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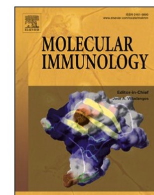

Short communication

Potency measurements of the complement system facilitated by antibodies targeting the zymogen form of complement factor D (Adipsin)

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ABSTRACT

The serine protease complement factor D is fundamental in the activation of the complement system. In addition, it was the first adipokine described (named Adipsin) and shown to improve beta cell function in diabetes. As part of an amplification loop of complement activation, factor D is a rate-limiting enzyme, and its accessibility contributes to the potency of complement activation. The dogma has been that conversion of the zymogen form, profactor D, to mature factor D occurred during secretion by adipocytes by uncharacterized proteases. However, recent findings demonstrated that the serine protease MASP-3 of the lectin pathway of the complement system mediated this conversion, suggesting that pattern recognition of pathogen/danger-associated molecular patterns could be a prior requirement for all complement activation. To facilitate studies addressing this hypothesis, we have developed monoclonal antibodies specific for human profactor D without binding to mature factor D. We demonstrate their applications in accessing the conversion of profactor D into mature factor D and in measuring levels of profactor D.

1. Introduction

The innate immune system comprises a range of defense mechanisms that serve to eliminate microorganisms from the host. The complement system is a part hereof, covering activation via the classical-, the lectin-, and the alternative pathway (Merle et al., 2015a, 2015b). All three pathways lead to the formation of complexes termed C3 convertases that catalyze the deposition of C3 fragments (C3b) on complement activating microbial surfaces. As an integrated part of the alternative pathway but also employed by the two other pathways is a positive feedback loop, also known as the amplification loop. The amplification loop accelerates the creation of C3 convertases and thus deposited C3b fragments that may form complexes with the serine protease factor B. Factor D (fD) serves as a key-serine protease for the alternative pathway/amplification loop by cleaving and thus activating factor B, resulting in the generation of the C3 convertase, C3bBb (Fig. 1A) (Schreiber et al., 1976). It only activates factor B when factor B is in complex with C3b (Forneris et al., 2010).

Deficiency of fD results in severely decreased activity of the complement system and increased risk of infections (Barratt and Weitz, 2021; Hiemstra et al., 1989; Sprong et al., 2006). The accessibility of fD is rate-limiting for complement activation, and the levels of fD affect the potency of the complement system (Hiemstra et al., 1989; Sprong et al., 2006; Uchibori et al., 2002).

Human fD is mainly synthesized by adipocytes, initially as a pre-proprotein of 253 a.a. residues, including a signal peptide for secretion spanning 19 residues (White et al., 1992). After secretion and removal of the signal peptide, it is referred to as a zymogen or simply, profactor D (pfD), with the six a.a. residue propetide (*N*-APPRGR) still attached to its *N*-terminal (Fig. 1B). For fD to obtain its mature form, the proeptide must be cleaved of, resulting in mature fD, characterized by the new *N*-terminal *N*-ILGGRW and comprising a total of 228 a.a. residues that fold into a single trypsin-like serine protease domain. In SDS-PAGE mature fD migrates with an MW corresponding to 26 kDa and pfD at 26.6 kDa, making it difficult to distinguish the two forms from each other. Macrophages, monocytes and astrocytes also produce fD (Volanakis and Narayana, 1996; White et al., 1992).

Until recently, the concept was that due to cleavage by uncharacterized proteases during secretion by the adipocytes, fD existed only in the circulation in its mature form (Volanakis and Narayana, 1996; White

