von Willebrand factor binds to native collagen VI primarily via its A1 domain

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Collagen VI is abundant in the arterial subendothelium. To investigate its mechanism of interaction with von Willebrand factor (vWF), collagen VI was isolated from human placenta and from the extracellular matrix of the human lung fibroblast cell line MRC-5. Purified vWF bound to non-digested collagen VI with moderately high affinity ($EC_{50} \approx 5$ nM) and could be inhibited by the *Hirudo medicinalis* collagen inhibitor calin. The anti-(human vWF A1 domain) monoclonal antibody (AJvW-2), as well as aurin tricarboxylic acid (ATA), at concentrations that saturate the vWF A1 domain, also inhibited this binding. In contrast, the monoclonal anti-(human vWF A3 domain) antibody (82D6A3) inhibited vWF binding to collagens I, III and IV, but had no effect on vWF binding to collagen VI. Likewise, vWF binding to collagen VI was not inhibited by the recombinant vWF domain D4. Polyclonal anti-(collagen VI) antibodies,

specifically neutralizing the binding of vWF to collagen VI, confirmed that in the intact endothelial cell extracellular matrix, collagen VI was accessible for interaction with vWF. This binding was only marginally affected by 82D6A3 but was dose-dependently inhibited by AJvW-2, ATA and the A1 domain analogue VCL (recombinant A1 domain of vWF), with IC_{50} values comparable to those found for the inhibition of vWF binding to isolated collagen VI. The weak interaction of isolated human platelets with collagen VI was mediated via the platelet collagen receptor (GPIa/IIa) and was competitively inhibited by vWF but not by VCL, suggesting that vWF and GPIa/IIa bind to neighbouring but distinct sites on collagen VI. We conclude that vWF binds to collagen VI primarily via its A1 domain, which distinguishes it from the vWF A3 domain-mediated binding to fibrillar collagens.

INTRODUCTION

As a consequence of vessel wall injury, thrombogenic subendothelial structures are exposed to circulating blood and trigger platelet adhesion and thrombus formation [1,2]. In addition to the direct binding of platelets to exposed collagen fibres via their main collagen receptor, the glycoprotein complex GPIa/GPIIa [3], platelet adhesion to the vascular subendothelium is also mediated via von Willebrand factor (vWF) [4], which interacts with a receptor on the platelet surface, i.e. the GPIb/IX/V protein complex, an interaction that leads to platelet activation and subsequent thrombus formation [4–6]. At high shear forces, vWF present in the subendothelium has been estimated to account for > 50% of total platelet adhesion, the residual binding being mediated via circulating and platelet vWF [6,7].

A study in parallel-plate flow chambers of the adhesion of platelets to subendothelium and to extracellular matrices produced by cultured cells [8] has shown that vWF mediates binding of platelets to several types of fibrillar collagens, and has identified the presence in subendothelium of a vWF receptor, different from collagen I and III [9]. Rand et al. have identified collagen type VI, abundant in human vascular subendothelium, as a putative receptor for vWF [10], and have confirmed by immunohistochemical investigations that, in human vascular subendothelium, vWF is found in association with collagen VI microfibrils [11].

Collagen type VI, found in most connective tissues including cartilage, might play a role in anchoring the basement membrane of non-epithelial cells to the underlying connective tissue, but also in bridging collagen I and III fibrils with a network of microfilaments [12]. Collagen VI is made up of three genetically distinct subunits: the $\alpha 1(VI)$ (M_r 150 000) and $\alpha 2(VI)$ (M_r 140 000) chains contain a short, 105 nm, triple helix flanked by N- and C-terminal globular modules, consisting of one and two repeated modules respectively of approx. 200 amino acid (aa) residues. The larger size of the $\alpha 3(VI)$ chain (M_r 250 000–300 000) stems from additional aa in the C-terminal end and is due to the presence of a very extended N-terminal region of approx. 1600 residues [13]. This region consists of at least eight repetitive 200-aa-long modules sharing sequence homology with vWF A domains [13,14].

Denis et al. [15] have reported that the dimeric T116 fragment of vWF, corresponding to the vWF aa residues 449–728, and comprising the entire vWF A1 domain, was capable of blocking vWF binding to human endothelial cell matrix (ECM). Furthermore these authors found that the SpI fragment of vWF (aa 911–1365) bound to ECM and collagen VI, but found no interaction for the C-terminal vWF segment (aa 1366–2050). In contrast, de Groot et al. [9] described a reactive monoclonal antibody (CLB-Rag 38) with an epitope located in the vWF C-terminal region, that could inhibit the binding of vWF to ECM. In this study, we have therefore investigated in more detail the contribution of several vWF domains to the binding of vWF to

Abbreviations used: vWF, von Willebrand Factor; ATA, aurin tricarboxylic acid; AJvW-2, monoclonal anti-(human vWF A1 domain) antibody; 82D6A3, monoclonal anti-(human vWF A3 domain) antibody; VCL, recombinant A1 domain of vWF; aa, amino acid(s); ECM, endothelial cell matrix; GdmCl, guanidinium chloride; DMEM, Dulbecco's modified essential medium; FCS, foetal calf serum.

