P-selectin–dependent platelet aggregation and apoptosis may explain the decrease in platelet count during Helicobacter pylori infection

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P-selectin expression has been shown in Helicobacter pylori–infected persons, an infection that has been clinically associated with platelet-related diseases, such as idiopathic thrombocytopenic purpura. However, the role of P-selectin expression during H pylori infection remains unclear. In this study, we hypothesized that P-selectin expression was associated with platelet aggregation during H pylori infection. Using flow cytometry, we examined the levels of adhesion between H pylori and platelets as well as the levels of P-selectin expression and platelet phosphatidylserine (PS) expression during H pylori infection. Significantly high levels of adhesion between proaggregatory bacteria and platelets were observed. We identified that H pylori IgG is required for bacteria to induce P-selectin expression and that a significant release of P-selectin is essential for H pylori to induce aggregation. In addition, cellular apoptotic signs, such as membrane blebbing, were observed in platelet aggregates. PS expression was also detected in platelets during infection with both pro-aggregatory and nonaggregatory strains of H pylori. These results suggest that the decrease in platelet counts seen during H pylori infection is the result of P-selection–dependent platelet aggregation and PS expression induced by the bacteria. (Blood. 2010;115(21):4247-4253)

Introduction

Many diseases associated with platelet aggregation have been described as being related to Helicobacter pylori infection. For example, H pylori–infected persons have a tendency toward suffering from myocardial infarctions,1,3 coronary heart disease,4,5 and stroke.6 It has also been suggested that H pylori may trigger the formation of thrombotic thrombocytopenic purpura (TTP) by inducing platelet aggregation through an interaction with the von Willebrand factor (VWF).7 There have also been implications that chronic H pylori infection may be associated with idiopathic thrombocytopenic purpura (ITP), as eradication of the bacteria from gastric mucosa has shown improvement in some ITP patients.8-16 There is ongoing interest in identifying the various H pylori virulence factors that may predict the risk of developing symptoms of ITP. Studies have primarily focused on 2 groups of putative bacterial virulence factors, the cag pathogenicity island (for which CagA is a marker) and the vacuolating cytotoxins, such as VacA17,18; however, CagA and VacA have not been suggested to be causes of H pylori–induced platelet aggregation.19

There has been evidence showing that associations exist between H pylori and platelet aggregation in vivo. Platelet aggregation was observed in rat gastric mucosal microcirculation in vivo after H pylori administration.20 An increase in arterial thrombosis was also found in chronic H pylori–infected mice.17 However, the mechanisms of how H pylori induces platelet aggregation are not clearly understood. Byrne et al proposed that the H pylori strain 60190 (ATCC 49503) induces platelet aggregation through interactions between H pylori, its antibody, and the platelet receptor FcγRIIA (CD32), as well as VWF and its receptor glycoprotein (GP) Ib/IX.19 VWF found in blood plasma is produced in the endothelium (in Weibel-Palade bodies) and megakaryocytes (α granules of platelets).21 Evidence suggests that VWF is one of the essential elements involved in H pylori–induced platelet aggregation.19 Recent work has shown that the D′-D3 domains of VWF can interact with the integral membrane protein P-selectin (CD62P) in Weibel-Palade bodies.21

P-selectin is a member of the selectin family of cell surface receptors, which primarily mediates tethering and rolling of leukocytes,22-24 Platelets are known to become activated when brought into contact with activators, including arachidonic acid (AA), adenosine diphosphate (ADP), collagen, and epinephrine. Once activated, platelets release several different coagulation factors and platelet-activating factors,25 such as P-selectin, usually found in the membrane of the platelet-secretory granules (α granules).26,27 This is known as degranulation, during which redistribution of these factors from the membrane of the granules to the plasma membrane occurs.22 H pylori infection may be associated with the increase of P-selectin expression; however, the function of P-selectin in this situation remains unclear. In this study, we explored the functions of P-selectin in the induction of platelet aggregation during H pylori infection.

Not all H pylori strains induce platelet aggregation, but H pylori infections are known to induce apoptosis in AGS cells of the human gastric epithelial cell line, AGS cells.28 Platelet apoptosis has also been reported.29,30 We therefore hypothesized that, aside from inducing platelet aggregation, H pylori may also induce platelet apoptosis, which may explain the decrease in platelet levels seen in...
some TTP and ITP patients. Interestingly, these patients showed improved platelet counts after eradication of the bacteria.

