Interactions of Ficolin and Mannose-Binding Lectin with Fibrinogen/Fibrin Augment the Lectin Complement Pathway

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Key Words

Blood coagulation • Complement • Fibrin • Fibrinogen •
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Abstract

Ficolin and mannose-binding lectin (MBL) are animal lectins that are involved in innate immunity by initiating the lectin complement pathway. Here, we report that interactions between these lectins and fibrinogen/fibrin augment the lectin pathway. An ELISA revealed that recombinant mouse ficolin A (rFcnA), rMBL-A and rMBL-C bind to fibrinogen in a dose-dependent manner. Affinity Western blotting showed that these lectins bind to the α - and β -chains of fibrinogen and the α - and β -chains of fibrin, but not to the γ -chain, and that rMBL-A and rMBL-C preferentially bind to the α - and β -chains. The C4 deposition activity on Fbg-coated plates was observed by using mouse serum, and the deposition on GlcNAc-coated plates was enhanced by fibrinogen supplementation and further enhanced by the addition of thrombin. Similar effects of fibrinogen and fibrin were observed in the bindings of these lectins to a Gram-positive pathogen, *Staphylococcus aureus*, and in the subsequent C3 deposition on the bacteria. In particular, the lectin pathway, through MBLs, seemed to synchronize with blood coagulation. There-

fore, it is suggested that the lectin pathway collaborates with the coagulation system in the first-line host defense against pathogens under conditions such as injury and inflammation.

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Introduction

The molecular basis of innate immunity lies in the ability of firstline host defense molecules to discriminate infectious agents from self. Lectin occupies a crucial position in this system through its recognition of pathogen-associated molecular patterns exposed on pathogens. Among the animal lectins, ficolin (FCN) and mannose-binding lectin (MBL) have a unique property in activating complement via MBL-associated serine proteases (MASPs) in the lectin pathway [1–4]. Upon the bindings of FCN and MBL to pathogens, the pro-enzymes of MASPs are converted to the active forms and, in turn, MASPs activate the complement.

FCN is a group of proteins containing both a collagen-like and a fibrinogen (Fbg)-like domain [5], and the latter is responsible for a common binding specificity for *N*-acetylglucosamine (GlcNAc) [6]. To date, 3 types of FCN

have been identified in humans, L-FCN [5], M-FCN [7, 8] and H-FCN [9], and 2 types in mice, FcnA [10] and FcnB [11]. FCN is roughly classified into 2 groups: the serum type, which includes L-FCN, H-FCN and FcnA, and the non-serum type, which includes M-FCN and FcnB [12]. We have previously demonstrated that all human FCN can form complexes with MASPs and sMAP, a truncated form of MASP-2, to consequently activate the lectin pathway [2, 13–15].

Recently, X-ray crystal analysis of FCN identified the 3D structure of its Fbg-like domain and the molecular architecture underlying GlcNAc-recognition, which are very similar to those of tachylectin 5A, a defense lectin in horseshoe crab [6, 16, 17]. Interestingly, tachylectin 5A, which is mainly composed of the Fbg-like domain, binds to the tetrapeptide GlyProArgPro, a well known inhibitor of blood coagulation, in addition to GlcNAc [18]. It is known that this tetrapeptide mimics the N-terminal sequences of α - and β -chains of fibrin (Fbn). Tanio et al. [16] reported that the binding of M-FCN to GlcNAc was inhibited by this tetrapeptide, indicating that FCN also interacts with this tetrapeptide. Based on these observations, it seems possible that FCN can interact with Fbg and/or Fbn. In addition, our phylogenetic analysis of Fbg-like sequences revealed that the Fbg-like domain of FCN is structurally related to the γ -chain of Fbg, and possibly evolved from a common ancestor [19, 20]. This suggests the plausible ability of FCN to bind to the α - and β -chains, because the N-termini of the α - and β -chains interact with the 'a- and b-pockets' of the γ -chain for fibrin clotting during blood coagulation.

