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Studies of the binding of ficolin-2 and ficolin-3 from the complement lectin pathway to *Leptospira biflexa*, *Pasteurella pneumotropica* and Diarrheagenic *Escherichia coli*

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ABSTRACT

Ficolins recognize pathogen associated molecular patterns and activate the lectin pathway of complement system. However, our knowledge regarding pathogen recognition of human ficolins is still limited. We therefore set out to explore and investigate the possible interactions of the two main serum ficolins, ficolin-2 and ficolin-3 with different Gram-negative bacteria. We used recombinant ficolin molecules and normal human serum, which were detected with anti-ficolin monoclonal antibodies. In addition we investigated the capacity of these pathogens to activate the lectin pathway of complement system. We show for the first time that human ficolin-2 recognizes the nonpathogenic spirochete *Leptospira biflexa* serovar Patoc, but not the pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm. Additionally, human ficolin-2 and ficolin-3 recognize pathogenic *Pasteurella pneumotropica*, enteropathogenic *Escherichia coli* (EPEC) serotype O111ab:H2 and enteroaggregative *E. coli* (EAEC) serogroup O71 but not four enterohemorrhagic *E. coli*, three EPEC, three EAEC and two nonpathogenic *E. coli* strains (DH5α and HB101). The lectin pathway was activated by *Pasteurella pneumotropica*, EPEC O111ab:H2 and EAEC O71 after incubation with C1q depleted human serum. In conclusion, this study provide novel insight in the binding and complement activating capacity of the lectin pathway initiation molecules ficolin-2 and ficolin-3 towards relevant Gram-negative pathogens of pathophysiological relevance.

1. Introduction

Ficolins are soluble pattern recognition molecules that interact with pathogen associated molecular patterns, such as acetylated monosaccharides like *N*-acetyl glucosamine and *N*-acetyl-galactosamine (Lu and Le, 1998; Sugimoto et al., 1998) and non-sugars such as *N*-acetyl-glycine, *N*-acetyl-cysteine and acetyl-choline (Krarup et al., 2004) on the surface of pathogens leading the activation to the lectin pathway of complement system by utilizing a set of associated serine proteases named manose binding lectin associated serin proteases (MASPs) (Endo et al., 2011; Degn and Thiel, 2013). Structurally, ficolins are oligomeric proteins formed by an amino terminal collagen-like domain and a carboxyl terminal fibrinogen-like domain. The former domain is present also in C1q and mannose-binding lectin and allows oligomerization and the later domain is required for pathogen recognition (reviewed in Degn and Thiel, 2013; Garlatti et al., 2007). Ficolins in serum are associated to MASP-1, -2 and 3 and two non-enzymatic proteins MAP-1 (MAp44 or MASP1 isoform 3) and MAP-2 (Map19 or MASP2 isoform 2) (reviewed in Dobó et al., 2014). After recognition of the pathogen, MASP-1 cleaves MASP-2, which subsequently cleaves C4 in two the fragments C4a and C4b. The component C2 binds to C4b and can be cleaved by both MASP-1 and MASP-2, generating fragments C2a and C2b, allowing assembly of the C3 convertase (C4b2a). This enzyme cleaves C3 in fragments C3a and

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C3b. Deposition of C3b on the pathogen surface facilitates its phagocytosis and also participates in the formation of the C5 convertase (C4b2aC3b) to yield C5b that initiates the polymerization of the membrane attack complex ($C5b6789_n$, MAC) leading to pathogen lysis (reviewed in Dobó et al., 2014; Matsushita et al., 2000, 2002). Additionally, C reactive protein (CRP) and pentraxin 3 (PTX3) can cooperate with ficolins in the recognition of pathogen associated molecular patterns (Ma et al., 2004; Ng et al., 2007; Zhang et al., 2009; Gout et al., 2011).

Three ficolins have been described in humans: ficolin-1 (M) (Endo et al., 1996; Lu et al., 1996a,b; Teh et al., 2000), ficolin-2 (L) (Matsushita et al., 1996) and ficolin-3 (H) (Sugimoto et al., 1998). Ficolin-1 is encoded by the 9 exon gene *FCN1* on chromosome 9q3.4, is expressed on the surface of peripheral blood monocytes, granules of peripheral neutrophils, alveolar type II epithelial cells, and is found at low concentrations (0.06–1.07 μg/ml) in serum (Rørvig et al., 2009; Honoré et al., 2008; Honoré et al., 2010; Wittenborn et al., 2009).

Ficolin-2 is encoded by the 8 exon gene *FCN2* on chromosome 9q3, is expressed in liver and is found in serum (3.3–5 μg/ml) (Krarup et al., 2004; Hummelshøj et al., 2005; Munthe-Fog et al., 2009 Kilpatrick and Chalmers, 2012). The *FCN2* gene is highly polymorphic with numerous reported single nucleotide polymorphisms (SNPs) throughout the promoter, introns and exons. Variations located in the promoter has been shown to significantly affect the the protein's serum concentration, whereas two SNPs in exon 8 increase or decrease ficolin-2 binding affinity to GlcNAc, respectively (Hummelshøj et al., 2005; Munthe-Fog et al., 2009).

When it comes to recognition and binding, ficolin-2 differ from many other pattern recognition molecules due to the wide variety of reported ligands. The fibrinogen-like domain contains no less than four distinct binding grooves (S1, S2, S3 and S4), all of which have different recognition specificity (Garlatti et al., 2007). In accordance, numerous ligands have been reported, many of which are in the category of acetylated compounds: GlcNAc, GalNAc, CysNAc, acetylated low density lipoproteins, 1,3-β-glucan. In addition to binding to pathogens, ficolin-2 has been shown to bind DNA and sequester apoptotic cells; suggesting a possible role as scavenger molecule (Jensen et al., 2007).