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isolated human collagen VI [16] and to the extracellular matrix of endothelial cells. Furthermore, a recent study reported that vWF can mediate platelet adhesion to collagen VI even at low shear forces [17], so we have also investigated the role of vWF in the interaction between isolated platelets and collagen VI.

MATERIALS AND METHODS

Isolation of vWF

vWF was isolated from human plasma cryoprecipitate by reprecipitation and bentonite-mediated fibrinogen depletion, followed by gel filtration on a Sepharose 4B-CL column (2.6×95 cm) as previously described [18]. On reducing SDS/PAGE, the isolated vWF showed a single band. The interaction between isolated vWF and human glycoprotein Ib (GPIb) was studied via an ELISA procedure, in which the botrocetin-mediated vWF binding to microtitre plate-coated GPIb was monitored by means of a polyclonal anti-vWF antiserum (Dako, Copenhagen, Denmark) [19].

Isolation of human collagen VI

Pepsin-digested collagen type VI was isolated from human placenta following extraction of minced and washed human placenta with 6 M guanidinium chloride (GdmCl) dissolved in 50 mM Tris/HCl buffer, pH 7.5, in the presence of aprotinin (100 units/ml) and 1 mM PMSF. This was followed by an acidic digestion with pepsin as described by Trüeb et al. [20]. Non-digested collagen type VI was prepared by ion-exchange chromatography of the GdmCl extract of human placenta on Q-Sepharose (Pharmacia, Uppsala, Sweden) upon dialysis against 50 mM Tris/HCl buffer, pH 8.0, containing 6 M urea and elution by using a gradient of NaCl (0–0.3 M) in this buffer, as described by Jander et al. [21] for the isolation of bovine collagen type VI. This was followed by gel filtration of collagen VI-containing fractions on Sepharose 4B-CL (Pharmacia). Collagen type VI-containing fractions, eluting in the void volume, were then dialysed against 50 mM acetic acid and freeze-dried. Collagen type VI was traced in each fraction by overnight coating at 4 °C on microtitre plates ($50 \mu\text{l} + 200 \mu\text{l}$ of 0.1 M Tris/HCl buffer, pH 8.0, per well) and incubation with a commercial rabbit anti-(collagen VI) antiserum (Chemicon, Temecula, CA, U.S.A.) (1/1000, v/v), and goat anti-(rabbit Ig) antibodies conjugated to horseradish peroxidase respectively following a classical ELISA procedure. Human collagen VI was also isolated from the lung fibroblast cell line MRC-5, a rich source of collagen VI [22,23] not expressing vWF. Cells, grown to confluency in Dulbecco's modified essential medium (DMEM), containing 10% (v/v) foetal calf serum (FCS), were removed by lysis for 5–10 min with 0.1 M NH_3 , and the residual extracellular matrix was extracted with 6 M GdmCl dissolved in 50 mM Tris/HCl buffer, pH 8.0, containing 0.1% (w/v) $\text{Na}_2\text{S}_2\text{O}_5$ /0.1 M ϵ -amino caproic acid/5 mM benzamidine/2 mM *N*-ethylmaleimide/2 mM EDTA/0.2 mM Pefabloc (non-toxic substitute for PMSF)/100 mM NaCl. Extracts were then concentrated by dialysis against water and freeze-drying, and gel-filtered on Sephacryl S-500 ($2.6 \text{ cm} \times 95 \text{ cm}$) in the extraction buffer. During these experiments, collagen VI was also detected with the monoclonal anti-(collagen VI) antibody 5C6 (kindly given by Dr. E. Engvall, La Jolla, CA, U.S.A.) conjugated to horseradish peroxidase and by staining with *o*-phenylenediamine. In some preparations, gel-filtered collagen VI was further purified by ion-exchange chromatography on Q-Sepharose in urea as mentioned above.

