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Plasmatic ADAMTS-13 metalloprotease and von Willebrand factor in children with cyanotic congenital heart disease

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Abstract

Changes in plasma von Willebrand factor concentration (VWF:Ag) and ADAMTS-13 activity (the metalloprotease that cleaves VWF physiologically) have been reported in several cardiovascular disorders with prognostic implications. We therefore determined the level of these proteins in the plasma of children with cyanotic congenital heart disease (CCHD) undergoing surgical treatment. Forty-eight children were enrolled (age 0.83 to 7.58 years). Measurements were performed at baseline and 48 h after surgery. ELISA, collagen-binding assays and Western blotting were used to estimate antigenic and biological activities, and proteolysis of VWF multimers. Preoperatively, VWF:Ag and ADAMTS-13 activity were decreased (65 and 71% of normal levels considered as 113 (105-129) U/dL and 91 ± 24% respectively, $P < 0.003$) and correlated ($r = 0.39$, $P = 0.0064$). High molecular weight VWF multimers were not related, suggesting an interaction of VWF with cell membranes, followed by proteolytic cleavage. A low preoperative ADAMTS-13 activity, a longer activated partial thromboplastin time and the need for cardiopulmonary bypass correlated with postoperative bleeding ($P < 0.05$). Postoperatively, ADAMTS-13 activity increased but less extensively than VWF:Ag (respectively, 2.23 and 2.83 times baseline, $P < 0.0001$), resulting in an increased VWF:Ag/ADAMTS-13 activity ratio (1.20 to 1.54, respectively, pre- and postoperative median values, $P = 0.0029$). ADAMTS-13 consumption was further confirmed by decreased ADAMTS-13 antigenic concentration (0.91 ± 0.30 to 0.70 ± 0.25 μg/mL, $P < 0.0001$) and persistent proteolysis of VWF multimers. We conclude that, in pediatric CCHD, changes in circulating ADAMTS-13 suggest enzyme consumption, associated with abnormal structure and function of VWF.

Key words: ADAMTS-13; von Willebrand factor; Congenital heart disease; Thrombosis; Cardiac surgery

Introduction

ADAMTS is an abbreviation for *a desintegrin and metalloprotease with thrombospondin type 1 motif*, and corresponds to a family of zinc metalloproteases with desintegrin and protease domains (ADAM-related), cystein-rich regions and a thrombospondin 1 repeat. ADAMTS-13, a member of the family, is synthesized in hepatic stellate cells and endothelial cells (1-3), and is the principal von Willebrand factor (VWF) cleaving protease. Mutations of the ADAMTS-13 gene are associated with the presence of abnormally large VWF multimers in plasma, which constitutes the basis for the thrombotic events that occur in subjects with congenital thrombotic thrombocytopenic purpura. Protease deficiency may also occur in the presence of autoimmune IgM and IgG inhibitors (4,5).

An imbalance between the circulating levels of VWF and ADAMTS-13 has been reported in a number of acquired diseases such as coronary artery disease and myocardial infarction, peripheral arterial disease, ischemic stroke, preeclampsia, inflammatory bowel disease, and liver cirrhosis (6-9). Elevated plasma VWF antigen (VWF:Ag) and/or decreased ADAMTS-13 activity are associated with negative outcomes of these disorders. In disseminated intravascular coagulation and sepsis, exceedingly high VWF:Ag has been reported in association with a progressive decrease in ADAMTS-13 activity as the severity of the disease increases. This reflects widespread thrombotic microangiopathy generally associated with circulating ultralarge VWF multimers (10,11).

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Abnormalities in coagulation parameters and platelet function have been reported in patients with cyanotic congenital heart disease (CCHD). These abnormalities tend to be more pronounced in the perioperative course, particularly in the presence of cardiopulmonary bypass (12-15). Albeit changes in ADAMTS-13 activity and VWF protein levels have been reported in subjects undergoing ON- as well as OFF-pump cardiac surgery (11,16-18), they have not been investigated in the pediatric population with CCHD undergoing surgical palliation or repair. Thus, the present study was planned to analyze the pre- and postoperative abnormalities in circulating ADAMTS-13 and VWF in this setting.