Ficolin-3 is encoded by the 8 exon gene *FCN3* on chromosome 1p35.3, is expressed by bile duct epithelial cells and hepatocytes, ciliated bronchial epithelial cells, alveolar type II epithelial cells, and is present in serum (18.4–25 μg/ml) (Krarup et al., 2004; Inaba and Okochi, 1978; Munthe-Fog et al., 2008; Akaiwa et al., 1999). Initially, ficolin-3 was shown to bind to *Aerococcus viridans* and specifically to the polysaccharide of this bacterium, but few ligand has been reported to date (Tsujimura et al., 2001). Apart from pathogens ficolin-3 also binds to late apoptotic cells (Honoré et al., 2007).

Interaction of ficolins with some Gram-positive and Gram-negative bacteria, protozoa and fungus has been established (Sugimoto et al., 1998; Matsushita et al., 2002; Zhang et al., 2009; Lu et al., 1996a,b; Teh et al., 2000; Matsushita et al., 1996; Tsujimura et al., 2001; Kjaer et al., 2011; Liu et al., 2005; Aoyagi et al., 2008; Krarup et al., 2005; Lynch et al., 2004; Carroll et al., 2009; Cestari et al., 2009; Evans-Osses et al., 2010; Swierzko et al., 2012). Nevertheless, the repertoire of pathogens recognized is limited, especially for ficolin-3. Therefore, it is necessary to evaluate additional pathogens to assess the real capability of ficolins to recognize specific pathogens and activate the lectin pathway of the complement system.

The aim of this study is to determinate if the human ficolin-2 and ficolin-3 are able to interact with Gram negative bacteria such as *Leptospira* spp., pathogenic *E. coli* and *Pasteurella pneumotropica*, and subsequently activate the lectin complement pathway. We focused on these particular group of bacteria because we have been working on the mechanisms employed by these pathogens to circumvent hostís innate immune responses in the last few years (Barbosa et al., 2009, 2010; Fraga et al., 2014; Sahagún-Ruiz et al., 2014; Abreu et al., 2015). Moreover, the diseases caused by pathogenic *Leptospira* and *E. coli* are of major public health importance in developing countries, and unravelling the mechanisms used by these bacteria to survive in the host may contribute to the development of therapeutic or preventive strategies that may interfere with bacterial infection and dissemination.

2. Materials and methods

Protocols were previously approved by the local institutional committee. (06/10/CEUA/ICB) from the Institute of Biomedical Sciences, University of São Paulo.

2.1. Bacteria

P. pneumotropica was isolated from hamster liver at the Institute of Biomedical Sciences, University of São Paulo, and identified using the API system at the Butantan Institute, São Paulo, Brazil (Sahagún-Ruiz et al., 2014). Enterohemorrhagic *E. coli* (EHEC): O157:H7 (prototype strain EDL933), O26:H11 (LB20), O111ac:H^- (LB2), O157:H7 (LB5); enteropathogenic *E. coli* (EPEC): O127:H6 (prototype strain E2348/69), O55:H6 (LB28), O111ab:H2 (LB6), O119:H6 (LB9); enteroaggregative *E. coli* (EAEC): O44:H18 (prototype strain O42), O64:H25 (BA83), O71 (BA114), ONT (BA120) and nonpathogenic *E. coli* DH5α and HB101 strains were obtained from a collection of the Laboratory of Bacteriology from the Butantan Institute (Bueris et al., 2007; Mairena et al., 2004). Frozen aliquots of *P. pneumotropica* and *E. coli* were prepared from overnight LB broth cultures from a single colony on LB agar, and stored in 40% glycerol at −80 °C until use. *Leptospira biflexa* serovar Patoc (nonpathogenic) and *L. interrogans* serovar Kennewicki strain Fromm (pathogenic) were obtained from the Faculty of Veterinary Medicine, University of São Paulo, and kept in culture at 30 °C at low passage (up to 5th passage). Leptospira were cultivated for seven days in modified EMJH medium at 29 °C under aerobic conditions, as previously described (Barbosa et al., 2006).

2.2. Purified ficolins, antibodies and pool of human serum

Recombinant (r) ficolin-2 and ficolin-3 were produced as previously described (Hummelshøj et al., 2007, 2008). Mouse monoclonal antibodies specific for ficolin-2 (FCN219) or ficolin-3 (FCN334) has previously been characterized (Hummelshøj et al., 2005; Munthe-Fog et al., 2007). Polyclonal goat antibody against human C4 was purchased from Complement Technology (Tyler, Texas, USA). Secondary anti-IgG antibodies conjugated with peroxidase for ELISA were purchased from KPL (Tyler, Texas, USA). A pool of normal human sera (NHS) was obtained from 47 healthy volunteers, after informed written and signed consent. All human serum samples were anonymized before use.

2.3. ELISA

Bacteria were cultivated in Luria Bertani (LB) media for 12–14 h at 37 °C at 185 rpm until reach an optical density ($OD_{600\,nm}$) of no greater than 0.5. The bacteria number was adjusted to 2×10^8 per tube (assuming that an $OD_{600\,nm}$ 0.25 contains approximately 2×10^8 bacteria/ml for *E. coli* or *P. pneumotropica*). *Leptospira* concentration was obtained by direct counting in a Petroff Hausser chamber on dark field microscopy. Subsequently, cells were collected by centrifugation (5500 × *g* for 15 min) and washed once with 20 ml PBS pH 7.4 at 21 °C. Bacteria were incubated at 37 °C at 185 rpm in 20 or 40% NHS in TBS-T Ca^{++} (50 mM Tris, 150 mM

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