Interaction of human complement factor H variants Tyr^{402} and His^{402} with Leptospira spp.

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**INTRODUCTION**

Leptospirosis is a zoonosis caused by pathogenic bacteria from the genus *Leptospira*. The disease represents a serious public health problem in underdeveloped tropical countries. Leptospires infect hosts through small abrasions in the skin or mucous membranes and they rapidly disseminate to target organs. The capacity of some pathogenic leptospiral strains to acquire the negative complement regulators factor H (FH) and C4b binding protein correlates with their ability to survive in human serum. In this study we assessed the functional consequences of the age macular degeneration-associated polymorphism FH His^{402} or FH Tyr^{402} on FH–Leptospira interactions. In binding assays using sub-saturating amounts of FH, the FH Tyr^{402} variant interacted with all the strains tested more strongly than the FH His^{402} variant. At higher concentrations, differences tended to disappear. We then compared cofactor activities displayed by FH His^{402} and FH Tyr^{402} bound to the surface of *L. interrogans*. Both variants exhibit similar activity as cofactors for Factor I-mediated cleavage of C3b, thus indicating that they do not differ in their capacity to regulate the complement cascade.

Complement FH, a 155-kDa plasma glycoprotein composed of 20 globular domains (termed short consensus repeats, SCRs), is the major soluble complement regulator that controls AP activation and the amplification reaction at the C3 level. It inhibits the complement AP by preventing binding of Factor B to C3b, accelerating decay of the C3-convertase C3bBb and acting as a cofactor for the cleavage of C3b by Factor I (Weiler et al., 1976; Whaley and Ruddy, 1976; Pangburn et al., 1977). FH, encoded by the *HF1* gene, is as a single chain with 1231 amino acid residues. The regulatory function on complement amplification is conferred by the first four domains (Gordon et al., 1993; Kühn and Zipfel, 1996), while the remaining 16 SCRs present several binding sites for different targets, including: self surfaces, thereby preventing complement activation on host cells (Sharma and Pangburn, 1996); C3 fragments (Zipfel et al., 1999); C-Reactive Protein (Mold et al., 1999; Ormsby et al., 2006); fibromodulin (Sjöberg et al., 2005); adrenomedullin (Pio et al., 2001); cell receptors (Jarva et al., 1999; Zipfel et al., 2002); M protein and heparin (Sharma and Pangburn, 1997; Giannakis et al., 2003).

The polymorphism (Tyr^{402}His) located in SCR7 of both FH and factor H-like protein 1 (FHL-1) has been associated with age macular degeneration (AMD) by different groups (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005; Teixeira et al., 2010). The frequency of the His^{402} allotype of FH in the population is quite relevant. Approximately 35% of European descendants carry this allele (Clark et al., 2010). In Brazilian...
subjects (a highly ethnically mixed population) the frequency of this allele is 13% (Teixeira et al., 2010). Additionally, this same FH polymorphism was associated with other chronic diseases, such as Alzheimer disease (Zetterberg et al., 2008).

The hypothesis that this FH polymorphism could affect FH binding properties was approached by several groups (Laine et al., 2007; Skerka et al., 2007; Yu et al., 2007; Haapasalo et al., 2008) in studies that evaluated the effect of the Tyr402His substitution on binding of FH to heparin, C-reactive protein, and bacterial surface proteins. Using SCR 5–7 domains from FH His402 variant, Laine et al. (2007) observed a weaker binding to C-reactive protein when compared to SCR 5–7 from FH Tyr402. They obtained similar results when purified human FH variants were employed. In addition, no differences in the binding to heparin were observed (Laine et al., 2007). On the other hand, the binding of the Tyr402 and His402 allotype to C-reactive protein did not differ in a study reported by Yu et al. (2007). However, this group observed that the Tyr402 allotype binds much better to M6 protein from Streptococcus pyogenes. A study by Haapasalo et al. (2008) demonstrated that the FH Tyr402 and FH His402 allotypes display different binding affinities for several group A Streptococcus (GAS) strains: binding of the FH His402 allotype to GAS is significantly weaker compared to that exhibited by the FH Tyr402 allotype. As a consequence, GAS strains are more susceptible to phagocytosis in the presence of the His402 allele due to increased C3b deposition on the bacterial surface (Haapasalo et al., 2008).

