Erythrocytes and Von Willebrand Factor in Venous Thrombosis
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Summary and concluding remarks

A continuous circulation of blood and a tight regulation of blood coagulation is essential for human life. Disturbed blood coagulation may result in bleeding when coagulation is impaired, or thrombosis, when blood undesirably clots within the circulation. In both events, deregulated cell-cell or cell-protein contact as well as changes in protein function play an important role.

Circulating cells, for example platelets, leukocytes and erythrocytes, contribute significantly to blood coagulation. Platelets, with their specialized functions in primary hemostasis, are the key cells that mediate blood clotting. When the endothelium is damaged, platelets can bind to collagen via the receptor glycoprotein IV (GPIV) and the integrin $\alpha_2\beta_1$ (GPIa/GPIIa) and to von Willebrand factor (VWF) via the receptor GPIb-V-IX and the integrin $\alpha_{IIb}\beta_3$ (GPIIb/GPIIIa).[1-7] Once bound to collagen or VWF, a rise in cytosolic calcium levels activates the platelets causing α-granule secretion, shape changes, increased adhesiveness, and phosphatidylserine exposure.[1,8,9] The negatively charged surface of phosphatidylserine exposing platelets promotes the formation of tenase (FVIIIa-FIXa) and prothrombinase (FVa-FXa) complexes, thrombin generation, and eventually fibrin production.[10-12]

Leukocytes also significantly contribute to blood clotting. In arterial thrombosis, monocytes are recruited to activated endothelial cells, migrate through the intima, differentiate into macrophages, take up lipoproteins, and cause local inflammation which eventually may trigger the rupture of an atherosclerotic plaque and the release of thrombogenic material into the circulation.[13] In venous thrombosis leukocytes are also recruited by activated endothelial cells, but instead of migrating through the intima, monocytes trigger FVII-dependent coagulation by tissue factor (TF) expression and neutrophils promote FXII-dependent coagulation through the release of neutrophil extracellular traps (NETs).[14-17]

In contrast, the role of erythrocytes in hemostasis is less understood. Hematocrit levels are strongly associated with the efficiency of hemostasis. Anemia increases the risk of bleeding while erythrocytosis, on the other hand, increases the risk of thrombosis.[18,19] This is mostly explained by erythrocyte-induced platelet margination.[20] Similar to platelets, erythrocytes can also expose phosphatidylserine on the outer leaflet of the cell membrane and promote the formation of tenase (FVIIIa-FIXa) and prothrombinase (FVa-FXa) complexes and thrombin formation.[21,22] Phosphatidylserine exposure on erythrocytes can be triggered by a calcium influx or conditions that stress the erythrocytes (e.g. sickle cell disease or storage in blood banks).[23-25] Furthermore, erythrocytes can also bind platelets via ICAM-4 and the platelet integrin $\alpha_{IIb}\beta_3$, they can bind to activated neutrophils, and they can bind to fibrin at low shear stress.[26-28] The most studied thrombotic event involving erythrocytes is the adhesion of sickle erythrocytes to endothelial cells. Sickle erythrocytes can bind to endothelial-derived VWF strings, which may cause vaso-occlusive events.[29,30] However, this interaction has hardly been described with non-sickle erythrocytes. Other mechanisms by which erythrocytes could contribute to hemostasis remain to be investigated.

Where leukocytes are part of the initiation process of both arterial and venous thrombosis and platelets are the main cells found in arterial thrombi, erythrocytes form the bulk of venous thrombi.[31] For a long time, erythrocytes were thought to be innocent bystanders that become tangled up in the fibrin mesh of

venous thrombi. Although it is known that venous thrombi develop in the absence of endothelial injury by the recruitment of leukocytes and platelets, it remains uncertain when erythrocytes come into play during the development of a venous thrombus. Whether erythrocytes can bind to the activated endothelial cells is currently not well studied. Another question that needs to be addressed is whether erythrocytes are passively trapped in the fibrin network or are actively recruited by a specific binding mechanism. Interestingly, in mouse models of venous thrombosis it was shown that reducing erythrocyte retention produced smaller clots and that erythrocyte retention depended on FXIIIa-mediated fibrin fibre formation and clot stiffening.[32,33] Reducing erythrocyte retention in venous thrombi could thus be beneficial for patients suffering from venous thrombotic events. However, the mechanisms that drive erythrocyte retention in venous thrombi are currently not fully understood. Therefore, understanding how erythrocytes contribute to the development of venous thrombosis is of great importance.