**Methods**

**Study subjects**

Fifteen healthy study subjects with platelet counts ranging between 150,000 and 400,000/mm³ were enrolled into this study. *H. pylori* (Hp) infection status was assessed by the capsule ¹³C-urea breath test (UBT; INER-Hp C-tester) and anti-Hp antibody test using Hp Rapid Test Strip (ACON Laboratories). UBT was performed according to previous study protocols. Six of 15 study subjects tested positive for both UBT and anti-Hp antibodies, and were grouped as Hp IgG (+); the remaining 9 subjects, who tested negative for both UBT and anti-Hp antibodies, were grouped as Hp IgG (−). Informed consent was obtained from all study subjects in accordance with the Declaration of Helsinki, and study protocols were approved by the Institutional Review Board of the Kaohsiung Municipal Hsiao-Kang Hospital (KMHK-IRB no. A96040270).

**H. pylori culture and LPS extraction**

*H. pylori* strains ATCC 49503, 51932, and 43504 were cultured on CDC ANA blood agar (BD Biosciences) at 37°C in a microaerobic chamber for 3 days. *H. pylori* was scraped from the agar, resuspended in phosphate-buffered saline (PBS), and used within 4 hours. This is true for all experiments apart from those assessing the effect of bacteria storage time, where bacteria were kept at room temperature for up to 72 hours. The amount of *H. pylori* cultured was determined by optical disturbance (OD) at 600 nm, where 1 OD was equivalent to 1 × 10⁸ colony-forming units (CFUs)/mL. *H. pylori* lipopolysaccharide (LPS) extraction was performed using the LPS extraction kit (iNtRON Biotechnology) according to the manufacturer’s instructions. Finally, the extracted LPS was spun at 3000g for 15 minutes and then resuspended in PBS.

**Platelet preparation**

Platelets were isolated from blood collected in blood collection tubes containing 3.2% (0.109M) sodium citrate of both Hp IgG⁻ and Hp IgG⁺ study subjects. Platelet-rich plasma (PRP) was obtained by spinning blood samples at 1000g for 10 minutes, whereas platelet-poor plasma, used as reference for the aggregation assays, was obtained by further spinning at 3000g for 15 minutes. Washed platelets were prepared by adding one-sixth volume of acid-citrate-dextrose solution (2.5% trisodium citrate, 1.4% citric acid, and 2% D (+)-glucose) to PRP. The platelets were spun down (3500g, 8 minutes) and the pellets resuspended in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (10mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 137mM NaCl, 2.68mM KCl, 0.42mM NaH₂PO₄, 1.7mM MgCl₂, 1mM CaCl₂, 5mM D(+) -glucose, pH 7.35). The platelet count was adjusted to the original concentration of the blood donor (~ 250 000/mL). Platelets were kept in a sealed, air-permeable bag and oscillated in a flat-bottomed shaking device at the constant speed of 70 cycles per minute at room temperature for 24 hours. These platelets were then used for the stored platelet P-selectin activation and aggregation assays.

**Platelet aggregation assays**

The aggregation assay was applied by incubating a PRP (100 × 10⁹ platelets) and 0.4 OD *H. pylori* (resuspended in PBS, 4 × 10⁹ bacteria) mixture (platelet/bacteria ratio = 25:1) at 37°C for 5 minutes and assessed by a Platelet Aggregometer (Chrono-Log). Aggregation was expressed as a variation in light transmission, using the light transmitted through platelet-poor plasma as a baseline. The ability for platelet aggregation was analyzed before each individual experiment using platelet activators, such as 0.5 mg/mL AA, 20μM ADP, 10 μg/mL collagen, and 0.3mM epinephrine (Helena Laboratories), as positive controls. Pooled immunoglobulins (Igs) were obtained from the serum of subjects with positive anti-*H. pylori* antibody test and purified. The serum was mixed with an equal amount of wash/bindng buffer (0.1M sodium phosphate, 0.15M NaCl, pH 7.4) and then incubated with protein A/G beads at 4°C for 1 hour, after which they were collected. The pooled Igs were then eluted with elution buffer (0.2M glycine, pH 3); after this, they were concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore) and then adjusted to the final concentration of 10 mg/mL in each sample solution.

