von Willebrand Factor Directly Interacts With DNA From Neutrophil Extracellular Traps

Sandra Grässle, Volker Huck, Karin I. Pappelbaum, Christian Gorzelanny, Camilo Aponte-Santamaria, Carsten Baldauf, Frauke Gräter, Reinhard Schnepfenreich, Tobias Obser, Stefan W. Schneider

Objective—Inflammatory conditions provoke essential processes in the human vascular system. It leads to the formation of ultralarge von Willebrand factor (VWF) fibers, which are immobilized on the endothelial cell surface and transform to highly adhesive strings under shear conditions. Furthermore, leukocytes release a meshwork of DNA (neutrophil extracellular traps) during the process of the recently discovered cell death program NETosis. In the present study, we characterized the interaction between VWF and DNA and possible binding sites to underline the role of VWF in thrombosis and inflammation besides its function in platelet adhesion.

Approach and Results—Both functionalized surfaces and intact cell layers of human umbilical vein endothelial cells were perfused with isolated, protein-free DNA or leukocytes from whole blood at distinct shear rates. DNA–VWF interaction was monitored using fluorescence microscopy, ELISA-based assays, molecular dynamics simulations, and electrostatic potential calculations. Isolated DNA, as well as DNA released by stimulated leukocytes, was able to bind to shear-activated, but not inactivated, VWF. However, DNA–VWF binding does not alter VWF degradation by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13. Moreover, DNA–VWF interaction can be blocked using unfractionated and low-molecular-weight heparin, and DNA–VWF complexes attenuate platelet binding to VWF. These findings were supported using molecular dynamics simulations and electrostatic calculations of the A1- and A2-domains.

Conclusions—Our findings suggest that VWF directly binds and immobilizes extracellular DNA released from leukocytes. Therefore, we hypothesize that VWF might act as a linker for leukocyte adhesion to endothelial cells, supporting leukocyte extravasation and inflammation. (Arterioscler Thromb Vasc Biol. 2014;34:1382-1389.)

Key Words: endothelium ■ heparin ■ inflammation ■ leukocytes ■ von Willebrand factor

Inflammation causes a release of von Willebrand factor (VWF), an adhesive glycoprotein stored in endothelial cells and platelets. It was shown that luminal-secreted VWF from Weibel–Palade bodies forms ultralarge fibers, which are immobilized on the endothelial cell surface. In this context, shear flow mediates VWF uncoiling, which transforms VWF to a highly adhesive protein by exposing the A1- and A2-domains, which features specific binding sites for heparin, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), and glycoprotein Ibα (GP1bα). The latter is involved in VWF–platelet interaction. Platelet adhesion to VWF has been shown to play an essential role in primary hemostasis and the formation of deep vein thrombosis in mouse models. Also, heparin binds to parts of the A1-domain. Besides naturally occurring unfractionated heparin, there are different kinds of low-molecular-weight heparins on the market, which differ in molecular properties and in their mode of action. The length of these heparins varies significantly. For example, unfractionated heparin has a molecular weight of 15 kDa, whereas the smallest low-molecular-weight heparin, Fondaparinux, contains only 5 sugar residues. However, all heparins are highly negatively charged.

Besides the aforementioned VWF release, inflammatory conditions provoke the generation of extracellular DNA traps, which have recently been discovered as large DNA fibers generated by leukocytes, especially neutrophils (neutrophil extracellular traps, NETs). The recently described mechanism, called NETosis, which is distinct from apoptosis or necrosis, implies that leukocytes are able to release a meshwork of chromosomal DNA, which includes histones and granular antimicrobial...
proteins, such as myeloperoxidase or neutrophil elastase, to trap and kill microorganisms. Extracellular DNA traps have been linked to several diseases, including sepsis, small-vessel vasculitis, or preeclampsia. Other studies showed that both extracellular DNA traps and VWF are significantly involved in deep vein thrombosis. It has been demonstrated that there is a correlation between levels of circulating nucleosomes, activated neutrophils, and the occurrence of deep vein thrombosis. Also, immunostained sections of venous thrombi from animal models indicate a connection of inflammation and thrombosis by the fact that both NETs and VWF seem in close proximity to each other. This association of both molecules was linked to the capability of VWF to bind histones.

Here we show that VWF and pure, isolated DNA from human whole blood are able to interact in a shear-dependent manner, but independently of histones or other proteins. We examined the interaction of ultralarge VWF and extracellular DNA traps under flow conditions and analyzed the characteristics of the binding process by applying an immunobased binding assay and immunostaining. We hypothesize that VWF, by binding DNA, might act as a linker for leukocyte adhesion to the endothelium and thereby underline the role of VWF in inflammation, besides its function in platelet adhesion.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

DNA Binds to VWF Under Shear Conditions

To investigate whether VWF can bind to isolated DNA molecules, we studied the binding of DNA to physiologically released ultralarge VWF fibers on a histamine-stimulated, confluent endothelial cell layer under shear flow conditions of 2 to 20 dyne/cm². Additionally, we applied a microfluidic channel system using VWF functionalized surfaces, which were perfused with pure, isolated DNA from whole blood. Protein contamination of isolated DNA was excluded by

