Identification of Domains Responsible for von Willebrand Factor Type VI Collagen Interaction Mediating Platelet Adhesion under High Flow*

(Received for publication, May 28, 1998, and in revised form, August 21, 1998)

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We have identified type VI collagen (Col VI) as a primary subendothelial extracellular matrix component responsible for von Willebrand factor (vWF)-dependent platelet adhesion and aggregation under high tensile strength. Intact tetrameric Col VI was the form of the collagen found to be capable of promoting vWF-mediated platelet adhesion/aggregation under this shear condition, whereas removal of the predominant portion of the terminal globules by pepsin treatment abrogated its activity. The inability of the pepsin-digested Col VI to support any platelet interaction at high flow was because of the failure of the A3(vWF) domain to bind to this form of collagen, suggesting a stringent requirement of a tridimensional conformation or of intactness of its macromolecular structure. In contrast, the A1(vWF) domain bound to both intact and pepsin-digested Col VI tetramers but, in accordance with the cooperating function of the two vWF domains, failed to support platelet adhesion/aggregation under high shear onto Col VI by itself. The putative A1(vWF) binding site resided within the A7(VI) module (residues 413–613) of the globular amino-terminal portion of the a3(VI) chain. Soluble recombinant A7(VI) polypeptide strongly perturbed the vWF-mediated platelet adhesion to Col VI under high shear rates, without affecting the binding of the vWF platelet receptor glycoprotein Ibα to its cognate ligand A1(vWF). The findings provide evidence for a concerted action of the A1(vWF) and A3(vWF) domains in inducing platelet arrest on Col VI. This is accomplished via an interaction of the A1(vWF) and A3(vWF) domains with a site contained in the a3 chain A7(VI) domain and via a conformation-dependent interaction of the A3(vWF) domain with the intact tetrameric collagen. The data further emphasize that Col VI microfilaments linking the subendothelial basement membrane to the interstitial collagenous network may play a pivotal role in the hemostatic process triggered upon damage of the blood vessel wall.

Platelet adhesion to the exposed vascular subendothelial matrix proteins at the injury site is a crucial step in initiating the hemostatic and thrombotic processes. Hemodynamic forces play a significant role in the process of thrombus formation. There is a high shear stress opposing platelets adhesion, and they are forced toward the vascular lining surface and adhere at sites of vascular damage. Rapid formation of the initial platelet layer involves bridging between collagens and maybe other components (1–8) of the subendothelium on one side and platelet membrane receptors on the other side (GPIbα and GPIIbβ3). A pivotal role in this hemodynamic process is played by von Willebrand factor (vWF)1, which is the major adhesive protein mediating the bridging interaction as a function of shear forces. vWF, recognized by the platelet surface glycoprotein GPIbα, is particularly efficient in capturing platelets under high flow rates (9), and the participation of vWF in hemostasis is fundamental for thrombus formation under high shear stress conditions such as those present in small arteries or in pathological vessel affections such as stenosed arteries in arteriosclerosis. As extracellular matrix-assembled vWF of the subendothelium may not always be freely accessible to platelets in areas of rapid flow (10), the circulating vWF pool may be rather important in initiating platelet cohesion (11). Consistent with this idea is the observation that soluble plasma vWF binds rapidly and tightly to an underlying extracellular matrix, even when this latter is produced by fibroblasts (12). Moreover, high blood flow rates have been suggested to modulate the vWF release from the endothelium such as to provide additional available vWF for platelet interaction (13). High shear forces also induce a conformational transition in vWF, which converts it from a globular state to an extended chain structure. This structural transition is believed to result in the exposure of the intramolecular domains and in a reorientation of the polymers in the direction of the stress field (14).