Antibody production, isolation and characterization

Polyclonal antisera were produced in rabbits, to both the purified non-digested and the pepsin-digested collagen VI. The specificity of these antisera was investigated by incubation on microtitre plates coated with collagens I, III and IV, or with native or pepsin-digested collagen VI ($25 \mu\text{g}/\text{ml}$ in 50 mM acetic acid), and detection of bound antibodies. In addition, the anti-(pepsin-solubilized collagen VI) antibodies were validated in Western blots on purified non-digested collagen VI. The specificity of the antisera was further validated by means of metabolic labelling studies during which endothelial cells were grown for 2 h in DMEM lacking FCS and then for an additional 24 h in the presence of 0.5% FCS and $50 \mu\text{Ci}$ [^{35}S]Met/ml [22]. The medium was then immunoprecipitated by different anti-(collagen VI) antisera (1/200, v/v) for 3 h at 37 °C and goat anti-(rabbit Ig) antiserum overnight ($200 \mu\text{g}/\text{ml}$) at 4 °C, and immunoprecipitates were subjected to SDS/PAGE and autoradiography.

Rabbit anti-(pepsin-solubilized collagen VI) antibodies and monoclonal anti-vWF [monoclonal anti-(human vWF A3 domain) (82D6A3) and monoclonal anti-(human vWF A1 domain) antibody, AJvW-2] or anti-(collagen VI) (5C6, 4B10) antibodies were purified from murine ascites by Protein A chromatography.

Production of endothelial extracellular matrix

ECM was obtained by growing passage II human umbilical vein endothelial cells till confluency on 96-well microtitre plates, in DMEM, containing 20% (v/v) human serum. After treating the plates with 0.1 M NH_3 for 5–10 min and rinsing with PBS, they were stored at -20 °C until use. Only microtitre plate cultures were selected containing low levels of ECM-associated vWF, i.e. yielding A_{492} values at least 3 times lower than those observed following the binding of vWF ($1 \mu\text{g}/\text{ml}$) to these plates.

Static binding assays

vWF binding (0 – $20 \mu\text{g}/\text{ml}$) to microtitre plates coated with collagen VI ($20 \mu\text{g}/\text{ml}$) and to ECM-containing microtitre plates, blocked with 1% (w/v) BSA in PBS, was studied at room temperature. After incubations for 60 min, bound vWF was detected by peroxidase-conjugated rabbit anti-(human vWF immunoglobulins) (Dako). During competition studies, vWF ($3 \mu\text{g}/\text{ml}$) binding to human collagens type I, III, IV and VI purified from placenta (Sigma) and to the extracellular matrix derived from endothelial cells was studied in the presence of isolated collagen VI (0 – $20 \mu\text{g}/\text{ml}$), the vWF fragment Leu³⁰⁴-Ser⁷²⁸ [24] [recombinant A1 domain of vWF (VCL), 0 – 200 nM], aurin tricarboxylic acid (ATA; Sigma, St. Louis, MO, U.S.A.; 0 – $300 \mu\text{M}$), the anti-(vWF domain A3) antibody 82D6A3 (0 – $20 \mu\text{g}/\text{ml}$) produced in our laboratory, the anti-(vWF domain A1) antibody AJvW-2 [25] (Ajinomoto Co, Yokohama, Japan; 0 – $20 \mu\text{g}/\text{ml}$), the collagen inhibitor calin (Biopharm, Sussex, U.K.), derived from the saliva of the leech *Hirudo medicinalis* [26] (0 – $100 \mu\text{g}/\text{ml}$) and the vWF-derived D4 domain (0 – $50 \mu\text{g}/\text{ml}$), and the monoclonal anti-(human vWF D4 domain) antibodies RU9, RU10, RU11 and RU12 (0 – $20 \mu\text{g}/\text{ml}$). The VCL fragment was kindly given by Dr. Garfinkel, Biotechnology General, Rehovot, Israel, and the vWF-derived D4 domain and the anti-D4 monoclonal antibodies RU9–RU12 were generously provided by Dr. Sixma, University Hospital, Utrecht, The Netherlands. In addition, collagen VI-coated and ECM-containing microtitre plates were pre-incubated with various dilutions of anti-(collagen VI) antisera (1/100–1/12800, v/v) or Protein A-purified anti-(collagen VI) antibodies (0 – $300 \mu\text{g}/\text{ml}$). Following washing, vWF

(3 $\mu\text{g/ml}$) was added, and bound vWF was detected as indicated above.

