Sepsis-Induced Coagulation in the Baboon Lung Is Associated with Decreased Tissue Factor Pathway Inhibitor

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Increased tissue factor (TF)-dependent procoagulant activity in sepsis may be partly due to decreased expression or function of tissue factor pathway inhibitor (TFPI). To test this hypothesis, baboons were infused with live *Escherichia coli* and sacrificed after 2, 8, or 24 hours. Confocal and electron microscopy revealed increased leukocyte infiltration and fibrin deposition in the intravascular and interstitial compartments. Large amounts of TF were detected by immunostaining in leukocytes and platelet-rich microthrombi. TF induction was documented by quantitative reverse transcriptase-polymerase chain reaction, enzyme-linked immunosorbent assay, and coagulation assays. Lung-associated TFPI antigen and mRNA decreased during sepsis, and TFPI activity diminished abruptly at 2 hours. Blocking antibodies against TFPI increased fibrin deposition in septic baboon lungs, suggesting that TF-dependent coagulation might be aggravated by reduced endothelial TFPI. Decreased TFPI activity coincided with the release of tissue plasminogen activator and the peak of plasmin generation, suggesting that TFPI could undergo proteolytic inactivation by plasmin. Enhanced plasmin produced in septic baboons by infusion of blocking antibodies against plasminogen activator inhibitor-1 led to decreased lung-associated TFPI and unforeseen massive fibrin deposition. We conclude that activation of TF-driven coagulation not adequately countered by TFPI may underlie the widespread thrombotic complica-

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Sepsis is a serious medical condition caused by a severe infection leading to a systemic response syndrome that includes widespread activation of inflammation and coagulation and may progress to dysfunction of the circulatory system, acute respiratory distress syndrome, and onset of multiple organ dysfunction,^{1,2} which are leading causes of morbidity and mortality in sepsis.³

Although the pathogenesis of septic acute respiratory distress syndrome is not precisely understood, it is well accepted that inflammation, coagulation, and apoptosis are intimately linked in sepsis.⁴ Activation of tissue factor (TF)-dependent coagulation leads to formation of thrombin and subsequent deposition of fibrin.^{5,6}

Tissue factor pathway inhibitor (TFPI) is the main inhibitor of the serine proteases involved in the TF-driven pathway *in vivo*. Two forms of TFPI are produced through alternative mRNA splicing. TFPI- α contains three Kunitz-type domains,⁷ whereas TFPI- β has the Kunitz-3 domain and C terminus of TFPI- α replaced with an unrelated C-terminal domain directly attached to the membrane via a glycosyl phosphatidylinositol anchor.^{8,9} In the mouse, TFPI- β mRNA has a similar tissue distribution as TFPI- α mRNA, but the encoded TFPI- β protein has yet to be detected.⁸ TFPI can be cleaved and inactivated by plasmin¹⁰ or by neutrophil elastase.¹¹

Although heightened TF-induced coagulation is a consistent finding in disseminated intravascular coagulation and multiple organ dysfunction associated with sepsis, there are controversial reports on the changes of TFPI in plasma during sepsis, ranging from increased^{12–14} to decreased^{15,16} or

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unchanged levels.¹⁷ Moreover, despite being widely accepted that the endothelium is the most important source of TFPI *in vivo*,^{18,19} the information concerning the role and dynamics of *Escherichia coli* (EC)-associated TFPI during sepsis is still scant. This renders the pathophysiological role of TFPI in sepsis elusive.

Because endothelial dysfunction plays a key role in the pathogenesis of sepsis²⁰ and because the lung is rich in microvessels and expresses large amounts of TFPI,²¹ we examined the time course changes of TF and TFPI in the lung and plasma of baboons challenged with *E. coli*. Our specific objective was to determine the role of TFPI in the pathophysiology of sepsis and the time frame in which the balance between the TF-dependent procoagulant and the TFPI anticoagulant activities are impaired in the baboon lung during sepsis. Our results revealed that i) TFPI immunodepletion leads to increased fibrin deposition in the lung; ii) bacterial infusion leads to a large drop in TFPI activity after 2 hours, which also coincides with the maximum release of tissue plasminogen activator (t-PA) from EC and the peak of plasmin generation; and iii) augmented plasmin generation achieved through inhibition of plasminogen activator inhibitor-1 (PAI-1) associates with decreased TFPI antigen and activity and increased fibrin deposition in the lung. We suggest that plasmin-dependent proteolysis of TFPI may be partly responsible for the detected loss of TFPI function. Our study analyzed, for the first time, the TFPI functional activity, antigen, and mRNA levels during the initial phases of sepsis.

Materials and Methods

Reagents

Antibodies and suppliers used were as follows: monoclonal antibody (mAb) against Kunitz 3 domain of human recombinant TFPI (r-TFPI) and full-length human r-TFPI expressed in Chinese Hamster Ovary cells²² [gifts from Dr. T. Hamuro (KAKETSUKEN, Kumamoto, Japan)]; rabbit anti-human r-TFPI₁₋₂₄₉ IgG and mAbs against human full-length r-TFPI (raised and characterized in-house); mAb 10H10 anti-human TF [gift from Dr. J. Morrissey (University of Illinois at Urbana-Champaign, Urbana, IL)]; rabbit anti-human TF IgG [gift from Dr. W. Ruf (Scripps Research Institute, La Jolla, CA)]; mAb anti-human t-PA and rabbit anti-hirudin IgG (American Diagnostica Inc., Greenwich, CT); rabbit anti-human neutrophil elastase (Calbiochem, San Diego, CA); rabbit anti-human plasmin- α_2 antiplasmin complex (Boehringer, Mannheim, Germany); mAb anti-CD68, mAb anti-GPIIb-IIIa and rabbit anti-human myeloperoxidase (DakoCytomation, Carpinteria, CA); antibodies anti-human t-PA [gift from R.H. Lijnen (University of Leuven, Leuven, Belgium)]; and peroxidase- or fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Human coagulation factors VIIa, X, and Xa were from Enzyme Research Laboratories (South Bend, IN). Protein G-purified mAb anti-human PAI-1 (CLB-2C8) has been described previously.²³ Chromogenic substrate S-2765 for factor Xa (FXa) was purchased from DiaPharma

(Westchester, OH). Human r-TF (Innovin, 0.22 μ g/vial) was from Dade Behring (Deerfield, IL). The primers were synthesized by the core facility of the University of Oklahoma Health Science Center. Triton X-100, pepstatin A, leupeptin, pefabloc SC, calpain inhibitor I, calpain inhibitor II, aprotinin, benzamidine, sodium *o*-vanadate, 1,10-phenanthroline, *o*-phenylenediamine, *n*-octyl β -D-glucopyranoside, native protein deglycosylation kit, and normal goat serum were obtained from Sigma Chemical (St. Louis, MO). *p*-Aminodiphenylmethylsulfonyl fluoride was from Calbiochem. Bovine serum albumin was obtained from Equitech-Bio (Kerrville, TX). *E. coli* organisms (serotype B7-086a:K61; American Type Culture Collection, Rockville, MD), stored in the lyophilized state at 4°C after growth in tryptic soybean agar, were reconstituted and used as described previously.²⁴ To eliminate differences due to *E. coli* strain variations, all animals were infused with *E. coli* from this single isolate.

