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Abnormal degradation of von Willebrand factor main subunit in pulmonary hypertension

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Abnormal degradation of von Willebrand factor main subunit in pulmonary hypertension. A.A.B. Lopes, N.Y. Maeda. ©ERS Journals Ltd 1995.

ABSTRACT: We wished to investigate whether the abnormalities in multimeric structure and biological function of von Willebrand factor (vWF) observed in pulmonary hypertensive patients could be related to increased proteolytic degradation.

We therefore analysed plasma vWF subunit composition in 24 pulmonary hypertensive patients, aged 1.2–45 yrs. After immunoisolation, vWF was subjected to 5.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting. vWF intact subunit (225 kDa) and four proteolytic fragments (189, 176, 150 and 140 kDa) were visualized by peroxidase staining and analysed in a laser densitometer.

In pulmonary hypertensive patients, the relative density of the intact subunit was decreased, and this was associated with an increase in the 176 kDa proteolytic fragment. The mean densities of the other fragments were not significantly changed, but in some patients the 150 and 140 kDa polypeptides were markedly increased. Abnormalities in multimeric structure of vWF (loss of high molecular weight multimers and increase in low molecular weight forms in comparison with controls), were associated with a significant decrease in biological activity (62–92% activity, 95% confidence interval (CI) for the mean). Total proteolytic fragment density correlated positively with multimeric abnormalities (r_S=0.45), and negatively with biological activity of vWF (r_S=-0.49).

Thus, in pulmonary hypertension, multimeric abnormalities and decreased biological activity are related to proteolytic degradation of vWF main subunit, possibly reflecting extensive endothelial dysfunction.

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The von Willebrand factor (vWF) is a large multimeric adhesive glycoprotein synthesized in megakaryocytes and endothelial cells. In addition to serving as a carrier for coagulation factor VIII, it plays an important role in platelet and endothelial cell adhesion to the sub-endothelium [1, 2]. There is general agreement that vWF is an acute phase reactant, since both constitutive and stimulated endothelial vWF release are modulated by mediators of the acute phase response [3–5]. Circulating vWF consists of an array of multimers with a molecular mass ranging from 500 to more than 10,000 kDa. When analysed under reduced conditions, the main subunit (225 kDa) and at least three proteolytic fragments (189, 176 and 140 kDa) are identified [6, 7]. Plasma concentration of vWF has been used as an index of endothelial dysfunction. Increased release of vWF from endothelial cells has been reported in several conditions, including connective tissue disorders [8], sepsis syndrome [9], pregnancy-induced hypertension [10], thrombotic thrombocytopenic purpura [11], diabetes [12], and percutaneous transluminal coronary angioplasty [13].

Endothelial release of vWF is also increased in vascular disorders associated with heightened shear stress [14]. In pulmonary hypertension, high vWF plasma concentration is associated with enhanced endothelial vWF production, as demonstrated by immunoperoxidase stain applied to lung tissue [15]. However, there is evidence that dysfunctional endothelial cells synthesize defective molecules, with increased susceptibility to proteolytic cleavage. Plasma vWF biological activity is decreased in pulmonary hypertensive patients, and this is associated with a loss of high molecular weight multimers [15]. In addition, we have recently observed that the concentration of dimeric vWF is decreased in some patients with high vWF plasma levels [16]. Although these findings are suggestive of proteolytic cleavage *in vivo,* no definite proof of ongoing vWF degradation in this disorder has been presented. We therefore decided to investigate whether abnormal proteolysis of vWF occurs in pulmonary hypertension, by examining its subunit composition. The protein was isolated from plasma by means of immunoprecipitation. Reduced vWF was then subjected to electrophoresis in polyacrylamide gels, followed by Western immunoblotting. Protein was visualized by peroxidase staining and final analysis was carried out using laser densitometry.