To confirm the interaction of FCN with Fbg and/or Fbn, we assessed the binding of FcnA to Fbg- and Fbn-coated microtiter plates and the C4 deposition activity of serum on the plates. In a comparison, the bindings of MBL-A and MBL-C to Fbg- and Fbn-coated plates were also assessed. Next, using *Staphylococcus aureus* as a non-self target, we assessed the effects of Fbg and Fbn on the bindings of these recognition molecules to the bacteria and on the C3 deposition activity of serum on the bacteria. We report here the enhanced bindings of the recognition molecules to the targets and the subsequent, augmented complement activation in the presence of Fbg and/or Fbn. We also discuss the possible collaboration of the lectin pathway with the blood coagulation system and its physiological significance in host defense of innate immunity.

Materials and Methods

Recombinant FcnA, Recombinant MBLs and Mouse Serum

The recombinant FcnA (rFcNA) was prepared as previously described [15], and used for the assays of its bindings to Fbg- and GlcNAc-BSA-coated microtiter plates and to *S. aureus*. The recombinant mouse MBLs (rMBLs), termed rMBL-A and rMBL-C, were purchased from R&D Systems (Minneapolis, Minn., USA) and used for the similar binding assays. The pooled serum was obtained from C57BL/6 mice, and used for C4- and C3-deposition assays to determine the activity of the wild type (WT) mouse serum, as described below. The pooled FcnA-deficient serum was obtained from FcnA-deficient mice, which was established by gene targeting in a C57BL/6 background [manuscript in preparation]. To prepare the FcnA- and MBL-depleted serum (FcNA/MBL-depleted serum), the FcnA-deficient serum was incubated with 1/10 volume of mannan-agarose gel slurry at 4°C for 2 h, and then centrifuged to remove the agarose gel.

All DNA recombination and animal studies were conducted according to the guidelines of Fukushima Medical University.

ELISA for the Bindings of Recombinant rFcNA and rMBLs to Fbg and Fbn

Microtiter plates (Sumilon, Sumitomo Bakelite, Tokyo) were coated with human Fbg (>80% of clottable protein; Sigma-Aldrich, St. Louis, Mo., USA) or with mouse Fbg (>80% of clottable protein, Sigma Aldrich) at a concentration of 10 μ g/ml in 0.25 M Na-phosphate, pH 7.5, and incubated overnight at 4°C. The plates were then washed with 50 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl, 2 mM CaCl_2 and 0.5% Tween-20 (TBS-Ca/T) and blocked with 0.1% BSA in TBS-Ca at 37°C for 3 h. rFcNA, rMBL-A and rMBL-C were incubated in the Fbg-coated microtiter plates at amounts of 0.031 to 2 μ g per well in 100 μ l of reaction mixture, at 37°C for 1 h. After washing with TBS-Ca/T, the microtiter plates were incubated with anti-FcnA [15], anti-MBL-A and anti-MBL-C Abs (HyCult Biotechnology, Uden, Netherlands), followed by the biotinylated second Abs (DakoCytomation, Glostrup, Denmark), and finally with the avidin-biotinylated HRP complex (ABC reagents; Vector Lab, Burlingame, Calif., USA). HRP activity was determined by incubation with TMB (KPL Co., Gaithersburg, Md., USA) and H_2O_2 at room temperature for 1–10 min, which was stopped with 1 M H_3PO_4 . The developed color was monitored in a Multimode detector DTX880 (Beckman Coulter, Fullerton, Calif., USA) at 450 nm. In a comparison, the bindings of above recombinants to GlcNAc and their nonspecific bindings were evaluated by using GlcNAc-BSA-coated and non-coated microtiter plates, respectively.

In a similar experiment to assess their bindings to Fbn, the Fbg-coated microtiter plates were washed with TBS-Ca/T and then treated with 20 mU of human thrombin (>2,000 NIH units/mg protein; Sigma Aldrich) at 37°C for 30 min in 100 μ l of TBS-Ca per well. The plates were washed again, blocked and then used for the binding assay described above. In an inhibition experiment, rFcNA, rMBL-A and rMBL-C were incubated in the Fbg-coated microtiter plates in the presence of up to 150 mM GlcNAc or mannose.

