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Research paper

A novel assay to quantitate MASP-2/ficolin-3 complexes in serum

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ABSTRACT

Ficolin-1, -2 and -3 are recognition molecules in the lectin complement pathway and form complexes with serine proteases named MASP-1, -2 and -3 and two nonenzymatic proteins. MASP-2 is the main initiator of lectin pathway activation, while ficolin-3 is the most abundant ficolin molecule in the circulation. The significance of lectin pathway complexes in the circulation is unknown. Thus, we established an assay for the measurement of circulating MASP-2/ficolin-3 complexes. A quantitative sandwich ELISA was developed for the measurement of the MASP-2/ficolin-3 complexes in serum based on monoclonal antibodies against MASP-2 for coating and anti-ficolin-3 for detection. In addition, we assessed the serum concentrations of ficolin-3 and MASP-2 and the extent of ficolin-3 mediated C4 deposition on acetylated BSA in samples from 97 healthy donors. The median concentration of MASP-2/ficolin-3 complexes was found to be 119.7 AU/ml (range: 2.9–615.5 AU/ml). Significant correlations were found between the level of MASP-2/ficolin-3 complexes and the concentration of ficolin-3 (Spearman $r=0.2532$, $p=0.0124$), and MASP-2 (Spearman $r=0.4505$, $p<0.0001$), as well as the degree of C4 deposition (Spearman $r=0.671$, $p<0.0001$). When ficolin-3 deficient (homozygous for the rs28357092 polymorphism) and MASP-2 deficient (homozygous for the rs72550870 polymorphism) sera were incubated together, complex formation was induced between MASP-2 and ficolin-3. The complex formation disappeared in the presence of EDTA. An assay allowing quantitative measurement exclusively of MASP-2/ficolin-3 complexes in serum is described. This method may add further insight into the pathophysiology of disorders associated with the deficiency or abnormal activities of MASP-2 and ficolin-3.

1. Introduction

The classical pathway, the alternative pathway and the lectin pathway are the initiating routes of the complement system, which have important roles in innate and adaptive immune defence (Walport, 2001). The lectin pathway may be activated by mannose-binding lectin (MBL), ficolin-1 (M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin) in varying degrees when they recognize and bind to sugar structures or acetylated compounds present on microorganisms or on altered-self cells (Hummelshoj et al., 2008). However, MBL and the ficolins exhibit differences in tissue expression, ligand-binding specificity and recognition of pathogens, indicating that each molecule has a specific role

Abbreviations: acBSA, acetylated bovine serum albumin; AU, arbitrary unit; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCN334-Bio, biotinylated monoclonal antibody against ficolin-3; MASP-2, mannose-binding lectin-associated serine protease-2; MBL, mannose-binding lectin; NHSP, normal human serum pool; OPD, orthophenylenediamine; PBS, phosphate buffered saline; PBS-T, PBS containing 0.05% Tween-20; PRM, pattern recognition molecule; rficolin-3, recombinant ficolin-3; SPS, sodium polyanethole sulfonate; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05% Tween-20; TCC, terminal complement complex; TMB, 3,3′,5,5′-Tetramethylbenzidine

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(Turner, 2003; Endo et al., 2007). Among the recognition molecules in the lectin pathway ficolin-3 is the most abundant serum component with a median concentration in healthy Caucasians of 25 μg/ml, followed by ficolin-2 (5 μg/ml) and MBL (1 μg/ml), respectively (Munthe-Fog et al., 2007, 2008; Garred et al., 1992b). Serum levels of ficolin-1 are normally low (1440 ng/ml according to Sallenbach et al., 2011 or 60.5 ng/ml according to Honore et al., 2008). MBL and the ficolins form complexes with MBL/ficolin-associated serine proteases named MASP-1, MASP-2 and MASP-3 and two non-enzymatic proteins named sMAP (MAp19) and MAP-1 (MAp44) (Yongqing et al., 2012). MASP-2 has a clearly defined role in the cleavage of C4 and C2 which leads to the formation of the C3 convertase, C4b2a (Thiel et al., 1997).

On the other hand, it is suggested based on a mouse model that MASP-1 enhances complement activation by direct activation of MASP-2 but cannot induce C3-convertase formation itself (Takahashi et al., 2008). Regarding MASP-3 it has been reported that activated MASP-3 in a mouse model triggers the alternative complement pathway (Iwaki et al., 2011). Initiation of any of the three pathways of the complement system is associated with the cleavage and deposition of C3b and culminates in activation of the terminal complement pathway, which initiates the formation of the terminal C5b-9 complement complex (TCC).

