Proteolysis of von Willebrand Factor and Shear Stress–Induced Platelet Aggregation in Patients With Aortic Valve Stenosis

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Background—Excessive bleeding may complicate congenital cardiac defects. To explain the pathogenesis of this abnormality, we evaluated selected parameters of primary hemostasis in patients with aortic valve stenosis before and after corrective surgery.

Methods and Results—We examined shear-induced platelet aggregation with the filter aggregometer test and von Willebrand factor (vWF) structure by evaluating the multimeric distribution and extent of subunit proteolysis. The platelet count was reduced before corrective surgery, and shear-induced platelet aggregation was impaired. Moreover, vWF multimers of higher molecular mass were decreased, and proteolytic subunit fragments were increased. After correction of the cardiac defect, all of these parameters returned to normal.

Conclusions—Alterations of vWF and platelet function may contribute to the bleeding diathesis in patients with aortic valve stenosis. Improvement after corrective surgery suggests that the passage of blood through a stenosed aortic valve may result in shear forces that induce vWF interaction with platelets in the circulation and, in turn, trigger platelet clearance, vWF degradation, and the impairment of primary hemostasis. (Circulation. 2000;102:1290-1295.)

Key Words: von Willebrand factor ■ platelets ■ stenosis ■ valves

Bleeding from gastrointestinal angiodysplasia may complicate aortic valve stenosis.1–4 Supportive therapy is often inadequate to control such episodes, and replacement of the stenotic valve, rather than resection of the affected bowel tract, provides the most effective treatment.5 In nearly all cases, the largest multimers of plasma von Willebrand factor (vWF) are decreased.5,6 Patients with other congenital cardiac defects, mainly of the ventricular septum, occasionally exhibit a similar abnormality.7 The structural alteration of vWF may be relevant in the pathogenesis of hemorrhage because large multimers are also deficient in individuals with type 2A von Willebrand disease (vWD),8,9 in whom bleeding from the intestinal wall, often associated with angiodysplasia,4,5 is a frequent complication. The vWF abnormality in type 2A vWD is caused by congenital defects10,11 that enhance the susceptibility of the molecule to proteolysis.12 Under normal conditions, cleavage of a distinct bond in the vWF subunit,13 presumably resulting from the activity of a specific protease,14,15 is a physiological process that regulates the size of circulating multimers.16 Thus, vWF in normal plasma contains fragments of the 225-kDa native subunit,17 mainly with apparent molecular masses of 176 and 140 kDa, along with a minor component of 189 kDa. Patients with type 2B vWD8 also exhibit enhanced vWF proteolysis12 that results from binding of a distinctly abnormal vWF to circulating platelets,18 with subsequent microaggregation and rapid clearance.19 Fragments of the vWF subunit may also be increased in individuals with certain acquired clinical conditions, mostly as a consequence of enhanced activity of specific proteolytic enzymes.20–23

Blood components are subjected to increased shear stress when passing at greater than normal velocity through a stenosed aortic valve.24 Such a condition may contribute to molecular changes of vWF that increase the risk of bleeding associated with this cardiac defect. Previous experimental work has indicated that shear forces resulting from the circulation of blood may be relevant in rendering vWF susceptible to proteolytic cleavage,25 possibly by changing the shape of the molecule from coiled coil to elongated filament26 and exposing the bond between residues Tyr842 and Met843 to specific protease activity.13 Moreover, high shear stress may induce binding of the high-molecular-weight multimers of plasma vWF to the platelet membrane27 with subsequent aggregation.28–30 Thus, elevated shear exerts mul-
tiple effects on vWF and could account for the disappearance of the larger multimeric forms, possibly with increased subunit proteolysis, in patients with aortic valve stenosis. The purpose of the present study was to evaluate this hypothesis by analyzing the occurrence of vWF subunit degradation and the characteristics of shear-induced platelet aggregation in such individuals. The results demonstrate enhanced proteolysis of vWF associated with increased platelet turnover but decreased aggregation of circulating platelets. These anomalies, which can all contribute to a heightened hemorrhagic risk, were reversed by surgical correction of the stenotic valve.

