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OPSONIC FUNCTION AND CONCENTRATION OF
HUMAN SERUM FICOLIN/P35

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Abstract : Collectins, C-type (Ca^{2+} -dependent) animal lectins with both collagenous and carbohydrate recognition domains, function as opsonins against pathogens. We previously described an *N*-acetylglucosamine (GlcNAc)-binding lectin (ficolin/P35) with a collagen- and a fibrinogen-like sequence present in human serum. In this report we show that ficolin/P35 can serve as an opsonin and enhance the clearance of pathogens having surface GlcNAc. Ficolin/P35 bound to an Ra chemotype strain of *Salmonella typhimurium* (TV119) which has an exposed GlcNAc at the non-reducing termini of the polysaccharide. On the other hand, ficolin/P35 did not bind to LT2, a smooth type strain of *S. typhimurium* with additional O-polysaccharides covering GlcNAc. Ficolin/P35 enhanced the uptake of TV119 by monocytes or polymorphonuclear leukocytes but had no opsonic activity towards LT2. These results suggest that, like collectins, ficolin/P35 is a collagenous lectin which has a role in innate immunity against certain pathogenic organisms by acting as an opsonin.

We prepared monoclonal antibodies against ficolin/P35 and developed an enzyme-linked immunosorbent assay (ELISA) for measuring ficolin/P35 concentrations in humans. The mean serum concentration of ficolin/P35 from 130 normal individuals was estimated to be 13.7 $\mu\text{g/ml}$.

Key words : ficolin, opsonin, ELISA, phagocytosis, serum concentration

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INTRODUCTION

Polymorphonuclear leukocytes and monocytes have important roles in host defense against infecting bacteria by phagocytizing them. Certain animal lectins function as opsonins against microorganisms bearing certain oligosaccharides on their surfaces. Collectins^{1,2}, a group of oligomeric C-type (Ca^{2+} -dependent)³ animal lectins, include mannose-binding lectin (MBL)⁴, conglutinin, CL-43⁵, and the pulmonary surfactant proteins⁶, SP-A and SP-D. These collectins play an important role in host defense. With carbohydrate-recognition domains they bind to oligosaccharides on the surfaces of microorganisms⁷ and through their collagenous regions act as opsonins by binding to the receptors on a wide range of phagocytic cells^{2,8}.

We previously reported a novel type of human serum *N*-acetylglucosamine (GlcNAc)-binding lectin termed ficolin/P35⁹. Ficolin/P35 is the same protein as elastin-binding protein (EBP-37)¹⁰ and hucolin¹¹ and belongs to the ficolin family which is characterized by the presence of a collagenous domain and a fibrinogen-like domain^{12,13}. Ficolin/P35 forms a homopolymer through intermolecular disulfide bonding as do the collectins. More recently, we reported that ficolin/P35 activates the complement system via the lectin pathway in association with MBL-associated serine protease (MASP), suggesting that it may play a role in innate immunity¹⁴.

In this report we describe the binding of ficolin/P35 to *Salmonella typhimurium* possessing GlcNAc at the non-reducing termini of their lipopolysaccharide, its opsonic function and the serum concentration of ficolin/P35 determined by ELISA.

MATERIALS AND METHODS

Reagents

Mannan from *Saccharomyces cerevisiae* and bovine serum albumin (BSA) were purchased from Sigma Chemical, GlcNAc from Wako Pure Chemical Industries and CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals. Coupling of mannan to CNBr-activated Sepharose was performed according to the manufacturer's instructions. [¹²⁵I]NaI was purchased from Amersham International. Ficolin/P35 was labeled using [¹²⁵I]NaI and Iodo-Gen from Pierce Chemical.

Purification of human ficolin/P35

Highly purified ficolin/P35 was prepared as described previously⁹. Protein concentration was determined by measuring the optical density at 280 nm or with a BCA protein assay kit (Pierce Chemical).