**Adhesion of *H. pylori* to platelet assays**

To avoid aggregational depletion of platelets induced by *H. pylori* infection (platelet/bacteria ratio = 25:1), an *H. pylori* concentration of 0.1 OD (platelet/bacteria ratio = 100:1) was chosen for this experiment. This allows a low level of aggregation to occur while still permitting the use of flow cytometry to analyze the Hp-conjugated platelets. After incubation with *H. pylori*, stained with a Bacteria Counting Kit (Invitrogen), platelets were labeled with phycoerythrin (PE)–conjugated mouse anti-CD41 IgG (BD Biosciences). The association between *H. pylori* and platelets was examined by flow cytometry, and 1 × 10⁹ platelets were analyzed. An association was considered present when the platelets stained positive for both *H. pylori* (FL1) and PE (FL2).

**Polymerase chain reaction amplification of *H. pylori* urease**

DNA extraction was performed on the *H. pylori*, PRP, and aggregate mixtures by spinning down at 3000g for 15 minutes. The primers used were derived from the internal 412-bp fragment of the urease A gene: 5’-GCCAATGGTAAATTAGTT-3’ and 5’-CTCCT-TAATGGTTTATAC-3’. Polymerase chain reaction was performed, and the products were analyzed on a 2% agarose electrophoresis gel stained with ethidium bromide.

**Determination of VWF levels**

After platelet aggregation assays, the supernatant was collected and the VWF antigen determined by an immunoturbidimetric assay with the STA Liatest VWF kit (Diagnostica Stago) on a Sysmex CA-1500 analyzer (Sysmex).

**P-selectin expression analysis using flow cytometry**

The expression of P-selectin on platelets was determined by flow cytometry with monoclonal antibodies, PE-conjugated mouse anti-CD62P IgG (BD Biosciences) was used to assess α granule degradation. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD41 IgG (BD Biosciences) was used as an independent marker for the activation of platelets. Anti-CD62P-PE antibodies were added to the resuspended platelets, which were then collected by spinning the 1% formaldehyde prefixed PRP and *H. pylori* mixture (platelet/bacteria ratio = 100:1) at 3000g for 15 minutes. After incubation in the dark at room temperature for 30 minutes, anti-CD-FITC antibodies were added and incubated in the dark at room temperature for a further 30 minutes. The fluorescence intensity was analyzed on the EPICS XL-MCL flow cytometer (Beckman Coulter). The level of platelets expressing P-selectin was defined as a fraction of the 10 000 platelets sorted exhibiting specific binding (ie, CD62p⁺) minus that exhibiting nonspecific binding (ie, the percentage defined with IgG-FITC conjugate). Each experiment was repeated at least 3 times. The mean level of P-selectin expression and fold increase in *H. pylori*-infected samples, compared with noninfected control samples, was recorded.

**Examining apoptotic membrane blebbing using scanning electron microscopy**

Platelet aggregates were collected after incubating PRP with *H. pylori* for 5 minutes (platelet/bacteria ratio = 25:1). The reaction was terminated with 2.5% glutaraldehyde (Sigma-Aldrich) in PBS after 2 hours. The aggregates were then rinsed with PBS 3 times and fixed with 1.33% osmium tetroxide (Fluka) in PBS for 1 hour. The aggregates were again rinsed with PBS 3 times and fixed with 1.33% osmium tetroxide (Fluka) in PBS after 2 hours. The aggregates were then collected by spinning the 1% formaldehyde prefixed PRP and *H. pylori* mixture (platelet/bacteria ratio = 100:1) at 3000g for 15 minutes. After incubation in the dark at room temperature for 30 minutes, anti-CD-FITC antibodies were added and incubated in the dark at room temperature for a further 30 minutes. The fluorescence intensity was analyzed on the EPICS XL-MCL flow cytometer (Beckman Coulter). The level of platelets expressing P-selectin was defined as a fraction of the 10 000 platelets sorted exhibiting specific binding (ie, CD62p⁺) minus that exhibiting nonspecific binding (ie, the percentage defined with IgG-FITC conjugate). Each experiment was repeated at least 3 times. The mean level of P-selectin expression and fold increase in *H. pylori*-infected samples, compared with noninfected control samples, was recorded.
Fixed onto metal discs and viewed with a FEI Quanta 400 F (FEI) environmental scanning electron microscope under secondary electron imaging mode.