<table>
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<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>ADAMTS13: a disintegrin and metallocproteinase with a thrombospondin type 1 motif, member 13</td>
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<td>NET: neutrophil extracellular trap</td>
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<td>VWF: von Willebrand factor</td>
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Figure 1. DNA binds to von Willebrand factor (VWF) under shear conditions. A. Protein-free DNA was perfused at 10 dyne/cm² over a histamine-stimulated, intact endothelial cell layer (thereby releasing VWF fibers) and labeled with Sytox Green (visualized in red). DNA colocalized with released VWF strings (arrowheads). Also, nuclei of human umbilical vein endothelial cells were stained during the fixation process because of excessive dye in the supernatant. Green indicates VWF; and red, DNA. Scale bar, 10 μm. B. Isolated DNA was perfused over a functionalized VWF surface at 2 dyne/cm². 4',6-Diamidino-2-phenylindole–labeled DNA (visualized in red) binds to VWF fibers only (green), but not globular, nonactivated VWF, under flow conditions. Scale bar, 50 μm. C. Binding of DNA to VWF is Ristocetin-dependent: increasing DNA concentrations (0–5 μg/mL) were incubated with 1 μg/mL VWF in the presence (solid line) or absence (dotted line) of 1.5 mg/mL Ristocetin. The binding of DNA to VWF was assessed by fluorescence intensity measurements using Sytox Green labeling. Data are expressed as mean±SD (*P<0.05; n=6).
absorbance measurements of 260/280 nm ratio, Bradford assay, and gel electrophoresis. In both cases, immunofluorescence staining showed a clear colocalization of perfused DNA with either cellular-released VWF fibers or surface-coated VWF (Figure 1A and 1B; Movie 1 in the online-only Data Supplement). DNA did not interact with BSA-coated surfaces or globular, nonactivated VWF (data not shown). No difference in binding could be observed at different levels of shear stress between 2 and 20 dyne/cm², so we assumed that already 2 dyne/cm² is sufficient to promote DNA–VWF interaction (data not shown). These results indicate that DNA is able to bind directly to VWF under shear conditions without the involvement of other proteins.

To characterize the binding of DNA to VWF in more detail, we performed an immunobased quantitative assay with and without Ristocetin, an antibiotic thought to expose the binding sites of the A1-domain of VWF in the absence of high shear forces. Both DNA and VWF molecules were incubated in increasing DNA/VWF ratios in the absence or presence of Ristocetin at different concentrations, and DNA binding was examined. DNA binding to VWF was only detectable when both molecules were coincubated with 1.5 mg/mL Ristocetin (Figure 1C) and decreased with descending Ristocetin concentrations (Figure I in the online-only Data Supplement). These data are in line with our abovementioned data and demonstrate that DNA binding to VWF is dependent on an exposed VWF A1-domain and occurs only on activated, but not on globular, VWF.

DNA Binding to VWF Is Blocked by Heparin

Because of our previous results, we hypothesized that the VWF A1-domain is mainly involved in the binding process of DNA to VWF. To delineate the contribution of heparin-binding sites in the A1-domain, we assessed whether DNA binding to VWF can be blocked using unfractionated heparin. Therefore, plasmatic VWF was immobilized on a microfluidic
channel and was preincubated with 50 U/mL unfractionated heparin before perfusion with isolated DNA. Indeed, immunofluorescence staining confirmed that the binding of DNA to VWF was completely abolished compared with a control channel (Figure 3A and 3B). Because the DNA was proven to be protein-free, the involvement of histones could be excluded. DNA that was once immobilized on a VWF surface could be removed with DNAse I, but not with heparin (Figure IIIA and IIIB in the online-only Data Supplement), indicating that the binding occurs with a high affinity. This result could also be accomplished in an ELISA-based binding assay (Figure IIIC in the online-only Data Supplement).

In addition, we analyzed whether autogenic DNA of Staphylococcus aureus– or phorbol 12-myristate 13-acetate (PMA)–stimulated neutrophils from whole blood can attach to a VWF functionalized surface under flow conditions, and whether this interaction can be blocked by heparin. For this experiment, neutrophils were stimulated to release NETs and afterward perfused over a microfluidic channel. In the control channel, both S. aureus– (data not shown) and PMA-treated neutrophils released a massive network of DNA fibers, which were captured by the VWF surface. Thereby, the DNA meshwork and several neutrophilic cells were immobilized on the surface, and secondary neutrophils were trapped (Figure 3C). Perfusion of 100 U/mL DNAse I over the formed DNA network completely dismantled all DNA fibers and removed all attaching neutrophils (Movie II in the online-only Data Supplement). However, if the VWF-coated surface was blocked with 50 U/mL heparin beforehand, neutrophils that released their DNA could not attach to the surface, and leukocyte adhesion to VWF was almost completely impaired (Figure 3D). Because, for this experiment, it was an autogenic release of NETs from neutrophils, an involvement of histones cannot be excluded in this case. Nevertheless, this shows that heparin is able to block the DNA and leukocyte binding to VWF, indicating the same binding site of heparin and DNA within the A1-domain.

