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Fast form alpha-2-macroglobulin - A marker for protease activation in plasma exposed to artificial surfaces

Daniel Biltoft[a,b,*], Jørgen B. Gram[a,b], Anette Larsen[a,b], Anna-Marie B. Münster[a,b], Johannes J. Sidelmann[a,b], Karsten Skjoedt[c], Yaseelan Palarasah[a,b,c]

[a] Unit for Thrombosis Research, Department of Regional Health Research, University of Southern Denmark, Esbjerg, Denmark
[b] Department of Clinical Biochemistry, Hospital of South West Jutland, Esbjerg, Denmark
[c] Department of Cancer and Inflammation Research, Institute for Molecular Medicine, University of Southern Denmark, Odense, Denmark

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ABSTRACT

Objectives: Investigation of the blood compatibility requires a number of sensitive assays to quantify the activation of the blood protein cascades and cells induced by biomaterials. A global assay measuring the blood compatibility of biomaterials could be a valuable tool in such regard. In this study, we investigated whether an enzyme-linked immunosorbent assay (ELISA), that specifically measures the electrophoretic "fast form" of α_2-macroglobulin (F-α_2M), could be a sensitive and global marker for activation of calcium dependent and in-dependent proteases in plasma exposed to biomaterials *in vitro*.

Methods: A F-α_2M specific monoclonal antibody was generated and applied in an ELISA setup. Using the F-α_2M ELISA, we investigated activation of calcium dependent and in-dependent proteases by polyvinylchloride (n = 10), polytetrafluoroethylene (n = 10) and silicone (n = 10) tubings as well as glass tubes (n = 10).

Results: We found that F-α_2M is a sensitive marker for activation of both calcium dependent and in-dependent proteases. A significant difference between F-α_2M concentrations in the control sample and plasma exposed to the artificial surfaces was found ($p > 0.001$). This was observed both in the presence and absence of calcium. Furthermore, the highest F-α_2M concentration was in both cases found in plasma incubated with glass.

Conclusions: Our findings demonstrate that F-α_2M is a sensitive marker for detection of protease activation in plasma by artificial surfaces. Potentially, levels of F-α_2M could be a global marker of the blood compatibility of biomaterials.

1. Introduction

Exposure of human blood to artificial surfaces in clinical settings is frequently associated with an increased risk of thrombotic and inflammatory reactions [1–3]. These complications arise from a complex inter-play between surface induced protein adsorption, cell adhesion, and a series of blood protein cascades: the contact system, coagulation, and the complement system [4]. Protein adsorption of contact factors to artificial devices or the action of tissue factor from blood cells has been suggested to be initiators of coagulation [2,5,6]. Activation of the complement system induced by artificial surfaces is reportedly mediated through the alternative and classical pathways [7,8] and the contact system [4]. Currently, the use of biomaterials in clinical settings require administration of anticoagulants to minimize thrombotic complications. Such an approach, however, does not completely prevent the complications triggered by interaction of blood with artificial surfaces [2,4]. Furthermore, studies investigating inhibition of the complement cascade or the contact activation system, as a new approach to improve the blood compatibility of biomaterials, highlight the complexity of the interaction between blood and artificial surfaces [2,4]. Investigations of the blood compatibility and development of new biomaterials therefore call for the use of several assays measuring initiation of the contact system, coagulation, and the complement cascade as well as markers for platelet and leukocyte activation. The International Standardization Organization (ISO) 10993-4 standard describes a number of assays to study specific analytes that reveal the activation status of these biological systems. However, no global and sensitive immunoassay exists for detection of activation of proteases induced by biomaterials. Such assay could be useful in search of biomaterials with improved blood compatibility.

The broad-spectrum inhibitor, α_2-macroglobulin (α_2M), interacts with a vast number of proteinases in blood including those involved in contact activation and coagulation [9]. In human blood, α_2M circulates as a 725 kDa tetramer in a concentration of about 2.4 mg/mL [10].

* Corresponding author at: Unit for Thrombosis Research, Department of Regional Health Research, University of Southern Denmark, Niels Bohrs vej 9, 6700 Esbjerg, Denmark.
E-mail address: dbiltoft@health.sdu.dk (D. Biltoft).