Materials and methods

Patient group

The patient population consisted of 12 consecutive children and 12 consecutive adults, who were admitted to the Heart Institute in São Paulo, Brazil, for treatment of either primary pulmonary hypertension or pulmonary hypertension associated with congenital heart defects. None of these patients had clinical or laboratory evidence of disseminated intravascular coagulation or enhanced systemic proteolysis, and none had been under anticoagulant or thrombolytic therapy. Patients with pulmonary hypertension associated with other disorders, such as chronic obstructive airway disease, thromboembolic pulmonary disease, autoimmune disease, and hepatic or renal failure were not included. All patients were informed that blood samples were obtained for research purposes and gave informed consent. The study protocol was approved by the Scientific Committee of the Heart Institute, São Paulo, Brazil.

Reagents

Ristocetin and protease inhibitors, edetic acid (EDTA), N-ethylmaleimide (NEM), phenylmethylsulphonyl fluoride (PMSF), leupeptin and aprotinin were acquired from Sigma (St Louis, MO, USA). Electrophoresis pure reagents, protein A-sepharose CL4B and protein G-Sepharose 4 Fast Flow were purchased from Pharmacia Fine Chemi-cals (Piscataway, NJ, USA). Assera-Plate vWF electroimmunodiffusion kit was obtained from Diagnostica Stago (Asnieres, France). Affinity purified rabbit anti-human vWF polyclonal antibody, biotinylated swine anti-rabbit and anti-mouse immunoglobulin G (IgG) and avidin-biotin-peroxidase complex were obtained from Dako Corporation (Carpinteria, CA, USA). The mono-clonal anti-human vWF antibodies AvW-16 and AvW-17 were kindly provided by R. Montgomery from The Blood Center of Southeastern Wisconsin (Milwaukee, WI, USA). The monoclonal anti-human vWF antibody ECA-4 was a gift from M.S. Ferreira, Laboratory of Immunology, Heart Institute (São Paulo, Brazil). All other reagents were of the highest grade available.

Blood collection and inhibition of proteolysis ex-vivo

Peripheral venous blood was collected in 1:10 volumes of 3.8% sodium citrate, in the presence of protease inhibitors: 5 mM EDTA, 6 mM NEM, 1 mM PMSF, 0.25 mM leupeptin and 20 $U \cdot ml^{-1}$ aprotinin. Immediately after collection, blood was subjected to centrifugation at 3,000×g for 20 min. Plasma was stored at -70°C and thawed only once for use. vWF immunoisolation was carried out at 4°C and protease inhibitors were used at the same final concentrations in all buffers.

Immunoisolation of plasma vWF

Isolation of vWF from plasma was performed by immunoprecipitation. Identical results were obtained using either anti-vWF polyclonal antibody or anti-vWF ECA-4 monoclonal antibody. Plasma was incubated with the antibody (108 $\mu g \cdot ml^{-1}$, final concentration) in NET-gel buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA pH 8.0, 0.25% gelatin, 0.02% sodium azide) containing all protease inhibitors described previously, for 1 h at 4°C. The mixture was then incubated with protein A-Sepharose or protein G-Sepharose (780 μl of beads$\cdot mg^{-1}$ of antibody) for 1 h at 4°C. The beads were washed twice with NET-gel buffer and once with 10 mM Tris pH 7.5, 0.1% Nonidet P-40. vWF was eluted by incubation with 50 mM Tris pH 6.8, 2% SDS, 100 mM dithiothreitol, 30% glycerol, 0.02% bromophenol blue for 20 min at 60°C. Beads were removed by centrifugation at 12,000×g for 2 min at 4°C and vWF was subjected to electrophoresis or stored at -20°C.

