CD44 mediates the catch-bond activated rolling of HEPG2Iso epithelial cancer cells on hyaluronan

Maximilian Hanke-Roos, Katharina Fuchs, Stojan Maleschlijski, Jonathan Sleeman, Véronique Orian-Rousseaud, and Axel Rosenhahn

aDepartment of Medicine V, University Hospital Heidelberg, Heidelberg, Germany; bAnalytical Chemistry – Biointerfaces, Ruhr-University Bochum, Bochum, Germany; cInstitute of Functional Interfaces, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany; dInstitute of Toxicology and Genetics, Karlsruhe Institute of Technology, Karlsruhe, Germany; eCentre for Biomedicine and Medical Technology Mannheim (CBTM), Universitätsmedizin Mannheim, University of Heidelberg, Mannheim, Germany

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ABSTRACT
The attachment of cancer cells to the endothelium is an essential step during metastatic dissemination. The cell surface receptor CD44 is capable of binding to hyaluronan (HA) produced by tumor cells and by cells of the tumor microenvironment, including blood endothelial cells. Here, we investigated the role of CD44 in the interaction between the liver cancer cell line HepG2Iso and HA surfaces. The rolling interaction was quantitatively analyzed using a microfluidic shear force setup. It was found that rolling of the liver cancer cells on HA depends on CD44, which mediates a catch-bond interaction and thus a flow-induced rolling of the cells. Reduction of CD44 expression by means of siRNA, inhibition of the interaction of CD44 with HA by antibody blocking, and treatment with low molecular weight HA inhibited liver cancer cell rolling on HA-coated surfaces. The results not only clearly show the dependency of the shear-induced catch-bond interaction of HepG2Iso cells on CD44 and HA, but also for the first time demonstrate CD44-mediated rolling for epithelium-derived cells that are typically adherent.

Introduction
Rolling of cells on the surface of blood vessels is a physiologically important process that can be observed for a variety of different cell types. Leukocyte extravasation from the blood stream by selectin-mediated rolling at sites of inflammation has been extensively studied.1-5 Early studies reported that increased wall shear stresses reduced the lifetime of tethers of PSLG-1-expressing cells to P-selectin-coated surfaces,6-10 a behavior consistent with a slip-bond interaction.11 Single molecule atomic force microscopy (AFM) experiments investigated this interaction in further detail and revealed that this receptor-ligand pair interacts via a catch-bond mechanism.11,12 In contrast to the more common slip-bond interaction, the catch-bond interaction is induced by tensile forces that act on the receptor. Under flow conditions this is equivalent to the requirement of a minimum shear stress for the interaction to take place. It has been shown that shear forces promote the P-, E-, or L-selectin-mediated rolling of lymphocytes.13,14

Similar to inflammatory responses, metastatic dissemination also involves the entry of tumor cells into the blood stream and their extravasation at sites of secondary tumor formation.15 After release into the blood stream, circulating tumor cells (CTC) experience a high rate of cell death due to the presence of immune cells, the high velocity of blood flow and detachment-induced cell death or anoikis.16 Only a small proportion of CTCs is able to extravasate from the blood vessels and to successfully metastasize.15,17-19 Metastatic extravasation is thought to involve steps similar to those observed for leukocytes at sites of inflammation (tethering, rolling, arrest and transmigration through the endothelium). Usually the luminal surfaces of endothelial cells are coated by a glyocalyx that contains a mixture of different glycoproteins, glycolipids and polysaccharides. The glyocalyx controls many cell-cell recognition and cell adhesion processes. Hyaluronan (HA) is not only an important component in the extracellular matrix20 but is also a major component of the endothelial cell glyocalyx of
CD44 and its binding partner HA have been reported to be involved in many different physiological processes such as lymphocyte extravasation,22,23 and rolling,23,24 homing and engraftment of haematopoietic stem cells25 and also in pathological processes including tumor progression and metastasis.26-28 Furthermore, the expression of CD44 variant isoforms in several types of cancer cells has been directly linked to tumor progression.26,27,29,30