Material and Methods

Patients, diagnostic data and postoperative events

Pediatric patients with CCHD undergoing cardiac surgery (first operation or reoperation) were enrolled consecutively. Diagnostic data were collected on a hospital basis, one day before surgery, at the Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil. Neonates, patients under intensive care and those undergoing emergency cardiac surgery were excluded. Preoperative (baseline) data were registered, including demographic and general laboratory parameters, and the principal diagnosis as established by Doppler-echocardiography and, in some instances, angiography. The need for cardiopulmonary bypass as well as relevant postoperative events (infection, sepsis, bleeding, and fatal outcome) are reported. Relevant postoperative bleeding was defined as 10 mL kg^{-1} h^{-1} during the first hour and 5 mL kg^{-1} h^{-1} subsequently, with the need for blood product transfusion. The study was approved by the Scientific and Ethics Committee of the Instituto do Coração and Hospital das Clínicas, Universidade de São Paulo, Brazil (CAPPesq #07106).

Biochemical analyses

Peripheral venous blood was collected 1 day before and 48 h after surgery. Blood was collected [1:10 (v/v) into 3.2% sodium citrate] for analysis of plasma VWF:Ag and VWF biological activity (VWF:CB), ADAMTS-13 antigen (ADAMTS-13:Ag) and ADAMTS-13 activity. Protease inhibitors were added for the analysis of VWF multimeric structure (19).

Plasma VWF:Ag and ADAMTS-13:Ag were determined by ELISA (Diagnostica Stago, France, and Technoclone, Austria, respectively). Data are reported as U/dL and μg/mL, respectively. Plasma VWF:CB was measured as the ability of the VWF protein to bind to collagen, according to Siekmann et al. (20). An in-house enzyme immunoassay was developed using a peroxidase-conjugated rabbit anti-human VWF polyclonal antibody (Dako Corporation, USA). Data are reported as percent activity relative to normal pooled plasma used in the same assay.

ADAMTS-13 activity was measured by the collagen binding method proposed by Rick et al. (21). Briefly, an in-house enzyme immunoassay was developed, where plasma was used as the source of both enzyme and substrate. Plasma was processed before (baseline sample) and after dialysis against 1.5 M urea. Diluted plasma samples were added in triplicate to plate wells pre-coated with collagen (Vitrogen, Cohesion Corp., USA). The binding of VWF to collagen was measured using peroxidase-labeled rabbit anti-human VWF polyclonal antibody (Dako Corporation). Plates were analyzed

Table 1. Baseline laboratory data.

	Patients (n = 48)	Healthy children* (n = 9)	P
SpO_2 (%)	80 (53-91)	98 (95-99)	<0.0001
Hematocrit (%)	47 ± 7	37 ± 3	<0.0001
Hemoglobin (g/dL)	15.9 ± 2.3	12.7 ± 1.2	<0.0001
Platelets (x10^9/L)	313 ± 114	301 ± 56	0.8438
Leukocytes (cells/mm^3)	10631 ± 3613	5667 ± 1222	<0.0001
VWF:Ag (U/dL)	73 (51-159)	113 (105-129)	<0.0001+
VWF:CB (%)	48 ± 21	63 ± 9	0.0004+
ADAMTS-13:Ag (μg/mL)	0.91 ± 0.29	1.11 ± 0.09	0.0002+
ADAMTS-13 activity (%)	65 ± 24	91 ± 24	0.0029+
VWF:Ag/ADAMTS-13 activity	1.17 (0.62-4.84)	1.13 (1.1-1.69)	0.7427
VWF multimers	0.44 (0.11-0.56)	0.36 (0.25-0.55)	0.0081+

