Characteristics and Biological Variations of M-Ficolin, a Pattern Recognition Molecule, in Plasma

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Abstract

The three human ficolins, H-ficolin, L-ficolin and M-ficolin, are pattern recognition molecules of the innate immune system. All three ficolins can activate the lectin pathway of the complement system after binding to pathogens. H- and L-ficolin are serum proteins with an average concentration of 18 and 3 $\mu\text{g/ml}$, respectively. M-ficolin has been described as a membrane-associated pattern recognition receptor of monocytes, being also present in granulocytes; recently, minuscule amounts of M-ficolin have been found in serum, too. No assay specific for M-ficolin has yet been described and biological variations are unknown. We have raised specific monoclonal anti-human M-ficolin antibodies and have developed a quantitative assay for M-ficolin. M-ficolin elutes as a large, 900-kDa protein upon gel permeation chromatography of serum. Analysis of M-ficolin levels in serum samples of 350 blood donors reveals a mean concentration of 1.07 $\mu\text{g/ml}$, ranging from 0.28 to 4.05 $\mu\text{g/ml}$. Analyses of consec-

utive acute phase serum samples from major surgery patients indicated a complex response. Ontogeny was investigated through cord blood samples from healthy full-term babies, which showed adult levels, with sequential samples showing no increase from birth to 1 year of age. We suggest that M-ficolin should also be considered as a humoral pattern recognition molecule.

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Introduction

Ficolins are a group of proteins capable of recognizing pathogen-associated molecular patterns and are hence supposed to play a role in the body's innate immune defense [1]. Ficolins have been identified in species ranging from ascidian [2], over *Xenopus* [3] to mammals: rodents [4], pigs [5] and humans. Humans have three ficolins: H-ficolin (Hakata antigen, ficolin-3) [6], L-ficolin (ficolin/P35, ficolin-2) [7] and M-ficolin (ficolin/P35-related protein, ficolin-1) [8], whereas other mammals only have two ficolins, which are paralogues of L-ficolin and M-ficolin [9].

The structural composition of the ficolins is similar to that of the collectins mannan-binding lectin (MBL) and

the lung surfactant proteins, SP-A and SP-D. Ficolins thus consist of polypeptides that trimerize into subunits, which in turn oligomerize into larger macromolecules [9]. The polypeptide chain of the ficolins consists of an N-terminal cysteine-containing segment followed by a collagen-like region and a C-terminal globular domain. The globular domain resembles that of fibrinogen β and γ polypeptide chains and is referred to as a fibrinogen-like domain; it is responsible for the ligand-binding properties of the ficolins [5]. The ficolins have been shown to associate with the MBL-associated serine proteases (MASPs) in a manner similar to MBL [10–12]. When the ficolin/MASP complexes are bound to a suitable pattern of ligands, the MASPs will become activated and are now able to activate the complement system via the lectin pathway.

H-ficolin is synthesized by bronchial epithelial cells and type II alveolar epithelial cells in the lung, by bile duct epithelial cells and by liver cells. From the latter site H-ficolin is secreted into the blood, where it is reported to have a median plasma concentration of 18 $\mu\text{g/ml}$ [13]. H-ficolin possesses binding specificity for a polysaccharide from the bacterium *Aerococcus viridans* and for N-acetylglucosamine (GlcNAc) [6, 13, 14]. H-ficolin has been proposed to mediate the clearance of late apoptotic cells since it was found to bind apoptotic Jurkat cells [15, 16].

L-ficolin is produced by hepatocytes and secreted into the bloodstream. It shows a median plasma concentration of 3 $\mu\text{g/ml}$ [13, 17]. L-ficolin has been shown to bind some capsulated bacteria and, like H-ficolin, it may play a role in mediating clearance of apoptotic cells [13, 15]. The ligands for L-ficolin appear to be acetyl groups presented by acetylated compounds as diverse as GlcNAc, N-acetyl galactosamine (GalNAc), N-acetylcysteine (CysNAc) and acetylcholine [18, 19].

The last of the three human ficolins, M-ficolin, differs from the other two ficolins in several ways. Unlike the other ficolins, M-ficolin has been found to be mainly synthesized by and presented on the surface of peripheral blood monocytes and was until recently not detected in serum [8, 20–22]. When monocytes differentiate into macrophages or dendritic cells, their M-ficolin production is gradually reduced and finally ceases [21, 23]. The structure and ligand specificity of M-ficolin is very similar to that of L-ficolin, with an amino acid sequence identity of 77% and a preference for acetylated compounds such as GlcNAc, GalNAc, CysNAc and acetylated human serum albumin [10, 24]. The crystal structure of the globular domain of M-ficolin has recently been solved and this

unveiled a single calcium-dependent binding site. The structure also revealed that the binding site functions as a pH-sensitive switch rendering the binding site incapable of ligand-binding when the pH is below 5.6 [25, 26].

We have synthesized recombinant M (rM)-ficolin in a mammalian cell line and exploited this to develop a mouse monoclonal anti-human M-ficolin antibody useful for the development of a sandwich assay for quantification of M-ficolin. We have used the assay to describe biological variations of M-ficolin in plasma as well as to characterize the protein.