Detection of surface exposure of PS using flow cytometry

Platelet-surface exposure of phosphatidylserine (PS) was determined using flow cytometry with the Annexin V-FITC Apoptosis Detection Kit (BioVision) according to the manufacturer’s instructions. Platelets were labeled with PE-conjugated mouse anti-CD41 IgG and mixed with annexin V–FITC after incubation with H. pylori (platelet/bacteria ratio = 100:1). Platelet apoptosis was determined by flow cytometry where both annexin V–FITC and PE-conjugated mouse anti-CD41 IgG (FL2) stains were positive.

Results

Limitations of H. pylori to induce platelet aggregation

Dramatically high percentages of platelet aggregation were found when 0.2 OD pro-aggregatory H. pylori strain ATCC 49503 (H. pylori 49503; 20 x 10^6 CFU/ml) was applied (platelet/bacteria ratio = 50:1) to the assay. This trend continued for concentrations up to 1.0 OD H. pylori (platelet/bacteria ratio = 10:1; Figure 1A). These results indicate that the concentration threshold for H. pylori–induced platelet aggregation is where platelet/bacteria ratio is 50:1. Levels of H. pylori–induced platelet aggregation decreased with increasing bacteria storage time (Figure 1B). In accord with these results, double the threshold concentration of H. pylori (0.4 OD; platelet/bacteria ratio = 25:1) and fresh H. pylori (≤ 4 hours) were used in the following experiments. Platelet aggregation occurred in response to infection with H. pylori 49503 (CagA+/VacA+); this strain was therefore used as the pro-aggregatory H. pylori strain. Aggregation was occasionally observed in response to the H. pylori strain ATCC 51932 (H. pylori; CagA+/VacA–), and no aggregation was seen with the H. pylori strain ATCC 43504 (H. pylori; CagA+/VacA+). The latter was therefore used as the nonaggregatory H. pylori strain (Figure 1C).

Adhesion of H. pylori to platelets induces platelet aggregation

A significantly higher binding ability using flow cytometric assays between platelets and bacteria was observed in pro-aggregatory H. pylori 49503 compared with that of nonaggregatory H. pylori 43504 and that of H. pylori 51932 (Figure 2A). The firm adhesion between platelets and H. pylori was further demonstrated by amplifying the H. pylori specific urease A gene fragment extracted from both the aggregates and the PRP mixtures. A 412-bp DNA fragment was observed in the H. pylori 49503 aggregates (Figure 2B lanes 2 and 3); however, none was observed from the nonaggregatory H. pylori 43504 PRP reaction mixture (Figure 2B lanes 4 and 5). AA-induced platelet aggregates were used as a negative control (Figure 2B lane 1), and DNA fragments amplified from bacterial DNA extracts were used as positive indicators (Figure 2B lanes 6 and 7). These results provide new evidence with regards to the significance of adhesion between the pro-aggregatory H. pylori and platelets in initiating platelet aggregation.

P-selectin activation is essential during platelet aggregation induced by H. pylori infection

LPSs extracted from these H. pylori strains (H. pylori 49503, H. pylori 43504, and H. pylori 51932) showed no signs of aggregation induction (Figure 3A) and were therefore used as negative controls, and AA was used as a positive control for P-selectin activation assays. Results
49503. Platelet aggregation resumed when pooled Igs of Hp 3, thus suggesting that washed platelets are not activated by aggregation when anti-CD62P was added (Figure 4B bar 4 vs bar 3). There was also no further decrease in the levels induce platelet aggregation in the washed platelet assays (Figure 4A bar 8 vs bar 7). However, among these other stimuli, such as AA (Figure 4A bar 2 vs bar 1) and epinephrine (Figure 4A bar 8 vs bar 7). It was not only observed with Hp 49503 infection. Significantly high levels of PS exposure were observed in infections with both the pro-aggregatory strain (Hp 49503) and non-aggregatory strains (Hp 43504 and Hp 51932) of H. pylori.

