Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3.

Callum N. Watson  
*University of Birmingham*

Steven W. Kerrigan  
*Royal College of Surgeons in Ireland, skerrigan@rcsi.ie*

Dermot Cox  
*Royal College of Surgeons in Ireland, dcox@rcsi.ie*

Ian R. Henderson  
*University of Birmingham*

Steve P. Watson  
*University of Birmingham*

*See next page for additional authors*

Citation  
Abstract

Gram-negative Escherichia coli cause diseases such as sepsis and hemolytic uremic syndrome in which thrombotic disorders can be found. Direct platelet–bacterium interactions might contribute to some of these conditions; however, mechanisms of human platelet activation by E. coli leading to thrombus formation are poorly understood. While the IgG receptor FcγRIIA has a key role in platelet response to various Gram-positive species, its role in activation to Gram-negative bacteria is poorly defined. This study aimed to investigate the molecular mechanisms of human platelet activation by E. coli, including the potential role of FcγRIIA. Using light-transmission aggregometry, measurements of ATP release and tyrosine-phosphorylation, we investigated the ability of two E. coli clinical isolates to activate platelets in plasma, in the presence or absence of specific receptors and signaling inhibitors. Aggregation assays with washed platelets supplemented with IgGs were performed to evaluate the requirement of this plasma component in activation. We found a critical role for the immune receptor FcγRIIA, αIIbβ3, and Src and Syk tyrosine kinases in platelet activation in response to E. coli. IgG and αIIbβ3 engagement was required for FcγRIIA activation. Moreover, feedback mediators adenosine 5’-diphosphate (ADP) and thromboxane A2 (TxA2) were essential for platelet aggregation. These findings suggest that human platelet responses to E. coli isolates are similar to those induced by Gram-positive organisms. Our observations support the existence of a central FcγRIIA-mediated pathway by which human platelets respond to both Gram-negative and Gram-positive bacteria.

Keywords

Blood platelets, Escherichia coli, Fc gamma receptor IIA, immunity, thrombosis

Introduction

Platelets have been long known to be activated by bacteria [1]. This is likely to contribute to a balanced immune response [2], but it is also associated with pathological conditions such as infective endocarditis, atherothrombosis, and sepsis [3–5]. In the latter, disseminated microvascular thrombosis has a role in pathophysiology of sepsis and might be mediated through direct platelet–bacterium interactions. Recently, great emphasis has been placed on understanding the molecular mechanisms by which platelets are activated by bacterial cells. Elucidation of such mechanisms would provide opportunities to regulate them during infection. These mechanisms are diverse and include activation by whole bacteria or their released products [1, 6]. Despite multiple bacterial-strain specific molecular interactions, human platelet FcγRIIA is required for activation by a number of different Gram-positive species [7–15] and might contribute to the thrombotic complications found in infective diseases [15]. FcγRIIA is a low-affinity receptor for the constant region of IgGs that recognizes IgG-coated bacteria or their products through avidity. Upon ligand engagement, FcγRIIA signals through Src and Syk tyrosine kinases via a dual YxxL sequence known as an immunoreceptor tyrosine-based activation motif (ITAM) that is present in its cytoplasmic tail [16].

Gram-negative E. coli are commensal bacteria of the human and other mammalian gastrointestinal tracts. They rarely cause disease, except in cases of damaged gastrointestinal barriers or immunocompromised hosts. However, pathogenic strains of E. coli can cause three general clinical syndromes: enteric/diarrheal disease, urinary tract infections, and sepsis/meningitis [17]. In the latter, E. coli strains are the most common Gram-negative bacteria isolated from patients with bacteremia, sepsis, and neonatal meningitis [17–20], causing a major clinical burden and thousands of deaths per year. However, scarce information is available on the molecular interactions between E. coli and platelets [21].

The aim of this study was two-fold: to investigate human platelet activation by whole E. coli clinical isolates, and to investigate if FcγRIIA mediates platelet activation.
Materials and methods

Reagents

All reagents were from described sources [14]. Fibrinogen was from Calbiochem (Merck Millipore, Nottingham, UK) and was depleted of IgGs by incubation with protein A (rec-Protein A-Sepharose 4B Conjugate, Life Technologies [Paisley, UK]).

