Abnormal endothelial factor VIII associated with pulmonary hypertension and congenital heart defects

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ABSTRACT In patients with pulmonary hypertension associated with congenital heart defects, ultrastructural abnormalities are observed in endothelial cells, which suggest heightened metabolic function. If endothelial production of the von Willebrand factor (vWF) is increased, this may be associated with abnormal interactions with platelets leading to worsening of the pulmonary hypertension. We therefore evaluated vWF in 30 patients with pulmonary hypertension (25 with congenital heart defects) and in 30 individuals with normal pulmonary arterial pressure (12 with congenital heart defects). We measured the antigenic (vWF: Ag) and biologic (VWF: rist) activity of vWF in plasma and assessed endothelial vWF: Ag directly by an immunoperoxidase stain applied to lung biopsy tissue. Because of considerable variance and small size, the group of five patients with pulmonary hypertension and without congenital heart defects were excluded from statistical analyses. Patients with pulmonary hypertension and congenital heart defects had significant higher vWF: Ag levels than individuals with normal pulmonary arterial pressure without congenital heart defects (p < .05), whereas values in those with normal pressure and congenital heart defects were intermediate. In lung biopsy tissue available from 29 patients in this study and from 11 others we previously reported, immunostain of pulmonary arterial endothelium for vWF was intense (suggesting increased production) in 29 of 32 with pulmonary hypertension and congenital heart defects and in only one of eight with normal pulmonary arterial pressure and congenital heart defects (p < .01). Only three patients with congenital heart defects and pulmonary hypertension and increased vWF: Ag, however, had increased vWF: rist. Compatible with this discrepancy was a loss of vWF high–molecular weight forms as determined by both crossed immunoelectrophoresis and multimeric analysis. Our results suggest that increased vWF in most patients with congenital heart defects and pulmonary hypertension is associated with increased production of a biologically deficient molecule lacking high–molecular weight forms.


IT HAS BEEN SUGGESTED that abnormalities in platelet endothelial interaction may be important in the pathogenesis of pulmonary vascular disease and may contribute to the heightened pulmonary vascular reactivity observed in patients with pulmonary hypertension related to congenital heart defects.1–4 We5 as well as others6, 7 have reported structural changes in the pulmonary vascular endothelial cells of these patients, which imply increased metabolic function. The von Willebrand Factor (vWF) is a large multimeric plasma glycoprotein produced by endothelial cells,8 which may be important in the adherence of platelets during vascular injury.9 We hypothesized that vWF synthesis would be increased in patients with pulmonary hypertension, in particular the group with associated congenital heart defects. This might contribute to abnormal interactions with platelets, perhaps resulting in increased adherence,1 increased release of vasoactive substances3 and smooth muscle mitogens,10 and worsening of the pulmonary hypertension. We therefore measured the circulating components, vWF: Ag and vWF: rist, reflecting antigenic and biologic activity of vWF, analyzed the structure of the molecule by crossed...
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<th>Group and patient No.</th>
<th>Age (mo/yr)</th>
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<th>Ppa (mm Hg)</th>
<th>Psa (mm Hg)</th>
<th>Rp (µm²)</th>
<th>Rs (µm²)</th>
<th>Qp:Qs</th>
<th>Biopsy grade</th>
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<td>AVSD</td>
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<td>I</td>
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<td>68</td>
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<td>B severe</td>
<td>I</td>
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<td>15</td>
<td>1:1</td>
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</table>

For convenience in both tables 1 and 2 acyanotic patients with congenital heart defects are listed first; cyanotic patients follow.

Ppa, Psa = mean pulmonary and systemic arterial pressure; Rp, Rs = pulmonary and systemic vascular resistance; Qp: Qs = pulmonary-to-systemic flow ratio; M = morphometric grade; H-E = Heath-Edwards grade; aV/v/c = artery, vein, capillary; vWF: Ag, vWF: Ant = antigenic and biological components of vWF Factor VIII; PH = pulmonary hypertension; CHD = congenital heart disease; PV = pulmonary vein; CoA = coarctation; PPS = peripheral pulmonary stenosis; AS = aortic stenosis; s/p = status post; VSD = ventricular septal defect; AVSD = atrioventricular septal defect; PDA = patent ductus arteriosus; L-TGA, D-TGA = L and D transposition of the great arteries; MVR = mitral valve replacement; MS = mitral stenosis; ASD II = secundum atrial septal defect; TOF = tetralogy of Fallot.
immunoelectrophoresis and multimeric pattern, and assessed endothelial content by an immunoperoxidase vWF stain applied to lung biopsy tissue.

