Supranormal von Willebrand Factor Multimers in Scleroderma

By Pier Mannuccio Mannucci, Rossana Lombardi, Antonella Lattuada, Elena Perticucci, Rossano Valsecchi, and Giuseppe Remuzzi

Platelet adhesion-aggregation reactions play an early and pivotal role in the pathogenesis of systemic sclerosis in scleroderma, but the mechanisms are incompletely understood. We determined whether or not plasma from 11 consecutive patients with scleroderma contained a subset of larger than normal ("supranormal") multimers of von Willebrand factor (vWF) that are potent inducers of platelet aggregation and adhesion. Supranormal multimers were found in all patients on at least one of two different occasions 9 to 12 months apart, whatever the duration and severity of the disease, but in none of the normal controls.

Microangiopathy is an early hallmark of scleroderma, the third most frequent rheumatic disorder after rheumatoid arthritis and systemic lupus erythematosus. Probably triggered by circulating agents injurious to human endothelial cells, microangiopathy initiates with endothelial cell damage in small arteries and arterioles followed by platelet adhesion to exposed subendothelium and platelet thrombus formation. Von Willebrand factor (vWF), a large multimeric glycoprotein with a major role in supporting platelet-subendothelial interaction and platelet thrombus formation, is synthesized and stored in endothelial cells, from which it is secreted into plasma. In scleroderma, high levels of plasma vWF were found by Kahaleh et al., who purported that they might be due to leaky endothelial cells and reflect the degree of ongoing endothelial damage. Platelets, however, are another possible source for high plasma vWF because the protein is also synthesized by megakaryocytes and stored in the platelet organelles, called alpha granules, from which it is released into plasma during adhesion-aggregation reactions. In endothelium and platelets, the multimeric organization of vWF differs from that in plasma. Plasma vWF is organized into multimers that range from dimers of the 225-Kd subunit to very large multimers containing more than 20 subunits. Endothelial and platelet vWF have an additional subset of multimers larger than those normally present in plasma. These "supranormal" multimers, unlike regular multimers, are potent inducers of platelet aggregation under conditions of high shear stress like those found in the microcirculation of scleroderma.

Administration of low-dose aspirin (40 mg) to five of the 11 patients for ten days to inhibit the platelet release reaction slightly reduced the amounts of supranormal multimers, suggesting that they might originate in part from platelets. Supranormal multimers may contribute to the pathogenesis of systemic sclerosis by inducing platelet aggregation and enhancing adhesion to subendothelium under the conditions of elevated shear stress occurring in the partially occluded vessels of the arterial microcirculation of scleroderma.

Moreover, supranormal multimers should facilitate vWF-supported bridging of circulating platelets to the vascular subendothelium because they bind the most avidly to the extracellular matrix in an in vitro model for the basement membrane. In this study we demonstrated that plasma from 11 patients with scleroderma often contains a subset of supranormal vWF multimers and that in five of the patients the proportion of supranormal multimers is slightly reduced by short-term administration of low-dose aspirin.

