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Tissue Factor-Dependent Coagulation Is Preferentially Up-Regulated within Arterial Branching Areas in a Baboon Model of *Escherichia coli* Sepsis

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Endothelium plays a critical role in the pathobiology of sepsis by integrating systemic host responses and local rheological stimuli. We studied the differential expression and activation of tissue factor (TF)-dependent coagulation on linear versus branched arterial segments in a baboon sepsis model. Animals were injected intravenously with lethal doses of *Escherichia coli* or saline and sacrificed after 2 to 8 hours. Whole-mount arterial segments were stained for TF, TF-pathway inhibitor (TFPI), factor VII (FVII), and markers for endothelial cells (ECs), leukocytes, and platelets, followed by confocal microscopy and image analysis. In septic animals, TF localized preferentially at branches, EC surface, leukocytes, and platelet aggregates and accumulated in large amounts in the subendothelial space. FVII strongly co-localized with TF on ECs and leukocytes but less so with subendothelial TF. TFPI co-localized with TF and FVII on endothelium and leukocytes but not in the subendothelial space. Focal TF increases correlated with fibrin deposition and increased endothelial permeability to plasma proteins. Biochemical analysis confirmed that aortas of septic baboons expressed more TF mRNA and protein than controls. Branched segments contained higher TF protein levels and coagulant activity than equivalent linear areas. These data suggest that site-dependent endothelial heterogeneity and rheological factors contribute to focal procoagulant responses to *E. coli*. (*Am J Pathol* 2005, 167:1161–1172)

Endothelium is a cell monolayer strategically located between blood and the underlying tissue. Its multiple func-

tions are essential to normal vascular biology and endothelial dysfunction can play critical roles in the genesis of several vascular disorders.¹ Endothelial functions are differentially regulated in distinct segments of the vascular tree by integrating different local biochemical signals and biomechanical forces created by the local blood flow. Normal arterial flow is laminar, while secondary flows generated at curves and branches cause wall stresses to be nonuniform.² Studies have shown that hemodynamic shear stress influences endothelial function and phenotype.³ Arterial-level laminar shear stress favors endothelial anti-thrombotic phenotype, whereas low shear stress—which is prevalent at branching sites of the arteries—stimulates a prothrombotic state.⁴ *In vitro* exposure to physiological shear stress stimulates endothelial cells (ECs) to release factors that can regulate the coagulation cascade both directly and indirectly, by up-regulating tissue factor pathway inhibitor (TFPI),⁵ thrombomodulin, tissue plasminogen activator, prostacyclin, NO, and decreasing plasminogen activator inhibitor-1.⁶ In addition, changes in shear stress can influence leukocyte adhesion by regulating the expression of several adhesion molecules and chemoattractants, such as ICAM-1, VCAM-1, and MCP-1.⁷ The *in vivo* heterogeneity of large artery endothelium is highlighted by the focal distribution of the atherosclerotic lesions at sites that are associated with complex flow separations and disturbances, such as arterial branches and curvatures.⁶

ECs play a major role in sepsis, a deadly pathological condition that has a mortality rate of 30 to 50%, representing the most common cause of death among hospitalized patients in noncoronary intensive care units.⁸ Local responses of ECs to invading pathogens include release of inflammatory mediators, leukocyte recruitment,

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and induction of a procoagulant activity.⁹ It has been suggested that the functions of microvascular endothelium are altered heterogeneously by severe sepsis in different organs.⁹

Our group has developed and used for many years a model of severe sepsis involving the administration of a lethal dose (LD₁₀₀) of *Escherichia coli* in baboons.¹⁰ The hallmark of this pathological condition is represented by EC dysfunction, characterized as an excessive, sustained, and generalized activation of the endothelium.⁹ We hypothesized that localized changes of endothelial function in the areas of the arterial tree exposed to perturbed flow may contribute to the severe sepsis phenotype. In this study we compared the expression and function of pro- and anti-thrombotic proteins in straight arterial segments versus branches of healthy and septic baboons. Our data demonstrate that endothelial responses to *E. coli* differ according to the spatial geometry of the arteries, showing that branches display an increased tissue factor (TF)-dependent coagulant function, when compared to the straight segments.

Materials and Methods

Animals

Papio cynocephalus baboons were purchased from the breeding colony at Oklahoma University Health Sciences Center. The animals had normal hematological parameters (leukocytes, platelet counts, and hematocrits) and were free of tuberculosis. Experiments were performed on eight baboons. Five were injected with lethal doses [LD₁₀₀; 10⁹ colony-forming units (cfu)/kg] of *E. coli* (type B7 086a:K61, no. 33985; American Type Culture Collection, Rockville, MD),¹¹ and three animals were used as controls. Animals were sedated with ketamine hydrochloride (14 mg/kg, intramuscular) and anesthetized intravenously with sodium pentobarbital (2 mg/kg). Two animals were euthanized after 2 hours and three after 8 hours after *E. coli* infusion by intravenous administration of 50 mg/kg of pentobarbital. The protocol was approved by the Institutional Animal Care and Use Committee.

