Cyr61 is a target for heparin in reducing MV3 melanoma cell adhesion and migration via the integrin VLA-4

Patrick Schmitz[1]*; Ursula Gerber[1]*; Norbert Schütze[2]; Eva Jüngel[3]; Roman Blaheta[3]; Annamaria Naggi[4]; Giangiacomo Torri[4]; Gerd Bendas[1]

[1]Department of Pharmacy, Rheinische Friedrich Wilhelms University Bonn, Bonn, Germany; [2]Orthopedic Center for Musculoskeletal Research, University Würzburg, Würzburg, Germany; [3]Frankfurt University Hospital, Center of Surgery, Research Laboratory, Frankfurt am Main, Germany; [4]"G. Ronzoni" Institute for Chemical and Biochemical Research, Milano, Italy

Summary

The integrin VLA-4 is important for the metastatic dissemination of melanoma cells. We could recently show that heparin can block VLA-4 binding, which contributes, next to blocking P- and L-selectin, to the understanding of antimetastatic activities of heparin. The matricellular ligand Cyr61, secreted by numerous tumours, is responsible for increased tumourigenicity and metastasis. This has been attributed to Cyr61 binding to, and thus activating integrins. However, a VLA-4/Cyr61 axis has not yet been reported. Since Cyr61 possesses heparin binding capabilities, Cyr61 can be supposed as potential target for heparin to indirectly interfere with integrin functions. The present *in vitro* studies address (i) the existence of a Cyr61/VLA-4 axis and (ii) the functional relevance of heparin interference via Cyr61. The C-terminal module III of Cyr61 could be exposed as nanomolar affine binding site for VLA-4. A shRNA-based knockdown of Cyr61 in MV3 human melanoma cells reduced VLA-4-mediated cell binding to VCAM-1, migration on fibronectin, and integrin signalling functions significantly. Using a biosensor approach we provide insight into heparin interference with this process. The low-molecular-weight heparin tinzaparin, but not the pentasaccharide fondaparinux, binds module IV of Cyr61 with micromolar affinity. But tinzaparin cannot interfere with Cyr61 accumulation onto syndecan-4, indicating different Cyr61 binding sites for heparin and other GAGs. Nonetheless, tinzaparin affects the VLA-4 binding and signalling functions selectively via Cyr61 already at very low concentration most likely by blocking the cellular secreted free Cyr61. This study emphasises Cyr61 as promising, and hitherto not considered target for heparin to selectively influence integrin functions.

Keywords

Cyr61/CCN-1, heparin, melanoma cell adhesion, migration, VLA-4

Correspondence to:
Prof. Dr. Gerd Bendas
Department of Pharmacy
Rheinische Friedrich Wilhelms University Bonn
An der Immenburg 4, 53121 Bonn, Germany
Tel.: +49 228 735250, Fax: +49 228 734692
E-mail: gbendas@uni-bonn.de

* These authors contributed equally.

Received: February 21, 2013
Accepted after major revision: July 18, 2013
Prepublished online: September 5, 2013

doi:10.1160/TH13-02-0158
Thromb Haemost 2013; 110: 1046–1054

Introduction

Cysteine-rich protein 61 - Cyr61 (CCN-1) is one of the six members of the CCN family of matricellular proteins (1). These secreted proteins possess a non-structural role in modulating the cell-extracellular matrix (ECM) interaction and thus exert regulatory functions via a variety of molecular mechanisms in a non-redundant and cell-specific manner. Like all CCN proteins, Cyr61 is formed by four characteristic domain structures comprising an N-terminal insulin growth factor domain, a von Willebrand factor C domain, a thrombospondin-1 homology domain (TSP1) and a cysteine knot-containing C-terminal domain (2–4).

Although the specific function of Cyr61 in cancer remains controversial (3), numerous studies confirm that Cyr61 provides signals for increased tumourigenicity and cell proliferation and supports tumour cell activities in the metastatic cascade, such as migration, angiogenesis, escape of the immune surveillance and survival of anoikis (5, 6). An aberrant expression of Cyr61 has been reported in various human tumour entities, and elevated Cyr61 levels have been correlated with advanced adenocarcinoma, pancreatic cancer, and glioma (7, 8).

Many of these activities arise through a direct binding of Cyr61 after its cellular secretion to distinct subtypes of integrins. Integrins are α/β heterodimeric adhesion receptors with ubiquitary cellular expression patterns and different functions in cell/cell and cell/ECM communication and signalling.

The Cyr61–integrin connection has first been demonstrated by the binding of Cyr61 to integrin αvβ3 to mediate endothelial cell adhesion and migration (9). This interaction has also been shown to be responsible for enhanced tumourigenicity and proliferation of glioma by activating integrin-linked kinases (7) or for the regulation of breast cancer proliferation (10) mediated through the focal adhesion kinase (FAK)/PI3K/PKB signalling pathway (11). The Cyr61 interaction with further integrins, such as αvβ5 (12),

α6β1 (13, 14), αMβ2 (15) has been related to cellular adhesion, migration, chemotaxis and proliferation. The α2β1-mediated peritoneal dissemination of gastric cancer cells could be correlated with Cyr61 levels (16). A more comprehensive summary of Cyr61/integrin interactions with relevance to tumourigenicity is given as Suppl. Table 1 (available online at www.thrombosis-online.com).

Interestingly, the binding sites within the Cyr61 molecule for the diverse integrins are different (2). Concluding, the growing insight into the Cyr61/integrin axis makes Cyr61 attractive as a target for interference with various tumour activities.

However, other members of the integrin familiy with potential importance in the metastatic process of tumours have not been related to Cyr61 activities. For example, a regulation of the integrin α4β1/VLA-4 by Cyr61, to our knowledge, has not yet been reported. VLA-4 is not only a vital receptor on certain leukocytes, but is also expressed by different tumour entities. In case of melanoma cells, VLA-4 was shown to contribute dominantly to the metastatic dissemination in the body (17–19). Therefore, the attenuation of the VLA-4 binding to the endothelial ligand VCAM-1 appears promising to block metastatic dissemination of melanomas.

In this regard, we have recently demonstrated that heparin can block VLA-4 mediated melanoma cell binding activities (20). In light of the effort to explain the potential antimetastatic activities of heparin in numerous preclinical approaches (21) and selected clinical trials (22), this finding could contribute to the understanding of anti-adhesive functions of heparin in addition to the well established blocking activities on P- and L-selectin (21, 23, 24).