Methods

Patients

Twenty-two patients, 9 men and 13 women between the ages of 37 and 86 years (mean, 65 years), with stenosis of the aortic valve were enrolled in the study. All underwent evaluation before and 6 months after corrective surgery. For shear-induced platelet aggregation experiments, the patients were compared with 48 healthy volunteers aged between 24 and 86 years (mean, 61 years). All patients and control subjects signed an informed consent, according to the Declaration of Helsinki, after approval of the protocol by the Ethics Committee of the University of Milan. Blood for testing vWF antigen (vWF:Ag) as well as multimer and subunit structure was collected from an antecubital vein into a polypropylene tube containing a 1/10 final volume of an anticoagulant and protease inhibitor mixture, to yield 125 mmol/L trisodium citrate, 5 mmol/L EDTA, and 6 mmol/L N-ethylmaleimide. This blood was centrifuged at 3600g for 20 minutes, and the resulting plasma was frozen in ethanol/dry ice and stored at −70°C until tested. Shear-induced platelet aggregation was evaluated with 20 μL of whole blood collected into polypropylene tubes containing 500 U/mL of recombinant hirudin (HV1, IKETON Farmaceutici Srl) and tested within 3 hours of collection. Cells were counted with an automated instrument (Coulter T-890, Instrumentation Laboratory) in 5 mL of whole blood containing EDTA.

Measurements of vWF

Plasma vWF:Ag was measured with an enzyme immunoassay with the monoclonal antibody LJ-C3 (10 μg/mL) for capture and the peroxidase-conjugated monoclonal antibody LJ-2.2.9 (5 μg/mL) for detection.11 The concentration of vWF was calculated from a standard curve obtained with pooled, normal plasma.11 The multimeric structure of vWF was evaluated by SDS-agarose gel electrophoresis on a low-resolution gel system (0.8% low-gelling-temperature agarose). The percentage of high-molecular-weight vWF proteolysis, the protein was isolated from plasma by using the monoclonal antibody RG 5.5.72 coupled to CNBr-activated Sepharose CL 4B (Pharmacia) at a density of 4 mg of IgG per milliliter of beads, as described.12 Purified vWF was reduced with 65 mmol/L DTT in the presence of 2% SDS for 15 minutes at 69°C. Electrophoresis was performed in 5% SDS-polyacrylamide gels. The protein was then transferred to nitrocellulose membranes, incubated with a pool of monoclonal antibodies directed against multiple epitopes in vWF,13,14 and visualized with 125I-labeled rabbit anti-mouse IgG. Samples were also probed with the monoclonal antibodies M7 and M31, which react with epitopes located between Lys185 and Met288 or Leu1481 and Met1693, respectively.15 In the presence of reduced vWF from normal plasma, both antibodies bind to the intact subunit of 225 kDa. Moreover, antibody M7 specifically recognizes the normal proteolytic fragment of 140 kDa and the fragment of 176 kDa generated by plasmid digestion of vWF. Antibody M31 specifically recognizes the normal proteolytic fragments of 189 and 176 kDa as well as the fragment of 145 kDa generated by plasmin digestion.15 Numbering of vWF residues is given here according to the sequence of the mature subunit, from which numbering according to the sequence of the pre-pro-vWF precursor is obtained by adding 763. All stated molecular masses are apparent as calculated from relative mobility in SDS-polyacrylamide gels. The relative proportion of vWF fragments was determined by excising individual bands from the nitrocellulose membrane, using the autoradiograph as a template, counting the corresponding radioactivity, and expressing it as a percentage of the total counts from all bands.

Shear-Induced Platelet Aggregation

This measurement was performed as previously described by O’Brien and Salmon.14 Five milliliters of whole blood containing hirudin was pushed through the capillary-size channels of polycarbonate glass fibers (Pall U100, Pall Process Filtration) at a constant pressure of 100 mm Hg. The glass fibers have a diameter ranging from 0.1 to 3.4 μm, and the filter retains particles having a diameter >10 μm. The drops of blood passing through the filter in 20 seconds were enumerated by an automatic drop counter and collected into tubes containing EDTA. The percentage of retained platelets was calculated from the counts in blood before and after passage through the filter.

Glycocalcin Assay

Plasma glycosaccharin (GC) was measured as previously described.35 Values were expressed both as concentration in platelet-poor plasma and as an index relative to the platelet count (GC index=sample GC concentration in μg/mL×(250×10^3/L)/(sample platelet count)).