Two CD44 variant isoforms, namely CD44v3 and CD44v6, that have been implicated in various signaling pathways20 are also of interest in normal and pathological contexts. In the apical ectodermal ridge of the developing limb bud the heparan sulfate-modified CD44v3 isoform can bind fibroblast growth factor (FGF) in order to present FGF to its receptor that is expressed in the underlying mesenchymal cells, thereby promoting limb development.31 Furthermore, CD44v3 can act as a coreceptor in breast cancer cells for heparan-binding EGF-like growth factor (HB-EGF) during activation of the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR).32 In addition, high expression of this isoform is correlated with tumor growth and metastasis.33 CD44v6 also functions as a co-receptor during ligand-induced activation of the RTKs (MET) and vascular endothelial growth factor receptor 2 (VEGFR-2). This collaboration is important for tumor progression and metastasis, and in particular in pancreatic cancer.27,34,35 Recently the expression of CD44v6 has also been connected to the metastatic potential of colorectal cancer stem cells,30 highlighting the relevance of this receptor.

We recently found that several haematopoietic cells show a flow induced (catch-bond) activation of the CD44-HA interaction. Several leukemic cell lines, as well as CD44-positive leukemic blasts, and haematopoietic progenitor cells exhibited a flow-induced, catch-bond like interaction with HA-coated substrates under physiologic flow conditions.36,37 Similar to selectin-mediated rolling,13 the interaction with the HA surface was activated by shear forces of >0.2 dyn/cm² (hence “flow-induced”). With increasing shear force, the tendency of the cells to roll on synthetic HA surfaces also increased. Once the shear force was too strong to maintain the surface contact (>1 dyn/cm²), cells detached from the interface.13,36,37

While the CD44-HA mediated rolling of cells is well documented15,22-24 a catch-bond activation of the CD44-mediated interaction of epithelial tumor cells with HA has not been demonstrated. In this study we investigated the flow-induced interaction of the CD44 positive, metastatic cancer cell line HepG2iso with HA coated surfaces under physiological flow conditions. For analysis and characterization, a custom-built microfluidic shear force setup was used.36,38 Microfluidic experiments allow dynamic investigation of the interaction of cells with their microenvironment, and have been successfully used to show that substrate viscoelasticity determines HepG2 cell migration and spreading.39 Here we applied microfluidic techniques to demonstrate that a flow-induced interaction between HepG2 and HA occurs, and explored whether the CD44 receptor is predominantly responsible for the catch-bond interaction. siRNA knock-down and antibody blocking experiments were performed to understand if specific CD44 variant isoforms are involved in the interaction. In addition, the effect of treatment with various concentrations of short chain length HA on the rolling interaction with HA was also investigated.

Results
CD44 positive metastatic HepG2iso liver carcinoma cells (Fig. 1A) were used to characterize the interaction of cancer cells with HA under shear forces that are typically present in post-capillary venules (approximately 1–4 dyn/cm²).23 and to investigate whether the binding is catch-bond activated. For the HA specific study we used spectroscopically well characterized, chemically grafted hyaluronic acid thin films. The HepG2Iso cell line was chosen as model cell line as it expresses the standard CD44 isoform (CD44s) and to a lesser extent various CD44 variant isoforms (CD44v)40 (Fig. 1E). A related liver carcinoma cell line HepG2 serves as a negative control, as it is not metastatic and does not express any CD44 isoform41 (Fig. 1F). Comparative experiments between HepG2Iso and HepG2 (Fig. 1B) showed that an interaction with the HA surface could only be observed for the CD44⁺ cell line HepG2Iso. To determine the potential of the cells for catch-bond interactions, the very low initial shear rate of approximately 0.01 dyn/cm² was stepwise increased by 2.33% every 0.5 s. Few cells interacted with the HA-coated surface at low shear rates. With increasing shear stress an increase in the fraction of CD44⁺ cells that interacted with the HA surface was observed. Of these cells, nearly all rolled over the HA substrate, as visually determined from the recorded time-lapse videos. Similar to the flow-induced interaction observed for haematopoietic cells,36,37 the fraction of rolling cells increased with increasing shear stress, reaching its maximum at approximately (0.7–1) dyn/cm² (Fig. 1B). At higher shear stress the fraction of rolling cells decreased, as the force exerted by the flow exceeded the binding strength of the cells, leading to their detachment from the surface.
The specificity of rolling of HepG2Iso cells on HA-coated surfaces was examined by a comparison with chondroitin sulfate (CS)-coated surfaces. CS is a sulphated glycosaminoglycan (GAG) consisting of D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) and is structurally very similar to HA (glucuronic acid and glucosamine). Fig. 1C shows that no interaction took place with the CS-coated substrates, demonstrating the specificity of the adhesion of HepG2Iso to HA.