Experimental Procedures

The study protocol received prior approval by the Institutional Animal Care and Use Committees of both Oklahoma Medical Research Foundation and the University of Oklahoma Health Science Center. *Papio cyanocephalus* baboons were held for 30 days at the University of Oklahoma Health Science Center animal facility, and only animals with a negative blood culture were included in the study. Two experimental *E. coli* groups were studied. One group of 13 animals was infused with live *E. coli*, using mostly sublethal doses (Supplemental Table 1 at <http://ajp.amjpathol.org>) as described previously.²⁴ The time point at which the infusion was started is further indicated as T₊₀, a time point of *n* hours thereafter referred to as T_{+n} hours. Time points before the start of the challenge are indicated as T_{-n} hours. Three animals per time point were sacrificed at T₊₂, T₊₈, and T₊₂₄ hours after infusion. A subgroup of *E. coli*-treated animals received inhibitory antibodies anti-TFPI or anti-PAI-1. Two animals received 5 mg/kg mAb anti-human TFPI administered intravenously 10 minutes before the start of the *E. coli* challenge, followed by a second injection with the same amount at T₊₆ hours after *E. coli* infusion, and the animals were sacrificed at T₊₂₄ hours. Another two animals were injected with mAb anti-human PAI-1 (2C8) at T₋₃₀ minutes before *E. coli* challenge. The control group comprising three animals received saline infusion only. Lung tissue samples were snap frozen in liquid nitrogen and stored at -80°C.

Preparation of Lung Homogenates

Lung tissue was homogenized on ice with 1% Triton X-100 and 60 mmol/L *n*-octyl β -D-glucopyranoside in 50 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 8.5, containing protease inhibitors (1.5 μ mol/L pepstatin A, 10.5 μ mol/L leupeptin, 0.25 mmol/L pefabloc SC, 20.9 μ mol/L calpain inhibitor I, 20 μ mol/L calpain inhibitor II, 1.5 μ mol/L aprotinin, and 1 mmol/L each of benzamidine, sodium *o*-vanadate, and 1,10-phenanthroline). The ex-

Table 1. Sequences Primers for TF, TFPI- α , TFPI- β , and β -Actin

Name	Forward primers	Reverse primers
TF	5'-TGCTTTTACACAGCAGACACAGAGT-3'	5'-AAGACCCGTGCCAAGTACGT-3'
TFPI- α	5'-TGAGGGCATGTAAAAAGGTTTC-3'	5'-TTCACTCTCTGCTTCTTTCTTTTCTT-3'
TFPI- β	5'-TGGAAGAATGCGGCTCATATT-3'	5'-TGCTATCCAATCCTAGAAAGACATG-3'
β -Actin	5'-TGGAAGAATGCGGCTCATATT-3'	5'-TGCTATCCAATCCTAGAAAGACATG-3'

tracts were centrifuged at $14,500 \times g$ for 15 minutes, and the supernatants, representing the lung lysates, were stored at -80°C .

TFPI Antigen and Anticoagulant Activity Assays

For TFPI antigen measurement in the lung extracts, we developed a sandwich-type enzyme-linked immunosorbent assay (ELISA), using a cocktail of mAbs against r-TFPI as capturing layer and the rabbit anti-human TFPI IgG for detection. The concentration of TFPI was extrapolated from a standard curve made of serial dilutions of human full-length r-TFPI.

For the TFPI activity assay, homogenates were dialyzed overnight against 50 mmol/L Tris-HCl buffer, pH 7.4, to remove the detergents. Next, *p*-amidinophenylmethylsulfonyl fluoride (2.7 mmol/L final concentration, pH 5.0) was added to inhibit endogenous serine proteinase activities. TFPI activity was then measured, by evaluating the ability of TFPI to inhibit the activation of FX by TF-FVIIa. In selected experiments, TFPI activity was measured on tissue cryosections.²⁵ TFPI activity was extrapolated from a standard curve constructed with serial dilutions of human full-length r-TFPI.

TF Antigen and Activity Assays

TF antigen was determined by ELISA using a matched-pair antibody set from Affinity Biological Inc. (Ancaster, ON, Canada). TF activity was measured through the ability of TF in lysates to shorten the clotting time of plasma. Briefly, tissue homogenates were dialyzed to remove the detergents as above, and a clotting time assay was performed using a STArt 4 coagulometer (Diagnostics Stago, Asnieres, France) after adding normal pooled citrated plasma and 25 mmol/L CaCl_2 . TF activity of the samples was determined by reference to a standard curve made with known amounts of recombinant TF (Innovin; Dade Behring, Marburg, Germany).

Bleeding Time

To determine the time required for the bleeding to stop in the animals treated with anti-TFPI antibodies, a lancet was used to make a standardized 3-mm stab wound on the skin of the forearm. A stopwatch was started immediately on skin incision and blood drops were removed every 15 seconds with the use of a paper filter. If bleeding did not recur within 30 seconds of cessation, it was considered stopped. Bleeding time was measured at T_0 and then every 30 minutes, until 2 hours after treatment with the anti-TFPI antibody.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Lung lysates treated or not with the deglycosydases contained in the E-DEGLY kit according to Piro and Broze²⁶ were subjected to nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE MES 4 to 12% polyacrylamide gradient gels; Invitrogen, Carlsbad, CA), and TFPI was detected by Western blotting using rabbit anti-TFPI antibodies, as described previously.²⁷

Quantitative Reverse Transcriptase-Polymerase Chain Reaction-Based Gene Expression Analysis

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine the relative amount of TF, TFPI α , TFPI β , and β -actin mRNA in the lung. Primers were designed by using Primer Express software (Applied Biosystems, Foster City, CA). The sequences of the primers are listed in Table 1.

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer. For each sample, 2 μg of total RNA was reverse-transcribed by using the Omniscript Reverse Transcription System (Qiagen, Valencia, CA) with oligo-dT primers. Real-time PCR was performed in duplicate with 1 μl of the 20- μl RT reaction products by using iTaq SYBR Green Supermix with ROX kit (Bio-Rad, Hercules, CA) in an ABI Prism 5700 sequence detector (Applied Biosystems). Relative quantification of gene expression with the standard curve method was performed following the manufacturer's protocol (Applied Biosystems). The relative expression of target genes was normalized with β -actin mRNA level as housekeeping gene.