Preparation of monoclonal antibodies against ficolin/P35

Female BALB/C mice were injected subcutaneously with purified ficolin/P35 (10

μg) in complete Freund's adjuvant. After 3 weeks, the mice were boosted with 10 μg of ficolin/P35 and 3 days later, spleens were removed and fused with NS-1 cells at a spleen cell to myeloma cell ratio of 5 : 1 using PEG 1500 (Boehringer Mannheim). Hybridoma cells were seeded in 96-well tissue culture plates at a concentration of 10^5 cells/well in RPMI 1640 medium containing 15% fetal calf serum and HAT supplement. Hybridoma cells producing anti-ficolin/P35 antibodies were obtained by ELISA or Western blotting screening of culture supernatants for those which reacted with ficolin/P35. Six of the positive monoclonal antibodies (2F5, GN1 ~GN5) were purified from ascites fluid of mice bearing the hybridoma by precipitation with 50% saturated ammonium sulfate, followed by chromatography on DEAE-Toyoparl. All monoclonal antibodies were of the IgG1 isotype.

Radioiodination of purified ficolin/P35

Radioiodination was carried out by the Iodo-Gen (Pierce Chemical) method with 14.8 MBq of Na^{125}I and 160 μl of protein solution (50 $\mu\text{g}/\text{ml}$) in 0.2 M phosphate buffer, pH 7.2. Excess free ^{125}I was removed with an Excellulose GF-5 desalting column (Pierce Chemical) eluted veronal buffer. The specific activity of radioiodinated ficolin/P35 was 0.24 MBq/mg.

Bacterial strains

Three strains of *Salmonella typhimurium*, TV119, TV160 and LT2, were kindly provided by Dr. K. Yasunaka, (Department of Microbiology, Fukuoka University School of Medicine). TV119 belongs to an Ra chemotype strain lacking O-polysaccharide, but bearing normal Lipid A and a complete core polysaccharide with an exposed GlcNAc, TV160 has incomplete core polysaccharides, lacking GlcNAc and LT2 has additional O-polysaccharides covering GlcNAc. Bacteria were cultivated overnight at 37°C in LB broth and then diluted 1 : 50 in 5 ml of fresh broth. After incubation at 37°C for 3 hr, bacteria at the mid-log phase were washed 3 times with HBSS containing 0.1% gelatin (GHBSS) and resuspended in GHBSS containing 10 mM CaCl_2 at an A_{660} of 0.5 (1×10^8 colony-forming units/ml).

Binding of radiolabeled ficolin/P35 to S. typhimurium

S. typhimurium (1×10^7 CFU of TV119, TV160, or LT2) in 100 μl of GVB containing 2 mM CaCl_2 in 1.5 ml Eppendorf tubes were treated with 10 to 50 μl of ^{125}I -ficolin/P35 ($7 \sim 35 \times 10^4$ cpm) for 30 min on ice and then washed 3 times by centrifugation at $7,000 \times g$ with GVB containing 2 mM CaCl_2 . The pellets were transferred to new microfuge tubes for the last wash and counted in a gamma counter.

Preparation of polymorphonuclear neutrophils (PMNs) and monocytes

Heparinized whole blood from healthy volunteers was centrifuged at 300 g for 10 min. After removal of the supernatant, blood cells were diluted two-fold with PBS.

Cells were layered on Polymorphoprep (Nycomed Pharma) and centrifuged at 350 g for 30 min at 20°C. After the mononuclear leukocyte fraction was washed twice with RPMI 1640, residual erythrocytes were removed by hypotonic lysis. The cells were resuspended at 1×10^7 /ml in RPMI 1640 containing 10% FCS and seeded into 8-chamber Lab-Tek slides (Nunc) at 300 μ l/well were incubated overnight at 37°C in a 5% CO₂ atmosphere. The polymorphonuclear leukocyte fraction was washed with GHBSS, and residual erythrocytes were then removed by hypotonic lysis. The cells were resuspended at 1×10^7 /ml in GHBSS containing 10 mM CaCl₂.

