Human Neutrophils Support Thrombin Generation in Peritonitis

KEY WORDS: Dialysis; inflammation; neutrophils; peritonitis; tissue factor.

In septic peritonitis, polymorphonuclear neutrophils (PMNs) are abundant in the peritoneal cavity, as they play a crucial role in the innate host defense (1). For sepsis-induced recruitment, PMNs have to migrate from the circulation to the site of infection in response to chemotactic factors including CXCL8 (interleukin-8, or IL-8), a very powerful chemotactic mediator (2). Fibrin deposition is a major mechanism for bacterial entrapment (3). This fibrin deposition is likely the result of thrombin generation. In the context of peritoneal dialysis (PD), fibrin deposition was recognized early on as a determining event in peritoneal sclerosis (4). Furthermore, patients on PD are prone to septic peritonitis. Extravascular coagulation leading to fibrin deposition has been the subject of limited investigation. Experimental models have documented the formation of thrombin in the peritoneum, indicating a plasmatic extravasation (5). Thrombin inhibition is effective in preventing fibrin deposition in such models. However, the trigger of thrombin generation remains to be fully defined. As it has been recently described that PMNs play a major role in the initiation of thrombosis (6), we hypothesize that migrated PMNs directly support thrombin generation in peritonitis. To document this hypothesis, we had the opportunity to analyze neutrophils from peritonitis fluid. Polymorphonuclear neutrophils were obtained from 3 consenting patients presenting end-stage kidney disease and currently treated with PD. We also analyzed the expression of tissue factor (TF) by purified

PMNs obtained from healthy consenting subjects. As it is known that circulating PMNs do not express TF, PMNs were stimulated by IL-8 and tumor necrosis factor (TNF) in order to mimic inflammatory conditions. Furthermore, lipopolysaccharide (LPS)-stimulated monocytes were used as positive control of TF expression.

METHODS

Peritonitis fluid was obtained in patients presenting clinical symptoms of septic peritonitis as a complication of dialysis. Bacterial analysis of the fluid confirmed the presence of *Enterococcus* (in patient 1), *Streptococcus* (in patient 2), *Staphylococcus* and *Escherichia coli* (in patient 3). Neutrophils were studied before antibiotic treatment. For neutrophil isolation, monocytes were depleted using magnetic microbeads conjugated to mouse monoclonal anti human CD-14 antibodies (MACS, Miltenyi Biotec S.L., CA, USA). Purity was 99 %.

Polymorphonuclear neutrophils were isolated from consenting healthy volunteers using Polymorphprep (Cosmo Bio USA, Inc., CA, USA). Polymorphonuclear neutrophils were stimulated with TNF- α (100 ng/mL), or IL-8 (500 ng/mL) + TNF (100 ng/mL).

To assess procoagulant activity, a thrombin generation (TG) test was performed as previously described (7). Platelet poor plasma was used, and supplemented with aprotinin (Sigma, France) at 300 kallikrein inhibitory units (KIU)/mL in order to block contact system. Ten μ L of cell suspension (5.10^6 cells/mL) were added to 80 μ L of PPP. Thrombin generation was performed in the presence of a specific antibody blocking the coagulant activity of TF (4509 American Diagnostica, MA, USA). The fluorogenic substrate, Z-Gly-Gly-Arg-AMC (Bachem, Switzerland) was added, and fluorometric determination of TG was performed using Fluoroskan Ascent plate reader (Thermo-Labsystems, Finland). Thrombinoscope software (Synapse BV, The Netherlands) was used to calculate TG parameters: lag-time (min), thrombin peak (nM), endogenous thrombin potential (ETP, nM.min).

We next measured the ability of PMNs to convert FX in FXa using a home-made chromogenic method (chromogenic substrate S-2765 [Chromogenix, Milan, Italy]).

For TF mRNA identification, total mRNA was extracted (RNeasy Mini kit, [QIAGEN, MD, USA]) and reversely transcribed into cDNA (iScript cDNA synthesis Kit, [Biorad, CA, USA]). The following primers and TaqMan probes were used: TF (Hs00175225_m1 [Applied Biosystems, CA, USA]) and β 2 microglobulin (endogenous controls) (Hs00187842_m1 [Applied Biosystems, CA, USA]). PCR mixture: PCR Master mix (Applied Biosystems, CA, USA). Applied Biosystems 7500 Fast Real-Time PCR System – software V2.03. The expression levels of TF gene were normalized to those of the reference gene, thus giving rise to δ Ct values (Ct: threshold cycle). The number of fold changes in the TF mRNA levels were calculated with the comparative Ct method $2^{-(\Delta Ct1 - \Delta Ct2)}$; corresponding to: $\Delta Ct1 = Ct_{TF} - Ct_{\beta 2m}$; $\Delta Ct2 = Ct_{TF} - Ct_{\beta 2m}$ (1: Target, 2: control).

