Vimentin Exposed on Activated Platelets and Platelet Microparticles Localizes Vitronectin and Plasminogen Activator Inhibitor Complexes on Their Surface*

Received for publication, October 5, 2001, and in revised form, December 14, 2001
Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M109675200

Thomas J. Podor‡§§, Davindra Singh‡§§, Paul Chindemi‡§§, Denise M. Foulon‡§§, Robert McKelvie‡§, Jeffrey I. Weitz‡§§, Richard Austin‡§§, Ghislain Boudreaux, and Richard Davies‡§§

From the Departments of §Pathology and Molecular Medicine, and ¶Medicine, McMaster University and the Hamilton Civic Hospitals Research Centre, Hamilton, Ontario L8V 1C3, Canada, ¶Pfizer Pharmaceuticals, Montreal, Quebec H3J 2M5, Canada, and the ¶¶Ottawa Heart Institute, University of Ottawa, Ottawa, Ontario K1Y 4W7, Canada

Type 1 plasminogen activator inhibitor (PAI-1), the primary inhibitor of tissue-type plasminogen activator (t-PA), is found in plasma and platelets. PAI-1 circulates in complex with vitronectin (Vn), an interaction that stabilizes PAI-1 in its active form. In this study, we examined the binding of platelet-derived Vn and PAI-1 to the surface of isolated platelets. Flow cytometry indicates that, like P-selectin, PAI-1, and Vn are found on the surface of thrombin- or calcium ionophore-activated platelets and platelet microparticles. The binding of PAI-1 to the activated platelet surface is Vn-dependent. Vn mediates the binding of PAI-1 to platelet surfaces through a high affinity (Kd of 50 nM) binding interaction with the NH2 terminus of vimentin, and this Vn-binding domain is expressed on the surface of activated platelets and platelet microparticles. Immunological and functional assays indicate that only ~5% of the total PAI-1 in platelet releasates is functionally active, and it co-precipitates with Vn, and the vimentin-enriched cytoskeleton fraction of activated platelet debris. The remaining platelet PAI-1 is inactive, and does not associate with the cytoskeletal debris of activated platelets. Confocal microscopic analysis of platelet-rich plasma clots confirm the co-localization of PAI-1 with Vn and vimentin on the surface of activated platelets, and platelet microparticles. These findings suggest that platelet vimentin may regulate fibrinolysis in plasma and thrombi by binding platelet-derived Vn-PAI-1 complexes.

Most acute coronary syndromes are caused by thrombosis superimposed on disrupted atherosclerotic plaque (1). Thrombi formed at sites of arterial injury are comprised of platelets and fibrin. The extent of fibrin formation at these sites depends on the dynamic balance between the coagulation and fibrinolytic pathways. Intravascular fibrinolysis is initiated by tissue-type plasminogen activator (t-PA) that converts plasminogen to plasmin. The process is regulated by type 1 plasminogen activator inhibitor (PAI-1), the physiological inhibitor of t-PA (2). PAI-1 circulates in two distinct pools (3). Only 10% of circulating PAI-1 is in the plasma where it is bound to vitronectin (Vn) (4–6), an interaction that stabilizes PAI-1 in its active conformation (7, 8). The remaining PAI-1 is stored in platelet α-granules (3, 9, 10) and released by platelet activation. The observation that only a small proportion of platelet PAI-1 is functionally active (9–11) is difficult to reconcile with the fact that levels of platelet-derived PAI-1 in thrombi determines the susceptibility of these thrombi to lysis by t-PA (12, 14–20).

Recent studies with PAI-1 and Vn-deficient mice indicate that the incorporation of both PAI-1 and Vn into thrombi formed at sites of carotid artery injury prevent premature thrombolysis (23, 24). Interestingly, the initial thrombotic response of Vn-deficient mice to arterial injury was similar to that of wild-type controls, but their thrombi were unstable and frequently embolized (24). Consequently, the patency rate of the injured arteries 30 min after injury were as high in Vn-deficient mice as in PAI-1-deficient mice, which demonstrate progressive thrombolysis, and significantly greater than wild-type mice. These findings may reflect an effect of Vn on the regulation of platelet PAI-1 activity, or the Vn-dependent binding of PAI-1 to other proteins such as fibrin (25).

Platelet α-granule PAI-1 is derived from de novo megakaryocyte biosynthesis (26, 27). In contrast, Vn internalized from plasma is targeted to platelet α-granules (27). Although the platelet storage pool of PAI-1 is reportedly stabilized by its interactions with calcium, and other α-granule proteins, it is not clear if α-granule Vn regulates platelet PAI-1 function (28). Based on size fractionation and immunoblot analysis, some PAI-1 released by activated platelets is complexed with high molecular weight forms of Vn (13, 28, 29), and almost half of the platelet Vn remains cell-associated after platelet activation (30). Immunogold electron microscopic studies have revealed Vn and PAI-1 localized within the surface connected system, and on the surface of resting (31) and activated platelets (32).
Vitronectin Mediates PAI-1 Binding to Platelet Vimentin

33. Although these finding suggest that Vn and PAI-1 bind to the surface of activated platelets, such interactions have not been defined.

