Supplemental Data

for

The endothelial glycocalyx anchors von Willebrand factor fibers to the vascular endothelium

Short title: Glycocalyx anchors von Willebrand factor fibers

Thejaswi Kalagara[1,2,#], Tracy Moutsis[1,#], Yi Yang[3], Karin I. Pappelbaum[1], Anne Farken[1], Lucia Cladder-Micus[2], Sabine Vidal-y-Sy[4], Axel John[5], Alexander T. Bauer[4], Bruno M. Moerschbacher[2], Stefan W. Schneider[4], Christian Gorzelanny[1,4,*]

[1] Department of Dermatology, Medical Faculty Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

[2] Institute for Biology and Biotechnology of Plants, University of Münster, Schlossplatz 8, 48143 Münster, Germany

[3] Department of Orthopedics, Tongji Hospital, Tongji University School of Medicine, 389 Xincun Road, 200065 Shanghai, China

[4] University Hospital Hamburg-Eppendorf, Department of Dermatology and Venereology, Martinistraße 52, 20246 Hamburg.

[5] Department of Urology, University of Ulm, Prittwitzstraße 43, 89075 Ulm, Germany

# contributed equally

* Corresponding author: Christian Gorzelanny
Medical Faculty Mannheim, Heidelberg University
Experimental Dermatology
Theodor-Kutzer-Ufer 1-3
68167 Mannheim, Germany
tel.: +49 621 383 6904
fax.: +49 621 383 6903
Email: christian.gorzelanny@medma.uni-heidelberg.de

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) from collagenase-digested human umbilical veins were isolated as described previously[1]. The cells were cultured at 37°C/5% CO_2 in M199 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS), 5 U/ml heparin (Biochrom, Berlin, Germany), 1% penicillin/streptomycin and 1% growth supplement derived from bovine retina[1]. HEK293 cells were maintained at 37°C/5% CO_2 in EMEM supplemented with 10% FCS. Where indicated, cells were pre-treated for 16 h with COS (2 µg/ml) or for 30 min with Heparinase-I (50 mU/ml, Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence staining

Cells and tissue slides were fixed with ice cold 4% paraformaldehyde in PBS for 20 min. Cells were washed and blocked with 2% bovine serum albumin (BSA) in incubation buffer (100 mM sodium phosphate buffer containing 4% sucrose) for 1 h at room temperature. Immunofluorescence staining was performed with primary anti-human VWF antibody (DAKO) and anti-SDC-1 antibody (R&D systems, Minneapolis, MN, USA) at a final dilution of 1:200 in incubation buffer containing 0.5% BSA overnight at 4°C. A secondary fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody (0.5 mg/ml, BD, Heidelberg, Germany) and/or Alexa555-conjugated donkey anti-goat antibody was used at a final dilution of 1:400 at room temperature for 1 h. Endothelial glycocalyx was stained with Texas Red conjugated wheat germ agglutinin (WGA; Invitrogen, Darmstadt, Germany), diluted 1000-fold in incubation buffer. Nuclei were stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) in phosphate buffered saline (PBS) for 10 min. Cover slips were embedded into Mowiol-glycol solution containing 50 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich). Fluorescence images were acquired with an Observer.Z1 (Zeiss, Jena, Germany).

Preparation of chitosan and partially acetylated chitosan oligosaccharides (COS)

Chitosan (Mahtani Chitosan Pvt. Ltd, India) with a degree of acetylation (DA) of 27% and a degree of polymerization (DP) of 818 was prepared and characterized as previously reported[2-4]. Partially acetylated chitosan oligosaccharides (COS) were prepared from chitosan by enzymatic hydrolysis using human recombinant chitotriosidase at 37°C in 150 mM

ammonium acetate buffer[5]. COS with a DP between 2 and 12 were separated by size exclusion chromatography on three HiLoadTM 26/60 SuperdexTM 30 prep-grade columns. Collected fractions were analyzed by MALDI-TOF mass spectrometry using a UV laser and 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Cell transfection

HEK293 cells were transfected with vector pDsRed-N1 (Addgene plasmid #54493) containing the full-length human SDC-1 gene cloned from a HUVEC cDNA library. Stably transfected cells were obtained by selection with G418 (500 µg/ml).

