Neutrophil cathepsin G modulates platelet P-selectin expression and inhibits P-selectin-mediated platelet–neutrophil adhesion

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1. Close contact between platelets and neutrophils modulates their cellular interactions in thrombotic and inflammatory states, with stimulation of P-selectin expression on platelets by agonists such as thrombin and neutrophil-derived cathepsin G being critical in mediating platelet–neutrophil adhesion. This study compared the effects of thrombin and cathepsin G on platelet P-selectin expression and on P-selectin-mediated platelet–neutrophil adhesion.

2. Washed platelets and platelet–neutrophil mixed cell suspensions (platelet/neutrophil ratio, 10:1) were incubated with either the supernatant of activated neutrophils, purified cathepsin G or thrombin. Platelet P-selectin expression and platelet adhesion to neutrophils was quantified by flow fluorocytometric analysis.

3. The supernatant from activated neutrophils stimulated platelet P-selectin expression comparable to that produced by purified cathepsin G or thrombin. P-selectin expression induced by both activated neutrophil supernatant and purified cathepsin G was completely inhibited by α-antichymotrypsin, a specific inhibitor of cathepsin G. Unlike thrombin, which induced maximum platelet P-selectin expression by 10 min, sustained to 120 min, cathepsin G induced an initial large increase in platelet P-selectin expression, followed by a progressive reduction over 30–60 min to baseline levels.

4. Co-incubation of neutrophils with thrombin-stimulated platelets resulted in a significant increase in P-selectin-mediated platelet–neutrophil adhesion, which was completely inhibited by pre-incubation of neutrophils with anti-sialyl Lewis* monoclonal antibody. Thrombin produced maximum platelet–neutrophil adhesion by 10 min which remained stable over 120 min. In contrast, cathepsin G-stimulated platelets did not adhere to neutrophils over 120 min of co-incubation. Addition of cathepsin G to thrombin-stimulated platelets caused a progressive reduction over 30–60 min to baseline levels of platelet–neutrophil adhesion.

5. Neutrophil-derived cathepsin G is a potent platelet activator, but unlike thrombin it causes a time-dependent loss of platelet P-selectin expression and inhibits P-selectin-mediated platelet–neutrophil adhesion. Therefore, cathepsin G may modulate thrombin-mediated platelet–neutrophil adhesive interactions in inflammation and thrombosis.

INTRODUCTION

A close association between platelets and neutrophils has been described at sites of inflammation and thrombosis [1–4]. In such settings, bidirectional cellular interactions mediated by the release of soluble mediators and intercellular adhesion potentially modulate platelet and neutrophil functions. Previous studies in vitro have shown that activated neutrophils can enhance platelet thromboxane formation and 5-hydroxytryptamine release as well as platelet aggregation [5–7], while activated platelets have been shown to adhere to neutrophils and stimulate superoxide production [8, 9]. Other studies have demonstrated a co-dependency for platelet–neutrophil metabolism of arachidonic acid metabolites such as platelet-activating factor (PAF) and leukotriene C4 [10, 11] which are likely to further amplify the inflammatory and thrombotic responses in various disease states.

P-selectin is a membrane glycoprotein that is translocated to the surface of platelets after α-granule release in response to a variety of agonists including thrombin, PAF and collagen [12–14]. Platelet P-selectin expression mediates platelet adhesion to neutrophils via interaction with one or more glycoproteins bearing sialylated, fucosylated lactosamine O-linked oligosaccharides, such as sialyl Lewis*, and expressed on the surface of neutrophils and monocytes [15]. P-selectin glycoprotein ligand-1 (PSGL-1) is a quantitatively minor neutrophil glycoprotein that shows high-affinity binding to P-selectin by presenting <1% of the surface sialyl Lewis* [16, 17]. Previous studies have shown that the interaction

Key words: adhesion, cathepsin G, neutrophil, platelet, P-selectin, thrombin.
Abbreviations: HBSS, Hanks balanced salt solution; FMLP, N-formyl-L-Met-L-Leu-L-Phe; MCF, mean channel fluorescence; PAF, platelet-activating factor; PE, phycoerythrin; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; RSF, relative specific fluorescence.
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between platelet P-selectin and its neutrophil counter-receptor(s) is critical in platelet–neutrophil binding and is likely to be important in promoting platelet–neutrophil interactions [18–21].