Antibody and Special Reagents

Monoclonal antibody (mAb) against human TF (clone TF9-10H10) and sheep anti-human FVII IgG were gifts from Dr. James H. Morrissey, University of Illinois, Urbana-Champaign, IL. Rabbit anti-human FVII IgG was kindly

provided by Dr. Wolfram Ruf, Scripps Research Institute, La Jolla, CA. Mouse mAb anti-human TFPI was a gift from Dr. Tsutomu Hamuro, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan, and rabbit anti-human TFPI IgG was produced as described.¹² mAb anti-human antithrombin-serine protease complexes were from Diagnostica Stago (Asnières, France). Rabbit anti-human PSGL-1 IgG was from Dr. Kevin Moore, Oklahoma Medical Research Foundation, Oklahoma City, OK. mAbs anti-human CD31, CD68, and glycoprotein IIb-IIIa (CD41), as well as rabbit IgG anti-human myeloperoxidase were from DakoCytomation (Carpinteria, CA). Fluorophore-conjugated secondary antibodies (fluorescein isothiocyanate/goat anti-rabbit IgG, fluorescein isothiocyanate/goat anti-mouse IgG, Cy₃/goat anti-mouse IgG, and Cy₃/goat anti-rabbit IgG) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-mouse IgG conjugated with 10-nm colloidal gold was from Electron Microscopy Sciences (Washington, PA). Human FVIIa and FX(a) were from Enzyme Research Laboratories (South Bend, IN). Chromogenic substrate S2756 was purchased from Chromogenix (Molndal, Sweden). Innovin (relipidated human recombinant TF) was from Dade (Miami, FL). Trizol was from Invitrogen (Carlsbad, CA). All molecular biology reagents, tubes, and tips were nuclease-free.

Immunofluorescence

Whole Mount en Face Staining

Aortas were removed, rinsed in phosphate-buffered saline (PBS), and placed in 4% paraformaldehyde in PBS at 4°C for 4 hours. The vessel segments were gently cleaned of fat and adventitia, and opened longitudinally to expose the lumen. Segments—approximately 5 × 5 mm in size—were cut from both linear and intercostal branch point flow divider regions of the aortas (Figure 1a), washed in PBS, cryoprotected in 15% sucrose in PBS for 2 hours, snap-frozen in liquid nitrogen cooled isopentane (−160°C), and stored at −80°C.

For indirect immunofluorescence, segments were thawed in PBS at 20°C and free aldehyde groups were quenched with 0.1 mol/L glycine in PBS (15 minutes). The tissue samples were permeabilized with 0.1% saponin in PBS (PBS/SAP) (10 minutes at 20°C) and incubated in 3% bovine serum albumin in PBS/SAP (1 hour at 20°C). Saponin was kept in all incubation buffers throughout the staining procedure, to ensure a proper penetration of the

Figure 1. Alterations of the arterial wall after *E. coli* sepsis. **a:** Diagram mapping the location of the two arterial areas examined: the straight areas were located at least 1 cm distal to intercostal bifurcation, whereas the branch areas were located adjacent to the lateral wall of the intercostal artery. **b:** Combined diagrammatic and micrographic representation of the *en face* deep tissue confocal imaging on the *z* axis. **c** and **d:** Representative *en face* confocal images of CD31 staining (green) in ECs covering the straight (**c**) and branch (**d**) areas. Note the elongated shape and orientation in the flow direction (**arrows**) of the ECs and their nuclei in the straight segments, as compared to the irregular shape and orientation in the branch area. Frank denudations of the endothelial lining are marked with **asterisks**. Throughout this article, all specimens were labeled with TO-PRO3 as nuclear counterstaining (shown in blue). **e–h:** Light and electron micrographs of the arterial intima of control (**e** and **g**) versus septic baboons (**f** and **h**). The arterial wall of *E. coli*-challenged animals displays dramatic morphological changes, including massive infiltration of leukocytes (L) into the subendothelial space (**f, inset**, and **h** versus **e, inset**, and **g**), large intercellular gaps and extensive interstitial edema (**f, inset**, and **h**). Images shown in **e** and **f** are semithin acrylic resin sections stained with toluidine blue. High magnifications of the intima layer are shown in **insets**. **i–k:** Fibrinogen (red)—as a marker of endothelial permeability—was detected in much larger amounts within the subendothelial space of the arterial branches (**k**) than in the straight vessel segments from septic animals (**j**), but was absent in similar areas from healthy baboons (**i**). Scale bars: 50 μ m (**c, d, i–k**); 100 μ m (**e, f**); 5 μ m (**g** and **h**).

