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Regular Article

Procoagulant tissue factor activity on microparticles is associated with disease severity and bacteremia in febrile urinary tract infections ☆

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ABSTRACT

Introduction: Inhibition of tissue factor, the primary initiator of coagulation in sepsis, attenuates morbidity in primates infused with *Escherichia coli*. In a human endotoxemia model, microparticles expressing procoagulant TF (MP-TF) are released in blood concurrently with markers of inflammation and coagulation. We investigated whether the release of MP-TF into blood is accompanied by procoagulant and inflammatory changes in patients with *E. coli* urinary tract infection.

Materials and methods: In a multicenter cohort study, we determined clinical disease severity using APACHE II scores and measured plasma MP-TF activity, TAT, sE-selectin, sVCAM-1, procalcitonin and monocyte count in blood of 215 patients with community-acquired febrile *E. coli* urinary tract infections.

Results: Plasma MP-TF activity on admission corresponded with clinical disease severity (APACHE II score; $P = 0.006$) and correlated significantly but weakly with plasma markers of disease severity (sE-selectin, sVCAM-1, procalcitonin). Additionally, median plasma MP-TF activity was higher in patients than in healthy controls (197 vs. 79 fM Xa/min; $P < 0.0001$), and highest in bacteremic patients (325 fM Xa/min). MP-TF activity showed a weak inverse correlation with monocyte count (r_s -0.22; $P = 0.016$) and a weak correlation with TAT (r_s 0.23, $P = 0.017$). After 3 days of antibiotic treatment, upon resolution of the infection, plasma MP-TF activity and TAT concentrations declined.

Conclusions: Microparticle-associated procoagulant tissue factor activity is related to disease severity and bacteremia in febrile *E. coli* UTI patients and may contribute to the prothrombotic state in gram-negative sepsis.

Introduction

Sepsis is associated with activation of the coagulation cascade, which may range from subclinical to widespread microvascular thrombosis and disseminated intravascular coagulopathy. Blood microparticles are highly mobile carriers of pro-inflammatory mediators and procoagulant proteins and could play a major role in the onset of sepsis-related morbidities and mortality.

Tissue factor (TF), the primary initiator of coagulation *in vivo*, is thought to play an important role in sepsis. In primates, inhibition of the tissue factor pathway with anti-TF monoclonal antibodies, tissue factor pathway inhibitor (TFPI) or active site-inactivated FVIIa attenuated coagulopathy and prevented acute lung injury, renal failure and mortality in septic shock caused by *Escherichia coli* [1–3]. Furthermore, in human endotoxemia models, coagulopathy improved following infusion of TFPI or recombinant human IL-10 (known to inhibit LPS-induced TF activity and monocyte TF-expression, respectively) [4,5].

In a kinetic study of healthy volunteers challenged intravenously with purified *E. coli* lipopolysaccharide, we demonstrated that microparticles bearing functional procoagulant TF (MP-TF activity) are concurrently released with markers of inflammation and coagulation [6]. Interestingly, the subject with the most prominent clinical response to endotoxin also had the highest MP-TF activity. The role of microparticles expressing tissue factor (MP-TF) in the pathogenesis of sepsis has not yet been elucidated.

Abbreviations: intensive care unit, ICU; microparticles expressing procoagulant tissue factor, MP-TF; tissue factor, TF; thrombin-antithrombin complex, TAT; urinary tract infection, UTI.

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As inflammatory conditions caused by an intact pathogen may similarly induce shedding of MP-TF, we investigated the relation between MP-TF activity, bacteremia and clinical disease severity using the APACHE II score in a large cohort of patients with febrile *E. coli* urinary tract infection (UTI). In addition, we assessed whether MP-TF activity decreased upon resolution of the infection and explored the association between MP-TF activity, markers of disease severity (sE-selectin, sVCAM-1, procalcitonin) and blood monocyte count. Finally, we examined levels of thrombin-antithrombin complex (TAT) as a marker of coagulation in a subset of patients.

Materials and Methods

Study Design

From January 2004 to December 2009, we enrolled 787 consecutive patients presenting with community-onset febrile urinary tract infections at the emergency departments of seven hospitals and 35 affiliated primary healthcare centers in the western part of The Netherlands [7]. This multi-center cohort study was approved by the Medical Ethical Committees of the participating centers and all patients gave written informed consent.

Inclusion criteria were age ≥18 years, ear temperature ≥38.0 °C or a history of fever and rigors within 24 hours prior to presentation, at least one symptom of UTI (dysuria, frequent or urgent urination, perineal pain, flank pain or costovertebral tenderness), and positive nitrite dipstick test or leukocyturia. Leukocyturia was defined as a positive leukocyte esterase dipstick test or the presence of >5 leucocytes per high-power field in the urine sediment. Exclusion criteria were current treatment for urolithiasis or hydronephrosis, hemodialysis, kidney transplantation or polycystic kidney disease.