**Discussion**

It has previously been described that virulence factors of H. pylori, such as CagA and VagA, have no influence in H. pylori-induced samples were added (Figure 4B bar 5 vs bar 3) and were again inhibited with the addition of anti-CD62P (Figure 4B bar 6 vs bar 5). No aggregation was observed in stored Hp IgG+ PRP infected with Hp 49503 (platelet/bacteria ratio = 25:1; Figure 5A bar 8), although standard agonists (AA, ADP, and collagen shown in Figure 5A bars 5-7, respectively) were able to induce aggregation. No significant increase in P-selectin expression in the stored PRP (Figure 5B bar 8 vs bar 7) was observed during H. pylori infection (platelet/bacteria ratio = 100:1). Furthermore, Figure 6 showed that VWF depletion occurs only in Hp 49503-induced platelet aggregation mixtures.

**PS exposure and membrane blebbing were observed during H. pylori infection**

PS exposure in platelets (Figure 7A) and the apoptotic sign of membrane blebbing on platelet aggregates (Figure 7B) were observed during H. pylori infection. Significantly high levels of PS exposure were observed in infections with both the pro-aggregatory strain (Hp 49503) and non-aggregatory strains (Hp 43504 and Hp 51932) of H. pylori.

showed that significant P-selectin activation occurred only in response to the pro-aggregatory Hp 49503 (Figure 3B). Neither Hp 43504 nor Hp 51932 was able to induce platelet activation. P-selectin activation seen with pro-aggregatory Hp 49503 appeared to occur in a concentration-dependent manner. However, no increase in P-selectin was seen with Hp 51932, despite raising the bacterial concentration to 1.0 OD (Figure 3C). The comparisons of P-selectin expression between Hp IgG+ and Hp IgG− during H. pylori infection are shown in Figure 3D. No significant difference in P-selectin expression was observed between the 2 groups (Figure 3D bar 1 vs bar 3). No change in P-selectin expression was observed in Hp IgG− in response to H. pylori infection (Figure 3D bar 2 vs bar 1), although a significant increase was observed in Hp IgG+ (Figure 3D bar 4 vs bar 3). These results indicate that Hp IgG may have an essential role in P-selectin expression during the bacteria infection.

The aggregation induced by pro-aggregatory Hp 49503 is inhibited when anti-CD62P was applied (Figure 4A bar 10 vs bar 9). It was not only observed with Hp 49503 infection but also with other stimuli, such as AA (Figure 4A bar 2 vs bar 1) and epinephrine (Figure 4A bar 8 vs bar 7). However, among these assays, full inhibition was only observed where Hp 49503 applied. Despite being a pro-aggregatory Hp strain, Hp 49503 was unable to induce platelet aggregation in the washed platelet assays (Figure 4B bar 3 vs bar 1). There was also no further decrease in the levels of aggregation when anti-CD62P was added (Figure 4B bar 4 vs bar 3), thus suggesting that washed platelets are not activated by Hp 49503. Platelet aggregation resumed when pooled Igs of Hp IgG+ were added (Figure 4B bar 5 vs bar 3) and were again inhibited with the addition of anti-CD62P (Figure 4B bar 6 vs bar 5). No aggregation was observed in stored Hp IgG+ PRP infected with Hp 49503 (platelet/bacteria ratio = 25:1; Figure 5A bar 8), although standard agonists (AA, ADP, and collagen shown in Figure 5A bars 5-7, respectively) were able to induce aggregation. No significant increase in P-selectin expression in the stored PRP (Figure 5B bar 8 vs bar 7) was observed during H. pylori infection (platelet/bacteria ratio = 100:1). Furthermore, Figure 6 showed that VWF depletion occurs only in Hp 49503-induced platelet aggregation mixtures.

**PS exposure and membrane blebbing were observed during H. pylori infection**

PS exposure in platelets (Figure 7A) and the apoptotic sign of membrane blebbing on platelet aggregates (Figure 7B) were observed during H. pylori infection. Significantly high levels of PS exposure were observed in infections with both the pro-aggregatory strain (Hp 49503) and non-aggregatory strains (Hp 43504 and Hp 51932) of H. pylori.

