Changes in ADAMTS13 (von-Willebrand-factor-cleaving protease) activity after induced release of von Willebrand factor during acute systemic inflammation

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Summary
Von Willebrand factor (VWF) is synthesized in endothelial cells, stored in the form of high molecular weight multimers and released after stimulation. After release, the multimers are cleaved by ADAMTS13 (von-Willebrand-factor-cleaving protease). We studied healthy volunteers in a double-blind, placebo controlled inflammation model. Ten male volunteers received 2 ng/kg endotoxin intravenously, and 5 volunteers placebo. Endotoxin infusion induced systemic inflammation and coagulation activation. After 4 hours the observed increase in neutrophils reached a maximum (273±34 % of baseline; mean±SEM) and the platelet count dropped (81±2 %). These parameters returned to baseline values after 24 hours. VWF antigen increased to 259±16 % of baseline after 4 hours, remained elevated (192±15 %) after 24 hours and returned to baseline after 7 days. Unusually large VWF multimers occurred in the plasma 4 hours after endotoxin infusion. ADAMTS13 activity (measured with a collagen-binding assay) decreased to 64±5 % of baseline (P<0.001) after 4 hours, was still reduced after 24 hours (86±6 %; P=0.008) and returned to normal after 7 days. VWF multimer analysis showed pronounced satellite bands in the 4-hour samples, indicating cleavage of VWF by ADAMTS13. No apparent changes of the analyzed parameters were observed in the placebo group. The reciprocal course of ADAMTS13 and VWF after short-term VWF release induced by systemic inflammation is similar to that observed after induction of VWF release by desmopressin.

Keywords
von Willebrand factor, ADAMTS13, systemic inflammation, endotoxin

Introduction
Von Willebrand factor (VWF), a glycoprotein necessary for platelet adhesion and aggregation, is synthesized in endothelial cells and megakaryocytes as a multimeric protein. Upon stimulation of these cells (i.e. by thrombin, endotoxin, cytokines, during acute phase reactions, or by infusion of desmopressin), VWF is released into the plasma. Immediately thereafter, the large multimeric forms are processed by proteolysis by the VWF-cleaving protease (ADAMTS13) on the surface of the endothelial cells, resulting in the generation of the normal multimeric pattern of VWF (1–3). Infusion of desmopressin into healthy volunteers induces an increase in VWF levels, together with the appearance of unusually large (UL) VWF multimers, while the activity of ADAMTS13 is reduced to about half of the initial value (4).

In the absence or complete functional inactivation of ADAMTS13, UL-VWF multimers accumulate and can induce platelet aggregation under high fluid shear rates, leading to the clinical picture of thrombotic thrombocytopenic purpura with thrombocytopenia, haemolysis and disturbance of microcirculation (5, 6). In addition, due to its physiological role in haemostasis, VWF might be involved in coagulation activation and platelet consumption in sepsis, leading to disseminated intravascular coagulation and contributing to multiple organ failure (7). However, the time course of ADAMTS13 has not been evaluated under such pathological conditions. Therefore, the aim of this study was to characterize the regulation of ADAMTS13 and VWF during systemic inflammation. We used an established model with endotoxin infusion into healthy volunteers (8), which is known to induce acute release of VWF (9, 10). We hypothesized that ADAMTS13 might be consumed during the stimulated
increase of UL-VWF multimers, similar to that observed after infusion of desmopressin (4).

Methods

Study design and study subjects

This study was conducted in 15 healthy male volunteers (age 28 ± 5 years) with a body mass index of 23.2 ± 2.5 kg/m² (mean±SD) as a part of a previously published randomized, placebo-controlled trial (11). Physical health was defined as the absence of disease detectable by medical history, physical examination, routine laboratory, virologic variables, and drug screening. The study was approved by the Ethics Committee of the Medical University of Vienna and performed according to the principles of the Declaration of Helsinki. All participants gave written informed consent prior to enrollment in the study.