Affinity Western Blotting for Fbg and Fbn with rFcNA, rMBL-A and rMBL-C as Probes

To prepare Fbn, Fbg was treated with 20 mU of thrombin at 37°C for 10 min. The deglycosylated Fbg was prepared by treating

Fbg with endoglycosidase F (endo F) as described [21]. The same amounts of Fbg, deglycosylated Fbg and Fbn were subjected to SDS-PAGE under reducing conditions, and then transferred to an Immobilon-P filter (Millipore, Billerica, Mass., USA). The filter was probed with rFcna, rMBL-A and rMBL-C at concentrations of 0.05 µg/ml in TBS-Ca/T. The bound recombinants were detected by incubation with primary Abs against Fcna, MBL-A and MBL-C. The signal was visualized by further incubation with biotinylated second Abs (DakoCytomation), ABC reagents (Vector Lab), and a chemiluminescence substrate (ECL; Amersham Biosciences, Amersham, UK). The chemiluminescence image was read in a LAS-3000 (Fujifilm, Tokyo).

C4 Deposition on Fbg-Coated or GlcNAc-Coated Microtiter Plate

To evaluate the complement activation through the lectin pathway, C4 deposition was determined by an ELISA, as previously described [15]. Briefly, up to 2 µl of mouse serum was incubated in a Fbg-coated microtiter plate in 100 µl of TBS-Ca at 37°C for 10 min. The plate was then incubated with human C4 on ice for 30 min, and the bound C4b was detected with HRP-sheep anti-human C4 Ab (Biogenesis, Poole, UK). Color was developed using TMB (KPL Co.) and H₂O₂, and monitored in a DTX880 Multimode detector (Beckman) at 450 nm. In another experiment to evaluate the effects of Fbg/Fbn on C4 deposition on GlcNAc, 0.75 µl of the mouse serum was incubated with GlcNAc-BSA-coated microtiter plates in 100 µl of TBS-Ca containing various amounts of Fbg in the presence or absence of 20 mU thrombin at 37 °C for 10 min, and bound C4b was detected as above.

FACS for the Bindings of rFcna, rMBL-A and rMBL-C to S. aureus

The bindings of rFcna, rMBL-A and rMBL-C to *S. aureus* were evaluated as previously described [14]. Briefly, 1 × 10⁷ cells of heat-killed *S. aureus* were incubated with 0.45 µg of rFcna, rMBL-A, and rMBL-C in 60 µl of Hanks balanced salt solution containing Ca²⁺ and Mg²⁺ (HBSS⁺⁺; Sigma Aldrich) at 4°C for 1 h. After washing with HBSS⁺⁺, *S. aureus* was further incubated with anti-Fcna, anti-MBL-A and anti-MBL-C Abs, and then with FITC-labeled second Abs (DakoCytomation). In an inhibition experiment, rFcna, rMBL-A and rMBL-C was incubated with 150 mM GlcNAc or mannose prior to incubation with the bacteria. The binding activity was evaluated on a FACScalibur flow cytometer (BD Biosciences, San Jose, Calif., USA).

In another experiment to evaluate the effects of Fbg/Fbn, the heat-killed *S. aureus* was pretreated with Fbg in the presence or absence of 10 mU thrombin at 37 °C for 20 min in 60 µl of HBSS⁺⁺, then washed with HBSS⁺⁺. *S. aureus* treated was then subjected to the binding assay, where it was incubated with 0.45 µg of rFcna, rMBL-A and rMBL-C at 4 °C for 1 h. The recombinants bound to the bacteria were detected as above.