Given the small number of investigations assessing the functional consequences of the age-related macular degeneration-associated polymorphism Tyr402His on FH—pathogen interactions and the fact that leptospires may cause eye diseases such as uveitis, the main purpose of the present study was to compare binding of Tyr402 and His402 FH variants to pathogenic Leptospira strains. We also evaluated cofactor activity displayed by both variants bound to the leptospiral surface in order to assess if they differ in their capacity to regulate the complement cascade.

MATERIALS AND METHODS

PURIFICATION OF FH (His402) AND FH (Tyr402) VARIANTS

The two allotypes were isolated from 100 ml EDTA plasma collected from homozygous healthy donors after previous genotyping, according to Teixeira et al. (2010). Briefly, the material was treated with 10 mM CaCl2 and 0.6 mM phosphoethanolyl fluoruride (final concentrations) in order to block the protease cascade, followed by dialysis in 8 mM EDTA and 10 mM benzamidine, pH 5.8, for 18 h at 4°C. The precipitate was washed twice in the same solution and solubilized in 10 mM Tris–HCl, 150 mM NaCl, 100 mM dextrose, 100 mM glycine, and 0.01% sodium azide, pH 8.5. The material was then separated by gel filtration chromatography using a Sephacryl S-200 column (GE Healthcare Life Sciences). The fractions containing FH were pooled and dialyzed against 40 mM NaH2PO4 pH 8.1 overnight and loaded in citrate phosphate buffer (pH 5.0) plus 0.01% H2O2. The reaction was allowed to proceed for 10 min and was then interrupted by the addition of 50 μl of 8 M H2SO4. The absorbance at 492 nm indicated, both variants of FH were purified exclusively by affinity column.

LEPTOSPIRA STRAINS AND CULTURE

Leptospira interrogans serovar Pomona strain Fromm, L. kirschneri serovar Grippotyphosa strain Moskva V and L. borgpetersenii serovar Javanica strain Veldrat Bataviae 46 were used in the assays. All strains were obtained from the Laboratório de Zoonoses, Faculdade de Medicina Veterinária e Zootecnia, University of São Paulo, Brazil. Moskva V and Veldrat Bataviae 46 strains were culture-attenuated by successive passages in artificial medium. Virulence of the strain Fromm was maintained by iterative passages in hamsters. Leptospires were cultured at 29°C under aerobic conditions in liquid EMJH medium (Difco – USA) with 10% rabbit serum, enriched with 0.015% (w/v) l-asparagine, 0.001% (w/v) sodium pyruvate, 0.001% (w/v) calcium chloride, 0.001% (w/v) magnesium chloride, 0.03% (w/v) peptone, and 0.02% (w/v) meat extract.

IMMUNOELECTRON MICROSCOPY

The following protocol is modified after Barbosa et al., 2010. L. interrogans serovar Pomona strainFromm cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 60 min at 22°C. After two washes with PBS, bacteria were incubated with 5 μg/ml of purified FH (His402) or FH (Tyr402) for 60 min at 22°C with gentle agitation. After two washes with PBS to remove unbound FH, bacteria were applied to electron microscopy grids and then incubated for 60 min with a polyclonal goat anti-human FH (Quidel) diluted 1:500 in PBS/1.5% BSA/0.05% Tween 20. After four washes with PBS/1% BSA, they were incubated for 60 min with rabbit anti-goat antibody–colloidal gold (10-nm particles; Sigma-Aldrich, Co., St Louis, USA) and after successive washes in PBS/1% BSA, 0.85% NaCl, and bi-distilled water, bacteria were stained with 2% uranyl acetate. Grids were examined with an electron microscope (Zeiss EM 109; Carl Zeiss, Inc., Oberkochen, Germany) at an accelerating voltage of 80 kV.

ASSESSMENT OF SURFACE ACQUISITION OF FH VARIANTS ON LEPTOSPIRES BY ENZYME IMMUNOASSAY

Leptospires grown to mid-log phase were harvested by centrifugation at 5400 g for 30 min and gently washed in PBS (pH 7.4) twice. Bacteria were incubated with 0–20 μg/ml of purified FH His402 or FH Tyr402 for 60 min at room temperature. After centrifugation at 10000 g for 10 min at 4°C, the pellets were washed three times with a buffer containing 1.0 mM MgCl2, 0.6 mM CaCl2, and 1% glucose, and then suspended in 0.1 M NaHCO3, pH 9.6. Bacteria were used for coating ELISA plate wells overnight at 4°C. The wells were washed twice with PBS/0.05% Tween 20 (PBST) and then blocked with 200 μl of 0.5% BSA for 2 h at 37°C. Bound FH was detected by adding 100 μl of a 1:10000 dilution of goat anti-human FH (Quidel) in PBS. Incubation proceeded for 60 min at 37°C and after three washes with PBST, 100 μl of a 1:10000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG in PBS was added to each well and incubated for 60 min at 37°C. The wells were washed three times and o-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H2O2. The reaction was allowed to proceed for 10 min and was then interrupted by the addition of 50 μl of 8 M H2SO4. The absorbance at 492 nm
Silva et al. Binding of FH to Leptospira was determined in a micro plate reader (Labsystems Uniscience, Multiskan EX).

