]

Here, we provide evidence that latent heparanase is able to activate the binding of an integrin by adding a recombinant latent form of the enzyme to melanoma cells. Integrins fulfill multiple functions in the process of tumor cell metastasis. Beside the matrix-binding characteristics and thus enabling cell migration, various interactions of the tumor cells with cellular components in the hematogenous distribution or settlement at distant sites are mediated by integrins.[1] Furthermore, matrix binding of integrins confers a "cell adhesion-mediated resistance" against chemotherapeutic treatments to tumor cells which helps to survive long periods of dormancy in term of "minimal residual disease."[42–44]

With this background, our findings refer to a dual and synergistic function of heparanase in term of "autoregulation" of tumor cell invasiveness or control of their binding properties. While active heparanase secreted by the cells of a primary tumor helps to transit the tissue by ECM degradation and remodeling, upregulated integrin function is a factor in cell migration, when leaving the slightly acidic tumor tissue. Both activities appear synergistic as a spatially and timely well-balanced process. The tight time window for integrin activation by latent heparanase found in our study indicates this sensitive regulation.

We focused our study on VLA-4, an integrin with strong impact on melanoma metastasis, which acts as a matrix-binding receptor in cell migration and also mediates cell–cell interactions. This provides an indication for the comprehensive impact

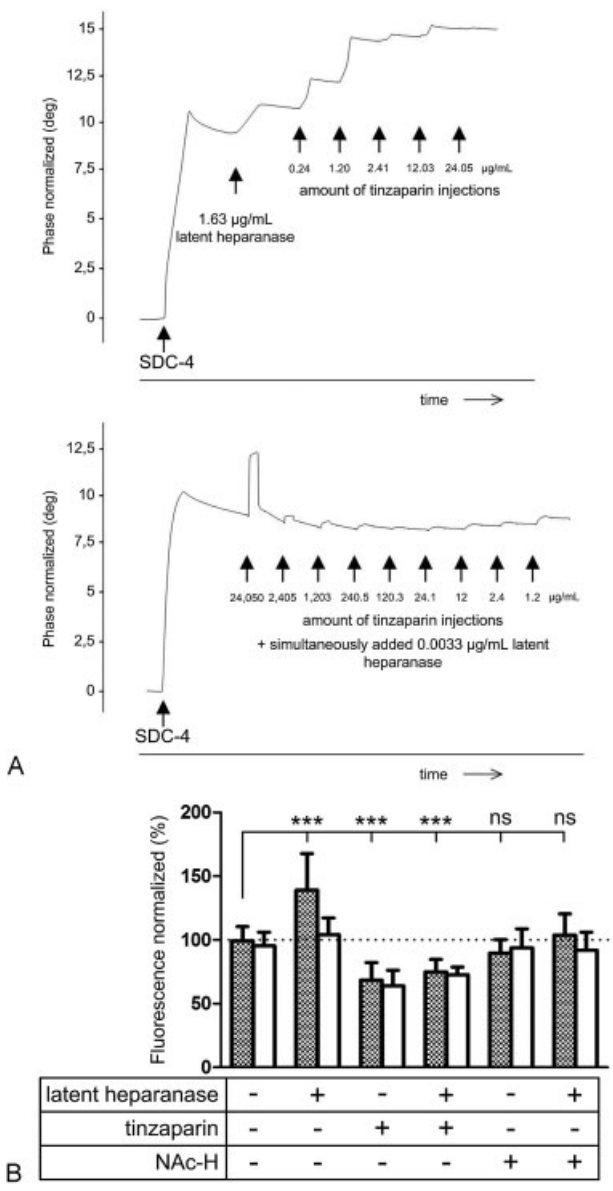

Fig. 4 LMWH interference with latent heparanase activation of VLA-4 binding. (**A**) SAW biosensor measurement of heparanase binding to immobilized SDC-4 and subsequent addition of tinzaparin (upper graph). Already bound heparanase cannot be replaced from its SDC-4 binding by tinzaparin. Contrary, tinzaparin additionally sticks to heparanase, indicated by the further increases in phase changes. Heparanase preincubated with decreasing concentrations of tinzaparin is held off from SDC-4 binding until going below a threshold level of tinzaparin (12 µg/mL) (lower graph). (**B**) Flow cytometry data on VCAM-1 binding of MV3wt and aSDC-4MV3kd cells in presence of tinzaparin or *N*-acetylated heparin derivative (NAc-H, 50 µg/mL). Tinzaparin reduced the binding of both cell types below the level of heparanase activation representing heparanase inhibition next to a competitive VLA-4 blockade. NAc-H, which is unable to block VLA-4 directly, reduces VLA-4 binding to the level of the unstimulated cells indicating the relevance of heparanase inhibition for reduced cell adhesion.

on multiple processes and relevance of these proadhesive effects of heparanase. Considering the intensity of integrin activation, our data display an increase in binding comparable to manganese ions, the common experimental approach to shift integrins into a state of maximal affinity.