In this thesis, we aimed to generate insight in how and when erythrocytes can bind to endothelial cells and to the glycoprotein VWF. First, we started out with a brief overview about thrombosis and hemostasis **(Chapter 1)**. Next, we described a double hit model to investigate erythrocyte adhesion to endothelial cells. Using this model we showed that erythrocytes, which were activated by a calcium influx, could bind to endothelial-derived UL (ultra large)-VWF strings **(Chapter 2)**. In addition, we showed that erythrocytes exposed to low shear stress can specifically bind to immobilized recombinant VWF. This interaction was independent of platelets. We also provided new insight into the structural composition of venous thrombi and suggested that the erythrocyte-VWF-fibrin interaction contributes to this architecture **(Chapter 3)**. We could not find VWF on circulating erythrocytes, but found small erythrocyte populations that were positive for fibrin **(Chapter 4)**. Finally, we discussed the link between the ABO blood groups and VWF **(Chapter 5)**.

Erythrocytes adhere to endothelial cells in a double hit model

As mentioned before, venous thrombi develop in the absence of endothelial injury.[31] Local hypoxia activates the endothelial cells upon which they release the content of Weibel-Palade bodies and express adhesion molecules.[34] ULVWF strings released by Weibel-Palade bodies recruit platelets.[15] Simultaneously, P-selectin, which is translocated from the Weibel-Palade bodies to the plasma membrane, recruits leukocytes.[14] Next, tissue factor-positive monocytes and microparticles initiate FVII (factor FVII) dependent coagulation.[14] Platelet-neutrophil complexes induce neutrophil extracellular trap (NET) formation, which provides a surface for FXII-dependent coagulation.[14] Finally, initiation of coagulation results in fibrin formation, which together with VWF and NETs, forms a scaffold for platelets and erythrocytes.[35] It has been well established that erythrocytes form the bulk of venous thrombi and contribute to the propagation of the thrombus. However, whether erythrocytes can adhere to activated endothelial cells like platelets and leukocytes, thereby contributing to the initiation of a venous thrombus, remains unclear. It is well described that sickle erythrocytes adhere to endothelial cells and thereby can induce thrombotic and vaso-occlusive events.[36] Multiple receptor ligand combinations for sickle cell adhesion to endothelial cells have been identified.[36] The adhesion of sickle erythrocytes to endothelial cells can be mediated by the binding of integrin $\alpha_4\beta_1$ on the erythrocyte membrane to its endothelial ligand vascular cell adhesion molecule-1, by bridging of CD36 expressed by erythrocytes and

endothelial cells via thrombospondin, or by the interaction between the Landsteiner-Weiner blood group glycoprotein (ICAM-4) with the integrin $\alpha_V\beta_3$.[36-42] On the contrary, non-sickle erythrocytes have been described to show only minor adhesion to endothelial cells.[30,43] However, the major drawback of these studies is that the experimental setup was optimized to investigate sickle erythrocyte adhesion to endothelial cells. Variables that affect non-sickle erythrocyte adhesion to endothelial cells, like shear stress, endothelial cell activation, or erythrocyte stimulation, were only individually tested. How their combined effect influences non-sickle erythrocyte adhesion to endothelial cells is currently not known. Therefore, we used in **Chapter 2** a double-hit experiment design to investigate under which circumstances non-sickle erythrocytes adhere to endothelial cells. In contrast to earlier findings, we were able to show that non-sickle erythrocytes can adhere to endothelial cells, provided that (1) endothelial cells are activated and (2) erythrocytes are exposed to a stressor that induces a calcium influx. Although calcium-loaded erythrocytes also showed increased binding to non-activated endothelial cells, significant adhesion was mainly observed when calcium-loaded erythrocytes were perfused over endothelial cells that were activated by thrombin or histamine. The increased binding of calcium-loaded erythrocytes to resting endothelial cells can be explained by the increase of phosphatidylserine exposure on the erythrocyte membrane. Phosphatidylserine is a ligand for CXCL16/SR-PSOX and has been shown to mediate calcium-loaded erythrocyte adhesion to endothelial cells.[44]