All volunteers reported to the study ward at 08.00 h, after an overnight fast. Throughout the entire study period all volunteers had to lie in bed and were kept fasting for 2 h after endotoxin infusion. Vital variables (ECG, heart rate and oxygen saturation, blood pressure) were monitored on an automated monitoring system (Care View System, Hewlett Packard, Böblingen, Germany). Our endotoxin model has been described in detail (9, 11, 12). Ten study subjects received 2 ng/kg of endotoxin (National Reference Endotoxin, Escherichia coli, United States Pharmacopoeial Convention Inc., Rockville, MD, USA) as an intravenous bolus infusion for 1 to 2 min, and 5 volunteers received physiological saline as placebo. A continuous infusion of 200 mL/h saline was given during the following 8 h. All volunteers were requested to attend the clinic in the morning of the following day for blood sampling and to report of any adverse events.

Sampling and analysis

The blood samples were obtained by fresh venipunctures before drug injection and at 1, 2, 3, 4, 6, 8, 24 h (and 7 days) after lipopolysaccharide (LPS) infusion on the arm opposite the LPS injection point. Care was taken to avoid trauma and stasis at the venipuncture site. Citrated platelet poor plasma for the coagulation assays was obtained by centrifugation at 2000 x g for 15 min at 4°C and stored at -80°C until analysis. Blood cells were counted from EDTA blood with a Sysmex Counter (Milton Keynes, UK).

Von Willebrand factor antigen (VWF:Ag) was measured with a commercial ELISA assay using polyclonal rabbit antihuman VWF antibodies (Asserachrom VWF, Boehringer-Mannheim, Mannheim, Germany) and expressed in units per milliliter (U/mL) using the standard preparation from the test kit. ADAMTS13 activity was determined according to Gerritsen (13) with some modifications as follows: Patients’ plasma samples were diluted in 1/20 to 1/60 with 5mM Tris, 1.5 M Urea, pH 8.0 and incubated with 9.3 mM BaCl₂ for 30 min. This activated plasma was mixed with purified recombinant VWF (Baxter BioScience, Vienna, Austria) (14) and the mixture was incubated at 37°C for 2 h. The reaction was stopped by the addition of 50 mM Na₂SO₄ and the incubation mixtures were centrifuged for 5 min at 2500 g. The supernatant was used for measuring the residual VWF-collagen-binding activity, which was determined according to Siekmann et al. (15) with some modifications: The wells of a microtiter plate were coated overnight at 4°C with pepsin-digested type III collagen from human placenta (Southern Biotechnology Associates, Inc., Birmingham, USA) followed by blocking (Superblock, Pierce, Rockford, Illinois, USA) for 1 h at room temperature. The samples were added to the blocked wells. After incubation for 2 h at room temperature, the plates were washed and further incubated for 1 h with polyclonal horse-raddish peroxidase-conjugated anti-VWF antibody (DAKO A/S, Glostrup, Denmark). A color reaction was achieved by addition of ImmunoPure TMB Substrate (Pierce, Rockford, Illinois, USA), and after 4 min of incubation the reaction was terminated by the addition of H₂SO₄. The absorbance was read at 450 nm using an ELISA reader 3550 (Bio-Rad Laboratories, Hercules, CA, USA).

The ADAMTS13 activity of tested plasma samples was read from a calibration curve established with pooled normal human plasma, prepared from the plasma samples of 20 healthy individuals. One U/mL ADAMTS13 activity is defined as the amount of ADAMTS13 in the pooled normal human plasma. Thus, 1 U/mL represents 100% ADAMTS13 activity. The detection limit of the assay was 0.1 U/mL. Von Willebrand factor multimers were analyzed on standard (1%) or high resolution (2.5%) SDS-agarose gels as described (16). Plasma samples were adjusted with buffer to 60 ng VWF:Ag per lane.

Data analysis

Data are expressed as means and the standard errors of the mean (SEM) unless otherwise stated. All statistical comparisons of continuous variables were made with non-parametric tests. Comparisons were done with the Mann Whitney U-test or the Wilcoxon signed rank test only for the time point of maximum effects to avoid multiple comparisons. A two-tailed p-value of less than 0.05 was considered statistically significant.