Bacterial culture and preparation

E. coli strains, CFT073 (isolated from a patient with acute pyelonephritis and bacteremia [22]) and RS218 (isolated from a case of neonatal meningitis [23]), were cultured aerobically at 37°C overnight in an LB broth. Bacteria were washed and adjusted in PBS to an optical density (OD) of 1.6 at a wavelength of 600 nm. Bacteria were used at a 10-fold dilution in aggregation assays unless otherwise indicated.

Assays of platelet function

Platelet preparation from healthy volunteers was performed as previously described [14]. The study design was approved by the relevant ethics committee (ERN_11-0175). Platelet aggregation was assessed by light transmission in a PAP-8 aggregometer for up to 30 min. Time-matched controls were run alongside. Stimulation by cross-linking of FcγRIIA was performed by pre-incubation of platelets for 3 min with mAb IV.3 (4 μg/mL) followed by anti-mouse IgG F(ab’)_2 (30 μg/mL). When indicated, concentrations of both mAb IV.3 and anti-mouse IgG F(ab’)_2 were doubled or reduced to half. ATP release was assessed at the end of the recording using a luciferin–luciferase based assay [9]. Eptifibatide (9 μM), dasatibib (4 μM), and PRT-060318 (10 μM) were used at supramaximal concentrations.

Cell lysates and protein phosphorylation studies were performed as previously published [14].

Statistical analysis

Statistical analysis was performed using GraphPad (Prism). Data are presented as mean ± standard deviation (SD), and comparisons between mean values were performed using Student’s t-test or ANOVA when multiple samples were compared. p < 0.05 (two-tailed) was considered to be significant.

Results and discussion

E. coli bacteria stimulate αIIbβ3-mediated platelet aggregation via FcγRIIA and Src and Syk tyrosine kinases

Previous studies have shown a characteristic pattern of platelet activation by Gram-positive bacteria, i.e. they induce “all-or-nothing” aggregation of platelets following a lag time that decreases with increasing concentrations of bacteria [14]. We hypothesized that Gram-negative organisms could trigger platelet aggregation in a similar manner. To test this, we investigated two blood-borne isolates of E. coli, CFT073, which was isolated from a patient with urinary tract infection and bacteremia, and RS218, which was isolated from a child with meningitis. We found that both strains induced “all-or-nothing” platelet aggregation in plasma after a lag phase (Figure 1A.i and B.i). In contrast, as exemplified in Figure 1C for cross-linking of mAb IV.3 to cluster FcγRIIA, most platelet agonists cause rapid activation, which can give rise to partial aggregation when low concentrations of agonist are used. This suggests that bacteria have a unique positive feedback mechanism that gives rise to an “all-or-nothing” response.

The rest of the study was performed with the intermediate bacterial concentration; e.g. bacterial suspensions at OD_{600nm} = 1.6 were used at a 10-fold dilution in aggregation assays. Under these experimental conditions and performing the reactions in the presence of plasma, both strains induced platelet aggregation in six out of seven donors tested. For E. coli CFT073, the lag time for the onset of aggregation varied from 120 to 270 sec (mean ± SD: 180 sec ± 58, n = 6) (Figure 1A ii). E. coli RS218 induced aggregation with lag times ranging from 120 to 270 sec (mean ± SD: 180 sec ± 58, n = 6) (Figure 1B ii).

As shown in Figure 2A and B, platelet aggregation to E. coli CFT073 and RS218 was blocked in the presence of the αIIbβ3
antagonist epifibatide, which confirmed that the change in light transmission was due to dllb3-mediated platelet–platelet binding rather than passive agglutination.

To analyze whether FcyRIIA and its signaling pathway components, Src and Syk tyrosine kinases, have a role in E. coli induced platelet aggregation, specific inhibitors were employed. Aggregation induced by E. coli CFT073 or RS218 strains was abolished when platelets were pre-incubated with either mAb IV.3 (FcyRIIA inhibitor, Figure 2A and 2B), dasatinib (Src inhibitor, Figure 2C and 2D), or PRT-060318 (Syk inhibitor, Figure 2C and 2D) in the presence of plasma.

