Abstract—Inflammatory cytokines alter the hemostatic balance of endothelial cells (ECs). Alternatively spliced human tissue factor (asHTF), a soluble isoform of tissue factor (TF), has recently been detected in ECs, possibly contributing to procoagulability. Agonists regulating asHTF expression and release are yet unknown. This study examines the effect of TNF-α and IL-6 on the endothelial expression of both TF variants and delineates the impact of asHTF on the procoagulability of extracellular fluids. asHTF and TF mRNA were assessed by real-time PCR, and asHTF, TF, and tissue factor pathway inhibitor (TFPI) proteins by Western blot and/or fluorescence microscopy before and after stimulation with TNF-α (10 ng/mL) or IL-6 (10 ng/L). The procoagulability of cell supernatant was analyzed by a chromogenic assay with or without phospholipid vesicles. We found asHTF mRNA to be maximally increased 10 minutes after TNF-α and 40 minutes after IL-6 treatment (asHTF/GAPDH ratio 0.0223±0.0069 versus 0.0012±0.0006 for control, P<0.001 and 0.0022±0.0004 versus 0.0012±0.0007, P<0.05, respectively). Not only was asHTF increased, but also TFPI decreased after cytokine treatment. asHTF was found in the supernatant as early as 5 hours after TNF-α stimulation, supporting factor Xa generation after repiliation (6.55±1.13 U versus 2.99±0.59 U in control supernatant, P<0.0001). Removal of asHTF from supernatants by immunoprecipitation diminished its procoagulability to baseline. The soluble TF isoform expressed and released from ECs in response to inflammatory cytokines becomes procoagulant in the presence of phospholipids. Thus, asHTF released from ECs is a marker for and a contributor to imbalanced hemostasis. (Circ Res. 2005;96:0-0.)

Key Words: tissue factor ■ procoagulability ■ cytokines ■ inflammation ■ endothelial cells

The proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) orchestrate inflammatory responses within the vessel wall.1 They can cause severe endothelial dysfunction by induction of the angiotensin II type 1 receptor2 and C-reactive protein, and by direct inhibition of endothelial nitric oxide synthase.1 The plasma levels of TNF-α and IL-6 are significantly elevated in patients with severe coronary artery disease as well as with heart failure because of other causes.3 TNF-α and IL-6 are both noted for inducing the expression of procoagulant proteins such as tissue factor (TF) in cardiovascular cells.4–6 Among them are endothelial cells known to express not only full-length TF,7,8 the initiator of the extrinsic coagulation system,9,10 but also to release its inhibitor, tissue factor pathway inhibitor (TFPI),11 in response to TNF-α.12 Increased levels of TNF-α and IL-6 in plasma are associated with increased TF protein in blood.13 Alternatively spliced human tissue factor (asHTF) has recently been described to be a soluble isoform of TF, which is present in blood.14 Endothelial and epithelial cells as well as leukocytes were documented as possible sources for asHTF in extracellular fluids.14–16 The physiological role of asHTF is thus far not understood. It was suggested by Bogdanov et al that the soluble TF isoform may contribute to the thrombogenicity of extracellular fluids.14 The findings of others challenged this hypothesis that asHTF is procoagulant.17 Only 1 of the 2 TF variants, found in the plasma of patients with coronary artery disease, was shown to exhibit procoagulative properties.14 The microparticle (MP)-associated full-length TF supported thrombin generation but not the soluble TF isoform.18 Moreover, it is presently not known how the expression and release of the soluble TF isoform are regulated. In this study, we investigated whether the soluble TF isoform, asHTF, is expressed in and released from human endothelial cells in response to the inflammatory cytokines TNF-α and IL-6. Secondly, we examined the extent to which cytokine-induced asHTF contributes to extracellular procoagulability in proportion to TF-bearing MPs.