Platelet adhesion to collagen VI and ECM

Human platelets were isolated from antecubital vein blood collected on acid dextrose citrate buffer and washed twice by centrifugation and resuspension in acid dextrose buffer, followed by a final resuspension in 50 mM Tris/HCl buffer, pH 8.0. Platelets were then incubated at 400 000/ μl on microtitre plates coated with collagen VI or containing ECM (150 $\mu\text{l/well}$) for 2 h at room temperature. Bound platelets were detected by the addition of 10 mM *p*-nitrophenyl phosphate in citrate buffer [27] and stained for 2 h at room temperature. Following addition of 50 μl of 2 M NaOH, A_{405} was read and corrected for specific binding by comparison with platelet deposition in non-coated wells. Platelet binding was studied in the presence of increasing concentrations of the RGD-containing cyclic peptide G4120 (Genentech, San Francisco, CA, U.S.A.; 0–0.5 $\mu\text{g/ml}$), 82D6A3 (0–20 $\mu\text{g/ml}$), AJvW-2 (0–20 $\mu\text{g/ml}$), calin (0–5 $\mu\text{g/ml}$), vWF (0–20 $\mu\text{g/ml}$) and VCL (0–10 $\mu\text{g/ml}$) or after pretreatment of the microtitre plates with purified anti-(collagen VI) antibodies (0–300 $\mu\text{g/ml}$).

RESULTS AND DISCUSSION

Isolation of non-digested and pepsin-digested collagen type VI

SDS/PAGE of reduced pepsin-solubilized placental collagen VI revealed the characteristic collagen VI triplet [21]. However, in addition to protein bands with expected M_r values of approx. 230 000 and 140 000, non-digested placental collagen VI yielded low- M_r bands between 50 000 and 70 000 that were reactive in Western blots with anti-(collagen VI) antibodies, which is indicative of some proteolysis during the extraction. These preparations were used for the production of rabbit antisera. When isolated from the extracellular matrix produced by MRC-5 cells, during gel filtration on Sephacryl S-500 (Figure 1), the main peak

Figure 1 Isolation of polymerized human collagen VI

Collagen VI elution profile [detected via ELISA, following gel filtration on Sephacryl S-500 (2.6 cm $\times$ 95 cm) in 50 mM Tris/HCl buffer, pH 8.0, containing 6 M GdmCl] of GdmCl-solubilized ECM from MRC-5 lung fibroblasts. Inset: SDS/PAGE [4–15% (w/v) polyacrylamide gradient gel] of the main collagen VI fractions (lanes 2 and 3) and the right shoulder fractions (lanes 4 and 5) under non-reducing (lanes 2 and 4) and reducing (lanes 3 and 5) conditions. M_r values ($\times 10^{-3}$) (lane 1) are specified.

Figure 2 Affinity of vWF for collagen VI isoforms

Binding of increasing concentrations of purified vWF to microtitre plates coated with pepsin-solubilized collagen VI ($\square$), with non-digested collagen VI isolated from placenta ($\bullet$) and from human MRC-5 extracellular matrix ($\circ$).

contained highly polymerized collagen VI, yielding the expected bands upon reduction (Figure 1, inset), as well as low- M_r bands. Shoulder fractions eluting at slightly lower size, however, lacked the $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ chains and were not collected for the interaction studies reported below, even though these fractions were equally capable of vWF binding (results not shown). Western blots of the purified collagen VI showed that the anti-(collagen VI) antisera described below reacted with the M_r -140 000 band as well as with the low- M_r degradation bands, but not with the M_r -230 000 band (results not shown).

Affinity of vWF for isolated collagen VI

Binding of vWF to microtitre plates coated with non-digested collagen VI purified from placenta ($\text{EC}_{50} \cong 8 \text{ nM}$), or isolated from the extracellular matrix of MRC-5 cells ($\text{EC}_{50} \cong 5 \text{ nM}$, Figure 2), occurred with a severalfold higher affinity than that to pepsin-solubilized collagen VI ($\text{EC}_{50} \cong 90 \text{ nM}$).

vWF A1 domain interaction with collagen VI

In view of the reported inhibition of vWF binding to collagen VI by the vWF fragment T116 (aa 449–728), vWF binding to collagen VI was investigated in the presence of ATA, which binds to the vWF A1 domain [28]. Low concentrations of ATA, up to 10 μM , totally prevented vWF binding to non-digested collagen VI ($\text{IC}_{50} \cong 0.6 \mu\text{M}$), but approx. 300-fold higher ATA concentrations were required to block its binding to pepsin-digested collagen VI ($\text{IC}_{50} \cong 200 \mu\text{M}$, Figure 3). The binding of vWF to fibrillar collagens, in contrast, was only somewhat modulated by ATA, i.e. an apparent 2-fold increase in the affinity of vWF for collagens I, III and IV occurred, upon gradual saturation of the A1 domain with ATA (results not shown). The botrocetin-mediated binding of vWF to microtitre plate-coated glycoprotein Ib [19] (Figure 3) was inhibited by very similar concentrations of ATA, lending further support to the interpretation that low concentrations of ATA inhibit vWF binding to collagen VI as a consequence of ATA binding to the A1 domain of vWF. Similarly, the anti-(vWF domain A1) antibody AJvW-2 inhibited the binding of vWF to native collagen VI with an IC_{50} of approx. 0.5 $\mu\text{g/ml}$ (results not shown), although this antibody did not interfere with vWF binding to fibrillar collagens or to pepsin-solubilized collagen VI. The importance of the A1 domain for the interaction of vWF with collagen VI was further