vWF subunit analysis

Following reduction, 90 µl of supernatant (obtained from 1 ml of plasma) was subjected to 5.5% SDS-PAGE. Molecular weight markers were: fibronectin, 220 kDa; myosin, 205 kDa; beta galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66 kDa. After electrophoresis, proteins were transferred to nitrocellulose membrane in 48 mM Tris, 384 mM glycine, 0.037% SDS, 20% (v/v) methanol, pH 8.3. After blocking, the membrane was reacted overnight with 1:150 dilution of anti-vWF polyclonal antibody or 1:25 dilution of anti-vWF monoclonal antibodies (AvW-16, AvW-17). Biotinylated swine anti-rabbit or anti-mouse IgG was used as the secondary antibody at 1:500 dilution for 2 h. The membrane was then incubated with 1:2,500 dilution of avidin-biotin-horseradish peroxidase for 1 h and the enzyme activity was detected with 0.1% (w/v) 3,3'-diaminobenzidine, 0.01% (w/v) $NiCl_2$ and 0.3% (v/v) H_2O_2 in phosphate buffer pH 7.3. Western blots were subjected to densitometric analysis using a Ultroscan XL laser densitometer (Pharmacia LKB, Bromma, Sweden). Membranes with least background stain used for densitometric scanning were sometimes different from those used to make the figures. The densities of vWF polypeptides relative to total subunit density were calculated.

Reliability of the peroxidase staining method for semi-quantifying vWF fractions has been tested in our laboratory previously [17]. In the present study, densitometric measurements of vWF intact subunit and fragments in replicate experiments resulted in coefficients of variation ranging from 0.7–11.0%. In addition, when total vWF subunit was analysed using the peroxidase staining method, results correlated well with vWF antigenic concentration measured by electroimmunodiffusion ($r=0.63$; $p<0.001$).

vWF multimeric analysis

Plasma was diluted 1:20 in 10 mM Tris, 2% SDS, 1 mM EDTA, 8 mM urea, 0.02% bromophenol blue, pH 8.0. After incubation for 1 h at 56°C, the samples were

subjected to electrophoresis in 1% SDS-agarose gel. Immunoglobulin M (IgM) (950 kDa) was used as the molecular weight marker. Protein transfer to nitrocellulose membrane was performed in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol, pH 8.3. An immunoperoxidase stain was applied to the membrane, as described for vWF subunit analysis. The blots were subjected to densitometric analysis, and the density of four major bands migrating close to IgM (assumed as low molecular weight multimers) was expressed as percentage of total. These faster moving bands were of adequate resolution and were easily individualized in all blots.

vWF antigenic and biological activities

vWF antigenic activity (vWF:Ag) was measured by electro-immunodiffusion [18]. vWF biological activity was measured as the ability of plasma to promote platelet agglutination in the presence of ristocetin (ristocetin cofactor (RiCof) activity), [19]. Briefly, normal platelets (5×10^7 cells) were agglutinated in the presence of either normal plasma or plasma from pulmonary hypertensive patients (50 µl) and threshold ristocetin concentrations of 0.35–0.5 $mg \cdot ml^{-1}$. Agglutination response was determined by the change in light transmission at 4 min. Results were compared with normal values in the same assay and expressed as percentage activity.

Statistical analysis

Results are expressed as mean±SD. Differences between groups were analysed using the Student's t-test. Results of experiments in which patient data had to be expressed as percentage of control value are presented as 95% confidence intervals (95% CI) for the means. The influence of vWF subunit abnormalities on its multimeric structure and biological activity is shown in correlation graphs. These correlations were tested using nonparametric (Spearman's coefficient (r_S)) statistics. The correlations of vWF subunit abnormalities with clinical and laboratory variables were tested in the same way. A significance level of 0.05 was assumed.

Results

General diagnostic and laboratory data are summarized in table 1. Thirteen patients had decreased systemic arterial oxygen saturation associated with increased haematocrit level. Pulmonary hypertensive patients had increased vWF:Ag values in comparison with controls (124±45 and 91±27% activity, respectively; $p<0.01$). vWF biological activity was decreased (RiCof=75–96% activity, 95% CI for the mean). Values where even lower when biological activity was related to vWF antigenic concentration (RiCof/vWF:Ag=0.62–0.92, 95% CI for the mean).