The prevailing importance assigned to collagens in the initial steps of the vWF-mediated thrombogenic events has led to the pinpointing of two collagen binding sites in vWF: one located in the A1(vWF) and one in the A3(vWF) domains (15–17). However, the binding site contained within the A3(vWF) domain, rather than that of the A1(vWF) domain, has been proposed to

1 The abbreviations used are: vWF, von Willebrand factor; vWD, von Willebrand’s disease; rVWF, recombinant polypeptide of vWF; Col VI, type VI collagen; mAb, monoclonal antibody; GP, glycoprotein; BSA, bovine serum albumin; GuHCl, guanidine HCl.

2 According to the nomenclature proposed by Bork and Koonin (64) for the vWF type A domains (65).
be the predominant collagen binding site, at least for collagen type III (15, 18, 19). Possibly, this is a consequence of the fact that the Al(vWF) domain may also be engaged in the binding to the GPIbα, glycosaminoglycans, and sulfatides (16, 20). On the other hand, the Al(vWF) domain has been recently proposed to mediate vWF interaction with Col VI (21). Accordingly, in addition to the main fibrillar collagen types I, III, and V, previous studies have suggested that the microfibrillar Col VI could similarly be effective in stimulating a vWF-dependent platelet aggregation at low but not at high shear rates (6, 22, 23).

Our results are consistent with a pivotal role of Col VI in mediating vWF-dependent platelet cohesion at high shear forces and shed light on the complex mechanisms of Col VI-vWF-platelet interaction by unravelling the occurrence of a cooperative binding of the Al(vWF) and A3(vWF) domains of vWF to multiple sites of Col VI. Among these, one located within the A7(VI) vWFA module at the amino-terminal portion of the α3(VI) chain (amino acids 413–613) was found to be a critical A1(vWF) binding site required for optimal platelet adhesion/aggregation at high shear rates.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Specificities of the mouse monoclonal antibodies (mAbs) used in this study were as follows. mAbs LJ-IbI (24) and LJ-IbII (25) are directed against the platelet GPIbα; mAbs LJ-CP5 (26) and LJ-P5 (27) are against the αIIbβ3 integrin complex. mAb LJ-IbIII blocks binding of GPIbα to vWF, whereas mAb LJ-IbII displays only a minimal inhibitory effect on the binding of GPIbα to vWF but completely obliterates the α-thrombin binding to the same receptor. mAb LJ-CP8 binds the binding of the αIIbβ3 integrin to both vWF and fibrinogen, whereas mAb LJ-P5 selectively inhibits the binding of the αIIbβ3 integrin to soluble vWF but not to fibrinogen. mAbs NMC-4 and MR5 (kindly provided by L. Hoyer, Holland Laboratory, American Red Cross, Rockville, MD) react with the Al(vWF) domain (28) and the A3(vWF) domain (29, 30), respectively. mAb LJ-C3 is directed against the amino-terminal portion of vWF corresponding to residues 1–272 of the mature subunit, and it is known to inhibit the interaction of vWF with coagulation factor VIII (31). Purified IgG and F(ab')2 were prepared as described previously (27). All mAbs were used at saturating concentrations.

**Extracellular Matrix Molecules**—Intact tetramers of Col VI were purified from embryonic chick gizzard, human placenta, and adult bovine aorta by extraction in Tris-HCl, pH 7.6, with 6 M urea and protease inhibitors and separated by gel filtration chromatography on Sepharose CL-4B columns as described previously (32, 33). These Col VI preparations were analyzed by SDS-agarose gel electrophoresis and were found to be composed mainly of tetramers, with an estimated Mr of >2,000 kDa. Chick Col VI tetramers deprived of their amino-terminal globular domains and the predominant portion of the carboxy-terminal domains were produced by treatment of the purified intact Col VI with 1% (w/v) pepsin as described previously (34–37). Intact and pepsin-digested tetramers purified from human placenta following extraction with 6 M guanidine HCl were kindly supplied from Huey-Ju Kuo (The Shriners Hospital for Crippled Children, Portland, OR). Human vWF was purified from plasma obtained from healthy donors after informed consent by gel filtration chromatography on Sepharose CL-4B columns (Amersham Pharmacia Biotech) as described previously (37). The ristocetin cofactor activity in this purified vWF was determined to be 155 units/mg (38). The ristocetin cofactor activity in this purified vWF was determined to be 155 units/mg (38).