**Inhibition Efficiency of DNA–VWF Binding Varies for Different Heparins**

Using the abovementioned immunobased assay together with Ristocetin, we applied different commercially available heparins to block DNA binding to VWF in vitro. Unfractionated heparin almost completely inhibited DNA–VWF interaction, whereas low-molecular-weight heparins such as Tinzaparin (6.5 kDa on average) or Fondaparinux (a pentasaccharide)
were less effective (Figure 4A and 4B). Preincubation of VWF with Tinzaparin significantly reduced the binding of DNA, whereas Fondaparinux, the smallest heparin available, kept DNA–VWF binding unchanged. After partial digestion of unfractionated heparin using heparinase I, the DNA–VWF interaction could be restored (Figure 4C and 4D). These results indicate that an inhibition of DNA–VWF binding depends on the length of negatively charged molecules. To further confirm the charge-dependent interaction of negative DNA with the positive A1-domain, we neutralized the negative charge of the DNA by polycationic chitosan. It is well known that chitosans are able to interact with polyanions such as DNA. Indeed, chitosan-mediated blockage of negative charges on DNA inhibits its binding to VWF in a concentration-dependent manner (Figure V in the online-only Data Supplement), which strengthens our hypothesis that DNA binding to VWF is primarily charge-based.

**VWF A1-Domain Is a Potential Binding Site for DNA**

Experiments suggest a competitive binding between GP1bα, heparin, and DNA to VWF. Because binding sites of GP1bα

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**Figure 4.** Binding of DNA to von Willebrand factor (VWF) is blocked by low-molecular-weight heparins. A, Increasing concentrations (0–5 μg/mL) of DNA were incubated with 1 μg/mL VWF, which was preincubated with 50 U/mL unfractionated heparin (UFH), 50 U/mL Tinzaparin, or 29 μg/mL Fondaparinux (both low-molecular-weight heparins). Binding of DNA to VWF was assessed by Sytox Green fluorescence intensity increase. The dotted box is represented as a bar graph in B to indicate significant blockage. B, Preincubation of VWF with UFH and Tinzaparin, but not with Fondaparinux, causes a significant decrease in DNA binding. Data are expressed as mean±SD (*P<0.05; n=3). C, Increasing concentrations (0–5 μg/mL) of DNA were incubated with 1 μg/mL VWF, which was preincubated with 50 U/mL UFH, or UFH that has been digested by heparinase I beforehand. Binding of DNA to VWF was assessed by Sytox Green fluorescence intensity increase. The dotted box is represented as a bar graph in D to indicate significant blockage. D, Preincubation of VWF with UFH causes a significant decrease in DNA binding, which can be restored by partial heparinase I digestion of UFH. Data are expressed as mean±SD (*P<0.05; n=3).
and heparin are located within the A1-domain, we calculated the electrostatic potential produced by this domain to search for positively charged regions where the negatively charged DNA could potentially bind. The electrostatic potential revealed a positively charged region at the A1-domain (Figure 5A), which remained approximately constant during the course of a 195-ns molecular dynamics simulation (Figure IVA in the online-only Data Supplement). This positively charged area intersects with a part of the GP1bα-binding site (compare blue in Figure 5A with orange in Figure 5B) and contains the residues associated with the heparin-binding site (compare blue in Figure 5A with green in Figure 5C). This suggests that DNA interacts with VWF at the same regions that GP1bα and heparin do.

**DNA Does Not Block the Cleavage Site for ADAMTS13**

Because we found that DNA binds to the VWF A1-domain, we intended to ascertain whether this interaction could possibly block also parts of the A2-domain, which contains the cleavage site for ADAMTS13. Therefore, we performed in vitro flow experiments for DNA binding to VWF fibers released by a confluent human umbilical vein endothelial cell layer with or without the addition of ADAMTS13. After immunofluorescence staining, we could see that even in the presence of DNA, VWF fibers were removed by ADAMTS13 activity (Figure 6A and 6B). Although we saw the same amount of fibers in the end point observation of the microfluidic experiments, we observed a decreased cleavage capacity of ADAMTS13 in the time course of an ELISA activity (Figure 6C). In addition, electrostatic potential calculations revealed that the surface around the A2-domain is mostly negatively charged (see Figure IVB in the online-only Data Supplement), which may disfavor the interaction of this domain with DNA. Surprisingly, in the presence of DNA, more VWF fibers were detectable. Using DNase I, the increase in VWF string formation could be significantly reduced (Figure 6A). The latter results indicate that the DNA may support the activation and stabilization of VWF fibers on shear flow conditions, similar to previous results where platelet-bound VWF has been shown to be a better ADAMTS13 substrate.