Previously, we have demonstrated that PAI-1 and Vn co-localize with the Triton X-100-insoluble vimentin intermediate filaments of damaged cells (34). Because vimentin also is a component of the platelet cytoskeleton (35, 36), we hypothesized that vimentin exposed upon platelet activation may serve to localize Vn-PAI-1 complexes on the platelet surface. Using recombinant vimentin fragments, we have (a) identified a high affinity Vn-binding site on the amino-terminal domain of vimentin, and (b) by developing an antibody against this fragment, have demonstrated that the expression of this binding site on activated platelets and platelet microparticles serves to localize Vn-PAI-1 complexes to their surface. Our results are the first demonstration that the active form of platelet PAI-1 is selectively bound to the surface of activated platelets, and that flow cytometry is a useful method for measuring its surface expression in vitro and in vivo.

MATERIALS AND METHODS

Chemicals and Reagents—Alkaline phosphatase-conjugated streptavidin, p-nitrophenyl phosphate, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride were purchased from Invitrogen. Bovine vimentin was obtained from Roche Molecular Biochemicals. Human PAI-1 derived from human HT 1080 fibrosarcoma cells was obtained from American Diagnostica. Human α-thrombin was purchased from Enzyme Research Laboratories. Bovine serum albumin (BSA), casein, Triton X-100, reduced glutathione, ethylenediamine, dithethanolamine, caprylic acid, bovine protein kinase, thrombin receptor activating peptide (TRAP), prostaglandin E1, normal goat immunoglobulin (IgG), normal rabbit IgG, and ascitic fluids for monoclonal antibodies (mAbs) to vimentin (clone V.9) and isotype-matched, non-specific mouse IgG were obtained from Sigma. Phycoerythrin (PE)-conjugated mAb directed against CD42b (GP1b-α), fluorescein isothiocyanate (FITC)-conjugated mAb directed against CD62P (P-selectin), and FITC-conjugated streptavidin were purchased from Immunotech Inc. Monospecific antisera directed against bovine vimentin was raised in rabbits as described (37). Affinity purified sheep anti-human Vitronectin IgG (SAH-Vitronectin), anti-human vimentin IgG (SAH-VIM133), and normal sheep IgG were obtained from Affinity Biologicals. Monoclonal antibody to PAI-1 (M22) was purchased from Biopool AB. Purified vitronectin, PAI-1, and the various antibodies were biotinylated with biotinyl-N-amino caproic-N-hydroxysuccinimide ester (Roche Molecular Biochemicals) (27, 37). Rabbit polyclonal antibody directed against residues 35–50 in the amino-terminal domain of murine vimentin (Rab 484) was kindly provided by Dr. Wally Ip (University of Cincinnati). High binding 96-well microtiter plates were obtained from Costar Science Corp. Tween 20, Coomassie Brilliant Blue R-250, urea (electrophoresis grade), acrylamide:bis 37.5:1, molecular weight markers, glycine, TRIS, SDS, G-25M Sepharose, and gelatin were purchased from Bio-Rad.

Preparation of Washed Platelets—Blood was collected from the ante-cubital vein of healthy volunteers into one-sixth volume of acid citrate dextrose. After centrifugation at 200 × g for 15 min at 22°C, platelet-rich plasma (PRP) was harvested. Platelets were then pelleted by subjecting PRP to centrifugation at 1,000 × g for 10 min at 22 °C. The platelet pellet was washed twice with calcium-free Tyrodes buffer, pH 7.4, containing 0.35% BSA, 10 mM prostaglandin E1, 0.1 mg/ml apyrase, and one-tenth volume of Diabute H vacutainer tube solution (Becton Dickinson) containing citrate, theophylline, adenosine, and dipipyridamole. Platelets were then resuspended at 108 platelets/ml in calcium-free Tyrodes buffer containing 0.01 mg/ml apyrase and stored at 23 °C until used.

Flow Cytometry—Immuno-fluorescence flow cytometry was used to detect platelet-associated PAI-1, vitronectin, and vimentin. Washed platelets (106/ml) were incubated for 10 min in the absence or presence of thrombin, A23187, or TRAP prior to analysis using a Coulter EPICS C Flow Cytometer or FACScan. Platelet-forward and side angle light scatter, and for relative FITC and PE fluorescence intensities. Platelets and platelet microparticles were then identified by their characteristic light scatter profiles on dot plots of forward light scatter versus right angle side light scatter. Flow cytometry data were analyzed using WinMDI software version 2.8.

Isolation and Labeling of Vn from Plasma—Vn was isolated from normal platelet-poor plasma by non-denaturing heparin-Sepharose and immuno-affinity chromatography (27). Oligomeric Vn preparations were obtained by incubating plasma with 8 μl of P-selectinase in PBS for 1 h at 37 °C, followed by extensive dialysis against PBS. Isolated Vn was characterized using SDS-PAGE, and native-PAGE and by its affinity for heparin and the conformation-sensitive antibody, mAb86E. Total Vn protein was quantified using a Vn-specific enzyme-linked immunosorbent assay (38). For some experiments, Vn was radiolabeled using 125I-Bolton-Hunter reagent (22) to a specific activity of 800 cpm/ng.