**Preparation of Recombinant Vascular Endothelial Growth Factor—**Recombinant human vascular endothelial growth factor (vascular EC50 '2n M); pepsin Col VI/vWF (EC50 '3 mM).

**Preparation of Col VI and vWF Substrates and Flow Chamber Assembly**—Intact and pepsin-digested Col VI tetramers were dissolved in 0.05 M bicarbonate buffer, pH 9.6, to 5–100 μg/ml and coated onto a central area of glass coverslip (24 × 50 mm, 100 μl of Col VI solution/coverslip), that was delimited by a 15-mm silicon ring (Flexiperm-Disc Heraeus Instruments). The amount of immobilized collagen (either chick or human) was estimated by independent coating with [125I]-labeled Col VI and was determined to range between 0.15 and 4.36 μg/cm2 for the coating concentrations used. In experiments in which vWF was used in immobilized form, coverslips were coated with 100 μl of vWF at 100 μg/ml in 0.04 M phosphate buffer, pH 7.4, containing 0.15 M NaCl. Coated coverslips were placed in a humid chamber at 4 °C overnight, followed by washings in phosphate-buffered saline and saturation with 1% BSA in 20 mM Tris-HCl, pH 7.4, with 0.15 M NaCl for 60 min at room temperature. Saturated coverslips were then assembled in a parallel-flow chamber (modified Richardson’s flow chamber) (31), which was then filled with isotonic saline. A syringe pump (Harvard Apparatus, Boston, MA) was used to aspirate the fluid through the chamber at a constant flow rate for 1–10 min before being perfused with platelet-containing solutions. Flow rates utilized were 0.13, 0.26, 0.53, 2.66, 5.33, 7.99, and 10.66 ml/min, which produced 25, 50, 100, 500, 1,000, 2,500, and 5,000 s wall shear stresses, respectively. Before being tested, blood samples were incubated at 37 °C for 30 min as to re-equilibrate the system to physiological temperature.

**Blood Sampling**—Blood from healthy volunteers and from a patient affected by a severe form of vWD (less than 1% of ristocetin cofactor and undetectable or barely visible vWF multimers after SDS-polyacrylamide gel electrophoresis) was obtained after informed consent. All donors were healthy individuals, free of current medication, and were not taking aspirin or nonsteroidal anti-inflammatory drugs for at least 2 weeks before blood sampling. Blood was collected from the antecubital vein through a 18-gauge needle into syringes containing 400 units/ml (final concentration) thrombin inhibitor hirudin (Iketon, Italy) as anticoagulant.

**Preparation of Platelets**—Platelets were prepared by a modification of a previously described method (41). To prevent unwanted platelet activation, apyrase III (Sigma), an ADP scavenger, was added to the blood samples at a final concentration of 10 units/ml. Blood samples were divided into 5-ml aliquots and centrifuged at 800 × g for 14 min, the plasma was removed, and the sedimented cells, including platelets and leukocytes residing at the top of the erythrocyte cushion, were resuspended in divalent cation-free HEPES-Tyrode buffer (10 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 0.4 mM Na2PO4, 10 mM NaHCO3, and 5 mM dextrose), pH 6.5, containing 5 units/ml apyrase (final concentration) and centrifuged at 800 g for additional 14 min. This procedure was repeated four times, and after the last centrifugation cycle, the pellet was resuspended in HEPES-Tyrode buffer containing 2 mM CaCl2, 2 mM MgCl2, and 1% BSA (BSA buffer). In some experiments, 10 μM POE (Sigma) was added to the platelet suspension to prevent platelet activation. Platelets and leukocytes counts ranged from 1–1.5 × 109/ml and from 4–6 × 109/ml, respectively, and the hematocrit ranged from 44–48%.