The molecular mechanisms of latent heparanase in upregulating integrin activity are not fully elucidated, but the focal adhesion complex formation by restructuring the cytoskeleton, a quite general prerequisite for integrin binding function, appears as underlying principle. By using a knockdown procedure, SDC-4 could clearly be identified as key player for transducing the signals of extracellular heparanase binding to the cell to activate the focal adhesion complex formation, as schematically depicted in ►**Fig. 5**. This is in line with numerous findings on the role of SDC-4 as the preferred HSPG linked to focal adhesion complex formation.[37,45] The remarkable high binding affinity of latent heparanase to SDC-4, confirmed in our biosensor approach, underlines this outstanding role of SDC-4 and closes a gap in interpretation of former studies on heparanase-induced cell spreading.[23] However, we cannot exclude a comparable high binding affinity of heparanase to other HSPGs at the cell surface and thus further implications on signaling. Nevertheless, the much lower affinities of heparanase to VLA-4 or to VCAM-1 make, at least, a direct effect of heparanase on the adhesion receptors unlikely. The obvious independence of VLA-4 activation from PKC activity is unusual, and in contrast to earlier findings of T cell activation.[24] However, VLA-4 was reported to be able to bypass PKC by direct binding to paxillin in the process of cytoskeleton remodeling.[40]

Our findings are also remarkable in light of numerous approaches to figure out the molecular mode of action of heparin in blocking tumor cell metastasis. Several postulations and experimentally well-confirmed data exist that display adhesion receptors as targets for heparin.[6] Beside the primary focus on P- and L-selectin, VLA-4 has also been considered as a target, as VLA-4–mediated cell adhesion could efficiently be blocked by heparin, LMWH, or heparin derivatives.[27] LMWH also blocked experimental metastasis of VLA-4 positive B16F10 melanoma cells by attenuating VLA-4 binding.[7]

Here, we extend this view by postulating latent heparanase as another, not yet considered target of heparin. Although the enzymatically active heparanase attracted much attention in heparin targeting approaches,[30] binding to the latent form of the enzyme has not been figured out yet. However, the biosensor binding data confirm that latent heparanase binds LMWH, and even better to some semisynthetic heparin derivatives. The insight into the binding affinities gives reason to interpret how heparin can interfere with the proadhesive effects of heparanase. Although heparin will not be able to compete heparanase that was already bound to SDC-4, our data confirm that heparin binds the free form of latent heparanase and thus keeps it away from SDC-4 and subsequently alleviates VLA-4 activation.

In case of heparanase-positive tumor cells, the activation of integrins by secreted latent heparanase is supposed to be a permanent, but not yet considered, process. Consequently, the blockade of latent heparanase appears as an unnoticed "side" effect in the antimetastatic approaches using heparin. We confirmed here that interference with heparanase proadhesive function by heparin has functional consequences for cell adhesion. The upregulation of VLA-4 activity by

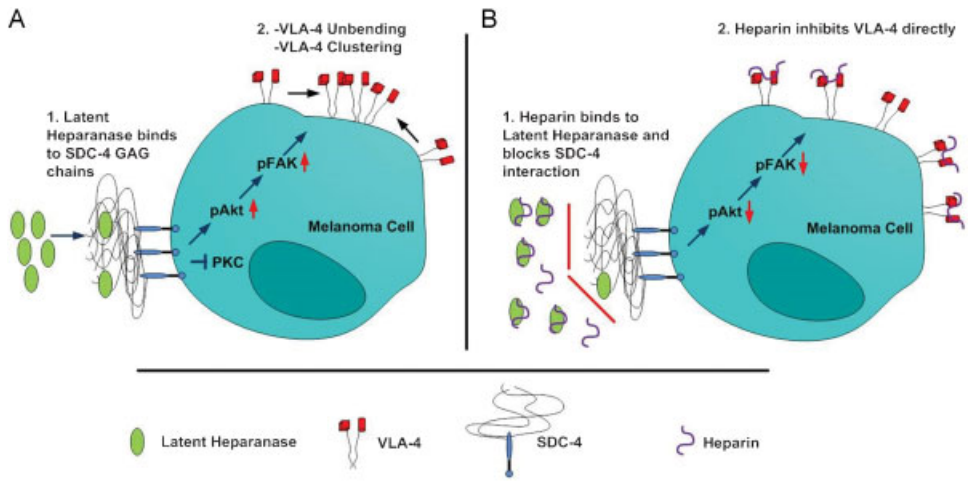

Fig. 5 Latent heparanase-mediated signaling mechanisms. (**A**) Latent heparanase binding to SDC-4 GAG chains on melanoma cells initiates an intracellular signaling involving Akt and FAK. Increased levels of phosphorylated Akt (pAkt) and FAK (pFAK) give rise to VLA-4 clustering, unbending, and finally elevated ligand binding. (**B**) Heparin inhibits VLA-4 activation by two different mechanisms. On the one hand, heparin directly binds to VLA-4 and blocks ligand binding. On the other hand, heparin interacts with latent heparanase and prevents binding to SDC-4 GAG chains. Thus, SDC-4–mediated intracellular signaling is disrupted and VLA-4 activation is alleviated.

heparanase was traced back using a heparin derivative that specifically blocked heparanase but left VLA-4 unaffected.

Finally, inhibition of VLA-4–mediated adhesion by binding latent heparanase is a novel, not yet regarded target of heparin with vital functional consequences, which should also go beyond the primary cell adhesion focus with relevance for angiogenesis, chemoresistance, and myeloid cell recruitment.

Conflict of Interest

None declared.

Acknowledgments

The authors thank Dr. Annamaria Naggi and Dr. Giangiacomo Torri, Ronzoni Institute for Biochemical Research, Milano, Italy, for providing semisynthetic heparin derivatives, and Dr. Patrick Schmitz for helpful guidance in the SDC-4 knockdown procedure.

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