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Full Length Article

Endothelial cell surface limits coagulation without modulating the antithrombin potency☆

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ABSTRACT

Antithrombin (AT) binds *in vitro* and *in vivo* to endothelial cells through various receptors, including heparan sulphate glycosaminoglycan (HSPG) that could modulate the AT activity. A thrombin generation assay (TGA) was set up at the surface of HUVEC and HMVEC evaluating their participation in the coagulation-anticoagulation processes. TGA induced by 0.5 pM Tissue Factor was performed in normal or AT-deficient plasma spiked with various amounts of recombinant or plasma-derived AT (0, 0.1, 0.5 and 1.0 U/ml). To evaluate the role of HSPG or cellular anticoagulant receptors, cells were treated or not with heparin, a mix of heparanase I, II and III, a neutralizing anti-Endothelial Protein C Receptor (EPCR) or with an anti-Tissue Factor Pathway Inhibitor (TFPI) antibody. The presence of the cells diminished the TG in normal plasma and maintained anticoagulation in AT-deficient plasma. Spiking the AT-deficient plasma with different doses of AT demonstrated that the cells did not amplify the anticoagulant activity of AT. The recombinant AT binds the cells with a higher avidity than the plasma-derived one but this did not affect its anticoagulant potency. Moreover both bindings are independent of the HSPG. The antithrombotic activity kept in absence of AT was not inhibited by blocking antibodies directed against EPCR or TFPI. Our data did not reveal a major co-factor activity for AT from endothelial cells that could have been mediated by HSPG. In contrast, it reveals the presence of alternative anti-coagulant system(s) in two venous cell types that maintain an antithrombotic activity.

1. Introduction

The process of coagulation is tightly and permanently controlled within blood vessels by the plasma and the cellular components. Antithrombin (AT) is the main coagulation inhibitor, targeting several enzymes participating in the clotting process [1]. It efficiently inhibits thrombin and factor Xa and, in a lesser extent factor VIIa and factor IXa [2].The anticoagulant function of AT is amplified by two to three orders of magnitude in the presence of heparin or heparin derivatives. Heparin is known to process by different molecular mechanism of action, either modulating the efficacy of AT for its target or bridging the target with AT [3]. AT was shown *in vitro* and *in vivo* to bind endothelial cells and the extracellular matrix with an affinity close to its affinity for heparin [4]. Heparan-sulphate proteoglycans (HSPG) are heparin-like molecules constituents of the endothelial cells glycocalix, lining the vessel wall and also present in the endothelial extracellular matrix. A binding of AT to HSPG was thus suspected to favor the anticoagulant activity of AT by increasing *in situ* its potency.

The interaction of AT with the vessel wall was shown maintaining the vascular homeostasis and protecting the vessel wall constituents. It helps in preserving the vessel surface integrity by limiting the HSPG shedding during ischemia/reperfusion [5]. Through the binding of members of the syndecan family, AT was also demonstrated to support other functions than its main anticoagulant property such as anti-inflammatory, anti-angiogenic and anti-chemiotactic properties [6–10].

The development of global assays evaluating coagulation helps measuring the influence of specific parameters or components during the clotting. Among these global assays, the thrombin generation assay (TGA) consists in measuring the appearance of thrombin in function of time in a plasma-phase, sometimes in the presence of platelets [11]. This technic is now widely used to identify and quantify blood defects as well as assessing the efficiency of pharmaceutical compounds. The relevance of this assay can be illustrated, for example, by its use with success *ex vivo* to individualize treatments prior to clinical interventions on severe hemophilia A patients [12].

Recently, a TGA was performed in the presence of an endothelial

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Anticoagulant potency of the endothelium.
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cell line evaluating their participation to the coagulation process [13]. This elegant assay demonstrated the possibility of combining a living endothelial cell surface and the measurement of the thrombin generation. As the endothelial cells express at their surface, the tissue-factor plasma inhibitor (TFPI) and the endothelial protein C receptor (EPCR), this combination brought the cellular components of the anticoagulation in an assay that usually evaluate only the plasmatic components of the coagulation [14–16]. Coll et al. were thus able to show that the anticoagulant potential of the cells was more pronounced than spiking the plasma with thrombomodulin. Their anticoagulant potential was mediated through the activated protein C pathway (APC) whereas the TFPI had a negligible influence in this system [13].

To evaluate the role of the endothelial cell surface, and in particular the HSPG, in functionally modulating the anticoagulant potency of AT, we set up a similar assay using human umbilical vein endothelial cells (HUVEC) and microvascular endothelial cells from derma (HMVEC-D) as a recipient surfaces. The potencies of a plasma-derived AT (pdAT) and a recombinant AT (recAT) were comparatively evaluated in this assay [17].

2. Material and methods

2.1. Material

HUVEC (ref. C2519A) and HMVEC-Dermal (ref. CC-2543) and their specific culture media (EGM2 and EGM-2MV, respectively) were purchased from Lonza (Levallois-Perret, France). Cells are a pool from different donors. Antithrombin depleted plasma was from Cryopep (FDPAT-10, Montpellier, France). Anti-EPCR is from Millipore (MABS1271, Molsheim, France) and anti-TFPI is from SEKISUI (ADG4903, Roermond, The Netherlands). Enzymes heparanase I (H2519-50UN), heparanase II (H6512-10UN) and heparanase III (H8891-10UN) were purchased from Sigma Aldrich (L'Isle d'Abeau, France). Anti-HSPG is from Amsbio (ref. 370255-1) and its secondary antibody anti-mouse IgM (ref. 626820) from Life Technology. Heparin CHOAY is from (Sanofi-Aventis, Paris, France). Atryn®, a recombinant AT (recAT) produced in transgenic goat milk and Aclotine®, a human AT (pdAT) purified from plasma, were freeze-dried and were provided by LFB USA (Framingham, MA, USA) and LFB Biomédicament (Les Ulis, France), respectively [17]. Protein concentrations were determined with Coomassie® plus Protein Assay Reagent Kit (Thermo Fischer Scientific, Illkirch, France).