**Platelet Aggregation Assay**—Platelet-rich plasma from whole blood, obtained from healthy donors after informed consent, was prepared by dilution 1:6 (v/v) into citric acid/citrate/dextrose, pH 4.5, and differential centrifugation. Platelets were washed free of plasma constituents.
platelet adhesion at high shear rates although interference with \( \alpha IIb \beta 3 \) integrin receptor by antibody mAb LJ-P5 (not shown) and LJ-CP8 (Figs. 2A) caused a substantial abrogation of platelet arrest and the subsequent tethering and aggregation. However, interference with \( \alpha IIb \beta 3 \) activity did not impede the vWF-induced transient contact of platelets with the substrate, which was noticed to be of variable duration. Thus, at each given time point of analysis, a certain coverage of the substrate area under consideration was ob-

![Image](321x237 to 541x729)

**Fig. 1. Platelet adhesion to intact Col VI tetramers.** A, dose-dependent platelet adhesion to intact tetramers of Col VI in flow conditions. Whole blood containing recombinant hirudin as anticoagulant and treated with the fluorescent dye mepacrine to label flowing platelets was perfused through a parallel flow chamber at 37 °C under the indicated shear rates. Data points report the percentages of surface coverage by platelets in an area of 0.037 mm\(^2\) after 1 min of perfusion in a representative case. B, effect of wall shear rate on platelet adhesion to intact Col VI. Percentage of surface coverage as a function of perfusion time is shown at 1,000 s\(^{-1}\) (gray circles) and 2,000 s\(^{-1}\) (black squares) shear rates. These data are representative of three separate experiments that gave similar results and were obtained with the same blood sample employed for experiments described in A, C, real time observation of platelet adhesion and aggregation onto Col VI tetramers: effect of large globular domain destruction. Representative single-frame images showing direct comparisons of platelet adhesion and aggregation onto intact and pepsin-digested tetramers of Col VI under conditions of high and low shear rate and when perfused in whole blood.
served. In accordance with the prerequisite of a platelet GPIIb-αIIbβ3 interaction to bring through thrombus formation under high tensile strength (9, 11, 44), platelet adhesion/aggregation was completely blocked by mAb LJ-Ib1 (Fig. 2A), known to perturb the GPIIb binding to vWF. mAb LJ-Ib10 specifically interfering with the GPIIb binding to α-thrombin did not have any effect, ascertaining the specificity of the GPIIb interaction with vWF in the system. In analogy, no platelet adhesion was observed with vWF-deficient blood from a patient with severe vWD (Fig. 2A) or when GPIIbα function was inhibited with 5 μM rvWF(445–733) polypeptide, a recombinant containing A1(vWF) domain responsible for GPIIb binding site of vWF (Fig. 2A).

To further define the shear rate-related involvement of soluble vWF in cases when flowing platelets were confronted with an underlying Col VI substrate, platelets were perfused at different shear forces, ranging from those found in larger veins (e.g. 25 s⁻¹) to those found in small arterioles (e.g. 1,500–2,000 s⁻¹), in the presence or absence of the anti-GPIIbα antibody LJ-Ibl. These experiments demonstrated that, contrary to what was previously reported for collagen type I (and possibly also type III), Col VI supported vWF-dependent platelet adhesion/aggregation down to a shear force of about 100 s⁻¹ (Fig. 2B). Shear rates of 50 s⁻¹ or lower still promoted some platelet adhesion and aggregation, which, however, was independent of the participation of vWF (Fig. 2B).