antibodies. Saponin exclusively removes cholesterol molecules from lipid-containing structures, leaving stable holes large enough for penetration of antibodies.¹³

Next, the vessel segments were placed in mixtures of mAbs and polyclonal IgGs (10 μ g/ml and 20 μ g/ml, respectively) for 1 hour at 20°C or overnight at 4°C. The samples were washed 3 $\times$ 10 minutes in PBS/SAP, and incubated for 1 hour at 20°C with combinations of appropriate detection antibodies conjugated with fluorescein isothiocyanate and Cy₃ diluted 1:100 in 1% bovine serum albumin in PBS/SAP. After washing as above, the arterial segments were mounted with the endothelial side up between glass slides and coverslips using Vectashield hardset mounting medium (Vector Laboratories, Burlingame, CA) containing TO-PRO-3 iodine (Molecular Probes, Eugene, OR) as nuclear counterstain.

As negative controls for polyclonal antibody staining, the primary antibodies were replaced with equivalent amounts of rabbit or sheep nonimmune serum. mAb anti-digoxigenin, a hapten antigen that occurs only in plants, was used as control for mAb staining. Specimens were examined by epifluorescence confocal imaging¹⁴ using a Nikon C1 confocal laser-scanning unit equipped with a three-laser launcher (488, 543, and 633 nm emission lines) installed on an Eclipse TE200-U inverted microscope (Nikon, Melville, NY). Images were taken with either a $\times$ 20 plan achromat objective (NA 0.46) or a $\times$ 60 apochromat oil immersion objective (NA 1.4). Image collection parameters (neutral density filters, pinhole, and detector gains) were kept constant during image acquisition, to make reliable semiquantitative comparisons between the linear and branched regions of the arteries.

Vessel segments were analyzed by optical sectioning, and z-stacks were reconstructed using Imaris volume rendering software (Bitplane AG, Zurich, Switzerland). Several rendering methods were used, including maximum intensity projection, an approach that involves generation of two-dimensional extended focus images by integration of the fluorescence signal collected within a defined three-dimensional structure.

The measurement of fluorescence intensity in z-stacks was done using the EZ-C1 software (Nikon). Briefly, single-channel grayscale images (12 bit, 4095 gray levels/pixel) were collected at 5- μ m z-steps and the average fluorescence intensity of each image was integrated. In total, 12 z-stacks per experimental condition (branch, straight, and control), obtained from four independent experiments were collected and analyzed in a blind manner. Co-localization of multichannel images was done using Imaris co-localization module.

Tissue Immunostaining

For immunostaining on cryosections, the artery segments were fixed, cryoprotected, mounted in Tissue-Tek OCT compound, and snap-frozen as above. Tissue sections were immunostained and analyzed by confocal microscopy, as described for whole mount aortas.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

Apoptotic cells were visualized using an *in situ* fluorescence TUNEL assay (Roche, Indianapolis, IN), according to the manufacturer's instructions.

Electron Microscopy

Tissue preparation and immunogold labeling of TF on Lowicryl-embedded tissues was done as described.¹⁵ For quantitative evaluation, sections were prepared (three sections/block; three tissue blocks/condition) from the branch and straight aorta segments collected from septic animals. Ten digital electron micrographs for each condition were taken at a standardized magnification of 20,000-fold. The relative membrane length and areas were determined using Image J image analysis software (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>). The linear labeling density was defined as the ratio of gold particles per 100- μ m membrane. The intracellular and extracellular gold label distribution of TF was defined as the ratio of gold particles per 100 μ m². Gold particles within 20 nm of a membrane were considered membrane-associated. Mean values and SE (SEM) were calculated and a paired *t*-test was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA). Statistical significance was set at a *P* value <0.05 .

Biochemical Analysis

Extraction of RNA and cDNA Synthesis

Total RNA was extracted from rapid-frozen aorta segments using Trizol according to the manufacturer's instructions. The concentration of RNA was estimated with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the purity and integrity were assessed by capillary gel electrophoresis using an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). Equal amounts of RNA from different tissue samples were used for cDNA synthesis by reverse transcription using SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocol.

SYBR Green-Based Quantitative Real-Time Polymerase Chain Reaction

Each 25- μ l SYBR Green reaction consisted of 5 μ l of cDNA (12.5 ng/ μ l), 12.5 μ l of iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), and 7.5 μ l of 200 nmol/L forward and reverse primers. The primer sequences were designed using Primer Express 2.0 (ABI). The primers for baboon TF (GenBank no. AY685127) are as follows: 5-TGCTTTTACACAGCAGACACAGAGT-3' (forward) and 5-AAGACCCGTGCCAAGTACGT-3' (reverse). The primers for human 18S rRNA (GenBank no. AJ844646) are as follows: 5'-CCCGAAGCGTTTACTTTGAA-3' (for-

ward) and 5'-CGCGGTCCTATTCCATTATTC-3' (reverse). Optimization was performed for each primer to ensure that no nonspecific primer-dimer amplification signal in no-template control tubes occurred. Assays were performed with an ABI Prism 7000 sequence detector (ABI) using the default program provided by the manufacturer.