Methods

Patient population. Factor VIII metabolism was assessed in 60 individuals at The Hospital for Sick Children, The Toronto General Hospital, and McMaster University Medical Centre between March 1983 and June 1984, under a protocol approved by the Human Ethics Committee. The study was designed to include 32 consecutive patients in whom a lung biopsy would be performed to assess the severity of pulmonary vascular disease. Additional patients and control subjects in the same age range were studied over a slightly extended time frame. Pulmonary hypertension (mean pulmonary arterial pressure > 18 mm Hg) was present in 30 of 60 subjects (age range 5 months to 40 years), and normal pulmonary arterial pressure was present in the remaining 30 (age range 11 months to 38 years). Thirty-seven of the 60 subjects had congenital heart disease. (25 of the 30 patients with pulmonary hypertension and 12 of the 30 with normal pressure). Further diagnostic details are given in tables 1 and 2.

Assessment of presence and severity of pulmonary hypertension. Cardiac catheterization was carried out in 41 of the 60 study subjects. The presence and severity of pulmonary hypertension was established in 29 patients and the presence of normal pressure in the remaining 12. Of the 19 patients who did not undergo cardiac catheterization, 18 without cardiorespiratory disease were presumed to have normal pulmonary arterial pressure and one 5-month-old infant in whom a two-dimensional echocardiogram established the diagnosis of a complete atrioventricular septal defect was presumed to have pulmonary hypertension because the two are invariably associated.

In 32 of the 60 subjects, lung biopsy tissue was taken and analyzed according to our previously described protocol. Specimens were obtained from (1) patients who were being considered for a Fontan procedure, i.e., right atrial–to–pulmonary arterial anastomosis, to establish that the pulmonary vascular bed was normal or that changes were minimal and (2) from patients in whom severe vascular disease was suspected based on a high preoperative level of pulmonary vascular resistance. Thus a wide spectrum of pulmonary vascular abnormalities was included in the group in whom biopsy tissue was analyzed (tables 1 and 2). To assess the severity of the vascular changes, both the morphometric quantitative and the Heath-Edwards qualitative methods of analysis were used. In addition, each tissue section was inspected thoroughly for evidence of vascular microthrombi, which may be related to increased platelet adhesion and aggregation.

The morphometric grades given are as follows: grade A, extension of muscle into normally nonmuscular peripheral arteries, ± mild increase in medial wall thickness of normally muscular arteries, > 10% < 15% external diameter; grade B, extension of muscle + more severe increase in medial wall thickness of normally muscular arteries, subdivided into B mild when it is ≥ 15% < 20% external diameter and B severe when it is > 20%; grade C, features of B severe + decreased arterial concentration relative to alveoli, subdivided into C mild when there is half or more than half of the normal number of arteries present and C severe when less than half the normal number is present. The Heath-Edwards grades are as follows: grade I, medial hypertrophy; grade II, cellular intimal hyperplasia; grade III, occlusive fibrous intimal hyperplasia; grade IV, dilatation; grade V, angiomatoid formation. Structural abnormalities in pulmonary veins were described qualitatively.

Analysis of vWF. Blood samples for assessment of vWF activity were taken in all patients at the time of routine blood drawing, i.e., before intracardiac repair or cardiac catheterization. A 6 ml blood sample was drawn into 3.8% sodium citrate/0.1M epsilon amino caproic acid and centrifuged at 1200 g at 4°C for 15 min. Multiple aliquots of plasma were frozen at −70°C. A quantitative measurement of vWF antigen (vWF: Ag) was performed with an immunoelectrophoretic technique and vWF ristocetin cofactor activity (vWF: rist).
was assessed by ristocetin-induced aggregation of formalin-treated human platelets.\textsuperscript{15} vWF:Ag was also assessed by crossed immunoelectrophoresis and multimeric analysis in 2.0% and 1.3% sodium dodecyl sulfate gels.\textsuperscript{16} The anti-hemophilic factor VIII:C was measured by modification of the activated partial thromboplastin time.\textsuperscript{17} Antithrombin III was measured by fluorescent substrate assay.\textsuperscript{18} Routine prothrombin, partial thromboplastin times, and platelet counts were performed in all patients.

