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Full Length Article

Tissue factor expressed by adherent cells contributes to hemodialysis-membrane thrombogenicity

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ABSTRACT

End-stage renal patients present a high risk of thrombosis and bleeding. Consequently, it is challenging to prevent clotting during hemodialysis. If a contact system induces thrombin generation in the extra corporeal circuit, recent data suggest a role of tissue factor (TF) in hemodialysis-associated thrombosis. Using a method of elution, we collected adhering cells to an acrylonitrile membrane layered by polythyleneimine (AN69-ST). Using optic microscopy and flow cytometry, we observed that adherent cells were mainly constituted by activated polymorphonuclear neutrophils (PMNs). Using a sensitive fluorogenic method of thrombin generation, we found that adhering cells triggered thrombin generation in a TF-dependent manner. We next identified the presence of TF mRNA (Q-PCR) in adhering cells. Using immunofluorescence, we observed the presence of TF in PMNs and of TF-decorated neutrophil extracellular traps (NETs). As TF triggers thrombin generation after binding to serine protease FVIIa, we evaluated the effect of an inactivated human recombinant factor VIIa (hrFVIIai) in a sheep model of hemodialysis (HD). One single bolus of hrFVIIai maintained the full patency of the hemodialysis circuit without any measurable systemic anticoagulant effect. TF is a promising target for preventing thrombosis during HD.

1. Introduction

The prevention of clotting is a crucial issue in hemodialyzed-ESRD patients [1]. Early attempts at extracorporeal circulation used hirudin to prevent coagulation until heparin became the drug of choice. The major drawback of heparin is that it increases bleeding in ESRD patients who frequently present combined hemostatic disorders [2]. Low molecular weight heparins and fondaparinux have been used in such situations but remain contraindicated in ESRD patients [3]. In parallel, the community has focused on understanding the biocompatibility of hemodialysis (HD) devices. Selective adsorption of plasmatic high molecular weight kininogen and prekallicrein on "negatively-charged" HD membranes has been found to trigger [4] the so-called "intrinsic coagulation pathway". However, it was reported that thrombin generation induced by extracorporeal circulation was comparable in factor XII (FXII)-deficient and -non deficient patients [5]. This suggests the paradigm that the TF-dependent pathway of initiation of coagulation may apply to dialysis membranes. Indeed, tissue factor (TF), the major trigger of coagulation *in vivo*, binds with a very high affinity to coagulation factor VII(a) to form the so-called "extrinsic tenase complex" which subsequently catalyzes the conversion of factor X to FXa and of factor IX to IXa.

As the contact of blood with biomaterials activates leukocytes and platelets, these cells may be the source of TF. It is well-established that monocytes represent a major source of TF. TF expression is not constitutive but is induced by strong stimulation such as increased adhesion [6,7]. Neutrophils and leukocyte-derived microparticles may also be a source of TF.

Indeed, it was recently reported that PMNs from ESRD patients expressed TF during the course of HD sessions, in response to complement anaphylatoxin C5a [8].

The aim of this study is to analyze which blood cells have adhered to an acrylonitrile membrane during HD and if this cellular material could trigger thrombin generation. As TF is the main trigger of coagulation, we extensively searched for its expression in the adherent cell material.

2. Methods

2.1. Reagents and antibodies

Reagents and antibodies used in the study include phosphate buffered saline (PBS) (Biomerieux™), paraformaldehyde (PFA) (Electron

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Microscopy Sciences), mouse anti-human H3-Cit monoclonal antibody clone (7C10) (Abcam), and mouse anti-human TF monoclonal antibody clone (IIID8) (4509 American Diagnostica).

2.2. Elution of adherent cells from the hemodialyzer membrane

The hemodialyzer membrane that we studied is an acrylonitrile membrane layered by polythyleneimine (AN69-ST, Hospal-Gambro); this membrane is not coated. Elution was adapted from Grooteman et al. [9,10]. At the end of HD, the dialysis circuit (lines and AN69-ST dialyzer) was rinsed with 2.5 L PBS, through the arterial line, at 200 mL/min. The circuit was filled with 250 mL PBS containing 3 mmol/L EDTA. This solution circulated in a closed circuit for 20 min. After centrifugation, pellets were pooled for cell counting.

2.3. Western blotting

Ten-microgram protein samples were loaded and separated by 10% SDS-PAGE gel. Proteins were transferred to a PVDF membrane (Biorad), and probed with a mouse anti-human TF antibody (4509). Membranes were incubated with an anti-mouse secondary antibody conjugated to horseradish peroxidase. Detection of the labeled proteins was performed by chemiluminescence using ECL from GE Healthcare.

2.4. Fluorescence microscopy

Adherent cells were cytocentrifugated for 10 min at 125 g and fixed for 15 min at 4 °C in PBS containing 4% PFA, then blocked for 30 min in PBS BSA 3% buffer and incubated at 4 °C overnight with an anti-human TF antibody or isotype control, followed by 2 h incubation with an Alexa 488-conjugated secondary antibody. Between each step, cells were rinsed with PBS. Images were visualized by fluorescence microscopy (Zeiss Axiovert 200, Metamorph).