Materials and Methods

Production of rM-Ficolin

A cDNA clone (imageID 4717731) encoding the full length cDNA of human M-ficolin was introduced into the *pcDNA3.1(-)* vector [10]. Freestyle™ HEK293F cells (R79007, Invitrogen) were used for transient expression of rM-ficolin. The cell line has been adapted to grow in suspension in a serum-free medium (Freestyle Expression Medium, Invitrogen). The *pcDNA3.1(-)M-ficolin* expression construct was introduced using a cationic lipid-based transfection reagent (293fectin™, 12347-019, Invitrogen) according to the manufacturer's protocol. In brief, for each transfection reaction, 30 μg of purified *pcDNA3.1(-)M-ficolin* construct and 30 μl 293fectin were each mixed with 1 ml transfection optimizing buffer (Opti-MEM, 31985-047, Invitrogen) and incubated for 5 min at room temperature (RT). The two reagents were combined and allowed to form complexes for 20 min at RT. The complexes were added to 30×10^6 HEK293F cells in 28 ml Freestyle Expression Medium, seeded in a 150- cm^2 incubation flask (TC flasks sterilized by γ -irradiation, 90150; TPP, Switzerland) and incubated for 48–72 h at 37°C, 5% CO_2 , under constant gentle mixing. Supernatants were harvested after centrifugation (200 g, 5 min), preserved by adding 15 mM NaN_3 and stored at 4°C. A batch of HEK293F cells, treated only with empty liposomes, was run in parallel and served as a control supernatant.

Purification of rM-Ficolin

Two milliliters of CysNAc beads (prepared as reported by Krup et al. [19]) were packed on a column and washed with binding buffer [10 mM Tris, 145 mM NaCl, 15 mM NaN_3 , pH 7.4 (TBS) with 0.05% (v/v) Tween 20 and 5 mM CaCl_2]. Cell culture supernatant containing rM-ficolin was mixed with an equal volume of binding buffer and applied to the column. After washing, bound rM-ficolin was eluted with binding buffer containing 200 mM GlcNAc. Fractions were collected, dialyzed against PBS (140 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , pH 7.4) with 15 mM NaN_3 and stored at 4°C. Purified rM-ficolin used for immunizing animals was dialyzed against PBS and prepared <1 week before the immunization. The protein concentration was determined by optical density measurement using the conversion factor of $E_{280\text{ nm}}$ at 1 mg/ml = 1.60 as calculated from the amino acid composition. The concentration was confirmed by quantitative amino acid analysis. The appearance of this rM-ficolin has been described in detail by Frederiksen et al. [10].

Table 1. Immunization protocol

Day	Antigen dose and route of administration
1	50 µg pcDNA3.1(-)M-ficolin construct, i.m. 20 µg rM-ficolin in FCA, s.c.
32	25 µg pcDNA3.1(-)M-ficolin construct, i.m. 15 µg rM-ficolin in FIA, s.c.
134	50 µg pcDNA3.1(-)M-ficolin construct, i.m. 10 µg rM-ficolin in FIA, s.c.
267	7.5 µg rM-ficolin in FIA, s.c.
330	4 µg rM-ficolin in FIA, s.c.
389	1 µg rM-ficolin in FIA, s.c.
410	2.5 µg rM-ficolin in PBS/0.9% NaCl, i.v.
413	Sacrificed, spleen and enteric lymph nodes removed

FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant.

Commercial Anti-M-Ficolin Antibodies

Two monoclonal anti-M-ficolin antibodies (ABS 036-05 and ABS 036-01) were obtained from AntibodyShop, Copenhagen. The antibodies were stated to react specifically with rM-ficolin coated on micro wells and to be useful for ELISA. One polyclonal rabbit anti-human M-ficolin antibody [20] is commercially available through Hycult Biotechnology (catalogue No. HP9039). As we found this antibody to react equally well with L- and M-ficolin (see below), it shall be referred to as anti-L/M antibody.

Production of Monoclonal Anti-M-Ficolin Antibodies

Female BALB/c mice were immunized with the pcDNA3.1(-)M-ficolin expression construct as well as with rM-ficolin. Naked plasmid DNA in 10 mM Tris-HCl, pH 8.5, was administered intramuscularly in the upper thighs of the mice, whereas rM-ficolin protein was emulsified with Freund's complete adjuvant (195187, ICN Biomedicals) for the first injection, or Freund's incomplete adjuvant (F-5506, Sigma-Aldrich) for subsequent subcutaneous injections in the neck region. The immunization protocol is summarized in table 1. The presence of anti-M-ficolin antibodies in the sera was determined as described below.

Spleen and enteric lymph node cells from the mouse with the highest anti-M-ficolin antibody titer were fused with mouse myeloma cells (X63-Ag8.653) and seeded on BALB/c peritoneal macrophages [27]. Hybridoma supernatants were assessed for anti-M-ficolin antibodies, and positive hybridomas were cloned by limiting dilution. Five hybridomas were generated (7E5, 7C9, 7F6, 7G1 and 9H3), one of which, 7G1 (IgG₁ isotype), is used for the assay presented in this report.

Assay for Anti-M-Ficolin Antibody

Sera and hybridoma supernatants were tested for anti-M-ficolin antibodies by time-resolved immunofluorometric assay (TRIFMA). FluoroNunc microtiter wells (437915/437958, Nunc) were coated with 1 µg acetylated BSA (B2518, Sigma-Aldrich) in 100 µl coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 15 mM Na₂SO₄, pH 9.6) overnight at RT. After blocking residual binding

sites with 200 µg human serum albumin in TBS, the wells were incubated overnight at 4°C with 100 ng rM-ficolin in 100 µl binding buffer. After washing, serum or hybridoma supernatants, diluted in binding buffer, were added to the wells and incubated for 3 h. After washing in binding buffer, biotin-conjugated rabbit anti-mouse Ig antibodies (Z0259, DakoCytomation, biotinylated in-house with 167 µg biotin-N-hydroxy-succinimide per mg protein) were added at 0.25 µg/ml. Incubation for 3 h and washing was followed by adding europium-labelled streptavidin (1244-360, Perkin Elmer) at 0.1 µg/ml TBS/Tween, 25 µM EDTA. After 1 h at RT and washing, bound europium was quantified by adding 200 µl enhancement buffer and reading the fluorescence on a fluorometer (Victor™, Perkin Elmer). Data were assessed using the WorkOut 2 version 2.0 software (Perkin Elmer).