Table 1. – Diagnosis and general laboratory data

Patient No.	Sex M.F	Age yrs	Diagnosis	Ppa mmHg	Hct %	Sao_2 %	vWF:Ag %	RiCof %
1	M	1.2	IAA/VSD	65	45	>90	124	100
2	M	3	PPH	30	41	>90	141	108
3	F	3	VSD	56	37	>90	95	35
4	F	5	VSD	58	43	>90	105	88
5	M	7	PPH	45	40	>90	226	130
6	F	9	PDA	54	43	>90	133	109
7	F	10	ASD	22	43	>90	126	121
8	F	10	MS	61	36	>90	90	80
9	F	11	PPH	65	40	>90	177	71
10	F	13	VSD	51	61	84	65	52
11	F	13	PPH	100	55	75	178	88
12	M	14	VSD	81	61	74	171	64
13	M	17	VSD/PDA	87	64	80	127	62
14	F	20	VSD	105	51	89	124	60
15	F	21	VSD	73	54	85	61	101
16	M	24	SV	68	66	72	73	58
17	M	26	SV	77	52	84	90	68
18	M	26	SV	66	54	85	64	112
19	F	27	PPH	88	47	>90	109	89
20	F	29	SV	52	61	81	98	89
21	F	34	PPH	60	45	>90	197	119
22	F	35	PPH	45	56	87	177	102
23	F	39	PDA	98	61	81	134	53
24	M	45	VSD	104	63	88	86	95

M: male; F: female; ASD: atrial septal defect; Hct: haematocrit; IAA: interruption of the aortic arch; MS: mitral stenosis; Ppa: mean pulmonary arterial pressure; PDA: patent ductus arteriosus; PPH: primary pulmonary hypertension; RiCof: ristocetin cofactor activity; Sao_2: systemic arterial oxygen saturation; SV: single ventricle; VSD: ventricular septal defect; vWF:Ag: plasma von Willebrand factor antigenic activity.

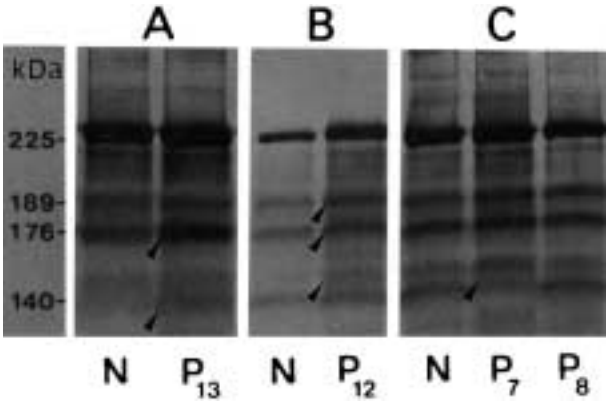

Fig. 1. – Representative analysis of vWF subunit in pooled normal plasma (N) and in plasma from pulmonary hypertensive patients (Pts). Patient numbers correspond to those of table 1. After 5.5% SDS-PAGE, Western immunoblotting was performed using affinity purified rabbit anti-human vWF polyclonal antibody. Relative mobilities are expressed in kilodaltons. The intact vWF subunit is represented by the 225 kDa protein. Fragments with relative density different from control value are pointed out by the arrowheads. a) the density of the 176 kDa fragment is markedly increased in patient No. 13. b) both 189 and 176 kDa fragments are markedly increased in comparison with control. c) no significant changes in fragment density are seen, except for the increased density of the 150 kDa protein and degradation of the 140 kDa fragment in patient No. 7. The 225 and the 176 kDa polypeptides were further identified by the monoclonal antibodies, AvW-16 and AvW-17 (not shown). vWF: von Willebrand factor; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