**Discussion**

In this work, we could show that protein-free DNA is able to directly bind to VWF. Our data extend to the already existing in vivo measurements in deep vein thrombosis mouse models, that plasma DNA levels are increased and that VWF and DNA released by leukocytes (NETs) overlap in thrombus sections. However, these previous findings were correlated to the observation that proteins such as histones are still present on the autolog-released NETs and that these proteins are then able to interact with VWF. In contrast, our findings demonstrate that the colocalization of VWF with DNA is not necessarily dependent on other proteins, but instead pure isolated DNA, which is protein- and RNA-free, is able to bind directly to VWF and mediate leukocyte adhesion under physiological blood flow conditions (Figures 1A and 3C). Consequently, the DNA–VWF interaction could not only lead to thrombus formation but additionally promote leukocyte extravasation. In this context, it was recently demonstrated that blocking of VWF decreases leukocyte extravasation in different inflammatory mouse models and in immune complex–mediated vasculitis, which may open a new anti-inflammatory treatment option. What is more, we previously showed that *S. aureus* itself binds to the VWF A1- and A3-domains, which supports *S. aureus* adhesion to the vessel wall and, in turn, can be blocked using heparin. Besides this process, DNA release by leukocytes can be induced by *S. aureus* infection. Hence one may imagine a scenario where *S. aureus*–induced NET formation leads to DNA–VWF–*Staphylococcus* binding, followed by leukocyte activation.
adhesion to the vessel wall and consecutive extravasation. Furthermore, a deficiency in the VWF-regulating enzyme, ADAMTS13, leads to an increased leukocyte adhesion and enhanced extravasation of neutrophils. The binding of leukocyte-derived extracellular DNA to VWF, therefore, represents a new possibility for leukocytes to be recruited to the vessel wall, besides the suggested direct binding to VWF via P-selectin glycoprotein ligand-1 and β2 integrins.

However, our data show that this interaction strongly depends on shear flow. Uncoiling of the VWF molecule occurs under flow conditions above a critical shear rate, which can expose binding sites such as the A1-domain. In the absence of flow conditions, Ristocetin is an antibiotic to mimic this state-function relationship without the application of shear flow in an in vitro setup. In both cases, we could detect a binding of DNA to VWF; however, if both prerequisites are absent, an interaction of the molecules is not possible. These findings suggest that DNA binds only to an uncoiled VWF and, therefore, to its A1-domain.

Our studies suggest that binding occurs via electrostatic interactions between the polyanionic molecule (DNA) and the positively charged VWF A1-domain, but has no effect on the VWF A2-domain, because the specific enzyme, ADAMTS13, is still able to cleave VWF fibers. It is well known that the A1-domain of VWF exhibits a main role in the function of the large VWF molecule. Many binding sites are buried at this location, especially the binding sites for both platelet GP1bα and heparin. All these partners, examined in our experiments, are negatively charged or have at least several negatively charged residues, so that they may be classified as polyanionic molecules that are able to bind to the positively charged VWF A1-domain. In particular, the phosphate groups of the DNA backbone and the sulfate groups linked to the heparin sugars make up the similar polyanionic character of both biopolymers. However, low-molecular-weight heparins and heparinase-digested unfractionated heparin instead show less capability to block DNA–VWF binding, which suggests that the impaired interaction is because of molecule size and the amount of negative charges.

Heparins are known to be multifunctional glycosaminoglycans, which are preferred anticoagulants for venous thromboembolism treatment and the first choice of therapy for venous thromboembolism in patients with cancer. The classic mode of action is that heparins are able to bind antithrombin III and thereby accelerate the blockage of thrombin and mainly factor Xa. However, as shown in this work, heparins are also able to directly interact with VWF, thereby blocking DNA binding to VWF, which demonstrates that the prothrombotic effect of extracellular DNA traps could be diminished by heparin. In conclusion, our data add a new aspect to the anticoagulatory mechanisms of heparin, which may further explain the beneficial effect of heparin in the treatment of venous thromboembolism, especially in patients suffering from cancer.

Our experiments propose a new general function of the VWF molecule, namely to act as a polyanion-binding molecule after uncoiling by interacting with highly negatively charged molecules such as heparin or DNA, however being nonspecific but with a high affinity. This DNA–VWF interaction may play a pivotal role in leukocyte adhesion and extravasation on inflammation and coagulatory conditions.

Figure 6: DNA binding does not inhibit von Willebrand factor (VWF) cleavage by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). A, DNA significantly increases the amount of VWF strings in the absence of ADAMTS13, which can be prevented by DNase I. However, both in the presence and absence of DNA on VWF fibers, the number of VWF fibers per field of view is significantly decreased by ADAMTS13. There is no significant difference in the amount of strings after the addition of ADAMTS13 in both cases. Data are expressed as mean±SD (*P<0.05; n=3). B, Immunofluorescence images showing that even in the presence of DNA, ADAMTS13 is able to cleave VWF fibers at 10 dyne/cm². Without ADAMTS13, large VWF strings are visible on human umbilical vein endothelial cells. These fibers are degraded in the presence of ADAMTS13. White indicates VWF. Scale bar, 50 μm. C, ADAMTS13 activity was measured using an ELISA that quantifies cleavage by glutathion-S-transferase–tagged VWF73 (GST-VWF73) products. In the presence of DNA, ADAMTS13 cleavage is slowed down (open dots) but, however, reaches the same level after 30 min as without DNA (filled dots).
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Disclosures

None.