The antibody in the hybridoma supernatants was tested for cross-reactivity with L-ficolin on wells coated with L-ficolin purified from plasma [19] and for false positives with a control supernatant (described above) incubated in the acetylated BSA-coated wells. The antibodies were further tested for reaction with ficolins on Western blots as described below.

Purification and Biotinylation of Antibodies

Hybridoma supernatants were centrifuged for 30 min at 10,000 g and the harvested supernatant was supplemented with 10 mM in EDTA and 10 mM in Na₂HPO₄. The solution was passed through a 5-ml HiTrap protein G-Sepharose column (17-0405-01, GE Healthcare) prewashed with 0.1 M glycine, pH 2.5, and re-equilibrated with washing buffer (10 mM EDTA in PBS, pH 7.4). The column was washed and bound antibody eluted using 0.1 M glycine, pH 2.5. Fractions of 1 ml were eluted into 80 µl neutralizing buffer (1 M Tris-HCl, pH 8.5) and the purified antibodies were dialyzed against PBS. For biotinylation, the antibody was dialyzed against PBS, adjusted to pH 8.5, biotinyl-N-hydroxy-succinimide (Sigma-Aldrich) in DMSO was added (167 µg/mg antibody) and the mixture was incubated for 20 h before dialysis against TBS.

M-Ficolin Assay

The concentration of M-ficolin was determined by TRIFMA. TRIFMA is a sandwich assay similar to ELISA, taking advantage of the long-lasting fluorescence of the europium ion, which can thus be measured with great sensitivity by photon counting without interference from the rapidly fading background fluorescence. This provides TRIFMAs with greater sensitivity, greater reproducibility, greater range and greater signal-to-noise ratios in comparison to ELISAs.

The wells of FluoroNunc microtiter plates were coated with 100 ng monoclonal anti-M-ficolin antibody (MAb 7G1) in 100 µl of coating buffer overnight at 4°C. The wells were blocked as described above and subsequently washed thrice with TBS/Tw [TBS with 0.05% (v/v) Tween 20]. Samples were diluted in sample buffer, 100 µl was added per well and the plates were incubated overnight at 4°C. The sample buffer was composed of 50 mM Tris-HCl, 0.05% (v/v) Tween 20, 1 M NaCl, 5 mM EDTA, 100 µg heat-aggregated normal human immunoglobulin per milliliter (prepared by incubating human IgG; Statens Serum Institut, Denmark) at 63°C for 30 min, followed by removal of heavily aggregated IgG by centrifugation), 50 µg bovine immunoglobulin/ml (purified from ox serum using a protein G column as described above) and 1 mg human serum albumin/ml. After washing with

TBS/Tw, the wells were incubated with 100 ng biotinylated MAb 7G1 in 100 µl TBS/Tw for 2 h at RT. Wells were washed in TBS/Tw and developed with europium-labelled streptavidin, as described above. Dilutions of pooled serum were included on each plate for the construction of a standard curve, also in addition to three internal control sera and a negative buffer control. For routine assays, serum samples were tested 40-fold diluted.

Affinity Purification of Ficolins from Serum

Twelve FluoroNunc microtiter wells were coated with 100 ng MAb 7G1, 100 ng monoclonal anti-L-ficolin antibody (MAb GN5, HM2091, Hycult Biotechnology) or 100 ng monoclonal nonspecific mouse IgG₁ κ (purified from mouse ascites, M-7894, Sigma-Aldrich, on protein G beads as described above) in 100 µl of coating buffer. Wells were blocked by incubation with TBS/Tw and washed as described above. Serum diluted twofold with TBS/Tw was added to all wells and incubated overnight at 4°C. Wells were sequentially washed and bound material eluted for 10 min with 150 µl SDS-PAGE sample buffer [30 mM Tris-HCl, 10% (v/v) glycerol, 8 M urea, 3% (w/v) SDS, 0.1% (w/v) bromophenol blue, pH 8.9] diluted 1:1 in binding buffer. The content from the first well was transferred to the next washed well, thus accumulating released proteins. The eluates were analyzed reduced and non-reduced by Western blotting using either polyclonal rabbit anti-human L/M-ficolin, diluted 1 to 2,500, or 1 µg MAb 7G1 or 1 µg MAb GN5 per milliliter as primary antibody, and HRP-conjugated secondary antibody (goat anti-rabbit Ig for the rabbit antibody; P0448, DakoCytomation) or rabbit anti-mouse Ig for the two MABs (Z0260, DakoCytomation). The blots were developed using a chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate, 34075, Pierce) and analyzed using a CCD camera (Fuji 3000).