Although neutrophils produce a variety of inflammatory mediators including PAF, elastase and oxygen free radicals, platelet activation by neutrophils appears to be mainly mediated by the release of the neutrophil serine protease, cathepsin G [5–7, 20, 21]. This has been supported by studies demonstrating that purified cathepsin G induces platelet calcium mobilization, thromboxane formation, 5-hydroxytryptamine release and platelet aggregation as well as P-selectin expression [5–7, 22]. Therefore, platelets stimulated by cathepsin G released from activated neutrophils might be expected to adhere to neutrophils in a P-selectin-dependent manner. However, there is contrary evidence to suggest that activated neutrophils may actually inhibit platelet–neutrophil adhesion and promote time-dependent dissociation of platelet–neutrophil aggregates [23]. Previous studies have shown that cathepsin G can cause extensive proteolysis of the glycoprotein GPIba subunit within the platelet GPIb-IX complex on the platelet surface [24, 25]. A similar proteolytic effect of cathepsin G on P-selectin or its neutrophil ligand may explain the inhibition by activated neutrophils of platelet–neutrophil adhesion.

Therefore the aims of this study were first to compare the effects of supernatant from activated neutrophils, cathepsin G and thrombin on platelet P-selectin expression, and to determine the time course of these effects. The second aim was to determine whether neutrophil-derived cathepsin G inhibits P-selectin-mediated platelet–neutrophil adhesion or dissociates platelet–neutrophil aggregates via effects on platelet P-selectin or its neutrophil ligand.

METHODS

Materials

Cathepsin G, z1-antichymotrypsin, cytochalasin B and N-formyl-L-Met-L-Leu-L-Phe (fMLP) were purchased from Calbiochem, Sydney, Australia. Bovine serum albumin (BSA) was from CSL (Melbourne, Australia), phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Co. (Sydney, Australia), thrombin from Armour Pharmaceuticals Co. (Kansas City, IL, U.S.A.), and prostaglandin E1 from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Mouse isotype control IgG1–fluorescein isothiocyanate/IgG2a–phycoerythrin (PE), PE-conjugated monoclonal antibodies to P-selectin (CD62P, IgG1 isotype) and integrin (CD11b, IgG2a isotype) and unconjugated monoclonal antibody to sialyl Lewisx (IgM isotype, containing 0.01% azide) were purchased from Becton Dickinson, Rutherford, NJ, U.S.A.

Subjects and blood collection

Twenty-eight healthy subjects (14 males and 14 females), mean age 27.7 (range 23–44) years, with no history of infection, trauma or use of medication, including aspirin, in the preceding 10 days, were recruited and each subject gave written informed consent. The study was approved by the Committee for Human Rights of the University of Western Australia. Blood was taken at 09.00 h with the subjects having fasted overnight and having undertaken no strenuous exertion that morning. Blood was collected from an antecubital vein without use of a tourniquet via a 19-gauge butterfly needle. The first 2 ml was discarded, and 20 ml was then drawn directly into acid–citrate–dextrose (1.5 ml/10 ml) tubes. A further 40 ml of blood was drawn directly into heparin (1 i.u./ml) tubes.

Preparation of cells

Washed platelets. Whole blood anticoagulated with acid–citrate–dextrose was centrifuged (150 g, 12 min) and the resulting platelet-rich plasma was aspirated and washed with Tyrode's buffer (138 mmol/l NaCl, 29 mmol/l KCl, 12 mmol/l NaHCO3, 0.4 mmoll/l NaHPO4, 5.5 mmol/l glucose, 0.35% (w/v) BSA, pH 6.5) containing 50 ng/ml prostaglandin E1. After centrifugation (2000 g, 10 min) the platelet pellet was resuspended in Tyrode's buffer containing 2 mmol/l Ca2+. Platelets were counted, diluted to 25 x 10⁶ cells/ml in Heps-buffered Hanks balanced salt solution (HBSS), pH 7.4, containing 0.25% (w/v) BSA and kept at room temperature.

