Effects of Inflammatory Cytokines on the Release and Cleavage of the Endothelial Cell-derived Ultra-large von Willebrand Factor Multimers under Flow†

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ABSTRACT

ADAMTS-13 cleaves ultra-large and hyper-reactive von Willebrand factor (ULVWF) freshly released from activated endothelial cells to smaller and less active forms. This process may be affected by the amount of ULVWF released and the processing capacity of ADAMTS-13, contributing to the development of thrombotic diseases. We determined the effects of inflammatory cytokines on the release and cleavage of ULVWF to evaluate potential links between inflammation and thrombosis. Human umbilical vein endothelial cells were treated with IL-6, IL-8, or TNF-α, and the formation of platelet-decorated ULVWF strings was quantitated. IL-8 and TNF-α significantly stimulated the release of ULVWF in a dose-dependent manner. IL-6 induced ULVWF release only when it was in complex with the soluble IL-6 receptor. IL-6, but not IL-8 nor TNF-α, inhibited the cleavage of ULVWF strings by ADAMTS-13 under flowing, but not static, conditions. These results suggest that inflammatory cytokines may stimulate the ULVWF release (IL-8 and TNF-α) and inhibit the ULVWF cleavage (IL-6), resulting the accumulation of hyper-reactive ULVWF in plasma and on the surface of endothelial cells to induce platelets aggregation and adhesion on the vascular endothelium. The findings describe a potential linkage between inflammation and thrombosis that may be of therapeutic importance.
INTRODUCTION

Von Willebrand factor (VWF) multimers mediate platelet adhesion to subendothelium exposed at the site of vessel injury. VWF is synthesized and stored in α-granules of megakaryocytes/platelets and the Weibel-Palade bodies of endothelial cells. Endothelial cells, the major source of plasma VWF, release VWF multimers both constitutively and upon stimulation. VWF forms released through stimulation are rich in the ultra-large (UL) multimers that are hyper-reactive, capable of forming high strength bonds with the platelet GP Ib-IX-V complex in the absence of any modulators. These hyper-reactive ULVWF multimers are normally cleaved by a VWF-cleaving enzyme in plasma into smaller and less active form before their release into plasma. The cleavage occurs in vitro at a single peptide bond between Y842 and M843 in the VWF-A2 domain, generating 176-kDa and 140-kDa fragments that are found in the normal circulation. The VWF cleaving enzyme has recently been characterized and found to be a member of the ADAMTS (A Disintegrin and Metalloprotease with Thrombospondin motif) metalloprotease family (ADAMTS-13). Despite of recent progress in characterizing conditions in which ADAMTS-13 acts optimally, how this metalloprotease cleaves ULVWF in vivo remains largely unknown.

We have recently demonstrated that ULVWF multimers secreted from endothelial cells are anchored to the cell surface as extraordinarily long string-like structures capable of inducing platelet adhesion and aggregation. These ULVWF multimeric strings are rapidly cleaved by ADAMTS-13 in vitro at a kinetic that is more than 1000-fold faster than that observed under static conditions, suggesting that ULVWF processing may occur on or near the surface of the vascular endothelial cells in vivo.
The process of ULVWF proteolysis is in a constant balance between the amount of ULVWF released from endothelial cells and the processing capacity of ADAMTS-13. Factors that disrupt this balance result in pathological conditions ranging from bleeding to thrombosis. For example, a sustained deficiency of this metalloprotease, either congenital or acquired, results in a systemic thrombotic microangiopathy called thrombotic thrombocytopenic purpura. Increased proteolysis is associated with bleeding disorders such as the type 2A von Willebrand disease. In addition to these permanent defects, ULVWF/ADAMTS-13 control mechanism may also be influenced either by rapid and excessive ULVWF release or a transient inhibition of the rate of ULVWF cleavage by ADAMTS-13. Increased plasma levels of VWF have been reported in a wide variety of disease states, such as bacterial or viral infections, trauma, autoimmune diseases, and coronary and peripheral artery diseases. Although these diseases cannot be linked by a common cause, they are, by the common pathology of inflammation, suggesting that inflammation may be the shared stimulus for release of endothelial cell-derived ULVWF. The large amount of newly released ULVWF may lead to a consumptive deficiency of ADAMTS-13. Reiter et al. showed that administration of 1-deamino-8-D-arginine vasopressin (DDAVP), which stimulates VWF release from endothelial cells, resulted in transient appearance of ULVWF in plasma and decrease in ADAMTS-13 activity, indicating that overwhelming release of ULVWF may, at least transiently, exhaust ADAMTS-13 activity. The question is whether inflammatory mediators such as cytokines and chemokines, which are often strong agonists to endothelial cells, may have similar effects. This determination may provide a critical link between inflammation and thrombosis.
We have examined three inflammatory cytokines IL-6, IL-8, and TNF-α for their effects on the release and cleavage of ULVWF. These cytokines are chosen because they are released from endothelial cells and leukocytes during the early stage of systemic inflammation\textsuperscript{25-27}. Although their effects on the release of ULVWF from vascular endothelial cells remain to be investigated, they have been demonstrated to have profound stimulatory effects on endothelial cells\textsuperscript{27,28}. 
MATERIALS AND METHODS