Morphological Analysis

For immunofluorescence, tissues were fixed in 4% paraformaldehyde, washed with phosphate-buffered saline containing 20% sucrose, embedded in ornithine carbamoyltransferase, snap-frozen, and stored at -80°C .

Staining with hematoxylin and eosin, double immunolabeling for TFPI or TF and cell markers (CD68 for macrophages, myeloperoxidase or elastase for neutrophils, and gpIIb-IIIa for activated platelets) and fibrin were performed as described previously.²⁵ As negative control for polyclonal antibody staining, the primary antibodies were replaced with equivalent amount of rabbit nonimmune serum. mAb anti-digoxigenin (IgG1; Roche Diagnostics, Indianapolis, IN), a hapten antigen that occurs only in

plants, was used as isotype-matched control for mAb staining.²⁵

Specimens were examined by epifluorescence confocal imaging using a Nikon C1 confocal microscope (Nikon USA, Melville, NY). The measurement of fluorescence intensity was done as previously described.²⁵ In brief, 10 to 15 single-channel grayscale images (12-bit, 4095 gray levels/pixel) were collected for each experimental condition, and the average fluorescence intensity of each whole image was integrated using the EZ-C1 software (Nikon). Image collection parameters (neutral density filters, pinhole, and detector gains) were kept constant during image acquisition, to make reliable comparisons between specimens.

Statistical Analysis

For statistical analyses, we used InStat (GraphPad Software, Inc., San Diego, CA). Values are given as mean $\pm$ SEM. The differences between *E. coli*-challenged groups were compared against the control by one-way analysis of variance, followed by single comparison with control by using Dunnett test. Differences were considered as significant when $P < 0.05$. All experiments were performed in duplicate.

Results

Clinical Evidence of Sepsis after *E. coli* Challenge

The main clinical and hematological parameters of the animals before *E. coli* challenge (T_0) and at the time of sacrifice (T_s) are summarized in Supplemental Table 1 (available at <http://ajp.amjpathol.org>). The challenge led to progressive systemic hypotension, paralleled by increased heart rate (tachycardia) and respiratory rate (tachypnea).

Alterations of the hemostatic and hematological parameters include variable decrease in fibrinogen levels, slight prolongation of the activated partial thromboplastin time, and increased levels of fibrin-degradation products, which reached maximum levels at T_{+24} hours. White blood cell counts dropped at T_{+2} and T_{+8} hours and recovered or increased at T_{+24} hours. Platelet counts gradually decreased and reached the lowest level at T_{+24} hours.

Sepsis Induces Structural Changes in the Lung

Morphological evaluation of lungs was performed by histology and electron microscopy. The lung structure changed gradually and reached the most extensive alterations at T_{+24} hours. In contrast to the normal architecture of the alveolar septae in healthy baboons (Figure 1, a, c, and d), the lungs of *E. coli*-challenged animals showed thickened septae (Figure 1, b, e, and f) and marked accumulation of inflammatory cells (especially

neutrophils and monocytes) within the microvasculature (Figure 1e).

Patchy edema due to capillary leakage into the alveolar space and accumulation of eosinophilic proteinaceous material in the alveolae were observed at T_{+24} hours (Figure 1b), especially in the animals with poor clinical prognosis. The same animals also displayed inflammatory cells and hemorrhage in the alveolar space (Figure 1f).

Quantitative Analysis of Neutrophil and Macrophage Accumulation in the Lung

Quantification performed on lung cryosections after staining for neutrophil elastase and CD68 revealed that both neutrophils (Figure 1, g and h) and macrophages (Figure 1, i and j) gradually accumulated in the lung, attaining maximum numbers at T_{+24} hours.

TF-Dependent Coagulation Increases in the Lung of Septic Baboons

Cell type-specific localization of TF was investigated by double immunofluorescence labeling for TF and cell markers. In septic baboon lungs, TF was mainly detected in macrophages (Figure 2a) and EC (Figure 2, a and d) and to a lesser extent in neutrophils (Figure 2b).

Strong TF staining was associated with intravascular microthrombi (Figure 2, c and d), especially colocalizing with activated platelets, as shown by staining with anti-gpIIb-IIIa IgG (Figure 2c). The activation of coagulation in the lung parenchyma led to local generation of thrombin, revealed through binding of its specific inhibitor hirudin, followed by immunodetection with rabbit anti-hirudin IgG (Figure 2, e–g). The presence of intravascular and/or interstitial fibrin deposits was detected by immunostaining with fibrin-specific antibodies, by phosphotungstic acid staining (Figure 2h), or by electron microscopy (Figure 2i).

TF mRNA expression was increased by 4.5-fold over controls at T_{+2} hours ($P < 0.01$) and stayed slightly elevated at T_{+8} and T_{+24} hours (Figure 3a). The levels of both TF antigen and activity were significantly higher than in controls at T_{+8} ($P < 0.05$) and T_{+24} hours ($P < 0.01$) (Figure 3, b and c). The specificity of the activity assay was confirmed through incubation of the samples with inhibitory antibodies, which produced almost total inhibition of TF clotting activity (Figure 3c).

TFPI Inhibition with Blocking Antibodies Increased Fibrin Deposition in the Lung

The baboons treated with blocking mAb anti-human TFPI plus *E. coli* survived the first 24 hours after challenge and were sacrificed for tissue analysis. The treatment resulted in massive intravascular deposition of fibrin (Figure 4a), compared with the animals treated with *E. coli* alone (Figure 4b). No fibrin staining was detected in nonchallenged animals (Figure 4c). Isotype-matched control im-

Figure 1. Morphological analysis and dynamics of inflammatory cell accumulation in the lung of *E. coli*-challenged baboons. **a** and **b**: Light microscopy of the lung in control (**a**) and septic (**b**; 24 hours) baboons. The lung from a healthy baboon shows normal morphology, whereas the organ from a septic animal displays massive interstitial edema, intra-alveolar accumulation of eosin-stained proteinaceous material (asterisk), and inflammatory cell infiltrates within the intra- and extravascular compartments. **c–f**: Electron microscopy images reveal that, in contrast to normal ultrastructure of the lung from control animals (**c** and **d**), *E. coli*-challenged baboons (**e** and **f**) showed massive accumulation of leukocytes (predominantly neutrophils) in the microvasculature (**e**). The alveolar spaces collapsed, and the alveolar walls are thick and rich in interstitial fibroblasts, extracellular matrix, and inflammatory cells (**f**, M). **g–j**: Immunofluorescence detection of neutrophils (**g**, red, rabbit anti-elastase IgG/donkey anti-rabbit IgG-Cy3) and monocyte/macrophages (**i**, green, mAb anti-CD68/donkey anti-mouse IgG-fluorescein isothiocyanate). Nuclei (N) were counterstained with TOPRO-3 (blue). **h** and **j**: Histograms showing the time course of neutrophils and macrophages accumulation in the lung during sepsis. Twelve microscopic fields per time point were counted, and data were analyzed by analysis of variance (* $P < 0.05$; ** $P < 0.01$). av, alveolae; c, capillaries; EC, endothelial cells; M, macrophages; mv, microvessels; PMN, neutrophils; rbc, red blood cells. Magnification bars: 100 μ m (**a**, **b**, **g**, and **i**); 10 μ m (**c**, **d**, **e**, and **f**).