Neutrophil suspension. Neutrophils were prepared from whole blood, anticoagulated with heparin, by centrifugation on discontinuous Percoll gradients [26]. After centrifugation (400 g, 30 min), plasma and lymphocytes were discarded and the neutrophil layer was aspirated. The cells were sedimented by centrifugation (400 g, 10 min) and erythrocytes in the pellet were lysed with hypotonic saline. Neutrophils were finally resuspended in Heps/HBSS containing 2 mmol/l Ca2+ and 0.25% (w/v) BSA, counted and diluted to 25 x 10⁶ cells/ml. Neutrophil purity and viability were greater than 95% as determined by differential count (Coulter Counter) and Trypan Blue exclusion respectively.

Stimulation of platelet P-selectin expression by neutrophil supernatant

Cytochalasin B was used to prime neutrophils for fMLP-induced degranulation. The neutrophil suspension (0.4 ml, 25 x 10⁶ cells/ml) was incubated at room temperature with cytochalasin B (5 μg/ml) for 2 min followed by fMLP (10 μmol/l) for a further 5 min. Non-activated neutrophils were similarly
incubated for a total of 7 min with buffer alone. The non-activated and activated neutrophil suspensions were centrifuged (400 g, 2 min) to sediment cells and obtain non-activated or activated supernatants respectively.

Washed platelet suspension (40 μl, 25 × 10⁶ cells/ml) and neutrophil supernatant (40 μl of activated or non-activated supernatant) were incubated with buffer (10 μl) and a specific PE-labelled monoclonal antibody to P-selectin (10 μl, 1/40 dilution) for 40 min at room temperature before fixation with 0.5% (v/v) formaldehyde. PMA (10 μl, 1.6 μmol/l) or thrombin (10 μl, 0.01 i.u./ml) was added to separate tubes each containing washed platelets and the supernatant from non-activated neutrophils. PMA and thrombin served as positive controls and the concentrations used were the lowest that produced maximum platelet activation. In a similar set of experiments purified cathepsin G (0.1, 10 and 50 μg/ml) was incubated with washed platelet suspension for 40 min before fixation with formaldehyde and assessment of platelet P-selectin expression. To confirm that platelet P-selectin expression was induced by cathepsin G in activated neutrophil supernatants, α1-antichymotrypsin, the specific human plasma inhibitor of serine proteases such as cathepsin G, was incubated for 10 min at concentrations of 10, 50 and 100 μg/ml with activated neutrophil supernatant before the incubation with platelet suspension for 40 min. To determine the time course of the effect of cathepsin G on P-selectin expression, platelet activation by cathepsin G (50 μg/ml) was stopped after 1, 10, 30, 60 and 120 min by the addition of 0.5% (v/v) formaldehyde before incubation with the PE-labelled monoclonal antibody to P-selectin. Finally, to examine the possible effect of cathepsin G on sialyl Lewis¹, neutrophils were incubated with cathepsin G (50 μg/ml) for 30 min before incubation with the mouse, anti-human sialyl Lewis¹ monoclonal antibody followed by a fluorescein isothiocyanate-labelled anti-mouse Fc-specific antibody.

**Flow cytometry**

Platelet P-selectin expression was assessed by measuring the fluorescence of the PE-labelled P-selectin-specific monoclonal antibody using a FACScan flow cytometer (Becton Dickinson) [13, 14, 27]. The platelet population was identified by setting analytical gates on the basis of forward- and side-angle light scatter with data collected in log mode using single parameter analysis of 5000 platelets. Binding of the PE-labelled antibody to platelets was quantified as the percentage of platelets exhibiting PE fluorescence as well as the mean channel fluorescence (MCF). The relative density of P-selectin receptors per platelet was deemed to be directly proportional to the level of P-selectin-specific fluorescence per platelet. Therefore, the relative density of P-selectin receptors is expressed either as the relative specific fluorescence (RSF) or as MCF (in arbitrary units). The RSF was calculated from the formula: RSF = Antilog [(n − m)/channels per log] − 1, where n is the MCF of platelets incubated with the test antibody and m is the MCF of platelets incubated with the negative control antibody [28].