Interestingly, the structure of Cyr61 incloses a potential heparin binding site in the TSP1 domain, which is related to the ECM binding characteristics of Cyr61 (25). The heparin binding site, next to a further GAG binding site of Cyr61 could also contribute to Cyr61 accumulation at the cell surface after secretion by binding to HSPGs, most likely syndecan-4 (SDC-4) (14). Consequently, heparin could bind to secreted Cyr61 and interfere with the cellular accumulation and integrin activation. Although a potential blockade of Cyr61 activities by heparin has been reported (26), direct inhibitory approaches and detailed data on heparin/Cyr61 binding do not exist. However, the potential of heparin as Cyr61 inhibitor warrants further investigations in this field.

The aim of this study is to shed light on a potential VLA-4/Cyr61 axis in melanoma cell binding to evaluate, whether anti-adhesive heparin effects on this integrin are partly related to a blockade of the Cyr61 activation. This should open a view on Cyr61 as novel potential target for heparin in anti-cancer approaches.

Therefore, we selected the VLA-4 dependent metastatic human melanoma cell line MV3, created a Cyr61 knockdown clone by shRNA and compared their VLA-4 activities in cell binding and migration in *in vitro* assays with respect to heparin inhibition. We provide evidence that the VLA-4 mediated binding and migration of MV3 is controlled by Cyr61. Supplemented by biosensor-based binding affinity measurements of Cyr61 to heparin, SDC-4, and VLA-4, we explain the ability of heparin to block Cyr61 and postulate a mechanistic model. These findings justify Cyr61 as novel attractive target for heparin in term of antimetastatic approaches.

Materials and methods

Chemicals and proteins

Recombinant human (rh) VCAM-1-Fc chimera, rh VLA-4 (his-tag) and rh syndecan-4 (histag) (SDC-4) were purchased from R&D Systems (Wiesbaden, Germany). Fibronectin was from Roche Diagnostics GmbH (Mannheim, Germany), bovine serum albumin (BSA), cyanuric chloride, heparitinase I, and chondroitinase (ABC from Proteus Vulgaris) were from Sigma-Aldrich (Deisenhofen, Germany). Chloroform was obtained from Riedel-de Haen (Seelze, Germany). DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DOGS-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, USA). Tinzaparin was from Leo Pharma GmbH (Neu Isenburg, Germany). A commercial preparation from pig mucosa (Laboratori Derivati Organici, Trino Vercellese, Italy; molecular weight 18,000) was used for the formulation of the following heparin derivatives: Partially (50%) 2-O-desulfated (2O-DS-H, molecular weight about 16,000); N-acetylated heparin 100% (NAc-H, molecular weight about 16,000); as described in details recently (27). All samples were recovered after desalting by freeze-drying and characterised by NMR spectroscopy.

All salts and buffers were of analytical grade.

Production of rCyr61

SF-21 caterpillar (lepidopteran) derived cells were used to express recombinant Cyr61 as Fc-tagged protein (rCyr61) (28). The recombinant vector was mixed with linearised DNA which contains a modified Autographa californica multiple nucleopolyhedrovirus (AcMNPV) genome. SF-21 cells were transfected according to the supplier's information in six-well plates. From two rounds of transduction and amplification, aliquots were prepared and stored at -20°C for further purifications of the protein. To purify the rCyr61 protein, protein G sepharose (1 ml) columns were used. Columns were equilibrated with phosphate-buffered saline (PBS) (pH 7), the supernatant was applied (volumes between 15-45 ml) at a flow rate of 2 ml per minute (min). Columns were washed with 10-fold column volumes of PBS, and the protein was eluted with elution buffer (0.1 M glycine, pH 2.5). Thereafter eluted fractions were neutralised using 3 M Tris/HCL pH 8. The purity of the protein was checked by silver gel electrophoresis and Western blotting as described previously (28). In addition, mutated proteins were similarly purified from supernatants of SF-21 cells transduced with a construct that lacked exon 5 of the Cyr61 sequence corresponding to a recombinant protein lacking C-terminal domain IV (rCyr61 MI-MIII-Fc), or lacking domains III and IV (rCyr61 MI-MII-Fc) or domains II, III, and IV (rCyr61 MI-Fc).

Cell culture

The human melanoma cell line MV3 was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) as described recently (19). For decreasing the Cyr61 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 carried out by using FuGene®6 (Roche Applied Science), efficient DNA-lipid concentration has previously been tested by GFP-plasmid transfection. After transfection, cells stably expressing antiCyr61-shRNA were selected under permanent puromycin treatment. Cyr61 expression of MV3 cells was measured by flow cytometry (FACS Calibur, BD Heidelberg, Germany) using an antiCyr61 mAb (SantaCruz Biotechnology Inc., Heidelberg, Germany) after cell permeabilisation using Tween20 and detecting a FITC coupled antiFc antibody.

SDS-PAGE and Western blotting

For protein separation, MV3 wild-type (MV3wt), aCyr61MV3 clone cells, and clone cells of a non-target shRNA-plasmid were lysed by a commercial cell extraction buffer (Invitrogen) supplemented with phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail according to manufacturer´s instructions. Total protein yield was determined by amido black assay (29). SDS-PAGE was performed as described (30). Separated proteins were transferred to polyvinyldifluorid membranes using a tank blot system at 100 V, 350 mA for 60 min. After blocking unspecific binding sites on the membrane with a solution of 5% skim milk protein in TBS with 0.2% tween, Cyr61 was detected using antiCyr61 mAb and anti mouse-IgG-HRP (Calbiochem) as a secondary antibody. Chemiluminescence was detected after treating the membrane with Immun-Star™ WesternC™ chemiluminescence kit (BioRad) according to manufacturer´s protocol.

Flow chamber assay

To determine adhesive interactions of MV3wt and aCyr61MV3 clone cells with the ligand VCAM-1 under physiological flow conditions, glass slides were coated with 0.2 µg VCAM-1 Fc chimera and subsequently incorporated into a parallel plate flow chamber as described (31) using an inverted microscope and PBS as flow medium at a shear rate of about 200 s^{-1}.

Experiments were performed after treatment of $1x10^6$ MV3wt or clone cells suspended in FCS-free RPMI medium with 1 mM Mn^{2+} for 5 min at 37°C (stimulated cells) in comparison to unstimulated cells. To determine the contribution of exogenous recombinant Cyr61 to aCyr61MV3 clone cells, 0.25 µg to 1.75 µg rCyr61 were added to Mn^{2+} stimulation and mixed with cell suspension. For inhibition experiments either tinzaparin or 2O-DS-H (27) were applied in amounts of up to 100 µg per $1x10^{-6}$ cells in 100 µl, incubated for 5 min at 37°C with gentle shaking and subsequently injected into the flow chamber system. Cell adhesion was monitored over a period of 5 seconds (sec) while capturing a video sequence with a CSC-795 camera (Pacific Corporation, Tokyo, Japan). Videos were analysed with Imagoquant MultiTrack-AVI-2 software (Mediquant GmbH, Lützen, Germany).