References


Significance

It was recently shown that von Willebrand factor–DNA interaction plays an important role in inflammation and deep vein thrombosis. Using advanced microfluidics, molecular biological tools, and electrophoretic potential calculations, the present work unravels the molecular mechanism of this interaction. We could show, for the first time to our knowledge, that isolated, pure (protein-free) DNA binds to the A1-domain of von Willebrand factor, which can be blocked by heparins. Hence, heparin treatment might be of superior benefit for patients with thrombosis known to exhibit high amounts of DNA and von Willebrand factor, as also reflected in recent therapeutic guidelines for cancer-related thrombosis. Therefore, our study emphasizes the effect of von Willebrand factor’s binding capacity to highly negatively charged molecules and considerably underlines the role of von Willebrand factor in both inflammation and thrombosis.
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**MATERIALS AND METHODS.**

*Von Willebrand factor directly interacts with DNA from Neutrophil Extracellular Traps*

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VWF directly interacts with DNA

Material and Methods

Blood sample collection
Venous blood was drawn from healthy volunteers into sodium citrated blood vacuum collection tubes. The study was conducted in conformity to Declaration of Helsinki and was approved by the Ethics Committee of Heidelberg University (Mannheim, Germany). From all subjects, appropriate informed consent was obtained.

Recombinant proteins
Recombinant proteins were produced using plasmids with the following deletions: VWFdelA1 (deletion p. Glu 1260 – Gly 1479), VWFdel A2 (deletion p. Asn 1493 – Glu 1673), VWFdel A3 (deletion p. Gly 1672 – Gly 1874) as described previously. The deletions were introduced by site directed mutagenesis using a QuikChange Site-Directed Mutagenesis Kit (Agilent, Waldbronn, Germany; catalog number: 200518). For the isolated VWF A1-domain we used a pIRESneo2 VWF A1-6xHis-Furin cleavingsite-A2 construct. Therefore, we cloned the VWF cDNA nucleotide c.3688-5016 (p.Val 1230-Gly 1672) into a pIRESneo2 vector (Clontech, Saint-Germain-en-Laye, France; catalog number: 6938-1) containing the VWF-signalpeptide and the first 3 aminoacids of the VWF-propeptide. Site directed mutagenesis was carried out by using a QuikChange Site-Directed Mutagenesis Kit (Agilent, Waldbronn, Germany; catalog number: 200518) to introduce a BamHI-site after the nucleotide c. 4389 (p. Ala 1463). An oligonucleotide with the sequence of the 6x His-tag and the furin cleaving-site was cloned into the BamHI-site. 293 cells were stable transfected with 4µg plasmid by liposomal transfer (Lipofectamine 2000, Invitrogen, Darmstadt, Germany; catalog number: 11668019). After 24h the cells were selected using 500µg/ml G418h. Expression was done in Optipro-SFM (Invitrogen, Darmstadt, Germany; catalog number: 12309019) for 72h. Stable cells which express the A1-6xHis-furin-site-A2 construct and the endogene furin were cleaved by the protein between VWFA1 and A2 domain. The VWF A1-domain was purified using Ni-NTA affinity chromatography.

DNA isolation from whole blood
DNA was isolated from human whole blood using the QIAamp DNA Blood Mini Kit 50 (Qiagen, Hilden, Germany; catalog number: 51104) according to manufacturer’s instructions. Blood samples were pretreated with Proteinase K from the kit and 4 µl RNaseA (100 mg/ml, Qiagen, Hilden, Germany; catalog number: 1007885) per 200 µl blood sample. Isolated DNA was tested for purity by absorbance measurements of 260/280 nm ratio, Bradford assay and gel electrophoresis, dissolved in Tris-EDTA (TE) buffer and stored at -20°C.

Cell culture of HUVEC
Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords (kindly provided by the Department of Gynaecology at the University Medical Centre Mannheim and Sankt Hedwig Hospital Mannheim) using collagenase (PAA, Pasching, Austria; catalog number: K21-240) and cultured in M199 medium (PAA, Pasching, Austria; catalog number: E15-834) supplemented with 10% fetal calf serum and antibiotics penicillin and
VWF directly interacts with DNA

streptomycin and used for experiments up to passage two, as described before.

**Isolation of neutrophils from human peripheral whole blood**

Neutrophils were isolated from freshly drawn human peripheral whole blood using the MACS whole blood column kit (Miltenyi Biotec, Bergisch Gladbach, Germany; catalog number: 130-093-545) utilizing whole blood CD15 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany; catalog number: 130-091-058) according to manufacturer’s instructions. Isolated neutrophils were kept in RPMI 1640 (Sigma, Steinheim, Germany; catalog number: R0883).