Materials and Methods

Primers and probes for quantitative real-time RT-PCR were synthesized by TibMolbiol (Berlin, Germany). TaqMan Universal Master Mix was purchased from Applied Biosystems (Darmstadt, Germany). Protease inhibitor cocktail, bovine brain phosphatidyl serine

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From the Department of Cardiology and Pneumology (B.S., S.A., W.P., H.-P.S., U.R.), Charité—University Medicine Berlin, Campus Benjamin Franklin, Berlin, Germany; and the Institute of Pharmacy (B.S.), Free University of Berlin, Germany.
Correspondence to Dr Ursula Rauch, Medical Clinic II, Charité—Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail ursula.rauch@charite.de
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(PS), egg yolk phosphatidyl choline (PC), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were obtained from Sigma-Aldrich (Munich, Germany). Human factor X and a monoclonal antibody against full-length tissue factor (clone TFE) were from Enzyme Research Laboratories (South Bend, Ind). CD62E-FITC antibodies (clone BBIG-E5, IgG1) were bought from R&D Systems (Wiesbaden, Germany) and control antibodies (clone DAK-G01, IgG1) from DakoCytomation (Hamburg, Germany). Protein G-plus agarose was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif). Recombinant factor VIIa (NovoSeven) was a gift from Novo Nordisk (Mainz, Germany). Spectrozyme FXa antibodies and antibodies against tissue factor pathway inhibitor (TFPI; clone 2H8) were purchased from American Diagnostica GmbH (Pfungstadt, Germany). Polyclonal asHTF antibodies were raised against the unique C-terminal asHTF domain (Pineda Antikörper-Service, Berlin, Germany). The specificity of these antibodies have been described before.15

Cell Culture
Human umbilical vein endothelial cells (HUVECs) and endothelial cell culture media were purchased from PromoCell (Heidelberg, Germany). HUVECs were maintained in endothelial cell growth medium (containing 5% fetal calf serum) in cell culture flasks at 37°C in a humidified incubator (5% CO2, 95% air). Cells were split at the ratio of 1:3 every passage. Cells from 3 to 6 passages were used in this study. For stimulation experiments, HUVECs were grown to confluence, serum-starved for 2 hours, washed in PBS, and afterward stimulated with TNF-α (10 ng/mL) or IL-6 (10 ng/mL) in endothelial basal medium for either 10, 40, 60, and 180 minutes for mRNA analysis or 3, 5, 8, and 18 hours for analysis of proteins.

TF Isoform Specific Real-Time PCR (TaqMan)
Real-time PCR using full-length TF, asHTF, and GAPDH specific primers and probes was performed as previously described.45

Western Blot
Cell lysates and supernatants of stimulated or unstimulated HUVECs were subjected to immunoprecipitation. Fifty μg of protein or 2 mL of supernatant were incubated overnight at 4°C with either a monoclonal antibody against TF or an anti-TFPI antibody and the appropriate detection either monoclonal anti-TF antibodies, polyclonal anti-TF antibodies, or anti-TFPI antibodies, and the appropriate horse radish peroxidase conjugated secondary antibodies were used.

Isolation of Cell-Derived Microparticles
Cell culture supernatants were collected at various time intervals after cytokine treatment and were then centrifuged for 10 minutes at 200g to remove whole cells and cell debris. MPs were isolated from the cell supernatant by ultracentrifugation at 17 000g for 30 minutes as previously described.19

Flow Cytometric Analysis
After MP isolation, cell supernatants were analyzed in a FACScan flow cytometer (Becton Dickinson) to confirm the supernatants to be free of MPs. MPs were identified on binding of FITC-conjugated monoclonal CD62E antibodies (1.5 μg/mL) as previously shown.92 Fluorescence thresholds for the monoclonal antibodies were set in terms of binding of isotype-matched control antibodies (1.5 μg/mL).

Measurement of Tissue Factor Activity in Stimulated and Unstimulated HUVECs, Cell Culture Supernatant, and Cell-Derived Microparticles
Stimulated or unstimulated HUVECs were washed twice with ice-cold PBS. Cells were incubated for 15 minutes at 37°C with 0.1 mol/L n-octyl-β-D-glycopyranosid in HEPES buffer (10 mmol/L HEPES, pH 7.5, 5 mmol/L CaCl2, 1 mg/mL BSA) (200 μL final volume). TF activity was measured by adding 100 μL of the sample to a solution containing 2 nM FVIIa, 150 nM factor X, and 5 mmol/L CaCl2. At intervals, samples were transferred to a microtiter plate, containing EDTA buffer (50 mmol/L. Bicine, pH 8.5, 100 mmol/L NaCl, 25 mmol/L EDTA, 1 mg/mL BSA), which terminated the generation of Xa. A chromogenic substrate (0.5 mmol/L final concentration) was added to each well. The increase in OD was measured at 405 nm for 30 minutes by using a kinetic ELISA plate reader at 37°C (Molecular Devices). TF activity units were assessed by a standard curve.