Platelet and plasma preparations
Human blood was obtained from 32 healthy donors under a protocol approved by the Institutional Review Board of the Baylor College of Medicine. These donors had no history of thrombosis and had not been on medications for at least two weeks. All donors signed consent forms. There were 18 females and 14 males in the donor pool, ranging from 21 to 56 years of age.

To obtain washed platelets, blood was drawn into acid-citrate dextrose anticoagulant (ACD, 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid, 10% v/v) and centrifuged at 150 × g for 15 min at 24°C to separate platelet-rich plasma (PRP). PRP was then centrifuged at 900 × g for 10 min to obtain platelets. The platelet pellets were washed once with a CGS buffer (13 mM sodium citrate, 30 mM glucose and 120 mM sodium chloride, pH 7.0), and resuspended in Ca++-free Tyrode’s buffer (138 mM sodium chloride, 5.5 mM glucose, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, and 0.36 mM sodium phosphate dibasic, pH 7.4).

For most of the studies, platelet-poor plasma (PPP) was used as the source of ADAMTS-13 metalloprotease. For this, blood drawn into 0.38% sodium citrate, instead of ACD, was first centrifuged at 150 × g for 15 min at 24°C to obtain PRP and then at 900 × g for 10 min to obtain PPP.

Endothelial culture
Under a protocol approved by the Institutional Review Board of the Baylor College of Medicine, endothelial cells were obtained from human umbilical veins (HUVECs) as described previously. Briefly, the umbilical cords were first washed with phosphate...
buffer (140 mM NaCl, 0.4 mM KCl, 1.3 mM NaH$_2$PO$_4$, 1.0 mM Na$_2$HPO$_4$, 0.2% glucose, pH 7.4), and then infused with a collagenase solution (0.02%, Invitrogen Life Technologies, Carlsbad, CA). After 30 min incubation at room temperature, the cords were rinsed with 100 ml of the phosphate buffer. Endothelial cells were collected by centrifugation and plated on a culture dish in Medium 199 (Invitrogen Life Technologies) containing 20% heat-inactivated fetal calf serum and 0.2 mM of L-glutamine.

To induce the release of ULVWF multimers, HUVECs were stimulated with 25 µM histamine (Sigma-Aldrich, St. Louis, MO). Effects of cytokines on the release of ULVWF were determined by incubating HUVECs with one of the three cytokines for up to 24 hrs before the perfusion experiments. Cytokines tested in the studies were interleukin-6 (recombinant human IL-6, R&D systems Inc. Minneapolis, MN), interleukin-8 (recombinant human IL-8, R&D systems Inc.), and tumor necrosis factor-α (recombinant human TNF-α, R&D systems Inc.). The reagents were reconstituted in PBS, aliquoted, and stored in –20 °C until use.

The formation and cleavage of VWF strings was studied under flow in a parallel-plate flow chamber system and observed by phase-contrast video microscopy as previously described $^{13}$.