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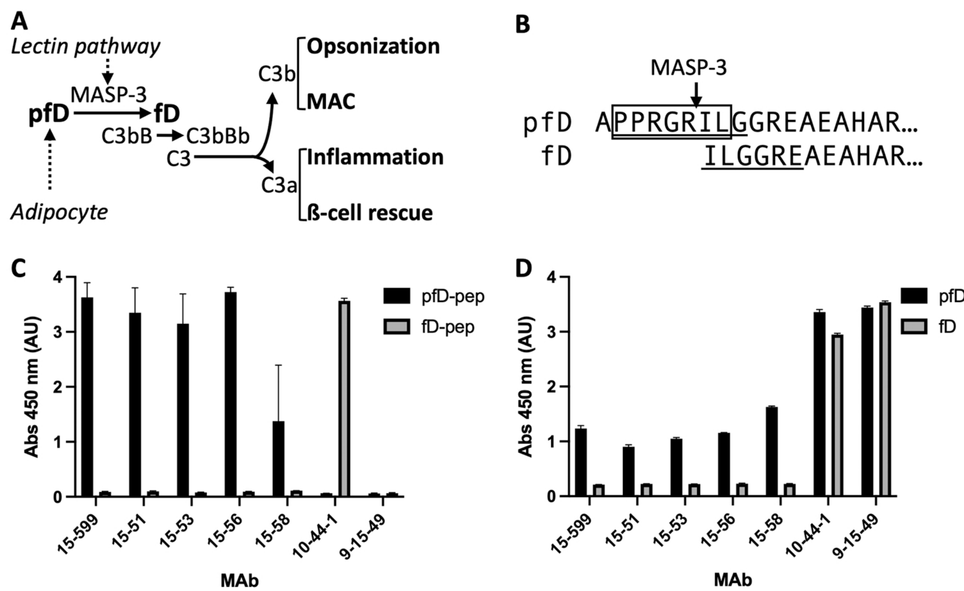

Fig. 1. Profactor D (pfD) vs. mature factor D (fD) and characterization of anti-pfD Mabs. A) Role and activation of fD (Adipsin), synthesized by adipocytes, and converted from the zymogen to mature form by MASP-3 of the lectin pathway of the complement system. Mature fD is rate limiting for complement activation via the alternative pathway and amplification loop, where it cleaves factor B in complex with C3b and generates a C3-convertase (C3bBb) to elicit the complement effector functions of deposited C3b and soluble C3a, and C3a's function in controlling differentiation of insulin-producing pancreatic ß-cells. B) N-termini of pfD and mature fD. Upon conjugation to diphtheria toxoid, the peptide indicated with boxed residues was used for immunization. The peptides indicated with underlined residues were upon conjugation to ovalbumin used in the initial ELISA screening process. C) Screening of anti-pfD Mabs using pfD and fD peptides conjugated to ovalbumin and coated in ELISA wells. D) Reactivity of Mabs with intact proteins in solution was tested by ELISA, by catching recombinant pfD or mature fD isolated from serum, using an anti-fD $F(ab')_2$ fragment for coating and detecting bound antibodies with HRP-anti-mouse-Ig-Fc specific antibody. Mab 9–15–49 and 10–44–1 served in both setups as controls and both recognize a common pfD-fD epitope. Mab 10–44–1 recognizes an epitope located in the proximity of the N-terminal of mature fD but excluded in the pfD peptide used for initial screening. Bars represent the average of triplicate measurements, and error bars represent max and min values.

et al., 1992; Yamauchi et al., 1994). pfD, has so far been indetectable or detectable at minuscule concentrations in human blood (Jing et al., 1999; Volanakis and Narayana, 1996; White et al., 1992; Yamauchi et al., 1994).

Recently, it was demonstrated by several groups that mannan-binding lectin-associated serine protease 3 (MASP-3) of the lectin pathway serves as a key serine protease for the activity of the alternative pathway, due to its ability to cleave pfD into mature fD (Fig. 1A) (Dobo et al., 2016; Hayashi et al., 2019; Iwaki et al., 2011; Pihl et al., 2017). So far MASP-3 is the only protease reported to cleave pfD, although, in its absence, other uncharacterized proteases may also contribute hereto (Pihl et al., 2017). In MASP-3 deficient individuals, characterized by the developmental syndrome named 3MC, the majority of fD in the circulation was found as pfD, although minor amounts of mature fD also was detected and provided some activity of the alternative pathway (Pihl et al., 2017). For MASP-3 to become an active protease, which cleaves pfD, MASP-3 may be activated by the proprotein convertase subtilisin/kexin (PCSK) 5 and PCSK6 (Oroszlan et al., 2021). For mature fD to become active, it must also undergo ligand-induced conformational changes, involving residues in the N-terminal of mature fD and the catalytic triad of the protease domain found C-terminally (Jing et al., 1999).