Statistical Methods

Comparisons between measurements before and after surgery were performed with Student’s t test for paired values, with the assumption that the differences between the paired observations were normally distributed. We based our approach on the consideration that before and after measurements are commonly acknowledged to be naturally paired data.

Results

Structure of Plasma vWF in Patients With Aortic Valve Stenosis

The concentration of plasma vWF:Ag was within normal limits in all patients tested before correction of the aortic valve stenosis (Figure 1). After surgery, a significant increase was observed in all but 4 of the 22 cases studied, in whom the vWF:Ag concentration actually decreased (Figure 1). In all individuals, the corresponding values remained within the normal range except in 2 cases, in whom plasma vWF was above the upper limit of normal (Figure 1). By comparison, there was no significant difference in the plasma concentration of fibrinogen tested before and after surgery (data not shown), indicating that the changes in vWF were not a reflection of a generalized disturbance of plasma proteins.

The relative proportion of large vWF multimers was significantly decreased in most patients before treatment, but complete correction of this defect was observed 6 months after valve replacement (Figure 2A). The loss of large vWF multimers was accompanied by a decrease in the proportion of intact subunits relative to the proteolytic fragments of 176 and 140 kDa, a structural alteration that was normalized after surgical correction of the stenosis (Figure 2B). Of note, the relative proportion of the minor 189-kDa fragment remained unchanged (not shown). Immunoblotting analysis of reduced plasma vWF demonstrated that the enhanced proteolysis...
observed before replacement of the diseased valve generated the same vWF subunit fragments seen in normal plasma, without evidence for novel species with distinct immunochemical characteristics (Figure 3).

**Platelet Function in Patients With Aortic Valve Stenosis**

The platelet count was significantly increased ($P<0.05$) after correction of the aortic valve stenosis but remained within normal limits (Figure 4A). There was no difference in leukocyte and red blood cell count before or after surgery (data not shown). The plasma concentration of soluble GC, a marker for platelet membrane destruction, was determined in 17 patients before and after valve replacement and was found to be within normal limits in both instances (Figure 4B). Shear-induced platelet aggregation was significantly increased after correction of the aortic valve stenosis, and this was particularly true for the patients whose values were outside the normal range before surgery (Figure 5). The enhanced function after valve replacement was evident both as an increase in the percentage of retained platelets and as a decrease in the amount of blood flowing through a device in normal limits (Figure 4A).
which blood was pushed through a filter under conditions generating elevated shear stress (Figure 5).

**Discussion**

These results identify changes of vWF structure and alterations of platelet function that may contribute to a defect of primary hemostasis in patients with aortic valve stenosis. The decreased concentration of large plasma vWF multimers may reduce the interaction of platelets with exposed tissues at sites of vascular injury, because vWF with normal structure is required for platelet thrombus formation under arterial flow rate conditions. Lack of large vWF multimers is the cause of bleeding in type 2A vWD patients, in whom defective hemostasis persists even when plasma levels of the altered, endogenous vWF are raised by treatment with desmopressin. A normal concentration of vWF protein, therefore, is not sufficient to support platelet adhesion and aggregation if the structure of the molecule is not preserved. The majority of patients evaluated in this study had frankly reduced large vWF multimers before correction of their valvular defect, thus explaining the occurrence of a bleeding diathesis despite a total plasma vWF concentration that was within the control range.

The existence of an abnormally enhanced proteolysis of the constitutive subunit may explain the structural defect of vWF in patients with aortic valve stenosis. It is well established that a single peptide bond cleavage in the vWF subunit, between Tyr842 and Met843, leads to a significant reduction in multimer size. Of note, the subunit fragments detected in the patients had immunochemical characteristics indistinguishable from those of fragments found at lower concentrations in all normal individuals. In other pathological situations, eg, acute pancreatitis and decompensated cirrhosis, enzymes such as elastase may be responsible for the digestion of vWF. Moreover, in patients with myocardial infarction treated by thrombolytic therapy, enhanced vWF proteolysis depends on the action of plasmin. In all of these cases, the fragments of vWF subunits may be similar to the ones present in normal individuals with respect to molecular mass, but they can be differentiated by immunochemical analysis in that they originate from different regions of the subunit and contain distinct epitopes. Our findings rule out the presence of unusual vWF fragments in aortic stenosis patients and suggest that a physiological vWF-cleaving protease is involved in the pathogenesis of the bleeding tendency that appears in some of these individuals.