The lentiviral vector pLentiADR3/IRP-SDC-1 was produced by subcloning the SDC-1 gene from pDsRed-SDC-1. Viruses were produced as previously reported.[6] Knockdown of Ext1 was conducted with shRNA (pGFP-C-shLenti, Origene, Rockville, USA). Two different shRNAs (shExt1-A: CAGACACCAGGAATGCCTTATATCACGTC; shExt1-B: GAGCGGAGAGTTTGAAGTCTTTACAGGCG) were tested. Vector shExt1-A showed the strongest silencing activity (shExt1-A: 59.6 ± 4.9%; shExt1-B: 50 ± 4.0% by qRT-PCR) and was therefore used for subsequent experiments. Transduced HUVECs were selected for 2 d with 0.5 µg/ml puromycin.

Enzyme linked immunosorbent assay (ELISA)

VWF ELISA was performed as previously described[7]. The release of VWF from HUVECs was induced by stimulation with 50 µM histamine for 15 min. Cell supernatants were collected and added for 1 h at 37°C to a 96-well plate pre-coated with polyclonal rabbit anti-human VWF antibody (DAKO, Hamburg, Germany). Immobilized VWF was detected with a polyclonal rabbit anti-human VWF antibody conjugated to horseradish peroxidase (HRP). The HRP substrate (ABTS, Roche Diagnostics, Mannheim, Germany) was incubated for 0.5 h and the absorption was measured at 405 nm. Serially diluted human plasma was used to prepare a standard curve.

Quantitative real time PCR (qRT-PCR)

Total RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany) and qRT-PCR was performed as previously reported using the Reverse Transcription System and the GoTaq® qPCR Master Mix (Promega, Heidelberg, Germany)[8]. The following primer pairs were used: Ext1: 5ô-GAG ACA ATG ATG GGA CAG ACT TC-3ó and 5ô-CTC TGT CGC

TGG GCA AAG-3'; SDC-1: 5'-GCT CAC ACA CCT GTA GCA CT-3' and 5'-TGC TGT CTC CCG ACC ATA GA-3'; SDC-2: 5'-TCG GCG GAG TCG AGA GC-3' and 5'-CGT CGT GGT TCC CAC TTT TGG-3'; SDC-3: 5'-AAC TTC GAG AGA CCC GTG GA-3' and 5'-GCT CTT CCG GGA CTT CTG TC-3'; SDC-4: 5'-CCC CAA GAG AAT CTC ACC CG-3' and 5'-ATT GGT GGG GGC TTT CTT GT-3'. Expression levels were normalized to the endogenous β-actin gene (5'-AGA AAA TCT GGC ACC ACA CC-3' and 5'-CCA TCT CTT GCT CGA AGT CC-3').