The findings of a specific conversion mechanism of pfD into mature fD by activated MASP-3 guided the attention to the importance of the lectin pathway and pattern recognition in potentiating or even controlling the overall activity of the complement system. Hence, the perception that fD always and immediately after secretion by adipocytes circulates as mature fD vs. pfD ought to be readdressed by focusing on how pfD conversion into mature fD is affected by or affects disease pathologies. In immunodiagnostic investigations of diseases, levels of fD in the circulation, measured in form of total fD (conc. of mature fD + pfD), were increased in patients with inflammatory diseases i.e. age-related macular degeneration (AMD) (Scholl et al., 2008; Silva et al., 2012; Stanton et al., 2011) and coronary artery disease (Ohtsuki et al., 2019; Shahini et al., 2017).

In the present work, we developed monoclonal antibodies (Mabs) specific for human pfD. These can be implemented in assays to characterize the conversion of pfD to mature fD, and in accessing the potency of the complement system in immunodeficiencies and autoimmune diseases associated with uncontrolled complement activation. In a conceptual framework, the data presented here and also by others suggest that when MASP-3 is so actively involved in factor D processing and activation (Dobo et al., 2016), one may not entirely consider MASP-3 as component of the lectin pathway. The activity of MASP-3 merits it as a general complement element, acting in the alternative, and likely also in the classical complement pathway via the amplification loop.

2. Materials and methods

2.1. Generation of anti-pfD Mabs

Mabs specific for pfD were generated by immunizing NMRI mice with the peptide NH_2-PPRGRILC-COOH, corresponding to the N-terminal of human pfD (Fig. 1B), conjugated to diphtheria toxoid via an additional C-terminal cysteine residue, using the heterobifunctional crosslinker sulfosuccinimidyl 4-(N-malerimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce), accordingly to the manufacturer's recommendations. Prior to subcutaneous immunizations of the mice, the conjugated peptide was mixed with Gerbu adjuvants (GERBU Biotechnik GmbH), using 50 μg conjugate in 100 μl Gerbu solution per immunization. Three days prior to fusion, the mice were immunized intravenously in the absence of adjuvants, and spleen cells were harvested and fused with myeloma cells, as described in detail previously and in general following the principles of Köhler and Milstein (Bjerrum et al., 2020; Kohler and Milstein, 1975; Aagaard et al., 2018). Supernatants from hybridomas were initially screened by ELISA with Maxisorb ELISA plates coated with 5 μg/ml of pfD (NH_2-PPRGRIL) or fD (NH_2-ILGGRE) peptides conjugated to ovalbumin (Fig. 1C), followed by at least three rounds of cloning by limiting dilution, ensuring clonality of the final hybridoma.

2.2. Recombinant pfD and purification of fD from serum

A pcMV6-XL4 vector encoding the full-length pfD sequence,

including the original signal peptide and an additional C-terminal 6 histidine tag, was provided by professor Gregers Rom Andersen and colleagues and transfected into Expi 293FTM, according to the supplier's instructions (Thermo Fisher Scientific) (Pihl et al., 2017). Three days post-transfection, the culture supernatant was harvested, centrifugated (1000xg, 10 min), and pfD purified by Nickel-chelate chromatography. Mature fD was enriched by polyethylene glycol precipitation (12–24% PEG W/V) from a serum pool ($n = 10$) in the presence of 0.5 M NaCl. The washed pellet was resuspended in TBS and purified by immunoaffinity chromatography using a matrix conjugated with Mab 1–49, recognizing a common fD and pfD epitope.

2.3. ELISAs

2.3.1. ELISA 1

The reactivity of peptide-directed Mabs with the proteins pfD and mature fD was analyzed by means of a sandwich ELISA. For this, we coated Maxisorb wells with F(ab')$_2$ fragments (0.5 μg/ml in 15 mM Na_2CO_3, 35 mM $NaHCO_3$), generated by pepsin digestions of a Mab 9–15–46 (reacting with both pfD and mature fD). After blocking in TBS with 0.05% emulfogen (TBS-E), pfD or mature fD (1 μg/ml TBS-E) was incubated in the wells for 1 hr, washed thrice with TBS-E, and incubated with anti-pfD Mabs (1 μg/ml) in TBS-E. Wells were washed with TBS-E, and bound Mabs were detected with HRP-conjugated rabbit anti-mouse-IgG-Fc antibody (0.25 μg/ml Southern Biotech), devoid of reactivity with the light chain part of mouse IgG, and developed with TMB-One (Kem-En-Tec Nordic).