vWF subunit composition

In normal plasma, vWF intact subunit was identified as a 225 kDa protein. A major proteolytic fragment of approximately 176 kDa and less prominent fragments of 189, 150 and 140 kDa were identified (fig. 1). vWF subunit composition was abnormal in most patients. In general, the densities of proteolytic fragments relative to total subunit were increased. However, fragment abnormalities were not uniform. For example, whilst the 189 and 176 kDa polypeptides were clearly increased in patient No. 12, the marked increase in 176 kDa polypeptide in patient No. 13 was associated with normal immunoreactivity of the 189 kDa fragment (fig. 1a and b). When the patient group was analysed as a whole, the mean density of the 176 kDa fragment was significantly increased ($p<0.05$) (Fig. 2). Although the mean densities of the other fragments were not significantly changed, the concentrations of 150 and 140 kDa polypeptides were importantly increased in some patients (fig. 2). As a result, the relative density of the intact vWF subunit was significantly decreased ($p<0.05$) (fig. 2).

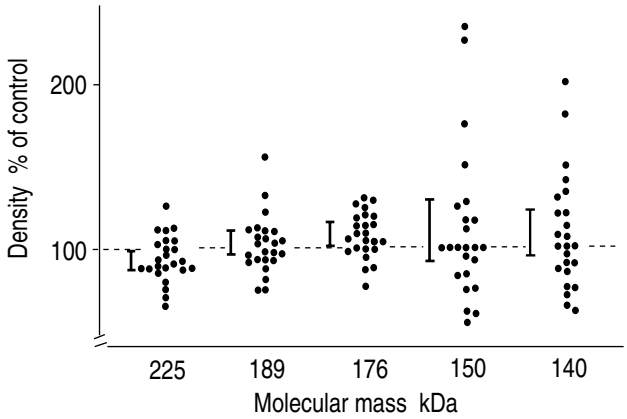

Fig. 2. – Semiquantitation of plasma vWF main subunit (225 kDa) and proteolytic fragments (189, 176, 150 and 140 kDa) by densitometric analysis of Western blots in 24 pulmonary hypertensive patients. The density of each vWF fraction was related to total subunit density and expressed as percentage of control value in the same assay; dotted line is 100% line. Bars represent 95% confidence intervals for the means. A significant decrease in the intact vWF subunit was associated with a significant increase in the 176 kDa proteolytic fragment ($p<0.05$). vWF: von Willebrand factor.