**Enzyme-linked immunosorbent assay (ELISA)**

The formation of IL-6 and soluble IL-6 receptor (sIL-6R, Sigma Aldrich, St. Louis, MO) *in vitro* was determined by an ELISA method. IL-6 (100 ng/ml of coating concentration) was first incubated in wells of a microtiter plate at room temperature for 2 hr. The wells were then washed with PBS three times and incubated with sIL-6R (80 ng/ml) for additional 30 min. After washing to remove the unbound molecules, wells were incubated
with 3% bovine serum albumin (BSA) to block non-specific binding. Bound sIL-6R was detected by incubating wells with a mouse monoclonal anti-IL-6R antibody (BD Bioscience, San Jose, CA) for 60 min, followed by additional 30 min incubation with HRP-conjugated rabbit anti-mouse IgG. Positive color was developed with o-Phenylenediamine and detected in a spectrophotometer (Bio-Rad laboratories, Hercules, CA) at 490 nm.

**Purification of ADAMTS-13**

ADAMTS-13 was partially purified on a DEAE column from factor VIII/VWF concentrate using the method described earlier \(^3^0\). The total protein content of the ADAMTS-13 preparation was 126 μg/ml. Purified ADAMTS-13 cleaved VWF after a 24 hr incubation at 37 °C in the presence of 1M urea and BaCl₂, yielding 176-kDa and 140-kDa fragments on reduced SDS-PAGE gels.

**Cleavage of ULVWF under static condition**

The ADAMTS-13 activity was measured under static conditions using ULVWF as substrate \(^3^1\). This method is modified from that of Furlan et al \(^9\). Briefly, normal plasma as the source of ADAMTS-13 was diluted (1:5) with a low ion strength Tris-saline buffer and activated by 5 min incubation with 1 mM BaCl₂. The pretreated plasma was then mixed with ULVWF. The mixture was dialyzed in 1.5 M of urea for 24 hr at 37 °C, and then separated on 1% agarose gel at 50 V constant voltage until proteins had traveled 1 cm. The separated proteins were transferred to a PVDF membrane and VWF multimers detected by western blot using a polyclonal anti-VWF antibody (Dakocytomation, Carpinteria, CA).
Statistical analysis

All the experimental data were all presented as mean ± SE. The unpaired 2-tailed Student’s $t$ test was used for all data analysis and a $p$ value less than 0.05 was considered statistically significant.
RESULTS

**IL-8 and TNF-α, but not IL-6, stimulated HUVECs to release ULVWF under flow.**

We tested the effects of cytokines on the release of ULVWF from endothelial cells by measuring the formation of ULVWF-platelet strings under flow. When HUVECs stimulated with IL-8, TNF-α, or histamine (as positive control) were perfused with washed platelets, ULVWF-platelet strings were detected 30 sec after perfusion (Figure 1). The numbers of ULVWF strings formed in the presence of IL-8 or TNF-α increased with increasing doses of the cytokines, with maximal effects observed at 100 ng/ml and 10 ng/ml for IL-8 and TNF-α, respectively (Figure 2A). At the maximal concentration, the numbers of ULVWF strings formed on endothelial cells stimulated with TNF-α reached a plateau after 60 min incubation (Figure 2B). In contrast to IL-8 and TNF-α, IL-6 (10 – 100 ng/ml) induced the secretion of fewer ULVWF strings (Figures 1 and 2).

Although normal plasma concentrations of these cytokines are very low, they increase up to several hundred to thousand folds during myocardial infarction\(^2\):\(^3\) and other conditions with acute inflammation\(^4\). For example, Ueda et al reported that plasma IL-6 levels increased to 27 ng/ml from less than 10 pg/ml\(^5\). The concentrations tested in this study represented the likely plasma levels of these inflammatory cytokines during severe diseases.