2.3.2. ELISA 2

Total fD (mature fd + pfD) was measured using Mab 1–49 (2 μg/ml), coated in Maxisorb ELISA wells. Wells were blocked in TBS-E, washed thrice, and incubated for 3 hrs. with dilutions of protein samples or serum samples diluted in TBS-E. Plates were washed in TBS-E, and incubated for 2 hrs. with biotinylated Mab 1–46, also recognizing an epitope common for both mature fD and pfD, at a concentration of 0.5 mg/ml TBS-E. Wells were washed thrice, and incubated for 30 min with streptavidin-HRP (Zymed, 0.1 μg/ml) in TBS-E, washed thrice, and developed as above. Purified mature fD obtained from Complement Technology Inc. was used to calibrate a serum pool, which afterward served as standards in all the ELISAs for estimating the concentrations of total fD (mature fD+ pfD).

2.3.3. ELISA 3

pfD was measured using essentially the same procedure as in ELISA 2, with the substitution of anti-pfD Mab 15–599 (2 μg/ml) as coating antibody. Purified recombinant pfD, served as calibrator.

2.4. MASP-3 mediated cleavage of pfD into mature fD

Maxisorb ELISA plates were coated o/n with 5 μg mannan (Sigma-Aldrich)/ ml in 1 M of NaCl. After blocking and washing in TBS-E, wells were incubated with 200 ng of recombinant mannan-binding lectin (MBL, Enzon Pharmaceuticals) /ml in TBS-E with 2 mM $CaCl_2$ for 6 hrs. Plates were washed thrice and incubated for 6 hrs. with varying concentrations (0−100 ng/ml) of activated MASP-3 diluted in TBS-E with calcium (Pihl et al., 2017). Plates were washed thrice, and wells incubated with 200 ng recombinant pfD /ml for 1–4 hrs. at 37 °C. Supernatants were removed and diluted 10–300 times and analyzed in ELISAs specific for pfD (ELISA 3) and total fD (ELISA 2).

3. Results and discussion

Five Mabs with the desired specificity for pfD, as compared to mature fD, were generated and characterized (Fig. 1C). All five Mabs demonstrated specificity for pfD using N-terminal peptides derived from pfD and mature fD, respectively. To ensure reactivity with the corresponding