Specificity of the amplification product was confirmed by examination of dissociation reaction plots, and end-reaction products were visualized on ethidium bromide-stained 1.4% agarose gels. Each sample was tested in triplicate, and samples obtained from two independent experiments were used to calculate the means and SEM. All data were normalized to an internal standard (18S ribosomal RNA).

Proteolytic Activity of TF-FVIIa Complex

TF-FVIIa-dependent activation of FX was determined on segments prepared as above using a modification of the two-stage chromogenic assay.¹⁶ In brief, the segments were incubated with 10 nmol/L FVIIa for 30 minutes at 37°C, then the activation was initiated with 200 nmol/L FX. After 15 minutes at 37°C the supernatant was removed and quenched in ice-cold TEB (50 mmol/L Tris-buffered saline, pH 8.8, supplemented with 25 mmol/L ethylenediamine tetraacetic acid and 0.1% bovine serum albumin). Total FXa generated was determined from the hydrolysis of the chromogenic substrate S-2765.

Whole Mount Assay of TF Antigen Levels

Quantification of TF was performed on the same corresponding segments used for the activity assays. To dissociate any coagulation complexes formed with TF and TFPI, the segments were first incubated with ice-cold TEB (30 minutes at 4°C), then fixed with 4% para-formaldehyde in PBS (1 hour at 20°C) and assayed by enzyme-linked immunosorbent assay essentially as described.^{5,12} TF concentrations were extrapolated from standard curves constructed with serial dilutions of recombinant TF (0.3 ng to 20 ng/well). Statistical analysis of biochemical data were performed with one-way analysis of variance with Tukey-Kramer multiple comparisons post test. (InStat, GraphPad). Statistical significance was set at a *P* value <0.05.

Results

En Face Deep Tissue Confocal Imaging of Baboon Arteries

We studied the differential three-dimensional distribution of proteins involved in the TF pathway of coagulation in the straight versus intercostal branch segments of the aorta in baboons challenged with LD₁₀₀ doses of *E. coli*, as compared to control animals (Figure 1a). For this purpose, we developed a whole-mount immunofluorescence staining approach, which allows the visualization of proteins located in the deepness of the upper arterial

intima. The approach consists of *en face* staining of arterial whole-mount segments followed by three-dimensional confocal imaging. Z-stacks of xy optical sections were collected and three-dimensional rendering was performed using specialized software (Figure 1b). Images are presented either as single optical section or as maximal intensity projections of multiple serial images acquired in Z-stacks. This method allowed us to visualize much larger areas of the arteries than it is achievable on tissue sections, a feature that had particular importance when linear and branched vessel segments were analyzed in parallel. Using this technique we could perform high-resolution analysis of the differential staining patterns of proteins located in the thickness of the intima (upper three to four cell layers). *En face* staining for CD31, an EC protein that preferentially locates at the cell borders, revealed that cells covering the straight segments were elongated and aligned in the direction of blood flow (Figure 1c), whereas those covering the branch areas had variable shapes and random orientations (Figure 1d).

Structural Changes of the Arterial Wall Induced by Severe Sepsis

Both light microscopy and electron microscopy studies revealed that *E. coli* sepsis induced marked changes in the structure of the aortic intima. Although aorta of normal baboons displayed a continuous endothelial monolayer with a quiescent phenotype [Figure 1; c, e (inset), and g], *E. coli*-induced changes included cell contraction, marked attenuation in places, or focally swollen cells [Figure 1, f (inset) and h].

En face staining for CD31 revealed the presence of frank denudations, especially but not exclusively at branching points (Figure 1d, asterisks). *E. coli* sepsis caused increased adhesion and transmigration of the leukocytes into the subendothelial space, as well as massive extracellular edema [Figure 1, f (inset) and h]. Immunostaining with anti-fibrinogen IgG showed considerable accumulation of fibrinogen/fibrin in the intima of branches (Figure 1k) and to a lesser extent in the straight arterial segments of the septic baboons (Figure 1j), whereas no staining was observed in similar areas of the normal animals (Figure 1i).