To induce a calcium influx into the erythrocytes, we used a non-physiological stimulus (ionomycin), however physiological calcium influxes into non-sickle erythrocytes have also been described. Erythrocytes express NMDA receptors which, upon stimulation, mediate calcium influxes.[25,45] Also the lipid signalling molecule lysophosphatidic acid (LPA) was shown to cause a calcium influx into erythrocytes.[46] As described in **Chapter 3**, LPA is able to promote erythrocyte adhesion and this effect depends on an increase of intracellular calcium. Interestingly, cell activation by calcium influxes is a common mechanism among many cell types. Platelets, for instance, also rely on increased cytosolic calcium levels for proper activation.[8,47] It would be interesting to see whether, beside lysophosphatidic acid, other platelet-activating stimuli (e.g. ADP, serotonin, thromboxane A2) can also induce calcium fluxes into erythrocytes. In addition, outside-in signalling can activate platelets once they adhere to a substrate.[47] Despite the differences between erythrocyte and platelet adhesion, it would be of interest to investigate whether erythrocyte-VWF interaction causes a similar outside-in signalling. Moreover, investigating the downstream effectors in erythrocytes that respond to increased cytosolic calcium levels and how they modulate erythrocyte adhesiveness could shed more light on the role of erythrocyte adhesion in thrombosis and hemostasis.

VWF is a substrate for erythrocyte adhesion

While sickle erythrocytes can adhere via many different mechanism to endothelial cells, we describe in **chapter 2** that non-sickle erythrocytes mainly adhere to ULVWF strings that are released from activated endothelial cells. The adhesion of non-sickle erythrocytes to VWF has, to our knowledge, only been described by Wick *et al*. In this study, the authors investigated the adhesion of sickle and non-sickle erythrocytes to endothelial cells with or without the addition of endothelial cell supernatant. However, they did not look at the effect of endothelial cell activation and ULVWF string secretion on

erythrocyte adhesion. Moreover, to our knowledge we are the first group that investigated erythrocyte adhesion to VWF in the context of venous thrombosis.

The adhesion of erythrocytes to protein substrates other than VWF has often been described. For instance, erythrocytes from patients with polycythemia vera were shown to adhere to laminin α5 via Lu/BCAM.[48] Erythrocytes were also shown to adhere to fibrinogen and fibrin, however conflicting results have been reported about this interaction. Aleman and co-workers previously reported that erythrocytes adhered to fibrinogen and this adhesion persisted at a shear rate of 1000 s^{-1}.[32] However, prior to the flow assay, erythrocytes were statically incubated on the fibrinogen layer during which they can settle and adhere.[32] Static incubation is a valid approach, keeping in mind that a venous thrombus develops during blood stasis or at near static conditions. However, in a different study by Goel *et al*. only minor erythrocyte adhesion could be observed when they were perfused at near static shear rates (50 s^{-1}) over fibrin.[28] Based on their observations they concluded that erythrocyte adhesion to fibrin was mediated by secondary plasma proteins that bound to fibrin.[28] In **chapter 3** we used a robust method to examine the interaction between erythrocytes and purified proteins. Using this method, we showed that erythrocytes bind to VWF and that the adhesion increased significantly when the wall shear stress approached stasis. However, little binding to fibrinogen or fibrin could be observed which is in line with the results from Goel *et al.* In addition, we observed little binding of erythrocytes to collagen or fibronectin. This preferential binding to VWF suggests that the erythrocyte-VWF interaction is either mediated by a highly specific receptor or via an interaction that depends on distinct epitopes that are mainly present within VWF.