To test whether the binding of CD44 to HA accounts for the rolling process, the anti-CD44 antibody BU52 that specifically blocks HA binding by CD44 was applied. Fig. 1D shows that in the presence of BU52 no rolling of the HepG2Iso cells was observed on HA. Treatment with an isotype control IgG had no significant effect on the interaction of the cells with the HA surface.

To further confirm the involvement of CD44 in the rolling of HepG2Iso cells on HA, and to determine whether CD44v3 or CD44v6 isoforms may account for this interaction, downregulation of all CD44 isoforms or just the exon v3 or exon v6-containing CD44 isoforms was achieved by means of siRNA (Fig. 2A–C). The efficiency of the knock-down was verified by western blot analysis. The membrane was probed with CD44pan, CD44v3 or CD44v6 antibodies. An Erk antibody served as loading control (Fig. 2A–C). All three siRNA approaches resulted in the repression of the targeted CD44 isoforms.

Comparison of the knock-down cells with the untreated cells revealed that while the control siRNA transfected cells showed a flow-induced rolling interaction with HA analogous to the untreated cells, the knock-down of all isoforms in HepG2Iso cells inhibited the rolling completely (Fig. 2A). In contrast, knockdown of the exon v3 or exon v6-containing CD44 isoforms had no effect on the interaction of the cells with the HA surfaces (Fig. 2B, C). Of note the HepG2Iso cells expresses several CD44 isoforms as demonstrated by run off analysis (Fig. 1D) and western blot analysis (Fig. 2E). The main CD44 isoforms detected were CD44s, just the exon v3 or exon v6-containing CD44 isoforms was achieved by means of siRNA (Fig. 2A–C). The efficiency of the knock-down was verified by western blot analysis. The membrane was probed with CD44pan, CD44v3 or CD44v6 antibodies. An Erk antibody served as loading control (Fig. 2A–C). All three siRNA approaches resulted in the repression of the targeted CD44 isoforms.

Figure 1. Rolling only occurs on HA and only for CD44+ cells. (A) Scanning electron microscopy (SEM) image of HepG2Iso cells rolling on HA. Cells were fixed with 10% paraformaldehyde (PFA) in PBS under flow at approximately 1 dyn/cm² for 10 min. (B) CD44+ HepG2iso and CD44- HepG2 cells on an HA-coated surface. Only HepG2iso cells showed a flow-induced interaction with HA (n = 4; ≥ 150 cells/FOV). The fraction of interacting cells ranged from 71-79% for the untreated HepG2iso cells and from 4-9% for the HepG2 cells. (C) HepG2iso cells exposed to HA-/CS-coated surfaces. Rolling only occurred on HA (n = 4; ≥ 130 cells/FOV). The fraction of interacting cells ranged from 72-92% for the cells interacting with HA and from 4-22% for the cells interacting with CS. (D) Exemplary curves for the suppression of the rolling interaction with HA by blocking of CD44 using BU52 (untreated: n = 9; with BU52: n = 7; with IgG1: n = 4; each treatment with > 200 cells/FOV). The fraction of interacting cells ranged from 56-72% for the untreated HepG2Iso cells, from 47-51% for the HepG2Iso cells incubated with the IgG1 control antibody and from 1-3% for the HepG2Iso cells incubated with BU52. All error bars represent the SD. (E) A runoff RT-PCR was performed to identify all CD44 isoforms expressed by HepG2iso cells. The runoff RT-PCR revealed the presence of CD44s the smallest CD44 isoform, a long variant isoforms containing the exons v4-v10, the CD44v3 variant isoform and a CD44 isoform containing the variant exons v4-v6. (F) A western blot analysis was performed to detect CD44 in HepG2iso cells and HepG2 cells. In HepG2iso cells the western blot showed a band for CD44s at 95 kD detected by the pan CD44 antibody Hermes 3 and a band for CD44v6 at 170 kD detected by a CD44v6 specific antibody (BIWA). The analysis of HepG2 cells showed that these cells do not express any CD44. The antibody against PCNA was used as loading control.
CD44v3, CD44v4-v6 and CD44v4-v10 (Fig. 1D). The v3 exon is therefore not expressed in the same isoform as the v6 exon. The CD44s isoform, which is highly abundant, was detected using a pan CD44 antibody. In addition, a high molecular weight CD44 isoform that might correspond to the CD44v4-v10 isoform was detected using an antibody against the CD44v6 peptide sequence.