**Discussion**

It has previously been described that virulence factors of H. pylori, such as CagA and VagA, have no influence in H. pylori–induced

![Figure 3. The pro-aggregatory strain of H. pylori stimulates the expression of P-selectin. Platelets were fixed with formaldehyde and labeled with anti-CD41 IgG and P-selectin, as well as the anti-CD62P antibody. They were then analyzed using flow cytometry.](image-url)

![Figure 4. Anti-P-selectin antibodies completely inhibit H. pylori-induced platelet aggregation. Before the addition of H. pylori (platelet/bacteria ratio = 25:1) and aggregation activators, anti-CD62P antibodies were incubated with PRP for 1 minute. (A) Addition of anti-CD62P antibodies was found to inhibit aggregations induced by pro-aggregatory Hp 49503, AA, and epinephrine. PRP, washed platelets, and pooled Igs were obtained from the anti-Hp antibody-positive samples. Washed platelets were prepared by resuspending the pellets of PRP mixture in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer and were then adjusted to the original concentration (~250 000/mL). Pooled Igs purified with protein A/G beads were adjusted to the final concentration of 10 mg/mL. (B) Platelet aggregation induced by Hp 49503 was inhibited in PRP and in washed platelets with pooled Igs where anti-CD62P antibodies applied.](image-url)
platelet aggregation\(^1\) and that \textit{H pylori}–induced platelet aggregation may be strain-dependent.\(^3\) After assessing different strains of \textit{H pylori} for their ability to induce platelet aggregation, the \textit{H pylori} strain ATCC 49503 and \textit{H pylori} strain ATCC 43504 were chosen as the pro-aggregatory strain and nonaggregatory strain, respectively, for this study. By analyzing \textit{Hp IgG}/H\(1001\) PRP, \textit{Hp IgG}/H\(1002\) PRP (Figure 1C), and washed platelets of \textit{Hp IgG}/H\(1001\) samples (Figure 4B), we further strengthened evidence previously described\(^1\) that \textit{H pylori}–induced platelet aggregation requires the presence of \textit{Hp IgG}s.

Human platelet aggregation has also been associated with bacterial LPS.\(^\text{40}\) LPS from \textit{Escherichia coli} is known to bind to platelets,\(^\text{41}\) and the LPSs of some specific \textit{H pylori} strains carry the sialy\(\text{L}1\text{H}1\) antigen.\(^\text{42}\) The sialy\(\text{L}1\text{H}1\) structure can be recognized by 3 members of the selectin receptor family.\(^\text{43}\) However, the LPSs extracted from the specific \textit{H pylori} strains used in this study were unable to induce aggregation or activate P-selectin; we therefore used these as negative controls for P-selectin expression assays. Further investigation is needed into whether the specific \textit{H pylori} LPS has a role in adhesion between living bacteria and P-selectin of activated platelets.

In this study, we showed the limitation of pro-aggregatory strains of \textit{Hp 49503} in platelet aggregation induction. Aggregation induction appears to be concentration- and storage time-dependent (Figure 1), and certain amounts of living bacteria are necessary to interact with platelets for the induction of platelet aggregation. Once the amount of living bacteria reaches the threshold, aggregation can be initiated. This theory is supported by our adhesion assays, where strong adhesions between platelets and the pro-aggregatory strain \textit{Hp 49503} (Figure 2A) were observed. The appearance of pro-aggregatory strain \textit{Hp 49503} DNA in platelet aggregates (Figure 2B) also demonstrates a high level of adhesion between platelets and the pro-aggregatory strain \textit{Hp 49503}. These results indicate that a threshold of bacterial bound platelets exists and is necessary to initiate platelet aggregation. Despite the absence of \textit{H pylori} from routine blood and serum sample cultures, \textit{H pylori} has been detected in atherosclerotic plaques by the polymerase chain reaction method.\(^\text{4}3\) This indicates that bacteria from a chronic localized \textit{H pylori} infection in the gastric mucosa may have, at some point, had the chance to leak into the blood,
inducing platelet aggregation within the blood, thus forming multiple emboli. This may have eventually led to the development of subtle cardiovascular diseases.