**Shear-induced DNA-VWF binding**

Channels of a 48 well microplate (BioFlux 200, IUL Fluxion, San Francisco, USA; catalog number: 910-0004) were coated with 100 µg/ml plasmatic VWF (Calbiochem, Darmstadt, Germany; catalog number: 681300) and perfused with 10 µg/ml isolated DNA from whole blood in TE buffer at 2 dyne/cm² for 15 min. Binding of DNA to the VWF surface was assessed by a 4′,6-Diamidin-2-phenylindol (DAPI) staining (1:2000; Sigma, Steinheim, Germany; catalog number: D8417-1MG) and anti-VWF-FITC labelling (1:200; GeneTex, Irvine, USA; catalog number: GTX28B22). When indicated, DNA-bound VWF was perfused after the staining with 100 U/ml DNAse I (Thermo Scientific, Schwerte, Germany; catalog number: EN0521) or 50 U/ml heparin (Biochrom, Berlin, Germany; catalog number: L6510) until DNA was removed. Images were acquired using a Zeiss AxioObserver Z1 equipped with an AxioCam MRc (both Carl Zeiss AG, Oberkochen, Germany).

For determination of the blocking capacity of heparin, the coated VWF surfaces were preincubated with 50 U/ml heparin (Biochrom, Berlin, Germany; catalog number: L6510) or 2% BSA (PAA, Pasching, Austria; catalog number: K41-012) as negative control before the subsequent perfusion of DNA.

**DNA-binding to VWF released from HUVEC**

A confluent monolayer of HUVEC cultured on a µ-Slide I Luer IBIDI-slide (IBIDI GmbH, Munich, Germany; catalog number: 80166) as described previously was stimulated with 10 µM histamine and perfused at 10 dyne/cm² with 10 µg/ml isolated DNA, Sytox Green (1:1000; Invitrogen, Darmstadt, Germany; catalog number: 910575) and 100 U/ml DNAse I (Thermo Scientific, Schwerte, Germany; catalog number: EN0521) when indicated. A hematocrit of 25% in HEPES-buffered saline was applied. After perfusion, cells were fixed using 4% PFA and blocked with 2% BSA (PAA, Pasching, Austria; catalog number: K41-012). VWF fibers were stained using a rabbit anti-human VWF antibody (DAKO, Hamburg, Germany; catalog number: A0082). Nuclei of HUVEC were stained during the fixation process due to excessive Sytox Green dye in the supernatant.

For ADAMTS13 studies, HUVEC were subsequently perfused with 430 ng/ml recombinant ADAMTS13 (R&D systems, Wiesbaden, Germany; catalog number: 6156-AD-020) for 15 min at 37 °C before fixation. Images were acquired using a Zeiss AxioObserver Z1 equipped with an AxioCam MRc (both Carl Zeiss AG, Oberkochen, Germany).
**Immunobased DNA-VWF interaction assay**

A 96 well microtiter plate was coated with anti-human VWF (1:500; DAKO, Hamburg, Germany; catalog number: A0082) and blocked with 4 % BSA (PAA, Pasching, Austria; catalog number: K41-012). Isolated DNA from whole blood (0-5 µg/ml) was incubated with 1 µg/ml plasmatic VWF (Calbiochem, Darmstadt, Germany; catalog number: 681300) or 1 µg/ml recombinant A1-domain and 0.15 - 1.5 mg/ml Ristocetin (Moelab, Langenfeld, Germany; catalog number: 10966183) in TE buffer. After coincubation DNA binding to VWF was quantified using Sytox Green (1:1000; Invitrogen, Darmstadt, Germany; catalog number: 910575). Fluorescence intensity was analyzed at a fluorescence microplate reader (Tecan Trading AG, Männedorf, Switzerland) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

For heparin blocked DNA-VWF binding, 1 µg/ml VWF and 1.5 mg/ml Ristocetin were preincubated in the presence of 50 U/ml unfractionated heparin (Biochrom, Berlin, Germany; catalog number: L6510), 50 U/ml Tinzaparin (LEO Pharma, Neu-Isenburg, Germany; catalog number: FC0291), 29 µg/ml Fondaparinux (GlaxoSmithKline, Greenford, UK; catalog number: 6084) or pre-digested unfractionated heparin using 0.15 U/µl heparinase I over night (catalog number: H2519) before the addition of indicated DNA concentrations as described above.

When indicated, 50 U/ml unfractionated heparin (Biochrom, Berlin, Germany; catalog number: L6510) or 100 U/ml DNase I (Thermo Scientific, Schwerte, Germany; catalog number: EN0521) were added to the reaction only after the addition of indicated DNA concentrations.

For chitosan blockage of negative charges, 1.25 µg isolated DNA was incubated with 0.7 µg (high amount), 0.35 µg (medium amount) or 0.175 µg (low amount) of chitosan (MW 4.3*10^5 g/mol; degree of acetylation 10%) before DNA was used for VWF binding as described above.