Monocytes were prepared as follows; mononuclear cells prepared as described above were seeded into 8-chamber Lab-Tek slides and cultured overnight in RPMI 1640 containing 10% FCS. After adherent cells were carefully washed 3 times with GHBSS, they were suspended in 100 μ l of GHBSS containing 10 mM CaCl₂ and used as monocytes.

Phagocytosis of S. typhimurium by monocytes

S. typhimurium (1×10^8 CFU of TV119) suspended in 500 μ l of GHBSS containing 10 mM CaCl₂ were incubated in the presence or absence of ficolin/P35 (10 μ g/ml) at 4°C for 30 min in 1.5 ml Eppendorf tube. The bacteria were then washed three times with GHBSS and resuspended in 500 μ l of GHBSS containing 10 mM CaCl₂. One hundred microliter suspensions of bacteria (2×10^7 CFU) were added to monocytes and incubated at 37°C for 5, 10, 20, and 40 min. Phagocytosis was stopped by addition of ice-cold PBS and the cells were washed with ice-cold PBS 3 times. The number of bacteria phagocytized by 400 monocytes was counted by light microscopy after fixation with methanol and staining with May-Giemsa. The phagocytic index was defined as the ratio of the number of ingested bacteria incubated with ficolin/P35 per 400 monocytes to the number of ingested bacteria incubated without ficolin/P35 per 400 monocytes.

Phagocytosis of S. typhimurium by PMNs

S. typhimurium (2×10^7 CFU of TV119) in 200 μ l of GHBSS were incubated at 4°C for 30 min in the presence (50 μ g/ml) or absence of ficolin/P35. Bacteria were then washed 3 times with GHBSS containing 10 mM CaCl₂ and resuspended in 200 μ l of GHBSS containing 10 mM CaCl₂. Two hundreds microliters of suspended PMNs (2×10^6) were added to the bacteria. The mixture of bacteria and PMNs was incubated at 37°C for 20 min with rotational agitation. Phagocytosis was stopped by addition of ice-cold PBS and the cells were washed with PBS 3 times, and cytocentrifuged on a glass plate. They were stained with May-Giemsa and phagocytosis of bacteria by PMNs was observed by light microscopy.

Biotinylation of monoclonal antibody

Anti-ficolin/P35 monoclonal antibody (GN5) was dialyzed against PBS, and diluted to 1 mg/ml with PBS. *N*-hydroxysuccinimidobiotin (BNHS, Sigma, St.

Louis, MO) was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. A mixture of 60 μ l of BNHS and 1 ml of antibody was kept at room temperature for 4 hr. Free BNHS was removed by gel filtration with a using a Sephadex G-25 column and then dialysis against PBS.

Measurement of ficolin/P35 concentration in normal human serum

Microtiter plate (Sumilon S type plate, Sumitomo Bakelite) wells were coated with 100 μ l of anti-ficolin/P35 monoclonal antibody (10 μ g/ml GN4 in 0.25 M phosphate buffer, pH 7.5) by incubating at 4°C overnight. The wells were washed with PBS containing 0.1% Tween 20 and then blocked at 37°C for 2 hr with 1% BSA in 0.1 M phosphate buffer (pH 7.5). Sera were diluted 1/10 with dilution buffer (0.1% BSA in 0.1 M phosphate buffer, pH 7.5), and a standard ficolin/P35 dilution series and serum dilution series were prepared by successive 1 : 2 dilutions with 0.1% BSA in 0.1 M phosphate buffer (pH 7.5). The wells were washed and 50 μ l aliquots of sample solutions and the standard ficolin/P35 solutions were added and the plates were incubated at 37°C for 2 hr. Samples or standards were loaded into the wells in duplicate. Serum samples from 130 normal individuals were provided by Dr. Terai (Department of Laboratory Medicine, Hokkaido University Medicine). After washing, 50 μ l of biotinylated anti-ficolin/P35 monoclonal antibody (GN5, 5 μ g/ml, in dilution buffer) was added to the wells and the plates were incubated at 37°C for 2 hr. After washing, 50 μ l of avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector lab) which had been mixed with dilution buffer 30 min before use, was added to the wells and the plates were incubated at 37°C for 2 hr. The color was developed by addition of 100 μ l of ABTS solution and the absorbance at 405 nm was measured. From these data, we plotted a sigmoid curve on semi-log graph paper for standard ficolin/P35 and serum dilution series. Serum concentrations of ficolin/P35 were calculated as follows: (the concentration of standard ficolin/P35) $\times$ (the dilution of serum) at the parallel region of the sigmoid curve. The results are expressed as means $\pm$ standard error (SE). Statistical analyses were made using the Student's paired and unpaired *t*-tests and Tukey's method after one-way analysis of variance.