Figure 2. TF expression, thrombin generation, and fibrin deposition in the baboon lung at 24 hours after *E. coli* challenge. **a–c:** Double immunostaining for TF (red, rabbit anti-human TF or mAb 10H10 anti-TF, followed by the appropriate donkey secondary IgG conjugated with Cy₃) and cell markers (green, donkey secondary IgGs conjugated with fluorescein isothiocyanate): mAb anti-CD68 for monocyte/macrophages (**a**), rabbit anti-myeloperoxidase IgG for neutrophils (**b**), and mAb anti-gpIIb/IIIa for platelets (**c**). Colocalization of the red and green fluorophores is shown in yellow (**arrows**). **d:** TF immunostaining (red, as for **a**) of an intravascular microthrombus (**asterisk**) and of the vascular endothelium (**arrow**). **e–g:** Double immunostaining for hirudin, used as thrombin probe (**arrows**, green, rabbit anti-hirudin IgG/donkey anti-rabbit IgG-fluorescein isothiocyanate) and TF (**arrows**, red, mAb 10H10 as above). Colocalization of thrombin and TF appears as yellow (**g, arrows**). For **a–g**, TOPRO-3 nuclear counterstaining is shown in blue. **h** and **i:** Phosphotungstic acid-hematoxylin (PHTA) staining (**h**) and transmission electron microscopy (EM) (**i**) show the presence of intravascular and interstitial fibrin deposits in the lung (**h: asterisks**, dark blue; **i: F**, black staining). av, alveolae; coll, collagen; EC, endothelial cells; MPO, myeloperoxidase; mv, microvessels. Magnification bars: 100 μ m (**a–h**); 10 μ m (**i**).

Figure 3. Analysis of the expression and activity of TF in lung extracts from control or *E. coli*-challenged baboons. **a:** TF mRNA levels determined by real-time RT-PCR. **b:** TF antigen assayed by ELISA. **c:** TF activity measured through clotting time assay. * $P < 0.05$; ** $P < 0.01$.

munostaining (mAb anti-digoxigenin, IgG₁) was negative (Figure 4d).

Quantitative analysis of TF immunostaining revealed significant increase of TF in the lung of the baboons treated with anti-TFPI plus *E. coli* versus *E. coli* alone or controls (Figure 5). TFPI activity in lung homogenates was almost completely abolished by the treatment with TFPI blocking antibody: 3.06 ± 1.26 ng/mg total protein ($n = 2$ animals; each in duplicate) compared with 59.4 ± 5.36 ng/mg ($n = 3$ animals, each in duplicate) in controls and 39.7 ± 11.37 ng/mg ($n = 3$ animals, each in duplicate) in animals challenged with *E. coli* only. In addition,

the animals receiving the blocking mAb exhibited consumption of fibrinogen and decreased platelet count at T_{+8} hours compared with T_0 (Supplemental Table 1 at <http://ajp.amjpathol.org>). Bleeding time was reduced to zero during the first hours after treatment with anti-TFPI antibody (0 from T_{+30} minutes to T_{+2} hours versus 2.5 ± 0.5 minutes at T_0), suggesting full abrogation of TFPI function, and was increased to more than 9 minutes at T_{+24} hours, probably reflecting the decreased platelet counts.

TFPI Expression and Activity Are Decreased in the Lung of Septic Baboons

Immunofluorescence labeling showed large amounts of TFPI in the normal baboon lung (Supplemental Figure 1a at <http://ajp.amjpathol.org>). In contrast, TFPI in EC baboon lungs was considerably decreased (Supplemental Figure 1b at <http://ajp.amjpathol.org>), showing and approximately threefold decrease of TFPI fluorescence intensity at T_{+2} hours (Figure 6a). This coincided with a concurrent decrease in t-PA immunostaining (Supplemental Figure 1, c and d, at <http://ajp.amjpathol.org>; Figure 6b) and was paralleled by a peak of plasmin generation, as identified by immunostaining with an antibody directed to a neo-epitope of plasmin- α_2 antiplasmin (Supplemental Figure 1, e and f, at <http://ajp.amjpathol.org>; Figure 6c).

TFPI- α mRNA levels were decreased at T_{+2} and T_{+8} hours and reached a significant difference ($P < 0.05$) at T_{+24} hours (Figure 7a). Although expressed at lower

Figure 4. Immunostaining for fibrin in lungs of baboons challenged with *E. coli* plus mAb anti-TFPI or mAb anti-PAI1, *E. coli* alone, and nonchallenged controls. **a–d:** The lungs of animals treated with *E. coli* and mAb anti-TFPI display much stronger staining for fibrin (**a**) compared with animals exposed to *E. coli* only (**b**). No fibrin was detected in the nonchallenged animals (**c**). Tissue sections incubated with mAb anti-digoxigenin, a hapten antigen that does not exist in animal tissues, was used as negative control for immunological specificity of the mouse mAbs (**d**). **e** and **f:** Massive fibrin deposition was detected in the lungs of animals challenged with *E. coli* and anti-PAI-1 antibody (**e**, phosphotungstic acid staining; **f**, fibrin-specific immunostaining). Magnification bars = 100 μ m.

Figure 5. Quantitative analysis of the fluorescence intensity of TF staining in lungs of baboons challenged with *E. coli* and mAb anti-TFPI, *E. coli* alone, and nonchallenged controls. Fifteen randomly selected microscopic fields [five images per animal, three animals per group for nonchallenged control and EC groups and two animals per group for *E. coli* plus anti-TFPI mAb (EC+anti-TFPI)] were collected, and the mean fluorescence intensity values of each image were averaged. The values are expressed as arbitrary units (AU) of gray levels on a 12-bit scale (* $P < 0.05$; *** $P < 0.001$).