METHODS AND PATIENTS

The methods for collecting blood, preparing platelet-poor plasma (PPP), and assaying vWF antigen and ristocetin cofactor activity in plasma were those published, except that 5 mmol/L EDTA, 6 mmol/L N-ethylmaleimide, and 1 mmol/L leupeptin were added to the sodium citrate anticoagulant to inhibit the in vitro action of calcium-dependent protease. The multimeric composition of vWF was analyzed by sodium dodecyl sulphate (SDS) agarose gel electrophoresis using low-resolution gels and a discontinuous buffer system that partially resolves larger vWF multimers. After electrophoresis, the gels were overlaid with rabbit IgG, and multimers were visualized by autoradiography. On autoradiographs the multimeric pattern of vWF was judged visually by an observer who was unaware of the source of the samples. As controls, normal plasma and plasma known to have aberrant vWF multimeric structures were run in the same gels (ie, plasma from patients with type IIB von Willebrand's disease, lacking the larger forms of normal multimers, and normal plasma collected after infusion of 1-deamino-8-D-arginine vasopressin, containing a subset of supranormal multimers). By using these controls, the multimeric patterns of the unknown samples (from scleroderma patients and healthy subjects) were visually judged to be normal, supranormal, or lacking the larger multimers. The relative percentage of larger multimers was also calculated by scanning the autoradiographs with a densitometer. The area under the densitometric curves corresponding to the larger multimers (arbitrarily defined as the area comprising 30% of the length of each curve starting from the cathodal origin of each gel) was computed and expressed as percentage of the total area. The reproducibility of this method was evaluated by analyzing on seven different gels plasma taken from a normal individual before and after being infused with 1-deamino-8-D-arginine vasopressin (DDAVP; the latter as a control). The mean percentage of larger multimers (±SD) was 25.6% ± 1.5% (coefficient of variation: 5.9%) for pre-DDAVP plasma and 34.1% ± 2.1% (coefficient of variation: 6.2%) for post-DDAVP plasma. The subunit composition of plasma vWF was studied by immu-
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no affinity purification, reduction, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-vWF monoclonal antibodies (MoAbs; kindly provided by Dr TS Zimmerman, La Jolla, CA) and 125I-rabbit antinouse antibody. Relative proportions of vWF fragments were determined by cutting out radioactive bands from the nitrocellulose blots and quantitating radioactivity in a gamma scintillation counter. The reproducibility of this method was evaluated by calculating the relative proportion of vWF subunit and fragments in the same normal plasma fully processed as described above in eight different experiments. The coefficient of variations were 5.2% for the 225-Kd subunit, 12.5% for the 189-Kd fragment, 10.2% for the 176-Kd fragment, and 8.5% for the 140-Kd fragment.

Eleven consecutive patients with scleroderma (nine women and two men; median age, 54 years; range 44 to 62 years) were studied. The clinical diagnoses, made according to the criteria of Asboe-Hansen, were acrosclerosis for eight patients (defined as calcinosis, Raynaud's disease, esophageal dysmotility, sclerodactyly, and telangiectasia), diffuse systemic sclerosis for two patients (defined as involvement of internal organs including lungs, kidneys, heart), and involvement of the same gel. This number of cases is too small to justify statistical analysis of the results (Table 1). In five patients treated with aspirin, there was no important post-treatment change of vWF levels (not shown).

In ten patients reinvestigated after 9 to 12 months, vWF levels remained high (not shown) and the multimeric structure remained supranormal, with no significant differences from baseline values in the proportion of larger multimers (Table 1). In five patients treated with aspirin, there was no important post-treatment change of vWF levels (not shown). In all, however, there was a lower proportion of larger multimers than in their preaspirin plasma run in parallel in the same gel. This number of cases is too small to justify statistical analysis of the results (Table 1). Figure 3 shows vWF multimeric patterns before and after aspirin in plasma run in parallel in the same electrophoretic gels.

RESULTS

Plasma vWF antigen and ristocetin cofactor activity were high in the majority of patients with scleroderma (Table 1), and the differences between patient and control groups were highly significant (P < .001) (Table 2). By visual judgment, a subset of larger than normal (supranormal) vWF multimers was present in patient plasma but not in normal plasma (Fig 1). This abnormality was present in varied degree in all patients, whatever the duration and severity of scleroderma. By densitometry, eight of 11 patients had a higher proportion of larger multimers than control plasma (upper normal limit: 31%), the differences between patient and control mean values being statistically significant (P < .001) (Table 2).

Examination of the subunit composition of plasma vWF demonstrated the presence of the same 189-, 176-, and 140-Kd fragments that are present in controls. No new fragments were demonstrated, nor was there any evidence of uncleaved pro-vWF. The relative proportions of the intact 225-Kd subunit and of the 176-Kd fragment were not significantly different from those in control plasma (225 Kd: 82.1% ± 4.1% for 11 normals v 84.4% ± 5.5% for 11 patients; 176 Kd: 91 ± 2.1 v 9.6 ± 3.8; Fig 2). There was however a significantly lower proportion of both 189- and 140-Kd fragments in patients than in controls (189 Kd: 3.8 ± 1.2 for normals v 2.2 ± 1.4 for patients, P < .05; 140 Kd: 5.7 ± 1.3 v 3.4 ± 1.4, P < .01; Fig 2).