RESULTS

Ficolin/P35 binding to TV119, an Ra chemotype strain of S. typhimurium

Ficolin/P35 binds specifically to GlcNAc⁹. In order to elucidate the physiological importance of ficolin/P35, we investigated ficolin/P35 binding to three types of *S. typhimurium* (TV119, TV160 and LT2) using ¹²⁵I-labelled ficolin/P35. The Ra chemotype strain of *S. typhimurium* lacks O-polysaccharides but bears a complete core polysaccharide. TV119, an Ra chemotype strain of *S. typhimurium*, possesses an exposed GlcNAc at the non-reducing termini of the Ra polysaccharide. TV160 lacks a GlcNAc and LT2 possesses additional O-polysaccharides covering a

Fig. 1. Binding of ficolin/P35 to *S. typhimurium*. Binding of ficolin/P35 to three types (TV119, TV160 or LT2) of *S. typhimurium* was investigated using radiolabeled ficolin/P35.

Fig. 2. Phagocytosis of *S. typhimurium* by monocytes. *S. typhimurium*, TV119 were incubated with ficolin/P35 (10 μg/ml). After washing, the bacteria were incubated with monocytes for various times (5, 10, 20, 40 min) on 8-chamber Lab-Tek slides. The numbers of bacteria ingested by 400 monocytes were determined by light microscopy. Error bars indicate the SD of triplicate assays.

(A)

(B)

Fig. 3. Photomicrographs of PMNs which took up bacteria

(A) PMNs which took up *S. typhimurium* (TV119) treated with ficolin/P35 (50 µg/ml). (B) PMNs which took up *S. typhimurium* (TV119) not treated with ficolin/P35. Ficolin/P35 enhanced the uptake of TV119 by PMNs.

GlcNAc. As shown in Fig. 1, when various amounts of ^{125}I -ficolin/P35 were added to a fixed number of bacteria, its binding to TV119 increased in a dose dependent manner. On the other hand, ^{125}I -ficolin/P35 did not bind to TV160 and LT2.

Fig. 4. Differences in serum ficolin/P35 levels among age groups.

Ficolin/P35 concentrations were assayed by a sandwich ELISA using two kinds of monoclonal antibody (GN4, biotinylated GN5). The mean and standard error of ficolin/P35 levels in each group (10-year interval) is shown. Horizontal bars represent one standard error.

Effect of ficolin/P35 on uptake of an Ra chemotype S. typhimurium by phagocytes

We next examined the opsonic function of ficolin/P35 using monocytes. *S. typhimurium* (TV119) were preincubated with ficolin/P35 (10 μg/ml) and then with monocytes for up to 40 min. As shown in Fig. 2, ficolin/P35 enhanced the uptake of TV119 in a time-dependent manner. Similar enhancement of uptake of TV119 by ficolin/P35 was observed with PMNs⁹. Photomicrographs of PMNs which took up TV119 are shown in Fig. 3.