The level of platelet–neutrophil adhesion was determined by flow cytometry using anti-P-selectin rather than anti-GPIIb-IIIa as a platelet marker [19]. In brief, for selected samples the neutrophil population was gated on the basis of its characteristic forward- and side-scatter profile and expression of the integrin, CD11b (present on neutrophils, monocytes and natural killer lymphocytes) on at least 99% of cells as determined by a specific PE-labelled monoclonal antibody. The percentage of neutrophils positive for platelet P-selectin fluorescence was taken as an estimate of the percentage of neutrophils with at least one bound platelet [19].
The MCF of the platelet marker on neutrophils was also determined as this has been shown to correspond semi-quantitatively to the number of platelets bound per neutrophil [19] and also provides an indication of the degree of activation of the platelets bound to neutrophils. Activated platelets and neutrophils were co-incubated before addition of the anti-P-selectin, thus minimizing the possibility that the antibody would interfere with platelet-neutrophil binding. However, it was possible that this method would underestimate platelet-neutrophil binding because P-selectin molecules involved in binding to neutrophils would be inaccessible to the PE-labelled antibody. Nevertheless, this does not appear to have been a significant problem, since the levels of thrombin-induced platelet-neutrophil adhesion (60–80% P-selectin-positive neutrophils) were very similar to those observed in other studies using GPIIb-IIIa or GPIb as markers of adhesion [19, 23], indicating that P-selectin is also a valid marker of platelet-neutrophil adhesion.

**Statistical analysis**

Data for the percentage positive cells, the RSF and MCF were expressed as means±S.E.M. For multiple comparisons, analysis of variance was used and where significant differences were identified (P<0.05), pairwise comparisons were made using Tukey’s multiple comparison test (Instat 2 programme, GraphPad, San Diego, CA, U.S.A.).

**RESULTS**

**Effects of neutrophil supernatant and cathepsin G on platelet P-selectin expression**

After incubation with the supernatant from non-activated neutrophils, platelet P-selectin expression was 28±4%, while the supernatant from fMLP/cytochalasin B-activated neutrophils induced P-selectin expression on 59±5% of cells (P<0.001, n = 14). The latter response was approximately 70% of the maximum platelet P-selectin expression induced by PMA (84±3%) and thrombin (79±5%) (Figure 1a). Purified cathepsin G also produced a dose-dependent platelet activation, with concentrations of 10 and 50 µg/ml producing a significant increase in the percentage of platelets expressing P-selectin (P<0.01 and P<0.001 compared with baseline respectively, n = 8, Figure 1b). The supernatant from activated neutrophils produced approximately 70% of the platelet P-selectin expression induced by the highest concentration of cathepsin G (50 µg/ml). Although the supernatant from activated neutrophils, like thrombin, produced significant platelet activation at 40 min as determined by the percentage of platelets expressing P-selectin, the relative number of receptors per cell, as indicated by the RSF, was only 35±6% of the RSF for thrombin-stimulated platelets (P<0.001, n = 14). Similar results were obtained with cathepsin G (50 µg/ml) which produced a significant increase in the percentage of platelets expressing P-selectin whereas the RSF per cell after stimulation with cathepsin G for 40 min was only 20±4% of that for thrombin-stimulated platelets (P<0.001, n = 6).

**Effect of α1-antichymotrypsin on platelet P-selectin expression induced by cathepsin G and neutrophil supernatant**

The specific cathepsin G inhibitor, α1-antichymotrypsin, produced a dose-dependent inhibition of platelet P-selectin expression induced by purified cathepsin G (50 µg/ml) with significant inhibition at 100 µg/ml α1-antichymotrypsin (P<0.001, n = 8, Figure 2a). Equally, at concentrations of 50 and