To differentiate between the procoagulant potential of the soluble TF isoform and the TF-bearing MP, the cell-derived MPs were isolated as described above. FACS analysis was performed to confirm the supernatants to be free of MPs. The MP-free supernatant was split in 2 parts. Immunoprecipitation with anti-asHTF antibodies was performed on one part of the samples as described above. After recalcification (5 mmol/L final concentration), TF activity of the supernatant before and after immunoprecipitation was measured in the presence of antibodies directed against TFPI (5 μg/mL). It was the aim to avoid possible interactions between TFPI, full-length TF, or asHTF. Phospholipid vesicles were prepared as described,20 and TF activity was measured in the supernatant with and without the addition of PC:PS (10 μmol/L PC:PS, 80:20). To exclude unspecific antibody binding, immunoprecipitation was also performed with rabbit IgG and TF activity measurement were performed thereafter.

MPs obtained by ultracentrifugation as described above were resuspended in HEPES buffer containing 0.1 mol/L n-octyl-β-D-glucopyranosid. Without addition of phospholipid vesicles, TF activity was then measured as described above.

Fluorescence Microscopy
HUVECs were grown on glass coverslips and stimulated as described above. Slides were washed in PBS and fixed (Merck). After a brief washing, cells were treated with PBS containing 2% BSA, 5% goat-serum, and 0.1% Triton X-100. Slides were then blocked with Avidin/Biotin Blocking Kit according to manufacturer’s protocol (Vector Laboratories). The washed slides were first incubated with an anti-asHTF antibody (3 μg/mL; 1:50) and, after washing procedures, with a monoclonal anti-TF antibody (1 mg/mL; 1:100) for 60 minutes at RT. Negative controls were performed using normal rabbit immunoglobulins instead of primary antibodies. For the detection of the primary antibodies, slides were incubated with appropriate biotinylated (for the detection of asHTF) and rhodamin-red–conjugated (for the detection of TF) secondary antibodies for 45 minutes at RT. StreptAvidin-conjugated MPP-488 (10 μg/mL; MoBiTec, Göttingen, Germany) and DAPI (1 μg/mL; Anaspec, San Jose, Calif) for nuclei staining, were also added. Slides were then mounted in MobiGlow (MoBiTec, Göttingen, Germany). Immunofluorescence micrographs were taken on an Olympus BX60 microscope and captured using analySIS software version 3.2.

Statistical Analysis
SPSS statistical software version 11.0.1 was used for statistical analysis. All data were expressed as mean±SD. Significant differences between mRNA ratios obtained by real-time PCR were determined by using the Student 2-tailed t test. Data obtained from activity measurements were analyzed by 1-way ANOVA and were Bonferroni-corrected for repeated measures over time. All experiments were performed at least 4 times and representative results are shown. A probability value ≤0.05 was regarded as significant.

Results
Effect of TNF-α and IL-6 on the Expression of TF and Its Isoform in HUVECs
Real time PCR was performed to investigate whether TNF-α and IL-6 altered mRNA expression levels of full length TF and its soluble isoform in endothelial cells. For the asHTF isoform, maximum mRNA levels were already reached 10
minutes after TNF-α stimulation (P<0.001; Figure 1A). Compared with baseline expression, asHTF mRNA increased \( \approx 45 \text{-fold.} \) AsHTF mRNA levels were still elevated 40 and 60 minutes after stimulation with TNF-α (P<0.001 [0 versus 40 minutes]; P<0.01 [0 versus 60 minutes]; Figure 1A). IL-6 treatment of HUVEC led to a small fold increase in asHTF mRNA levels 40 minutes after stimulation compared with baseline expression level (P<0.05; Figure 1B).