For NET staining, the dialysis membrane was extracted after the dismantling of the hemodialyzer. Adherent cells were fixed for 15 min at 4 °C in PBS containing 4% PFA. Cells were blocked for 30 min with PBS BSA 3% buffer and incubated at 4 °C overnight with the anti-human TF antibody or an antibody against H3-Cit, followed by incubation with secondary antibodies coupled to Alexa Fluor 488. To label DNA 4,6-diamidino-2-phenylindole (DAPI) was used.

2.5. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was extracted using the RNeasy Mini kit® (Qiagen). One microgram RNA was reversely transcribed into cDNA with the iScript cDNA synthesis Kit® (Biorad).

The following primers and Taqman probes were used as endogenous controls: TF (Hs00175225_m1; Applied Biosystems) and β2 microglobulin (Hs00187842_m1; Applied Biosystems). The PCR mixture used was PCR Master mix® (Applied Biosystems). Applied Biosystems 7500 Fast Real-Time PCR System-software V2.03. Results were normalized using the following formula: $2^{-\Delta Ct}$ corresponding to: $\Delta Ct = (\text{target} - \beta 2m)$.

2.6. TF activity assays

Actichrome ®TF activity assay (American Diagnostica) was used to determine the TF activity of the lysates of adherent cells. Protein samples were applied as described in the manufacturer's manual. A standard curve was constructed by TF standard. The results are expressed as TF nM/g of protein.

2.7. Fluorogenic measurement of thrombin generation

TG tests were performed as previously described [11]. PPP was supplemented with aprotinine (Sigma) at 300 kallikrein inhibitory units (KUI)/mL. Ten microliters of cell suspension ($5 \cdot 10^6$ cells/mL) were added to 80 μL of PPP. The fluorogenic substrate, Z-Gly-Gly-Arg-AMC (Bachem, Switzerland) was added, and fluorometric determination of TG was performed using a Fluoroskan® Ascent plate reader (ThermoLabsystems, Finland). Thrombinoscope™ software (Synapse BV, The Netherlands) was used to calculate the following TG parameters: lag-time (min), thrombin peak (peak, nM), and endogenous thrombin potential (ETP, nM·min).

2.8. In vivo sheep hemodialysis model

All animals received care in compliance with the European convention on animal care. The study was approved by the institutional Ethics committee of the Veterinary School of Lyon, France. Experiments were conducted on 4 clinically healthy, female Texel adult sheep, weighing 68 kg–73 kg. To conduct the vascular HD experiments, access was obtained by placing an arteriovenous shunt (catheters connected the carotid artery and the jugular vein), under general anaesthesia. Experiments were performed with a HD device using an AN69-ST® hemodialyzer. The circuit was previously rinsed with isotonic saline and the dialysate compartment was filled with saline in the absence of flow. Blood circulated in this circuit at 37 °C at a flow rate of 250 mL/min. HD was performed after the injection of either unfractionated heparin (UFH: 50 UI/kg bolus, followed by continuous infusion at the dose of 800 UI/h) or a single bolus of hrFVIIai (1 mg/kg) or saline. To assess circuit clotting, ΔP was monitored, with an increase in ΔP indicating clotting (Fig. 5A). During HD, blood samples were collected. The first blood sample was collected before the start of a hemodialysis session. Further samples were collected after the administration of hrFVIIai bolus.

The concentration of hrFVIIai in the sheep plasma was determined with an enzyme-linked immunosorbent assay (Asserachrom® VII:Ag (Diagnostica Stago)).

2.9. Coagulation studies

To determine Prothrombin time (PT) and activated partial thromboplastin time (APTT), blood was collected in sodium citrate and centrifuged at 2500*g* for 10 min to separate plasma. The following reagents were used: APTT: CK Prest® Reagent (Diagnostica Stago); PT: Neoplastine® CI Plus 5 (ISI = 1.3), and Neoplastine® CI 5 (ISI: 2) (both from Diagnostica Stago). Measurements were performed within 30 min after sampling.

The experimental conditions for TG measurement are described above. Sheep PPP was spiked with 10 μL of TF human recombinant Innovin® (Siemens) at 1 pM.

2.10. Statistical analysis data

Results were expressed as mean ± SEM or box plots. Comparisons within the same groups were performed using a paired *t*-test and Wilcoxon test. Statistical analysis was performed with Prism 5 software (GraphPad Software Inc.). A *p*-value of <0.05 was considered as significant.

3. Results

3.1. Characterization of cells adhering to dialyzer membranes

The first step of our study was to determine whether circulating blood cells could adhere to the membrane of the AN69-ST dialyzer. Adherent cells ($22.10^6 \pm 10$ cells) mainly contained leukocytes and platelet aggregates as observed in optical microscopy (Fig. 1A). The distribution of leukocytes was: PMNs (84 ± 7%), monocytes (6 ± 3%) and ~1% of lymphocytes (Fig. 1B). PMN activation was assessed by the expression of CD63, CD66b and CD11b (Fig. 1C). As expected, PMNs