Figure 6. Quantitative analysis of the fluorescence intensity after immunolabeling of lungs from control and septic baboons. **a:** TFPI. **b:** t-PA. **c:** Plasmin- α_2 antiplasmin. Twelve randomly selected microscopic fields (four images per animal, three animals per group) and the mean fluorescence intensity values of each image were averaged. Values are expressed as arbitrary units (AU) of gray levels on a 12-bit scale (** $P < 0.01$).

levels than TFPI- α , TFPI- β mRNA followed the same temporal pattern after *E. coli* challenge (Figure 7b).

The time course of TFPI activity, measured as the ability of TFPI to inhibit activation of FX by TF-FVIIa, was different (Figure 7c). At T_{+2} hours, the inhibitory ability of TFPI dropped to 14% of the control levels ($P < 0.01$). The initial decrease was followed by a recovery trend, reaching levels of 38% ($P < 0.05$) and 67% of the control values at T_{+8} and T_{+24} hours (Figure 7c). TFPI antigen measured by ELISA decreased significantly in the septic

animals versus controls for all of the time points examined ($P < 0.05$; Figure 7d). Plotting the above results as TFPI activity-to-antigen ratio showed that the inhibitory potential of the lung-associated TFPI decreased dramatically at T_{+2} hours (Figure 7e).

The decrease of TFPI activity was confirmed in TF clotting assays, in which we preincubated the samples with inhibitory anti-TFPI IgG. The differences between values measured after neutralizing the available TFPI and the ones determined for noninhibited samples should give the measure of the functional potency of TFPI to inhibit TF-FVIIa procoagulant activity. In controls, TF activity was approximately three times higher in the presence of anti-TFPI IgG than in its absence (data not shown), thus confirming that TFPI had high inhibitory potential in normal conditions. In contrast, we did not find equivalent differences in the T_{+2} -hour lung samples, which substantiates the loss of inhibitory capability of the available TFPI at this time point.

Western blot analysis of TFPI from lung lysates by probing with a rabbit polyclonal anti-full-length TFPI showed a decrease in intensity of TFPI at all time points after *E. coli* challenge, as compared with controls, without detectable degradation bands (Figure 7f). Deglycosylation of lung extracts from control animals with an enzyme cocktail containing PNGase, α_2 -neuroaminidase, and α -glycosidase induces a shift of TFPI band from an apparent molecular mass of ~45 to ~37 kD, typical for TFPI- α (Figure 7g).²⁶ No other smaller molecular weight bands potentially corresponding to TFPI- β ²⁶ were observed, suggesting that the α -spliced form is the major TFPI isoform in the baboon lung.

Figure 7. Biochemical analysis of the time course of TFPI expression and activity in lung extracts from control and *E. coli*-challenged baboons. Analysis of TFPI- α (a) and TFPI- β (b) mRNA expression performed by real-time RT-PCR shows steady decrease in septic baboons over time. **c:** TFPI antigen determined by ELISA also decreases. **d:** TFPI activity, measured through its capability to inhibit FX activation by pre-formed TF-FVIIa, drops dramatically at T_{+2} hours, after which it recovers gradually. **e:** The ratio between TFPI activity and antigen defines the specific activity of TFPI at different time points. For **a–e**, * $P < 0.05$; ** $P < 0.01$. **f:** Western blot for TFPI in lung extracts from baboons challenged with *E. coli* at T_{+2} , T_{+8} , or T_{+24} hours compared with healthy animals (T_0). TFPI was probed with a rabbit antibody anti-TFPI. **g:** Western blot for TFPI in lung extracts at T_{+24} hours. Tissue lysates were treated (+) or not (–) with deglycosylation enzymes before sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer, and probing with rabbit anti-TFPI IgG, as above. Full-length human rTFPI was used as control.

Effect of Increased Plasmin Generation Achieved through Blocking of PAI-1 with Inhibitory Antibodies

Treatment with blocking mAb anti-human PAI-1 plus *E. coli* resulted in massive fibrin deposition in the lung, as demonstrated by phosphotungstic acid staining (Figure 4e) and immunostaining with anti-human fibrin antibodies (Figure 4f). Histopathology showed marked congestion, leukocyte influx, and massive capillary leak, characterized by edema and hemorrhage (not shown). These lesions were incompatible with animal survival, and the two animals died after 11 and 51 hours after challenge, respectively. Quantitative analysis of TFPI immunostaining revealed a significant decrease of lung-associated TFPI in animals treated with anti-PAI-1 antibody and *E. coli*

Figure 8. Quantitation of the fluorescence intensity for TFPI (a) and TF (b) in the lung of baboons challenged with *E. coli* and anti-PAI-1 blocking antibody, *E. coli* alone, and nonchallenged controls. Fifteen randomly selected microscopic fields [five images per animal, three animals per group for nonchallenged control and EC, and two animals per group for *E. coli* plus anti-PAI-1 mAb (EC+anti-PAI-1), and the mean fluorescence intensity values of each image were averaged. The values are expressed as arbitrary units (AU) of gray levels on a 12-bit scale (* $P < 0.05$; *** $P < 0.001$).

compared with *E. coli* alone, and both were significantly decreased compared with unchallenged animals (Supplemental Figure 2, a–c, at <http://ajp.amjpathol.org>; Figure 8a). TF staining was significantly increased in *E. coli*-challenged animals compared with controls but was not affected by anti-PAI-1 antibody treatment (Figure 8b).

Functional Activity of Plasma TFPI Is Decreased in Sepsis

In contrast to the lung, plasma TFPI antigen was found increased T_{+2} ($P < 0.01$) and T_{+4} hours ($P < 0.05$) (Figure 9a). After showing a temporary reversal at T_{+6} hours, the TFPI antigen increased again at T_{+24} hours. However, the activity of TFPI in the same plasma samples did not mirror the changes of the antigen (Figure 9b). TFPI activity-to-antigen ratio demonstrated that TFPI in the plasma of septic baboons was significantly less functionally potent than in control animals (Figure 9c).

Discussion

The pathology of sepsis results, in part, from a complex deregulation of normal hemostasis leading to activation of the procoagulant pathways concurrently with impairment of both anticoagulant²⁸ and fibrinolytic systems.²⁹

Analysis of coagulation plasma markers revealed that baboons infused with 10^8 to 10^9 cfu/kg *E. coli* exhibit a two-stage procoagulant response³⁰: an early stage (T_0 to T_{+6} hours) dominated by thrombin and plasmin production and a second stage (T_{+12} to T_{+24} hours) dominated

by peak elevation of soluble fibrin monomer, elastase, and soluble thrombomodulin.

Here, we used this model of non-human primate sepsis to study, for the first time, the regulation of TF-dependent coagulation both in the lung tissue and in plasma at three key time points: T_{+2} hours (first stage), T_{+8} hours (transition from first to second stage), and T_{+24} hours (second stage) after *E. coli* infusion.