Tissue factor protein was detected by western blot and immunoprecipitation (IP): 1=μg protein samples were loaded and separated by 10% SDS-PAGE gel. Proteins were transferred to PVDF membrane (Biorad, CA, USA) and probed with mouse anti-human TF antibody (4509 American Diagnostica, MA, USA). Membranes were incubated with anti-mouse secondary antibody conjugated to horseradish peroxidase. Detection of the labeled proteins was performed by chemiluminescence using ECL from GE Healthcare. Tissue factor immunoprecipitation (IP) was realized by ImmunoCruz IP/WB Optima B System (Santa Cruz Biotechnology, CA, USA), according to the manufacturer's instructions. Two μg IP rabbit antibody anti-human tissue factor (4509 American Diagnostica, MA, USA) was used. Immunoprecipitated TF was detected by western blot with mouse anti-human tissue factor antibodies (4509 American Diagnostica, MA, USA).

RESULTS AND DISCUSSION

Our observations indicate that PMNs purified from peritonitis fluid support thrombin generation in the absence of exogenous TF and in the presence of aprotinin (Figure 1). This suggests the involvement of TF in PMN-supported thrombin generation. In agreement with this hypothesis, we noticed a reduction of the velocity of thrombin generation with a prolongation of the lag time and a decrease of the thrombin peak and the extrinsic thrombin potential (ETP). Tissue factor protein was visualized by western blot and immunoprecipitation to improve specificity. In comparison, TF expression was weak in unstimulated purified PMNs from healthy subjects. In these cells, TF expression was strongly induced by TNF and IL-8. As it is well established that monocytes express large amounts of TF in response to LPS, we analyzed this expression

Figure 1 — Thrombin generation and TF expression by purified PMNs obtained from PD patients with peritonitis. A: Flow cytometry (FC500 Beckman Coulter): PMNs from peritonitis fluid, after monocyte depletion (cells are fluorescently stained with CD45-PC5). B: TF protein expression: Western blot analysis of protein extracts from purified human monocyte activated with LPS (100 ng/mL) (positive control), purified PMNs from peritonitis fluid and TF immunoprecipitation (IP), PMNs purified from healthy individuals (control), or stimulated with TNF-α (100 ng/mL) or the combination of IL-8 (500 ng/mL) + TNF (100 ng/mL) C: TF activity measured by a home-made Xa generation amidolytic assay was measured on Xa-substrate S-2765 (Chromogenix, Milan, Italy). One TF unit is defined as the activity of recombinant human TF (DadeInnovin, Siemens, PA, USA) diluted at 1/1000. Effect of anti TF antibody (AbTF, 10 μg/mL) (4509 American Diagnostica, MA, USA). D: TF activity measured by thrombin generation assay (calibrated automated thrombinography (CAT), effect of anti TF antibody (AbTF, 10 μg/mL) (4509 American Diagnostica, MA, USA). PMN = polymorphonuclear neutrophils; PD = peritoneal dialysis; TF = tissue factor; LPS = lipopolysaccharide; IP = immunoprecipitation; TNF = tumor necrosis factor; IL = interleukin; IgG = immunoglobulin G.

in monocytes highly purified by elutriation (7). We next identified TFmRNA in purified PMNs and quantified the level of mRNA in comparison with unstimulated PMNs from healthy individuals. Delta Ct ratios were: Patient 1:63 Patient 2: 169, Patient 3: 247. Taken together, our results indicate that PMNs present in septic peritonitis fluid are procoagulant in a TF-dependent manner. To the best of our knowledge, it is the first time that it has been shown that TF is the main trigger of thrombin generation in human peritonitis. Animal models indicate that increased vascular permeability leading to plasma exudation is among the earliest signs of inflammation (5). In peritoneal sclerosis, a “vasculopathy” has been described as a result of inflammatory stimulation due to PD and resulting in progressive peritoneum deterioration. Infectious peritonitis could represent a second “hit” in the pathogenesis of encapsulating peritoneal sclerosis. Peritoneal macrophages could also play a role in peritonitis and peritoneal sclerosis, as evidenced in a model of thioglycollate-elicited peritonitis. In this model, it was shown that thrombin generation regulated chemokine and cytokine production. In our experiments, we carefully selected PMNs, discarding CD14-positive cells.