Gel Permeation Chromatography

Two hundred microliters of normal human serum (NHS) or 100 µl NHS mixed with 100 µl crude rM-ficolin supernatant were subjected to gel permeation chromatography (GPC) on a Superose 6 HR column (10 × 300 mm, 23.5 ml, GE Healthcare). The column was pre-equilibrated with TBS, 0.01% (v/v) emulfojen (polyoxyethylene 10 tridecyl ether, P-02393, Sigma) and 1 mM CaCl₂, which was also the running buffer. Fractions of 250 µl were collected in 96-well polystyrene microtiter plates (249570, Nunc) preblocked by incubation with TBS/Tw. M- and L-ficolin were quantified in the fractions by TRIFMAs. The assay for M-ficolin is described above and the assay for L-ficolin, a sandwich assay based on monoclonal antibodies, has been described previously [13]. In order to establish relevant molecular size markers, fractions were also analyzed for IgM, MBL, IgG and human serum albumin.

Serum Samples

Except for the samples used for generating the data shown in figures 1, 2 and 4a, b, all serum and plasma samples were previously collected and described [28–30]. All samples were obtained after informed consent was obtained and according to the Declaration of Helsinki.

To test the influence of sampling conditions, blood was collected into EDTA and distributed into 8 vials which were left at RT. At the given time points, a vial was centrifuged and the EDTA plasma recovered and frozen. However, two samples were taken

without additives and left to coagulate at room temperature. These two samples were centrifuged after 60 or 1,500 min, and the serum was recovered and frozen.

The samples used for the evaluation of the ontogeny of the ficolins were tested for L- and M-ficolin using the above-described assays, but also for H-ficolin using a previously described assay [13].

Results

Production of Hybridomas

In order to test the antisera from the DNA and protein of immunized mice and to make a selection of hybridoma antibodies sensitive for antibodies reacting with native M-ficolin epitopes, we chose to select on microtiter wells coated indirectly with M-ficolin through the binding of M-ficolin to a primary coat of acetylated BSA. Four mice were immunized. One reached a titer of 25,000, as judged by 50% of the maximum signal, and was selected for the generation of hybridomas. Five hybridomas were judged selective for M-ficolin (vs. L-ficolin or H-ficolin) as judged by Western blotting. One, 7G1, was chosen for the development of an M-ficolin assay since the antibody production was by far the most efficient in this hybridoma.

Specificity of Anti-M-Ficolin MAb 7G1

The specificity of 7G1 for M-ficolin is illustrated by the results of affinity purification of M-ficolin from serum and analysis by Western blotting shown in figure 1. The microtiter wells used for affinity purification were coated with either 7G1 (anti-M-ficolin), GN5 (anti-L-ficolin) or nonspecific monoclonal antibody. All these antibodies are of the IgG₁ isotype. Serum was incubated in the wells and after washing the bound proteins were eluted with SDS-PAGE sample buffer. The eluates were analyzed by SDS-PAGE Western blotting. Three different antibodies were used for development, 7G1 (anti-M-ficolin), GN5 (anti-L-ficolin) or a polyclonal anti-M/L antibody. Figure 1a illustrates the specificities of the two MABs 7G1 and GN5 as evaluated by non-reduced Western blotting: 7G1 only captures M-ficolin from serum, and GN5 only L-ficolin. Using the strongly denaturing SDS-PAGE sample buffer for elution from the wells, a portion of the antibody-coated wells was also eluted. These antibodies are thus in the samples and the secondary HRP-labelled rabbit anti-mouse Ig antibody will recognize them, giving rise to the bands at ~150 kDa (marked with stars in the figure). This is most pronounced in eluates from the non-specific IgG and the GN5-coated wells and much less

Fig. 1. Analysis of the specificity of antibodies. **a** Ficolins were caught from serum using either MAb GN5 (anti-L-ficolin antibody) or MAb 7G1 (anti-M-ficolin antibody) as catching antibody in microtiter wells. Wells coated with nonspecific mouse monoclonal IgG₁ served as control. Bound ficolins (together as it happens, with some of the coated antibody, see below) were eluted with SDS-sample buffer, applied to non-reduced SDS-PAGE and subsequently analyzed by Western blotting using either 7G1 or GN5 as the primary detecting antibody. **b** Ficolins were captured from serum using either 7G1 or GN5 as catching antibody and eluted with SDS-sample buffer. The samples were applied, reduced and non-reduced, to SDS-PAGE and analyzed by Western

blotting using a polyclonal rabbit anti-L/M-ficolin antiserum. The arrows indicate the position of the M- or L-ficolin bands. The bands marked by stars represent mouse IgG bands originating from coating antibody (mouse IgG isotypes) eluted from the wells, since these are also developed by the secondary rabbit anti-mouse antibody. **b** No mouse IgG bands are seen since this blot was developed using goat anti-rabbit IgG as the secondary antibody. The polyclonal antibody used in **b** is more sensitive at the conditions used, thus bands of lower molecular weight are also seen in the lane with non-reduced M-ficolin (catching antibody: 7G1) compared to the non-reduced M-ficolin lane in **a**.

from the 7G1-coated wells. The same IgG bands originating from the coating of antibodies are seen if we elute from antibody-coated wells if buffer instead of human serum had been incubated in the wells.

MAbs 7G1 and GN5 do not react with the reduced form of M-ficolin and L-ficolin, respectively, but a commercially available polyclonal antibody shows excellent reactivity with both reduced and non-reduced L- and M-ficolin (fig. 1b). Due to the higher sensitivity of this polyclonal antibody, this blot reveals remarkable similarity of the polymer distribution of serum L-ficolin and serum M-ficolin with the most marked band positioned at 300–400 kDa. L-ficolin, but not M-ficolin, showed the pres-

ence of a free polypeptide chain in the non-reduced run. Upon much longer exposure (fig. 1a, right half), lower-molecular-weight bands are also starting to appear in the lane with non-reduced M-ficolin (catching antibody: 7G1), but this is not shown since the background becomes a considerable problem under these conditions. The reduced polypeptide of M-ficolin is just a fraction faster (at ~34 kDa) than the reduced polypeptide of L-ficolin (at ~35 kDa). This blot lacks the mouse IgG bands since an anti-rabbit Ig antibody is used as developing antibody, compared to the anti-mouse Ig used in figure 1a.