The highest increase in TF mRNA detected by us at 2 hours matches the peak plasma levels of lipopolysaccharide, tumor necrosis factor- α , and other inflammatory cytokines,³¹ which are potent TF-inducers in monocytes,³² EC,³³ and neutrophils,³⁴ and could generate procoagulant microparticles.³⁵ The second TF mRNA increase observed at 24 hours (second stage) coincides with the second plasma peak of TF antigen.³⁶ Lung TF protein and its procoagulant potential increased during the 24-hour period, reflecting the gradual accumulation of leukocytes and platelets and TF production by EC and epithelial cells.³⁷ The detection of platelet-associated TF supports a recent report demonstrating TF pre-mRNA splicing and translation in activated platelets.³⁸ TF up-regulation results in thrombin generation, as reflected by increased plasma levels of TAT²⁹ and tissue-bound active thrombin, platelet activation, and fibrin deposition in the lung of septic baboons.

A major finding of our study is the significant impairment of TFPI anticoagulant activity in the lungs of septic baboons. Both TFPI- α and TFPI- β mRNA expression levels decreased during the first and mid-stages and reached their lowest levels during the second stage. Similarly, TFPI antigen decreased steadily during the time course of the experiment. Interestingly, however, the abrupt decrease of TFPI activity detected during the first stage of *E. coli* challenge did not match entirely the changes in the antigen levels. Because the TFPI inhibitory activity toward TF-FVIIa-FXa reflects mainly the amount of full-length TFPI, the difference between TFPI antigen and activity may indicate the presence in the tissue of truncated, functionally inactive forms of TFPI. Although the activity recovered in part at 8 and 24 hours after *E. coli* challenge, both the antigen and the activity of TFPI remained significantly lower in the septic animals than in the controls.

To confirm the effect of decreased TFPI on the hemostatic function of the lung during sepsis, two *E. coli*-challenged baboons were injected with an inhibitory mAb anti-human TFPI. This led to almost complete blocking of

Figure 9. TFPI antigen and activity in plasma from control and *E. coli*-challenged baboons. The time course of TFPI antigen determined by ELISA (a) and TFPI activity assayed against added TF-FVIIa and FX (b) indicate opposite tendencies of the parameters studied. c: Specific activity of TFPI, calculated as described for Figure 6c, decreased significantly throughout the time course (* $P < 0.05$; ** $P < 0.01$).

TFPI activity and to strong increase of TF and fibrin levels in the lung.

The decrease of tissue-associated TFPI during sepsis may result from multiple causes: i) proteolytic release from the cell surface into the plasma; ii) consumption during anticoagulation through formation of the FXa-TFPI-FVIIa/TF complex; or iii) decreased expression in the endothelium, either because of EC dysfunction or through inhibition of gene expression, as supported by our mRNA results. Most likely, all three processes are probably involved at different time points in the TFPI down-regulation.

We observed that the decrease of TFPI content in the lung was paralleled by an increase of plasma levels of TFPI antigen,¹² suggesting an enhanced release from the endothelium, possibly because of local thrombin generation.^{39,40} However, increased plasma TFPI may not necessarily reflect equivalent enhancement of plasma anticoagulant properties, because most of the TFPI in plasma has low activity, presumably because of C terminus truncation.⁴¹ This may explain why the increased plasma TFPI antigen found in the septic baboons actually had poor anticoagulant activity. So far, there are conflicting data about how sepsis mediators can affect the expression of TFPI by EC *in vitro*⁴² or *in vivo*⁴³ or levels of TFPI protein in the plasma.¹⁶ It was suggested that increased TFPI in plasma reflects endothelial injury and release of endothelial-bound TFPI into the plasma, where the protein is truncated, and as a result, has reduced anticoagulant activity.⁴⁴ The available TFPI may inadequately balance the increased TF-dependent coagulation in sepsis patients.⁴⁵ The resulting imbalance perpetuates the procoagulant state and predicts a poor outcome. Because TFPI could bind lipopolysaccharide directly and thus block the binding of the endotoxin to CD14 and prevent adverse cellular effects,⁴⁶ decreased levels of TFPI may also mean less protective anti-inflammatory effects in addition to the loss of anticoagulant function.

It has been shown that TFPI can be degraded, with loss of function potency by several proteases, including leukocyte elastase,^{11,47} metalloproteinases,⁴⁸ or plasmin.^{49–51} We showed that the loss of TFPI activity at 2 hours coincides with the release of t-PA from the tissue and the consequent peak of plasmin generation but not with the time course of neutrophil elastase release or neutrophil accumulation into the tissue. Net pulmonary release of t-PA could reach 15-fold increase during the first 2 hours.⁵² Because we have not detected elastase^{11,47} and/or MMP-specific⁴⁸ degradation products by Western blot analysis and because it was shown that plasmin can completely cleave TFPI without leaving degradation products,⁵⁰ we believe that plasmin, rather than elastase, may be responsible for proteolytic degradation of TFPI during the early stages of sepsis. Actually, it was shown that plasmin decreases the surface-associated TFPI on circulating monocytes after thrombolytic therapy and may contribute to thrombotic complications after fibrinolysis in acute myocardial infarction.⁵¹ Concomitantly, we show here that enhanced plasmin generation by blocking of PAI-1 with inhibitory antibody also significantly decreased the amount of lung-associated TFPI without increasing the TF levels and, paradoxically, promoted mas-

sive fibrin deposition in the lung, among other pathological effects that were incompatible with animal survival.

Our data suggest that the decrease of lung-associated TFPI in the advanced stages of sepsis could be responsible for the vascular and perivascular fibrin deposition that may lead to microthrombotic organ failure. We further speculate that the dysfunction of TFPI that occurs in the lung of septic animals may explain the association of this disease with pulmonary intravascular coagulation and acute respiratory distress syndrome.

In view of these observations, restoration of the TF-TFPI balance is likely to correct the derangement of coagulation in sepsis. In fact, it was shown that TFPI infusion in animals with severe sepsis modulates the development of TF-induced disseminated intravascular coagulation and can reduce mortality.^{46,53} These observations apparently contradict the results of the Phase III multicenter trial that found no substantial impact of r-TFPI administration on survival at 28 days. Some of the factors that could explain why the trial failed were recently reviewed.⁵⁴ We believe, however, that the main problem resides with the major differences existing between r-TFPI and the endogenous form of the inhibitor with regard to clearance from the circulation,⁵⁵ distinct membrane anchorage and subsequent mechanism of action on cell surfaces,^{56–58} or control of protease-activated receptor signaling. These differences could not only explain why r-TFPI has limited therapeutic effects in systemic inflammation but may also point toward the necessity of developing strategies to enhance the expression and activity of endogenous TFPI as a more promising approach to improve the outcome of patients with sepsis.