**IL-6 in complex with soluble IL-6 receptor stimulated the formation of ULVWF strings under flow.** The absence of the IL-6 receptor on HUVECs may be one reason for IL-6’s failure to induce HUVECs to release ULVWF. To address this possibility, we tested the ability of IL-6 that complexed with soluble IL-6R (sIL-6R), which has previously been shown to act on cells that lack IL-6R\(^6\):\(^7\), to stimulate HUVECs. The binding of IL-6 to
sIL-6R was first demonstrated by an ELISA assay, showing that sIL-6 specifically bound to IL-6, but not to BSA (Figure 3A). We prepared the pre-formed IL-6-sIL-6R complex by incubating IL-6 with sIL-6R for 30 min at room temperature. The complex was used to stimulate HUVECs for 10-60 min. Under these conditions, ULVWF strings were detected on the IL-6-sIL-6R-stimulated HUVECs (Figure 3B), but the numbers of strings formed (19.6±6.4) were fewer than that by histamine (66.0±29.7), IL-8 (47.1±115), or TNF-α (59.6±14.9, Student’s t test, n = 12, p < 0.001, Figure 3B). Soluble IL-6R alone had no stimulatory effects on the release of ULVWF from HUVECs.

**IL-6, but not IL-8 and TNF-α, inhibited the cleavage of ULVWF-platelet strings on endothelial cells under flow.** In addition to their effects on the release of ULVWF from endothelial cells, we also determined whether these cytokines affected the ADAMTS-13-mediated cleavage of ULVWF-platelet strings formed on the stimulated-endothelial cells. We have previously demonstrated that ULVWF-platelet strings are immediately cleaved and consequently not detectable if normal PRP, which contain ADAMTS-13, is perfused over stimulated HUVECs. To determine the effects on ADAMTS-13 activity, we pretreated normal PRP with various concentrations of cytokines for 10-30 min before perfusion. Under these conditions, we could not detect the formation of ULVWF-platelet strings with PRP pretreated with either IL-8 or TNF-α (Figure 4). In contrast, ULVWF strings were not efficiently cleaved if PRP was pretreated with IL-6 (Figure 4). The inhibitory effect of IL-6 was non-linearly related with the amount of IL-6 used, with a maximal effect observed at 100 ng/ml. The inhibitory effect of IL-6 (100 ng/ml) was also observed if whole blood was treated with IL-6 (Figure 5A) and if IL-6 was in complex with sIL-6R (Figure 5A).
To determine whether the inhibitory effect of IL-6 was directly on ADAMTS-13 or indirectly through other molecules in plasma, we tested the effect of IL-6 on partially purified ADAMTS-13. For this, washed platelets were mixed with ADAMTS-13 (1:1 vol/vol), and then perfused over histamine-stimulated HUVECs under 2.5 dyn/cm² shear stress. In the absence of IL-6, the partially purified ADAMTS-13 cleaved 100% of the ULVWF strings within 2 min after perfusion was initiated, whereas IL-6 pretreatment of ADAMTS-13 resulted in approximately 50% inhibition of ADAMTS-13 activity (Figure 5B).

Kinetics of IL-6 inhibition on the cleavage of ULVWF-platelet strings under flow. To further determine the kinetics of IL-6 inhibition, we first perfused washed platelets suspended in Tyrode’s buffer over histamine-stimulated HUVECs to allow ULVWF-platelet strings to form and then a solution containing purified ADAMTS-13 with or without preincubation with IL-6 (100 ng/ml). As shown in Figure 6, the purified ADAMTS-13 cleaved the ULVWF-platelet strings within 2 min of perfusion, whereas plasma from patients with congenital TTP patients failed to do so even after prolonged 15 min perfusion. ADAMTS-13 pretreated with IL-6 cleaved ULVWF strings more slowly than untreated ADAMTS-13 with more than 80% of ULVWF-platelet strings remaining after 2 min perfusion (Figure 6). It took 15 min for IL-6-treated ADAMTS-13 to cleave ULVWF strings to the extent as untreated metalloprotease.