The traditional methodology to measure the level or function of the complement system has been performed either via determination of single components or activated components and via functional assays measuring the hemolytic activity of the system. Deposition of activated components on different matrices reflecting the binding of the recognition molecules and the presence of downstream components was also analyzed (Mollnes et al., 2007). Regarding the lectin pathway widely accepted methods have been used to measure MBL in serum or lectin pathway complement activity based on MBL binding to mannan (Garred et al., 1992a; Seelen et al., 2005). Recently, a novel functional method was described by Hein et al., which is aimed for measuring the ficolin-3 mediated complement activation in human serum (Hein et al., 2010). However, the direct serum measurement of the initiator molecules of the lectin pathway with the associated enzymes has not been implemented. Here, we describe a novel assay which allows accurate, quantitative measurement of the lectin pathway activation potential, expressed by the amount of the initiator complex of the ficolin-3 mediated lectin pathway activation (MASP-2/ficolin-3) in serum, independently of downstream complement components.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 7.5), 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 sulphuric acid were prepared in the hospital pharmacy (Region H Apoteket, Rigshospitalet, Copenhagen, Denmark).

Recombinant ficolin-3 (rficolin-3) and biotinylated monoclonal antibody from mouse (IgG1) against human ficolin-3 (FCN334-Bio) were generated as described previously (Munthe-Fog et al., 2008). Acetylated bovine serum albumin (acBSA) was prepared as described previously (Hein et al., 2010).

2.2. Sample collection

Serum samples were obtained from 97 blood donors with written informed consent. From another 7 healthy individuals' serum, as well as EDTA-, citrate-, hirudin- and heparinized plasma were collected. All samples were stored at −80 °C. A normal human serum pool (NHSP) was prepared based on eight healthy donors. Serum deficient of ficolin-3 was collected from a patient with inherited deficiency of ficolin-3 (Leu117fs, rs28357092) (Munthe-Fog et al., 2009). Serum was also collected from a healthy adult individual, which has been identified by Sanger sequencing with hereditary MASP-2 deficiency who is homozygous for a MASP2 variant allele (Asp120Gly, rs72550870) (Stengaard-Pedersen et al., 2003). This individual will be described in detail elsewhere. In order to demonstrate the applicability of the MASP-2/ficolin-3 complex assay, patients with cardiac arrest (at the time of admission to the hospital, after cardiopulmonary resuscitation), 20 patients with stroke (at the time of admission to the hospital), 20 patients with stable angina pectoris (before elective coronarography) and 20 patients with hereditary angioedema due to C1-inhibitor deficiency (in symptom-free period) were also included in our study.

2.3. Ethics

The study was approved by The Committees of Biomedical Research Ethics of the Capital Region of Denmark.

2.4. MASP-2/ficolin-3 complex ELISA

In order to determine the serum level of MASP-2/ficolin-3 complex, Maxisorb ELISA plates (NUNC™, cat. no. 439454) were coated with 2 μg/ml monoclonal antibody to human MASP-2, clone 8B5 from rat (IgG1) (Hycult Biotech, cat. no. HM2190-IA) in PBS overnight at 4 °C. Plates coated with a monoclonal antibody of the same isotype served as control for the presence of heterophilic interference in the serum samples. To quench possible heterophilic interference serum samples and negative controls (MASP-2- or ficolin-3-deficient sera) were diluted 1:20 in PBS containing 0.05% Tween-20 (Merck, cat. no. 655205) (PBS-T) and 1:500 bovine and mouse serum. Diluted samples were applied to the plates in triplicates and incubated overnight at 4 °C. A standard two-fold dilution series (1:2.5–1:320) of pooled human serum was included on each plate. Plates were washed with PBS-T subsequently and incubated with 0.5 μg/ml biotinylated anti-ficolin-3 antibody (FCN334-Bio) overnight at 4 °C. Streptavidin-horseradish peroxidase conjugate (GE Healthcare, cat. no. RPN1231V) 1:1500 diluted was added to the wells and incubated for 2 h at room temperature. After washing with PBS-T, 100 μl/well TMB Sensitive Substrate Solution (Kem-En-Tec Diagnostics, cat. no. 4850A) was added and the enzymatic reaction was terminated with 50 μl/well 1 M sulphuric acid. Optical density