Localization of TF and TFPI within the Arterial Intima

Using *en face* z-imaging of arterial endothelium in conjunction with computer-assisted three-dimensional rendering we observed that leukocytes, stained for PSGL-1 as specific marker, accumulated in high amount as clusters at the branch areas when compared to the straight arterial segments of the septic baboons. Confocal imaging through the aortic intima showed a topographical heterogeneity of TF. Figure 2 displays pairs of selected optical sections from the upper and lower part of the z-stacks shown as movie files (video 1 to 3 in

Figure 3. Semiquantitative analysis of fluorescence intensity of TF and TFPI. Mean fluorescence intensity (MIF) of the z-images collected from the branch and straight vessel segments was integrated as described in Materials and Methods. MIF values of the specimens stained with anti-digoxigenin antibodies (immune control) represents the tissue autofluorescence.

Supplemental Material section, available on line at <http://ajp.amjpathol.org>.

TF fluorescence intensity was increased in the upper optical sections (z-steps, 0 to 5 μm), which represent the luminal EC surface of the intercostals branches (Figure 2, a to c, top; videos 1 to 2), as compared to the straight arterial segments [mean fluorescence intensity values in arbitrary units (AU): 790 ± 48 versus 652 ± 18 ; $n = 12$; Figure 3a]. TF located on ECs showed a granular distribution.

A particular aspect observed almost exclusively at the arterial branches was TF accumulation in the sub-endothelium (z-steps, 15 to 40 μm). At these levels, MIF values were >1100 AU in the branches, which is significantly higher than the ≈ 450 AU, determined for the straight segments (Figure 3a). TF-positive fluorescent material was organized in extracellular granular structures (Figure 2; a to d, bottom; video 1 to 3). Whereas endothelial TF co-localized with TFPI on double-labeled specimens (seen as yellow, Figure 2b, top), the sub-endothelial extracellular microvesicles contained only TF (Figure 2b, bottom; Figure 3, a and b). Fluorescence intensity analysis revealed that TFPI was primarily located in the first 20 μm , which represent mainly the thickness of the endothelial layer. Interestingly, TFPI staining on straight segments was stronger than in the branch areas. MIF values >2000 AU for straight, and ≈ 1200 AU for branch segments (Figure 3b).

Part of the TF-positive cells and particles also co-stained for PSGL-1, confirming that some of the TF-containing cells located in the intima of septic baboons were leukocytes (Figure 2c, top). Detection of TF- and PSGL-1-positive particles on the endothelial surface and in even higher amounts in the subendothelium (Figure 2c, bottom) suggests that the particles may have originated, at least in part, from leukocytes. Control staining with an isotype-matched antibody to an antigen that occurs only in plants showed no significant background staining (Figure 2e) either on the EC surface or in the subendothelium.

Postembedding immunogold electron microscopy (Figure 4) demonstrated that TF was present mainly on the plasma membrane of ECs (straight, 4.82 ± 0.88 gold particles/100 μm^2 ; branch, 7.57 ± 1.22 particles/100 μm^2 ; $n = 6$; $P < 0.05$), extravasated leukocytes (straight, 6.14 ± 1.3 particles/100 μm^2 ; branch, 5.83 ± 0.71 particles/100 μm^2 ; $n = 5$; NS) (Figure 4a, insets), on elongated subendothelial cells with smooth muscle characteristics (straight, 3.75 ± 0.85 particles/100 μm^2 ; branch, 4.12 ± 0.93 particles/100 μm^2 ; $n = 4$; NS) (Figure 4, a and b), and scattered within the extracellular space of the intima (straight, 0.85 ± 0.07 particles/100 μm^2 ; branch, 5.58 ± 1.4 particles/100 μm^2 ; $n = 6$; $P < 0.01$) [Figure 4, a (insets) and b]. In the branches, higher numbers of gold particles were detected in the subendothelial space neighboring endothelial gaps as compared with areas covered by apparently intact endothelium (4.93 ± 0.84 particles/100 μm^2 versus 7.57 ± 1.2 particles/100 μm^2 ; $n = 6$; $P < 0.05$). Because the postembedding immunogold labeling procedure is not compatible with osmium

Figure 2. Detection of TF in arterial segments of septic animals demonstrates that TF accumulates in higher amounts on the arterial branches than on the straight segments of aorta. The image sets (a–e) illustrate z-stacks of xy images collected at 5- μm intervals. Selected xy optical sections through endothelial (top) and subendothelial (bottom) planes are illustrated. The location of the xy image in the stack is shown on the yz image (middle, dotted lines). Animations of these image stacks are shown as movie files at <http://ajp.amjpathol.org>. TF (green) was detected in higher amount at the branches (a–c) than on the straight segments (d), both on the endothelial surface (top) and within the subendothelial space (bottom). b: Double immunostaining for TF (green) and TFPI (red) shows that endothelial TF co-localizes in a large extent with TFPI (yellow, top), whereas the subendothelial space contains only TF (green, bottom). c: Double staining for TF (green) and PSGL-1 (red) shows TF co-localization with leukocytes (arrow) and microvesicular material adherent on the endothelial surface (yellow, top). Interestingly, the subendothelial space contained large amounts of TF, both cell-associated and as extracellular TF (yellow, bottom). e and f: Negative controls for immunological specificity of the mouse monoclonal antibodies (e) and rabbit primary antibodies (f). Tissues were incubated with mAb anti-digoxigenin, a hapten antigen that does not exist in animal tissues (e), or nonimmune rabbit IgG, followed by appropriate fluorescein isothiocyanate- or Cy3-labeled secondary antibodies, as described in Materials and Methods. Note the lack of nonspecific staining on EC surface (e and f, top) and the low but distinguishable autofluorescence of the matrix in the subendothelium (e and f, bottom). TO-PRO3 nuclear (N) staining is shown as blue. Scale bars, 50 μm .