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References

1. Kaplan RL, Sahn SA, Petty TL: Incidence and outcome of the respiratory distress syndrome in gram-negative sepsis. *Arch Intern Med* 1979, 139:867–869
2. Moore FA, Moore EE, Read RA: Postinjury multiple organ failure: role of extrathoracic injury and sepsis in adult respiratory distress syndrome. *New Horiz* 1993, 1:538–549
3. Brun-Buisson C, Doyon F, Carlet J, Dellamonica P, Gouin F, Lepoutre A, Mercier JC, Offenstadt G, Régnier B: Incidence, risk factors, and outcome of severe sepsis and septic shock in adults: a multicenter prospective study in intensive care units. French ICU Group for Severe Sepsis. *JAMA* 1995, 274:968–974

4. Liaw PC, Esmon CT, Kahn moui K, Schmidt S, Kahn moui S, Ferrell G, Beaudin S, Julian JA, Weitz JI, Crowther M, Loeb M, Cook D: Patients with severe sepsis vary markedly in their ability to generate activated protein C. *Blood* 2004, 104:3958–3964
5. Carraway MS, Welty-Wolf KE, Miller DL, Ortel TL, Idell S, Ghio AJ, Petersen LC, Piantadosi CA: Blockade of tissue factor: treatment for organ injury in established sepsis. *Am J Respir Crit Care Med* 2003, 167:1200–1209
6. Crawley J, Lupu F, Westmuckett AD, Severs NJ, Kakkar VV, Lupu C: Expression, localization, and activity of tissue factor pathway inhibitor in normal and atherosclerotic human vessels. *Arterioscler Thromb Vasc Biol* 2000, 20:1362–1373
7. Broze Jr GJ, Girard TJ, Novotny WF: Regulation of coagulation by a multivalent Kunitz-type inhibitor. *Biochemistry* 1990, 29:7539–7546
8. Chang JY, Monroe DM, Oliver JA, Roberts HR: TFPI β , a second product from the mouse tissue factor pathway inhibitor (TFPI) gene. *Thromb Haemost* 1999, 81:45–49
9. Zhang J, Piro O, Lu L, Broze Jr GJ: Glycosyl phosphatidylinositol anchorage of tissue factor pathway inhibitor. *Circulation* 2003, 108:623–627
10. Li Y, Spencer FA, Becker RC: Plasmin-mediated proteolysis of vascular endothelial cell heparin releasable tissue factor pathway inhibitor. *J Thromb Thrombolysis* 2003, 15:19–23
11. Higuchi DA, Wun TC, Likert KM, Broze Jr GJ: The effect of leukocyte elastase on tissue factor pathway inhibitor. *Blood* 1992, 79:1712–1719
12. Sabharwal AK, Bajaj SP, Ameri A, Tricomi SM, Hyers TM, Dahms TE, Taylor Jr FB, Bajaj MS: Tissue factor pathway inhibitor and von Willebrand factor antigen levels in adult respiratory distress syndrome and in a primate model of sepsis. *Am J Respir Crit Care Med* 1995, 151:758–767
13. Sandset PM, Roise O, Aasen AO, Abildgaard U: Extrinsic pathway inhibitor in postoperative/posttraumatic septicemia: increased levels in fatal cases. *Haemostasis* 1989, 19:189–195
14. Brandtzaeg P, Sandset PM, Joo GB, Ovstebo R, Abildgaard U, Kierulf P: The quantitative association of plasma endotoxin, antithrombin, protein C, extrinsic pathway inhibitor and fibrinopeptide A in systemic meningococcal disease. *Thromb Res* 1989, 55:459–470
15. Novotny WF, Brown SG, Miletich JP, Rader DJ, Broze Jr GJ: Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. *Blood* 1991, 78:387–393
16. Bajaj MS, Rana SV, Wysolmerski RB, Bajaj SP: Inhibitor of the factor VIIa-tissue factor complex is reduced in patients with disseminated intravascular coagulation but not in patients with severe hepatocellular disease. *J Clin Invest* 1987, 79:1874–1878
17. Warr TA, Rao LV, Rapaport SI: Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. *Blood* 1990, 75:1481–1489
18. Broze Jr GJ, Warren LA, Novotny WF, Higuchi DA, Girard JJ, Miletich JP: The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. *Blood* 1988, 71:335–343
19. Broze Jr GJ: The rediscovery and isolation of TFPI. *J Thromb Haemost* 2003, 1:1671–1675
20. Hack CE, Zeerleder S: The endothelium in sepsis: source of and a target for inflammation. *Crit Care Med* 2001, 29:S21–S27
21. Ameri A, Kuppuswamy MN, Basu S, Bajaj SP: Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. *Blood* 1992, 79:3219–3226
22. Nakahara Y, Miyata T, Hamuro T, Funatsu A, Miyagi M, Tsunasawa S, Kato H: Amino acid sequence and carbohydrate structure of a recombinant human tissue factor pathway inhibitor expressed in Chinese hamster ovary cells: one N- and two O-linked carbohydrate chains are located between Kunitz domains 2 and 3 and one N-linked carbohydrate chain is in Kunitz domain 2. *Biochemistry* 1996, 35:6450–6459
23. Biemond BJ, Levi M, Coronel R, Janse MJ, ten Cate JW, Pannekoek H: Thrombolysis and reocclusion in experimental jugular vein and coronary artery thrombosis: effects of a plasminogen activator inhibitor type 1-neutralizing monoclonal antibody. *Circulation* 1995, 91:1175–1181
24. Taylor Jr FB, Stearns-Kurosawa DJ, Kurosawa S, Ferrell G, Chang AC, Laszik Z, Kossanke S, Peer G, Esmon CT: The endothelial cell protein C receptor aids in host defense against *Escherichia coli* sepsis. *Blood* 2000, 95:1680–1686
25. Lupu C, Westmuckett AD, Peer G, Ivanciu L, Zhu H, Taylor Jr FB, Lupu F: Tissue factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of *Escherichia coli* sepsis. *Am J Pathol* 2005, 167:1161–1172
26. Piro O, Broze Jr GJ: Comparison of cell-surface TFPI α and β . *J Thromb Haemost* 2005, 3:2677–2683
27. Lupu C, Hu X, Lupu F: Caveolin-1 enhances tissue factor pathway inhibitor exposure and function on the cell surface. *J Biol Chem* 2005, 280:22308–22317
28. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, Laszik Z, Esmon CT, Heyderman RS: Dysfunction of endothelial protein C activation in severe meningococcal sepsis. *N Engl J Med* 2001, 345:408–416
29. de Boer JP, Creasy AA, Chang A, Roem D, Brouwer MC, Eerenberg AJ, Hack CE, Taylor Jr FB: Activation patterns of coagulation and fibrinolysis in baboons following infusion with lethal or sublethal dose of *Escherichia coli*. *Circ Shock* 1993, 39:59–67
30. Taylor Jr FB, Wada H, Kinasevitz G: Description of compensated and uncompensated disseminated intravascular coagulation (DIC) responses (non-overt and overt DIC) in baboon models of intravenous and intraperitoneal *Escherichia coli* sepsis and in the human model of endotoxemia: toward a better definition of DIC. *Crit Care Med* 2000, 28:S12–S19
31. Creasey AA, Stevens P, Kenney J, Allison AC, Warren K, Catlett R, Hinshaw L, Taylor Jr FB: Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal *Escherichia coli*. *Circ Shock* 1991, 33:84–91
32. Nilsen DW, Almdahl SM, Svensson B, Vaage J, Rasmussen K, Østerud B: Lipopolysaccharide induced monocyte thromboplastin synthesis and coagulation responses in patients undergoing coronary bypass surgery after preoperative supplementation with n-3 fatty acids. *Thromb Haemost* 1993, 70:900–902
33. Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, Semeraro N: Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* 1983, 71:1893–1896
34. Todoroki H, Nakamura S, Higure A, Okamoto K, Takeda S, Nagata N, Itoh H, Ohsato K: Neutrophils express tissue factor in a monkey model of sepsis. *Surgery* 2000, 127:209–216
35. Østerud B, Bjørklid E: The tissue factor pathway in disseminated intravascular coagulation. *Semin Thromb Hemost* 2001, 27:605–617
36. Taylor Jr FB: The inflammatory-coagulant axis in the host response to gram-negative sepsis: regulatory roles of proteins and inhibitors of tissue factor. *New Horiz* 1994, 2:555–565
37. Drake TA, Cheng J, Chang A, Taylor Jr FB: Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal *Escherichia coli* sepsis. *Am J Pathol* 1993, 142:1458–1470
38. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS: Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. *J Exp Med* 2006, 203:2433–2440
39. Lupu C, Lupu F, Dennehy U, Kakkar VV, Scully MF: Thrombin induces the redistribution and acute release of tissue factor pathway inhibitor from specific granules within human endothelial cells in culture. *Arterioscler Thromb Vasc Biol* 1995, 15:2055–2062
40. Lupu C, Kruithof EK, Kakkar VV, Lupu F: Acute release of tissue factor pathway inhibitor after in vivo thrombin generation in baboons. *Thromb Haemost* 1999, 82:1652–1658
41. Novotny WF, Girard TJ, Miletich JP, Broze Jr GJ: Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. *J Biol Chem* 1989, 264:18832–18837
42. Bajaj MS, Bajaj SP: Tissue factor pathway inhibitor: potential therapeutic applications. *Thromb Haemost* 1997, 78:471–477
43. Shimokawa T, Yamamoto K, Kojima T, Saito H: Down-regulation of murine tissue factor pathway inhibitor mRNA by endotoxin and tumor necrosis factor- α in vitro and in vivo. *Thromb Res* 2000, 100:211–221
44. Shimura M, Wada H, Nakasaki T, Hiyoyama K, Mori Y, Nishikawa M, Deguchi H, Deguchi K, Gabazza EC, Shiku H: Increased truncated form of plasma tissue factor pathway inhibitor levels in patients with disseminated intravascular coagulation. *Am J Hematol* 1999, 60:94–98
45. Gando S, Kameue T, Morimoto Y, Matsuda N, Hayakawa M, Kemmotsu O: Tissue factor production not balanced by tissue factor