Data are reported as means ± SD or median and range. ADAMTS-13:Ag = plasma ADAMTS-13 antigenic concentration; SpO_2 = peripheral oxygen saturation; VWF:Ag = plasma von Willebrand factor antigenic concentration; VWF:Ag/ADAMTS-13 activity = ratio of VWF antigenic concentration to ADAMTS-13 activity; VWF:CB = plasma VWF activity reported as collagen binding capacity; VWF multimers = ratio of low molecular weight multimer density to total multimer density of VWF. *Age 5.75 to 7.25 years (median 6.08 years). +Differences were found to be significant using both non-parametric and parametric statistics for the appropriate data (Mann-Whitney test and Student *t*-test).

on a plate reader at 492 nm. ADAMTS-13 activity was calculated taking into account the residual collagen binding activity after dialysis, and the collagen binding activity in the individual's baseline sample. Data are reported as percent activity.

The multimeric structure of VWF was detected and analyzed by Western blotting as previously described (19). Luminographs were subjected to densitometric analysis, and the relative density of low molecular weight VWF multimers was calculated. Low molecular weight VWF fractions were considered as the five lower bands migrating just above immunoglobulin M (950 kDa, used as a molecular mass marker). The density of these bands was calculated, and the sum was divided by total multimer density.

Biochemical analyses were also performed using samples collected from healthy pediatric subjects, and data are presented as control values. These were children suspected of having mild heart disease, but found to have normal cardiovascular status after diagnostic evaluation.

Statistical analysis

Data are reported as means and standard deviation or median and range. Comparisons between groups were performed using the Student *t*-test or the Mann-Whitney test. Preoperative and postoperative data were compared using the paired *t*-test or the Wilcoxon test. For correlations, the Spearman coefficient (r_s) was calculated. For variables with Gaussian distribution, linear regression and analysis of covariance (ANCOVA) were used to analyze pre- and postoperative associations. Logistic regression models were constructed to investigate possible associations with postoperative bleeding. A confounder was defined as a second variable causing a significant reduction in the hazard ratio associated with the variable under investigation. Multivariate analysis was carried out by including all variables with a P value of <0.10 in univariate and bivariate analysis. In all final procedures, 0.05 was adopted as the level of significance.

Results

Of 56 consecutive CCHD patients who were candidates for elective pediatric cardiac surgery, 48 were enrolled. Eight children could not be included, as the surgical procedure had to be postponed in view of an acute illness (generally, an acute viral insult). Age ranged from 0.83 to 7.58 years (median 2.54 years) and mean body weight was 11.9 ± 3.7 kg. Despite decreased peripheral oxygen saturation (Table 1), no patients had hematocrit above 55%. The leukocyte count was significantly elevated compared with healthy pediatric subjects, but there was no evidence of ongoing infectious or inflammatory disorders. The platelet count of the patients was normal, as were blood coagulation tests [activated partial thromboplastin time (APTT) and prothrombin time] (data not shown).

As shown in Table 1, preoperative VWF:Ag and VWF:CB were statistically less in patients. Lower ADAMTS-13:Ag and ADAMTS-13 activity in patients compared to controls (Table 1) were associated with a loss of high molecular mass VWF multimeric protein complexes, resulting in increased density of low molecular mass fractions (Table 1 and Figure 1), which might be interpreted as proteolytic degradation with enzyme consumption. In order to test for possible influences of ABO blood groups on the biochemical parameters (group ''O'' patients are known to have lower VWF levels compared to ''non-O'' subjects), VWF and ADAMTS-13 levels were