**Platelet binding to VWF**

100 µg/ml plasmatic wildtype VWF (Calbiochem, Darmstadt, Germany; catalog number: 681300) or 100 µg/ml recombinant A1-domain was immobilized on a microfluidic channel (BioFlux 200, IUL Fluxion, San Francisco, USA; catalog number: 910-0004) and perfused with 10 µg/ml isolated DNA from whole blood in TE buffer (or TE buffer only as negative control) at 2 dyne/cm² for 15 min. Platelet rich plasma (PRP) was isolated from citrated peripheral whole blood and perfused for 30 min at 2 dyne/cm². Movies were acquired at the Zeiss microscope. Attaching platelets were fixed using 4 % PFA and stained with an anti-CD42b antibody (1:200; ImmunoTools, Friesoythe, Germany; catalog number: 21270421).

**Platelet adhesion on VWF mutants**

Platelets were isolated from human citrated whole blood by centrifugation at 180 g for 15 min for PRP and subsequent centrifugation at 1200 g for 10 min. Platelets were prelabeled using Celltrace™ Calcein green AM (1:1000; Invitrogen, Darmstadt, Germany; catalog number: C34852) and perfused over a microfluidic channel at 2 dyne/cm². Channels were coated with VWF mutants VWFdelA1, VWFdelA2 and VWFdelA3 as previously described.  

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(VWF mutants were kindly provided by Prof. Schneppenheim, Hamburg, Germany). Adhering platelets were counted per field of view.
For physiological measurements, platelets were resuspended in HEPES-buffered saline supplemented with different amounts of washed erythrocytes ranging from 0-45%.

**Neutrophil flow assay**
Isolated neutrophils were resuspended to 4 million cells per ml in RPMI (PAA, Cölbe, Germany) and stimulated with 10 mM PMA (Sigma, Steinheim, Germany; catalog number: P8139-5MG) or 2 x 10^7 bacteria/ml of *Staphylococcus aureus* wt strain SA113 (ATCC 35556) (cultured as described previously ²) before perfusion over a VWF coated surface (100 µg/ml; Calbiochem, Darmstadt, Germany; catalog number: L6510) or 2% BSA (PAA, Pasching, Austria; catalog number: K41-012). DNA was stained with DAPI (1:5,000; Sigma, Steinheim, Germany; catalog number: D8417-1MG) and VWF was labelled using an anti-VWF-FITC antibody (1:200; GeneTex, Irvine, USA; catalog number: GTX28B22).

**Molecular dynamics simulations**
Molecular dynamics (MD) simulations of the VWF A1-domain and the VWF A2-domain were carried out by using the 4.5.5 version of the GROMACS package ⁴, ⁵. Initial atomic coordinates of the VWF A1-domain were taken from the X-ray structure determined by Emsley *et al.* ⁶ (Protein Data Bank ID code 1AUQ), and for the VWF A2-domain were extracted from the structure determined by Zhang *et al.* ⁷ (Protein Data Bank ID code 3GXB). The simulation systems contained one of the domains (either A1 or A2) solvated by approximately 23000 water molecules. In both cases, sodium and chloride ions were added to yield a salt concentration of 150 mM, and additional chloride ions were added ensuring an uncharged system. The Amber99sb-ildn* force field ⁸-¹⁰ was used for the protein, the TIP3P model ¹¹ for the water molecules, and Joung parameters ¹² for the ions. Electrostatic interactions were computed by using the particle-mesh Ewald method ¹³, ¹⁴. Short-range non-bonded interactions were considered and described by a Lennard-Jones potential within a cut-off of 10 Å. For the water molecules the Settle algorithm ¹⁵ was used to constraint both bond lengths and angles. For the protein the Lincs ¹⁶ algorithm was used to constraint the bonds lengths and virtual interaction-sites ¹⁷ were added to remove angular motions involving hydrogen atoms. The leap frog algorithm ¹⁸ was used to numerically integrate Newtonian equations of motion at discrete time steps of 4 fs for a total simulation time of 195 ns. Temperature was kept constant at 300 K by coupling the system to a velocity-rescaling thermostat ¹⁹ (coupling constant t = 0.5 ps). Pressure was maintained constant at 1 bar by using the Parrinello-Rahman barostat ²⁰ (coupling constant t = 5.0 ps). Before each 195 ns production run, a solvent equilibration of 1 ns, with the protein (either the A1-domain or the A2-domain) harmonically restrained (harmonic force constant of 1,000 kJmol⁻¹nm⁻²), was carried out. Principal component analysis (PCA) ²¹ was used to extract representative conformations of each domain. Each MD trajectory was projected onto the three dimensional space constituted by the
first three principal components. Subsequently, ten snapshots uniformly distributed over the 3D-PCA subspace were selected.

**Electrostatic calculations**
The electrostatic potential was computed for ten representative conformations of the VWF A1-domain, and separately for ten conformations of the VWF A2 domain. These conformations were extracted from the 195 ns molecular dynamics simulation mentioned above. The electrostatic potential was computed by solving the linearized Poisson-Boltzmann equation by using the Adaptive Poisson/Boltzmann Solver (APBS)\textsuperscript{22}. Calculations were carried out following a sequential focusing multigrid Poisson-Boltzmann approach. Coarse grid dimensions were equal to the protein dimensions expanded by a factor 1.7. Fine grid dimensions were obtained by adding 2 nm to the protein dimensions, yielding a grid spacing of approximately 0.05 nm. Single Debye-Hückel boundary conditions were imposed. A concentration of 150 mM of single-charged ions was included in the calculation. The dielectric constant was 2 for the protein and 78 for the solvent. The molecular surface was calculated by rolling a probe sphere of radius 0.14 nm over the protein and smoothed as described by Bruccoleri et al.\textsuperscript{23}. The electrostatic potential was computed at a temperature of 300 K. 10 grid points per Å\textsuperscript{2} were used at the surface interface between the protein and the solvent. The electrostatic potential was mapped on the protein surface by using the PyMOI software\textsuperscript{24}.