Vimentin Binding Assays—To demonstrate the Vn dependence of PAI-1 binding to vimentin, 96-well microtiter plates were coated overnight at 4 °C with various concentrations of purified bovine vimentin diluted in PBS, pH 7.4. After blocking with 3% BSA, the washed wells were incubated with 20 nM biotinylated PAI-1 (bt-PAI-1) diluted in PBS containing 3% BSA, 0.1% Tween 80, 5 mM EDTA, and 20 units/ml aprotinin. After washing, binding of the presence of competing unlabelled purified Vn. Binding of bt-PAI-1 was measured by monitoring the absorbance at 405 nm after the addition of streptavidin-conjugated alkaline phosphatase/ p-nitrophenyl phosphate substrate, and subtracting binding to BSA-coated wells. To examine the interaction of Vn with vimentin or recombinant vimentin peptide fragments, 96-well microtiter plate wells coated with bovine vimentin, vimentin peptides, or BSA were incubated for 1 h at 37 °C with varying concentrations of 125I-labeled native or urea-treated Vn in the absence or presence of a 20-fold molar excess of unlabeled ligand. In each case, specifically bound Vn was determined by subtracting the radioactivity bound to BSA-coated wells.

Construction of Recombinant Human Vimentin Peptide Plasmids—Human umbilical vein endothelial cells were isolated as described (39), and total RNA isolated using a single-step acid guanidium thiocyanate-phenol-chloroform method (40). A 0.5-kb cDNA fragment encoding the amino terminus head domain and a portion of helix IA (133 amino acids) of human vimentin was generated using the reverse transcription-PCR. Denatured RNA was reverse transcribed using Invitrogens Superscript I17 RNase H reverse transcriptase, and the PCR product was then gel purified using the QIAEX7 DNA Gel Extraction kit, subcloned into T-ended pBLUESCRIPT, and plated DH1O with Escherichia coli. Plasmid DNA was isolated as previously described (41) and digested with XhoI. This produced a 460-bp fragment that was subcloned into the XhoI site of pFL-1-(HMK) heart muscle kinase (HMK) recognition site-modified vector. After digesting the pFL-1-HMK-VIM133 plasmid with HindIII/Xhol to release the HMK-VIM133 DNA insert, the fragment was then ligated into the HindIII/Xhol site of the EcoRI/XhoI-transformed plasmid. The bacteriophage M13mp18-ampicillin-resistant transformed bacterial strain was then used to transform competent BL21(DE3) E. coli. Likewise, a 1.1-kb cDNA fragment encoding the central α-helical rod domain and nonhelical carboxy-terminal tail domain (367 amino acids) of human vimentin was amplified by standard reverse transcription as described above (42, 43). This cDNA was subcloned into the EcoRI site of a pET-21c vector to produce the pET-21c CT-VIM367 plasmid that was used to transform BL21(DE3) competent E. coli. Recombinant VIM133 and VIM367 fusion proteins were isolated under denaturing conditions using a Ni2+-His column system (Novagen), and then dialyzed into TBS buffer, pH 7.4, containing 8 μl urea. For some studies, the pFL-1-HMK-VIM133 fusion peptide was radiolabeled with 50 units of protein kinase and 50 μCi of [32P]ATP (ICN Biomedicals) to a specific activity of 950 cpm/nmol.

Flow Cytometry Detection of In Situ Platelet Activation—To demonstrate a clinical example of vimentin-mediated expression of PAI-1 on activated platelets in situ (44, 45), nine patients with documented coronary artery disease (CAD) were recruited from the Hamilton General Hospital Cardiac Rehabilitation Program. CAD patients were habitually sedentary, and all had a clinical history of CAD, and electrocardiographically-light exercise induced ischemic heart disease. The control group consisted of four age-matched control subjects with no documented history of CAD, as well as the absence of electrocardiographic or chest pain symptoms during a symptom limited exercise treadmill test. Patients were receiving enteric-coated aspirin 325 mg daily, and had been withdrawn from all anti-anginal medications. Written informed consent was obtained...
from all subjects, and all protocols were approved by the Hamilton Health Sciences Human Ethics Committee. All subjects were instructed to avoid vigorous physical activity and meals for at least 2 h on the morning of the testing. A pre-exercise blood sample was obtained from an antecubital vein saline lock 20 min prior to beginning the treadmill exercise test. Patients then performed a symptom limited treadmill exercise test using the ACIP protocol (46), with repeat blood samples being drawn immediately after peak exercise, and 3 h post-exercise. For whole blood flow cytometry, blood samples were collected into Dia- tube-H vacutainer tubes, incubated with saturating concentrations of fluorescent-labeled, or biotinylated antibodies plus streptavidin-FITC, and then briefly fixed with 1% formalin, diluted with HEPES-Tyrodes buffer, and processed for flow cytometry as described above. The expression of Vn on the surface of platelets was not evaluated in whole