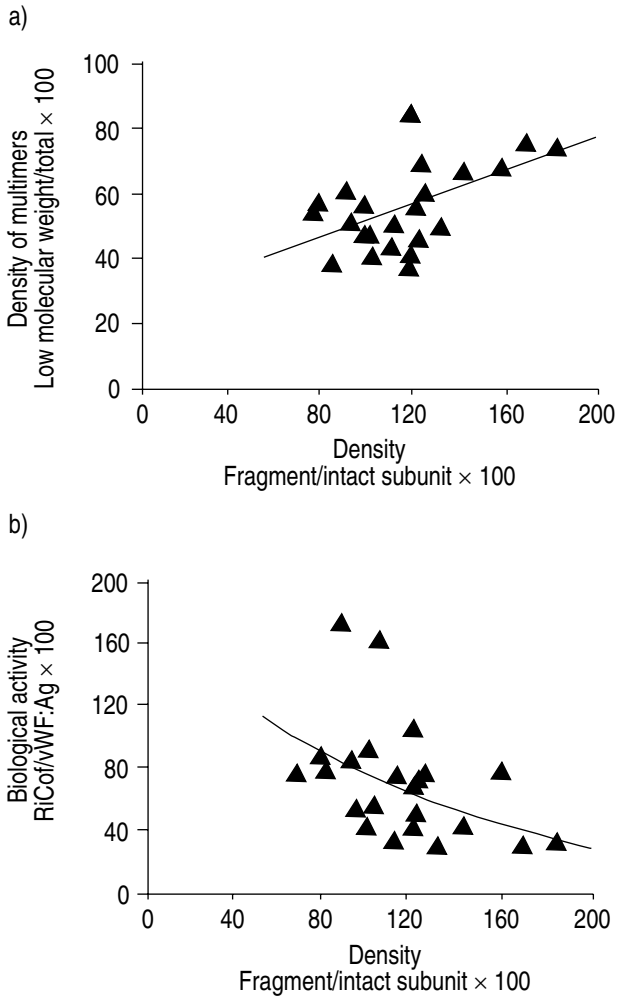

Fig. 3. – a) Positive correlation between proteolytic subunit fragmentation and multimeric vWF abnormalities in pulmonary hypertensive patients. Results were obtained by laser scanning of Western blots. Subunit fragmentation is expressed as total fragment to intact subunit density ratio. Multimeric abnormalities are expressed as the relative density of low molecular weight multimers (four faster migrating bands in 1%-SDS agarose gel electrophoresis). The correlation was significant using the nonparametric Spearman rank-order test ($r_s=0.45$; $p=0.033$) b) Negative correlation ($r_s=-0.49$; $p=0.017$) of vWF biological activity with proteolytic subunit fragmentation. Biological function is expressed as the ratio of ristocetin co-factor to vWF antigenic activity (RiCof/vWF:Ag). SDS: sodium dodecyl sulphate.

vWF multimeric structure

Analysis of vWF multimers in pulmonary hypertensive patients showed decreased concentration of high molecular weight polymers associated with increased density of low molecular weight forms. The ratio of low molecular weight to total multimers was 55±13% in patients *versus* 31±8% in controls ($p<0.001$).

Relationship of different findings

Abnormalities in vWF multimeric structure correlated positively with subunit fragmentation abnormalities ($r_s=0.45$; $p=0.033$) (fig. 3a). In addition, a negative correlation was observed between vWF biological activity and subunit fragmentation abnormalities ($r_s=-0.49$; $p=0.017$) (fig. 3b). There were no significant correlations of vWF subunit abnormalities with patient age, sex, haematocrit level or systemic arterial oxygen saturation. In particular, when patients with primary and secondary pulmonary hypertension were compared, no remarkable qualitative differences were detected in the structure of vWF protein. In regard to the severity of the disease, patients No. 9 and 12, with the highest values of vWF fragment concentration relative to the intact subunit, had advanced pulmonary hypertension with right ventricular failure and died a few months after laboratory tests.

Discussion

Our findings with regard to plasma vWF structure and function in patients with pulmonary hypertension can be summarized as follows: 1) increased density of proteolytic fragments (in particular the 176 kDa polypeptide) relative to total vWF subunit; 2) increased density of low molecular weight multimers relative to high molecular weight forms; and 3) increased vWF:Ag associated with decreased vWF biological activity.

TSAI *et al.* [20] have demonstrated that an apparent increase in the amount of vWF proteolytic fragments may result from mechanisms unrelated to proteolysis. In their study, fragment concentration was found to be higher in smaller multimers. Therefore, ristocetin-induced adsorption of the larger multimers onto platelets resulted in apparent increase in the density of proteolytic fragments relative to the intact subunit. On the basis of these findings, the authors proposed that an apparent increase in vWF fragments may occur in clinical conditions associated with depletion of the larger multimers. In pulmonary hypertension, the hypothesis that vWF associates with platelet microaggregates cannot be excluded. It is known that platelets bind large vWF multimers under physiological stimuli [21]. In addition, we have demonstrated circulating platelet aggregates indicative of *in vivo* platelet activation in these patients [22].