Analysis of possible cross-reactivity of the antibodies was also carried out using solid phase assays. We coated

Fig. 2. Analysis of M-ficolin after GPC. **a** NHS (200 μ l) was passed through a Superose 6 column and fractions were analyzed for M-ficolin (●) and L-ficolin content (▲) by TRIFMA. Total protein content (mAU) was estimated by absorption at OD_{280 nm} (—). **b** 100 μ l NHS were mixed with 100 μ l crude rM-ficolin supernatant and passed through the same column. Fractions were analyzed for M-ficolin content (■) by TRIFMA. The graph for M-ficolin in NHS from **a** is also shown here (●) for comparison. Note the extra peaks in the graph of the fractionation of rM-ficolin supernatant containing NHS, originating from the lower oligomeric forms of rM-ficolin. Arrows indicate the elution volume of IgM (970 kDa), MBL (800 kDa), IgG (150 kDa) and human serum albumin (HSA, 67 kDa).

microtiter wells with 7G1 (anti-M-ficolin) or GN5 (anti-L-ficolin), added serum, washed and subsequently used biotinylated 4H5 (anti-H-ficolin), biotinylated 7G1 (anti-M-ficolin) or biotinylated GN5 (anti-L-ficolin) as developing antibodies. We found no signal in wells coated with 7G1 and developed with biotinylated 4H5 or GN5, but only observed a signal when developed with

biotinylated 7G1 (data not shown). A similar scenario was observed when the coating antibody was GN5: in this case only biotinylated GN5 gave a signal. Regarding H-ficolin specificity, only the combination of coated 4H5 and developing biotinylated 4H5 gave a signal in such a setup. Thus neither 7G1 nor GN5 detects H-ficolin.

Fig. 3. M-ficolin assay on serum and on rM-ficolin. **a** The dilution factor is given on the abscissa. The resulting time-resolved fluorescence signals are given on the ordinate as photon counts per second. ● = Dilutions of serum; ◆ = dilutions of rM-ficolin, starting at a concentration of 190 ng/ml; ■ = dilutions of rM-ficolin added to NHS; the first dilution contains 76 ng rM-ficolin/ml and 10% serum. **b** A sample of reduced rM-ficolin applied to SDS-PAGE analysis followed by silver staining to visualize the protein. The molecular weights of the markers are indicated.

Size of M-Ficolin in Serum

The result of the analysis by GPC of M-ficolin in native configuration as present in NHS (fig. 2a) further stresses the selectivity of the assay based on MAb 7G1. M-ficolin appears in two closely overlapping peaks, the major peak eluting just 1 ml after the void volume. The estimated apparent M_r of the first peak is higher than that of IgM (970 kDa), whereas the estimated apparent M_r of the second peak is just lower than IgM but higher than MBL (~800 kDa). Figure 2a illustrates the analysis of L-ficolin in the fractions, which shows two distinct peaks, both eluting later than M-ficolin. The first peak of L-ficolin appears just after MBL with an apparent M_r of ~400 kDa, and the second peak coincides with IgG, i.e. an apparent M_r of 150 kDa.

Specificity of Commercial Anti-M-Ficolin Antibodies

A polyclonal rabbit anti-M-ficolin IgG was tested in various assays and proved to react in all assays more or less equally well with M- and L-ficolin (fig. 1b). When our investigation was nearly finished, we noted that monoclonal anti-M-ficolin antibodies had also become commercially available. The two available antibodies were first tested on rM-ficolin coated onto microtiter wells and both reacted strongly. However, ABS-3601, but not ABS-3605, reacted equally strongly when the coating comprised L-ficolin purified from serum. This was supported by Western blot analysis, where both reacted strongly with blotted M-ficolin, and ABS-3601, but not ABS-3605,

also with blotted L-ficolin. However, we could detect no reaction with fluid phase, native M-ficolin. We tested this by coating the two antibodies onto microtiter wells (in parallel with 7G1), incubated with dilutions of rM-ficolin or dilutions of serum. The wells were then developed with biotinylated MAbs in checkerboard configuration, followed by europium-labelled streptavidin as described. While strong reactions were seen with 7G1 as catcher and developer, no reactions above background were seen with the two commercial MAbs, also not when combined with 7G1 as catching or developing antibody.