An immunoperoxidase stain for vWF:Ag\textsuperscript{19} was applied to tissue sections in all patients who underwent lung biopsy. The sections were taken from paraffin-embedded tissue blocks and stained for vWF:Ag by the triple-bridged immunoperoxidase technique. The primary rabbit polyclonal monospecific antibody to human vWF:Ag was applied at 1:50 dilution (Behring Diagnostics). This was followed by swine antirabbit immunoglobulin (Dako) at a 1:25 dilution, then subsequently linked to rabbit peroxidase antiperoxidase (Dako) at a 1:30 dilution; 3,3-diaminobenzidine (DAB) (1 ng/100 ml Tris buffer) with added H\textsubscript{2}O\textsubscript{2} (0.02 ml/100 ml DAB) was used as the chromogen. Normal rabbit serum (Dako) at 1:50 dilution was applied instead of the rabbit vWF for a negative control. The density of

### TABLE 2
Clinical details and factor VIII values in individuals with normal pulmonary arterial pressure

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<th>Group and patient no.</th>
<th>Age</th>
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<th>Biopsy grade</th>
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<th>vWF activity (%)</th>
<th>vWF:Ag</th>
<th>vWF:rist</th>
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<td>116</td>
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<td>11 yr</td>
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<td>7</td>
<td>16 yr</td>
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<td>104</td>
<td>154</td>
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<tr>
<td>4</td>
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<td>68</td>
<td>62</td>
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<td>Control               — — — —</td>
<td>80</td>
<td>70</td>
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<td>± 17.8</td>
<td>± 17.6</td>
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Abbreviations as in table 1.

CIRCULATION
staining observed in endothelium of pulmonary arteries, veins, and capillaries was graded semiquantitatively as either 1+, 2+, or 3+.

Analysis of data. Because of the small size and considerable variance, the group of five patients with pulmonary hypertension and without congenital heart defects were excluded from statistical analyses. Nonetheless, the findings in these patients are of interest and are therefore discussed and included in the tables and figures. One-way analysis of variance was used to compare vWF levels in the remaining three groups of patients: those with pulmonary hypertension and congenital heart defects (group 1), those with normal pulmonary arterial pressure and congenital heart defects (group 2), and those with normal pressure without congenital heart defects (group 3). Tukey’s test was used to establish which groups were different. Since only patients from groups 1 and 2 underwent lung biopsy, Fisher’s exact test was used to compare vWF peroxidase staining in patients with normal or increased circulating levels. In all analyses $p < .05$ was considered statistically significant. All the biochemical analyses were performed without knowledge of the subject’s status. However, in assessing the lung biopsy tissue sections, it could not be discounted that those in which the arteries were thick walled likely came from patients with pulmonary hypertension.

Results

Circulating vWF. Normal vWF: Ag, vWF: rist, and VIII: C values in our laboratory are consistently under 130% activity. Group 1 patients had significantly higher vWF: Ag values than group 3 patients ($p < .05$). Values were intermediate in group 2 and not significantly different from those in the other two groups (figure 1). Among the five patients with pulmonary hypertension and without congenital heart defects there was considerable variance in vWF: Ag levels, but the mean value was similar to that in group 1 (figure 1). Abnormally increased vWF: Ag values (>130% activity) were present in 16 of 30 patients with pulmonary hypertension and only two of 30 with normal pressure.

Among the subgroups, vWF: Ag values were elevated in 14 of 25 group 1 patients, two of five with pulmonary hypertension and without congenital heart defects, two of 12 group 2 patients, and none of 18 group 3 patients (figure 1).

For technical reasons, vWF: rist could be assessed in only 54 of 60 patients. Only four of 13 patients studied with increased vWF: Ag and pulmonary hypertension had an accompanying increase in vWF: rist, suggesting that in most cases the molecule was functionally abnormal (figure 2). Compatible with this, crossed immunoelectrophoresis demonstrated increased anodal mobility in 12 of 20 patients with pulmonary hypertension studied (11 with congenital heart defects) and in only six of 17 with normal pressure (all six with congenital heart defects). Increased anodal mobility was present in patients with both increased vWF: Ag and normal vWF: Ag (figure 3). Multimeric analysis confirmed the loss of high–molecular weight

[Figure 1: Percentage of vWF antigenic activity (vWF: Ag) in individual patients in the four groups. Dashed line denotes upper limits of normal vWF: Ag activity in our laboratory. Next to individual values is mean ± SE for the group. Patients with congenital heart defects and pulmonary hypertension have significantly higher values ($p < .05$) than individuals without congenital heart defects with normal pulmonary arterial pressure. Mean value in group with pulmonary hypertension without congenital heart defects is similar to that in group with both pulmonary hypertension and congenital heart defects. Values in patients with normal pulmonary arterial pressure and congenital heart defects are intermediate between those in patients with pulmonary hypertension and congenital heart defects and those in patients with normal pulmonary arterial pressure without congenital heart defects.]