To test the specificity of the binding of HepG2Iso cells to HA, we pre-treated the cells with HA or various other GAGs including chondroitin sulfate (CS), heparan sulfate (HS) and keratan sulfate (KS). Only pre-incubation with HA led to a significant decrease in the number of cells interacting with the HA surface, demonstrating the specificity of the binding (Fig. 3A, B).

In addition to high molecular weight HA, short HA oligosaccharides (sHA) of 6-10 disaccharides in length were also able to block HepG2Iso cell rolling on HA-coated surfaces in a concentration dependent manner (Fig. 3C, D). A concentration of 10 μg/mL sHA did not influence the rolling of HepG2Iso cells on HA-coated surfaces.
surfaces, whereas treatment with 30 \( \mu g/mL \) and 50 \( \mu g/mL \) sHA significantly suppressed the rolling of HepG2Iso. To exclude toxic effects of high sHA concentrations, HepG2Iso cells were incubated with different concentrations of sHA under normal cell culture conditions. Microscopy images were recorded after 1 h of incubation, which is much longer than the duration of the microfluidic assay. No morphological differences between cells incubated without any HA and with 50 \( \mu g/mL \) sHA were detected (data not shown).

Figure 3C shows that pre-treatment of the cells with different concentrations of sHA had no effect on the shear stress \( \tau_{\text{max}} \) at which the maximum fraction of cells interacted with the HA-coated surface. This raised the question of whether the pre-treatment had any effect at all on the rolling process, e.g. the rolling velocity. To quantify rolling velocities, we used computer-aided cell tracking. For the analysis, a constant wall shear stress of approximately 1 dyn/cm\(^2\) was applied, as it was demonstrated above that the CD44-HA interaction is highest under these conditions. Figure 4A shows the rolling velocities of the cells under the presence of different sHA (6-10 disaccharide units, DS) concentrations. For comparison, the \( \tau_{\text{max}} \) values obtained for different sHA concentrations are also included in the graph. The figure shows that the rolling velocity increased continuously with rising sHA (6-10 DS) concentrations in the medium. Above 50 \( \mu g/mL \), the rolling velocity did not increase any further and remained constant (Fig. 4B).

**Discussion**

Our study has revealed that metastatic HepG2Iso liver carcinoma cells are capable of undergoing a flow-induced, catch-bond activated rolling interaction with HA-coated surfaces. We could clearly demonstrate that CD44 is the essential receptor in this catch-bond process, as the CD44 positive cell line HepG2Iso could interact...
with and roll on HA-coated substrates, while the CD44 negative cell line HepG2 could not. A further indication of the importance of CD44 for the rolling of HepG2Iso cells on HA came from the siRNA experiments. Indeed downregulation of the overall CD44 expression in Hep-G2Iso by means of CD44pan siRNA led to a loss of the ability of the cells to catch-bond to HA-coated surfaces. The importance of the HA binding domain in the CD44 receptor was proven by treatment of the cells with a selective antibody (BU52), which also suppressed the flow-induced rolling interaction. While the catch bond interaction of HepG2Iso cells is reported here for the first time, our observation that the cell surface receptor CD44 mediates cell rolling on its ligand HA is in good agreement with previous literature reports.15,23 Our data showing that a shear stress above approximately 1 dyn/cm² is capable of removing rolling cells from the surface is consistent with the values reported in the literature for leukocytes and various cancer cell lines.15,23

