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Ficolin-2 reveals different analytical and biological properties dependent on different sample handling procedures

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ABSTRACT

Ficolin-2 (L-ficolin) is a germ line encoded pattern recognition molecule circulating in the blood, and functions as a recognition molecule in the lectin complement pathway. However, consistent and reliable measurements of Ficolin-2 concentration and activity have been difficult to achieve. After recurrent observations of deviations in Ficolin-2 properties between different blood sample procedures, we decided to investigate this closer. Blood samples from ten healthy donors were collected in various serum and plasma tubes and Ficolin-2 properties were evaluated by different ELISA setups. We found that serum prepared from tubes containing the clot activator silica used as a standard technique in many routine laboratories held a significantly lower concentration of Ficolin-2 as compared to the other sample types. Furthermore, Ficolin-2 binding and complement activation potential in this type of serum was impaired when using an acetylated compound as matrix. On the other hand, Ficolin-2 in serum made without clot activator and in plasma irrespective of additive used, had the same concentration and was capable of initiating the lectin pathway measured as C4 and C3 deposition on the ligand. No Ficolin-2 mediated formation of the terminal complement complex was observed under the applied assay conditions. In conclusion, our results show that Ficolin-2 is a promiscuous molecule and that care should be taken during sampling, handling and matrix chosen for measurement of Ficolin-2 levels and activity.

1. Introduction

Ficolin-2 (L-ficolin) is a multimeric recognition molecule in the lectin pathway of complement a feature it shares with mannose-binding lectin (MBL), Ficolin-1 (M-ficolin), Ficolin-3 (H-ficolin) (Garred et al., 2009) as well as Collectin-11 (CL-11 or CL-K1) (Ma et al., 2013). Of the recognition molecules in the lectin pathway Ficolin-3 holds the largest complement activation potential followed by MBL and Ficolin-2 and finally Ficolin-1, which is only a poor initiator of complement (Hummelshoj et al., 2008). The exact quantitative nature of the complement activating capacity of CL-11 has not been determined yet. Functional complement activation assays has been established for evaluating the MBL (Roos et al., 2003; Seelen et al., 2005) and Ficolin-3 (Hein et al., 2010) pathways, but none easily available exist for Ficolin-2.

After translation from the *FCN2* gene several peptides coil up and form the characteristic oligomeric, bouquet-like structure of the Ficolin-2 molecule with a molecular weight of several hundred kD (Hummelshoj et al., 2007). Ficolin-2 is primarily produced in the liver and from here secreted to the blood stream (Matsushita et al., 1996). The mean concentration of circulating Ficolin-2 in the blood of healthy controls has been determined in several different studies to be: 3.7 μg/ml (Kilpatrick et al., 1999), 3.95 μg/ml (Atkinson et al., 2004), 3.3 μg/ml (Krarup et al., 2005), 5.4 μg/ml (Munthe-Fog et al., 2007), and 8.7 μg/ml (Watanabe et al., 2012). In the globular region of the molecule where the fibrinogen-like domain is located, Ficolin-2 recognizes and binds ligands through four distinct binding grooves: S1, S2, S3 and S4 all of which have different specificities. This feature is unique for Ficolin-2 since Ficolin-1 and Ficolin-3 only contain one binding groove, S1 (Garlatti et al., 2007a, 2010). Many ligands have been described for Ficolin-2 including: acetylated carbohydrates in the form of GlcNAc, GalNAc, CysNAc, acetylated low density lipoproteins (Faro et al., 2008), 1,3-β-glucan (Ma et al., 2004; Garlatti et al., 2007b,a), lipoteichoic acid (LTA) from the cell wall of Gram positive bacteria (Lynch et al., 2004), microorganisms such as capsulated *Staphylococcus aureus*, *Streptococcus pneumoniae* (Krarup et al., 2005), Gram-negative *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, and the pathogenic fungus *Aspergillus fumigatus* (Ma et al., 2009).

Abbreviations: acBSA, acetylated bovine serum albumin; ELISA, enzyme linked immunosorbent assay; MASP, MBL/ficolin/collectin associated serine protease; MBL, mannose-binding lectin; SPS, sodium polyanethole sulfonate; TCC, terminal complement complex.

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Activation of the lectin pathway of complement via Ficolin-2 happens in association with the MASPs where especially MASP-2 is important for the cleavage of C2 and C4 (Matsushita et al., 2000; Lacroix et al., 2009). For many of the listed ligands, Ficolin-2 has been reported to exhibit complement activation potential, however only in the form of cleavage and deposition of exogenous added C4. Furthermore, Ficolin-2 has been shown to interact with the long pentraxin PTX3 on the surface of *A. fumigatus* and hereby boost the complement response (Ma et al., 2009).

The *FCN2* gene is polymorphic and contains a number of SNPs in both the promoter as well as in the coding region (Hummelshoj et al., 2005). Several SNPs located in the promoter of *FCN2* is associated with inter individual differences in serum concentration of Ficolin-2 (Hummelshoj et al., 2005, Munthe-Fog et al., 2007). Several reports have shown that variation in the Ficolin-2 serum concentrations and genetic variation in the *FCN2* gene are associated with different infectious and inflammatory diseases (Kilpatrick and Chalmers, 2012).