Results

Baseline values

Table 1 shows the baseline values of the volunteers who received 2 ng/kg LPS in comparison with the placebo group. All variables were within normal ranges and not significantly different between the two groups.

Effects of LPS infusion

Infusion of 2 ng/kg LPS into healthy volunteers resulted in a well-known systemic inflammation reaction as evidenced by a significant increase of the neutrophil counts to 273 ± 34 percent of the baseline value (mean ± SEM) 4 h after LPS injection (P<0.001; Wilcoxon test) (Fig. 1). Neutrophil counts remained unchanged in the placebo group. Neutrophilia persisted for at least 6 h and returned to normal after 24 h. Platelet counts decreased in the LPS and placebo group 1 h after the start of infusion, most likely due to hemodilution (Fig. 1). From this time on platelet counts decreased to 81 ± 2 % of the baseline values (P<0.001) 4 h after LPS infusion and returned to normal values after 24 h. In the placebo group platelet counts remained stable and returned to normal values after 24 h. At the time of the maximum drop of the platelet counts (3 h) the difference between LPS...
Table 1: Baseline characteristics of male volunteers receiving endotoxin (LPS; 2 ng/kg body weight) and a placebo group (mean values ± standard errors of the means; Mann-Whitney U-test).

<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
<th>Placebo</th>
<th>P</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>White blood cell count (G/L)</td>
<td>3.3 ± 0.4</td>
<td>3.1 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Platelet count (G/L)</td>
<td>239 ± 9</td>
<td>264 ± 18</td>
<td>ns</td>
</tr>
<tr>
<td>von Willebrand factor antigen (U/mL)</td>
<td>0.93 ± 0.06</td>
<td>0.86 ± 0.04</td>
<td>ns</td>
</tr>
<tr>
<td>ADAMTS13 activity (U/mL)</td>
<td>1.20 ± 0.13</td>
<td>1.08 ± 0.12</td>
<td>ns</td>
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and placebo groups was highly significant (P<0.0001; Mann-Whitney U-test).

**Von Willebrand factor antigen (VWF:Ag)**
In the LPS group VWF:Ag started to increase 2 hours after LPS infusion. A maximum increase was observed after 4–6 h: VWF:Ag increased to 259 ± 16% of the baseline value (P=0.005 vs baseline) and remained elevated even after 24 h (192 ± 15% of baseline; P=0.005 vs baseline; Wilcoxon test). VWF:Ag levels returned to baseline values after 7 days. In the placebo group VWF:Ag levels remained unchanged. The differences in VWF:Ag levels between the LPS and the placebo group were statistically significant (P=0.001; Mann-Whitney U-test) (Fig. 2).

**ADAMTS13-activity**
After infusion of LPS, ADAMTS13 activity showed an inverse time course compared with VWF:Ag. ADAMTS13 activity started to decline after 2 h (82 ± 5% of baseline values) with a minimum activity of 64 ± 5% of baseline after 4–6 h (P<0.001; Wilcoxon test). ADAMTS13-activity was still reduced after 24 h (86 ± 6% of baseline; P=0.008). It returned to normal values after 7 days. In contrast, in the placebo group ADAMTS13-activity was not altered. The difference between the LPS and the placebo-group was statistically significant (P=0.001; Mann-Whitney U-test) (Fig. 2).

**Von Willebrand factor multimer analysis**
Figure 3 shows the analysis of VWF multimers in 5 volunteers receiving LPS (baseline and 4 hour values). Figure 3A (1% agarose gel) suggests the occurrence of UL multimers during the VWF release induced by LPS. Figure 3B (high-resolution gel) shows that the tripllet structure and the satellite bands are more pronounced in the 4-hour lanes, indicating the breakdown of large VWF multimers by ADAMTS13.