Because there was no significant change in the surface coverage above 1,000 s⁻¹, all subsequent high shear rate experiments were carried out under these conditions. The results obtained after blockage of the vWF-GPIIbα interaction underscored that vWF was an active component in the system but did not rule out a possible cooperation between vWF and other blood factors. We therefore perfused washed platelets in a BSA-containing Tyrode buffer suspension of red and white cells in which increasing amounts of purified vWF were added. In these cases, a substantial platelet adhesion and aggregation on Col VI was noted in a manner that was both dose-dependent and saturable (Fig. 3). Depending upon the amount of vWF added, microaggregates, small and large aggregates (see “Experimental Procedures”) could be observed, with the formation...
of the large aggregates starting at a vWF concentration of 2.5 µg/ml (Fig. 3). Apart from promoting platelet tethering to a molecular or cellular substrate, vWF has also been suggested to mediate the subsequent platelet-platelet interaction leading to αIIbβ3 integrin-dependent aggregate formation. To establish whether this was the case also on a Col VI substrate, washed platelets were similarly perfused in the presence of mAb LJ-P5, known to inhibit only the αIIbβ3 integrin binding to vWF. In such conditions, single platelets could still be detected on the Col VI substrate, but no aggregates formed (not shown).

**Identification of the vWF Domains Responsible for Platelet Interaction with Col VI and of the vWF Binding Sites on Col VI**—To identify the vWF domains responsible for platelet adhesion and aggregation on Col VI and to determine their relative importance, we utilized mAbs directed against the vWF domains A1(vWF), A2(vWF), A3(vWF), and A7(vWF). In solid-phase binding assays (i.e. static conditions) with no platelets, mAbs NMC-4 and MR5 inhibited the molecular interaction of vWF to intact Col VI tetramers to about 75 and 60%, respectively, whereas when added together, the inhibitory action was complete (Fig. 4A). Conversely, the addition of the anti-A1(vWF) and anti-A3(vWF) mAbs to platelets perfused at high shear rates abrogated adhesion, irrespectively of the mAb added, whereas addition of the functionally unrelated mAb LJ-C3 did not disturb platelet adhesion (Fig. 4B). These findings indicated that binding of both A1(vWF) and A3(vWF) to Col VI was a prerequisite for optimal vWF-mediated platelet-Col VI interaction at high shear rates. Because mAb NMC-4 also interferes with the GPIbα binding to the A1(vWF) domain, the complete blockage of platelet adhesion/aggregation observed after addition of this mAb could be attributed to a dual blockade of the Col VI and GPIbα receptor binding to the A1(vWF). Thus, these observations indicated that the vWF-dependent platelet interaction with Col VI at high shear rates required integrity of its terminal globular domains. Intriguingly, however, when the molecular interaction of vWF with pepsin-digested Col VI tetramers was examined in solid-phase binding assays under the inhibitory influence of the anti-A2(vWF) domain antibodies, it was found that only the anti-A1(vWF) mAb NMC-4 was capable of abolishing the interaction (Fig. 4A). This finding demonstrated that despite the reported ability of the A3(vWF) domain to interact with interstitial collagens, this domain failed to contribute to binding of vWF to the triple-helical region of Col VI.

To identify the vWF binding sites within the globular regions of Col VI, which could account for the binding activity exceeding that given by the collagenous region, we employed amino-terminal α3(VI) chain recombinant polypeptides (Fig. 5A) both in static and dynamic phase. In static phase, about 70% of the maximal vWF binding activity displayed by the intact tetramers was detected for the recombinant Col VI polypeptide pB10, encompassing almost the entire amino-terminal portion of the α3(VI) chain, *i.e.* the A8-A3(VI) modules. The prevailing binding activity of polypeptide pB10 could further be pinpointed to the A7(VI) module, as determined by direct binding of purified vWF to the polypeptide and the ability of this polypeptide, but not other A(VI) polypeptides, to compete for the binding of vWF to intact Col VI tetramers (Fig. 5B). The competition ability of soluble A7(VI) was dose-dependent, and the residual binding could be completely abrogated by simultaneous addition of the anti-A3(vWF) mAb MR5 (Fig. 5C). Further evidence for a specific role of A7(vWF) domain(s) in binding to the amino-terminal globular domain of Col VI was provided by the elected ability of the anti-A1(vWF) mAb NMC-4 to block the binding of vWF to pB10 and A7(VI) (Fig. 5D). In contrast to the A1(vWF) domain, the A3(vWF) failed to interact with the isolated region of the α3(VI) chain, as demonstrated by the lack of inhibition of the vWF binding to α3 recombinant polypeptides in the presence of anti-A3(vWF) antibodies (Fig. 5D). This finding suggested that the interaction site(s) of the A3(vWF) domain within the Col VI globular region was conformation-dependent and most likely required intactness of the quaternary structure of the fully assembled tetramer.