**FH ADSORPTION ASSAYS USING INTACT LEPTOSPIRES**

Freshly harvested leptospires (1 x 10^9) were washed twice with PBS (pH 7.4), and incubated with 5 μg/ml of purified FH His402 or FH Tyr402 for 60 min at 22°C with gentle agitation. After five washes with PBS (the last wash fraction was collected), proteins bound to the surface of bacteria were eluted with 100 μl of 0.1 M glycine–HCl pH 2.0 and supernatants were collected after centrifugation. One fifth (20 μl) of the wash and eluate fractions was subjected to 10% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. As a control, purified FH (Calbiochem) was also loaded in the same gel. Non-specific binding sites were blocked using 10% (w/v) dried milk in PBS–Tween (0.05%; pH 7.4) overnight at 4°C. Subsequently, membranes were rinsed three times in PBS–Tween (0.05%) and were incubated for 60 min at room temperature with a polyclonal goat anti-human FH (Quidel) at a 1:10000 dilution. Following three washes with PBS–Tween (0.05%), membranes were incubated with a secondary peroxidase-conjugated anti-rabbit IgG Ab (Sigma) for 60 min at room temperature at a 1:10000 dilution. The positive signals were detected by enhanced chemiluminescence (Super Signal, West Pico, Pierce).

**COFACTOR ACTIVITY OF LEPTOSPIRA-BOUND FH**

The cofactor activity of surface-attached FH was assayed by measuring Factor I-mediated cleavage of C3b (Grosskinsky et al., 2009). One milliliter of freshly harvested leptospires (1 x 10^9) were washed with a binding buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.4) and incubated with purified FH His402, FH Tyr402 (0.5–10 μg/ml), or 10% NHS–EDTA (from blood samples genotyped homozygous for the FH His402 allele or the FH Tyr402 allele), or binding buffer for 60 min at room temperature with gentle agitation. Bacteria were washed three times with washing buffer (100 mM NaCl, 50 mM Tris–HCl, 0.05% Tween 20, pH 7.4), and incubated with purified C3b (Calbiochem; 250 ng/assay) and Factor I (Calbiochem; 150 ng/reaction) for 60 min at 37°C. The samples were centrifuged and the supernatants were subjected to 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Blocking treatment and incubations with specific antibodies were performed as described above. Cleavage products of C3b were detected using polyclonal goat anti-human C3 (Calbiochem) at a 1:10000 dilution.

**RESULTS**

**BINDING OF FH His402 AND FH Tyr402 TO LEPTOSPIRA SPP.**

After incubation of *L. interrogans* Pomona strain Fromm with purified human FH His402 or FH Tyr402, immunoelectron microscopy was performed with anti-FH in order to evaluate binding of both FH variants to this pathogenic strain. Colloidal gold particles were detected on the leptospiral surface in both cases. Representative electron micrographs are shown in Figure 1. Apparently, no binding differences were observed at microscopical level thus suggesting that both variants may interact equally with...
L. interrogans. Organisms incubated in PBS did not present surface colloidal gold binding (data not shown).

To assess the interaction of human complement FH variants with Leptospira on a quantitative basis, we first incubated L. interrogans serovar Pomona strain Fromm with increasing amounts of purified FH His\textsuperscript{420} or FH Tyr\textsuperscript{420} (over a range of 0.1–2 \(\mu\)g/ml) and then immobilized the bacteria on microtiter wells. Bound FH was detected with a specific antibody. FH Tyr\textsuperscript{402} binding to strain Fromm was significantly stronger when compared to FH His\textsuperscript{402} binding within the range of 0.1–2 \(\mu\)g/ml (Figure 2). Since FH is found in the serum at high levels, we decided to extend this range up to 20 \(\mu\)g/ml using three pathogenic leptospiral strains (Figure 3). At all FH concentrations tested, FH Tyr\textsuperscript{402} binding to L. interrogans serovar Pomona strain Fromm was significantly stronger when compared to FH His\textsuperscript{402} binding to this virulent strain (\(P < 0.05\); Figure 3). L. borgpetersenii serovar Javanica strain Veldrat Bataviae 46 and L. kirshneri serovar Grippotyphosa strain Moskva V also bound FH Tyr\textsuperscript{402} with an apparently higher affinity at lower FH concentrations (Figure 3).