The level of shear stress determines the adhesion of erythrocytes to VWF

As mentioned before, we showed that the adhesion of erythrocytes to VWF increased significantly when the wall shear stress approached stasis. This condition is characteristic for venous thrombosis. Our data showed that erythrocytes begin to adhere to VWF at a wall shear stress below 1 dyne/cm^2 and when the wall shear stress approaches stasis, the adhesion of erythrocytes to VWF significantly increases. Although the adhesion mechanism that mediates erythrocyte binding to VWF is yet unknown, the observation that erythrocytes only bind to VWF at a very low wall shear stress suggests that the initial binding to VWF is either mediated by a low affinity interaction or by an interaction partner that is present at a low copynumber. In contrast, platelets can adhere to VWF at high shear stress (>20 dyne/cm^2). This is accomplished by an initial interaction between the platelet receptor GPIb-V-IX and VWF which slows the platelets down and causes platelet activation, followed by firm adhesion to VWF via the integrin $\alpha_{IIb}\beta_3$. As described by Joneckis *et al.* and observed by our group **(Chapter 3)** erythrocytes do not express common adhesion receptors for VWF (GPIb, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_V\beta_5$).[41] Thus, erythrocytes bind to VWF via one or more undiscovered mechanisms what makes it an interesting topic for future research. Although nothing is known about these binding mechanisms, we can argue about some potential candidates. One potential candidate receptor for VWF on erythrocytes is the Duffy antigen also known as the Duffy antigen receptor for chemokines (DARC). DARC acts as a multi-specific receptor for chemokines that contain the C-C or C-X-C motif.[49] One of the molecular characteristics of VWF is the abundant presence of cysteine residues within each monomere.[50] These cysteine residues are not only important for the structure and multimerization of VWF, but also determine its clearance rate, binding to

and cleavage by ADAMTS13, and binding to the platelet receptor GPIb-V-IX.[50-54] Interestingly, VWF (NCBI Reference Sequence: NP_000543.2) contains 8 C-C motifs and 32 C-X-C motifs which makes it a potential candidate ligand for DARC. While in contrast, the proteins to which only minor erythrocyte adhesion was observed, have 0-1 C-C motif (fibrinogen), 0-1 C-C and 0-3 C-X-C motifs (collagen), or 12 C-X-C motifs (fibronectin). In case erythrocytes use DARC to bind to VWF and the other proteins, the variable amount of C-C and C-X-C motifs between each protein could explain the difference in erythrocyte adhesion.

Another candidate group of molecules that could mediate erythrocyte adhesion to VWF are sulfated glycolipids. These sulfate ester-containing glycolipids are common constituents of cell membranes and can be found in cells from the brain, kidney, and spleen, but are also found in the membranes of platelets and erythrocytes.[55] Sulfated glycolipids have been shown to mediate cell adhesion.[55] More interestingly, VWF has been shown to bind specifically and with high affinity to sulfatides isolated from human erythrocytes and platelets.[56] This sulfatide-VWF interaction is known to contribute to the adhesion of sickle erythrocytes to VWF and could possible also mediate the adhesion of non-sickle erythrocytes to VWF.[38] Although, compared to platelets, erythrocytes contain relatively low amounts of sulfatide in their membranes, this could explain why erythrocytes only adhere to VWF at a reduced wall shear stress **(Chapter 3)**.[57] As discussed earlier, we propose that the adhesion of erythrocytes to VWF is mediated by either a low affinity interaction or a high affinity interaction partner that is expressed at a low copynumber on the erythrocyte membrane.

Inhibiting erythrocyte adhesion to VWF with inhibitors that specifically prevent interactions between sulfated glycolipids and their ligands, could be the first step to prove the involvement of sulfated glycolipids in the binding of erythrocytes to VWF. The most potent inhibitor for VWF binding to sulfatide is high molecular weight dextran sulfate.[38,55] Although high molecular weight dextran sulfate indeed did prevent erythrocyte binding to VWF (data not shown), it caused severe agglutination of erythrocytes which prevented us to use this method for further investigation. To circumvent this problem, it may be considered to test whether sulfated monosaccharides are able to inhibit the binding of erythrocytes to VWF.