Figure 3 A1 domain-mediated vWF binding to collagen VI

Inhibition by ATA of the binding of vWF (3 $\mu\text{g/ml}$) to microtitre plates coated with placental non-digested collagen VI (○) and with pepsin-solubilized collagen VI (●). Inhibition by ATA of the binding of vWF (1 $\mu\text{g/ml}$) to microtitre plate-coated platelet glycoprotein Ib, mediated by 0.4 nM botrocetin (△).

Figure 4 Role of vWF A3 domain in binding to collagen VI

Inhibition by the monoclonal anti-(vWF A3 domain) antibody 82D6A3 of the binding of vWF (5 $\mu\text{g/ml}$) to microtitre plates coated with collagen I (▲), or collagen III (△). Lack of inhibition by 82D6A3 of the binding of vWF (3 $\mu\text{g/ml}$) to plates coated with placental non-digested collagen VI (○).

illustrated by the use of the recombinant A1 domain analogue Leu⁵⁰⁴-Ser⁷²⁸, designated as VCL [24]. This fragment dose-dependently competed with vWF for binding to microtitre plates coated with non-digested collagen VI ($\text{IC}_{50} \cong 20 \text{ nM}$; results not shown). In contrast, VCL interfered considerably less with vWF binding to microtitre plate-coated collagens I, III and IV and pepsin-solubilized collagen VI. From the weak inhibition by ATA resp. AJvW-2 of the vWF binding to pepsin-digested collagen VI, it is clear that upon pepsin digestion of collagen VI, specific interactions between vWF and this collagen are no longer possible. Both in our study and in that of Denis et al. [15], vWF binding to collagen VI could be blocked by an A1 domain analogue, albeit that inhibition by T116 ($\text{IC}_{50} \cong 3 \mu\text{M}$) required a roughly 150-fold higher concentration than inhibition by VCL ($\text{IC}_{50} \cong 20 \text{ nM}$) in the present study.

Involvement of other vWF domains

Our monoclonal anti-vWF antibody 82D6A3, by binding to the A3 domain (J. Sixma, personal communication), strongly inhibits vWF binding to fibrillar collagens. As expected (Figure 4) the

binding of vWF to collagens I and III was dose-dependently inhibited with IC_{50} values of approx. 0.5 $\mu\text{g/ml}$. In contrast, virtually no inhibition was observed for vWF binding to microtitre plate-coated collagen IV and collagen VI (pepsin-digested or not) (Figure 4). Given earlier reports that the SpI fragment of vWF (aa 911–1365) could bind to collagen VI, we have therefore investigated whether amino acid sequences located downstream of the A3 domain (which consists of aa 923–1109) would interact with collagen VI. However, a recombinant fragment encoding the vWF D4 domain (aa 1183–1535) located adjacent to the A3 domain and (with the exception of aa 1109–1182) covering the remainder of SpI, did not interfere with the binding of vWF to native collagen VI, nor did a panel of anti-D4 monoclonal antibodies (raised against the recombinant D4-fragment). Hence these data substantiate the absence of interaction sites in the vWF D4 domain and suggest that its A3 domain does not interact with collagen VI via those aa sequences that mediate binding to fibrillar collagens and that are shielded by 82D6A3. We cannot, however, exclude the possibility that aa sequences in between the A3 and D4 domain interact with collagen VI.

Role of microfibrillar sequences in vWF binding to collagen VI

Recently, we have shown that calin, a protein present in the saliva of the leech *Hirudo medicinalis*, interferes with vWF binding to fibrillar collagens [26]. Presently, a calin-containing saliva-fraction dose-dependently inhibited vWF binding to microtitre plate-coated collagen type I with an $\text{IC}_{50} \cong 10 \mu\text{g/ml}$. Similarly, the calin extract dose-dependently inhibited the binding of vWF to coated non-digested collagen VI with comparable efficacy ($\text{IC}_{50} \cong 10 \mu\text{g/ml}$; results not shown). However, up to 100 $\mu\text{g/ml}$ did not interfere with vWF binding to pepsin-solubilized collagen VI. These data strongly suggest that vWF binding occurs to the fibrillar sequences of collagen VI. However, the collagen VI globular A domains are equally important for the interaction between this microfibrillar collagen and vWF, given the low affinity of vWF for pepsin-digested collagen VI. The latter treatment removes most of the globular domains, thus preventing collagen VI from forming biological superhelices [14]. The removal of the collagen VI globular domains as a consequence of pepsin treatment is accompanied by a loss of the AJvW-2 and ATA inhibition during vWF binding, confirming that collagen VI globular domains influence its interaction with vWF.