**ADAMTS13 activity ELISA**
To measure the cleavage of VWF by ADAMTS13 activity in a time-dependent manner, we applied a commercially available Technozym ADAMTS13 activity ELISA (Technoclon GmbH, Vienna, Austria; catalog number: 5450701). This ELISA contains a GST-tagged VWF73 substrate, which was mixed with 10 µg/ml lambda DNA or TE buffer as control before addition to anti-GST coated wells. For the cleavage reaction we used recombinant ADAMTS13 (R&D systems, Wiesbaden, Germany; catalog number: 6156-AD-020) at a concentration of 430 ng/ml. ADAMTS13 reaction was stopped at different time-points ranging from 5 to 30 min, afterwards the ELISA was performed according to manufacturers instructions. VWF73 cleavage products were detected by an HRP-conjugated monoclonal anti-N10 antibody and following TMB reaction was measured at 450 nm.

**Statistical analysis**
Mean data of experiments are given with standard deviation (SD). Statistical computation was performed with SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Differences between the groups were tested by the unpaired Student’s t-test. Values below $P < 0.05$ were considered to be statistically significant.
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References
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SUPPLEMENTAL MATERIAL.

Von Willebrand factor directly interacts with DNA from Neutrophil Extracellular Traps

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Supplemental Figures

Supplementary Figure I.

DNA binding to VWF is dependent on the Ristocetin concentration. **A.** Different concentrations of Ristocetin ranging from 1.5 mg/ml to 0.15 mg/ml were applied to induce a binding of VWF to DNA, which was measured by the fluorescence intensity increase of DNA labeled with Sytox Green. Increasing DNA concentrations (0-5 µg/ml) were incubated with 1 µg/ml VWF. The dotted box is represented as bar graph in **B** to indicate significant binding induction. **B.** Ristocetin concentrations ranging from 0.15 mg/ml to 1.5 mg/ml significantly induce binding of DNA to VWF. Data are expressed as means +/- SD (* P < 0.05).
Supplementary Figure II.

Hematocrit-dependent platelet adhesion to a DNA blocked VWF surface.  
A. Without hematocrit, DNA significantly blocks platelet adhesion to a VWF coated surface. However, this binding can be restored upon addition of 45% hematocrit. White: platelets. Scalebar: 200 µm.  
B. +DNA/-DNA ratio indicates the ratio of the amount of adhering platelets to a VWF coated channel in the presence and absence of DNA, respectively. A ratio of 1 indicates equal adhesion on both channels. This figure illustrates a hematocrit dependence of the DNA blocking capacity on platelet binding to VWF.
DNA binding to VWF can be removed by DNAse I, but not by heparin. A. DNA binding to a VWF functionalized surface can be removed by perfusion with 100 U/ml of DNAse I, whereas the binding remains stable when perfused with 50 U/ml heparin. Black: Inversed image of DAPI stained DNA. B. Representative quantification of remaining DNA on the surface. C. ELISA-based assay in which DNA binding to VWF (induced by Ristocetin) can be removed by DNAse I but not by heparin. UFH = unfractionated Heparin.
Electrostatic potential generated by the VWF A1 domain (A, left panel) and the A2 domain (B, right panel). Potentials for ten representative conformations (numbered from 1 to 10) for each domain are shown. Representative conformations were extracted from 195 ns molecular dynamics simulations of these domains. The potential is depicted at a distance of 1.4 Å from the protein according to the color scale at the right. Two orientations (rotated 180°) are considered per conformation.
DNA binding to VWF is impaired by neutralizing DNA using Chitosan. Preincubation of DNA with different amounts of Chitosan ranging from low, medium to high amounts caused a concentration-dependent decrease of DNA adhering to VWF. Binding of DNA to VWF is Ristocetin dependent. The binding of DNA to VWF was assessed by fluorescence intensity measurements using Sytox Green labeling. Data are expressed as means +/- SD (n = 3).
Legends for Supplemental Movie files

Supplementary Movie I. Prelabeled DNA binds to HUVEC-released VWF fibers. Sytox-Green prelabeled DNA (white) is perfused over a histamine-stimulated HUVEC layer in HEPES-buffered saline using 45% hematocrit. DNA attaches to released VWF fibers under flow.

Supplementary Movie II. Perfusion of DNase I removes attaching neutrophils from VWF surface. PMA-stimulated neutrophils are perfused over a VWF-coated surface. DNA is stained with DAPI (white). After attachment, DNA and bound neutrophils are removed using 100 U/ml DNase.