Platelet activation by E. coli requires the interplay between dllb3 and FcyRIIA

A key role for FcyRIIA in platelet activation by E. coli is further supported by the observation that dense granule secretion was inhibited by mAb IV.3 (Figure 3A.i and A.ii) and that FcyRIIA became phosphorylated in response to both E. coli CFT073 and RS218 strains (Figure 3B). Furthermore, while washed platelets were not able to support bacteria-mediated aggregation, aggregation to E. coli RS218 was restored in the presence of human IgGs (e.g. pooled human IgGs purified from healthy donors) alone or with fibrinogen (Figure 3C.ii). Simultaneous addition of human IgGs and fibrinogen was necessary for E. coli CFT073 to induce aggregation in washed platelets (Figure 3C.i). These observations suggest that the initiating event in activation is engagement of FcyRIIA by plasma IgG bound to bacteria.

Research on Gram-positive bacteria has shown that platelet activation is often the result of multiple bacterium–platelet molecular interactions. These include the combination of bacterial strain-specific molecular interactions and shared IgG-FcyRIIA mediated signaling events [6, 14]. Among the former, strain-specific streptococci and staphylococci proteins are found that bind directly or indirectly (e.g. via fibrinogen) to platelet surface receptors such as dllb3 or GPIb [6, 24]. Our results suggest that the mechanisms of platelet activation by E. coli might also have a strain-dependent component. However, future work is necessary to characterize the exact molecular interactions between platelets and these two E. coli strains, including the identification of potential bacterial components binding (directly or indirectly) to platelet surface receptors.

Previous studies demonstrated an unexpected role for dllb3 in controlling platelet dense granule secretion and FcyRIIA phosphorylation in response to a wide range of Gram-positive bacteria [13, 14]. We analyzed whether the same pattern of regulation could take place for E. coli. Indeed, E. coli CFT073 and RS218-induced dense granule secretion was inhibited by epifibatide (Figure 3A.i and 3A.ii) demonstrating that secretion is dependent on dllb3 engagement. In contrast, secretion induced by cross-linked mAb IV.3 was not decreased by epifibatide (Figure 3A.iii). Moreover, bacteria-induced tyrosine phosphorylation of FcyRIIA was also dependent on dllb3 engagement as observed by the inhibition of phosphorylation in the presence of epifibatide (Figure 3B).

The inability to detect secretion and FcyRIIA tyrosine phosphorylation in the absence of dllb3 engagement might reflect the weak nature of the pathway initiated after FcyRIIA engagement by IgG-coated bacteria in the absence of feedback signals. The mechanism by which initial dllb3 engagement takes place is, however, unclear. Strain-specific events mediating dllb3 activation are thought to exist for Gram-positive bacteria. For most cases, it is likely that dllb3 inside-out activation is achieved by FcyRIIA signaling, as well as by signaling from other strain-specific bacterial ligand–platelet receptor pairs. However, some bacteria such as Streptococcus gordonii DL1 and Staphylococcus aureus Newman can bind directly or indirectly (e.g. via...
fibrinogen) to αIIbβ3, which could facilitate αIIbβ3 activation [1, 10, 25–27]. Further investigations are necessary to characterize the mechanisms that lead to αIIbβ3 activation by *E. coli* CFT073 and RS218, including the identification of potential *E. coli* ligands binding to αIIbβ3.

Platelet activation by *E. coli* is also dependent on secondary mediators ADP and TxA₂

Platelet activation is reinforced by secondary mediators, which include release of stored ADP from dense granules and de novo synthesis of TxA₂. Positive feedback mechanisms driven by secondary mediators are normally required for full and/or sustained platelet aggregation to low concentrations of agonists. This can be seen in platelets stimulated with an intermediate concentration of thrombin-related peptide (TRAP). Pre-treatment of platelets with the ADP-receptor P2Y₁₂ antagonist, Cangrelor, and/or with cyclooxygenase inhibitor, indomethacin, did not affect the initial TRAP-induced aggregation but was followed by slow deaggregation that was not seen in controls (Figure 4C).