The characteristics of the catch-bond interaction are surprisingly similar to those observed for the selectin-mediated rolling of lymphocytes.13,14 Lymphocytes are also activated at a minimum shear of roughly 0.2 dyn/cm² to initiate the rolling interaction, for example on peripheral node addressin (PNAd).13 Similar to the CD44/HA mediated rolling, a maximum is reached at approximately 0.7–1 dyn/cm², and decreases at higher shear.13,14,36,37

Although CS-binding by CD44 has previously been implicated in the rolling of lymphoma cells and Natural Killer cells on CS-coated surfaces and endothelial cells,42,43 we did not observe any binding of HepG2 cells to CS-coated surfaces. However, these previously published studies did not investigate which alternatively spliced variants of CD44 are expressed on the cells employed. Importantly, we have previously shown that CD44s does not bind to CS, whereas CD44v6/v7-containing proteins can bind to CS.44 The majority of CD44 expressed by HepG2Iso cells is CD44s, and variant-exons...
containing isoforms are expressed at much lower levels.40 Consistently, the CD44s protein and not the variant isoforms is mainly responsible for HA binding by the HepG2iso cells, as demonstrated by siRNA knockdown of the exon v3 or exon v6-containing CD44 isoforms, which had no effect on the interaction of the cells with the HA surfaces, while the knock-down of all isoforms in HepG2iso cells inhibited the rolling completely. As CD44s does not bind to CS,44 these observations explain why HepG2iso cells bind to HA-coated surfaces, but not CS-coated surfaces.

Aggregation of cancer cells in the blood stream has been discussed as a mechanism that contributes to entrapment and extravasation of CTCs at sites of metastases. These emboli are thought to get stuck in the small capillaries, then attach to the endothelium as an initial step for extravasation.45 However, given the high HA content of the luminal glycocalyx of blood vessels,21 our data suggest that CD44-mediated rolling on the HA within the luminal capillary surface could also be a relevant step in the extravasation of cancer cells. Typical wall shear stresses observed in postcapillary venules are in the range of 1-4 dyn/cm²,23 offering optimal conditions for induction of cell rolling. This suggests that extravasation is supported by suitable local hydrodynamic properties in these capillary vessels. In contrast, arteries have wall shear stresses greater than 8 dyn/cm²,23 far exceeding the optimum of 1 dyn/cm² required for cell rolling. Moreover, cells in the blood tend to accumulate in the center of large vessels, the region of highest flow velocity.46 In smaller vessels, such as the postcapillary venules, the flow rate and the corresponding wall shear stress are reduced. Furthermore, the effect of the Saffman forces is smaller due to the lower flow velocity and vessel diameter.46 Thus our results could be one explanation for the tendency of CTCs to extravasate at sites that are rich in small capillary vessels.

Treatment of cells with sHA reduced the fraction of rolling cells in a dose-dependent manner, while the rolling velocity increased. This is in agreement with previous work that showed that treatment of CD44 positive cells with an antibody against CD44 increased the rolling velocity.15 As sHA is known to compete with binding of hMW HA to CD44,47 the reduced fraction of rolling cells and the increased rolling velocities were likely caused by decreasing the numbers of CD44 receptors available for the surface interaction. The probability that the cells attach to HA in the matrix might depend on the distance between free receptors on the cell surface. Furthermore, fewer free receptors might also influence the attachment events during rolling and thus result in higher velocities of the cells. The rolling probability and velocity appear to be collective effects that require more than one receptor at a time. Macromolecular HA has been reported to stabilize complexes between CD44 and other cell surface receptors and thus facilitate rolling.48 Treatment with sHA may prevent such complexes and counteract rolling on HA.49,50

In strong contrast to the sHA dose-dependent modification of rolling velocity, the shear stress \( \tau_{\text{max}} \) at which the maximum number of interacting cells was detected was surprisingly independent of the concentration of soluble sHA. This means that while the rolling velocity changes with different degrees of blocking of CD44 by sHA, the \( \tau_{\text{max}} \) is not influenced by the fraction of available receptors. It thus seems that \( \tau_{\text{max}} \) is governed by the shear activation threshold, which is a property of each single receptor on the molecular scale.