FIG. 1. Vitronectin-dependent expression of PAI-1 on the surface of activated platelets and platelet microparticles. Washed, resting (panel A) human platelets were activated with thrombin (0.5 units/ml) for 10 min (panel B), briefly fixed with paraformaldehyde, washed, and then incubated with fluorescently labeled antibodies directed against PAI-1 (MAI-12), vitronectin (SAHVn IgG); or P-selectin (CD62P), plus GPIb-α (CD42b) prior to their analysis by fluorescence flow cytometry. Platelets were gated on GPIb-α positive events, and by forward and side scatter for size and shape. Histogram depiction of the relative fluorescence intensity (RFI) of the various antibodies on: panel C, resting platelets; panel D, thrombin-activated platelets; and panel E, the RFI for MAI-12-FITC binding to platelets activated in the presence or absence of SAHVn IgG, or preimmune sheep IgG. Histograms depicting the RFI of the anti-P-selectin (panel F) and anti-PAI-1 (panel G) binding to platelets microparticles that were detected by gating on GPIb-α positive events within the smaller forward and side scatter for size and shape (lower left quadrants in panels A and B).

Vitronectin Mediates PAI-1 Binding to Platelet Vimentin
blood flow cytometry because of the interference from the micromolar concentrations of plasma Vn.

Analysis of Vn, PAI-1, and Vimentin in Platelet Releasate—Resting and activated platelet releasates were subjected to differential centrifugation and Western blot analysis to examine the distribution of Vn and PAI-1 binding to platelet vimentin. Washed platelets were incubated for 10 min at 37°C in the absence or presence of thrombin (2 units/ml). After centrifugation at 12,000 × g for 10 min, the supernatant was subjected to centrifugation at 100,000 × g for 3 h to ensure complete precipitation of platelet debris. Resultant pellets were lysed with lysis buffer (PBS, pH 7.4, containing 100 mM NaCl, 300 mM sucrose, 10 mM benzamidine, 5 mM EDTA, 10 units/ml aprotinin, 10 mM phenylmethylsulfonyl fluoride, 0.05% sodium azide, and 0.5% Triton X-100). The Triton-insoluble pellet was then extracted with lysis buffer containing 2% SDS, and the SDS neutralized by the addition of 2% Triton X-100. For immunoblot analysis, samples were solubilized in

FIG. 2. Vitronectin-dependent binding of PAI-1 to purified bovine vimentin. Panel A, 96-well plates microtiter were coated with various concentrations of purified vimentin, and then incubated with 20 nM bt-PAI-1 in the presence of increasing concentrations of Vn, and the bound bt-PAI-1 was determined by measuring the change in A405 nm after the addition of streptavidin-conjugated alkaline phosphatase/p-nitrophenyl phosphate substrate. Specific binding to vimentin was calculated by subtracting the background bt-PAI-1 binding to BSA-coated wells; panel B, microtiter plates were coated with various concentrations of purified vimentin, and then incubated with 250 nM 125I-Vn alone (green circles), or equimolar concentrations of both Vn and PAI-1 (red triangles). After washing, the bound radioactivity was determined, and corrected for radioactivity bound to BSA-coated wells.
Laemmli sample buffer (47) (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 0.001% bromphenol blue) containing 5% 2-mercaptoethanol, subjected to boiling water for 1 h, and then fractionated by SDS-PAGE using 7.5% slab gels. Separated proteins were transferred to nitrocellulose membranes, and after blocking with blotting buffer (PBS, pH 7.4, containing 1% casein and 0.05% Tween 20), the membranes were incubated for 1 h with 5 μg/ml SAH-VIM133, SAH-Vn, or SAH-PAI-1 IgG. After washing with blotting buffer, membranes were incubated with a 1:3000 dilution of horseradish peroxidase-conjugated rabbit anti-sheep IgG for 1 h. The blots were washed again, immersed in chemiluminescence reagent (ICN Biomedicals), and briefly exposed to autoradiography film.

The quantity of PAI-1 in the Triton-soluble and insoluble extracts (10^9 platelets/sample) was determined immunologically using a sandwich enzyme-linked immunosorbent assay (39), and functionally using a two-step method in which immobilized t-PA was first used to bind active PAI-1, and the bound PAI-1 then quantified with an affinity purified, horseradish peroxidase-conjugated sheep anti-human PAI-1 (48).

**Immunochemistry and Confocal Microscopic Image Analysis—** Sample preparation for immunofluorescence confocal microscopic imaging of the distribution of PAI-1, Vn, and vimentin in PRP clots was previously described for platelet-poor clots (25). Briefly, 150 μl of PRP was placed on APTEX-coated coverslips and clotted by the addition of thrombin (2 units/ml) and CaCl_2 (10 mM, final concentration). After incubation at 37 °C for 1 h, the clots were fixed with cold 3% formaldehyde in PBS for 5 min, washed alternately with PBS and PBS containing 0.1 M NaCl/liter of glycine, and then incubated for 30 min with blocking buffer (PBS containing 0.5% BSA and 50 μg/ml normal goat IgG). Primary antibodies, including a monoclonal anti-PAI-1 IgG (MAI-12), SAH-Vn IgG, SAH-VIM133 IgG, and SAH-vWF IgG, were diluted in blocking buffer and incubated with the clots for 1 h at 37 °C. Control clots were stained with each primary antibody separately, stained without primary antibodies, or stained with nonspecific mouse and sheep IgG. After washing, clots were incubated for 1 h at 37 °C with Texas Red rhodamine-conjugated goat anti-sheep or FITC-conjugated goat antimouse IgG. The coverslips were washed, mounted on glass slides using Permafluor mounting medium, and then subjected to Z-plane optical sectioning (200 nm/section) using a Zeiss LSM 10 and MetaMorph software (Universal Imaging). Clots stained with nonspecific primary antibodies were used to threshold for background fluorescence intensity.