It has been shown that short chain glycolipids are often cryptic in the plasma membrane of cells and exposure can be regulated by membrane lipid composition or may change during the cell cycle or cell differentiation.[58] Moreover, Roberts *et al*. suggested that calcium and magnesium binding may increase the accessibility of sulfatides in the erythrocyte membrane.[57] Whether a calcium influx also promotes the accessibility of erythrocyte sulfatides is not known. However, a steep rise of the intracellular calcium level does interfere with the flip-flopping of phosphatidylserine, which results in increased phosphatidylserine exposure on the outer leaflet of the membrane.[59] Such a change of membrane lipid composition may enhance the accessibility of sulfatides and thereby promote erythrocyte adhesion to VWF. This theory could explain the enhanced adhesion of erythrocytes to VWF which we have observed after exposure to the calcium ionophore ionomycin **(Chapter 2 and 3)**.

Interestingly, the ABH substances of the ABO blood groups are attached to glycoproteins, but also to glycosphingolipids.[60] Whether the different blood groups result in slight changes in membrane lipid

composition and thereby contribute to variation in the accessibility of sulfatides is not known. However, independent from whether sulfatides regulate erythrocyte adhesion to VWF or not, it would be interesting to test if erythrocytes from different ABO blood groups show variation in their ability to bind to VWF. In **Chapter 4** we found such a difference when we performed a FACS analysis of erythrocytes and looked at the presence of VWF on the cell membrane. However, uncertainty remained whether our antibody cross-reacted with the A antigens or whether indeed VWF preferentially binds to the A-positive erythrocytes. The erythrocyte-VWF adhesion assay described in **Chapter 3** could be used to test whether erythrocytes from different ABO blood groups show differences in adhesion capabilities.

The unresolved link between the ABO blood group system, VWF, and erythrocyte adhesion

A link between the ABO blood group system and VWF is known already for a long time. We have reviewed this topic in **Chapter 5**. Briefly, ABO blood group influences plasma VWF:Ag levels and the VWF:Ag levels can be ordered according to the ABO blood group genotype in the order: Bombay O_h < OO << A2O < A1O < BO < A1A1, BB < AB. ABO blood groups can theoretically influence plasma VWF:Ag levels by having an effect on synthesis, secretion or clearance of VWF, but no effects on synthesis or secretion could be found. Thus, ABO blood groups affect VWF clearance rates. Interestingly, evidence has been presented that VWF clearance is not influenced by ABH antigens present on VWF itself, but depends on the ABO blood group status of the individual.

ABO blood group antigens can be found on several different platelet membrane glycoproteins including GPIa, Ib, IIb, IIa, IIIa, IV, V, and PECAM-1 which all play important roles in cell-substrate or cell-cell adhesion.[61-65] Despite the fact that the presence of ABO blood group antigens on platelets is known already for a long time and has enjoyed great interest in the field of transfusion research, the exact function of the ABO antigens on these receptors is not known.[66] However, it has been shown that ristocetin-induced platelet aggregation (VWF:RCo) is higher in non-O samples compared to blood group O samples.[67] More interestingly, after correcting for VWF:Ag levels the observed difference in VWF:RCo remained significant.[67] This implies that VWF-platelet binding is modulated by the ABO blood groups independent of the variation in VWF levels that have been described between the different blood groups. Although beyond the scope of our research, it would be interesting to see how the ristocetin cofactor assay responds when plasma purified VWF is combined with ABO (mis)matched platelets.