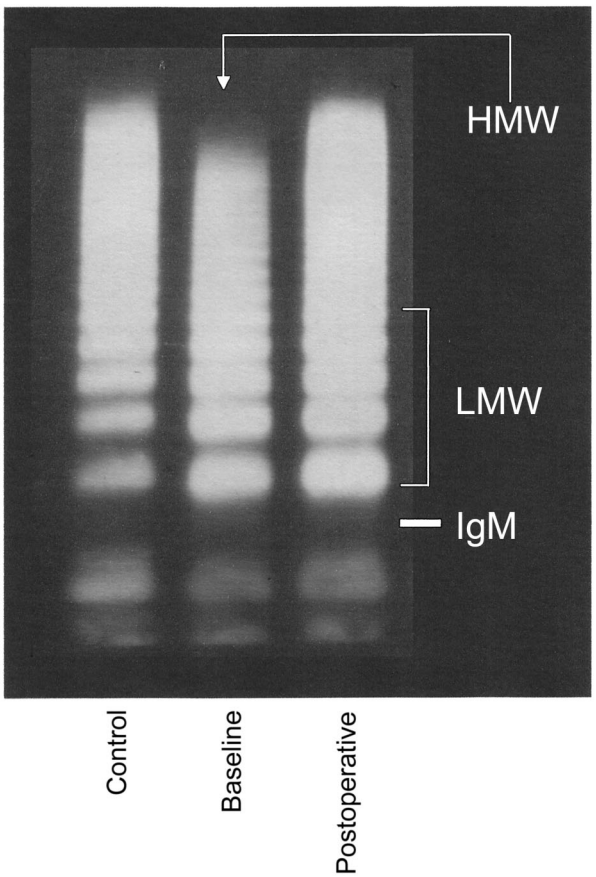

Figure 1. Representative Western immunoblot showing plasma von Willebrand factor multimeric structure (pattern observed in 36 of 48 children with cyanotic congenital heart disease). At baseline, reduction of high molecular weight multimers (HMW), with increased density of low molecular weight fractions (LMW, five bands migrating just above IgM) compared with control plasma (relative optical density of LMW multimers 0.44 (0.11-0.56) and 0.36 (0.25-0.55), respective median value and range in patients and controls, P = 0.0081). Postoperatively, the density of LMW fractions remained high [0.45 (0.17-0.63), P = 0.5360 versus baseline]. The migration of immunoglobulin M (IgM, 950 kDa) is indicated.

Table 2. Comparisons of VWF and ADAMTS-13 levels in patients according to ABO blood groups.

	Group O patients (n = 26)	Group non-O patients (n = 22)	P
VWF:Ag (U/dL)	71 (51-159)*	83 (51-143)+	0.0216
VWF:CB (%)	48 ± 18	48 ± 25	0.9446
ADAMTS-13:Ag (µg/mL)	0.91 ± 0.28	0.90 ± 0.31	0.8274
ADAMTS-13 activity (%)	62 ± 20	68 ± 28	0.4171

Data are reported as means ± SD or median and range. ADAMTS-13:Ag = plasma ADAMTS-13 antigenic concentration; VWF:Ag = plasma von Willebrand factor antigenic concentration; ADAMTS-13 activity = plasma ADAMTS-13 activity concentration; VWF:CB = plasma VWF activity reported as collagen binding capacity. *$P < 0.0001$ and +$P = 0.0004$ versus control level of 113 (105-129) U/dL (Mann-Whitney test).

analyzed in these groups. Table 2 shows that the only statistically significant difference between group ''O'' and group ''non-O'' patients was related to VWF:Ag level ($P = 0.0216$). However, in both blood groups VWF:Ag level was lower compared to controls. There were no significant associations of VWF and ADAMTS-13 levels with general laboratory data, except for a positive correlation of ADAMTS-13:Ag with peripheral oxygen saturation ($r_s = 0.39$, $P = 0.0059$).

Corrective and palliative procedures performed in 48 children included Glenn (n = 14) and Fontan (n = 9) operations, total repair of tetralogy of Fallot (n = 11) or pulmonary atresia (n = 7), Blalock-Taussig anastomoses (n = 6), and pulmonary artery banding (n = 1). Cardiopulmonary bypass was required in 31 cases (duration of 38 to 250 min). Peripheral oxygen saturation increased significantly in the entire patient group (Table 3). There were three immediate postoperative deaths associated with low cardiac output and circulatory collapse. None of the 45 remaining patients had any evidence of systemic infection or sepsis. Significant postoperative bleeding occurred in 13 children.