Fig. 1. Effects of neutrophil supernatant and cathepsin G on platelet P-selectin expression. (a) Platelet P-selectin expression after stimulation for 40 min with supernatant from non-activated neutrophils (○). PMA (1.6 µM, ●), thrombin (0.01 i.u./ml, □), or supernatant from activated neutrophils (▲). Data are means±S.E.M. (n=14). *P<0.001 compared with supernatant from non-activated neutrophils. (b) P-selectin expression in non-activated platelets (○) and after stimulation for 40 min with increasing concentrations of purified cathepsin G (▲) (n=8). *P<0.01 and **P<0.001 compared with non-activated platelets.
100 μg/ml, α1-antichymotrypsin completely inhibited platelet P-selectin expression induced by the supernatant from activated neutrophils (P < 0.01, n = 8, Figure 2b), indicating that stimulation of platelet P-selectin expression by neutrophil supernatant was likely to have been mediated by cathepsin G.

**Time course of the effects of thrombin and cathepsin G on platelet P-selectin expression**

In non-stimulated platelets, P-selectin expression did not change significantly at 1, 10, 30, 60 or 120 min, whereas within 10 min thrombin produced maximum platelet activation in terms of percentage of platelets expressing P-selectin and MCF, and there was no subsequent decline in the number of platelets expressing P-selectin or in the MCF up to 120 min post stimulation (Figure 3). In the first 10 min of stimulation, cathepsin G also increased the percentage and MCF of platelets expressing P-selectin to levels similar to that seen with thrombin. However, unlike thrombin, there was a significant decline in percentage and MCF of platelets expressing P-selectin within 30 min of stimulation with cathepsin G, which declined further to baseline levels by 60 min (P < 0.001 compared with 10 min, n = 6). When platelet P-selectin was maximally expressed after 10 min of thrombin stimulation, the addition of cathepsin G produced a similar progressive decline in percentage and MCF of P-selectin-positive platelets, falling to near baseline levels at 60 min (P < 0.001 compared with 10 min, n = 6).
Effect of thrombin on platelet adhesion to neutrophils

Thrombin stimulation of platelets co-incubated for 40 min with neutrophils resulted in a significant increase in platelet P-selectin-specific fluorescence on neutrophils, rising from a baseline of 24 ± 7% with unstimulated platelets to 56 ± 10% with thrombin-stimulated platelets (P < 0.001, n = 6, Figure 4). The thrombin-stimulated platelet P-selectin fluorescence on neutrophils was completely inhibited by preincubation of the neutrophils with a specific monoclonal antibody directed at the sialyl Lewis’ oligosaccharides on the neutrophil surface (P < 0.001, n = 6, Figure 4).

Time course of the effects of thrombin and cathepsin G on platelet–neutrophil adhesion

When incubated with non-stimulated platelets, the percentage of neutrophils exhibiting platelet P-selectin fluorescence increased to 20% at 10 min and did not change thereafter. In contrast, stimulation of platelets with thrombin in a mixed cell suspension of platelets and neutrophils produced a significant increase in the percentage and MCF of neutrophils exhibiting platelet P-selectin-specific fluorescence (P < 0.001, n = 7, Figure 5). The increased platelet–neutrophil adhesion occurred within 1 min of thrombin stimulation, reached a maximum by 10 min and did not decline over 120 min. This corresponded to the time course of the effect of thrombin on P-selectin expression in the washed platelet suspension (Figure 3). In contrast, cathepsin G stimulation of platelets in the mixed cell suspension produced no increase in platelet–neutrophil adhesion over 120 min, and in fact the percentage of neutrophils expressing platelet P-selectin fluorescence was significantly below base-line levels 120 min after cathepsin G stimulation (P < 0.05, Figure 5a). The lack of increased platelet–neutrophil binding after cathepsin G was discordant with the increased platelet P-selectin expression induced by cathepsin G in the washed platelet suspension within the first 10 min of stimulation (Figure 3). Similarly, in a mixed cell suspension, when platelet–neutrophil adhesion was induced by thrombin, subsequent addition of cathepsin G caused a significant decrease in the percentage and MCF of neutrophils expressing platelet-specific P-selectin by 30–60 min (P < 0.001, n = 7, Figure 5), indicating dissociation of platelet–neutrophil aggregates. Cathepsin G had no effect on the percentage of neutrophils expressing sialyl Lewis’ (95 ± 2%
DISCUSSION