Effect of anti-(collagen VI) antibodies on vWF binding to ECM

Rabbit antisera raised against non-digested and digested placental collagen VI showed comparable saturation curves on microtitre plates coated with purified pepsin-solubilized collagen VI or with non-digested collagen VI (results not shown), confirming that the lower vWF binding to pepsin-solubilized collagen VI (Figure 2) was not the result of a less efficient coating in the case of the pepsin-solubilized collagen VI. The anti-(collagen VI) antisera saturated endothelial cell extracellular matrix at only 4-fold higher concentrations (Figure 5A). Both our own and a commercial antiserum did not react with microtitre plate-coated collagens I, III and IV. To further validate antiserum reactivity with intact collagen VI synthesized by endothelial cells, human endothelial cells in culture were metabolically labelled with [³⁵S]Met and supernatants were immunoprecipitated with the anti-(pepsin-solubilized collagen VI) antiserum. Analysis of the immunoprecipitates by SDS/PAGE and autoradiography identified the presence in the immunoprecipitate of very-high- M_r material, not penetrating the gels, as well as two precipitated bands with M_r values of 230 000 and 150 000 (Figure 5A, inset).

Figure 5 Antibody (Ab)-mediated inhibition of vWF binding

(A) Dose-response of the binding of a rabbit antiserum, raised against purified placental pepsin-solubilized collagen VI, to microtitre plate-coated non-digested collagen VI (○) or to microtitre plates containing human endothelial cell extracellular matrix (●). Inset: SDS/PAGE (7.5% polyacrylamide gel) and autoradiography of [^{35}S]Met-labelled endothelial cell supernatant immunoprecipitated with the anti-(pepsin-solubilized collagen VI) antiserum. (B) Inhibition of binding of vWF (3 $\mu\text{g/ml}$) to microtitre plates coated with non-digested collagen VI (○) or containing ECM (●) by pretreatment of the microtitre plates with increasing concentrations of anti-(collagen VI) antibodies.

Similarly to the presence of a weak band with M_r 210000 in the shoulder fraction of the main collagen VI fraction during its isolation from MRC-5 extracellular matrix, a faint band of similar size was present in the immunoprecipitated supernatant. Pretreatment of coated non-digested collagen VI with anti-(collagen VI) antisera resulted in a dose-dependent inhibition of the binding of subsequently added vWF (Figure 5B), the degree of inhibition correlating well with the degree of collagen saturation by the antiserum. Likewise, pretreating endothelial cell ECM with the neutralizing anti-(collagen VI) antibodies equally inhibited the binding of added vWF dose-dependently (Figure 5B), whereas such pretreatment did not displace the low levels of ECM-associated vWF (results not shown). At comparable antibody concentrations, Protein A-purified anti-(collagen VI) antibodies were as inhibitory as the original antisera (results not shown), whereas non-specific rabbit sera did not interfere with vWF binding to ECM. In agreement with the absence of binding of the anti-(collagen VI) antisera to collagens I, III and IV, vWF binding with these collagens was only marginally affected by the antiserum pretreatment (results not shown). Antiserum pretreatment of pepsin-solubilized collagen VI-coated plates failed to prevent vWF binding to these plates, further substantiating the evidence that the binding to both variants of the same collagen was not identical.

vWF domain binding to native extracellular matrix collagen VI

Even though a strongly denaturing extraction of extracellular matrices with 6 M GdmCl could yield intact collagen VI, it is unlikely that such preparations would form microfibrils during the renaturation step following the removal of GdmCl. However, since anti-(collagen VI) antibodies were capable of blocking vWF binding to intact ECM (Figure 5B), the interaction of vWF with native collagen VI present in the extracellular matrices of endothelial cell and MRC-5 cultures could be investigated in greater detail. Saturation curves describing the binding of vWF to selected cultures of endothelial cell matrices with low endogenous levels of vWF were suggestive of a homogeneous saturation (Figure 6A). Double reciprocal plots of absorbance versus the vWF concentration (Figure 6A, inset) confirmed that this binding was monophasic and occurred with an affinity ($EC_{50} \cong 2\text{ nM}$) slightly higher than that derived for the binding of vWF to isolated non-digested collagen VI (Figure 2). This binding was inhibited by ATA ($IC_{50} \cong 1\text{ }\mu\text{M}$; Figure 6B) and by AJvW-2 ($IC_{50} \cong 3\text{ }\mu\text{g/ml}$; Figure 6C) at those concentrations of inhibitor that inhibit the binding of vWF to isolated collagen VI, confirming that in ECM also, native collagen VI interacts with vWF via its A1 domain. In agreement with the incapacity of 82D6A3 to inhibit the binding of vWF to isolated collagen VI, no inhibition of vWF binding to the ECM by 82D6A3 was observed (Figure 6D).