Investigation of Migration by Scratch assay

Migration analyses of MV3wt and clone type cells were performed as described by Liang et al. (32). Briefly, wells were coated with fibronectin [20 µg/ml] for 1 hour (h) at 37°C. Afterwards 1.2×10^5 cells were plated on the coated wells. After reaching 80% confluence, rCyr61 was added at a final concentration of 1 or 2 µg/ml. After an incubation time of 1 h the scratch was performed with a pipette tip. Images were taken in intervals of 2 h. The distance between the cells at different time points were used to determine migratory velocities. Each experiment was repeated at least three times.

SAW Biosensor measurements

Surface acoustic wave (SAW) measurements were performed to extract kinetic constants of heparin binding to immobilised rCyr61-Fc chimera, or the binding of rCyr61 to membrane-inserted VLA-4 or syndecan-4. Analyses were performed using a S-Sens™ K5 SAW biosensor supplied by SAW Instruments GmbH, Bonn, Germany as described before (20).

Fc tagged rCyr61 protein or Cyr61 mutants were immobilised on gold-coated sensors. Sensors were preincubated in an ethanolic solution of 11-mercaptoundecanoic acid (10 mM) forming a self-assembled monolayer. The terminal carboxyl groups were activated with a mixture of 200 mM *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) to bind 18 µg/ml rCyr61 Fc-chimera protein or equimolar amounts of Cyr61 mutated Fc-chimera, respectively. Excess of free activated NHS esters were blocked by 1 M ethanolamine (pH 8.5). After immobilisation of the proteins, a baseline equilibration in PBS was performed for 15 min. The flow rate was adjusted to 40 µl/min. For binding experiments the aforementioned heparins were injected in dilution series between 10^{-10} M and 10^{-3} M and binding events were measured as phase shifts of the acoustic wave.

For determination of rCyr61-Fc binding to VLA-4 or syndecan-4, SAW sensor chips were preincubated in a chloroform solution of 10 mM hexadecanethiol. After drying the quartz, a model membrane consisting of 20 mol% DOGS-NTA and 80 mol% DPPC was formed by the Langmuir-Blodgett technique and transferred to the sensors. The membrane coated sensors were subsequently incubated in an aqueous solution of 1.66 µM trehalose and dried overnight to allow a dry insertion of the intact model membrane into the measurement device. The dried membrane was reconstituted by starting the buffer flow (40 µl/min for baseline equilibration). Afterwards, either rh His-tagged VLA-4 or syndecan-4 (25 µg/ml) were injected with a flow rate of 20 µl/min for binding of the protein polyhistidine-tag to the DOGS-NTA lipid in the membrane. Subsequently, a dilution series of rCyr61-Fc between 10^{-13} M and 10^{-8} M was injected.

Statistics

Data are represented as mean ± standard deviation of at least three independent experiments. Comparisons were performed by the unpaired Student´s t-test.

Results

Cyr61 affects the VLA-4-mediated binding functions of MV3 melanoma cells

To investigate, whether Cyr61 has an impact on the cellular binding function of the integrin VLA-4, we selected the highly metastatic human melanoma cell line MV3, for which we recently demonstrated the importance of VLA-4 binding function in experimental metastasis (19), and performed a Cyr61 knockdown approach using shRNA. The resulting aCyr61MV3 clone displayed a tremendous downregulation of this signalling protein (►Figure 1A). Although the knockdown procedure did not affect the VLA-4 and other integrin expression levels (data not shown), a clear relation of reduced Cyr61 levels to attenuated focal adhesion kinase (FAK) pathway became evident (Suppl. Figure 1, available at www.thrombosis-online.com). As a further evidence of reduced VLA-4 signalling activity via the FAK pathway by diminished Cyr61 levels, a vinculin staining of cells seeded on VCAM-1 was performed (Suppl. Figure 2, available at www.thrombosis-online.com). This clearly indicates reduced vinculin spots in aCyr61MV3 clone cells, while addition of rhCyr61 upregulated the number of spots to the level of MV3wt cells.

The consequences of alleviated FAK activity by reduced cellular levels of Cyr61 on VLA-4 binding capacities were evaluated using a dynamic flow chamber approach for detecting the cell binding to immobilised VCAM-1. The kinetically followed adhesion behaviour, which reaches a saturation level after 4 sec, demonstrates a significantly reduced cell binding of the clone cells compared to MV3wt (►Figure 1B). To confirm that the binding deficiency of the clone is related to reduced Cyr61 levels, rCyr61-Fc chimera was gradiently added to the clone cells 5 min before the adhesion experiments. It is evident that amounts greater than 1 µg/1 x 10^6 cells of rCyr61-Fc added to the clone cells tend to restore the original binding capacity of the MV3wt cells. Longer preincubation periods were not efficient.

VLA-4 also contributes to cell migration by binding to fibronectin (34, 35), which appears as important step in the metastatic cascade of tumour cells. A modified wound healing assay was performed to investigate whether the aCyr61MV3 clones versus MV3wt cells display any differences in the migratory capacities on fibronectin surfaces. The dynamic process of closing the scraped gap by migrating cells was followed for 8 h, continuously taking microscopic images, and were evaluated by creating a velocity slope of the cells. Similar to the cell binding experiments, migratory velocity of the clone cells was also significantly reduced compared to the MV3wt cells (►Figure 1C; 21.4 µm/h vs 31.7 µm/h), but an identical migratory velocity as the MV3wt cells could be reached by the clone cells already after adding 1 µg/1 x 10^6 of rCyr61-Fc (32.2 µm/h). However, higher amounts of added rCyr61-Fc (5 µg) illustrate a further functional deficiency of the clone cells.