**Functional Identification of the vWF Binding Sites on Col VI**—The complete blocking effect exerted either by the anti-A1(vWF) or anti-A3(vWF) antibodies on the vWF-dependent platelet adhesion to intact Col VI tetramers at high shear rates suggested that both vWF domains could be equally important in mediating the molecular interaction (Fig. 4B). However, the
bivalency of the blocking effect caused by the anti-A1(vWF) mAb NMC-4, which affects both the binding to GPIbα and to Col VI, precluded the possibility of determining the reciprocal role of the two vWF domains in these experiments. We therefore analyzed the ability of the soluble A7(VI) polypeptide to prevent platelet adhesion to intact Col VI tetramers under high shear rates in the presence of soluble or immobilized vWF. In experiments in which washed platelets were perfused over intact Col VI tetramers at high shear rate in the presence of soluble vWF, the time-dependent platelet adhesion to the collagen substrate was markedly perturbed by the additional presence of competing soluble A7(VI), whereas the A6(VI) polypeptide had only a marginal effect (Fig. 6A). However, soluble A7(VI) polypeptide did not affect the ristocetin-induced platelet agglutination-aggregation in the simultaneous presence of the control antibody against the factor VIII binding domain of vWF (mAb LJ-C3) or the recombinant A6(VI) polypeptide (Fig. 7). In contrast, the addition of either of the anti-A1(vWF) and anti-A3(vWF) antibodies (mAbs NMC-4 and MR5, respectively) or the A7(VI) recombinant polypeptide strongly perturbed platelet adhesion to Col VI (Fig. 7). In the experimental situation in which platelets were perfused over immobilized vWF, solely the anti-A1(vWF) antibody NMC-4 was capable of blocking the platelet-vWF interaction. This finding indicated that neither the anti-A3(vWF) antibody nor the A7(VI) interfered with the GPIbα-A1(vWF) interaction, and hence the inhibitory effect was exerted exclusively on vWF-Col VI interaction.

DISCUSSION

In this study we provide definite evidence that Col VI may be a primary subendothelial ligand for vWF mediating the high shear rate-induced platelet adhesion and aggregation at sites of vascular injury. Binding of both the A1(vWF) and A3(vWF) domains to immobilized urea-extracted Col VI tetramers is essential for initiating the platelet adhesion/aggregation cascade, implying that none of the individual domains alone is capable of supporting platelet tethering and aggregation under high tensile strength. Although the A3(vWF) domain solely binds to the Col VI tetramers in which the terminal globular

FIG. 5. vWF binding to Col VI recombinant polypeptides of α3(VI) chain. A, schematic diagram illustrating the module organization of the α3(VI) chain, adopting the nomenclature of gene chick polypeptide (39), and the relative extension of the recombinant polypeptides produced from the corresponding human cDNAs. B, binding of vWF to immobilized recombinant Col VI polypeptides (vWF bound), when assessed as the percentage of bound protein in comparison to the amount bound to intact Col VI tetramers and binding of vWF to immobilized intact tetramers in the presence of soluble recombinant Col VI polypeptides (vWF displaced). In this latter case, values refer to percentage inhibition of vWF binding to Col VI by the various soluble competitors. Fibrinogen was used in both immobilized and soluble phase as a control protein. C, dose-dependent inhibition of vWF binding to immobilized intact tetramers by soluble A7(VI) recombinant polypeptide. At saturation of inhibition, further addition of the anti-A3(vWF) antibody MR5 completely abrogated the residual binding. D, binding of vWF to immobilized recombinant Col VI polypeptides in the presence of antibodies as in Fig. 4. Data represent mean ± S.E. from three independent experiments.
domains have been retained intact, the A1(vWF) domain interacts with both the triple-helical region and the amino terminus of the α3(VI) chain, and its binding is independent of the quaternary structure of the collagen (Fig. 8). A primary binding site of the A1(vWF) domain could be identified within the constitutively expressed A7 vWFA module of the α3(VI) chain (33) and shown to be distinct from that involved in the binding of the A1(vWF) domain to the platelet receptor GPIba (Fig. 8).