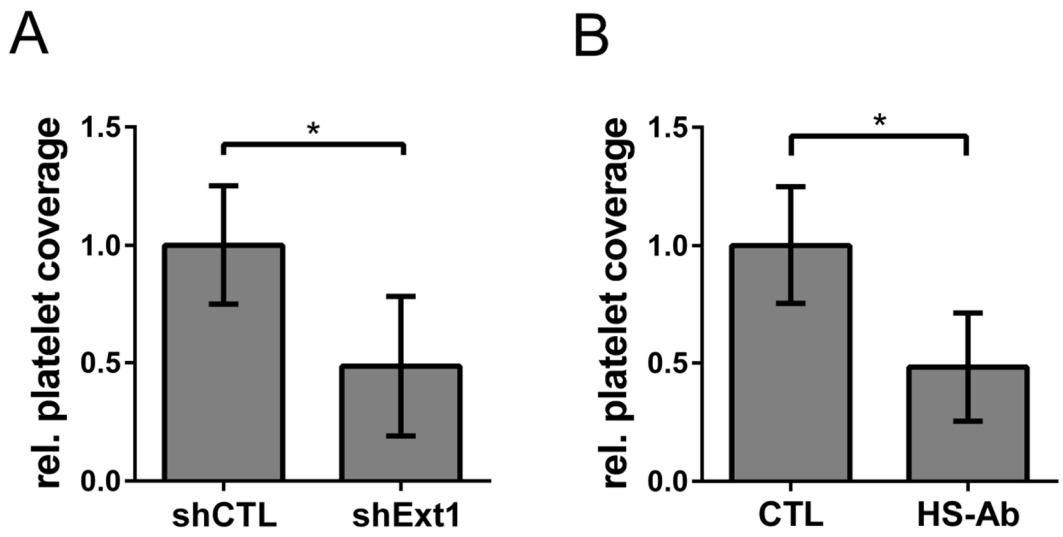
Preparation of platelets

Platelets were prepared as previously described[9]. Blood was drawn into sodium citrate-coated tubes (Sarstedt, Nuembrecht, Germany) and centrifuged at 120 x g for 15 min at room temperature to obtain platelet-rich plasma (PRP). PRP was supplemented with apyrase (75 microunits/ml; Sigma-Aldrich) and prostaglandin E1 (100 nM; Sigma-Aldrich) and centrifuged at 1200 x g for 15 min. The platelet pellet was resuspended in wash buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 103 mM NaCl, 2 mM $CaCl_2$, 75 microunits/ml apyrase, 100 nM PGE2, 3.5 mg/ml BSA, pH 6.5) containing 5 µM calcein red-orange (Invitrogen) and incubated for 30 min in the dark at room temperature. Subsequently, the platelets were centrifuged for 12 min at 1200 x g and washed twice with wash buffer. Finally, the pellet was resuspended in HEPES/Tyrode's buffer (137 mM NaCl, 2 mM KCl, 3 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM HEPES, 12 mM $NaHCO_3$, pH 7.4).

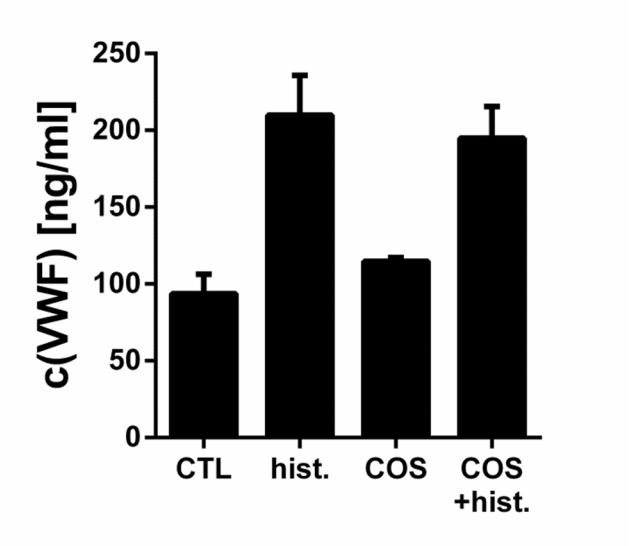
1. Desch A, Strozyk EA, Bauer AT, et al. Highly invasive melanoma cells activate the vascular endothelium via an MMP-2/integrin alphavbeta5-induced secretion of VEGF-A. *Am J Pathol*. 2012;181(2):693-705.
2. Vachoud L, Zydowicz N, Domard A. Formation and characterisation of a physical chitin gel. *Carbohydrate Research*. 1997;302(3-4):169-177.
3. Tian F, Liu Y, Hu K, Zhao BY. The depolymerization mechanism of chitosan by hydrogen peroxide. *Journal of Materials Science*. 2003;38(23):4709-4712.
4. Hirai A, Odani H, Nakajima A. Determination of Degree of Deacetylation of Chitosan by H-1-Nmr Spectroscopy. *Polymer Bulletin*. 1991;26(1):87-94.
5. Gorzelanny C, Pöppelmann B, Pappelbaum K, Moerschbacher BM, Schneider SW. Human macrophage activation triggered by chitotriosidase-mediated chitin and chitosan degradation. *Biomaterials*. 2010;31(33):8556–8563.
6. Evdokimov K, Biswas S, Schledzewski K, et al. Leda-1/Pianp is targeted to the basolateral plasma membrane by a distinct intracellular juxtamembrane region and modulates barrier properties and E-Cadherin processing. *Biochemical and Biophysical Research Communications*. 2016;475(4):342-349.
7. Gorzelanny C, Kmeth R, Obermeier A, et al. Silver nanoparticle-enriched diamond-like carbon implant modification as a mammalian cell compatible surface with antimicrobial properties. *Sci Rep*. 2016;6:22849.