- pathway inhibitor in sepsis promotes poor prognosis. *Crit Care Med* 2002, 30:1729–1734
46. Park CT, Creasey AA, Wright SD: Tissue factor pathway inhibitor blocks cellular effects of endotoxin by binding to endotoxin and interfering with transfer to CD14. *Blood* 1997, 89:4268–4274
47. Petersen LC, Bjorn SE, Nordfang O: Effect of leukocyte proteinases on tissue factor pathway inhibitor. *Thromb Haemost* 1992, 67:537–541
48. Belaaouaj AA, Li A, Wun TC, Welgus HG, Shapiro SD: Matrix metalloproteinases cleave tissue factor pathway inhibitor. Effects on coagulation. *J Biol Chem* 2000, 275:27123–27128
49. Li A, Wun TC: Proteolysis of tissue factor pathway inhibitor (TFPI) by plasmin: effect on TFPI activity. *Thromb Haemost* 1998, 80:423–427
50. Stalboerger PG, Panetta CJ, Simari RD, Caplice NM: Plasmin proteolysis of endothelial cell and vessel wall associated tissue factor pathway inhibitor. *Thromb Haemost* 2001, 86:923–928
51. Ott I, Malcouvier V, Schomig A, Neumann FJ: Proteolysis of tissue factor pathway inhibitor-1 by thrombolysis in acute myocardial infarction. *Circulation* 2002, 105:279–281
52. Nyberg A, Fagerberg A, Ahlqvist M, Jern C, Seeman-Lodding H, Aneman A: Pulmonary net release of tissue-type plasminogen activator during porcine primary and secondary acute lung injury. *Acta Anaesthesiol Scand* 2004, 48:845–850
53. Creasey AA, Chang AC, Feigen L, Wun TC, Taylor Jr FB, Hinshaw LB: Tissue factor pathway inhibitor reduces mortality from *Escherichia coli* septic shock. *J Clin Invest* 1993, 91:2850–2856
54. LaRosa SP, Opal SM: Tissue factor pathway inhibitor and antithrombin trial results. *Crit Care Clin* 2005, 21:433–448
55. Narita M, Bu G, Olins GM, Higuchi DA, Herz J, Broze Jr GJ, Schwartz AL: Two receptor systems are involved in the plasma clearance of tissue factor pathway inhibitor in vivo. *J Biol Chem* 1995, 270:24800–24804
56. Mast AE, Acharya N, Malecha MJ, Hall CL, Dietzen DJ: Characterization of the association of tissue factor pathway inhibitor with human placenta. *Arterioscler Thromb Vasc Biol* 2002, 22:2099–2104
57. Lupu C, Goodwin CA, Westmuckett AD, Emeis JJ, Scully MF, Kakkar VV, Lupu F: Tissue factor pathway inhibitor in endothelial cells colocalizes with glycolipid microdomains/caveolae: regulatory mechanism(s) of the anticoagulant properties of the endothelium. *Arterioscler Thromb Vasc Biol* 1997, 17:2964–2974
58. Sevinsky JR, Rao LV, Ruf W: Ligand-induced protease receptor translocation into caveolae: a mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway. *J Cell Biol* 1996, 133:293–304