Platelet binding to collagen VI

When coated on microtitre plates, collagen VI supported the adhesion of human platelets very weakly. Whereas A_{405} values > 1 were routinely observed for the adhesion of washed platelets to ECM, A_{405} values (after correction for specific binding) for the binding of washed platelets to collagen VI averaged 0.15–0.2 only, a finding in agreement with the poor aggregatory potential of collagen VI [29]. Platelet binding, however, was inhibited by pretreating the coated collagen VI with purified anti-(collagen VI) antibodies (results not shown), confirming the specificity of the binding. Also calin dose-dependently inhibited platelet binding to collagen VI (Table 1), substantiating the notion that, similarly to the binding of vWF, platelets also interact with the fibrillar sequences on collagen VI, most probably via their collagen receptor, GPIa/IIa. Platelet binding did not involve the platelet GPIIb/IIIa receptor, as is evident from the incapacity of G4120 to inhibit such binding (results not shown). Neither AJvW-2 nor 82D6A3 had any effect on platelet binding to collagen VI, showing that (in the absence of added vWF) platelet binding was not mediated via platelet vWF. Yet, although no inhibition was observed when using VCL, added vWF competed dose-dependently with platelet binding (Table 1), suggesting that vWF and the platelet collagen receptor (GPIa/IIa) bind to distinct but closely positioned fibrillar sequences on collagen VI. These results are at variance with recent observations [17] that vWF could mediate platelet adhesion to collagen VI in the absence of high shear forces. It may be noteworthy that calin blocks both vWF binding and platelet adhesion [26] to fibrillar collagens, which is suggestive of a functional similarity in the binding sites for vWF and GPIa/IIa. Control inhibition studies with calin, anti-(collagen VI) antibodies and anti-vWF antibodies under static conditions, however, showed that the stronger binding of washed platelets to ECM did not involve ECM collagens (including collagen VI), nor ECM-associated vWF. In agreement with earlier observations [30], we found that this binding depended on the involvement of matrix-associated fibronectin (results not shown).

Figure 6 Binding of vWF to endothelial extracellular matrix

(A) Binding of increasing vWF concentrations to the extracellular matrix produced in microtitre plates by human endothelial cells (passage II). Inset: Double reciprocal plot of this binding. (B) Inhibition of this binding by ATA; (C) inhibition by the anti-(vWF domain A1) antibody AJvW-2; (D) lack of inhibition by the anti-vWF antibody 82D6A3.

Table 1 Inhibition of the binding of washed human platelets to microtitre plate-coated non-digested collagen VI

*Means $\pm$ S.D. of three experiments.

Inhibitor	Residual binding (% of control)
Calin (dilution)	
1/160	86
1/80	100
1/40	62
1/20	25
vWF ($\mu\text{g/ml}$)	
10	$80 \pm 2^*$
15	$62 \pm 4^*$
20	$44 \pm 5^*$

Conclusion

The present study has demonstrated that native collagen VI has a moderately high affinity for vWF, thus being a major target molecule for vWF in the subendothelial extracellular matrix. In contrast with the binding of vWF to fibrillar collagens, which is mediated exclusively via its A3 domain, vWF binds to microfibrillar collagen VI primarily via its A1 domain. Under static conditions, platelet binding to collagen VI did not require vWF. Further investigations are necessary to delineate the role of

subendothelial collagen VI as a vWF receptor, and to determine whether collagen VI-associated vWF participates in the adhesion of platelets to ECM, taking into account the effect of shear forces on platelet binding.