Our observations are consistent with the notion that HA surfaces provided by the glycocalyx can facilitate binding of CD44-positive CTCs in the blood to endothelial cells. Although the HepG2 system is only a single cell line and thus generalization to other cancer cells has to be made with caution, we can speculate that increased circulating HA in the blood might saturate the CD44 receptors and thereby prevent binding of CTCs to the endothelium. Such a scenario could be pathophysiologically relevant. Notably, increased levels of HA in the circulation has been reported to suppress melanoma metastasis formation by inhibiting binding of the circulating melanoma cells to the lung endothelium.51 Furthermore, metastatic colonization is associated with sites of inflammation, and HA production by endothelial cells and surface deposition in vessels is increased by proinflammatory stimuli such as TNF-α, IL-1β and LPS, leading to increased CD44-dependent adhesive interactions,52,53 providing a mechanism through which HA-mediated arrest of CTCs may be increased at inflammatory sites.

**Material and methods**

**Cells and antibodies**

The human liver carcinoma cell lines HepG2 (ATCC/LGC Standards, Wesel, Germany) and HepG2iso were grown in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA Laboratories, Pasching, Austria). The CD44 antibody that blocks HA interactions (clone BU52) was obtained from AbD Serotec, Puchheim, the pan CD44 antibody against human CD44 Hermes 3 was a gift from S Jalkannen, Turku, Finland, the human CD44 exon v6-specific antibody VFF18 was a gift of Bender (Vienna, Austria) and the human CD44 exon v3-specific antibody was obtained from R&D Systems (Wiesbaden, Germany). The Erk antibody (K-23) was obtained from Santa Cruz (Heidelberg, Germany).
and the isotype control anti-mouse IgG1 from Sigma-Aldrich, Munich, Germany.

**Preparation of small hyaluronan oligosaccharides**

Hyaluronic acid (Healon, Abbott Medical Optics, IL, USA) for clinical application kindly provided by AMO (Ettlingen, Germany) was dissolved at 5 mg/mL in sodium phosphate buffer, pH = 5.3 and then sonicated. Small hyaluronan oligosaccharides (sHA) were generated by enzymatic digestion of HA with 200 U/mL bovine testis hyaluronidase (Sigma-Aldrich, Munich, Germany) for 6 h at 37°C. The fragments were separated on a Bio-Gel P-10 (Bio-Rad Laboratories, Inc., Hercules, California, USA) column with 115 cm length and 3.5 cm diameter overnight and 3 mL fractions were collected. To determine the HA concentration, the fractions were measured at 210 nm absorbance with reference to standards.

**FACE analysis of HA degradation**

To determine the HA fragment size, fluorophor-assisted carbohydrate electrophoresis (FACE) analysis of ANDS-labeled fragments was performed. Samples were briefly dried and resuspended in 5 mL 0.15 mol/L ANDS (in 0.15% Acetic Acid) and 5 μL 1 mol/l NaCNBH₄ (in DMSO) (both Sigma-Aldrich, Munich, Germany). The samples were incubated for 16 h, then dried again at 37°C and afterwards resuspended in 20% glycerol. The samples were then separated on a 30% polyacrylamide gel at 15 mA. UV light was used to visualize the bands in the gel. The size of the small hyaluronan oligosaccharides in each fraction was determined by comparison with similarly labeled, commercially available HA fragments of a defined size (Oligo HA₆, Sigma Aldrich, Munich, Germany).

**Exon specific RT-PCR**

Run off analysis using exon-specific PCR was performed according to König et al.³⁵ The v1 exon is not expressed in human cells due to the presence of a stop codon. Two sets of primers were used for the 2 spliced forms of exon v3 (designated CD44v3I and CD44v3II).

**Western Blot analysis**

Cells were lysed and boiled in SDS-sample buffer (125 mM TrisHCl pH 6.8; 4% SDS; 20% Glycerol; 0.01% bromophenol blue) containing 100 mM dithiothreitol (DTT) and electroblotted onto PVDF membranes. Blots were probed using the indicated antibodies. Staining was visualized using the enhanced chemiluminescence system (ECL, Thermo Fisher Scientific, Schwerte, Germany). After stripping (62.5 mM Tris, pH 6.8, 2% SDS, 0.8% DTT; 1 h; 55°C), the blot was probed with antibodies for loading controls.