8. Yang Y, Gorzelanny C, Bauer AT, et al. Nuclear heparanase-1 activity suppresses melanoma progression via its DNA-binding affinity. *Oncogene*. 2015;34(47):5832-5842.
9. De Ceunynck K, Rocha S, Feys HB, et al. Local elongation of endothelial cell-anchored von Willebrand factor strings precedes ADAMTS13 protein-mediated proteolysis. *J Biol Chem*. 2011;286(42):36361-36367.

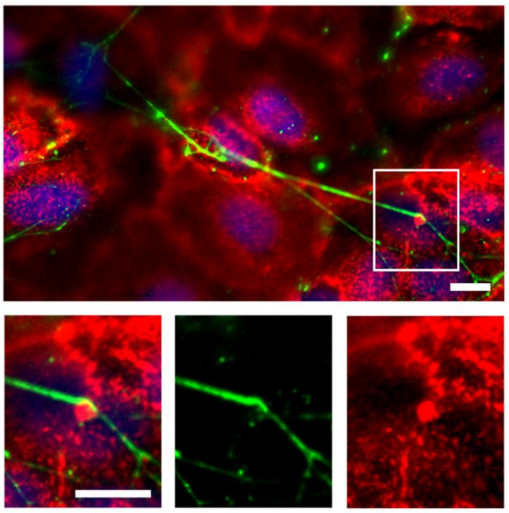
Figures



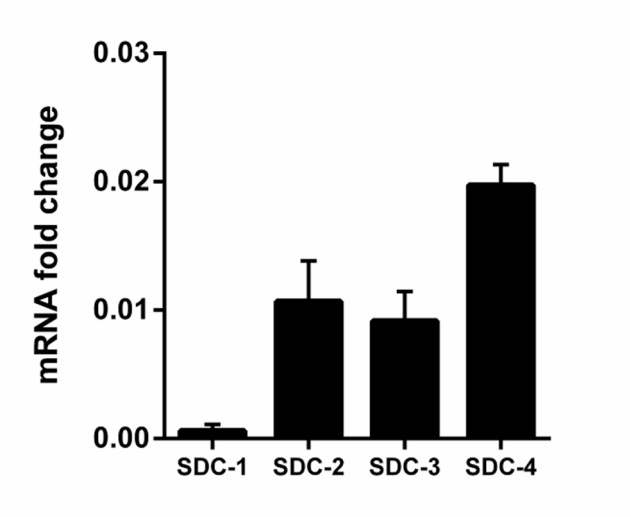
Supplemental Figure 1: HS dependent anchorage of VWF-platelet strings at the endothelial surface. A Knockdown of Ext-1 (shExt1) in HUVECs reduces VWF-mediated platelet binding in microfluidic experiments. HUVECs transduced with control shRNA vectors (shCTL) were used as control. **B** Reduced VWF-mediated platelet binding after blockage of HS by a specific antibody (HS-Ab; clone MAB2040, Merck-Millipore, Darmstadt, Germany). Mouse isotype IgG1 treated HUVECs were used as control (CTL). Release of VWF from the endothelial cells was induced with 50 µM histamine. n=4, mean ± SD, * P Ò0.05 (Studentôs t-test)



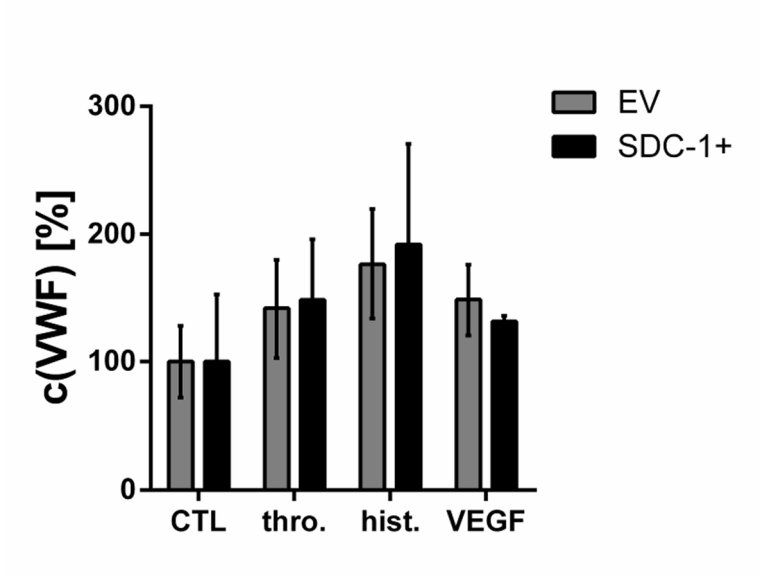
Supplemental Figure 2: Measurement of VWF in the supernatant of HUVECs. VWF release was induced by stimulation with 50 µM histamine (hist.) and measured by ELISA. To test whether COS treatment affects the amount of releasable VWF, HUVECs were pre-treated with COS prior to the addition of histamine. n=3, mean ± SD.