M-Ficolin Assay

We created a sandwich assay using 7G1 as the catching antibody and biotinylated 7G1 as the detecting antibody. Dilution curves for rM-ficolin, NHS and rM-ficolin in NHS are shown in figure 3. Clearly, the curves for rM-ficolin and NHS are not parallel. A number of dilution buffers were assessed and the most consistent results for plasma and serum were obtained with the complex buffer composition detailed above. This is a high ionic strength EDTA-containing buffer (this ensures disruption of the calcium-dependent M-ficolin/MASP complexes and also prevents coagulation) with proteins added to reduce background signals, similar to commercial additives for ELISAs. Aggregated IgG is included in order to avoid interference by rheumatoid factors. Ox IgG is included to avoid interaction between anti-bovine IgG antibodies (present at highly variable concentrations in human sera)

Fig. 4. Quantification of M-ficolin in serum and plasma. **a** A sample of blood collected into EDTA and a sample of blood collected without additives, withdrawn from one person, were stored at RT for the indicated time periods. The samples were centrifuged after the given time points and the supernatants (EDTA plasma and

serum) were analyzed in the M-ficolin assay. **b** Measurements of M-ficolin in corresponding serum and EDTA plasma samples from 50 individuals. **c** Stability of M-ficolin during the cycles (2, 6 or 11 cycles) of freezing and thawing of serum from 10 individuals.

and traces of ox IgG, which may originate from the FCS added to the cell culture medium and which co-purify with MAb. Parallelism was neither improved by other buffer compositions nor by diluting the rM-ficolin in various animal sera before further dilutions in buffer. The figure illustrates the addition of rM-ficolin to a human serum. We thus had to determine the concentration of M-ficolin in the standard serum by the arithmetic mean of the values for serum diluted 1/20, 1/40, 1/80 and 1/160. This was determined at 703 ng/ml. The coefficient of variation for the assay was determined from three internal control samples, always included in the assay, to be 17% at 120 ng/ml, 9% at 615 ng/ml and 5% at 1,595 ng/ml. This was based on 20 assays performed over a 4-week period. Adding rM-ficolin to NHS enabled us to determine recovery, but since the curves are not absolutely parallel, the results were not the same when testing at different dilution levels. Estimation of the recovery at the above-mentioned serum dilutions and calculation of the arithmetic mean reveals full recovery (100.1%). Looking at the middle of the curve, recovery near 100% is found.

In a previous study, a similar assay for MBL detected only higher oligomeric forms of this molecule [31]. To test whether the same would be true for the present M-ficolin assay, we tested fractions from GPC containing lower oligomeric forms of rM-ficolin. The resulting signal is illustrated in figure 2b where a crude supernatant of rM-

ficolin has been mixed with NHS and fractionated, and the fractions tested in the assay. Compared to serum alone (fig. 2a inserted in fig. 2b), this shows two additional peaks originating from lower oligomeric forms of rM-ficolin. Both peaks are below the apparent M_r of MBL and above that of IgG.

The concentration of M-ficolin was measured at different time points after blood collection with EDTA or without anticoagulant (fig. 4a). More M-ficolin is estimated in the EDTA plasma than in serum. The concentration of M-ficolin was stable over time in the supernatant from the coagulated sample (serum), whereas the concentration in the supernatant from the EDTA-stabilized blood (plasma) increases 40% after incubation at RT for 24 h.

Fig. 5. M-ficolin levels in blood donors. M-ficolin was determined in the serum of 350 blood donors. The values determined in each individual sample are depicted (**a**). **b** Statistical distribution with median (1,072 ng/ml, line in the box), 5–95 percentiles (whiskers) and arithmetic mean (1,209 ng/ml, cross inside the box). The box indicates the 25–75 percentiles. **c** Distribution of the values depicted on a linear scale. **d** Q-Q plot of the data. **e** Distribution of the values depicted after log transformation. **f** Q-Q plot of these data. **d** The data do not confirm a normal Gaussian distribution, but when the data are logarithmically transformed (**f**), they fit a normal distribution.

Fig. 6. Intra-individual variation in M-ficolin over time. The concentration of M-ficolin was estimated in plasma samples obtained over a 1-year period from 6 individuals.

Figure 4b shows the results of comparing the M-ficolin concentration in samples of EDTA plasma and serum taken at the same time from 50 persons. In this case, the serum was separated from the coagulum after 1 h at RT. While there is good correlation ($r = 0.90$), the plasma values are on average 33% higher than the serum values. Due to expected variations in sample collection and processing, we recommend that M-ficolin routinely should be estimated on serum rather than plasma samples.

A confounding factor often seen when testing biological samples is the influence of repeated freezing-thawing cycles. However, serum M-ficolin was stable through repeated freezing-thawing cycles (fig. 4c).

M-Ficolin Levels in Adults

The concentration of M-ficolin was estimated in serum samples from 350 Danish blood donors (fig. 5). The data were not distributed according to a normal Gaussian curve, but did so when log transformed. The concentration varied from 280 to 4,050 ng/ml, with a geometric mean of 1,069 ng/ml and an arithmetic mean of 1,085 ng/ml, both calculated from the log-transformed data. The 25 percentile is at 774 ng/ml and the 75 percentile at 1,471 ng/ml. The arithmetic mean calculated from the non-log-transformed data is 1,209 ng/ml.

Intra-individual variation in M-ficolin over time was evaluated by examining plasma samples collected regularly during a 1-year period from 6 individuals. Variations

of 15–30% in M-ficolin concentrations were observed for all 6 individuals during this time period (fig. 6).

Acute Phase Response

Sequential serum samples were obtained from patients to be operated for colorectal cancer. These samples were previously examined for IL-6, C-reactive protein (CRP), MBL and MASP-2 [28]. We selected samples from 6 patients with a low preoperative CRP level and high post-operative rise in CRP and a drop to near CRP baseline at the latest samples taken. The CRP response is depicted in figure 7a, and the values for M-ficolin in figure 7b. In general, there is a high variation in the response from the individual patients, but all 6 patients show an initial decrease in M-ficolin with trough levels on day 2 when CRP peaks. There may be a tendency to a sluggish increase after ~5 days in some of the patients, but there is no obvious correlation between the magnitude of the individual variation in M-ficolin concentration and the corresponding increase in CRP. The data do not suggest that M-ficolin behaves as a typical acute phase protein.