Acquisition of FH variants by pathogenic leptospires was also evaluated by Western blot. To this end, bacteria were incubated with the same amount of FH His\textsuperscript{402} or FH Tyr\textsuperscript{402} and after extensive washes the eluate fractions were separated by SDS-PAGE and subjected to Western blot with anti-FH antibody. Interestingly, FH His\textsuperscript{402} released by all three strains was more abundant when compared to FH Tyr\textsuperscript{402} probably reflecting a higher affinity of the Tyr\textsuperscript{402} variant for the Leptospira surface (Figures 4A,B). In addition to binding FH, the Leptospira strains also bound members of the FH-family, most probably FH-related protein 1 (FHR-1\(\alpha\) and FHR-1\(\beta\); Figure 4A) as has been previously reported (Meri et al., 2005).

**LEPTOSPIRA-BOUND FH His\textsuperscript{402} OR FH Tyr\textsuperscript{402} DISPLAY SIMILAR COFACTOR ACTIVITY**

We next compared the regulatory role of FH His\textsuperscript{402} and FH Tyr\textsuperscript{402} attached to the surface of Leptospira, by analyzing degradation of C3b mediated by Factor I. The serum-resistant L. interrogans serovar Pomona strain Fromm was incubated with purified FH His\textsuperscript{402} and FH Tyr\textsuperscript{402} (0.5–10 \(\mu\)g/ml), and also with 10% NHS (His\textsuperscript{402}) or 10% NHS (FH Tyr\textsuperscript{402}). After washing

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**FIGURE 2** Dose-dependent binding of FH variants to L. interrogans serovar Pomona strain Fromm. L. interrogans were incubated with increasing amounts of purified FH His\textsuperscript{420} or FH Tyr\textsuperscript{420} (0.1–2 \(\mu\)g/ml) and then immobilized on microtiter wells. The binding was assessed using a polyclonal anti-human FH. Each point represents the mean absorbance value at 492 nm \(\pm\) the SD of three independent experiments. \(* P \leq 0.05\) (Student’s two-tailed t-test). Both variants of FH were purified by gel filtration and affinity chromatography.

**FIGURE 3** Dose-dependent binding of FH variants to Leptospira. L. borgpetersenii, L. kirshneri, and L. interrogans were incubated with increasing amounts of purified FH His\textsuperscript{420} or FH Tyr\textsuperscript{402} (0–20 \(\mu\)g/ml) and then immobilized on microtiter wells. The binding was assessed using a polyclonal anti-human FH. Each point represents the mean absorbance value at 492 nm \(\pm\) the SD of three independent experiments. \(* P \leq 0.05\) (Student’s two-tailed t-test). Both variants of FH were purified only by affinity chromatography.
to remove unbound FH, C3b, and Factor I were added. The cleavage fragments of C3b in the supernatant were subjected to Western blot with anti-C3 polyclonal antibody. The presence of a ~46-kDa band indicates that acquired FH was able to promote Factor I-mediated cleavage of C3b (Figure 5). In the absence of FH, no degradation fragments were detected (Figure 5). Despite binding to Leptospira with different affinities, both variants exhibited similar cofactor activities against C3b. This indicates that they are equally efficient in regulating the complement cascade.

**DISCUSSION**

The AP of the complement system plays a pivotal role in the elimination of invading microorganisms. In order to survive in the host, pathogenic *Leptospira* have evolved mechanisms to avoid or, at least, minimize lytic complement attack. Acquisition of the host fluid-phase complement regulators FH and C4BP is one strategy adopted by these spirochetes to inhibit complement activation (Meri et al., 2005; Barbosa et al., 2009). Binding of FH to *Leptospira* spp. was first assessed by Meri et al. (2005). Serum-resistant and serum-intermediate strains were able to acquire FH and also the FH-family protein member FH-related protein 1 (FHR-1). Surface-bound FH remained functionally active as a cofactor for Factor I in the cleavage of C3b (Meri et al., 2005). *Leptospira*’s ability to bind either purified human FH or normal equine serum FH has been examined by indirect immunofluorescence analysis: *L. interrogans* bound this complement regulator while the non-pathogenic *L. biflexa* did not (Verma et al., 2006). Our immunoelectron microscopy data presented in the present study provide ultra-structural evidence consistent with these previous findings and demonstrate that FH His<sup>402</sup> and FH Tyr<sup>402</sup> variants interact with pathogenic *Leptospira*.