Platelet activation by neutrophil-derived cathepsin G has been proposed as a mechanism by which neutrophils are involved in thrombosis and can recruit and activate platelets in inflammatory disease [1, 2, 21]. In support of this, we have shown that the supernatant from neutrophils activated with fMLP/cytotochalasin B produced a significant increase in the percentage of platelets expressing P-selectin, indicating significant platelet activation and α-granule release. P-selectin expression on approximately 20% of unstimulated platelets appears to be an inevitable consequence of activation during preparation. However, the results suggest that this basal activation does not affect platelet responses to PMA, thrombin and cathepsin G which were close to maximum. Furthermore, the variation in mean basal P-selectin expression in different experiments would not influence the relative differences in stimulated P-selectin expression within a given experiment.

The supernatant from activated neutrophils induced platelet P-selectin expression equivalent to 70% of that induced by the highest concentration of pure cathepsin G (50 μg/ml) in the same experimental system. Although previous studies have suggested that cathepsin G concentrations of 0–10 μg/ml are within the physiological range [5, 6, 22], the results from this study suggest that local concentrations produced endogenously by degranulating neutrophils at sites of inflammation could be much greater. Alpha1-antitrypsin, present in human plasma, is the most potent and specific inhibitor of cathepsin G, and in this experimental model caused complete inhibition of platelet P-selectin expression induced by neutrophil supernatant, suggesting that cathepsin G released by activated neutrophils was the primary stimulus for platelet activation and P-selectin expression. Other investigators, however, have shown that elastase, released with cathepsin G from neutrophil azurophilic granules, can potentiate cathepsin G-induced platelet activation, although it does not directly stimulate platelets and is not inhibited by α1-antitrypsin [29].

A new and potentially important finding in this study was that, although activated neutrophil supernatant and purified cathepsin G induced expression of P-selectin on a high percentage of platelets, the relative receptor density per cell was low, being only 20–35% of that seen after 40 min of thrombin stimulation. Investigation of the time course of the effect of cathepsin G on platelet P-selectin expression revealed that within the first 10 min, cathepsin G increased the MCF and percentage of platelets expressing P-selectin to a level equivalent to that observed after thrombin stimulation, but unlike thrombin there was a subsequent decline in P-selectin expression over 30–60 min to baseline levels. A previous study of the effect of purified cathepsin G on P-selectin fluorescence over 30 min may not have detected a significant decline in P-selectin expression [22] because in the present study the cathepsin G-induced loss of P-selectin was most marked after 30–60 min. These results would be consistent with proteolytic cleavage by cathepsin G of P-selectin or at least the epitope recognized by the P-selectin-specific monoclonal antibody, although it is possible that cathepsin G may be causing a conformational change of the P-selectin molecule. Cathepsin G induced a rapid loss of P-selectin expression on platelets which had been maximally prestimulated with thrombin, and again this is consistent with a proteolytic effect of cathepsin G on P-selectin. In support of this, cathepsin G has been shown to cleave the von Willebrand factor binding site at the Leu275–Tyr276 peptide bond of the GPIbα subunit of the GPIb–IX complex [25, 30] to produce conformational changes in the fibrinogen binding site of GPIbα–IIIa [24] and to cleave the N-terminus of a separate protease-activated platelet receptor for thrombin at Arg41–Ser42 and at Phe55–Trp56 [31]. P-selectin, like GPIbα, is a heavily glycosylated protein with N-terminal extracellular lectin and epidermal growth factor domains [15] and it is quite likely to also contain one or more sites that are susceptible to cleavage by cathepsin G.