However, we believe that the abnormalities in vWF subunit illustrated in the present study cannot be explained on the basis of larger multimer depletion alone. Our patients had not only loss of high molecular weight polymers but also increased density of the smaller multimers which correlated positively with fragment concentration, suggesting proteolytic degradation. Moreover, in contrast to the data of TSAI *et al.* [20] the increase in proteolytic fragments in our patients was not uniform. Whilst the 176 kDa polypeptide was frequently increased, the concentration of the other fragments was largely variable. In addition, the density of the 140 kDa fragment was clearly decreased in one patient (fig. 1). Therefore, although consumption of the larger multimers might be a possible explanation for subunit abnormalities in some cases, our findings suggest proteolytic degradation of vWF. Since special care was taken to avoid proteolysis *ex vivo,* we believe that vWF degradation occurs *in vivo* in these patients.

The biological activity of vWF has been found to be decreased in pulmonary hypertension [15, 16]. Since the larger multimers are required for normal biological function [23], decreased vWF activity has been attributed to the loss of high molecular weight forms. Our data provide evidence that decreased vWF biological activity is related to proteolytic cleavage of the main subunit. In this case, the functional impairment may be explained by the loss of biologically active multimers and competitive inhibition by degradation products of the interactions between vWF and platelet glycoproteins.

Degradation of vWF has been reported in clinical conditions associated with heightened systemic proteolysis, such as acute leukaemia, decompensated cirrhosis, and acute pancreatitis [24]. Inability of degraded vWF to support haemostasis might contribute to the bleeding diathesis accompanying these states. This is unlikely to occur in pulmonary hypertensive patients, in whom there is no clinical or laboratory evidence of disseminated intravascular coagulation, systemic proteolysis or bleeding tendency. Instead, we would like to speculate that local protease activation at sites of pulmonary vascular injury may account for abnormal degradation of vWF and possibly other proteins. Firstly, physiological proteases, in particular those present in platelets and polymorphonuclear cells, can cause cleavage of vWF [25–28]. Secondly, there is evidence of abnormal interactions between circulating elements and damaged endothelium in pulmonary hypertension [29]. Thirdly, enhanced protease activity is present during pulmonary vascular remodelling [30–32].

Although we cannot establish whether vWF breakdown occurs within dysfunctional endothelial cells or after release into the circulation, the identification that the pattern of proteolysis was not uniform among the patients suggests that different types of proteases may be involved. The 176 and 140 kDa polypeptides result from a single cleavage between Tyr_{842} and Met_{843} in the main vWF subunit [33]. This site has been demonstrated to be a target of calcium-dependent neutral proteases (calpains), an important group of endopeptidases involved in the fragmentation of vWF [33]. Such a mechanism might explain subunit abnormalities in patient No. 13 (fig. 1a), in whom 176 and 140 but not 189 kDa fragments were increased. Another process might have operated in patient No. 12 (fig. 1b) in whom both 189 and

176 kDa polypeptides were markedly increased. Finally, oligosaccharides have been shown to protect vWF molecule from proteolytic cleavage [34, 35]. Therefore, abnormal carbohydrate processing of vWF in dysfunctional pulmonary endothelial cells, resulting in increased susceptibility to proteolysis, may also explain subunit abnormalities seen in our patients.

In conclusion, pulmonary hypertensive patients have increased vWF plasma levels, indicative of extensive endothelial dysfunction. However, the protein is structurally and biologically defective, lacking high molecular weight multimers. Subunit analysis provides evidence for abnormal degradation of vWF *in vivo*. This may have pathophysiological importance, since degradation products are known to stimulate synthesis and release of vWF, thereby explaining the increased plasma levels. Moreover, data suggest that multimeric abnormalities and decreased biological activity of vWF are related to degradation of the main subunit. It would be interesting to speculate whether structural and functional abnormalities of vWF have prognostic implications. Although we agree that further follow-up studies involving larger groups of patients would be necessary to answer this question, it is noteworthy that the highest degree of vWF abnormalities in this study, and also in our last series [16], was observed in two patients who died a few months after the tests. In this way, vWF characteristics should be analysed together with other biochemical and histopathological markers of severity of pulmonary vascular disease.

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