**Discussion**
The endotoxin model used for induction of the inflammatory response is well established and provides a unique model to evaluate pathophysiological responses to endotoxin (8, 17). Extensive reviews of endotoxin effects on coagulation and hemostasis have recently been published (7, 18). One of these effects is the release of VWF, which is a well-known acute-phase reactant (9, 20). Additionally, LPS infusion induces a short-term release of large VWF multimers after 2-4 h (10). The rise in VWF in response to an endotoxin infusion in healthy volunteers is similar to that observed during an acute phase reaction caused by a systemic inflammatory response to an infective organism. The increase of the leukocyte counts also fits in this model.

The current study demonstrates a drop of ADAMTS13 activity during the release of VWF stimulated by acute inflammation (Fig. 2). These results are very similar to that of our previous study on the release of VWF stimulated by desmopressin (4). In contrast to desmopressin, which causes a very fast release of VWF, peaking after 30 min, the LPS-induced inflammation produces a delayed increase of VWF, which reaches its maximum...
after 4 h (Fig. 2 and [21, 22]). Desmopressin, as well as inflammation, induced the transient release of UL VWF multimers (Fig. 3A). Of interest is the course of ADAMTS13, which is reciprocal to VWF:Ag in both studies: the largest decrease (to about 50% of baseline) occurred 30 min after desmopressin infusion, and during inflammation (to about 65% of normal) after 4 h. In both studies the triplet structure was pronounced at the time of maximum VWF release, clearly indicating that VWF is cleaved by ADAMTS13 (Fig. 3B). Reduced levels of ADAMTS13 have been found in various conditions associated with acute phase reactions: malignancy, infections, or after surgery (23). However, these studies reported data on single measurements, and the dynamics of ADAMTS13 and VWF have never been shown in acute inflammation. Our study demonstrates a tight association between the increase of VWF:Ag, the release of UL-VWF multimers, and the reciprocal drop of ADAMTS13 activity.

This association suggests that UL-VWF multimers, once released into the plasma, are rapidly cleaved by ADAMTS13 in order to dispose of these more platelet-adhesive and agglutinating forms of VWF. We cannot explain from our data whether ADAMTS13 activity is thereby exhausted by the excess of substrate, or is consumed and eliminated from plasma, for example by binding to the surface of endothelial cells. The more pronounced triplet structure in the high-resolution multimer gel indicates that UL-VWF multimers are actually cleaved by ADAMTS13 after release. A recent study by Mannucci and coworkers supports our hypothesis (24). Healthy persons with blood group 0 had lower plasma levels of VWF:Ag, but higher ADAMTS13 activity than individuals with other blood groups. Patients with type 3 VWD disease had significantly elevated ADAMTS13 activity, which decreased after infusion of VWF concentrates. As a confirmation of our previous study (4), Mannucci et al. also demonstrated that ADAMTS13 activity decreased after infusion of desmopressin in healthy volunteers (24). All these findings clearly indicate a direct regulatory mechanism between ADAMTS13 and its substrate, VWF.

There may be other possible reasons for a decreased activity of ADAMTS13 during inflammation: Crawley and coworkers demonstrated the susceptibility of ADAMTS13 to proteolytic enzymes like thrombin or plasmin (25). In our model of inflammation an activation of blood coagulation is induced via the tissue factor pathway and thrombin and plasmin are generated (8, 12, 18). Therefore, this effect may contribute to the drop of ADAMTS13 activity. However, our model resembles a low degree of inflammation, and the thrombin generated is partly blocked by antithrombin, as recognized by an increase of thrombin-antithrombin complexes (19). The recent study of Bernardo et al. (26) offers another explanation for the reduced activity of ADAMTS13 after endotoxin challenge: ADAMTS13 activity is suppressed by interleukin-6 (IL-6) under flow conditions. Injection of LPS in volunteers induces a considerably increase of IL-6. In the model used for this study the maximal increase of IL-6 occurs 4 h after LPS injection and reaches 1000 pg/ml, the 200-fold of the baseline value (19). The time course of IL-6 release follows that of VWF. Therefore, the reduction of ADAMTS13 activity may be caused by the high levels of IL-6. This model would also partly explain the reduction of ADAMTS13 activity after infusion of desmopressin (4), since this drug also causes a pronounced release of IL-6 (27). However, these hypotheses are contradicted by the studies of Mannucci et al. (24), who found a decline of ADAMTS13 activity in patients with severe, type 3 VWD after infusion of VWF concentrates, and in this setting there is no possibility for an increase of thrombin, plasmin or IL-6. Moreover, the occurrence of a pronounced triplet structure on the high resolution gels, indicating cleavage of VWF, suggests that there must be some residual active ADAMTS13 and therefore excludes a complete inactivation of ADAMTS13 by external factors. Therefore, our results support the proposed reciprocal relationship between ADAMTS13 and VWF. Whether this is a direct regulatory process, as suggested by Mannucci (24) or an exhaustion of the enzyme by excess of substrate, remains unanswered.