Cytokines had no inhibitory effects on the cleavage of ULVWF under static conditions. Having demonstrated an inhibitory effect of IL-6 on the cleavage of ULVWF strings on endothelial cells under a flow condition, we next tested the effects of this inflammatory cytokine on the cleavage of ULVWF under static conditions. Normal plasma was
incubated with each of three cytokines for 30 min at room temperature and the ADAMTS-13 activity then measured in the presence of barium and urea. As shown in Figure 7, the cleavage by normal plasma under static conditions was not altered by pretreating plasma with IL-6, IL-8, or TNF-α for 30 min in room temperature.
DISCUSSION

We have demonstrated that inflammatory cytokines have distinct effects on the release of ULVWF from HUVECs and the activity of the VWF cleaving metalloprotease, ADAMTS-13. IL-8, TNF-α, and, to a lesser extent, the IL-6-sIL-6R complex stimulate the release of ULVWF from endothelial cells in a dose-dependent manner (Figure 1 and 2). IL-6, but not IL-8 or TNF-α, inhibits the cleavage of ULVWF strings by ADAMTS-13 under flowing, but not static, conditions (Figure 3 and 7). These results suggest that inflammatory cytokines released during variety of pathological conditions may affect the balance between the quantity of ULVWF multimers released from endothelial cells and the rate of ULVWF cleavage by ADAMTS-13. As a result, ULVWF multimers may persist on vascular endothelial cells and in plasma long enough to induce platelet adhesion and aggregation, potentially leading to thrombosis. These findings may provide a linkage between inflammation and thrombosis.

Two cytokines that include significant release of ULVWF are known agonists for endothelial cells. IL-8, the prototype member of the CXC subfamily of chemokines is released from many different cell types in response to a variety of inflammatory stimuli. In endothelial cells, IL-8 is stored in Weibel-Palade bodies along with ULVWF multimers and P-selectin. Upon release, it activates endothelial cells, especially in microvascular endothelium and mediates the formation of platelet-leukocyte aggregates. TNF-α, predominantly derived from monocytes, activates endothelial cells to release IL-8, IL-6, and other cytokines.

IL-6 has been previously demonstrated to exert procoagulant effects on platelets, stimulate platelet production, enhance platelet response to thrombin, and stimulate the
hepatic release of C-reactive protein and fibrinogen, and promote the proliferation of vascular smooth muscle cells. We found that IL-6 alone does not stimulate the ULVWF release from HUVECs, probably because these cells lack the IL-6 receptor. However, the IL-6-sIL-6R complex induces a small, but significant amount of ULVWF release from HUVECs (Figure 3). Our data are consistent with previous reports that the IL-6-sIL-6R complex stimulates endothelial cells to express E-selectin, intracellular adhesion molecule-1, and vascular cellular adhesion molecule-1. The finding is also consistent with the reported mechanism that IL-6 acts on non-IL-6R expressing cells by forming a complex with its soluble receptor. The soluble IL-6 receptor can be produced during inflammation by membrane shedding from leukocytes into plasma. The IL-6-sIL-6R complex binds to gp130, the cytokine common signal-transducer of the IL-6 cytokine family that is expressed in most cell types. Upon binding, gp130 dimerizes and undergoes tyrosine phosphorylation, resulting in subsequent activation of the transcription factors STAT3.

Compared to the effect on endothelial cells, IL-6 significantly inhibits the rate of the cleavage of ULVWF-platelet strings by ADAMTS-13 (Figure 5 & 6). How IL-6 inhibits ADAMTS-13 activity remains to be further investigated. One of the potential mechanism for the inhibitory effect of IL-6 on ADAMTS-13 that IL-6 may impair docking of ADAMTS-13 to the ULVWF strings under flow, because this effect is only observed for cleaving ULVWF strings formed on endothelial cells under flow, but not in a static system involving long incubation of substrate with plasma. Potential effects of IL-6 on ULVWF cleavage have previously been suggested by clinical observations. First, serum levels of IL-6 have been reported to be significantly higher at the onset of TTP episodes.
than during remission and a high IL-6 level associates with a poorer prognosis in TTP. Second, individuals injected with DDAVP have a transient elevation of IL-6, along with detectable plasma ULVWF multimers and decreased ADAMTS-13 activity. Third, elevated levels of IL-6 have been detected in coronary arterial thrombi and atherosclerotic plaques. Finally, elevated IL-6 in serum is associated with increased 5-yr cardiovascular mortality, as well as with the presence of peripheral arterial occlusive disease.