From the initial cohort of 787 patients, we selected all 420 patients in whom *E. coli* was cultured from the urine sample obtained upon admission. We excluded 156 patients with pre-existing disorders or severe co-morbidity, i.e. cancer, autoimmune disease, diabetes, cerebrovascular accident or heart failure, because elevated plasma levels of microparticles and MP-TF activity have been reported in patients with these disorders [8–12]. This exclusion criterion resulted in a cohort of 264 fairly healthy patients at the onset of their urinary tract infection, since abovementioned disorders and co-morbidities were more prevalent amongst the majority of the critically-ill patients with the highest APACHE II scores. Another 49 patients were excluded because blood cultures or frozen plasma samples could not be retrieved, rendering a final study cohort of 215 patients.

As required by the study protocol, all patients received empirical intravenous or oral antibiotic treatment upon admission according to local hospital policy. Antimicrobial therapy consisted of intravenously administered cefuroxime (n = 89; of which 39 in combination with gentamicin) or oral treatment with ciprofloxacin (n = 106). A small number of patients (n = 20) was treated with other oral or intravenous antibiotic agents directed against gram negative micro-organisms.

Healthy volunteers without history of fever or infectious disease were recruited in the participating primary healthcare centers and amongst laboratory staff. Age and gender were similar to that of the study patients (63% female, median age 59 years [range 24-76]), as well as the protocol for blood collection and processing.

Procedures and Definitions

Clinical data and routine laboratory measurements were collected by the clinical investigators and qualified research nurses. Baseline data of the patients were obtained within 24 hours using a standardized questionnaire and by reviewing medical records. Double data entry was performed by two independent data managers and both entries were compared for discrepancies.

We calculated the most commonly used clinical disease severity score for septic patients, the Acute Physiology and Chronic Health Evaluation II (APACHE II) score. Patients were allocated to previously reported score categories, allowing assessment of the severity of the disease and providing an estimate of in-hospital mortality risk (an APACHE II score of 1-4 corresponded with a 4% mortality rate, whereas APACHE II scores of 5-9, 10-14, and 15-19 translated into an observed mortality rate of 8%, 15% and 25%, respectively) [13]. Clean midstream-catch or catheter-port urine were collected upon admission and cultured using local standard microbiological methods. Urine samples were considered infected in case of bacterial growth $>10^3$ CFU per ml urine or a bacterial monoculture $>10^2$ CFU per ml urine in the presence of leukocyturia.

Disease burden was systematically quantified in all 215 patients by taking blood cultures on admission. Patients were considered to have bacteremia when *E. coli* was cultured.

At baseline (day 0) and three days thereafter (day 3), venous blood samples were collected into EDTA [ethylenediaminetetraacetic acid] BD Vacutainer tubes (Franklin Lakes, NJ, USA) applying minimal venostasis and discarding the first tube. Plasma was prepared by removing cells through a single centrifugation step at 3500 x *g* (5 minutes at room temperature). Aliquots were transferred immediately to polypropylene tubes and frozen at -80 °C to enable future simultaneous analysis of day 0 and 3 samples. Sample processing time from venipuncture to storage at -80 °C was less than an hour for the majority of study patients and well within two hours for all patients as required by the study protocol. Plasma samples remained deep-frozen until analysis.

Isolation of Microparticles and MP-TF Activity Assay

After thawing of deep-frozen EDTA-anticoagulated plasma samples, microparticles were pelleted and repeatedly washed with pH 7.45 filtered 0.32% citrate/PBS buffer (30 minutes at 18,890 x *g* with minimum brake, 20 °C) thus diluting plasma constituents and EDTA more than 200-fold. The microparticle suspension was subsequently recalcified and incubated in a 1:5 ratio (v/v) with 10 mM pH 7.45 Hepes, 137 mM NaCl, 4 mM KCl, 5 mg/ml ovalbumin, 50 nM hirudin, 6 mM $CaCl_2$ and 25 μM negatively charged phospholipid vesicles (dioleoylphophatidylserine/dioleoylphosphatidylcholine 1/9). TF/FVII complex formation was initiated by the addition of FVII (Kordia, The Netherlands). The reaction was started by the addition of S2765 (Chromogenix, Italy) and FX (Kordia, The Netherlands). Subsequently, cleavage of the chromogenic substrate S2765 by the generated FXa was recorded during 90 minutes (absorbance at 405 nm). Parallel experiments were performed in the absence of FVII and in the presence of excess polyclonal sheep anti-human TF-IgG (Affinity Biologicals Inc., Canada) to demonstrate FVII and TF-dependency, respectively. MP-TF activity, defined as FVII- and TF-dependent FXa formation, was calculated as previously described [8] and expressed as fM Xa/min in plasma assuming a 100% microparticle recovery. None of the plasma samples used for isolation of microparticles had been thawed before and all samples were analyzed within one year after completion of the study. In previous experiments on plasma samples stored for more than 15 months, we did not observe degradation of active TF on microparticles after prolonged frozen storage; the same MP-TF activity was found in aliquotted samples from 16 patients after prolonged frozen storage.

Nineteen healthy volunteers were recruited to establish reference values for MP-TF activity, although power calculations indicated that a sample size of merely 9-10 for each group would suffice to detect a statistical significant difference. We defined elevated plasma MP-TF activity as levels >172 fMXa/min, indicating the 95th percentile of MP-TF activity of these 19 healthy controls (e.g. mean MP-TF activity + 2 SD).