A transient decrease of platelet counts is constantly observed in endotoxin models like that used in this study. Recent data indicate that platelets aggregate to leukocyte and to vascular endothelium (platelet margination) after endotoxin-induced inflammation (22, 28), explaining the transient decrease of platelet counts in our model. Platelet aggregation may be promoted by the occurrence of UL-VWF multimers (2, 26). In contrast, in pa-
tients with thrombotic thrombocytopenic purpura ADAMTS13 activity is always very low (below the detection limit of our assay), resulting in the formation of large amounts of UL-VWF multimers (5, 6). However, platelet aggregation and hemolysis occurs only in situations with additional high shear stress, and patients may have quite normal platelet counts over a long time during remission, although ADAMTS13 activity may be still low and UL-VWF multimers are present (29). Moreover, until now no study could demonstrate the persistence of UL-VWF multimers with enhanced platelet aggregation when ADAMTS13 activity is higher that 20–30% of normal, even not in situations with high shear stress. However, there may be clinical situations in which ADAMTS13 activity is decreased below that range. In critically ill patients with sepsis, for example, several factors can cause a reduction of ADAMTS13: the systemic inflammatory response is associated with a release of IL-6, which may abrogate the function of ADAMTS13 (26); systemic activation of coagulation with pronounced thrombin generation and hyperfibrinolysis may lead to direct degradation of ADAMTS13 (25); therapy with vasopressors (norepinephrine or vasoprosin) can induce the release of VWF, as well as the inflammatory response, resulting in a down-regulation of ADAMTS13 (4, 24); disturbance of liver function during multiple organ failure will result in a reduced synthesis of ADAMTS13 (6). Although every single factor alone will probably not be able to reduce ADAMTS13 activity below a critical limit, a combination of all in septic patients may result in considerably reduced levels. Recent, yet unpublished, data demonstrate a remarkable decrease of ADAMTS13 activity in patients with severe sepsis. Consecutively, cumulating UL VWF multimers may induce enhanced platelet aggregation, caused by shear stress and activation of endothelial cells during sepsis. Such platelet aggregates may, together with fibrin deposition (7, 18), contribute to septic organ failure.

This study is the first to show that ADAMTS13 activity decreases in a time-dependent manner during systemic inflammation in humans. Similar to our previous study in healthy men and patients with type 1 von Willebrand disease (4), the drop in ADAMTS13 activity may be due to the inhibition by IL-6 (26) or proteases (25) or to a direct interaction between the protease and its substrate. The excess of released UL-VWF multimers may lead to either exhaustion of ADAMTS13 or to consumption of ADAMTS13 and to elimination from plasma. Whether this effect has an impact on platelet aggregation, thrombosis or disturbance of microcirculation in inflammation, eventually leading to organ failure during severe sepsis, has to be determined in further studies.

Acknowledgments

We are grateful for the excellent assistance of the study nurses of the Department of Clinical Pharmacology, the skilful technical assistance of Birgitte Keil, Manfred Billwein, Ingrid Neunteufl and Sylvia Peyrer-Heimstätt (Baxter BioScience, Vienna, Austria), and the editorial assistance of Elise Langdon-Neuner. We also like to thank all volunteers for participating in this study. This trial was supported in part by grant #8387 from the “Jubiläumsfonds der Österreichischen Nationalbank”.

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