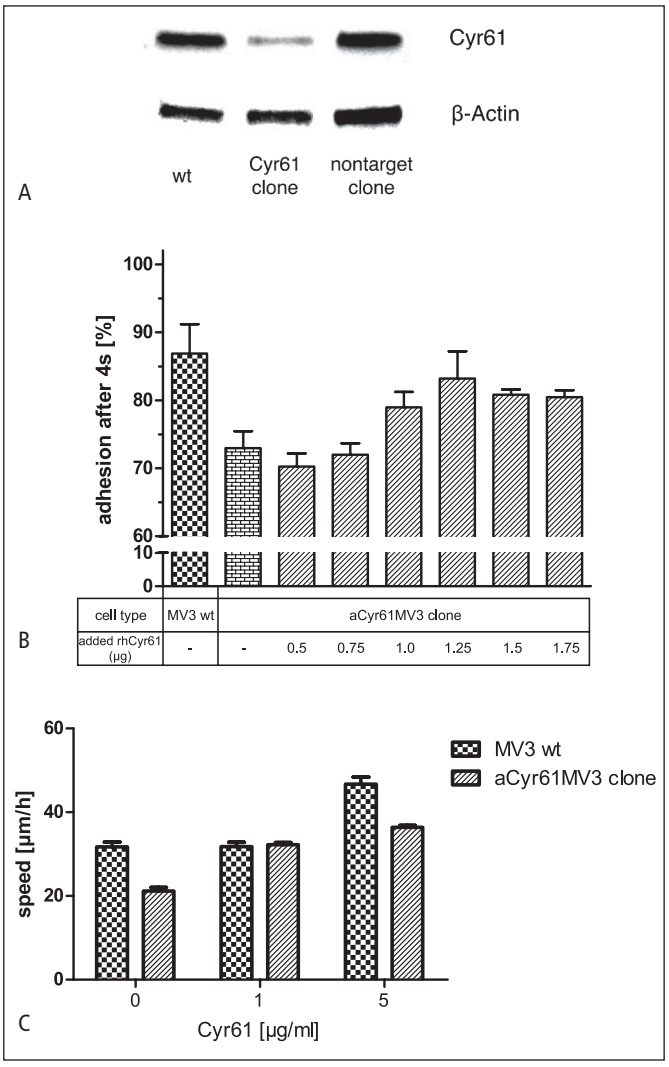

Figure 1: Functional consequences of a Cyr61 knockdown in MV3 human melanoma cells. A) Western blot protein characterisation of the aCyr61MV3 clone with respect to Cyr61 expression; left) MV3wt, middle) MV3 Cyr61 knockdown clone, right) MV3 non-target clone. B) Cyr61 stimulation of VLA-4 mediated aCyr61MV3 clone cell binding to VCAM-1 compared to MV3wt. Adhesion of 1 x 10^6 MV3 cells to immobilised VCAM-1 under flow conditions was determined after preincubation of the cells with 1 mM Mn^{2+} and/or the indicated amount of Cyr61 for 5 min. C) Comparison of MV3wt and aCyr61MV3 cell migratory velocity onto a fibronectin substrate and the effect of adding recombinant Cyr61. aCyr61MV3 cells display a significantly diminished migratory velocity compared to MV3wt cells, while adding 1 µg Cyr61 to 1 x 10^6 cells equilibrated the differences. Higher Cyr61 amounts facilitate the migration in MV3wt much more than in clone cells. Data were selected from migratory velocity slopes between 0 and 4 h of at least eight identical experiments.

Together, these data give clear indication for a Cyr61/VLA-4 activation axis with relevance to MV3 characteristics in metastatic behaviour.

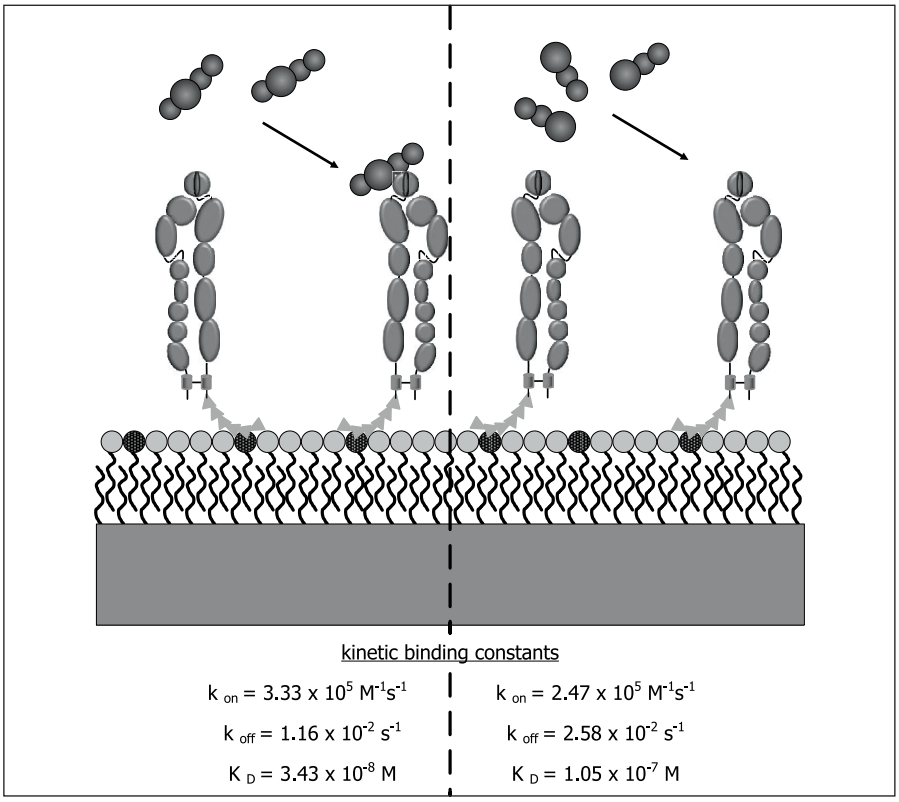

Figure 2: Binding of rCyr61-Fc or the mutated Cyr61 lacking module IV (rCyr61 MI-MIII-Fc) to VLA-4. VLA-4 was immobilised in a model membrane via histag-binding to the chelator-lipid DOGS-NTA at a SAW sensor chip followed by injection of either, rCyr61-Fc (left) or rCyr61 MI-MIII-Fc (right) for detecting the kinetic binding constants.

Insight into the Cyr61 binding to VLA-4

The Cyr61 effects for increased tumourigenicity were dominantly contributed to an activation of integrin binding and signalling functions. Although the binding properties of Cyr61 to various integrins have been evaluated showing differences in the binding domains situated within the modules II or III or IV of Cyr61, respectively (13, 36, 37), the nature and strength of Cyr61 binding to integrins is rather unknown.

To obtain a first insight into the Cyr61 binding to VLA-4 at a simulated cell surface, we applied a model membrane approach, which, in combination with a SAW biosensor device, allows for the detection of binding kinetics and affinity. Using Langmuir Blodgett technique, a model membrane was created at a SAW sensor chip, in which a site-directed membrane insertion of a His-tagged VLA-4 was achieved by binding a chelating lipid anchor (►Figure 2). It became evident that rCyr61-Fc binds to VLA-4 with an affinity in the mean nanomolar range.

For a further structural evaluation of this binding, we applied a series of mutated Cyr61 molecules gradually lacking the C-terminal modules IV; III and IV; or II, III and IV. While the lack in the cysteine-rich module IV (rCyr61 MI-MIII-Fc) has only a minor effect on binding affinity (►Figure 2), the absence of domain III prevents any binding of Cyr61. Consequently, domain III appears as the dominant binding site of the Cyr61 molecule for VLA-4.