*H pylori* has been reported to stimulate the expression of P-selectin; however, the functions of redistributed P-selectins remain unclear. Our data show that *Hp* 49503 can induce aggregation (Figure 1C) and stimulate high levels of P-selectin expression in *Hp* IgG+ PRP (Figure 3D). The inability of *Hp* 43504 to induce platelet aggregations may be a result of its inability to induce a significant release of P-selectins (Figure 3B-C). Again, platelet aggregation did not occur (Figure 5A) when stored platelets were applied, in which no significant release of P-selectin was observed (Figure 5B). There was a 2.5- to 3.0-fold increase (Figure 5B) in P-selectin expression in *Hp* IgG+ stored PRP compare with that of fresh PRP, with or without *Hp* 49503 infection; we therefore suspect that the high levels of P-selectin expression observed in the stored platelets were not induced by the bacteria infection. The necessity of P-selectin in *H pylori*-induced platelet aggregation was demonstrated in Figure 4 with the addition of anti-P-selectin antibodies (anti-CD62). This further confirms the indispensable role of P-selectin in *H pylori*-induced platelet aggregation. In this study, we have found that P-selectin is not only involved in *H pylori*-induced platelet aggregation, but also has as an essential role in the induction of platelet aggregation.

It has also been suggested that *H pylori* triggers the formation of TTP by inducing platelet aggregation through an interaction with the VWF. A previous study demonstrated that direct binding between VWF and *Hp* 49503 exists and that *H pylori*-induced platelet aggregation is inhibited by the anti-VWF antibody, aspirin, and by a GPIIb/GPIIIa antagonist. The D’-D3 domains of VWF in Weibel-Palade bodies have also been found to interact with membrane P-selectin. In this study, a significant consumption of VWF was observed when *Hp* 49503 was applied to PRP. Without further addition of VWF, platelet aggregation was also induced in washed platelets by adding pooled IgG. This indicates that newly released VWFs from activated platelets are enough to initiate aggregation. Based on these findings, we propose that *H pylori*-induced platelet aggregation is initiated with the binding of bacteria/antibodies to the platelet receptor FcγRIIA. Strong adhesions are necessary for the platelets to become active, after which there is a significant release of P-selectin and VWF. Adhesions between the bacteria/anti-*Hp* IgG/platelet complex, platelet-released VWF, and P-selectin finally induce aggregation.

*H pylori* infections have been associated with some adult patients with ITP; however, a few studies have found conflicting results in the pediatric field. Although the discrepancy in the clinical response to *H pylori* eradication therapy may be the result of differences in the bacterial strains, there has also been a suggestion that chronic infection with *H pylori* is a cause of secondary ITP, which differs from primary ITP (seen mostly in children). It is highly plausible that adult ITP patients, who respond to *H pylori* eradication therapy and show subsequent improvement in their post eradication platelet counts, have secondary ITP induced by long-term *H pylori* infection, although in cases, mostly childhood ITP, where patients do not respond to bacterial eradication, the ITP may not be secondary to *H pylori* infection. Accurate diagnosis of ITP is therefore essential.

In a study carried out by Ahn et al., eradication of *H pylori* from patients with ITP was found to reduce P-selectin expression but seldom to improve the subsequent post eradication platelet counts. This may partly be the result of the complex underlying etiology of ITP. However, it may also be because, after *H pylori* eradication therapy, *Hp* IgG+ patients will no longer carry P-selectin inducing *H pylori*, resulting in the reduction of P-selectin expression seen in the patients in this study. Other etiologies of ITP, excluding ITP secondary to *H pylori* infection, should be considered where post eradication platelet counts do not improve.

Not every strain of *H pylori* can induce platelet aggregation, but most *H pylori* strains can induce apoptosis in human gastric epithelial cells and its cell line, AGS cells. Our results demonstrate that both the pro-aggregatory strain *Hp* 49503 and the nonaggregatory strain *Hp* 43504 can induce PS exposure on the surface of platelets. Although PS expression does not necessarily imply certain apoptosis in platelets, the apoptotic membrane blebbing of PS on aggregates induced by the pro-aggregatory *Hp* 49503 further supports our hypothesis. The detailed mechanisms of *H pylori*-induced platelet apoptosis need further investigation.

In conclusion, this study provides an insight into the association between increased platelet P-selectin expression and *Hp* -induced platelet aggregation, as well as how a decrease in platelet count can be triggered both by platelet aggregation and platelet surface exposure of PS during *H pylori* infection.

Acknowledgments

This work was supported by the National Science Council (Taiwan; NSC96-2320-B-110-008) and Center for Nanoscience and Nanotechnology, the Aim for the Top University Plan, National Sun Yat-Sen University, Kaohsiung, Taiwan.

Authorship

Contribution: J.-J.Y., S.T., and J.-Y.W. designed and performed research; D.-C.W. prepared research; A.C. designed research, supervised the work, analyzed data, and prepared the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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