Ontogeny of M-Ficolin

In order to learn about the ontogeny of M-ficolin, umbilical cord blood samples and sequential blood samples over the 1st year of life were obtained from 6 healthy babies born at term and plasma M-ficolin levels were estimated. The results in figure 8a illustrate that (apart from two high values in the umbilical cords) the M-ficolin levels are stable from birth throughout the 1st year of life, with levels being comparable to adult levels.

In comparison, measurements of L- and H-ficolin on the same samples show these molecules to be lower at birth than after 6 months and onwards (fig. 8b, c). At birth, the level for L-ficolin is 65% of the 6-month sample (mean of serial samples from 15 babies) and for H-ficolin the mean level is 61% at birth compared to the 6-month sample. The 6-month samples are similar to adult levels for both L- and H-ficolin. In a previous study on the ontogeny of MBL [30], 37% of the 3-month level were found at birth, which was similar to adult levels.

Discussion

A number of molecules, membrane bound or soluble, are involved in triggering innate immune defense. The complement system contributes a large number of molecules involved in this process through the lectin pathway and the alternative pathway of complement activation.

Fig. 7. M-ficolin during acute phase responses. M-ficolin was estimated in sequential samples from patients undergoing surgery for colorectal cancer and compared to the CRP response. **a** CRP. **b** M-ficolin. The same signature is used for each patient in the two graphs. The value before surgery is given as day 0.

Fig. 8. Ontogeny of ficolins. The concentrations of M-ficolin (**a**), L-ficolin (**b**) and H-ficolin (**c**) were estimated in sequential samples from 8 babies during their 1st year of life. The values at time 0 are the estimates in serum from blood taken from the umbilical cord.

8

Four molecules activate the lectin pathway: H-ficolin, L-ficolin, M-ficolin and MBL. The present report presents some characteristics of M-ficolin in plasma and serum.

In order to investigate the biological function of M-ficolin and the possible clinical implications of a deficiency in this protein, we decided to raise MABs for the con-

struction of clinically applicable quantitative analyses. L- and M-ficolin are very similar molecules, and it has proven quite difficult to produce antibodies specific for M-ficolin. A complex and prolonged immunization procedure resulted in a modest anti-M-ficolin response in one mouse. Fortuitously, the hybridomas generated from this

mouse yielded an MAb (7G1) with excellent specificity and reactivity. Importantly, it reacted with native M-ficolin in solution. In comparison, a commercially available rabbit anti-M-ficolin antibody was found to react equally well with L- and M-ficolin, and we found that two commercially available anti-M-ficolin MAbs react excellently with M-ficolin when presented bound directly onto a surface, microtiter wells or blotting paper, i.e. partly denatured M-ficolin, while not binding M-ficolin presented in solution. One of them reacted equally well with L-ficolin. Two recently described MAbs bind to both solid phase M-ficolin and L-ficolin [22].

Using ELISA microtiter wells to immobilize 7G1, we found this MAb capable of retrieving M-ficolin from serum as evidenced by Western blot analysis of the eluate. When the blot was developed with 7G1, serum M-ficolin showed a major band at around 300 kDa, which could represent a disulfide-bonded complex of 9 of the 34-kDa M-ficolin polypeptide chains (i.e. 3 subunits each composed of three polypeptide chains) or 12 polypeptide chains (four subunits). However, when the blot was developed with a more sensitive antibody (the rabbit anti-L/M-ficolin antibody mentioned above) a range of lower oligomers could be seen. L-ficolin presented a similar pattern. The Western blot of reduced serum M-ficolin showed one band at about 34 kDa moving just a fraction faster than the polypeptide chain of L-ficolin.

For establishing a routine analysis of M-ficolin we used TRIFMA, as this assay in our hands is generally far superior to ELISA in terms of sensitivity, dynamic range and reproducibility. Basically, TRIFMA and ELISA are very similar, only the reporter in TRIFMA is the completely stable europium ion rather than the product of enzyme activity. The five anti-M-ficolin MAbs we generated were analyzed, and the use of 7G1 as both catching and developing antibody was found to yield superior results. In principle, using the same MAb as catching and developing antibody is only possible when, as for M-ficolin, the target molecule presents two or more identical target epitopes, and is thus capable of cross-linking the solid phase MAb and the biotinylated liquid phase MAb. The 7G1 antibody, when attached to microtiter wells as in TRIFMA, binds largely high-molecular-weight structures (see above).

The possibility may exist that individuals with mutations in the protein encoding part of the M-ficolin gene express M-ficolin with a varying degree of multimerization of the structural subunit. This is known to occur for MBL, another molecule with similar multimerization [1]. To test whether our M-ficolin assay can detect lower

oligomeric forms, we performed GPC on crude M-ficolin supernatant containing M-ficolin with such forms [10]. When we test the fractions, we see a signal also in fractions containing these lower oligomeric forms (fig. 2b). We thus believe that the assay will also pick up M-ficolin in such forms in human serum if this turns out to be an issue in the future.

In preliminary tests, a range of assay conditions were tested for routine quantification of M-ficolin. The chosen conditions are detailed in the Materials and Methods. Unfortunately, rM-ficolin and M-ficolin in serum at no condition show absolutely parallel dilution curves. The reason for this is not entirely clear, but one obvious element is that rM-ficolin shows a broad range of oligomers, most of which are lower oligomer forms [10], while most of the serum M-ficolin is seen as high oligomer forms. Whatever the cause may be, this renders defining the serum concentration somewhat arbitrary as the estimated concentration will vary with the dilution chosen for the calculation. We chose to use the mean of the calculated values at four dilutions and thus arrived at a concentration of 703 ng M-ficolin per milliliter of our standard serum pool, and this value was the basis for further quantifications.