The results obtained at 48 h postoperatively are presented in Table 3. There was an important (almost three-fold) increase in VWF:Ag, although the density of low molecular weight fractions remained high (Table 3 and Figure 1). VWF:CB increased from baseline. ADAMTS-13 activity increased, albeit not as impressively as VWF:Ag; this resulted in a significant increase in VWF:Ag/ADAMTS-13 activity ratio relative to baseline. ADAMTS-13:Ag actually decreased (Table 3). ADAMTS-13 and VWF activities correlated significantly with VWF:Ag at baseline and after surgery (Figure 2). Lowest preoperative levels of ADAMTS-13 activity were seen at VWF:Ag levels around 50 U/dL. Postoperative ADAMTS-13 activity and VWF:CB varied considerably at different concentrations of VWF. In addition, the VWF:CB line was considerably flattened after surgery versus baseline (Figure 2B, different slopes, $P = 0.0477$), suggesting lowered VWF biological activity relative to its

Table 3. Postoperative changes in laboratory parameters compared with baseline.

	Baseline (n = 45)	Postoperative (n = 45)	P
SpO_2 (%)	80 (53-91)	90 (71-99)	<0.0001
Hematocrit (%)	47 ± 7	36 ± 7	<0.0001
Hemoglobin (g/dL)	15.9 ± 2.4	11.7 ± 2.1	<0.0001
Platelets ($x10^9$/L)	317 ± 116	189 ± 113	<0.0001
Leukocytes (cells/mm^3)	10702 ± 3612	16067 ± 6826	<0.0001
VWF:Ag (U/dL)	75 (51-159)	212 (59-327)	<0.0001
VWF:CB (%)	48 ± 21	94 ± 33	<0.0001
ADAMTS-13:Ag (µg/mL)	0.91 ± 0.30	0.70 ± 0.25	<0.0001
ADAMTS-13 activity (%)	64 ± 25	143 ± 59	<0.0001
VWF:Ag/ADAMTS-13 activity	1.20 (0.67-4.84)	1.54 (0.51-5.13)	0.0029
VWF multimers	0.44 (0.11-0.56)	0.45 (0.17-0.63)	0.5360

Data are reported as means ± SD or median and range. ADAMTS-13:Ag = plasma ADAMTS-13 antigenic concentration; SpO_2 = peripheral oxygen saturation; VWF:Ag = plasma von Willebrand factor antigenic concentration; VWF:Ag/ADAMTS-13 activity = ratio of VWF antigenic concentration to ADAMTS-13 activity; VWF:CB = plasma VWF activity reported as collagen binding capacity; VWF multimers = ratio of low molecular weight multimer density to total multimer density of VWF. Data were collected at 48 h postoperatively from 45 patients who were alive at that time. The Wilcoxon test and the Student *t*-test for pairwise observations were used for statistical analysis.

antigenic concentration.

Preoperative levels of six specific variables (VWF:Ag, VWF:CB, ADAMTS-13:Ag, ADAMTS-13 activity, VWF:Ag/ADAMTS-13 activity ratio, and the relative density of low molecular weight VWF multimers) were tested for a possible association with postoperative bleeding. In univariate analysis, only preoperative ADAMTS-13 activity was significantly (negatively) associated with bleeding (P = 0.0265). The hazard ratio associated with levels of ADAMTS-13 activity below the default (mean) level of 64.6% was 11.33 (95%CI = 1.33-96.81). After testing for possible confounders and analyzing other variables, such as demographic data, oxygen saturation, coagulation tests, the need for cardiopulmonary bypass and its duration, and first operation versus reoperation, the final multivariate model included three variables potentially associated with postoperative bleeding, namely a low preoperative ADAMTS-13 activity, APTT and the need for ON-pump surgery. Significant postoperative bleeding occurred in 40% of patients with a preoperative ADAMTS-13 activity of <64.6% (versus 6% of patients above this level, P = 0.0170), 39% of patients undergoing ON-pump surgery (versus 6% of those undergoing OFF-pump procedures, P = 0.0180), and 55% of patients with both risk factors (versus 7% of individuals with one or none of them, P = 0.0010).