The correction of vWF structural abnormalities after surgical replacement of a diseased aortic valve indicates that disturbances of flow caused by the passage of blood through the constricted orifice, thereby generating high shear stress and turbulence, may be responsible at least in part for the heightened vWF proteolysis. All of the patients enrolled in our study had a pressure gradient between the left ventricle and aorta >50 mm Hg, a value at which marked hemodynamic alterations develop across the valve and corrective surgery is considered necessary. One possibility is that shear forces directly affect the molecular conformation of large vWF multimers, with exposure of the proteolytically sensitive bond between Tyr842 and Met843. Alternatively, but more likely in addition, blood flow with high shear rate may promote vWF A1-domain binding to platelet glycoprotein Ibα, an interaction that is typically short-lived unless coupled to activation. The immobilization of vWF on the platelet membrane may favor the formation of small aggregates that are temporarily sequestered from the circulation and promote degradation of larger multimers by action of the natural vWF-cleaving protease. The overall equilibrium of a process of this nature may explain the relative decrease in platelet count and total vWF plasma concentration observed in the patients before compared with after replacement of the stenotic valve. A similar mechanism has been proposed to explain the pathogenesis of type 2B vWD, in which large vWF multimers disappear from the plasma as a consequence of single point mutations that enhance the affinity of the A1 domain for platelet glycoprotein Ibα. In such a situation, vWF binding to platelets in the circulation may occur even in the absence of abnormally elevated shear rates but still leads to the formation of microaggregates, with transient platelet sequestration and multimer degradation. The occurrence of these pathogenetic events is documented by the rapidly reversible thrombocytopenia that develops when the plasma concentration of type 2B vWF is acutely increased after the administration of desmopressin. The relatively lower platelet count observed in the patients with aortic valve stenosis before compared with after correction of the defect is in agreement with the proposed consequences of vWF binding, although it could also be explained by direct mechanical disruption of platelets during passage through the stenotic orifice. The fact that soluble plasma GC was not increased in the samples obtained before surgery, when platelet counts were lower in the patients, favors the former hypothesis. Such a finding tends to exclude the possibility that platelet destruction, as seen in autoimmune thrombocytopenia with elevated plasma GC, is the cause of decreased platelet counts but is
compatible with the concept that reversible platelet sequestration is responsible for the phenomenon.

The measurement of shear-induced platelet aggregation ex vivo appears to be a sensitive labortory technique to document the abnormality of platelet function caused by aortic valve stenosis. The fact that aggregation induced by the passage of blood through the filter aggregometer at high shear rates was decreased indicates that the interaction between vWF and platelets is compromised in these patients. The alteration is explained by the absence of large-molecular-mass vWF multimers, which are known to play a key role in shear-induced aggregation.\(^{29}\) For example, when patients with type 3 severe vWD are treated to raise their plasma levels of vWF, the results of the filter aggregometer test remain abnormal if the therapeutic concentrates are deficient in large-molecular-mass multimers.\(^{31,41}\) Of note, the bleeding alteration is explained by the absence of large-molecular-mass multimers, which are known to play a key role in shear-induced aggregation.\(^{29}\) For example, when patients with type 3 severe vWD are treated to raise their plasma levels of vWF, the results of the filter aggregometer test remain abnormal if the therapeutic concentrates are deficient in large-molecular-mass multimers.\(^{31,41}\) Of note, the bleeding rate was decreased indicates that the interaction between vWF and platelets is compromised in these patients. The overall imbalance of primary hemostasis was mild. On the other hand, it is known that this test is not always an accurate predictor of a bleeding tendency,\(^{42}\) as has also been demonstrated in patients with mild vWD.\(^{43,44}\) We observed hemorrhagic complications characterized by intestinal bleeding in 2 patients with aortic valve stenosis before surgery. The parameters reflecting high-molecular-mass vWF multimer concentration and subunit proteolysis were as abnormal as those in patients without a bleeding tendency, stressing the predictable concept that alterations of vWF and platelet function are only 1 of several determinants of abnormal bleeding. Our findings, nevertheless, indicate that the treatment of hemorrhagic complications in patients awaiting valvular replacement should include the infusion of suitable concentrates to normalize the plasma levels of large vWF multimers. In these cases, a test of shear-induced platelet aggregation should be well suited for evaluating the effects of treatment.

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