Preparation of Fcna-MASP and MBLs-MASP Complexes from Mouse Serum

Fcna-MASP and MBLs-MASP complexes were prepared from the pooled mouse serum by GlcNAc-agarose chromatography, as the mannose- and GlcNAc-eluates, respectively. Briefly, the pooled mouse serum from WT mice was applied to a GlcNAc-agarose column, and the column was extensively washed with

TBS-Ca/T. The bound fraction was eluted with 0.3 M mannose and then with 0.3 M GlcNAc. The 2 eluates, mannose- and GlcNAc-eluates, were dialyzed against TBS-Ca and concentrated in a centrifugal filter (Amicon Ultra-4; Millipore). The protein concentration of the eluate was determined using a BCA protein assay kit (Pierce, Rockford, Ill., USA).

FACS for C3 Deposition on S. aureus

C3 deposition was assessed as previously described [22]. Briefly, 1 × 10⁸ cells of heat-killed *S. aureus* were incubated with 10 µl of the pooled mouse serum in the presence or absence of 30 µg Fbg and 100 mU thrombin in 45 µl of HBSS⁺⁺ at 37°C for 2 min. The reaction was terminated by adding 1 ml of chilled HBSS⁺⁺. The C3b on the bacteria was quantified using rat anti-mouse C3b (HyCult Biotechnology) and FITC-conjugated anti-rat IgG (DakoCytomation) Abs in a FACScalibur flow cytometer (BD Biosciences). In a similar experiment to evaluate the C3 deposition activities of Fcna-MASPs and MBLs-MASPs complexes, Fcna/MBL-depleted serum was supplemented with 6.0 µg of the mannose and GlcNAc eluates and subjected to C3 deposition assay as above.

Results

rFcna Bound to the Aα- and Bβ-Chains of Fbg, and the α- and β-Chains of Fbn

ELISA revealed that rFcna bound to the Fbg-coated microtiter plates in a dose-dependent manner (fig. 1a). A similar binding was observed when the Fbg-coated microtiter plate was treated with thrombin, in which a proportion of the coated Fbg is converted to Fbn, although the conversion was not experimentally confirmed. Another ELISA with mouse Fbg-coated plates instead of human Fbg-coated plates revealed very similar results (data not shown). As shown in figure 1b, affinity Western blotting demonstrated that rFcna recognized the Aα- and Bβ-chains of human Fbg, in addition to the α- and β-chains of Fbn, but not the γ-chain of Fbg/Fbn. Treatment of Fbg with endo F reduced the molecular sizes of the Bβ- and γ-chains but did not affect the α-chain. Treatment of Fbg with O-glycanase (endo-α-N-acetylgalactosaminidase) in the presence of neuraminidase, which cleaves O-linked sugars, showed no effect on the sizes of all 3 chains (data not shown). As shown in figure 1b, rFcna also bound to the deglycosylated Bβ-chain. These results indicate that rFcna binds to Fbg and Fbn not through the carbohydrate moiety of Fbg/Fbn. The binding of rFcna to Fbg was as high as that to GlcNAc (fig. 1c), although a precise comparison between the Fbg-coated and GlcNAc-coated microtiter plates is difficult. At least, the binding ability of rFcna to Fbg seems to be higher than those of rMBL-A and rMBL-C (compare fig. 1c with fig. 2a). As

Fig. 1. Binding of rFcnA to Fbg/Fbn. **a** Dose-dependent binding of rFcnA to Fbg-coated (open circle) and Fbg-coated/thrombin-treated microtiter plates (closed circle), as determined by ELISA. In a control experiment, nonspecific binding of rFcnA to non-coated (open square) and non-coated/thrombin-treated microtiter plates (closed square) was evaluated. **b** Affinity Western blottings of Fbg and Fbn probed with rFcnA. In Coomassie Brilliant Blue (CBB) staining (left panel) and affinity Western blotting (right panel), 1.2 μg each of Fbg (lane 1), endo F-treated Fbg (lane 2) and Fbn (lane 3) were subjected to SDS-PAGE on 8% polyacryl-

amide gel under reducing conditions. The deglycosylated Bβ- and γ-chains are marked with asterisks. **c** Comparison of the binding of rFcnA to Fbg-coated microtiter plates (closed circle) with that to GlcNAc-BSA-coated (open circle). Open squares show results from nonspecific binding to non-coated plates. **d** Dose-dependent inhibition of GlcNAc on Fbg-binding of rFcnA. rFcnA was used at 0.45 μg/well. Closed circles show results from blank without rFcnA and GlcNAc. **e** Affinity Western blotting of Fbn probed with rFcnA in the presence of 100 mM GlcNAc.