Cyr61 as a target for heparin

Recently, we were able to demonstrate that the VLA-4 binding function of MV3 cells can efficiently be blocked by heparin. Since we show here that VLA-4 binding function is partly regulated via Cyr61, the question arises whether the heparin blockade of VLA-4 function is, at least in part, related to an interference with Cyr61. Although former findings suggest a Cyr61 binding to heparin most likely via module IV (2), a detailed analysis of heparin interaction with Cyr61 in light of inhibitory approaches has not yet been described.

We first evaluated the binding affinities of unfractionated heparin (UFH) and the LMWH tinzaparin to Cyr61 using the SAW biosensor tool. rCyr61-Fc was immobilised at the sensor surface and interaction with increasing concentrations of heparin were evaluated. While UFH binds Cyr61 with low micromolar affinity, tinzaparin has a marginally lower affinity (►Table 1). This implies a size dependency of binding, which is further supported by the finding that the pentasaccharide fondaparinux did not show any binding to Cyr61.

Unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) binding can clearly be attributed to module IV of Cyr61, since the binding affinities of both heparins towards rCyr61 MI-MIII-Fc are significantly lower, roughly by one order of magnitude. These findings support the assumption that heparin can catch Cyr61 when released by the MV3 cells to interfere with the process of integrin activation. However, details of this interfer-

ence remained open. Therefore, the following approaches aimed to illustrate the options of heparin activity.

Cyr61 is known to be accumulated at the cell surface after exocytosis by a charge induced binding to surface-bound glycosaminoglycans, most likely syndecan-4 (SDC-4) (39). Using a comparable approach as illustrated in ►Figure 2, we performed a site-directed immobilisation of SDC-4 (via His-tag) into a model membrane to evaluate (i) how Cyr61 binds to SDC-4 and (ii) whether heparin can interfere with this binding. As a prerequisite, we confirmed the chondroitinase ABC and heparitinase I sensitivity of the rhSDC-4 to assure a natural-like decoration of the protein with GAG chains.

►Figure 3 provides an insight into this situation demonstrating that rCyr61-Fc binds to SDC-4 intensively with an affinity in the low nanomolar range (7.08 ± 1.49 nM; k_{on} 3.27 ± 1.21 x 10^6 M^{-1} s^{-1}; ►Figure 3, II). Our attempts to block this binding by tinzaparin were not successful. In a first approach, we preincubated rCyr61-Fc with 1.86 x 10^{-5}M tinzaparin, but binding of rCyr61-Fc to SDC-4 appeared unaffected showing comparable binding affinity data (not shown). To check in another approach, whether rCyr61-Fc bound to SDC-4 can be replaced by increasing amounts of tinzaparin, we found no indication for an interruption of Cyr61 binding to SDC-4 by heparin (►Figure 3, III). In contrast, tinzaparin tended to bind additionally to the membrane with a comparable affinity as shown in ►Table 1 for binding onto the immobilised rCyr61-Fc (36.5 ± 5.5 µM vs 16.6 ± 3.4 µM; ►Figure 3, IV). Since we could exclude a tinzaparin binding onto the SDC-4 containing membrane in absence of Cyr61 (not shown), heparin binding must be related to an interaction with rCyr61-Fc molecules already bound to SDC-4. This implies different binding sites of Cyr61 for heparin and the GAG side chains of SDC-4.

Table 1: Kinetic binding constants of UFH, tinzaparin, and LMWH derivatives binding to immobilised rCyr61 Fc-chimera.

	K_D [µM]	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]
UFH	3.68 ± 0.60	2.18 x 10^3 ± 0.19 x 10^3	0.80 x 10^{-2} ± 0.06 x 10^{-2}
Tinzaparin	16.6 ± 3.40	0.40 x 10^3 ± 0.08 x 10^3	0.65 x 10^{-2} ± 0.15 x 10^{-2}
2O-DS-H	1.72 ± 0.35	6.60 x 10^3 ± 0.07 x 10^3	1.13 x 10^{-2} ± 0.22 x 10^{-2}
NAc-H	0.84 ± 0.10	14.2 x 10^3 ± 0.20 x 10^3	1.20 x 10^{-2} ± 0.17 x 10^{-2}

To further elucidate this, we applied the mutated Cyr61 lacking the module IV (rCyr61 MI-MIII-Fc) in the SDC-4 and heparin interference approach. The mutated Cyr61 binds with a nearly identical high affinity as rCyr61-Fc to SDC-4 (33.1 ± 6.4 nM), but an additional binding of tinzaparin was not detectable. This supports the assumption of different binding sites of GAGs and heparin on Cyr61.