**siRNA oligonucleotides and transfection**

Aliquots of cells (2 × 10⁶) were seeded in 10 cm plates 24 h before transfection. The cells were transfected with Lipofectamin 2000 (Life technologies, Carlsbad, California, USA), according to the manufacturer’s protocol. Per plate, 18 μl of Lipofectamin 2000 were diluted in 547 μl of the corresponding serum free cell culture medium and incubated for 10 min at RT. 5 nmol/l of pan CD44 siRNA (5’-CTGAAATTAGGGCCAAATT-3’; 5’-AATGGTGCAATTGTTGAAAC-3’; 5’-CAGAAACTC-CAGACCAGTT-3’; Qiagen, Hilden, Germany), CD44v3 siRNA (5’-TGAAGATGAAAGAGACAGA-3’; 5’-AGGCATTGATGATGAA-3’; Eurofins MWG GmbH, Ebersberg, Germany), CD44v6 siRNA (5’-AGTAGTACAACGGAAAGAAA-3’; 5’-GGATATCCGCAAACACC-3’; Eurofins MWG GmbH, Ebersberg, Germany) or control siRNA (5’-UAAUGUAUUUGGAAAGC AUU-3’; 5’-AGGUGUAGUUGGAAUCCCUUUUU-3’; 5’-UGC GCUAGGCUGGCUUGCU-3’; Eurofins MWG GmbH, Ebersberg, Germany) were diluted with medium in a total volume of 547 μl. The two solutions were mixed gently together and incubated for 20 min at RT. In the meantime, the cell medium was replaced with 4.5 ml of fresh serum-containing medium. The siRNA-transfection reagent mixture (1 ml) was then applied to the cells. The cells were subsequently incubated with the siRNA mixture for 48 to 72 h. Knockdown was confirmed using Western blotting.

**Detachment of the cells**

The cells were seeded at a concentration of 3 × 10⁶ in 10 cm plates and cultured for 24 h. After 24 h of starvation, the cells were washed 3 times with phosphate buffered saline (PBS). The following steps were performed on ice. The cells were harvested using 5 mM EDTA in PBS and afterwards washed 3 times with PBS. After that, the cells were counted and diluted at a concentration of 1 × 10⁶ mL⁻¹ in DMEM without FCS. The cells were incubated with 9 μg/ml of the CD44 antibody (clone BU52, AbD Serotec, Puchheim, Germany, hereafter referred to as BU52), the isotype control anti-mouse IgG1 (Sigma-Aldrich, Munich, Germany), hereafter referred to as IgG1), with 1–50 μg/ml of sHA or 50 μg/ml macromolecular glycosaminoglycans (GAGs, HA, HS, KS and CS from Sigma-Aldrich, Munich, Germany) for 30 min. The number of experiments and where appropriate the number of
cells analyzed in the experiment are indicated in the respective figure legend. The statistical significance between different treatments was evaluated with an ANOVA test with a post-hoc Tukey analysis.

**GAG surface preparation**

The respective GAG (hyaluronic acid (HA) or chondroitin sulfate (CS); both from Sigma-Aldrich, Munich, Germany) was coupled to Nexterion® glass slides (Schott AG, Mainz, Germany) according to previously published protocols. Silicon wafers ([100], p-doped with boron, CrysTec, Germany) served as reagents and conductive surfaces for spectroscopic characterization. The substrates were cleaned and activated in an O2-plasma (Pci PCCE, Diener plasma GmbH + Co. KG, Ebhausen, Germany) at 150 W power and 0.4 mbar O2 pressure for 3 min. Functionalization with 3-Aminopropyltrimethoxysilane (APTMS, Sigma-Aldrich, Munich, Germany) was achieved by ultrasonication of the glass slides immersed in a solution of 5% APTMS in dry acetone for 30 min. The surfaces were subsequently immersed in a solution of the respective GAG (1 mg/ml), N-hydroxysuccinimide (NHS, 0.01 M, Sigma-Aldrich, Munich, Germany) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 0.05 M, Sigma-Aldrich, Munich, Germany) in 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid (HEPES)-buffer (10 mM, Sigma-Aldrich, Munich, Germany). During the reaction the immersed surfaces were placed on a vibrating table (60 rpm) at room temperature (RT) and quenched after 18 h by flooding with MilliQ® water (8x volume). To remove physisorbed GAGs, the samples were washed for 3 d in daily-replenished MilliQ® water before the slides were stored in MilliQ® water for further use. After each step of the reaction, the surfaces were analyzed by static contact-angle goniometry and spectral ellipsometry to verify successful grafting.