For routine analyses, it is important to know if the analyte is stable in serum and plasma, and if one can freely change between analyzing serum and plasma samples. The values estimated in plasma were quite consistently higher than the values estimated in serum – on average 30% higher. Thus, one must choose either serum or plasma when comparing different groups. We recommend using serum since tests showed that the time the blood sample spent on the bench before being centrifuged was unimportant for serum, but somewhat critical for blood collected with EDTA (fig. 4a). The increasing levels found in EDTA blood could possibly be due to the release of M-ficolin from monocytes and granulocytes. As the granulocytes are by far outnumbering the monocytes, and since (according to the literature and our own unpublished experiments) M-ficolin is stored inside the granulocytes, we assume that it is primarily released from granulocytes, which in addition are more fragile than monocytes. The treatment experiment (fig. 4a) was performed in order to examine assay conditions for clinical samples, where there is often some variation in the handling of the samples.

Evaluation of 350 serum samples from presumed healthy adult blood donors was used to establish a reference range for serum M-ficolin. The direct results did not show a Gaussian distribution; however, such distribution

was seen for the log-transformed values. In this case, the geometric mean of 1,069 ng/ml is of greater relevance than the arithmetic mean of 1,085 ng/ml, even though they are very close to each other. Of great interest is the finding that M-ficolin is a humoral protein present in serum at concentrations similar to those of MBL [32]. This differs remarkably from previous reports claiming that M-ficolin is not present in serum [20], and also from the recent paper by Honoré et al. [22] reporting the finding of only trace amounts of M-ficolin in serum (a mean of 60 ng/ml was estimated from 8 samples). The latter result is based on an assay measuring both M-ficolin and L-ficolin as the authors did not have access to an M-ficolin-specific antibody. The authors thus resort to absorbing L-ficolin from the samples (using anti-L-ficolin antibody and protein A Sepharose beads) before adding the samples to microtiter wells coated with a monoclonal anti-M/L-ficolin antibody, followed by incubation with the polyclonal anti-M/L-ficolin antibody from Hycult Biotechnology also used in the present report.

The observed interindividual difference of up to 15-fold and the comparable constant level of M-ficolin during a 1-year period indicate that there may be a genetic influence on the plasma concentrations. A number of polymorphisms of the M-ficolin gene are found in the databases and it would be logical to look for associations between M-ficolin levels and polymorphisms.

Analyses on samples obtained throughout the 1st year of life showed that M-ficolin was present at adult levels already at birth (cord blood) and no increase occurred during the 1st year. This is different from the cord blood level of roughly 2/3 of the 6-month samples seen for L- and H-ficolin. This difference in ontogeny between the ficolins probably reflects that the latter two are primarily produced by hepatocytes whereas M-ficolin is reported to be produced by monocytes. The fourth member of the molecules initiating the lectin pathway is MBL. This is also produced by hepatocytes and about one third was previously found at birth compared to 3-month samples [30].

Two of the cord blood samples presented higher M-ficolin levels than subsequent samples. We do not know the reason for this, but it may be that an admixture of the mothers' blood had occurred to some degree (although this is not expected from the medical records), with the higher level thus reflecting a mother with high M-ficolin levels. The two higher birth levels may also reflect a response towards stress induced by the birth. The birth of the 2 babies in question would then have to be different from the others tested. We do not see such a difference from the medical records, but this indicates that future

studies should have a closer look at the ontogeny of M-ficolin. The same two samples did not show elevated levels of H- or L-ficolin. It may be relevant to study samples taken at shorter intervals.

M-ficolin has been described as a protein being secreted from granulocytes [20, 33], and the concentration of the protein could thus be expected to increase in certain situations. We analyzed for a possible classical acute phase response of M-ficolin. Serum samples from patients undergoing resection for colon cancer were obtained before the operation and during the following days and weeks. We examined samples from the 6 patients showing most marked acute phase responses as judged by the increase in CRP. We found a modest drop in M-ficolin concentration on day 2. However, the concentration rebounds, reaching varying levels during the following 2 weeks, with some being equal to those before the operation. We would not suggest that M-ficolin is denoted an acute phase protein.

The native size of M-ficolin in serum, as judged by GPC, is comparable to a complex of ~900 kDa, i.e. larger than what we have previously observed for H-ficolin and L-ficolin [19, 34]. This indicates that M-ficolin is found in complex with other proteins apart from the MASPs or that M-ficolin is made up of more polypeptide chains than the other two ficolins. If a larger number of polypeptide chains are present, this leads to a higher number of ligand binding sites and thus to the possibility of an even stronger binding to patterns of ligands. We previously observed two peaks of L-ficolin in human serum [19], but the second peak was relatively smaller in area compared to in the present study. It seems likely that individuals differ in the balance between the two forms of L-ficolin. It may be recounted that the mobility of proteins on GPC reflects the Stoke's radius rather than the mass, and that collagenous proteins may have odd shapes leading to overestimation of their mass.

This report finds M-ficolin as a human serum protein, occurring at a broad range of concentrations in the adult Danish population. Epidemiological investigations, now made possible, are likely to further our understanding of the biological role(s) of this pattern recognition molecule.

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