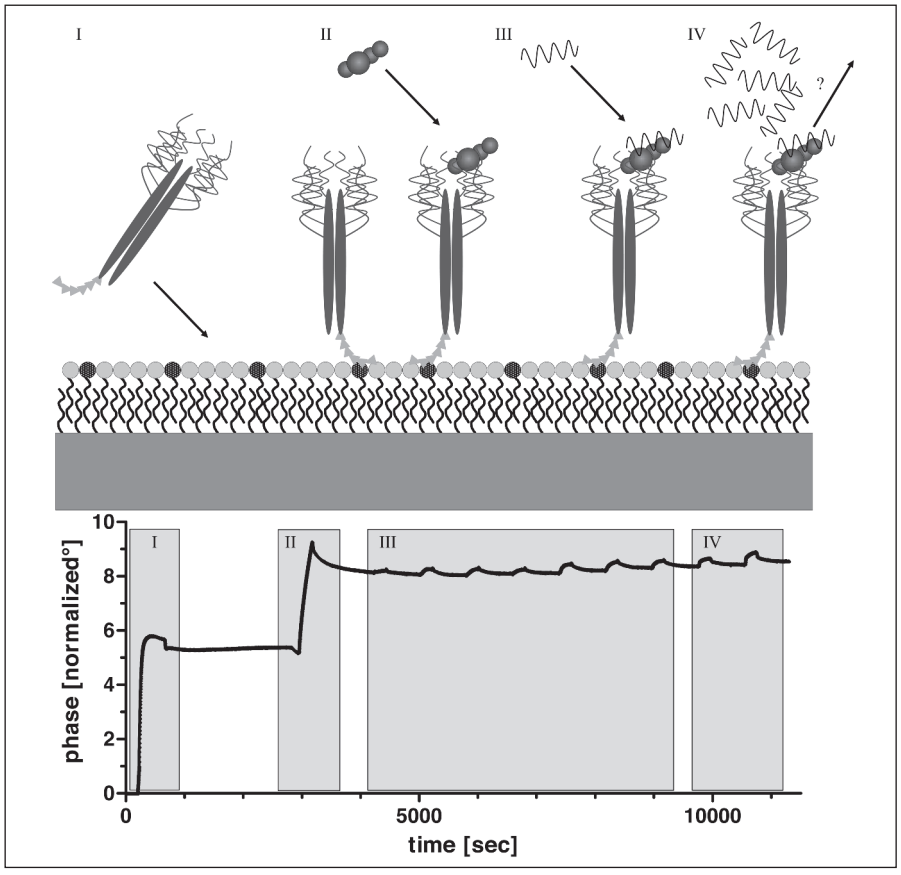

Figure 3: Simulation of Cyr61 accumulation at the cell surface and the potential interference of heparin thereof using a SAW biosensor approach. The schematic (above) and the corresponding phase shifts (below) indicate syndecan-4 immobilisation to the model membrane at a SAW sensor chip via histag binding to a membrane chelator (I), followed by detecting Cyr61 binding to syndecan-4 (II). Injecting increasing concentrations of tinzaparin (III) showed a comparable binding affinity to Cyr61 when compared to data in Table 1. Even at high tinzaparin concentrations a replacement of Cyr61 from syndecan-4 binding could not be achieved (IV).

To provide a further argument in this manner, we applied a semisynthetic heparin derivative with a 100% acetylation at the amino groups of glycosamines (27) as a model for structural convergency of heparin and heparan sulfate. This NAc-H binds rCyr61-Fc with an affinity of 0.84 ± 0.15 µM, more than 10-fold higher than tinzaparin affinity (►Table 1). One can argue that in addition to the higher MW compared to tinzaparin the higher affinity of NAc-H to Cyr61 results from a partial comprehension of the GAG binding site for interaction. Nevertheless, NAc-H was also not able to significantly replace Cyr61 from syndecan-4 binding in the above mentioned experimental assay.

Together, these data confirm a direct binding of heparin to Cyr61 with probably functional consequences for integrin activation, but heparin is not able to interfere with the Cyr61 accumulation at the cell surface. Consequently, heparin activities are restricted to free, but not to SDC-4 or VLA-4 bound Cyr61. This assumption is supported by the Western blot and vinculin staining data (Suppl. Figures 1 and 2, available online at www.thrombosis-online.com). Whereas addition of free Cyr61 induces a "re"activation of the FAK pathway in the aCyr61MV3 cell clones (Suppl. Figure 1, available online at www.thrombosis-online.com) or increases the number of vinculin spots (Suppl. Figure 2, available online at www.thrombosis-online.com), the simultaneous addition of tinzaparin and Cyr61 abolished this effect. In contrast, when adding Cyr61 and tinzaparin successively no inhibitory effect of the LMWH was detectable (Suppl. Figure 2, available online at www.thrombosis-online.com).

Functional consequences of heparin interference with Cyr61 for cell adhesion

Despite the heparin activity seems to be restricted to bind only free Cyr61, this should have consequences for VLA-4 activity. To tackle this issue we performed an initial dynamic binding experiment as shown in ►Figure 1B, but added 250 µg of tinzaparin to both, the MV3wt cells and the aCyr61MV3 clone cells. According to our previous binding studies (19), 250 µg of LMWH per 1 million cells are in an active mass range for reducing the VLA-4 mediated cell binding under these experimental conditions. Two hundred fifty µg of tinzaparin significantly reduced the binding of both cell populations to a nearly identical degree (19.4 % for MV3 cells vs 15.6 % of clone cells). One can propose that using this amount of LMHW, a potential blockade of Cyr61 has already been covered next to the direct VLA-4 receptor inhibition by tinzaparin.

To evaluate whether at lower LMWH amounts a differentiation between direct VLA-4 blockade and Cyr61 effects is possible, we performed the cell binding studies with tinzaparin amounts up to 100 µg (►Figure 4A). Although the initial significant difference in binding between clone and MV3wt cells became less pronounced and binding curves tend to approximate at higher tinzaparin amounts, a differentiation between direct receptor blockade and indirect deactivation of VLA-4 via Cyr61 depletion appears not possible using LMWH.

In a recent study (19) we underlined the structural modifications of LMWH which were not tolerated for VLA-4 inhibition. Amongst these, a desulfation at the 2O-position of uronic acids were shown not to affect VLA-4 binding. However, this 2O-DS derivative is able to bind rCyr61-Fc with an affinity of 1.72 ± 0.35 µM, comparable to UFH and 10-fold higher than tinzaparin (►Table 1). Therefore, this derivative appears suitable for a differentiation of Cyr61-related effects on VLA-4 and direct receptor blockade by heparin. Therefore, up to 100 µg of 2O-DS-H were applied in the cell binding studies (►Figure 4B). Actually, the clone cells were not affected by the 2O-DS derivative, but the MV3wt cells display an initial drop in binding up to 25 µg of heparin derivative and totally adapt the binding course of the clone cells at 50 µg. This strongly suggests that the initial significant difference is induced by the Cyr61 activity of the wt cells, which is equilibrated by the relatively low amount of about 50 µg heparin derivative.

Discussion

The heparin activity to attenuate experimental metastasis in numerous preclinical models of carcinoma and melanoma has attracted great attention during the last decade with respect to po-

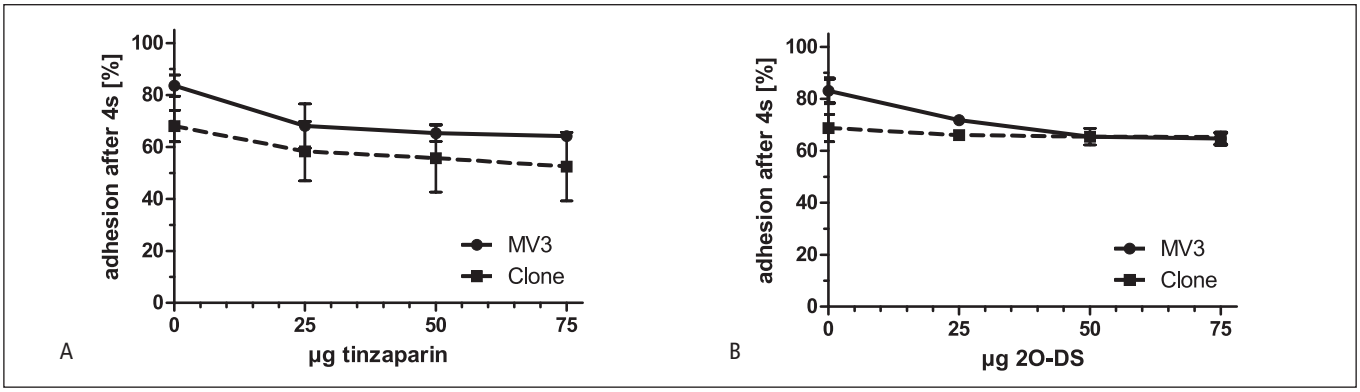

Figure 4: Inhibition of VLA-4 mediated MV3wt and aCyr61MV3 clone cell binding to VCAM-1 by heparin. Adhesion of 1 x 10^6 Mn^{2+} stimulated MV3 and aCyr61MV3 cells to immobilised VCAM-1 under flow conditions after 4 sec in presence of increasing amounts of A) the LMWH tinzaparin or B) the non VLA-4 binding heparin derivative 2O-DS-H.