Figure 4. Immunogold detection of TF on Lowicryl-embedded arteries. **a:** Low-magnification electron microscopy micrograph showing leukocyte (L) accumulation into the subendothelial space of aortic collected from septic baboons. High-magnification insets of the marked areas reveal immunogold labeling for TF (arrows) on the luminal and abluminal surface of ECs (oval inset) on the extravasated leukocytes (rectangular inset, L), as well as within the subendothelial extracellular space, especially close to interendothelial cell gaps (round inset). In addition, TF (arrows) is found in cells with smooth muscle aspect (SMC), and sometimes in association with the extracellular matrix (ECM). Cell debris can be frequently observed in the subendothelial area (**c**, asterisks). **d:** No TF-specific gold labeling is observed on the ECs of healthy baboons. Scale bars: 5 μm (**a**, **c**); 1 μm (**b**, **d**).

fixation of the phospholipids, we could not visualize the presumed vesicular material that contains the extracellular TF. Cell debris was frequently observed, especially in the branch arterial segments (Figure 4c, asterisks). TF-specific gold labeling was virtually absent on ECs of healthy baboons (Figure 4d). Double-immunolabeled TF and CD-31 partially co-localized in the intima, which suggests that some ECs contained TF (Figure 5a; co-localization in yellow, arrowheads). To identify the cell type of the TF-stained leukocytes, we performed double staining for TF and either CD68 (Figure 5b) or myeloperoxidase (Figure 5c) as specific markers of macrophages and neutrophils, respectively. We observed that both cell types stained for TF, but macrophages showed stronger signal than neutrophils.

Double labeling for TF and apoptotic cells, detected by fluorescence *in situ* TUNEL assay, revealed the presence of TF-positive apoptotic cells located almost exclusively within the intima of the arterial branches (Figure 5d; straight, 0.008 ± 0.0006 cells/1000 μm^2 ; branch, 3.6 ± 0.12 apoptotic cells/1000 μm^2 ; $n = 4$; $P < 0.01$). These data are supported by the presence of ultrastructural

signs of cell death (Figure 4c), suggesting that part of the subendothelial TF deposits may originate from apoptotic or necrotic cells entrapped within the subendothelial space.

Vascular-Associated TF Is Functionally Active

Double immunostaining showed that TF located in intimal cells and extracellular environment co-localized with FVIIa (Figure 5e) or FXa (not shown), suggesting that the functional sites of TF were intact. In addition, images of double or multiple staining for TF and thrombin-antithrombin (TAT) complexes (Figure 5f), activated platelets (Figure 5g), or fibrin (Figure 5h) suggest an active ongoing coagulation process in the arterial wall of septic baboons, especially at branching areas.

Septic Baboons Express Increased Levels of TF mRNA in the Vascular Wall

Quantitative real-time polymerase chain reaction revealed a 50% increase in TF mRNA in branch versus straight segments of healthy baboons. Compared to normal animals, TF mRNA was more than threefold overexpressed in septic animals euthanized after 2 hours after *E. coli* challenge (Figure 6). Animals euthanized 8 hours after challenge showed a general decrease in mRNA levels for a multitude of genes, including TF and several housekeeping genes (not shown), probably due to the profound cell and tissue dysfunction specific for severe sepsis.

Quantification of TF Antigen and Activity

Equal sized arterial wall samples were collected from straight or branch areas of normal and septic baboons and analyzed as described in the Materials and Methods section. The amount of TF was approximately fivefold increased in the linear segments and approximately eightfold at the branches of septic baboon arteries as compared to controls, whereas the normal baboons showed approximately similar TF content in the two arterial regions (Figure 7a). When TF-FVIIa activity was assessed, the branch segments of septic animals showed threefold increase as compared to equivalent tissue samples from normal animals, whereas the straight segments of septic animals showed only $\approx 70\%$ increase (Figure 7b).