Recent results by our group indicate that *Leptospira* interact with FH through two different binding sites, one located within SCRs 5–7 and other located at the C-terminal SCRs 18–20 (unpublished data). Considering that the His<sup>402</sup> or Tyr<sup>402</sup> polymorphism of FH is located in SCR7, we decided to evaluate whether these FH variants would display different binding affinities for this pathogen. According to the solid-phase binding assays, the FH Tyr<sup>402</sup> variant bound to the virulent strain Pomona more strongly than the FH His<sup>402</sup> did at all concentrations tested. Similar results were observed for the two other pathogenic strains when they were incubated with sub-saturating concentrations of FH. At higher concentrations, no difference in binding was detected. We speculate that intrinsic properties of each strain might affect binding to these FH variants. Since different binding affinities for FH His<sup>402</sup> and FH Tyr<sup>402</sup> may have functional consequences for the pathogen with regard to susceptibility to complement-mediated killing, we then compared cofactor activities of FH His<sup>402</sup> and FH Tyr<sup>402</sup> bound to the surface of *L. interrogans*. Both variants were
were incubated in blood from individuals homozygous for the FH genotype. Nevertheless, a number of studies report a high prevalence of the FH His402 allele (0.13) in Brazilian individuals (Teyixeira et al., 2010). This allele may confer a selective advantage by, for instance, limiting evasion from the immune system by pathogenic microorganisms or increasing susceptibility of the pathogen to opsonophagocytosis (Yu et al., 2007; Haapasalo et al., 2008; Ormsby et al., 2008).

In a previous study, Yu et al. (2007) analyzed the interactions of both FH variants with M6 protein from S. pyogenes, pneumococcal surface protein C (PspC) from S. pneumoniae, and complement regulator-acquiring surface protein 1 from Borrelia burgdorferi (BbCRASP-1). While PspC and BbCRASP-1 bound equally well to FH His402 and FH Tyr402, M6 protein interacted with the FH Tyr402 variant with greater affinity. Furthermore, reduced binding of the FH His402 variant of both FH and FHL-1 to GAS M6 protein has been observed by Ormsby et al. (2008). A comprehensive study by Haapasalo et al. (2008) involving almost 40 GAS strains strengthened these findings, showing that the majority of strains bound more weakly FH SCRs 5–7 allotype His402. Moreover, opsonophagocytosis was more pronounced when bacteria were incubated in blood from individuals homozygous for the FH (His402) allele (Haapasalo et al., 2008).

Factor H allotype His402 has been recognized as a leading risk factor for the development of age-related macular degeneration (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005). Nevertheless, a number of studies report a high prevalence of the homozygous genotype CC (that codes for His402) in different ethnic populations (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005). A study analyzing populations from different continents found that the frequency of FH allele (His402) is between 0.15 and 0.25 (Haapasalo et al., 2008) and a recent study reported a genotype frequency of 0.13 for CC (His402) in Brazilian individuals (Teyixeira et al., 2010). This allele may confer a selective advantage by, for instance, limiting evasion from the immune system by pathogenic microorganisms or increasing susceptibility of the pathogen to opsonophagocytosis (Yu et al., 2007; Haapasalo et al., 2008; Ormsby et al., 2008).

In conclusion, in this in vitro study we assessed the functional consequences of the AMD-associated polymorphism FH His402 or FH Tyr402 on FH–Leptospira interactions. While our data suggest that these variants have different binding affinities to these bacteria, both allotypes regulate the AP in a similar pattern; i.e., both act as cofactor of Factor I at similar efficiency. However, we cannot rule out the possibility that other FH polymorphisms may affect the interactions between this complement regulator and Leptospira. In this work we did not address this possibility. In addition, local tissue environment may directly influence the regulatory role of FH in vivo in the eye. For example, the variant FH His402 binds much less intensively to the Bruch’s membrane in the macula region of retina than the Tyr402 variant; which may be related to the etiopathogenesis of AMD (Clark et al., 2010). In equines, L. interagens is responsible for causing uveitis, a major
cause of blindness in these animals (Verma et al., 2005). A considerable fraction (18.4%) of infected patients from South India developed uveitis after acute leptospirosis (Pappachan et al., 2007). These considerations suggest that the role of FH polymorphism in patients with leptospiiral uveitis deserves further investigations.

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