shown in figure 1d, the binding of rFcnA to Fbg was inhibited by GlcNAc in a dose-dependent manner. Complete inhibition was observed at a concentration of GlcNAc exceeding 50 mM. A similar inhibitory effect of GlcNAc on the binding to Fbn was also observed in the affinity Western blotting (fig. 1e). These results confirmed the binding specificity of rFcnA to Fbg, and suggest that rFcnA recognizes Fbg and Fbn by the same or a very similar GlcNAc-binding site.

rMBL-A and rMBL-C Showed a Binding Preference for α- and β-Chains over Aα- and Bβ-Chains

As shown in figure 2a, rMBL-A and rMBL-C bound to Fbg, although their bindings appears to be very weak as compared with those to GlcNAc. Unlike rFcnA, the bindings of rMBLs were slightly increased by treating the Fbg-coated plates with thrombin (fig. 2b). The bindings of rMBLs to Fbg and Fbn (Fbg treated with thrombin) were reduced to 25–50% by excess amounts of GlcNAc and

Fig. 2. Binding of rMBL-A and rMBL-C to Fbg/Fbn. **a** Dose-dependent binding of rMBL-A (upper panel) and rMBL-C (lower panel) to Fbg-coated (closed circle), GlcNAc-BSA-coated (open circle) and non-coated microtiter plates (open square), as determined by ELISA. **b** Inhibitory effects of GlcNAc (G) and mannose (M) on the bindings of rMBL-A and rMBL-C. 1 μg/well each of rMBL-A (upper panel) and rMBL-C (lower panel) was incubated

in Fbg-coated and Fbg-coated/thrombin (Thr)-treated microtiter plates in the presence of 100 mM of GlcNAc (G) or mannose (M). Each binding level is expressed by subtracting the blank level without rMBLs. **c** Affinity Western blotting of Fbg/Fbn probed with rMBL-A (upper panel) and rMBL-C (lower panel). The Fbg and Fbn were blotted as in figure 1B.

mannose (fig. 2b), suggesting that rMBLs recognize Fbg and Fbn through similar sites to their sugar recognition. Affinity Western blotting revealed that, like rFcnA, rMBLs can bind to the Aα-, Bβ-, α- and β-chains, but not to the γ-chain (fig. 2c). Unlike rFcnA, rMBLs bound much more strongly to the α- and β-chains than to the Aα- and Bβ-chains. Similar to rFcnA, rMBL-A and rMBL-C also recognized the deglycosylated Fbg, suggesting that the recognition is not via the carbohydrate moiety of Fbg.

C4 Deposition was Observed on Fbg-Coated Microtiter Plates, and C4 Deposition on GlcNAc-BSA-Coated Microtiter Plates was Enhanced in the Presence of Fbg/Fbn

As shown in figure 3a, the pooled serum of WT mice revealed the C4 deposition activity on Fbg-coated microtiter plates, although the activity was much lower

compared with that on GlcNAc-BSA-coated plates. The addition of Fbg to the serum enhanced C4 deposition on the GlcNAc-BSA-coated plates in a dose-dependent manner (fig. 3b). The enhanced effect of Fbg was largely amplified by adding thrombin to the reaction mixture. Fbg/Fbn appeared to be effective in the physiological range of serum concentration.