However, as platelets do not adhere to VWF under normal physiological circumstances, any differences in VWF adhesion between platelets from different ABO blood groups would likely still not explain the variation in VWF levels between individuals from different blood groups. In the case ABO blood group antigens modulate platelet adhesion to VWF, it would be interesting to see whether the same is true for erythrocyte adhesion to VWF. VWF adhering to circulating erythrocytes could contribute to the differences in VWF:Ag levels between individuals from different ABO blood groups. Although, we studied circulating erythrocyte-VWF complexes and the results only suggested cross reactivity between the anti-VWF antibody (Dako A0082) with blood group A antigens, the presence of erythrocyte-VWF complexes could not be excluded **(Chapter 4)**. Binding to erythrocytes could potentially stabilize VWF in the

circulation similar to the stabilization of FVIII by VWF. Stabilization could protect it from ADAMTS13-mediated cleavage or clearance. This theory could explain the stratification of VWF:Ag levels between different ABO blood groups. The increased binding of VWF to non-O erythrocytes would also fit with the in **Chapter 5** described independent association between ABO blood groups and venous thrombosis. Where under physiological circumstances plasma VWF could be stabilized by erythrocytes, VWF-erythrocyte complexes could contribute to venous thrombosis at a pathologically low shear stress **(as seen in Chapter 3)**. Increased binding of VWF to non-O erythrocytes could explain the independent effects of ABO blood group on the risk for venous thrombosis. It would also explain the possibility to distinguish a low-risk group carrying genotypes O1O1, O1O2, O1A2, A2A2, or O2A2 compared to a high risk group which carries the genotypes A1A1, A1B, BB, A2B, O1A1, or O1B.

Human venous thrombi suggest that erythrocyte-VWF-fibrin complexes determine their composition

In **Chapter 3** we describe that erythrocytes, VWF, and fibrin show a striking pattern in human venous thrombi by forming erythrocyte-VWF-erythrocyte and erythrocyte-VWF-fibrin complexes. To our knowledge this is the first time that such complexes within human venous thrombi are visualized. Whether these complexes contribute to venous thrombus initiation, propagation, or stabilization is not known. However, our data suggests that VWF forms an important link between erythrocytes and fibrin and in this way could play a supporting role in the structure of venous thrombi.

Work from Wolberg AS and colleagues showed that transglutaminase factor XIII is critical for erythrocyte retention within venous thrombi and this directly affects thrombus size.[32] Moreover, they showed that FXIIIa-dependent erythrocyte retention in clots is mediated by fibrin α-chain crosslinking.[33] Wolberg and colleagues suggested that α-chain crosslinking results in increased clot stiffness and this could explain erythrocyte retention.[33] Besides fibrin crosslinking many other substrates for FXIIIa that are crosslinked to clots have been identified.[68] Interestingly, Hada *et al*. showed that FXIIIa also crosslinks VWF to fibrin and VWF is specifically crosslinked to the fibrin α-chain.[69] Based on these studies and the erythrocyte-VWF-fibrin complexes we described in **Chapter 3**, we would like to hypothesize that: in addition to increases in clot stiffness, FXIIIa-mediated fibrin α-chain crosslinking with VWF could mediate erythrocyte retention in venous thrombi. To test this hypothesis, it would be interesting to see whether differences in erythrocyte retention can be observed when clotting assays are performed with blood samples from WT mice and $VWF^{-/-}$ mice.

Summary and hypothetical model

To summarize, erythrocytes were thought to be innocent bystanders that become tangled up in the fibrin mesh of venous thrombi, but in this thesis, we describe that erythrocytes can bind to VWF and this interaction may contribute to the stabilization and propagation of a venous thrombus.

In contrast to arterial thrombi, venous thrombi develop in the absence of endothelial injury. We showed that non-sickle erythrocytes can adhere to endothelial cells, provided that (1) endothelial cells are

activated and (2) erythrocytes are exposed to a stressor that induces a calcium influx. Platelets also depend on increased cytosolic calcium levels for proper activation which could make this observation a similarity between arterial and venous thrombosis. We also described that erythrocytes adhere to ULVWF strings that are released from activated endothelial cells or to VWF that is immobilized on a surface. VWF as a substrate for cell adhesion is also a similarity between arterial and venous thrombosis, however platelets contribute to arterial thrombosis and erythrocytes contribute to venous thrombosis. Furthermore, we showed that erythrocytes preferentially bind to VWF and show only minor binding to fibrin(ogen), or other substrates. We also showed that the adhesion of erythrocytes to VWF increased significantly when the wall shear stress approached stasis. This fits with the difference between arterial thrombosis, which occurs at high wall shear stress and involves platelets, and venous thrombosis, which occurs at low wall shear stress and involves erythrocytes.