[Figure 2: Percentage of vWF biological activity (vWF: rist) in individual patients in the four groups. Note, only four patients, all with pulmonary hypertension had increased vWF: rist activity.]
forms in all patients with increased mobility and loss of both high- and intermediate-molecular weight forms in two patients (figure 4).

The antihemophilic factor VIII: C was assessed in 49 of 51 patients. Group 1 patients had significantly higher vWF: Ag values than group 3 patients (p < .05). Values were intermediate in group 2 patients and not significantly different from those in the other two groups. There was considerable variance in vWF: Ag levels among the five patients with pulmonary hypertension without congenital heart defects, but the mean value was similar to that in group 1 (figure 5). Elevated VIII: C activity was observed in patients with normal as well as increased vWF: Ag.

**Endothelial vWF.** Immunostain for vWF was carried out in all 32 patients in whom lung biopsy tissue was available and analyzed without knowledge of the circulating vWF: Ag level or the pulmonary arterial pressure. The intensity of immunostain was judged as 2+ or 3+ in 22 of 25 group 1 patients, in two of three with pulmonary hypertension without congenital heart defects, and in one of four group 2 patients (figure 6). Including our previously reported patients, in whom immunoperoxidase staining was carried out but not measurement of circulating vWF activity, 29 of 32 patients with pulmonary hypertension and congenital heart defects and only one of eight with normal pressure and congenital heart defects had 2+ or 3+ immunostain (p < .01). Staining was consistently highest in the arteries, less so in the veins, and least in the capillaries. See tables 1 and 2 for data in the individual patients. There was no evidence in any section studied of platelet fibrin microthrombi.

**Correlation with hemodynamic data and lung biopsy grade.** The two patients with the highest circulating vWF: Ag (>300% activity) both had unexplained pulmonary hypertension. Among the other patients with pulmonary hypertension, those with higher pulmonary arterial pressure or with more advanced pulmonary vascular changes on lung biopsy tissue (figure 7) were not distinguishable by their level of vWF: Ag or by the presence of increased vWF: Ag or by increased anodal mobility and an abnormal multimeric pattern. The
degree of left-to-right shunting (pulmonary-to-systemic flow ratio) did not correlate with the quantitative or qualitative abnormalities in vWF.

**Blood clotting studies.** Prothrombin time and partial thromboplastin times and platelet counts were normal (≤ 14 sec, ≤ 38 sec, ≥ 150,000) in all patients studied. None had symptoms of bleeding preoperatively, nor was excessive bleeding a problem postoperatively. Antithrombin III levels were normal (>50% activity) in all but two patients.

**Discussion**

The factor VIII complex contains two proteins: factor VIII, which functions as a cofactor for the activation of factor X and is absent in hemophilia, and vWF, a large multimeric protein that is involved in platelet adhesion. It is reduced in type I von Willebrand’s disease and qualitatively altered in type II von Willebrand’s disease. In the latter condition there is absence of the high–molecular weight components of the molecule. Qualitative abnormalities of vWF may be acquired in association with specific disease states, including the hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, the myeloproliferative syndromes, and disseminated intravascular coagulation.

Quantitative and qualitative abnormalities of vWF have been associated with pulmonary hypertension related to acute lung injury and high altitude. There is an increase in antigenic activity unaccompanied by an increase in biological activity and this is associated with a loss of the high–molecular weight forms. Among patients with unexplained (primary) pulmonary hypertension, an increase in the biological activity unaccompanied by an increase in antigenic activity has been described but with an associated loss of high–molecular weight forms. Since the latter would be expected to result in decreased biological activity, these findings are difficult to explain and differ from those in our patients with unexplained or secondary pulmonary hypertension. We observed abnormalities in vWF associated with increased antigenic rather than biological activity and, compatible with this discrepancy, loss of high–molecular weight forms.