In summary, we have demonstrated that the inflammatory cytokines IL-6, IL-8, and TNF-α have distinct effects on the endothelial cell release of ULVWF multimers, and on the processing of ULVWF by ADAMTS-13. IL-8- and TNF-α stimulate ULVWF from HUVECs in a dose-dependent manner. IL-6 in complex with sIL-6R is less effective in stimulating HUVECs to release ULVWF. In contrast to IL-8 and TNF-α, however, IL-6 (either alone or in complex with sIL-6R) significantly inhibits the rate of the cleavage of ULVWF strings by ADAMTS-13 under flow condition, but shows no affect on the cleavage of ULVWF under static condition with prolonged incubation of the metalloprotease with ULVWF. These results suggest that cytokines released during inflammation may alter the kinetics of converting the ultra-large and hyper-reactive ULVWF to the smaller and less active plasma forms of VWF. They may do so by increasing the ULVWF release, which could overwhelm the processing capacity of ADAMTS-13, or by directly inhibiting the ADAMTS-13 activity. The combined results will be the accumulation of the hyper-reactive ULVWF multimers in plasma to aggregate platelets and on endothelial cells to tether platelets and leukocyte to the vascular endothelium. Since IL-6 only partially inhibits ADAMTS-13 activity, thrombotic events...
may not occur as the result of a single episode of inflammation, but repeated inflammatory response will eventually lead to atherosclerosis and thrombosis. By the pro-thrombotic effects of IL-6, IL-8, and TNF-α, our results provide a possible mechanism linking inflammation to thrombosis.
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FIGURE LEGENDS

Figure 1. IL-8 and TNF-α, but not IL-6, stimulated the release of ULVWF multimers.
HUVECs were stimulated with TNF-α (50 ng/ml), IL-6 (100 ng/ml) or IL-8 (100 ng/ml) for 30 min and then perfused with washed platelets suspended in Tyrode’s buffer at 2.5 dyn/cm². The formation of the platelet-decorated ULVWF strings was detected on endothelial cells stimulated with TNF-α (A), IL-8 (B), but not on those stimulated with IL-6 (C). Histamine-stimulated endothelial cells (D) were used as positive control. No strings formed on untreated endothelial cells (E). The figure is a representative of 6 separate experiments (bar = 100 µm).

Figure 2. IL-8 and TNF-α induced the release of ULVWF from HUVECs. (A)
HUVECs were treated with various concentrations of cytokines for 30 min and then perfused with washed platelets suspended in Tyrode’s buffer at 2.5 dyn/cm². The ULVWF released from HUVECs stimulated by IL-8 or TNF-α had maximal effects at 100 and 10 ng/ml, respectively (mean±SEM, Student t test, n = 9). (B) HUVECs were treated with 50 ng/ml of TNF-α for up to 24 hrs before perfusion with washed platelets. The formation of ULVWF strings increased with incubation times, reaching plateau after 60 min (mean±SEM, n = 4).

Figure 3. IL-6 bound sIL-6R and stimulated ULVWF release from HUVECs.
Recombinant human IL-6 (100 ng/ml) was incubated with sIL-6R (80 ng/ml), and IL-6 binding to sIL-6R detected using a monoclonal IL-6 antibody. (A). sIL-6R bound to immobilized IL-6, but not BSA (mean±SEM, Student t test, n = 3, * p <0.001, as
compared to plates coated BSA). (B). HUVECs were treated with preformed IL-6-sIL-6R complexes for 30 min and perfused with washed platelets at 2.5 dyn/cm² shear stress. The platelet-decorated ULVWF strings formed on the complex-stimulated HUVECs, but the number of strings was significantly less than those of histamine-, IL-8, and TNF-α-stimulated cells (mean±SEM, Student t test, n = 3, ** p <0.01 compared to stimulation with histamine).

*Figure 4. IL-6, but not IL-8 and TNF-α, inhibited cleavage of ULVWF strings by ADAMTS-13 under flow.* Normal PRP samples were pretreated with each of the three cytokines and perfused over histamine-stimulated HUVECs at 2.5 dyn/cm² shear stress. The platelet-decorated ULVWF strings were mostly cleaved in the presence of IL-8 or TNF-α treated PRP, but not in the presence of IL-6 pretreated PRP. The maximal inhibitory effect on ULVWF cleavage was observed at 100 ng/ml of IL-6. As controls, there was no string formed with untreated PRP (100% cleavage), whereas the perfusion of washed platelets suspended in Tyrode’s buffer resulted in a significant number of strings (no cleavage). Figures were mean±SEM, Student t test, n = 6, * p <0.01, compared to IL-8 and TNF-α-treated PRP.