tential clinical applications (21). Several molecular mechanisms of heparin, beside the antithrombotic activity have been related to the antimetastatic efficiency (40–43). Amongst these, the blockade of P- and L-selectin, and thus the attenuation of tumour cell contacts with cellular and soluble blood components has been considered as primary targets of heparin (21, 24, 40). Furthermore, the anti-adhesive effects of heparin can be extended to selected integrins (44) which has attracted much less attention in that context. For example, the integrin VLA-4, which contributes dominantly to melanoma metastasis, can also be blocked by heparin, LMWH and semisynthetic heparin derivatives (19, 33).

However, integrins possess, based on their cell- and ECM-binding function, a bidirectional cellular signalling. This has been related as a functional key for the tumourigenic effects of a diverse group of signalling molecules referred as matricellular ligands, such as Cyr61. Cyr61 affects different pathways in tumour cells, such as the Wnt-β-catenin pathway, transforming growth factor or insulin-like growth factor pathways to promote tumourigenicity (45). Although the tumourigenic effects of Cyr61 are recognised, and a consensus is given that most of the Cyr61 effects were mediated via affecting integrins, the highly complex situation is far from well understood. A detailed analysis of these signalling events is beyond the scope of this article.

Here we focus on the mechanical understanding whether Cyr61 increases the binding affinity of VLA-4 as a potential effect to increase metastatic spread of VLA-4 containing cells. For the first time we confirm a Cyr61/VLA-4 axis with clear consequences for VLA-4 binding activity, but also for integrin signalling function via the FAK pathway. This is based on a high affinity binding of Cyr61 to this integrin. Furthermore, this sheds new light on the heparin activity to inhibit VLA-4. Although we have previously confirmed that heparin can bind to VLA-4 (19) and thus directly affect the receptor activity, the blockade of Cyr61 by heparin for interfering with the integrin activation process appears to be a permanent, but not yet a considered part of the general VLA-4 inhibition.

We postulate that heparin will only be able to snatch the free Cyr61, but can not abolish the Cyr61 binding to both, SDC-4 or VLA-4. Heparin binding is clearly restricted to module IV of the Cyr61 molecule. Compared to this, the binding affinities of Cyr61 to VLA-4 and SDC-4, both independent on module IV are higher by 2 or 3 orders of magnitude, mainly resulting from 100- or 1,000-fold higher association rates.

Nonetheless, heparin affects the MV3 binding selectively via blocking the Cyr61 activity, which became evident by applying the semisynthetic heparin derivatives. The heparin amounts needed to completely block the Cyr61 activity appears relatively low. Therefore, Cyr61 blockade has unknowingly been covered in our previous inhibitory investigations (19, 20) and could also be relevant in other studies of different intentions, but potential impact of VLA-4 (40, 46–48). Furthermore, heparin used in clinical dosages for antithrombotic prophylaxis or treatment regimes should also cover a Cyr61 blockade. In addition to the findings presented here, this could have also consequences for blocking other tumourigenic effects of Cyr61, such as angiogenesis or inducing epithelial to mesenchymal transition, and thus account to the spectrum of antimetastatic modes of action of heparin.

What is known about this topic?

- The integrin VLA-4 is essential for the metastatic dissemination of melanoma cells.
- Heparin possesses antimetastatic effects, which are partly related to inhibitory effects on cell adhesive functions, including VLA-4 binding.
- The matricellular ligand Cyr61/CCN-1 exhibits various effects for increased tumourigenicity.

What does this paper add?

- The data confirm the existence of a Cyr61 / VLA-4 signalling axis.
- Cyr61 affects the VLA-4 functions in MV3 melanoma cell binding, migration, and intracellular signalling.
- Heparin interferes with Cyr61 activities in directing VLA-4 adhesive and signalling functions and thus, Cyr61 appears as a novel, not yet considered target for heparin in antimetastatic approaches.

Concluding, we identified Cyr61 as a novel target of heparin with practical consequences for reduced VLA-4 activities in melanoma adhesion and migration. Due to the relatively low amount of heparin needed for a Cyr61 blockade, this has not yet been considered and identified. Further studies should be conducted to evaluate the impact of a Cyr61/heparin interplay on other integrins in tumourigenicity.

Acknowledgements

The authors thank Kapil Juvale for critically reading the manuscript.

Conflicts of interest

None declared.

Nomenclature and Abbreviations

Cyr61 (=CCN1), Cysteine-rich protein 61; ECM, extracellular matrix; EDC, *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOGS-NTA(Ni), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt); FCS, fetal calf serum; GAG, glycosaminoglycan; KD, equilibrium dissociation constant; k_{off}, dissociation rate constant (off-rate); k_{on}, association rate constant (on-rate); LMWH, low-molecular-weight heparin; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; rh, recombinant human; SAW, surface acoustic wave; SDS, sodium-dodecylsulfate; SDC-4, syndecan-4; TBS, tris-buffered saline; UFH, unfractionated heparin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