**RESULTS**

Vn-mediated PAI-1 Expression on the Surface of Activated Platelets and Microparticles—Flow cytometry studies have previously shown that activated platelets release plasma membrane-bound vesicles, called microparticles, which have a high density of prothrombotic proteins on their surface, including the adhesive receptor P-selectin (44, 45). We used immunofluorescence flow cytometry to determine whether the endogenous platelet PAI-1 and Vn are expressed on the surface of thrombin-activated platelets and platelet microparticles. Analysis of unstimulated platelets using the PE-CD42b (Gpib-α) reveals a single population of particles characterized by an ovoid forward and side light-scattering pattern (Fig. 1A). The unstimulated platelets also exhibited relatively low fluorescence intensity staining with FITC-MAI-12 (PAI-1), FITC-CD62P (P-selectin), SAH-Vn (vitronectin), and normal (preimmune) sheep IgG (Fig. 1C). Thrombin activation significantly alters the forward and side light-scattering characteristics of the Gpib-α positive platelets, and results in the generation of smaller platelet microparticles that are evident within the lower left quadrant of the dot plot (Fig. 1B). Platelet activation increases the relative fluorescence intensity of the FITC immunolabeling for P-selectin, PAI-1, and Vn (Fig. 1D). Activation also generates P-selectin and PAI-1 positive microparticles that were identified by virtue of their lower forward and side light scatter values, and their positive co-staining for Gpib-α (Fig. 1, F and G). Moreover, the presence of anti-Vn IgG during platelet activation virtually eliminates the binding of MAI-12 IgG to the activated platelet surfaces (Fig. 1E), and thereby indicates that Vn mediates the binding of PAI-1 to activated platelet surfaces.

**Vimentin Binds PAI-1 in a Vn-dependent Manner—** Building on our previous observation that PAI-1 and Vn co-localize with vimentin of damaged cells, we set out to determine whether PAI-1 and/or Vn bind to bovine vimentin. Hypothesizing that PAI-1 binding to vimentin would be Vn-dependent, we incubated vimentin-coated wells with a fixed concentration of bt-PAI-1 in the absence or presence of increasing concentrations of Vn, and then monitored PAI-1 binding. The binding of bt-PAI-1 to vimentin is negligible in the absence of Vn, but increases as a function of the Vn concentration (Fig. 2A). These results indicate that bt-PAI-1 binding to vimentin is Vn-dependent. Next, we examined the impact of PAI-1 on the binding of 125I-Vn to vimentin-coated plates (Fig. 2B). PAI-1 potentiates Vn binding to vimentin 2-fold, possibly reflecting the previously described formation of Vn dimers within a higher order Vn-PAI-1 complex (49).

**Identification and Characterization of the Vn-binding Domain on the Amino Terminus of Vimentin—** To identify the Vn-binding domain on vimentin, two overlapping recombinant human vimentin peptides were expressed (Fig. 3A). The first, a 133-amino acid peptide, is comprised of the amino-terminal head domain (residues 1–95) and 36 residues of the coil 1A within the central rod domain (residues 96–131). The second peptide is a 367-amino acid peptide analogue composed of the central rod domain, and the carboxyl-terminal rod domain. These peptides were designated VIM133 and VIM367, respectively. The VIM133 fusion protein migrated as a 20-kDa band on 12% SDS-polyacrylamide gels (Fig. 3B, lanes 2), and was detectable with a polyclonal antibody directed against bovine
vimentin (Rab37) and one directed against the vimentin head domain residues 35–50 (Rab484). In contrast, VIM133 did not stain with the monoclonal V.9 IgG that is directed against the carboxyl-terminal tail domain (Fig. 3B, lanes 2). VIM367 migrates as a 41-kDa band that stained with a polyclonal antibody against bovine vimentin and V.9 IgG, but not with Rab484 IgG, the antibody directed against the head domain of vimentin (Fig. 3B, lanes 3). Ligand blot analysis was used to identify the Vn-binding domain on vimentin. Native bovine vimentin and VIM133 bind bt-Vn (Fig. 3B, lanes 1 and 2), whereas VIM367 does not. These data reveal that the Vn-binding site resides in the amino-terminal region of vimentin.

We next examined the interaction of the VIM133 peptide with normal platelet-poor plasma that contains micromolar quantities of plasma Vn. First, varying doses of VIM 133, or the control VIM367 peptide were preincubated for 15 min with platelet-poor plasma containing trace amounts of 125I-labeled native Vn, and then subjected to nondenaturing native PAGE, and autoradiography to visualize changes in the molecular weight of Vn (Fig. 3C). The results indicate that in the absence of VIM peptide (Fig. 3C, lanes 1), plasma Vn predominantly migrates as the monomeric form (arrow c), with lesser quantities of Vn multimers apparent (arrow b). However, the VIM133 peptide induces formation of plasma Vn multimers in a dose-dependent manner (Fig. 3C, arrow a, lanes 2–4). In contrast, the VIM367 peptide did not effect the electrophoretic mobility of Vn. Western blot analysis of the plasma samples containing VIM133 indicate that the vimentin peptide co-migrates with the Vn multimers, and these multimers are resistant to SDS treatment (not shown).