*Figure 5. IL-6 inhibited the cleavage of ULVWF strings under different conditions.* (A) PRP treated with preformed IL-6-sIL-6R complexes inhibited the cleavage of ULVWF strings similarly as that of IL-6 alone. IL-6 also inhibited cleavage of ULVWF strings in whole blood. (B) Pretreatment of partially purified ADAMTS-13 with IL-6 inhibited the cleavage of ULVWF strings under flow by approximately 50%. Figures are
mean±SEM, Student $t$ test, n = 6, * p <0.01, compared to untreated PRP or purified ADAMTS-13).

**Figure 6. Time course of IL-6 inhibition on the cleavage of ULVWF strings.** The rates of cleavage of ULVWF strings by normal plasma, plasma pretreated with 100 ng/ml of IL-6, or plasma from patients with congenital TTP were measured by quantitating the numbers of ULVWF-platelet strings formed on HUVECs over a period of 15 min. Normal plasma completely cleaved and released ULVWF strings from endothelial cells within 2 min after the initiation of perfusion. Cleavage by plasma treated with IL-6 was significantly delayed with maximal cleavage achieved 15 min after perfusion initiation. TTP plasma failed to cleave ULVWF strings within 15 min of perfusion. Figures are mean±SEM, n = 4.

**Figure 7. IL-6 did not inhibit cleavage of ULVWF multimers under static conditions.** ULVWF multimers were incubated with normal plasma or plasma pretreated for 30 min at room temperature with IL-6, IL-8 or TNF-$\alpha$ in the presence of urea and barium. After a 24 hr incubation, plasma treated with each cytokine cleaved ULVWF substrate to the same extent as untreated plasma. The figure is a representative of 3 independent experiments.
FIGURES

Figure 1. IL-8 and TNF-α, but not IL-6, stimulated the release of ULVWF multimers.

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**Figure 2.**

**A**

![Graph showing IL-8 and TNF-α induced ULVWF release](image)

**B**

![Graph showing ULVWF strings on EC](image)

**Figure 2. IL-8 and TNF-α induced the release of ULVWF from HUVECs. (A)**

HUVECs were treated with various concentrations of cytokines for 30 min and then perfused with washed platelets suspended in Tyrode’s buffer at 2.5 dyn/cm². The ULVWF released from HUVECs stimulated by IL-8 or TNF-α had maximal effects at 100 and 10 ng/ml, respectively (mean±SEM, Student t test, n = 9). **(B)** HUVECs were treated with 50 ng/ml of TNF-α for up to 24 hrs before perfusion with washed platelets. The formation of ULVWF strings increased with incubation times, reaching plateau after 60 min (mean±SEM, n = 4).
**Figure 3.**

**A**  
Recombinant human IL-6 (100 ng/ml) was incubated with sIL-6R (80 ng/ml), and IL-6 binding to sIL-6R detected using a monoclonal IL-6 antibody. (A). sIL-6R bound to immobilized IL-6, but not BSA (mean±SEM, Student t test, n = 3, * p <0.001, as compared to plates coated BSA).  

**B**  
HUVECs were treated with preformed IL-6-sIL-6R complexes for 30 min and perfused with washed platelets at 2.5 dyn/cm² shear stress. The platelet-decorated ULVWF strings formed on the complex-stimulated HUVECs, but the number of strings was significantly less than those of histamine-, IL-8, and TNF-α-stimulated cells (mean±SEM, Student t test, n = 3, ** p <0.01 compared to stimulation with histamine).
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Figure 5. IL-6 inhibited the cleavage of ULVWF strings under different conditions.

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Effects of Inflammatory Cytokines on the Release and Cleavage of the Endothelial Cell-derived Ultra-large von Willebrand Factor Multimers under Flow

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