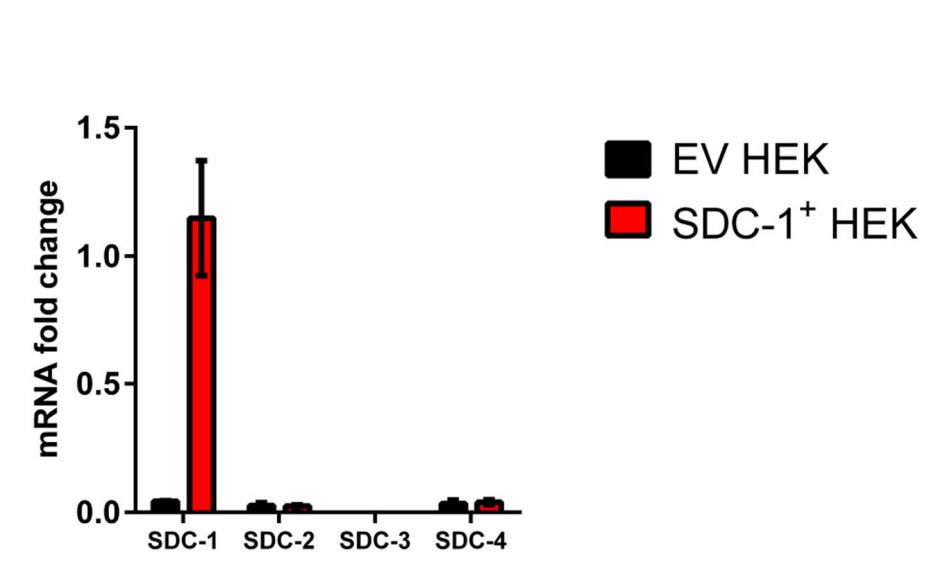
Supplemental Figure 3: Anchorage of VWF fibers at the surface of HUVECs. VWF was stained in green; the endothelial glycocalyx was stained with Texas-red conjugated wheat germ agglutinin in red. Nuclei were stained in blue. Scale bars correspond to 10 µm.



Supplemental Figure 4: Expression of SDCs in HUVECs. The expression levels of SDC-1, -2, -3 and -4 were measured by qRT-PCR. Data were normalized to the expression of ßï actin.



Supplemental Figure 5: Release of VWF from viral transduced HUVECs. VWF ELISA was used to control whether the over-expression of SDC-1 affects the releasable amount of VWF. HUVECs transduced with the empty vector (EV) or with SDC-1 vector (SDC-1^{+}) were stimulated with thrombin (thro.), histamine (hist.) or VEGF-A.



Supplemental Figure 6: Expression of SDCs in HEK293 cells. The expression levels of SDC-1, -2, -3 and -4 were measured by qRT-PCR in empty vector transfected cells (EV HEK) and cells overexpressing SCD-1 (SDC-1^{+} HEK). Data were normalized to the expression of ϐï actin.

Videos

Supplemental Movie 1: Binding of platelets to endothelial-released VWF. HUVECs were perfused at a constant shear stress of 6 dyne/cm².VWF release was induced by 50µM histamine. Platelets are shown in white, VWF fibers were stained in green using a FITC-conjugated antibody. The representative movie shows a sequence of 20 consecutive images recorded at a rate of 0.2 frames per second.

Supplemental Movie 2: Platelets trapped by VWF fibers in close proximity to the endothelial glycocalyx. HUVECs were perfused at a constant shear stress of 6 dyne/cm².VWF release was induced by 50µM histamine. Platelets are stained with CellTrace blue (false color coded in green), VWF fibers were stained with an FITC-conjugated antibody (false color coded in white). The endothelial glycocalyx was labeled with Texas Red-conjugated WGA in red. The representative movie shows a sequence of 20 consecutive images recorded at a rate of 0.2 frames per second.