References

1. Chen C-C, Lau LF. Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol 2009; 41: 771–783.
2. Walsh CT, Stupack D, Brown JH. G protein-coupled receptors go extracellular: RhoA integrates the integrins. Mol Interv 2008; 8: 165–173.
3. Dhar A, Ray A. The CCN family proteins in carcinogenesis. Exp Oncol 2010; 32: 2–9.
4. Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci 2006; 119: 4803–4810.
5. Sun Z-J, Wang Y, Cai Z, et al. Involvement of Cyr61 in growth, migration, and metastasis of prostate cancer cells. Br J Cancer 2008; 99: 1656–1667.
6. Tsai M-S, Bogart DF, Castañeda JM, et al. Cyr61 promotes breast tumorigenesis and cancer progression. Oncogene 2002; 21: 8178–8185.
7. Xie D, Yin D, Tong X, et al. Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/Lef signaling pathways. Cancer Res 2004; 64: 1987–1996.
8. Haque I, Mehta S, Majumder M, et al. Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis. Mol Cancer 2011; 10: 8.
9. Babic AM, Chen CC, Lau LF. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol 1999; 19: 2958–2966.
10. Menendez JA, Vellon L, Mehmi I, et al. A novel CYR61-triggered „CYR61-alphavbeta3 integrin loop" regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway. Oncogene 2005; 24: 761–779.
11. Menéndez JA, Mehmi I, Griggs DW, et al. The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives. Endocr. Relat. Cancer 2003; 10: 141–152.
12. Jandova J, Beyer TE, Meuillet EJ, Watts GS. The matrix protein CCN1/CYR61 is required for α(V) β(5) -mediated cancer cell migration. Cell Biochem Funct 2012; 30: 687–695.
13. Leu S-J, Liu Y, Chen N, et al. Identification of a novel integrin alpha 6 beta 1 binding site in the angiogenic inducer CCN1 (CYR61). J Biol Chem 2003; 278: 33801–33808.
14. Chen N, Chen CC, Lau LF. Adhesion of human skin fibroblasts to Cyr61 is mediated through integrin alpha 6beta 1 and cell surface heparan sulfate proteoglycans. J Biol Chem 2000; 275: 24953–24961.
15. Schober JM, Lau LF, Ugarova TP, et al. Identification of a novel integrin alphaMbeta2 binding site in CCN1 (CYR61), a matricellular protein expressed in healing wounds and atherosclerotic lesions. J Biol Chem 2003; 278: 25808–25815.
16. Lin B-R, Chang C-C, Chen L-R, et al. Cysteine-rich 61 (CCN1) enhances chemotactic migration, transendothelial cell migration, and intravasation by concomitantly up-regulating chemokine receptor 1 and 2. Mol Cancer Res 2007; 5: 1111–1123.
17. Okahara H, Yagita H, Miyake K, et al. 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: 3233–3236.
18. Garofalo A, Chirivi RG, Foglieni C, et al. Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastases. Cancer Res 1995; 55: 414–419.
19. Schlesinger M, Schmitz P, Zeisig R, et al. The inhibition of the integrin VLA-4 in MV3 melanoma cell binding by non-anticoagulant heparin derivatives. Thromb Res 2012; 129: 603–610.
20. Schlesinger M, Simonis D, Schmitz P, et al. Binding between heparin and the integrin VLA-4. Thromb Haemost 2009;102: 816–822.
21. Borsig L. Antimetastatic activities of heparins and modified heparins. Experimental evidence. Thromb Res 2010; 125: 66–71.
22. Akl EA, Gunukula S, Barba M, et al. Parenteral anticoagulation in patients with cancer who have no therapeutic or prophylactic indication for anticoagulation. Cochrane Database Syst Rev 2011; 1: CD006652.
23. Ludwig RJ, Boehme B, Podda M, et al. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res 2004; 64: 2743–2750.
24. Koenig A, Norgard-Sumnicht K, Linhardt R, et al. 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: 877–889.
25. Jun J-I, Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat Rev Drug Discov 2011;10: 945–963.
26. Walsh CT, Radeff-Huang J, Matteo R, et al. Thrombin receptor and RhoA mediate cell proliferation through integrins and cysteine-rich protein 61. FASEB J 2008; 22: 4011–4021.
27. Naggi A, Casu B, Perez M, et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. J Biol Chem 2005; 280: 12103–12113.
28. Schütze N, Kunzi-Rapp K, Wagemanns R, et al. Expression, purification, and functional testing of recombinant CYR61/CCN1. Protein Expr Purif 2005; 42: 219–225.
29. 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.
30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.
31. Fritzsche J, Alban S, Ludwig RJ, et al. The influence of various structural parameters of semisynthetic sulfated polysaccharides on the P-selectin inhibitory capacity. Biochem Pharmacol 2006; 72: 474–485.
32. Liang C-C, Park AY, Guan J-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007; 2: 329–333.
33. 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: 1166–1175.
34. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. Annu Rev Cell Dev Biol 2010; 26: 397–419.
35. Leiss M, Beckmann K, Girós A, et al. The role of integrin binding sites in fibronectin matrix assembly in vivo. Curr Opin Cell Biol 2008; 20: 502–507.
36. Chen N, Leu S-J, Todorovic V, et al. Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. J Biol Chem 2004; 279: 44166–44176.
37. Grzeszkiewicz TM, Kirschling DJ, Chen N, et al. CYR61 stimulates human skin fibroblast migration through Integrin alpha vbeta 5 and enhances mitogenesis through integrin alpha vbeta 3, independent of its carboxyl-terminal domain. J Biol Chem 2001; 276: 21943–21950.
38. Jedsadayanmata A, Chen CC, Kireeva ML, et al. Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue growth factor is mediated through integrin alpha(IIb)beta(3). J Biol Chem 1999; 274: 24321–24327.
39. Todorovic V, Chen C-C, Hay N, et al. The matrix protein CCN1 (CYR61) induces apoptosis in fibroblasts. J Cell Biol 2005; 171: 559–568.
40. Hostettler N, Naggi A, Torri G, et al. P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. FASEB J 2007; 21: 3562–3572.
41. Vlodavsky I, Mohsen M, Lider O, et al. Inhibition of tumor metastasis by heparanase inhibiting species of heparin. Invasion Metastasis 1994; 14: 290–302.
42. Sciumbata T, Caretto P, Pirovano P, et al. Treatment with modified heparins inhibits experimental metastasis formation and leads, in some animals, to long-term survival. Invasion Metastasis 1996; 16: 132–143.
43. Zhang C, Liu Y, Gao Y, et al. Modified heparins inhibit integrin alpha(IIb)beta(3) mediated adhesion of melanoma cells to platelets in vitro and in vivo. Int J Cancer 2009; 125: 2058–2065.
44. Bendas G, Borsig L. Cancer cell adhesion and metastasis: selectins, integrins, and the inhibitory potential of heparins. Int J Cell Biol 2012; 2012: 676–731.
45. Chong HC, Tan CK, Huang R-L, et al. Matricellular proteins: a sticky affair with cancers. J Oncol 2012; 2012: 351–389.
46. Niers TMH, Brüggemann LW, Klerk CPW, et al. Differential effects of anticoagulants on tumor development of mouse cancer cell lines B16, K1735 and CT26 in lung. Clin Exp Metastasis 2009; 26: 171–178.
47. Mousa SA, Linhardt R, Francis JL, et al. Anti-metastatic effect of a non-anticoagulant low-molecular-weight heparin versus the standard low-molecular-weight heparin, enoxaparin. Thromb Haemost 2006; 96: 816–821.
48. Ludwig RJ, Alban S, Bistrian R, et al. The ability of different forms of heparins to suppress P-selectin function in vitro correlates to their inhibitory capacity on bloodborne metastasis in vivo. Thromb Haemost 2006; 95: 535–540.