To quantify the binding of Vn to the amino terminus of vimentin, plate microtiter surfaces coated with VIM133 or BSA were incubated with 125I-labeled native or urea-treated oligomeric Vn in the absence or presence of a 20-fold molar excess of the unlabeled ligand. Both native and oligomeric 125I-Vn binds to VIM133 in a dose-dependent fashion (Fig. 3D). Whereas binding of urea-denatured oligomeric 125I-Vn is saturable, the binding of native 125I-Vn is not saturable at the concentrations used in these experiments. Scatchard analysis of the specific binding of urea-denatured Vn reveals the presence of a single class of high affinity binding sites with a $K_d$ of 80 nM. Preincubation with VIM133 slightly increases the binding of Vn to VIM133 by ~2-fold. Oligomeric Vn binds bovine vimentin with a $K_d$ similar to that for VIM133.

To further define the Vn-binding domain on VIM133, the peptide was subjected to limited proteolysis with thrombin and endoprotease Lys-C, and ligand blot analysis was used to identify the Vn-binding fragments. Thrombin, which cleaves the head domain of vimentin at Arg78 (50), generates a fragment that was recognized by Rab484 (Fig. 4A, lane 2, arrow e), an antibody directed against residues 35–50. This fragment binds bt-Vn (Fig. 4B, lane 2, arrow e). Bt-Vn also binds to thrombin within the digestion mixture (Fig. 4B, lane 2, arrow B). Endoproteinase Lys-C cleavages at residues Lys97 and...
Lys104 of VIM133, and generates two bands (51) that are both recognized by the Rab 484 antibody (Fig. 4A, lane 3, arrows d and f), and bind to bt-Vn (Fig. 4B, lane 3, arrows d and f). Taken together, these data suggest that the Vn-binding domain lies within residues 1–78 of vimentin.

Vimentin-dependent Expression of Active PAI-1 on the Surface of Activated Platelets and Platelet Microparticles—We next generated a sheep polyclonal antisera directed against the recombinant VIM133 peptide, and prepared affinity-purified, biotinylated anti-VIM133 IgG for flow cytometry analysis of vimentin expression on the surface of platelets that were activated with various agonists including thrombin, A23187, and TRAP (Fig. 5A). As with the antibodies directed against P-selectin, the anti-VIM133 IgG exhibits minimal or no binding to resting platelets. Activation of platelets with thrombin, A23187, and TRAP increases the expression of the VIM133 sequence on the surface of whole platelets (Fig. 5A), and platelet microparticles (Fig. 5B). The anti-VIM133 IgG binding to platelet surfaces increases rapidly (5–10 min) after thrombin stimulation, and the intensity of binding depends on the concentration of thrombin used to activate the platelets (Fig. 5C). Moreover, activating platelets in the presence of the anti-

![Graphs and tables showing flow cytometry data](image)
VIM133 IgG significantly attenuates PAI-1 expression (Fig. 5D) thereby confirming that vimentin mediates the binding of Vn-PAI-1 complexes on activated platelet surfaces.

Previous flow cytometry studies have demonstrated that in many patients with CAD, unaccustomed strenuous exercise can induce platelet activation and P-selectin expression, events that may contribute to exercise-induced myocardial ischemia (45). We performed flow cytometry analysis on blood samples taken from a small group of healthy individuals and CAD patients undergoing acute exercise stress to provide a clinically relevant example of elevated levels of vimentin and PAI-1 expression on activated surface of platelets activated in situ. Using P-selectin expression again as an index of platelet activation, there was no evidence of exercise-induced platelet activation in any of the four controls following exercise. In contrast, there was modest evidence of exercise-induced platelet activation in four of nine CAD patients, with the percentage of activated platelets increasing from <1% pre-exercise, up to 2.2–6.8% at the 1 min post-exercise time point, and returned back to baseline levels after 3 h. However, in one CAD patient, the platelet activation response was significantly more pronounced (Fig. 6). As in the healthy controls, the pre-exercise flow cytometry dot plots reveal that <1% of the gated events were positive for P-selectin, vimentin, and PAI-1 (Fig. 6A, first row panels). In contrast, 1 min after exercise, the percentage of circulating platelets that were positive for surface P-selectin, vimentin, and PAI-1 increased to ~15, 35, and 10% of the total events, respectively (Fig. 6A, second row panels). After resting for 3 h following the exercise test, the number of P-selectin and PAI-1 positive platelets had returned to baseline levels. However, the number of vimentin-positive events remained elevated (Fig. 6A, third row panels). Analysis of the microparticles present in the PRP prepared from these blood samples confirmed the transient increase in P-selectin and PAI-1, and the sustained elevation in the number of vimentin-positive platelet microparticles (Fig. 6B).