Discussion

We used a well characterized model of severe sepsis to study site-specific expression of TF and its natural inhibitor TFPI in the arterial wall. We found that TF is present in the vascular wall of septic animals, especially in areas exposed to disturbed blood flow. In septic baboons, the vascular content of TF mRNA was threefold higher than the healthy ones. In addition, we detected preferential accumulation of large amounts of TF in ECs, leukocytes,

Figure 5. Confocal imaging of TF-expressing cell types, *in situ* markers of apoptosis, and TF-dependent coagulation in the arterial branch area of septic baboons. Images in **a** and **b** are maximum intensity projections of *xy* images generated by *z*-stack rendering to integrate information from the thickness of the endothelium. The *xz* projections are shown on the **right** side of each maximum intensity projection image. The **double arrows** on the *xz* images show the rendered intima thickness. All micrographs are three-channel images (red, green, and blue). Several combination colors are obtained when two or all three fluorophores are co-localizing in the same place: red and green leads to yellow; red and blue to purple; green and blue to aquamarine; red, green, and blue to white, and so forth. In **a**, surface-located TF (red) co-localizes to a large extent with ECs labeled by anti-CD31 antibody (green; co-localization: yellow, **arrowhead**). Double labeling for TF (**b** and **c**, green) and CD68 (**b**, red) or myeloperoxidase (**c**, red) shows that TF is highly expressed by CD68-positive macrophages (**b**, yellow; **arrow**) and is present to a lower extent on PMNs stained for myeloperoxidase (**c**, **arrow**). **d**: Combined *in situ* TUNEL staining (green) and immunostaining for TF (red) reveals the presence of TF-positive apoptotic cells (**arrows**) within the endothelial layer and subendothelial area of arterial branches. **e**: Double immunostaining for TF (green) and factor VII (red) within the intima of arterial branches in septic baboons. TF co-localizes with factor VII (yellow/white) on the ECs and intima-located leukocytes. **f**: Double immunostaining for TF (green) and antithrombin-serine protease complexes (red, co-localization in yellow) demonstrates that thrombin was generated *in situ* on the surface of TF-bearing cells. The presence of TF-positive activated platelets (**g**) and fibrin (**h**), provides additional evidence of ongoing coagulant activity within the intima of septic baboons. TO-PRO3 nuclear (N) staining is shown as blue. Scale bars, 50 μ m.

platelets, and particles located in the subendothelial region of the branches, which contributes to a local increase in thrombin generation and fibrin formation.

Animal and human studies have established a critical role for the extrinsic pathway of coagulation in sepsis. In a nonhuman primate model, inhibition of TF leads to

Figure 6. Expression level of TF mRNA measured by quantitative real-time polymerase chain reaction in mRNA extracted from arterial segments of healthy and septic baboons (2 hours after *E. coli* challenge). The data were normalized to 18S ribosomal RNA. The values shown are the means of triplicate measurements $\pm$ SEM.

down-regulation of thrombin generation and improved survival.^{11,17–19} Administration of lipopolysaccharide in human volunteers results in TF-FVIIa-dependent generation of thrombin.²⁰ Endotoxemia leads to increased TF on the surface of inflammatory monocytes,²¹ neutrophils,²² and some ECs.²³ TF expression by ECs treated *in vitro* with lipopolysaccharide is well established, but the *in vivo* response is controversial. It was postulated that differential activation of endothelial TF expression might occur in distinct microvascular beds, particularly during severe sepsis.^{23,24} However, to date, there has been no experimental evidence supporting TF expression and activation in the large vessels in relation to arterial hemorheology and sepsis.

In this study we used a novel imaging method that allowed us to detect for the first time site-specific differences of TF expression and deposition within the intercostal branching points versus nonbranched regions of the aortas of septic baboons. Confocal analysis of whole-mount arterial segments after immunostaining revealed that subtle changes occurred on the endothelial surface

and within the thickness of the intima. We found that TF was located on the endothelial surface, platelet-rich microthrombi, and adherent or transmigrated leukocytes. Among these, monocytes/macrophages displayed considerably more staining than neutrophils. Whereas monocytes seem to represent the major source of induced intravascular TF expression *in vivo*,²⁵ the expression of TF by neutrophils and platelets is still controversial.^{22,26,27} Although we have clearly detected TF on neutrophils and platelet microthrombi in septic baboons, we cannot determine whether TF was locally synthesized or it was acquired from circulating particles via P-selectin/PSGL-1 interactions.²⁸ In ECs, TF appeared in granular structures heterogeneous in size, located mainly on the cell surface. Neither in this case did our data distinguish whether TF was produced by ECs or was transferred to the EC surface from blood-borne microvesicles. Finding that part of these particles also contains PSGL-1 represents a strong indication that leukocyte-derived microvesicles might deliver TF to the EC surface.