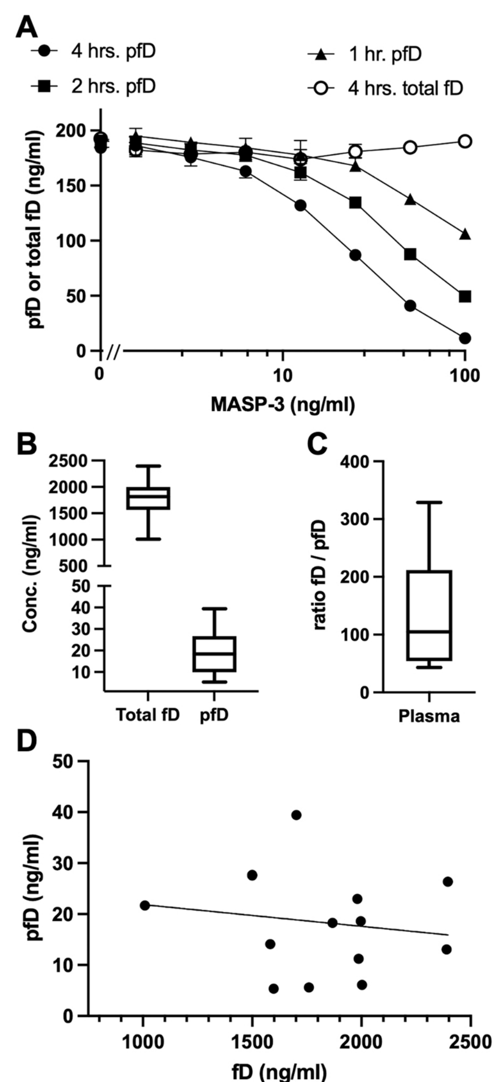

Fig. 2. Assessment of MASP-3-mediated conversion of profactor D (pfD) and analysis of plasma samples using ELISAs specific for pfD and total factor D (fD + pfD). A) Conversion of pfD by MASP-3. ELISA wells coated with MBL bound to mannan. Various concentrations of activated MASP-3 were bound by the MBL bound mannan and the wells were incubated with pfD at 37 °C for the indicated periods. Supernatants were measured by sandwich ELISAs specific for pfD and total fD, respectively (ELISA 3 & 2, Materials and methods). B) Concentration of pfD and total fD in 14 EDTA plasma samples obtained from healthy individuals. C) Ratio of the concentration of total fD:pfD in plasma samples (n = 14). D) Linear regression plot of the concentration of pfD as a function of total fD, with no significant sign of association between the two concentrations ($p = 0.6$, R=0.025, n = 14). The concentrations represent the average of triplicate measurements, and error bars/whiskers represent max and min values. Boxes represent the 25th and 75th percentile, with the median indicated as a line.

proteins and the ability to recognize native antigens in solution, the Mabs were also analyzed by their reactivity with recombinant pfD or mature fD purified from serum, immobilized via an anti-fD F(ab')$_2$ fragment (Fig. 1D). Again, all five Mabs demonstrated specific reactivity towards pfD relative to mature fD. Low signals of pfD were also observed when using mature fD derived from serum as a source of antigen. These low signals most likely represent small amounts of pfD found in the serum. Among the five anti-pfD specific Mabs, Mab 15–599 retained binding activity after coating in ELISA wells and did not cause unspecific reactions with a biotinylated detector antibody (not shown). Hence, Mab 15–599 was used as catching antibody in a sandwich ELISA specific for pfD. The consumption of pfD, during generation of mature fD by

MASP-3 was followed using two ELISAs, measuring pfD and total fD, respectively, and enabling us to calculate the ratio between the two. It was evident that the pfD ELISA measured the consumption of pfD in a dose-response relationship with the concentration of the applied MASP-3, complexed with mannan-binding lectin (MBL) bound to mannan; mimicking complement activation at the surface of a pathogen (Fig. 2A). The average concentration of total fD in plasma among the tested individuals was 1.8 μg/ml, corresponding well with previous observations (Fig. 2B) (Hecker et al., 2010; Maini et al., 2020; Scholl et al., 2008). The average concentration of pfD in EDTA plasma was 18 ng/ml, with a variation among individuals spanning 5 – 40 ng/ml. Comparing the two concentrations of total fD vs. pfD revealed that the average ratio between the two was 142 (total fD: pfD), with a relatively large variation among individuals, spanning a ratio of 43 – 328, and demonstrating that 0.3–2.2% of the total fD was in the form of pfD (Fig. 2C). We observed no relation between the measured concentration of total fD and that of pfD (Fig. 2D), suggesting that the conversion of pfD into mature fD in plasma is not a simple equilibrium but more likely regulated by the accessibility and activity of endogenous proteases, such as active MASP-3. Our reported concentrations ought to be taken with certain precautions because of the small samples size (n = 14). Future studies ought also to address how sampling and storage affects conversion to fD conversion in serum/plasma.

In summary, we succeeded in producing Mabs useful for the quantification pfD (zymogen form of fD). In combination with other assays, we expect the Mabs to be useful for accessing the potential role of zymogen and mature in the immunodiagnosis of diseases associated with uncontrolled complement activation.

CRediT authorship contribution statement

Yaseelan Palarasah: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Anne Sofie Løgstrup Henriksen**: Investigation, Writing – original draft. **Steffen Thiel**: Conceptualization, Methodology, Validation, Writing – original draft. **Maiken Henriksen**: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Søren W.K. Hansen:** Conceptualization, Methodology, Investigation, Validation, Data curation, Writing – original draft, Writing – review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) the current work.

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