Bindings of rFcnA, rMBL-A and rMBL-C to S. aureus were Enhanced by Fbg/Fbn

We next evaluated the effects of Fbg/Fbn using *S. aureus* as a non-self target. As shown in figure 4a, the binding of rFcnA to the bacteria was enhanced in the presence of Fbg. The binding was further enhanced by the addition of thrombin to the reaction mixture. Similar but slightly distinct effects of Fbg/Fbn were observed on the bindings of rMBL-A and rMBL-C to *S. aureus*. Fbg showed no or

Fig. 3. a C4 deposition activity of WT mouse serum on Fbg-coated microtiter plates (open circle). The various amounts of pooled mouse serum were incubated in Fbg-coated plates at 37 °C for 10 min. In a comparison, C4 deposition activity on GlcNAc-BSA-coated (open square) and that on non-coated microtiter plates (closed circle) were determined. **b** Effects of Fbg (open square) and Fbn (Fbg plus thrombin; closed square) on C4 deposition activity of mouse serum on GlcNAc-BSA-coated microtiter plates. The various amounts of Fbg were mixed with 0.75 μ l per well of serum in the presence (closed square) or absence (open square) of 20 mU of thrombin. The shaded area represents the physiological range of Fbg in the human serum, which was calculated from the amounts of serum and Fbg used.

only a small effect on the bindings of rMBL-A and rMBL-C, while the presence of both Fbg and thrombin in the reaction mixture largely enhanced the bindings of rMBLs (Fig. 4b, c). This profile was similar to that in the bindings of rMBLs to Fbg-coated and Fbg-coated thrombin-treated plates, as described above (fig. 2b).

In an additional experiment to evaluate the effect of Fbg/Fbn, *S. aureus* was pretreated with Fbg and Fbn (Fbg plus thrombin), and then subjected to binding with rFcnA. As compared with simultaneous mixing of rFcnA and Fbg, the pretreatment with Fbg showed a reduced binding of rFcnA to the bacteria (fig. 4d, center panel), suggesting that a population of the bound Fbg is washed out before the incubation with rFcnA. In contrast, pretreatment of *S. aureus* with Fbg and thrombin (fig. 4d, right panel) showed a very similar binding level to that observed by the simultaneous mixing. These results suggest that rFcnA binds to Fbg/Fbn on the surface of bacteria in addition to the carbohydrate ligands.

C3 Deposition on S. aureus was Enhanced in the Presence of Fbg/Fbn

C3 deposition on *S. aureus* was assessed using pooled mouse serum in the presence or absence of Fbg/Fbn. The addition of Fbg to the WT mouse serum enhanced C3 deposition on the bacteria (fig. 5a). The enhanced C3 deposition was amplified by the addition of thrombin. To evaluate the activities of FcnA-MASP and MBLs-MASP complexes, we assessed the C3 deposition on *S. aureus* using FcnA/MBL-depleted serum containing these complexes. As shown in figure 5b, C3 deposition activity of

the mixture containing the FcnA/MBL-depleted serum and FcnA-MASPs complex was enhanced in the presence of Fbg, while the addition of thrombin had a limited effect on C3 deposition. In contrast, C3 deposition of the mixture containing the FcnA/MBL-depleted serum and the MBLs-MASPs complex was less enhanced by Fbg, but further addition of thrombin largely enhanced C3 deposition on the bacteria (fig. 5c).

Discussion

In the present study, we experimentally verified the interaction of Fbg/Fbn with the recognition molecules of the lectin pathway, in which rFcnA and rMBLs directly bound to Fbg-coated plates, and Fbg/Fbn enhanced the bindings of rFcnA and rMBLs to *S. aureus* and the subsequent activation of complements C4 and C3. We also found that rFcnA and rMBLs bind to the α - and β -chains of Fbg, and the α - and β -chains of Fbn, but not to the γ -chain. A difference was observed in the binding property between rFcnA and rMBLs: rFcnA binds to the 4 chains with similar affinities, while rMBLs preferentially bind to the α - and β -chains. These binding profiles were observed throughout our present study, which included ELISA with Fbg/Fbn-coated microtiter plates, affinity Western blotting for Fbg/Fbn, and FACS for the binding to the bacteria. The subsequent complement activation showed a similar profile, which can be explained by the respective binding property of rFcnA or rMBLs (fig. 5b, c), or by the mixture of both (fig. 3b, 5a).