Erythrocytes do not express common receptors for VWF. Although the receptor for VWF remains unknown, we suggested two possible candidates: the Duffy antigen, based on the high amount of C-C and C-X-C motifs present in VWF, and sulfated glycolipids, because sulfatides have been shown to bind specifically and with high affinity to VWF. Compared to platelets, erythrocytes contain relatively low amounts of sulfatide in their membranes, which could explain why erythrocytes only adhere to VWF at a reduced wall shear stress. Moreover, it has been shown that short chain glycolipids are often cryptic in the plasma membrane of cells and can become more accessible under certain circumstances. This could explain why erythrocyte stimulation promotes adhesion to VWF.

A link between the ABO blood group system and VWF is known for a long time. ABO blood group influences plasma VWF:Ag levels by affecting VWF clearance rates and this depends on the ABO blood group status of the individual, but not on the blood group status of VWF itself. VWF-platelet binding is modulated by the ABO blood groups, but whether erythrocytes from different ABO blood groups show variation in their ability to bind to VWF is unknown. VWF adhering to circulating erythrocytes could contribute to differences in VWF:Ag, but the existence of circulating erythrocyte-VWF complexes is not yet proven. Binding to erythrocytes could potentially stabilize VWF in the circulation similar to the stabilization of FVIII by VWF, but could also promote venous thrombosis.

VWF-erythrocyte complexes could contribute to venous thrombosis at a pathologically low shear stress. We showed that erythrocytes, VWF, and fibrin show a striking pattern in human venous thrombi by forming erythrocyte-VWF-erythrocyte and erythrocyte-VWF-fibrin complexes. Other groups showed that transglutaminase factor XIII is critical for erythrocyte retention within venous thrombi and this directly affects thrombus size. FXIIIa-dependent erythrocyte retention in clots is mediated by fibrin α-chain crosslinking. Moreover, it was shown by other groups that FXIIIa can crosslink VWF to fibrin and VWF is specifically crosslinked to the fibrin α-chain.

This brings us to a final hypothetical model. Activated endothelial cells recruit leukocytes and platelets and release VWF from the Weibel-Palade bodies. At near static blood flow erythrocytes can adhere to VWF via sulfatides in their membranes. Simultaneously, leukocytes and platelets promote coagulation on the activated endothelial cells. While fibrin is formed, FXIIIa-mediated fibrin α-chain crosslinking enhances clot stiffening, but also promotes VWF crosslinking to fibrin α-chains. The combination of a

stiff clot, low shear stress, VWF crosslinked to fibrin, and an procoagulant environment that may activate erythrocytes and increases accessibility of sulfatides causes erythrocyte retention within the clot.

Future perspective

What the function of erythrocyte adhesion to VWF is, is yet unknown. However, our data and other recent developments show that erythrocytes are not the inert cells that only carry oxygen and carbon dioxide through our bodies without further interaction with their environment. As a next step, it is essential to define how important erythrocyte adhesion to VWF is in thrombogenesis. Questions that need to be answered are: Do erythrocyte-VWF complexes significantly contribute to (venous) thrombosis? How do erythrocytes bind to VWF? Are Duffy or sulfatides involved in the adhesion of erythrocytes to VWF? Is there a difference in erythrocyte-VWF binding between ABO blood groups? Do erythrocyte-VWF complexes determine plasma VWF levels and what does this mean for VWD type 1 diagnosis? And finally, can we modulate the erythrocyte-VWF interaction and thereby reduce erythrocyte retention in venous thrombi? Time will tell as both basic science and clinical studies will provide the necessary answers, but for now erythrocytes represent an interesting point of focus in thrombosis research.

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