Selective Expression of Active PAI-1 on the Surface of Activated Platelets—The use of the inhibitory antibody MAI-12 in our flow cytometry demonstration of the Vn-dependent expression of platelet PAI-1 supports the hypothesis that active PAI-1 is associated with the vimentin cytoskeleton on the surface of activated platelets and platelet microparticles. To further investigate this issue, we isolated the cellular debris from activated platelet releasates using differential centrifugation, and then analyzed the supernatants and pellets for the presence of PAI-1, Vn, and vimentin using Western blot analysis, and immunoassays for quantifying PAI-1 antigen and activity. The Western blots presented in Fig. 7 reveals that, in contrast to resting platelets, the PAI-1, Vn, and vimentin associated with activated platelet releasates remain in the low speed supernatant, and are then distributed between both the supernatant and Triton X-100-insoluble platelet pellet fractions after a second high speed (100,000 × g, 3 h) centrifugation step. Quantification of the specific activity of the PAI-1 in the high speed supernatant and pellet fractions reveals that ~6% of the total platelet PAI-1 antigen in the platelet releasate remains in the supernatant, and is not functionally active (Table I). In contrast, ~5% of the PAI-1 antigen released from activated platelets is precipitated in the Triton-insoluble platelet pellet fraction, and virtually all of this PAI-1 is functionally active.

Incorporation of Platelet PAI-1 and Vimentin with the Vimentin Cytoskeleton of Platelets in PRP Clots—We conducted dual-labeling immunofluorescence confocal microscopy of fixed, and unfixed PRP clots to examine whether platelet vimentin also regulates the deposition of platelet Vn-PAI-1 complexes in platelet-rich thrombi. The distribu-
tion of von Willebrand factor-positive platelets and platelet microparticles in PRP clots is evident in Fig. 8A. PAI-1 co-localizes with Vn on the surface of activated platelets and the smaller platelet microparticles that are associated with fibrin fibrils (Fig. 8, B–D). Similar images indicative of co-localization were obtained with PRP clots stained for PAI-1 and Vn (Fig. 8E–G), or Vn and vimentin (Fig. 8H–J), indicates co-localization of fluorochromes (yellow). Panels K and L are higher magnification pseudo-colored images of clot-associated Vn and vimentin from clots in panels H and I. The staining pattern in panel K illustrates the two distinct distributions of Vn within clots (arrows), namely fibrin-associated plasma Vn and platelet-derived Vn. Panel L demonstrates the punctuate distribution of platelet-associated vimentin, and the inset illustrates the overlapping distribution (yellow) of the platelet Vn and vimentin (platelet indicated by arrows in panels K and L) on the invaginated surface of the activated platelet surface (scale bar in panels A–J, 20 μm).

VITRONECTIN

VIMENTIN

Platelet-Associated Vitronectin

Platelet-Associated Vimentin

Fibrin-Associated Vitronectin

Fibrin-Associated Vimentin

FIG. 8. Immunofluorescence confocal microscopic localization of PAI-1, Vn, and vimentin in PRP clots. PRP clots were stained with primary antibodies directed against vWF, followed by FITC (green)-conjugated secondary antibodies (panel A). Alternatively, PRP clots were dual-labeled with primary antibodies directed against PAI-1 and Vn (panels B–D), PAI-1 and vimentin (panels E–G), or Vn and vimentin (panels H–L), and each primary detected with FITC (green) or Texas Red rhodamine (red), respectively. Digital overlay of green/red images (panels D, G, J, and inset L), indicates co-localization of fluorochromes (yellow). Panels K and L are higher magnification pseudo-colored images of clot-associated Vn and vimentin from clots in panels H and I. The staining pattern in panel K illustrates the two distinct distributions of Vn within clots (arrows), namely fibrin-associated plasma Vn and platelet-derived Vn. Panel L demonstrates the punctuate distribution of platelet-associated vimentin, and the inset illustrates the overlapping distribution (yellow) of the platelet Vn and vimentin (platelet indicated by arrows in panels K and L) on the invaginated surface of the activated platelet surface (scale bar in panels A–J, 20 μm).
significant co-distribution (yellow) of Vn (FITC; green) and vimentin (Texas Red rhodamine; red) over the invaginated surface of the activated platelet (Fig. 8L, inset).

**DISCUSSION**

Recently, we demonstrated that Vn mediates PAI-1 binding to fibrin (25). In this study, we provide evidence that Vn also mediates the binding of PAI-1 to the vimentin intermediate filament cytoskeleton that is exposed on the surface of activated platelets. Platelets activated by various agonists or by exercise generate platelet microparticles enriched in Vn-PAI-1 complexes. Moreover, confocal image analysis visually confirms that the Vn-PAI-1 complexes co-localize with the platelet vimentin cytoskeleton on the surface platelets in platelet-rich plasma clots suggesting vimentin also regulates the incorporation of active platelet PAI-1 into thrombi. Our evidence that Vn can bind PAI-1 simultaneously with other macromolecules such as vimentin and fibrin (25, 67) further supports our proposal that Vn-PAI-1 complexes bind to macromolecules rather than the individual proteins themselves, and that it is the Vn within these complexes that mediates these binding interactions.