Fig. 4. Binding of rFcnA (**a**), rMBL-A (**b**) and rMBL-C (**c**) to *S. aureus*. The bindings were determined by FACS in the presence of Fbg (thick gray line) and Fbg plus thrombin (Thr) (thick black line). The binding without Fbg and thrombin is depicted with thin black lines, and the blank with the bacteria alone is by a shaded area in each figure. **d** Binding of rFcnA to *S. aureus* pretreated

with Fbg. The bacteria were pretreated with HBSS⁺⁺ alone (left panel), Fbg (center panel) and Fbg plus thrombin (right panel), and then subjected to the binding assay with rFcnA. The result obtained (thick black line) is compared with that by the simultaneous mixing as in **a** (thick gray line). The shaded area shows the results from blank with bacteria alone.

Fig. 5. a C3 deposition activity of WT mouse serum on *S. aureus* in the presence of Fbg (thick gray line) and Fbg plus thrombin (thick black line). WT mouse serum was incubated with *S. aureus* in the presence of Fbg (thick gray line) or Fbg plus thrombin (black thick line) as described in 'Materials and Methods'. In a control experiment, the C3 deposition activity was determined in the absence of Fbg and thrombin (thin black line). The shaded area shows the results from blank with the bacteria alone. **b, c** C3

deposition activity of the FcnA/MBL-depleted serum supplemented with the GlcNAc-eluate (**b**) or the mannose-eluate (**c**). The FcnA/MBL-depleted serum was mixed with Fbg, the GlcNAc-eluate or the mannose-eluate and *S. aureus* in the presence (thick black line) or absence (thick gray line) of thrombin (Thr). In a control experiment, C3 deposition activity was determined for the same mixture without Fbg (**b, c**, thin black line) and for the FcnA/MBL-depleted serum alone (**b, c**, thin gray line).

Fig. 6. A schematic model showing the collaboration of the lectin pathway with blood coagulation. Each molecule of FCN or MBL binds to carbohydrates on the pathogen at the ligand-binding site(s) and also binds to Fbg/Fbn at the remaining binding site(s). Thrombin and possibly MASP-2 activate factor XIII, and the activated factor XIII (XIIIa) crosslinks between Fbn and pathogen, in addition to between Fbn. These bindings (depicted with circles) form a network on the surface of pathogen, which stabilizes the recognition molecule-target complex and therefore augments the lectin pathway by enhancing the activation of MASPs and the subsequent activation of complements such as C4, C2 and C3.

Our preliminary estimation of the concentrations of FcnA and MBLs in mouse serum suggests that the amount of FcnA is less than 10% of the total amount of MBL-A plus MBL-C (data not shown). Therefore, the complement activation of mouse serum would mainly reflect the sum total of the activities of MBLs rather than that of FcnA. The C4- and C3-deposition activities of WT mouse serum could be predominantly explored by MBLs, that explains the enhanced activity in the presence of Fbn.

It is well known that FCN recognizes the GlcNAc residue, but not the mannose residue, through its Fbg-like domain [5, 6, 23], whereas MBLs recognize both mannose and GlcNAc residues through their carbohydrate recognition domain. Our inhibition experiments revealed that rFcna recognizes the A α -, B β -, α - and β -chains through the same or a similar site to the GlcNAc-binding site, because the binding of rFcna to Fbg-coated microtiter plates and to *S. aureus* was inhibited by excess amounts of GlcNAc. Similarly, the bindings of rMBLs to Fbg/Fbn-coated microtiter plates were inhibited by GlcNAc and mannose, although the inhibition was in-

complete. This suggests that rMBLs recognize the four chains through their carbohydrate recognition domain, which overlaps with the sugar-binding sites, with a preferential affinity for the α - and β -chains. Another important observation is that rFcna and rMBLs recognize Fbg/Fbn not through the sugar moiety, because deglycosylation of Fbg did not affect their binding activity. Therefore, rFcna and rMBLs recognize some polypeptide regions of Fbg/Fbn through the Fbg-like and carbohydrate recognition domains, respectively, although the precise ligand sites on Fbg/Fbn are unknown.