For 3 patients who died postoperatively, preoperative levels of the biochemical parameters were within the range of the remaining patients. The multimeric structure of VWF was similar to the one shown as baseline in Figure 1. One of these patients (univentricular heart, 5.5 years of age) had postoperative bleeding, with a preoperative ADAMTS-13 activity of 59%.

Discussion

The congenital heart disease patients in this study had a common profile, namely hypoxemia and polycythemia combined with decreased ADAMTS-13 and VWF:Ag levels. Degradation of VWF multimers was demonstrated in the majority of patients, suggesting abnormal interaction of the VWF protein with membranes (platelets, endothelium) followed by enzymatic cleavage. Therefore, decreased ADAMTS-13 activity (associated with a mild decrease in ADAMTS-13:Ag) was likely a result of chronic enzyme consumption, although decreased production cannot be excluded (the ADAMTS-13 antigen was directly related to peripheral oxygen saturation). We speculate that in patients with CCHD, abnormal interactions of VWF with membranes may occur at sites of altered flow conditions (for example, systemic-to-pulmonary connections, either normally existing or surgically created). Although it has been acknowledged that ABO blood groups influence the circulating levels of VWF, with lower antigenic concentration being expected in group ''O'' subjects (22), this does not seem to be the main reason for decreased VWF in the present study, since low VWF:Ag was detected even in ''non-group O'' individuals. In this study, ADAMTS-13 levels were not influenced by ABO blood groups.

The changes in laboratory parameters observed postoperatively resemble those seen in conditions associated with acute phase (inflammatory) reaction (10,11,23). The intensity of these changes, however, varies considerably depending on the severity of the underlying disease. In sepsis with organ failure, cirrhosis and advanced liver dysfunction, importantly elevated VWF:Ag has been reported in association with decreased ADAMTS-13 activity, resulting in a marked increase in VWF:Ag/ADAMTS-13 activity ratio. In these instances,