2.2. Antithrombin preparation

To avoid potential effects on cells due to the different formulation buffers from Atryn and Aclotine, both AT were dialyzed against an HEPES buffer (25 mM HEPES, 175 mM NaCl, pH 7.4) known to have no effect on cells and on coagulation (data not shown). Freeze-dried AT were dissolved as recommended in 10 ml of water, 3-fold concentrated and dialyzed against the HEPES buffer using a Macrosep Advance centrifugal Device 30 K MWCO (Pall, Saint Germain en Laye, France). AT concentration was determined by the Bradford method and the activity of the molecules was controlled with a chromogenic method from Stago (STATCHROM ATIII, Stago, Asnières). The pdAT and recAT were also controlled to similarly restore the anticoagulant activity in AT-deficient plasma (Supplementary fig. 1).

2.3. Cell culturing

HUVEC were maintained in full EGM2 medium and sub-cultured at a density of 3000 cells/cm^2. HMVEC were maintained in full EGM-2MV medium and sub-cultured at a density of 3500 cells/cm^2. The cells were incubated at 37 °C in a humidified incubator under 5% CO_2. The medium was changed every 2 or 3 days until the cells reached 80% confluence. Cells were then trypsinized and re-seeded. Only cells that underwent 2 to 6 passages were used for the experiments.

2.4. Cell ELISA

HUVEC and HMVEC were respectively seeded at 5000 cells/well and 6000 cells/well in 96-wells plates. After reaching the confluence, AT was added into the wells. The cells were further incubated at different times at 37 °C in a humidified incubator under 5% CO_2. The medium was carefully removed. Cells were gently washed three times with PBS (300 µl) and fixed with 100 µl of 1% paraformaldehyde in PBS for 30 min at room temperature. All cell ELISA with HUVEC were also performed on living cells and the results were similar (data not shown). Cells were then washed four times with TBPS (PBS + 0.1% Tween-20) and incubated with PBS + 2% BSA overnight at 4 °C. After washing the wells twice with TPBS, the polyclonal goat anti-human AT (Ref. AF1267, R&D Systems, Lille, France) (600 ng/ml in PBS + 1% BSA) was added and the plate was incubated for 2 h at room temperature. After carefully washing the cells 4 times with TPBS an HRP-conjugated anti-goat IgG (40 ng/ml in PBS-1% BSA, Ref. 705-035-003, Jackson, L'Arbresle, France) was added and incubated for 1 h at room temperature under gentle agitation. The cells were washed 4 times with TPBS before TMB was added and incubated for 5 to 10 min at room temperature. The reaction was stopped with 2 N H_2SO_4 and the absorbance was read at 450 nm. Heparin used for the co-incubation with pdAT and recAT was controlled to totally inhibit the thrombin generation in a normal plasma at the dose between 0.01 and 0.1 U/ml and, to similarly inhibits an AT-deficient plasma reconstituted by 1 U/ml pdAT or recAT (Supplementary figs. 2 and 3).

2.5. Heparanase digestion of HUVEC

Heparanase digestion of the cell surface heparin sulphate (HS) was carried out for 2 h at 37 °C with 2 U/ml of heparanase I, 2 U/ml of heparanase II and 3 U/ml of heparanase III in EGM2 basal medium in a humidified incubator under 5% CO_2 [18]. The cells were next washed three times with medium. The presence of HSPG was detected using the Cell ELISA protocol with the dedicated antibodies.

2.6. TGA

The standard TGA operating mode is based on the CAT method (Calibrated Automated Thrombin Generation Assay) developed by Hemker et al. [11]. Briefly, 80 µl of sample or 80 µl of normal reference plasma from Siemens Healthcare (ORKE41, Saint-Denis, France) were dispensed into 96-wells plate; 20 µl of a mix of 0.5 pM of Tissue Factor and 4 µM of phospholipids were added. In order to correct for inner-filter effects and substrate consumption, each thrombin generation (TG) measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed amount of thrombin–α2-macroglobulin complex (thrombin calibrator; Stago, Asnières, France). The plate was put on a Fluoroscan Ascent fluorimeter (ThermoLabsystems, Issy les Moulineaux, France) and incubated for 10 min at 37 °C. Then, 20 µl of starting reagent FluCa Kit (Diagnostica Stago) containing the fluorogenic substrate and $CaCl_2$ was automatically dispensed. TG was followed by measuring the fluorescence intensity at 390 nm for the excitation and 460 nm for the emission. Thrombinoscope® (Diagnostica Stago), a software program, was used to calculate thrombin activity against the calibrator and display thrombin activity *versus* time. When the TGA was performed on cells, HUVEC were seeded at 5000 cells/well 96-wells plates. First, it was demonstrated that there was no difference in signals when TG without cells was performed in flat-bottom well compared to round-bottom well (data not shown). Thus, the former can be used to grow cells at their surface prior to initiate TG. The data of TG presented above were all performed in flat-bottom well.

After reaching the confluence, AT was added into the wells. The cells were further incubated for 2 h at 37 °C in a humidified incubator under 5% CO_2. The medium was carefully removed. Cells were gently washed three times with PBS and the TGA was performed on the culture