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DISCUSSION

While confirming that vWF plasma levels are often high in scleroderma, this study revealed a previously unreported aberration of the multimeric structure of the protein, consisting of the presence in plasma of a subset of supranormal multimers similar to those present in endothelial cells and platelets. Supranormal multimers are normally stored in endothelial cell organelles called Weibel-Palade bodies. As soon as they are secreted from endothelial cells, supranormal multimers are probably rapidly processed, so that they are not usually present in normal plasma. Perhaps in scleroderma larger amounts of supranormal multimers leak into plasma from Weibel-Palade bodies as a consequence of

Table 1. Main Demographic, Clinical, and Laboratory Data for 11 Patients with Scleroderma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Clinical Presentation</th>
<th>Disease Duration, Yr</th>
<th>vWF Antigen, U/dl</th>
<th>Ristocetin Cofactor, U/dl</th>
<th>Larger Multimers, % of Total Multimers</th>
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<tr>
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<td>252</td>
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</tr>
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<td>210</td>
<td>198</td>
<td>31 nd nd</td>
</tr>
<tr>
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<td>11</td>
<td>110</td>
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<tr>
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<td>Acrosclerosis</td>
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<td>28 34 nd</td>
</tr>
<tr>
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<td>Acrosclerosis</td>
<td>1</td>
<td>170</td>
<td>141</td>
<td>36 29 nd</td>
</tr>
<tr>
<td>11</td>
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<td>Acrosclerosis</td>
<td>4</td>
<td>182</td>
<td>216</td>
<td>37 35 —</td>
</tr>
</tbody>
</table>

Abbreviations: A, baseline value; B, after 9 to 12 months; C, after ten days of aspirin; nd, not done.
damage induced by agents injurious to human endothelial cells so that processing is overwhelmed and they are consistently detectable in plasma. However, since other vWF-containing cells (platelets and megakaryocytes) contain supranormal vWF multimers in alpha granules, one question that arises is whether at least a part of the supranormal multimers seen in scleroderma plasma is originating from activated platelets. That substances contained in platelet alpha granules are released into plasma of patients with scleroderma is demonstrated by the finding of high plasma levels of the platelet-specific protein beta thromboglobulin. The fact that aspirin, which inhibits the release of vWF from platelets, slightly but consistently decreased the proportion of larger multimers in five of our patients is, perhaps, indirect.
evidence that platelet vWF contributes to the supranormal multimers in scleroderma plasma. The daily dose of aspirin we gave (40 mg) was much smaller than that given by Sultan et al\textsuperscript{19} (1.5 g) to inhibit collagen- and thrombin-induced platelet release of vWF in normal volunteers. We chose this smaller dose because it produces fewer gastrointestinal (GI) side effects than the larger dose and yet inhibits collagen-induced release of vWF from normal washed platelets by more than 90\% (our unpublished observations).

Besides being due to increased leakage from platelets and endothelial cells, supranormal multimers might also be due at least in part to reduced processing. Zimmerman et al\textsuperscript{15} have demonstrated that in vivo proteolysis of vWF is a normal event, with a small proportion of plasma vWF being composed of 189-, 176-, and 140-Kd fragments cleaved from the 225-Kd subunit. Our study of the subunit structure produced evidence of reduced proteolytic processing of vWF, with a slightly but significantly lower proportion of the 189- and 140-Kd fragments in scleroderma than in normal plasma.

Whatever their source and cause, circulating supranormal multimers are likely to play a pathogenic role in the series of platelet-endothelial cell interactions that ultimately lead to systemic sclerosis. Also found in the chronic relapsing form of thrombotic thrombocytopenic purpura,\textsuperscript{7} an acquired disease that shares with scleroderma the presence of endothelial damage and enhanced platelet-adhesion aggregation, supranormal vWF multimers might contribute to the development of microangiopathy in both diseases because they are much more effective than regular multimers in supporting aggregation induced by shear stress\textsuperscript{9} and because they bind more avidly to the exposed subendothelium.\textsuperscript{10} The following sequence of events can be hypothesized in scleroderma: early endothelial cell damage results in platelet adhesion and aggregation in small arteries and arterioles; powerful shear stress is generated in the arterial microcirculation partially occluded by platelet thrombi and spasm; supranormal vWF multimers leaking from damaged endothelial cells and activated platelets cause further platelet aggregation induced by shear stress and enhance vWF-supported platelet adhesion to the subendothelium; mitogenic factors released, in turn, from platelets trigger intimal proliferation; the resulting vascular stenoses or occlusions lead to persistent ischemia and ultimately to sclerosis of the skin and visceral organs. Whether or not the reduction of supranormal multimers brought about by aspirin is of therapeutic significance for the disease remains to be established, but it is promising enough for further study.

**ACKNOWLEDGMENT**

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