Due to the accumulating data regarding crosstalk between the coagulation and complement systems (reviewed in Markiewski et al., 2007) the consideration on what type of blood sample to choose is highly relevant. Heparin, ethylenediaminetetraacetic acid (EDTA) and citrate are examples of conventional coagulation inhibitors used to make plasma for various purposes. Heparin indirectly inactivates thrombin through binding to antithrombin (Chuang et al., 2001) but is also known to interfere with the complement system (Mollnes, 1998). Furthermore, Ficolin-2 has been reported to bind heparin (Gout et al., 2010). Chelating Ca^{2+} in a blood sample with EDTA or citrate and by this way block coagulation is very efficient; however, this also obstructs complement function, which also is Ca^{2+} dependent. The anti-coagulant agent hirudin, a recombinant protein derived from lepirudin isolated from leaches, is a relatively new compound used for isolation of plasma. It is a direct and very potent inhibitor of thrombin and has not (yet) been shown to interfere with any other blood factors or complement function in general (Mollnes et al., 2002). Silica is an oxidized form of the element silicon and possesses the same properties as a glass vial in regard to activation of the coagulation system via factor XII in the intrinsic pathway and thus creating serum (Cochrane and Wuepper, 1971). In recent time, it has become commonly used as a coagulation activator in commercial blood collection systems.

After experiencing recurrent and inexplicable fluctuations in Ficolin-2 measurements, we wanted to thoroughly investigate the differences, if any, between serum and plasma in relation to Ficolin-2. As it turned out, Ficolin-2 was significantly affected by the presence of the clot activating mineral silica.

2. Materials and methods

2.1. Materials

Maxisorp microtiter plates were purchased from NUNC (Roskilde, Denmark). PBS-buffer (10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH = 7.4), Barbital-buffer (4 mM $C_8H_{11}N_2NaO_3$, 145 mM NaCl, 2.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, pH = 7.4) and 1 M sulfuric acid were all acquired from the hospital pharmacy (Rigshospitalet, Copenhagen, Denmark). Tween-20 was purchased from Merck (Hohenbrunn, Germany). Bovine serum albumin (BSA) (A3803), sodium acetate solution, acetic anhydride and sodium polyanethole sulfonate (SPS) (P2008-5G) were all purchased from Sigma–Aldrich (Copenhagen, Denmark). Polyclonal rabbit-α-C4c antibody (Q0369), Horseradish-peroxidase (HRP) conjugated rabbit-anti-mouse IgG and OPD substrate tablets were purchased from Dako (Glostrup, Denmark). Rabbit-anti-C3c polyclonal antibody was purchased from Dade-Behring (Marburg, Germany). Mouse-anti-human-C5b-C9 terminal complement complex (TCC) monoclonal antibody (DIA 011-01) was purchased from Bioporto Diagnostics (Gentofte, Denmark). Biotinylated polyclonal antibodies against Ficolin-2 and Ficolin-3 were purchased from R&D systems (Abingdon, UK). Donkey-anti-rabbit-HRP and streptavidin-HRP were purchased from GE Healthcare (Buckinghamshire, UK).

2.2. Monoclonal antibodies against ficolins

Well characterized monoclonal antibodies against Ficolin-2 (FCN216 and FCN219) and Ficolin-3 (FCN334) were used and has previously been described (Munthe-Fog et al., 2007, 2008).

2.3. Blood samples

Venous blood from 10 voluntary healthy donors was collected in four types of blood collection tubes: EDTA (Vacuette, BD Vacutainer) or hirudin (Monovette, Sarsted) coated tubes were used for plasma samples; serum samples were drawn in tubes coated with a clot activator in the form of silica particles (Vacuette, BD Vacutainer) or simple dry glass vials with no additive (The pharmacy at Rigshospitalet). All blood samples were left at room temperature for 2 h where after serum and plasma was isolated by centrifugation (1600 × *g* for 10 min), and stored at −80 °C.

2.4. Acetylated BSA (acBSA)

Acetylation of BSA was performed as previously described by Hein et al. (2010).

2.5. Concentration of Ficolin-2 and Ficolin-3 in serum and plasma

Concentrations of Ficolin-2 and Ficolin-3 in serum and plasma samples were determined in ELISA as described by (Munthe-Fog et al., 2007). Briefly, monoclonal antibodies directed against Ficolin-2 (FCN216) or Ficolin-3 (FCN334) were coated in Maxisorp plates over night (ON) 4 °C. Serum and plasma samples were diluted and incubated in the wells for 3 h at room temperature (RT), and furthermore a standard serial dilution of pooled human serum with known concentrations of Ficolin-2 and Ficolin-3 was added to each assay. Plates were washed in PBS-T and subsequently incubated with a biotinylated Ficolin-2 antibody (FCN219-biotin) or Ficolin-3 antibody (FCN334-biotin) ON at 4 °C. Finally, the plates were incubated with horseradish peroxidase (HRP) conjugated streptavidin for 2 h and developed with OPD substrate. Optical density (OD) were read at 490 nm on Microplate KC4 Signature EL808 (Biotek) and concentrations were calculated from the standard curve obtained of the NHSP.

2.6. Ficolin-2 and -3 binding to acBSA

Binding of the ficolins from serum and plasma samples was performed as described in Hein et al. (2010). Briefly, samples were added in serial dilutions to acBSA coated Maxisorp plates and incubated for either 30 min or 2 h at 37 °C. After washing in barbital-T (VBS-T) detection antibodies FCN219-biotin and FCN334-biotin, respectively, were added. Subsequently, plates incubated with streptavidin-HRP for 1 h 37 °C and were finally developed and read as described above. For evaluation of Ca^{2+}-dependency, 10 mM EDTA was added to the buffer in the sample incubation step.