Alterations in the vWF have been attributed to endothelial injury, the speculation being that the molecule is degraded by “activated” enzymes. In this study, we investigated the relationship between vWF and pulmonary hypertension mostly in patients with congenital heart defects. We found qualitative abnormalities in patients with congenital heart defects (loss of high–molecular weight forms) associated with significant quantitative abnormalities in those with pulmonary hypertension, i.e., increased antigenic activity unaccompanied by increased biological activity. Furthermore, our immunocytochemical studies support altered endothelial synthesis of vWF as at least one mechanism contributing to the abnormalities in circulating levels. That the increased staining was present primarily in the arteries, less so in the veins and capillaries, suggests a direct effect of increased pulmonary arterial pressure. That the severity or duration of vascular change did not correlate with the degree of qualitative or quantitative vWF abnormality implies substantial individual variation. It is also likely that this represents an alteration in endothelial metabolic function that occurs early in the course of the disease.

In our study, the majority of patients with pulmonary hypertension had increased circulating levels of vWF: Ag. Increased levels of vWF are associated with many states, even exercise, and are not a specific finding. However, the increased vWF antigen was only infrequently accompanied by increased circulating biological activity (vWF: rist) (tables 1 and 2). This suggested that qualitative abnormalities of the vWF molecule were also present in these patients. Further
FIGURE 6. Photomicrographs of factor VIII immunostaining. A, Faint immunostain of endothelium in preacinar (pa) and alveolar wall (aw) artery in a patient with normal vWF: Ag. B, Dense immunostain of endothelium in preacinar (pa) artery in a patient with increased vWF R: Ag. (Original magnification ×200 for both A and B.)

FIGURE 7. In the subgroup of patients with lung biopsy studies, there was no correlation between the grade of severity of vascular lesions (as defined in the text) and vWF: Ag activity.

analysis revealed that in most patients there was increased anodal mobility on immunoelectrophoresis confirmed by decreased high–molecular weight forms on sodium dodecyl sulfate gel analysis.

The etiology of the vWF abnormalities was further investigated. The increased endothelial vWF immunostaining seen in almost all patients with pulmonary hypertension suggests that heightened endothelial metabolism and increased synthesis may contribute to the quantitative abnormalities observed. The etiology of the qualitative abnormalities include the following possibilities: degradation of the molecule by an endothelial or circulating protease or increased “clearance” of the high–molecular weight forms in platelet aggregates. Since qualitative abnormalities were observed in some patients with congenital heart defects and normal pulmonary arterial pressure, both in our study and in that of Gill et al., we can only speculate that an abnormal hemodynamic condition may activate a protease that cleaves and degrades the high–molecular weight vWF forms. Indeed, in the study of Gill et al., the multimeric abnormality did not persist in the postoperative period when the hemodynamic abnormality
was corrected. Moreover, infusion of cryoprecipitate in the preoperative period corrected the abnormal bleeding time but not the multimeric abnormality. Both in our study and in that of Gill et al., abnormal vWF did not appear to be associated with excessive postoperative bleeding. This was analyzed retrospectively, however, and would need to be confirmed in a prospective study. The increased VIII: C (antihemophilic factor) we observed in patients with pulmonary hypertension has been reported previously in other conditions associated with increased vWF.  

The other possible explanation for the qualitative vWF abnormality, namely that the high–molecular weight forms are being “cleared” from the circulation by contributing to platelet adhesion to the altered endothelium or subendothelium, seems less likely. We would have expected to find the multimeric abnormality more consistently in the patients with the greatest hemodynamic and endothelial derangement and we might have expected to find some evidence of platelet fibrin microthrombi in the biopsy specimens. However, since abnormalities in the vWF molecule were not found in all patients with pulmonary hypertension, there appears to be a spectrum to the biochemical abnormality as well as the clinical problem. That is, there may be a subgroup of patients with congenital heart defects and pulmonary hypertension and abnormal vWF who do have platelet microaggregates and another perhaps much larger subgroup in which the abnormal vWF is associated with a “defective” circulating molecule that may actually be protective against increased platelet adherence.

Conclusion. Pulmonary hypertension related to congenital heart disease is associated with increased vWF antigenic activity. Results of immunostaining of lung biopsy specimens suggest increased endothelial production. Analysis of multimers, however, shows a loss of high–molecular weight forms, confirming decreased biological activity. The level of vWF antigen did not correlate with the severity of vascular changes on biopsy, suggesting that it may be an early feature in the development of the disease.

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