Platelet adhesion to neutrophils facilitates neutrophil superoxide production [8, 9] and transcellular metabolism of arachidonic acid and PAF [10, 11]. Close platelet–neutrophil contact may also create a microenvironment in which neutrophil-derived cathepsin G is protected from inactivation by antiproteases in plasma [7]. In this study the ratio of platelets to neutrophils (10:1) was chosen to simulate that likely to occur at sites of inflammation rather than the higher ratio found in blood, although other studies using whole blood [19] or a platelet–neutrophil ratio of 100:1 [23] found similar levels of platelet–neutrophil adhesion to those observed in the present study. Thrombin increased platelet P-selectin expression and induced a high and sustained level of platelet adhesion to non-activated neutrophils, as demonstrated by platelet-specific P-selectin fluorescence on neutrophils. Although previous studies have shown that P-selectin is critical to binding of activated platelets to neutrophils and monocytes [18–20] the nature of the ligand(s) for P-selectin on the neutrophil surface has been less clear [16]. This study provides further evidence supporting the importance of sialyl LewisX as a component of the neutrophil ligand for P-selectin by demonstrating complete inhibition of thrombin-stimulated platelet adhesion to neutrophils with a saturating concentration of anti-sialyl LewisX monoclonal antibody. Recent studies suggest that the glycoprotein PSGL-1 is likely to be the sialyl LewisX-bearing ligand on neutrophils that binds with high affinity to P-selectin [15, 16].
The time course of platelet–neutrophil adhesion after thrombin stimulation closely followed the thrombin-induced increase in platelet P-selectin expression. In contrast, the effect of cathepsin G was significantly different with no associated increase in platelet–neutrophil adhesion through 120 min of co-incubation. Furthermore, when platelet–neutrophil adhesion was induced by thrombin, cathepsin G caused dissociation of platelet–neutrophil aggregates. The inhibition of platelet–neutrophil adhesion by cathepsin G cannot be explained by loss of platelet P-selectin expression since there was no adhesion even before the reduction in P-selectin expression at 30 min. The explanation proposed by Rinder et al. [23] for their similar observation that neutrophil activation by FMLP inhibited P-selectin-mediated platelet–neutrophil adhesion was that neutrophil activation decreases the expression of the neutrophil ligand for P-selectin. However, similar activation of monocytes did not result in inhibition of monocyte–platelet adhesion, suggesting that neutrophil, but not monocyte, activation causes release of a product such as cathepsin G that modifies the P-selectin ligand on neutrophils [23]. In another study, cathepsin G was shown to inhibit E-selectin-mediated neutrophil adhesion to endothelial cells by a mechanism that did not involve cleavage of neutrophil L-selectin or modification of CD11b/CD18 expression [32]. However, other studies have suggested that neutrophil release products may cause degradation or redistribution of the P-selectin ligand on neutrophils [33–35]. In the present study, we were unable to demonstrate any reduction in sialyl Lewis* expression on neutrophils after 30 min of incubation with cathepsin G. However, it is possible that cathepsin G either cleaves or modifies the polypeptide backbone of the P-selectin ligand PSGL-1. Any resulting loss of sialyl Lewis* moieties would be insignificant relative to the total amount of this oligosaccharide present on neutrophils but high-affinity binding of P-selectin to sialyl Lewis* on PSGL-1 may well be disrupted. Thus, the precise mechanism for the lack of platelet neutrophil adhesion after stimulation by cathepsin G remains uncertain, but it seems likely that it involves an effect of cathepsin G on the neutrophil P-selectin ligand.

In summary, our investigations provide further insight into a complex range of platelet–neutrophil interactions that have been observed in vitro. This study has shown that cathepsin G released from activated neutrophils stimulates platelet activation with initial P-selectin expression, followed by a loss of P-selectin possibly due to the proteolytic effect of cathepsin G. This neutrophil-derived protease also inhibits platelet–neutrophil adhesion and can promote dissociation of platelet–neutrophil aggregates, but this effect may be due to the action of cathepsin G on the neutrophil P-selectin ligand. Although we can only speculate on the in vivo implications of our findings, the potential consequences of thrombin generation at sites of vascular injury may be to promote platelet adhesion to neutrophils, and thrombosis through platelet–leucocyte interactions. In predominantly inflammatory states, however, the balance may be towards neutrophil activation with release of cathepsin G, platelet activation, release of inflammatory mediators and platelet aggregation while subsequently inhibiting platelet–neutrophil adhesion.

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