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REFERENCES

- 1 Baumgartner, H. R. and Haudenschild, C. (1972) *Ann. N.Y. Acad. Sci.* **201**, 22–36
- 2 Gordon, J. L. and Pearson, J. D. (1987) in *Haemostasis and Thrombosis* (Bloom, A. L. and Thomas, D. P., eds.), pp. 303–311, Churchill Livingstone, Edinburgh
- 3 Saelman, E. V., Nieuwenhuis, H. K., Hese, K. M., de Groot, P. G., Heijnen, H. F. G., Sage, E. H., Williams, S., McKeown, L., Gralnick, H. R. and Sixma, J. J. (1994) *Blood* **83**, 1244–1250
- 4 Sakariassen, K. S., Bolhuis, P. A. and Sixma, J. J. (1979) *Nature (London)* **279**, 636–638
- 5 Sadler, J. E. (1991) *J. Biol. Chem.* **266**, 22777–22780
- 6 Bolhuis, P. A., Sakariassen, K. S., Sander, H. J., Bouma, B. N. and Sixma, J. J. (1981) *J. Lab. Clin. Med.* **97**, 568–576
- 7 Sixma, J. J., Pronk, A., Nieuwelstein, P. N. E. M., Zwaginga, J.-J., Hindriks, G., Tijburg, P. I. M., Banga, J.-D. and de Groot, P. G. (1991) *Ann. N.Y. Acad. Sci.* **614**, 181–192
- 8 Sakariassen, K. S., Aarts, P. A., de Groot, P. G., Houdijk, W. P. M. and Sixma, J. J. (1983) *J. Lab. Clin. Med.* **102**, 522–535
- 9 de Groot, P. G., Ottenhof-Rovers, M., van Mourik, J. A. and Sixma, J. J. (1988) *J. Clin. Invest.* **82**, 65–73

- 10 Rand, J. H., Patel, N. D., Schwartz, E., Zhou, S. L. and Potter, B. J. (1991) *J. Clin. Invest.* **88**, 253–259
- 11 Rand, J. H., Wu, X. X., Potter, B. J., Uson, R. R. and Gordon, R. E. (1993) *Am. J. Pathol.* **142**, 843–850
- 12 Colombatti, A., Ainger, K. and Colizzi, F. (1989) *Matrix* **9**, 177–185
- 13 Bonaldo, P. and Colombatti, A. (1989) *J. Biol. Chem.* **264**, 20235–20239
- 14 Colombatti, A. and Bonaldo, P. (1991) *Blood* **77**, 2305–2315
- 15 Denis, C., Baruch, D., Kielty, C. M., Ajzenberg, N., Christophe, O. and Meyer, D. (1993) *Arterioscler. Thromb.* **13**, 398–406
- 16 Hoylaerts, M. F., Nuyts, K., Vreys, I., Deckmyn, H. and Vermynen, J. (1995) *Thromb. Haemostasis* **73**, 1349 (Abstract 1723)
- 17 Ross, J. M., McIntire, L. V., Moake, J. L. and Rand, J. (1995) *Blood* **85**, 1826–1835
- 18 Ruggeri, Z. M., Zimmerman, T. S., Russell, S., Bader, R. and De Marco, L. (1992) *Methods Enzymol.* **215**, 263–275
- 19 Hoylaerts, M. F., Nuyts, K., Peerlinck, K., Deckmyn, H. and Vermynen, J. (1995) *Biochem. J.* **306**, 453–463
- 20 Trüeb, B., Schreier, T., Bruckner, P. and Winterhalter, K. H. (1987) *Eur. J. Biochem.* **166**, 699–703
- 21 Jander, R., Troyer, D. and Rauterberg, J. (1984) *Biochemistry* **23**, 3675–3681
- 22 Hessle, H. and Engvall, E. (1984) *J. Biol. Chem.* **259**, 3955–3961
- 23 Engvall, E., Hessle, H. and Klier, G. (1986) *J. Cell Biol.* **102**, 703–710
- 24 Gralnick, H. R., Williams, S., McKeown, L., Kramer, W., Krutzsch, H., Gorecki, M., Pinet, A. and Garfinkel, L. I. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7880–7848
- 25 Yamamoto, H., Nagano, M., Kitoh, M., Kobayashi, T. and Yoshimoto, R. (1995) *Blood* **86**, Suppl 1, Abstract 325, 84a
- 26 Harsfalvi, J., Stassen, J. M., Hoylaerts, M. F., Van Houtte, E., Sawyer, R. T., Vermynen, J. and Deckmyn, H. (1995) *Blood* **85**, 705–711
- 27 Bellavite, P., Andrioli, G., Guzzo, P., Angliano, P., Chirumbola, S., Manzato, F. and Santonastaso, C. (1994) *Anal. Biochem.* **216**, 444–450
- 28 Girma, J. P., Fressinaud, E., Christophe, O., Roualt, C., Obert, B., Takahashi, Y. and Meyer, D. (1992) *Thromb. Haemostasis* **68**, 707–713
- 29 Zangari, M., Kaplan, K. L., Glanville, R. W. and Rand, J. H. (1995) *Thromb. Res.* **79**, 5–6
- 30 Kawai, Y., Handa, M., Nagai, H., Kamata, T., Anbo, H., Kawono, K., Araki, Y., Yamamoto, M., Ikeda, Y. and Watanabe, K. (1989) *Acta Haematol. Jpn.* **52**, 1312–1321