**A New Paradigm for Platelet PAI-1 Function**—Platelet activation triggers cytoskeletal rearrangement that can lead to shape change, exocytosis, microparticle generation, adhesion, aggregation, and retraction (52). Using washed, resting and activated platelets, we measured surface expression of PAI-1 and Vn. PAI-1 was identified with the monoclonal antibody 5A1-12, which preferentially binds to the active form. Our studies suggest that only ~5% of the PAI-1 in platelet releasates is active, a concept supported by previous work (9–11). Only the active PAI-1 is associated with Vn and vimentin on the surface of activated platelets and platelet microparticles. We postulate that active PAI-1 in platelets reflects preformed Vn-PAI-1 complexes that are stored in the α-granule, and are released upon platelet activation. These complexes are bound to vimentin that is exposed during cytoskeleton rearrangement, membrane blebbing, and exocytosis. Given that Vn stabilizes PAI-1 in its active conformation, it is tempting to speculate that PAI-1-bearing platelets or microparticles that are generated in situ may have the potential to inhibit fibrinolysis for prolonged periods of time in the circulation, or at sites of vascular injury and thrombosis. Our findings are also consistent with the recent studies with arterial injury models in Vn-deficient mice that suggest that Vn promotes thrombosis at sites of arterial injury (23, 24).

Platelet activation in response to both weak and potent agonists results in the expression of Vn-PAI-1 complexes on the surface of activated platelets and platelet microparticles. Because we used washed platelets in these studies, the components of these surface-bound complexes are likely to be platelet-derived. This concept supports previous reports demonstrating the release of high molecular weight Vn-PAI-1 complexes from activated platelets (13), and the immunolocalization of these proteins on the surface of activated platelet and fibrin (31–33).

**Vimentin-type Intermediate Filaments**—Vimentin is the major type III intermediate filaments expressed in cells of mesenchymal (e.g. endothelium, fibroblasts, megakaryocytes), and myogenic (e.g. smooth muscle cells). Vimentin, a minor component of the platelet cytoskeleton (52), is associated with the Triton X-100 insoluble fraction of human platelets (35, 36), the same fraction that contains the active PAI-1 in activated platelet releasates. When examined by electron microscopy, vimentin forms a network of 10 nm intermediate filaments that form a ring close to the cell membrane, as well as a network in the body of cells. Our flow cytometry data indicate that this vimentin network is exposed when platelets are activated with thrombin or other agonists. Using recombinant vimentin fragments, we identified a high affinity Vn-binding site on the amino-terminal head domain of vimentin. The binding site is localized to the first 78 amino acids, a region that contains a non-α-helical domain with two motifs crucial for filament assembly: a nonapeptide with the sequence SSYRXFGG (53) and an arginine and proline-containing domain known as the RP-Box (54). The two arginine residues within the nonapeptide sequence are critical for filament assembly (50, 51, 54, 55). Likewise, the highly conserved RP-box sequence, which also is found in type III intermediate filaments, such as desmin, peripherin, and glial fibrillary acidic protein, contains serine phosphorylation sites, and arginine residues that also are critical for filament assembly (53–55).

The amino terminus of vimentin interacts with negatively charged phospholipid bilayers (56), thereby rationalizing its location near the cell membrane. Our studies reveal a novel role for the amino terminus of vimentin; namely, as a high affinity binding site for Vn.

The observation that the VIM133 peptide induces plasma Vn multimerization raises the possibility that vimentin exposure on the surface of activated platelets, or other cells may not only bind Vn, but may also modulate the structure and function of localized Vn polymers. Furthermore, our current findings represent a plausible explanation for our previous demonstration of increased expression of PAI-1 on the surface of endothelium exposed to bacterial endotoxin and other inflammatory mediators in vitro (57) and in vivo (34). Vimentin also may modulate inflammatory responses and atherosclerosis (58) through its interactions with plasma proteins such as complement (59, 60), IgG (61, 62), and fibrinogen (62). Cyto-keratin-type intermediate filaments that are also exposed on the surface of various cells interact with various hemostasis factors including kininogen (63, 64), plasminogen (65), and thrombin-antithrombin-Vn complexes (66). The potential clinical significance of our findings is underscored by the evidence of exercise-induced expression of vimentin and PAI-1 on the surface of circulating platelets and platelet microparticles in a small number of high-risk patients with CAD. Future studies are required to better understand the mechanisms regulating vimentin interactions with Vn, and the role of platelet surface-bound PAI-1 in fibrinolysis and in various prothrombotic states.

**REFERENCES**

Vitronectin Mediates PAI-1 Binding to Platelet Vimentin

Vimentin Exposed on Activated Platelets and Platelet Microparticles Localizes Vitronectin and Plasminogen Activator Inhibitor Complexes on Their Surface
Thomas J. Podor, Davindra Singh, Paul Chindemi, Denise M. Foulon, Robert McKelvie, Jeffrey I. Weitz, Richard Austin, Ghislain Boudreau and Richard Davies

doi: 10.1074/jbc.M109675200 originally published online December 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109675200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 35 of which can be accessed free at http://www.jbc.org/content/277/9/7529.full.html#ref-list-1