In contrast, secondary mediators are key for platelet activation to *Staphylococcus* and *Streptococcus* strains [14]. Here, inhibitors were used to investigate a role for the two feedback agonists in *E. coli*-induced platelet aggregation. Activation in response to *E. coli* CFT073 and RS218 was completely abrogated in the presence of either Cangrelor or indomethacin, or by the combination of both (Fig 4A and 4B). Thus, platelet activation by *E. coli* bacteria is also dependent on ADP and TxA₂.

Altogether, these data suggest that the combination of FcyRIIA activation upon recognition of IgG-coated bacteria plus αIIbβ3 engagement (e.g. either through binding to bacteria or as a result of inside-out platelet signaling events as discussed before) results in a weak signal leading to release of ADP and TxA₂. At this stage, feedback mechanisms are key in order to achieve full activation. Furthermore, ADP and TxA₂ signal to neighboring (bacteria-free) platelets and induce αIIbβ3 inside-out activation and consequent platelet–platelet aggregation.

Interestingly, FcyRIIA has also been shown to function as an adaptor protein amplifying αIIbβ3 signaling independent of extracellular IgG engagement [28, 29]. This suggests that FcyRIIA and αIIbβ3 could support both initial platelet–bacteria interaction and subsequent platelet–platelet aggregation by means of cooperative integrin/immunoreceptor tyrosine-based activation motif signaling.

In summary, in this study, we provide evidence that *E. coli* induces activation of platelets through the same shared pathway described for various Gram-positive *Staphylococcus* and *Streptococcus* species [14]. This pathway involves IgG-dependent FcyRIIA activation of Src and Syk kinases, and is reinforced by αIIbβ3 engagement and secondary mediators. Despite the fact that FcyRIIA-mediated aggregation was previously observed for Gram-negative *Helicobacter pylori* [30] and *Porphyromonas gingivalis* [31], the signaling pathway and role of αIIbβ3 in activation has been only evaluated in few Gram-positive species [10, 13, 14]. The demonstration of a common mode of platelet activation to Gram-positive and Gram-negative species further identifies FcyRIIA as a candidate receptor for prevention of bacteria-mediated platelet activation in thrombosis and related disorders.
E. coli CFT073 or E. coli RS218, other platelet receptors might be via toll-like receptor (TLR) 4 and CD62, leading to cell activation [36]. TLR4-mediated platelet cytokine secretion has been described in response to E. coli LPS [37]. While E. coli CFT073 and RS218 do not produce Shiga-toxin, a role for E. coli CFT073 and RS218 cell wall LPS in platelet activation cannot be discarded, either in relation to FcyRIIA-mediated events (via IgG) or independently of the IgG receptor. As a first approach to test the role of TLR4 in platelet activation in response to our E. coli strains, we used an inhibitory anti-TLR4 antibody, HTA125, and found that it had no effect on platelet aggregation, and did not prolong the lag time response to either E. coli CFT073 or E. coli RS218 using two different donors (data not provided). This suggests that TLR4 is not essential for platelet activation by the bacterial strains tested. However, it is still possible that E. coli LPS-IgG immune complexes are formed that can engage platelet FcyRIIA directly. In any case, the exact role of E. coli CFT073 and RS218 LPS and platelet TLR4 in mediating platelet activation still needs to be deciphered.

Interestingly, one previous study showed that FcyRIIA was required for platelet-mediated killing of IgG-opsonized E. coli K12 [38]. This suggests that platelet activation by bacteria might have different outcomes depending on the overall scenario: while unbalanced thrombi formation might have detrimental effects in cases such as HUS or sepsis, platelet activation by E. coli coated with IgG found in sera from healthy individuals (i.e. such ones used in this study) might contribute to host defense. Future investigations are necessary to further decipher the molecular interactions between E. coli and platelets, including potential synergism between IgG-FcyRIIA and LPS-TLR mediated pathways, and their role in homeostasis or disease. Furthermore, in view of the present study, care should be taken when using animal models for the study of platelet function during E. coli infections as FcyRIIA is not found in mice.

**Funding**

This work was supported by the British Heart Foundation (PG/13/42/30309).

**Declaration of interest**

The authors report no declarations of interest.

**References**