Full length TF mRNA was also increased on stimulation with TNF-α and IL-6. Compared with unstimulated controls, TF mRNA levels were still elevated 3 hours after stimulation (P<0.0005; Figure 1C). TNF-α as well as IL-6 induced maximal full-length TF mRNA expression 60 minutes after stimulation (for TNF-α, P<0.0005, Figure 1C; for IL-6, P<0.003, Figure 1D). However, the overall increase obtained by stimulation with IL-6 was less distinct compared with the one observed with TNF-α.

Detection of asHTF and TF Protein in Cells and Supernatants of Stimulated HUVECs

Compared with unstimulated controls (Figure 2C) stimulation with TNF-α (Figure 2D through 2F) as well as with IL-6 (Figure 2G through 2I) led to an increase in asHTF- and TF-associated immunofluorescence 5 hours after stimulation. Unstimulated cells stained only very faint for TF (Figure 2C). Staining of TF as well as asHTF was less increased after stimulation with IL-6 (Figure 2G through 2I) than TNF-α (Figure 2D through 2F).

Western blot analysis of cell lysates from TNF-α or IL-6 treated HUVECs revealed increased TF and asHTF protein levels compared with the unstimulated cells (Figure 3A, lane 3). HUVECs stimulated with TNF-α for 5 and 18 hours (Figure 3A, lanes 1 and 2) yielded intense signals for TF and asHTF compared with cells treated with IL-6 (Figure 3A, lanes 4 and 5).
The supernatants of stimulated HUVECs showed stronger bands for asHTF than for full length TF (Figure 3B), pointing to the presence of primarily asHTF in the supernatant. Only very faint bands for full length TF and asHTF protein were detected in the supernatants of unstimulated controls (Figure 3B, lane 1).

Redistribution of TFPI from Cells to Supernatant After Cytokine Stimulation

Stimulation with both TNF-α as well as IL-6 led to a depletion of TFPI in HUVECs. After cytokine stimulation TFPI was mainly present in the supernatant, but not within the cells. Figure 4A depicts the reduction in cellular TFPI after stimulation of HUVECs with TNF-α and IL-6. In the supernatant (Figure 4B) TFPI levels were increased on stimulation, whereas cell-associated TFPI was reduced (Figure 4A). Fluorescence microscopy (Figure 5) confirmed the results obtained from Western blotting (Figure 4). A reduction in cellular TFPI was observed on stimulation with TNF-α as well as with IL-6 (Figure 5E and 5H). In contrast to the reduction in cellular TFPI, TNF-α and IL-6 induced TF expression in HUVECs (Figure 5D and 5G) pointing to cytokines to influence the hemostatic balance of endothelial cells.

Effect of TNF-α and IL-6 on Cellular Procoagulability of HUVECs

A chromogenic assay was used to assess whether TF expression after cytokine stimulation was associated with an increased procoagulability of cells. Stimulation with TNF-α led to an ∼17-fold rise in cellular TF activity 3 hours after stimulation compared with unstimulated HUVECs (258.8±43.7 U versus 4295.3±376.5 U, P<0.0002; Figure 6A). Cell-associated TF activity was still elevated 18 hours after stimulation with TNF-α. IL-6 treatment also increased the procoagulant activity of HUVECs from 258.8±43.7 U at baseline to 1175.1±283.9 U 5 hours after stimulation (P<0.0003; Figure 6B). Overall, the increase in procoagulant TF activity induced by stimulation with TNF-α was more distinct than by stimulation with IL-6.

Role of Phospholipids and TFPI for the Procoagulability of the Supernatant

Cell culture supernatants of unstimulated and stimulated HUVECs were cleared from cell debris and phospholipid-rich MPs (Figure 7). MP-free supernatant was used to measure the TF activity. No difference in TF activity was detected in the supernatant of stimulated and unstimulated HUVECs (0.694±0.221 U in control supernatant versus 0.768±0.234 U in supernatant of stimulated HUVEC, ns). Because TF procoagulant activity depends on phospholipids to form an active complex with factor VII/VIIa, phosphatidyl choline/ phosphatidyl serine vesicles (PC:PS, 80:20) were added to the supernatants.