Large amounts of TF-bearing particles of multiple cell origin were detected in the subendothelial space of arterial branches. Similar to the particles that are attached to the endothelium, this subendothelial TF pool also co-localized to a large extent with PSGL-1, again suggesting a leukocyte origin. We could not determine whether the subendothelial particles were derived from blood- or tissue-located leukocytes. The compromised endothelial permeability at branching points, together with the fact that the TF-containing particles accumulated preferentially underneath the area with EC denudations support their blood origin. On the other hand, TF-bearing particles appeared in close proximity to the transmigrated leukocytes accumulated at the arterial branches, thus suggesting that these cells may contribute to the extracellular TF pool. The possible mechanisms of TF accumulation within the intima of arterial branches in septic baboons are summarized in Figure 8.

TF localized in the subintima may play a defensive role by promoting fibrin deposition, which supports the seal-

Figure 7. Biochemical analysis of TF production and activity. TF antigen (a) and activity (b) were significantly increased within the aortic intima of *E. coli*-challenged animals, particularly at the branching points.

Figure 8. Working diagram illustrating the possible sources of TF detected at the branching points of arteries in septic baboons. Cellular-associated TF may be produced by the circulating, adherent, or extravasated leukocytes, as well as by ECs. TF-rich particles could transfer TF from circulating leukocytes to platelets and ECs, as well as penetrate directly into the subendothelial space. In addition, the extracellular TF pool detected in the subendothelium may represent microparticles derived from adherent or extravasated leukocytes, platelets, and apoptotic cells. The TF detected at the branches of arteries in septic baboons is functionally active, supporting local thrombin generation, platelet activation, and fibrin deposition.

ing of endothelial gaps and protecting against bacterial invasion into tissues. Conversely, fibrin- and platelet-rich microthrombi could embolize to various organs, and consequently contribute to DIC.

Previous studies did not reveal the presence of TF in the aorta of baboons treated with *E. coli*.²³ This could be explained in part by the fact that the aortic branches were not systematically examined, and in part by differences in the methodology. Our novel whole-mount immunostaining approach provides much more information on TF localization than could be obtained from studies using conventional cross sections. Moreover, computer-assisted three-dimensional rendering of Z-sections permits the reconstruction of the complex three-dimensional morphology of aortic intima areas, as they appear *in situ*, and thus facilitates objective interpretation of variations in TF content, cell composition, and morphology.

Our confocal and electron microscopy data revealed that severe sepsis induced dramatic structural changes, including nuclear condensation or vacuolization, cell shape changes, such as cytoplasmic attenuation, shrinkage, or fragmentation, large gaps between ECs or frank endothelial denudation, and/or cell detachment. In addition, *E. coli* sepsis induced massive edema and leukocyte adhesion and trafficking through the intima, leading to a fivefold to eightfold increase of the intima's thickness at branch points. Whereas the vasculature of healthy baboons was covered by a continuous endothelium displaying normal tight junctions, the endothelium of septic animals was loosely connected and showed increased permeability to plasma proteins, such as fibrinogen, resulting in massive tissue edema. This aspect is well documented in the microvasculature but not in the large vessels, and can be induced by cytokines,²⁹ NO,³⁰ or thrombin,³¹ acting either alone or in a synergistic way.³¹

We demonstrate here that the combined effect of sepsis and focally perturbed hemodynamic forces at the branch points may contribute to a procoagulant state of the endothelium and its subjacent structures. In addition to their own procoagulant properties, the activated ECs covering the branching points can attract platelets, monocytes, and neutrophils, which further increase the local procoagulant potential. Also, ECs and other vascular cells undergoing apoptosis may express an increasingly procoagulant phenotype.^{32,33}

Our data show that the response of the endothelium to inflammation differs greatly in relation to the spatial location and the hemodynamic environment. The net procoagulant phenotype observed at branching points could result from the up-regulation of TF. ECs located in regions of altered hemodynamic forces show activation of nuclear factor- κ B and Egr-1 transcriptional networks, which control TF expression.^{34,35} Chronically decreased blood flow in rabbits stimulates VCAM-1 expression and enhances monocyte adhesion,³⁶ suggesting a connection between mechanical forces and leukocyte trafficking. In this context, we suggest that the low systolic pressure characteristic of sepsis may be responsible in part for the observed increase in leukocyte recruitment and TF expression.

In parallel with the focal up-regulation of TF, we observed decreased fluorescence intensity for TFPI at branching points. These findings correlate well with our previous *in vitro* results showing that ECs produced lower amounts of TFPI when exposed to low shear forces.³⁷ We can speculate that the development of a low blood-flow state in sepsis, whether secondary to reduced cardiac output, vasodilatation, or microthrombi occlusion, may decrease the shear forces, which would subsequently diminish TFPI expression and lengthen the clearance of activated serine proteases, and thus promote additional clotting.

Overall, our *in vivo* studies suggest that the combination of mechanical forces and sepsis challenge can induce morphological and functional changes to the endothelium at regions of disturbed flow, and may therefore contribute to the increased local thrombogenicity of the arterial wall.

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