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Upon interaction with a proteinase, α_2M undergoes an irreversible conformational change into a complexed or electrophoretic "fast form" (F-α_2M) [11] and exposes an otherwise hidden receptor-binding-domain (RBD) [12–14]. Based on this, we hypothesized that detection of F-α_2M could be sensitive and global approach for evaluation of the blood compatibility of biomaterials; an increase in F-α_2M levels would indicate activation of proenzymes from one or more enzymatic cascades.

In this study, we generated a F-α_2M specific antibody and applied this in an enzyme-linked immunosorbent assay (ELISA) setup to measure F-α_2M in plasma. Furthermore, we investigated whether the F-α_2M ELISA could detect activation of calcium dependent and in-dependent proteases in plasma after incubation with artificial surfaces.

2. Materials and methods

2.1. Buffers

Coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH = 9.6). PBS-TW buffer (PBS, 0.05% Tween-20, pH = 7.4). Substrate buffer (25 mM citric acid, 97 mM Na_2HPO_4, pH = 5.0). Sample dilution buffer (PBS-TW, 10 mM EDTA, 33.3 μM Phe-Pro-Arg-chloromethylketone (PPACK), pH = 7.4). TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH = 7.4 (at 37 °C)).

2.2. Plasma

A sodium citrate (0.109 M) stabilized plasma pool was obtained in-house from 20 healthy individuals (9 males, 11 females) who did not take hormone supplements. A serum pool was obtained from 22 healthy individuals (12 males, 10 females). Plasma and serum were stored at − 80 °C in 250 μL ampoules.

2.3. Reagents

Ortho-phenylenediamine (OPD) tablets were from Sigma-Aldrich Denmark ApS (Brøndby, Denmark). 3,3′,5,5′-tetramethylbenzidine (TMB) One™ ready-to-use substrate was from Kem-en-Tec Diagnostics A/S (Taastrup, Denmark). 30% H_2O_2 solution was from Merck (Darmstadt, Germany). 96-well polystyrene flat bottom MicroWell™ Maxisorp™ plates (Maxisorp plates) were from Thermo Fisher Scientific (Roskilde, Denmark). Methylamine (MA) was from Sigma.

Human native α_2M (N-α_2M) and human F-α_2M were from Sigma. Polyclonal goat anti-human α_2M IgG antibody (Cat. No. GAA2M-AP) was from Affinity Biologicals (Ancaster, Ontario, Canada). The antibody was dialyzed against PBS and subsequently biotinylated with *N*-hydroxydsuccinimide activated biotin (1 mg/6 mg antibody) for 3 h at RT. Finally, the antibody was dialyzed against PBS (biotinylated α_2M antibody). Horseradish peroxidase (HRP) conjugated streptavidin (HRP-Strp) was from Thermo Fisher Scientific. HRP conjugated rabbit anti-mouse IgG antibody (Cat. No. A9044) was from Sigma. Procoagulant phospholipid-TGT (TGT-lipid) was from Sweden). PPACK was from Haematologic Technologies Inc. (Essex Junction, Vermont, USA).

Recombinant RBD (rRBD) of human α_2M, cloned in vector pQE-30 with *E. coli* as expression system, was from Genscript (Piscataway, New Jersey, USA). The rRBD was expressed and purified as described elsewhere [15].

HiTrap™ Protein G HP 5 mL column (Protein G column) was from GE Healthcare Europe GmbH (GE Healthcare, Brøndby, Denmark).

MA-treated α_2M was prepared by incubating 1 mg/mL N-α_2M with 200 mM MA over-night (ON) at room temperature (RT). Subsequently, the sample was dialyzed against TBS buffer.

VWR Collection Silicone (Cat. No. 228-0706), Thermo Scientific Nalgene® polyvinylchloride (PVC) (Cat. No. 228-0178), and BOLA polytetrafluoroethylene (PTFE) (Cat. No. 228-0745) tubings, with a 3 mm internal diameter, were from VWR – Bie & Berntsen A/S (Søborg, Denmark). VWR Collection glass tubes, with a 10 mm external diameter and a U-bottom shape, were obtained from VWR.