In the presence of phospholipid vesicles TF activity in the MP-free supernatant was increased ∼2.2-fold 5 hours after stimulation with TNF-α (2.99±0.59 U in control supernatant versus 6.55±1.13 U in supernatant of stimulated HUVECs, P<0.00001; Figure 6D). 18 hours after TNF-α treatment TF activity was still increased in the supernatant compared with the corresponding supernatant of unstimulated cells (5.17±0.14 U versus 2.95±0.36 U in control supernatant, P<0.01; Figure 6D). In contrast, supernatants of IL-6–treated HUVECs did not show significant differences in TF activity at any time point, although phospholipids were added (Figure 6E).

All experiments described above were performed in the presence of anti-TFPI antibodies to avoid interactions between TFPI, full-length TF, or asHTF. With anti-TFPI antibodies present, TF activity in the supernatant was ∼16% higher than without anti-TFPI antibodies (Figure 6C).

Impact of asHTF on the Procoagulability Present in the Microparticle-Free Supernatant of HUVECs

To distinguish whether the soluble asHTF isofom or full-length TF contributed the procoagulability of the supernatant, immunoprecipitation with asHTF specific antibodies was performed to remove the soluble asHTF protein from the supernatant. Removal of asHTF from the supernatant by immunoprecipitation was confirmed by Western blotting (data not shown). After removal of asHTF, TF activity was
reduced to levels comparable with those supernatants obtained from unstimulated HUVECs, pointing to asHTF to be procoagulant in the MP-free supernatant. No significant difference in TF activity was found between the supernatants from controls before and after immunoprecipitation (Figure 6D and 6E). To exclude unspecific binding of the polyclonal asHTF antibodies, rabbit IgG instead of anti-asHTF antibodies was used for immunoprecipitation. Treatment of supernatants with rabbit IgG did not result in removal of asHTF from the supernatant or in a reduction of TF activity (data not shown).

Figure 5. Cytokine-induced TF expression and TFPI reduction/secretion in HUVECs. Fluorescent images of cellular TF (green), TFPI (red), and the nucleus (blue) in HUVECs before (A through C) and after 5 hour TNF-α (D through F) or IL-6 (G through I) stimulation. C, F, and I comprise the corresponding TF and TFPI images combined. Original magnification 1000×.

Figure 6. Procoagulant activity of cells (A and B), cell supernatant (C through E), and MPs (F) in response to stimulation with TNF-α (A, C, D, and F) and IL-6 (B and E). *P<0.00001, †P<0.00002, ‡P<0.00004, §P<0.001, ¶P<0.002, ‡P<0.003, †P<0.01, ¥P<0.03, and ¥P<0.05 TF activity of unstimulated (solid-filled bar) and cytokine-stimulated (open bar) HUVECs at different points in time (A for TNF-α, B for IL-6). Influence of TFPI-antibodies (solid-filled bars: with antibodies; open bars: without antibodies) on the TF activity of TNF-α-stimulated HUVECs (C). TF activity of cell culture supernatants obtained from unstimulated (solid-filled bars) and cytokine stimulated (checked bars) HUVECs and their corresponding asHTF immunoprecipitated samples (open bars: unstimulated control supernatant; spotted bars: cytokine stimulated) at different points in time (D for TNF-α, E for IL-6). TF activity of MPs shed from unstimulated (solid-filled bars) and TNF-α-stimulated (open bars) HUVEC at different time points (F).
Comparison of asHTF-Associated and Microparticle-Associated Procoagulability of Extracellular Fluids

To assess the impact of TF-exposing MPs on the procoagulability of extracellular fluids in comparison with soluble TF, TF activity measurements were performed on the previously isolated MPs. MP-associated TF activity significantly increased from 7.18 ± 0.30 U 5 hours after stimulation with TNF-α to finally 16.86 ± 1.93 U 18 hours after stimulation compared with the unstimulated control sample (P < 0.0004; Figure 6F). In contrast, the asHTF-associated procoagulant activity was highest 5 hours after stimulation with TNF-α and continuously decreased over time. The ratio of the procoagulant activity of TF-bearing MPs relative to that of asHTF increased over time, pointing to asHTF to be an early marker for a dysregulated endothelial hemostasis.

Discussion

This study demonstrated the proinflammatory cytokines TNF-α and IL-6 to induce the soluble TF isoform in HUVECs. AsHTF was released from endothelial cells into the supernatant, increasing its procoagulability in the presence of phospholipids. The soluble TF isoform contributed initially more to the procoagulability of extracellular fluids than TF-bearing MPs.