Although the participation of vWF in the high shear rate-induced platelet aggregation upon damage of the blood vessel wall is a critical step in aggregate formation, the ligands that favor optimal immobilization of the circulating globular vWF remain to be identified. A vast number of previous studies have suggested that interstitial collagen types I and III (and to a lesser extent, V) could be the candidate ligands. However, their localization in the connective tissues significantly below the zone interfacing the subendothelial basement membrane and the interstitium raises some doubts about their physiological relevance in the early phases of vascular repair. Instead, the intimate association of Col VI microfilaments with the basement membrane on one hand (46) and the interstitial collagen fibrils on the other (47) strongly supports an important role for this collagen in binding the circulating vWF at the sites of vascular injury. Furthermore, the colocalization of Col VI and vWF in the vascular subendothelium (48, 49), recently confirmed at the ultrastructural level (50), strongly points to the
possibility that Col VI may represent a central component of the subendothelial matrix contributing to platelet aggregation upon rupture of the blood vessel wall. However, previous studies utilizing pepsin-digested and/or guanidine HCl-extracted Col VI tetramers have failed to demonstrate a significant role for this collagen under conditions of high shear rates (23). At present, the nature of the discrepancy between urea- versus GuHCl-extracted Col VI is not clear, nor was it investigated in detail here, but several explanations may be considered. The loss of platelet aggregation-promoting activity of the GuHCl-extracted collagen may be attributed to a more severe unfolding of the molecule by exposure to GuHCl, or inhibitory contaminants may be present in the Col VI preparation based on GuHCl extraction. One such possible contaminant may be the proteoglycan decorin noted to remain associated with Col VI tetramers under such purification procedures (47). Finally, it remains possible that urea but not GuHCl extraction brings out a reactivity that does not occur in the native structure.

The mode and extent of platelet adhesion/aggregation onto Col VI at high shear rates was found to be similar to that reported for collagen type I (15, 51) and was strictly proportional to the amount of vWF that associated with the collagen substrate. Our findings are in disagreement with those previously reported and implicating the α2β1 integrin in the direct arrest and tethering of platelets on Col VI under high shear rates (6, 52) but do not preclude the possibility that both this receptor and glycoprotein VI (53) may be responsible for the transduction of specific intracellular signals essential for further activation of platelets, following the initial vWF/GPIbα-dependent contact with the collagen substrate.

Crystallographic analysis of the wild type and point-mutated A3(vWF) domain reveals that, opposite to the I domain of the α2β1 collagen binding integrin, disruption of the vestigial MIDAS motif of the A3(vWF) domain does not affect collagen binding (54, 55). Because the α2β1 integrin has an elected preference for the triple helix of collagens, the above finding is overtly in accord with our observations that the A3(vWF) domain is not involved in the vWF interaction with the triple-helical region of Col VI. Accordingly, the Col VI interaction site of the A3(vWF) domain may reside within the globular domains of the collagen and/or be dependent upon a specific macromolecular structure assumed by the intact, but not the pepsin-digested tetramer deprived of globular domains. This conclusion is in accordance with the A3(vWF)-mediated collagen type I-vWF interaction for which there is a strict requirement of a higher order-organized fibrillar structure to achieve maximal promotion of platelet aggregation (8). Nonetheless, the marked difference in macromolecular configuration between interstitial collagens and the microfilamentous Col VI indicates that the structural nature of the A3(vWF) binding sites must differ in these molecules. Yet, the versatility of this binding site is highlighted by the indiscriminate ability of the A3(vWF) domain to interact with fibrillar collagen type I, monomeric type III, and tetrameric Col VI. The A3(vWF) domain has recently been shown to contain a binding site(s) for both interstitial collagens (15, 19, 21, 54) and the basement membrane collagen type IV (21) but has been proposed to lack binding affinity for Col VI, for which a pivotal binding role has been attributed to the classical collagen-binding A1(vWF) domain (21). Our experiments, in static phase using intact Col VI tetramers purified from three different tissues avoiding the use of GuHCl, revealed that the A1(vWF)-Col VI binding accounted for about 60% of the interaction. Moreover, blockade of either the A1(vWF)-Col VI or the A3(vWF)-Col VI interaction demonstrated an essential role for both A(vWF) domains in promoting the high shear rate-induced platelet adhesion/aggregation.