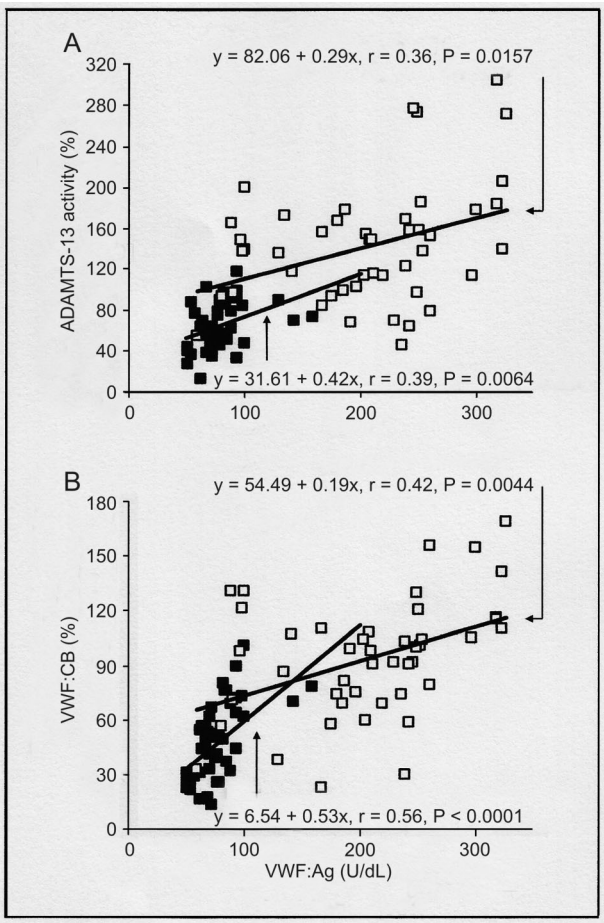

Figure 2. Preoperative (filled squares) and postoperative (open squares) correlations of plasma ADAMTS-13 activity (*A*) and von Willebrand factor activity (*B*, VWF:CB) with VWF antigenic concentration (VWF:Ag) in children with cyanotic congenital heart disease. Preoperative and postoperative mean values of ADAMTS-13 activity adjusted for VWF:Ag were 84 and 122%, respectively (P = 0.0066, ANCOVA). In *B*, the slopes are different (P = 0.0477), indicating lowered VWF:CB relative to VWF:Ag. Preoperative and postoperative adjusted mean values of VWF:CB were 62 and 79%, respectively (P = 0.0395, ANCOVA).

proteolysis of VWF becomes relatively reduced, and ultralarge VWF multimers are seen in the circulation in association with thrombocytopenia. Enzyme consumption and decreased synthesis likely account for the negative correlation of ADAMTS-13 activity with VWF:Ag (11,24). In the less severe situation of preeclampsia, increased VWF:Ag and unchanged (not decreased) ADAMTS-13 activity have been observed in the absence of ultralarge VWF multimers or thrombocytopenia (9). The postoperative changes observed in this study were not as dramatic as those reported in sepsis. We observed an important elevation of VWF:Ag with a mild increase in VWF:CB relative to baseline and continued cleavage of the VWF protein. In contrast to sepsis, ADAMTS-13 activity was increased. However, the VWF:Ag/ADAMTS-13 ratio was elevated compared with the preoperative value, and ADAMTS-13:Ag was actually decreased. We speculate that a fraction of ADAMTS-13 protein was converted to the active form, thus explaining an instantaneous increase in the protease activity even in the presence of total ADAMTS-13 decline. Ultralarge VWF multimers were not present in any patients, suggesting that the metalloprotease activity was sufficient to prevent disseminated thrombosis (there was a decreased platelet count, but not thrombocytopenia). Mannucci et al. (16) showed decreased ADAMTS-13 activity (and increased VWF:Ag) in adults undergoing cardiac surgery. The postoperative increase in the active/activatable fraction of VWF protease (relative to its antigenic concentration) observed here may be a characteristic of the pediatric population with CCHD undergoing surgery, although we must consider that we did not measure biochemical parameters immediately after surgery as done by the other investigators.

Many substances and conditions are known to cause endothelial cell activation and release of VWF from Weibel-Palade bodies, including hypoxia, epinephrine, thrombin, fibrin, cytokines, endotoxin, components of the complement system, and reactive oxygen intermediates (25-31). Many of these elements are probably present in the postoperative scenario of CCHD, and might explain the elevation of plasma VWF:Ag. Although hepatic stellate cell damage may be considered as a potential cause of altered ADAMTS-13 level (32,33), our postoperative findings are probably better explained by moderate protease consumption in the presence of elevated VWF:Ag. Altered synthesis (cytokinemia) and/or abnormal degradation (plasmin, thrombin) cannot be excluded (34,35).

Previously, preoperative abnormalities of VWF structure have been associated with postoperative bleeding in patients with aortic valve stenosis. Altered flow conditions through the aortic valve probably account for unfolding of the VWF molecule, thus facilitating its binding to platelet membrane glycoproteins, with subsequent proteolytic cleavage (36). Our data do not necessarily indicate a causal relationship between lowered preoperative ADAMTS-13 activity and postoperative bleeding. Rather, altered ADAMTS-13 levels should be viewed as part of an overall process of microvascular dysfunction, where abnormal functioning of endothelial cells and platelets, as well as inflammatory mediators contribute to an increased risk of bleeding. Further studies are necessary to clarify these intriguing and interesting relationships.

Thus, children with CCHD have abnormalities in circulating ADAMTS-13 and its substrate VWF that resemble those observed in disorders associated with microangiopathy and coagulopathy. Preoperative and postoperative data suggested abnormal proteolytic cleavage of VWF multimers associated with ADAMTS-13 consumption.

Acknowledgments

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