**Microfluidic cell detachment assay**

A custom-built microfluidic setup was used to apply a defined shear stress to the cells. The DC motor-driven syringe pump created an under-pressure and aspirated the medium, generating a well-defined volumetric flow of the liquid through the microchannels (dimensions: length ~25.0 mm, width ~1.5 mm and height ~135 μm) resulting in a wall shear stress that could be calculated using Poiseuille’s model under the Purday approximation. Incubation under non-flow conditions was avoided as the interaction of the non-adherent cells was the primary interest for this study. Prior to each experiment a 10 dyn/cm² pulse (for a duration of 0.5 s) was applied to detach all cells from the channel walls. The shear stress \( \tau_n \) at each time point \( t_n \) (\( t_n = n \Delta t, \Delta t = 0.5s, n = 0, 1, 2, 3 \ldots \)) was given by \( \tau_n = \tau_i * \left( R_t \right)^{\Delta t} \), with the initial shear stress \( \tau_i = 6 \mu Q / w h^2 \approx 0.01 \text{ dyn} / \text{cm}^2 \) with the liquid volumetric flow rate \( Q \), the channel dimensions \( w \) and \( h \) and the viscosity of the liquid \( \mu \) and the rate of shear flow increase \( R_t = 1.047 \). Starting at approximately 0.01 dyn/cm², the selected \( R_t \) led to an increase of the shear stress by 2.33% every 0.5 s.

During the experiment, the center of the channel was recorded by time-lapse microscopy using an inverted TE2000 microscope (Nikon, Tokyo, Japan) with a 4x PhL objective. The number of cells \( N(\tau) \) in the field of view that were in contact with the surface was counted at 5 s intervals. \( N(\tau) \) of each experiment was then normalized to the maximum number of cells in contact with the surface measured in the appropriate control experiment (untreated cells or cells treated with control siRNA, respectively) and \( N(\tau) \) was obtained as the average of at least 3 independent experiments. The number of experiments performed and where appropriate the number of cells analyzed in the experiment are indicated in the respective figure legend.

**Abbreviations**

- AFM: atomic force microscopy
- APTMS: 3-Aminopropyltrimethoxysilane
- CD44: cluster of differentiation 44
- CD44s: standard CD44 isofrom
- CD44v: CD44variant isofroms
- CS: chondroitin sulfate
- CTC: circulating tumor cells
- DS: disaccharide units
- DTT: dithiothreitol
- EGFR: epidermal growth factor receptor
- FACE: fluorophor-assisted carbohydrate electrophoresis
- FGF: fibroblast growth factor
- FOV: field of view
- GAG: glycosaminoglycan
- GalNAc: N-acetylglalactosamine
- GlcA: D-glucuronic acid
- HA: hyaluronic acid
- HB-EGF: heparan binding EGF-like growth factor
- HEPES: 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid
- hMW: high molecular weight
- HS: heparan sulfate
- KS: keratan sulfate
- Met: mesenchymal epithelial transition factor
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NHS N-hydroxysuccinimide
PBS phosphate buffered saline
PFA paraformaldehyde
PNAd peripheral node adressin
RT room temperature
RTK receptor tyrosine kinase
SD standard deviation
SEM Scanning electron microscopy
sHA hyaluronan oligosaccharides
VEGFR-2 vascular endothelial growth factor receptor 2
physiologic flow: A novel lymphocyte-endothelial cell primary adhesion pathway. J Exp Med 1996; 183:1119-30; PMID:8642254; https://doi.org/10.1084/jem.183.3.1119

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