Thus, our results are in disagreement with those of Hoylaerts et al. (21) and may reconcile to subtle structural alterations in the Col VI tetramers deriving from diverse extraction/purification procedures. These alterations may similarly be central for the differential loss of the platelet adhesion-promoting activity at high shear rates (23).

Mapping studies based upon the combined usage of pepsin-digested Col VI tetramers, recombinant polypeptides corresponding to the noncollagenous globular regions of the α3(VI) chain, and anti-A1(vWF)/A3(vWF) antibodies were instrumental in localizing the main A1(vWF) binding site within the globular portion of the Col VI molecule. This site could be identified within the A7(VI) module. This module inhibited platelet adhesion to the collagen under high flow conditions and, in contrast to the recombinant polypeptide rvWF(445–733) and to the anti-A1(vWF) antibody NMC-4, did neither affect the GPIbα-vWF binding nor interfere with the A3(vWF)-Col VI interaction. It has previously been reported that in the acquired autoimmune disease vWD, circulating autoantibodies reacting with both the A1(vWF) and A3(vWF) domains may be present that inhibit the vWF-collagen interaction without affecting the ristocetin-induced GPIbα-vWF interaction (57). Similarly, a number of experiments using recombinant mutated vWF polypeptides as well as studies on individuals carrying genetic mutations that result in substitutions of amino acids critical for the GPIbα-A1(vWF) interaction have also suggested that these two A1(vWF) binding functions can be attributed to disparate binding motifs (15, 18, 58–60). Recent crystallographic analysis of A1(vWF) (61) and A1(vWF)-NMC-4 Fab complex (62) provide structural evidence for a distinct localization of GPIbα and other functional sites in the crystal. These findings and our data suggest that the A1(vWF) binding sites for the platelet receptor GPIbα and collagens might be distinct.

In conclusion, we propose that to achieve maximal efficiency of vWF in initiating platelet adhesion onto Col VI under conditions of high shear rates, both A1(vWF) and A3(vWF) are necessary. Their concerted participation would provide the biomechanical conditions supporting reversible GPIbα-dependent platelet adhesion, αIbβ3 activation and its binding to the RGD sequence of vWF, and platelet arrest onto the Col VI. In the setting of vascular lesions, irreversible platelet adhesion to Col VI might enhance the platelet activation response and aggregate formation followed by the activity of αIbβ3, α2β1, and GP VI (63). Thus, our findings provide new prospects for the understanding of the molecular mechanism responsible for platelet adhesion phenomena and open new avenues for exploring the possible relevance of functional defects in the A1(vWF) and A3(vWF) domains of vWD and for developing new antithrombotic drugs.

Acknowledgments—We are indebted to Drs. Zaviero Ruggeri and L. W. Hoyer for providing monoclonal antibodies and recombinant vWF polypeptides and for helpful suggestions. Dr. Roberto Doliama and Bruna Wassermann are thanked for their assistance in the preparation of recombinant Col VI polypeptides, and Maria Teresa Mucignat is thanked for her supporting technical assistance.

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