Known to strongly induce full-length TF in endothelial cells, TNF-α was also revealed to be a potent inducer of asHTF expression. AsHTF expression was found to be increased 45-fold as early as 10 minutes after TNF-α treatment, subsequently followed by an early release of asHTF into extracellular space. In contrast to asHTF, full-length TF expression reached its maximal level 1 hour after stimulation with TNF-α. TNF-α was recently shown to induce the activation of the p38 mitogen-activated protein kinase (MAPK) pathway as early as 5 minutes after stimulation in endothelial cells. The p38 MAPK pathway was reported to influence pre-mRNA splicing on activation. p38-induced relocalization of a splicing factor, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), from the nucleus into the cytoplasm increases the nuclear ratio of SF2/ASF, a general splicing factor, relative to hnRNP A1. Modifications of this ratio, a major determinant of splice site selection, are very likely to induce rapid changes in pre-mRNA processing programs.

In contrast to TNF-α, stimulation with IL-6 resulted in an endothelial asHTF expression 1 order of magnitude lower than that obtained by TNF-α treatment of HUVECs. IL-6 itself can be released from endothelial cells via activation of the protease-activated receptor (PAR)-1. If stimulation with IL-6 led to a major asHTF and full-length TF expression in endothelial cells, factor Xa and thrombin would further activate endothelial PAR-1, subsequently leading to an overexpression of IL-6 and TF variants. To avoid an overexpression of thrombogenic proteins, TF in endothelial cells may not be as inducible by IL-6 as by TNF-α. In contrast, TNF-α is not known to be released from endothelial cells. asHTF is rapidly and strongly expressed by and released from endothelial cells in response to TNF-α, whereas full-length TF may still be encrypted within the cells. Thus, asHTF in extracellular fluids may serve as an early marker indicating upcoming thrombotic events.

Our results revealed asHTF to support factor Xa generation in the presence of factor VIIa and externally added phospholipid vesicles. After stimulation of HUVECs with IL-6, no TF activity was measurable in the supernatant despite repletion. This can be explained by the differential effect of inflammatory cytokines on endothelial asHTF expression and release, which is less distinct after stimulation with IL-6 than with TNF-α (Figure 1A and 1B). In accordance with others, we found TNF-α and IL-6 to release cell-associated TFPI protein from endothelial cells into the supernatant, thereby opposing TF activity in the extracellular space. Thus, proinflammatory cytokines differentially induced TF and decreased TFPI expression, thereby changing the hemostatic balance of endothelium. As shown here, TFPI was also able to diminish the procoagulant activity of the soluble TF isoform in the MP-free supernatant. To assess the procoagulant potential of asHTF, most experiments were performed in the presence of antibodies directed against TFPI. However, we cannot exclude that asHTF may indirectly affect the procoagulant activity by interacting with TFPI. The formation of an asHTF–TFPI complex via factor VIIa/VIIIa in vivo may be conceivable, indirectly leading to an increased procoagulability by opposing the inhibitory effect of TFPI on MP-associated full-length TF. With respect to thrombogenesis, circulating TF-exposing MPs are known to adhere to platelets in thrombi, indicating full-length TF to potentiate coagulation reactions in close proximity of thrombi. We observed in our in vitro experiments the ratio of TF-bearing MPs relative to the soluble TF isoform to increase over time on stimulation with TNF-α. The impact of the soluble TF isoform on the procoagulability may therefore be prominent only for a limited time at an early state of inflammation and will decrease in favor of the increasing number of TF-bearing MPs in the further course of the inflammatory process.

In conclusion, we showed asHTF to be present in the supernatant after stimulation of endothelial cells with TNF-α and IL-6. asHTF, released in response to TNF-α, supported factor Xa generation in the presence of phospholipids, pointing to the prothrombotic potential of soluble TF. Thus, the
soluble TF isoform is not only a marker for dysregulated hemostasis but also an early contributor to this imbalance.

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References

Procoagulant Soluble Tissue Factor Is Released From Endothelial Cells in Response to Inflammatory Cytokines

Björn Szotowski, Silvio Antoniak, Wolfgang Poller, Heinz-Peter Schultheiss and Ursula Rauch

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