The difference in the binding mode between rFcna and rMBLs suggests the distinct physiological significance committed to them. The strong binding of rFcna to both Fbg and Fbn suggests that this interaction is constitutively ready for host defense against non-self and is dependent on Fbg, but independent of coagulation. In contrast, the preferential affinity of rMBLs to Fbn suggests that this interaction is induced at times when the coagulation is triggered. Thus, the interaction of the recognition molecules of the lectin pathway with Fbg/Fbn

represents 2 types of collaboration between the complement and the blood coagulation systems. Of particular note, the lectin pathway through MBLs appears to be tightly coupled with blood coagulation.

FCN and MBL are oligomers composed of homologous monomer subunits and therefore have multivalent ligand-binding sites [9, 24]. Fbg is a dimer of 3 subunits, formulated as $(A\alpha)_2(B\beta)_2\gamma_2$, and Fbn is a polymer consisting of $(\alpha_2\beta_2\gamma_2)_n$. The interaction of 2 multivalent molecules, recognition molecules and Fbg/Fbn, would form a huge network overlapping targets, including pathogens. This network would serve as a powerful tool for host defense through preventing mobilization and infection of pathogens and ultimately leading to their phagocytosis and killing, especially in the cases such as injury, as illustrated in figure 6. It was reported that the commercially available Fbg preparation includes factor XIII as a contamination [25]. This might explain our present results, especially the result shown in figure 4d, where the mixing of Fbg, thrombin and *S. aureus* possibly activated the contaminated factor XIII, in addition to the conversion of Fbg to Fbn. The activated factor XIII would cross-link covalently between Fbn and the bacteria [25] (fig. 6). In addition, Fbg-binding proteins, which have been identified in *S. aureus* [26, 27], probably contribute to anchor the network to the surface of the bacteria. Therefore, each molecule of FCN and MBL is allowed to bind to both carbohydrates and Fbn on the bacteria. The network formed on the surface of bacteria results in the enhanced bindings of FCN and MBL, enhanced activation of MASPs and complements, and severe opsonization of the bacteria with C3b and C4b, which finally leads to the killing of bacteria.

Interestingly, it was reported that MASP-1, one of 3 kinds of MASP identified widely in vertebrates [28], showed thrombin-like activity [29, 30]. The physiological meaning of this activity is unknown. It is also unknown whether this activity is specifically endowed to MASP-1, because MASP-1 appears to have a broad range of substrate specificity. If MASP-1 actually has this type of activity in vivo, it implies that the activated MASP-1 can initiate coagulation. It was also reported that MASP-2, which was identified in relatively higher vertebrates [28], was capable of forming Fbn by cleaving prothrombin and factor XIII [25]. These observations suggest another collaboration between the lectin pathway and coagulation system. Because MASP-1 and MASP-2 form complexes with both FCN and MBLs, the reported activities of MASP-1 and MASP-2 could be fully displayed in close contact with Fbg/Fbn. Thus, it is possible that the lectin pathway and the coagulation system collaborate by initiating each other in a bidirectional manner.

It has also been reported that the purified human C-reactive protein can bind to *Salmonella enterica* and L-FCN, and that the interaction of C-reactive protein with L-FCN stabilized the binding of CRP to the bacteria, which therefore increased complement activation [31, 32]. This observation is a close analogy to our present study, in that the recognition molecule-pathogen complex is stabilized by another plasma protein and, in turn, the stabilization boosts the lectin pathway. Thus, in conjunction with the previous reports, the present study clearly indicates that the lectin pathway crosstalks and collaborates with other defense systems such as blood coagulation and acute phase inflammation, to eliminate non-self with high efficiency.

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