2.4. F-α_2M specific monoclonal antibody

Mouse anti-human F-α_2M monoclonal antibodies were produced essentially as described previously [16]. Briefly, NMRI mice were immunized subcutaneously, three times, with 25 μg MA-treated α_2M adsorbed to $Al(OH)_3$ and mixed in a 1/1 ratio with Freund's incomplete adjuvant. A last immunization of 25 μg antigen was administrated intravenously three days prior to fusion. Spleen cells were subsequently fused with SP2/0-AG14 myeloma cells using polyenthylene glycol, and the fused cells were cultured in enriched RMPI 1640 medium. To identify hybridoma clones expressing F-α_2M specific antibodies, the culture supernatants of hybridoma cells were contra-screened using Maxisorp plates coated with either 1 μg/mL N-α_2M or F-α_2M. Wells with superior signal generation on F-α_2M coated plates were identified, and the hybridoma clones corresponding to these wells were cloned using the limited dilution method. Once single clones were obtained, the cells were grown in large scale and antibodies were purified from culture supernatant using the Protein G column. The antibodies, 16-11-2, 16-11-15, and 16-11-17, were selected for further studies to demonstrate specificity for F-α_2M.

2.4.1. Demonstration of F-α2M specificity

Recognition of rRBD by 16-11-2, 16-11-15, and 16-11-17 was investigated by ELISA. Briefly, a Maxisorp plate was coated with 4 μg/mL rRBD or 1 μg/mL N-α_2M and incubated ON at 4 °C. After a washing step, 2 μg/mL of either 16-11-2, 16-11-15, 16-11-17, or mouse anti-human tissue plasminogen activator (t-PA) antibody (nonsense antibody developed in-house), diluted in PBS-TW, were added to the wells and the plate was incubated for 60 min at RT under agitation. Following another washing step, HRP conjugated rabbit anti-mouse IgG, diluted 1:4000 in PBS-TW, was added to the wells and the plate was incubated for another 60 min at RT under agitation. Finally, the plate was washed and color development was initiated by adding 100 μL of a solution containing 0.012% H_2O_2 and 0.6 mg/mL OPD in substrate buffer. After 15 min, 100 μL 1 M H_2SO_4 was added to stop the reaction. The color development was quantified by measuring the optical density (OD) at 492 nm using OD readings at 650 nm as reference.

The electrophoretic mobility of F-α_2M and N-α_2M in presence of 16-11-17 was investigated on native PAGE. Briefly, 100 μg/mL of either F-α_2M or N-α_2M was incubated in the presence of 40 μg/mL 16-11-17 or buffer for 2 h at 4 °C in PBS. Subsequently, the samples were diluted 1:1 in 2 × native sample buffer (62.5 mM Tris-HCl, 40% glycerol, 0.01% Bromophenol Blue, pH = 6.8) (Bio-Rad) and analyzed by native PAGE using a 4–15% MiniPROTEAN® TGX™ precast gel (Bio-Rad) with TG running buffer (Bio-Rad), as described by the manufacturer, for 80 min at 150 V. The proteins in the gel were then visualized using Bio-Safe™ Coomassie G-250 stain (Bio-Rad) as instructed by the manufacturer.

2.5. Assay development

During assay development, the following setup was used; Maxisorp plates were coated with 1 μg/mL 16-11-17 by adding 100 μL of antibody solution in coating buffer to each well and incubating the plates ON at 4 °C. The plates were washed three times with PBS-TW and blocked for 40 min in PBS-TW buffer. Samples were diluted in PBS-TW (with 10 mM EDTA) and 100 μL was added to the plates and incubated for 60 min at RT under agitation. Following another washing step, 100 μL of diluted biotinylated α_2M antibody (1:2000 in PBS-TW) was added to the wells and the plates were incubated for 60 min at RT under agitation. Next, the plates were washed 3 times with PBS-TW, and 100 μL HRP-Strp, diluted 1:4000 in PBS-TW, was added to each well. The plates were incubated for 60 min at RT under agitation. Color development was initiated by adding 100 μL of a solution containing