One limitation of this study is related to the small sample size due to the rarity of peritonitis. Furthermore, we did not have the opportunity to study circulating PMNs in patients during the course of peritonitis. However, it is known that TF is barely detectable in circulating PMNs. Here, we show that neutrophils that have migrated in the peritoneal cavity during peritonitis strongly express TF. We also show that TNF, an important cytokine during sepsis, strongly induced TF expression in normal PMNs. Interestingly, IL-8, a strong chemokine involved in PMN migration, reinforced the effect of TNF. In spite of the limited number of observations, these data prove the concept that PMNs express TF in inflammatory conditions. This is in agreement with the recent observation of TF expression by PMNs in human sepsis (8). These authors report that serum obtained from septic patients induced TF expression in association with NET (neutrophils extracellular TRAPs) formation. In peritonitis, TF expression is linked to the process of PMNs migration to the site of infection.

Several issues should be kept in mind when interpreting our data. It was recently demonstrated that the TF/FVIIa pathway was involved in a mouse model of abdominal sepsis induced by *Escherichia coli* (9) and that the inhibition of this pathway by recombinant nematode anticoagulant protein c2 (rNAPc2) did reduce thrombin generation and fibrin deposition without affecting the inflammatory or antibacterial response. In experimental polymicrobial peritonitis, it was demonstrated that a peritoneal lavage with recombinant human antithrombin (rhAT) inhibited coagulation and reduced neutrophil recruitment and local cytokine concentrations, ultimately improving survival (10). The inhibition of TF may therefore be useful to avoid fibrin deposition in organs without compromising the host defence. However, the role of intra-abdominal fibrin is ambiguous as it is an adaptive response precluding bacterial spread but it also enhances

abscess formation. Another issue regarding the importance of fibrin deposition is to elucidate whether PMNs and TF are involved in peritoneal sclerosis in PD, in the absence of sepsis. Peritoneal sclerosis is indeed a major complication of PD associated with fibrin deposition. It should be interesting to evaluate whether the inhibition of thrombin generation is effective in preventing peritoneal sclerosis. Our data are a good example of the cross-linking between coagulation and inflammation and illustrate that extravascular coagulation is involved in the pathogenesis of peritonitis, indicating possible therapeutic targets.

DISCLOSURES

The authors have no financial conflicts of interest to declare.

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REFERENCES

1. Broche F, Tellado JM. Defense mechanisms of the peritoneal cavity. *Curr Opin Crit Care* 2001; 7:105–16.
2. Craciun FL, Schuller ER, Remick DG. Early enhanced local neutrophil recruitment in peritonitis-induced sepsis improves bacterial clearance and survival. *J Immunol* 2010; 185:6930–8.
3. van Veen SQ, Meijers JC, Levi M, van Gulik TM, Boermeester MA. Effects of intra-abdominal administration of recombinant tissue plasminogen activator on coagulation, fibrinolysis and inflammatory responses in experimental polymicrobial peritonitis. *Shock* 2007; 27:534–41.
4. Honda K, Oda H. Pathology of encapsulating peritoneal sclerosis. *Perit Dial Int* 2005; 25(Suppl 4):S19–29.
5. Szaba FM, Smiley ST. Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion in vivo. *Blood* 2002; 99:1053–9.
6. Darbousset R, Thomas GM, Mezouar S, Frere C, Bonier R, Mackman N, et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood* 2012; 120:2133–43.
7. Nguyen P, Broussas M, Cornillet-Lefebvre P, Potron G. Coexpression of tissue factor and tissue factor pathway inhibitor by human monocytes purified by leukapheresis and elutriation. Response of non adherent cells to lipopolysaccharide. *Transfusion* 1999; 39:975–82.
8. Kambas K, Markiewski MM, Pneumatikos IA, Rafail SS, Theodorou V, Konstantonis D, et al. C5a and TNF-alpha up-regulate the expression of tissue factor in intra-alveolar neutrophils of patients with the acute respiratory distress syndrome. *J Immunol* 2008; 180:7368–75.
9. Weijer S, Schoenmakers SH, Florquin S, Levi M, Vlasuk GP, Rote WE, et al. Inhibition of the tissue factor/factor VIIa pathway does not influence the inflammatory or antibacterial response to abdominal sepsis induced by *Escherichia coli* in mice. *J Infect Dis* 2004; 189:2308–17.

10. van Veen SQ, Cheung CW, Meijers JC, van Gulik TM, Boermeester MA. Anticoagulant and anti-inflammatory effects after peritoneal lavage with antithrombin in experimental polymicrobial peritonitis. *J Thromb Haemost* 2006; 4:2343-51.

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