Measurement of the concentration of ficolin/P35 in normal serum

To clarify the physiological significance of ficolin/P35 measurement of ficolin/P35 concentrations is needed. We developed a sandwich ELISA using two kinds of monoclonal antibody (GN4 and biotinylated GN5) as described in MATERIALS AND METHODS and determined serum concentration of ficolin/P35. Serum samples were from 130 normal individuals consisting of 65 men and 65 women with a mean age of 47.3 years (range 12–91 years). The mean and standard deviation of ficolin/P35 levels in normal serum (10-year interval) was plotted. The mean concentration of ficolin/P35 was 13.65 μg/ml ± 1.17 and the values increased in younger and older people (Fig. 4).

DISCUSSION

We demonstrated in this report that purified human ficolin/P35 bound selectively to TV119, an Ra chemotype strain of wild type *S. typhimurium* that has an exposed GlcNAc residue at the non-reducing termini of its rough core polysaccharide. In contrast, ficolin/P35 did not bind to TV160, a strain lacking a GlcNAc, or to LT2, a smooth type strain bearing O-polysaccharide covering GlcNAc. Ficolin/P35 binding to TV119 induced enhancement of its phagocytosis by monocytes and PMNs, suggesting that ficolin/P35 has a role in host defense against pathogens possessing surface GlcNAc residues. MBL is a lectin with binding specificity for mannose and GlcNAc. Kawasaki and coworkers¹⁵⁾ showed that serum MBL binds to certain rough strains of *E. coli*, presumably via L-glycero-D-manno-heptose residues or GlcNAc associated with core oligosaccharides. In other studies, Kuhlman *et al.*¹⁶⁾ showed that serum MBL binds to the mannose-rich O-polysaccharide within the lipopolysaccharide of *S. montevideo*. Unlike MBL, ficolin/P35 does not bind to mannose⁹⁾. MBL may function as an opsonin against gram negative bacteria having mannose or GlcNAc at the nonreducing termini of their rough core polysaccharides. On the other hand, ficolin/P35 may be an opsonin against the latter bacteria.

We have not identified the cellular receptor that mediates the phagocytosis of ficolin/P35-opsonized bacteria. It is likely that the opsonization is mediated directly via the collagen-like regions of the pathogen-bound ficolin/P35 interacting with the C1q-receptor. Recently, Tenner *et al.* reported that the C1q-receptor, a 126,000 Mr cell surface protein, enhances C1q- or MBL- mediated phagocytosis¹⁷⁾. The structural similarity of ficolin/P35 with C1q and MBL suggests that the C1q-receptor may be a receptor for ficolin/P35. We are investigating the identity of the receptor involved in ficolin/P35-mediated phagocytosis. In addition to ficolin/P35, there is a GlcNAc-binding lectin termed Hakata antigen in human serum¹⁸⁾ which also belongs to the ficolin family. Although the function of Hakata antigen is unknown, it is likely that, like ficolin, it acts as an opsonin against certain bacteria.

To measure the serum concentration of ficolin/P35 we developed an ELISA using monoclonal antibodies against it. The mean serum concentration of ficolin/P35 from 130 normal individuals was estimated to be 13.7 $\mu\text{g/ml}$. This value is greater than that reported by Kilpatrick *et al.* (3.7 $\mu\text{g/ml}$)¹⁹⁾ who used the same antibodies as we used in this study. The reason for this difference is unclear. One possibility is that it may reflect ethnic differences between the populations studied. It has been reported that MBL levels are high in younger age²⁰⁾. The present study revealed that ficolin/P35 levels were also high in younger age groups. It is of particular interest that in addition to younger age ficolin/P35 levels are high in older age groups. The up-regulation of MBL synthesis during early childhood implies that MBL has an important role in the first line of host defense prior to acquired

immunity. Our findings suggest that ficolin/P35 has a similar role as MBL in host defense especially in both children and older people.

In conclusion, ficolin/P35 may play a role in innate immunity by enhancing phagocytosis of certain bacteria by monocytes or PMNs.

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