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Neutrophil Cathepsin G Promotes Prothrombinase and Fibrin Formation under Flow Conditions by Activating Fibrinogen-adherent Platelets*

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Human neutrophil proteases cathepsin G and elastase can directly alter platelet function and/or participate in coagulation cascade reactions on the platelet or neutrophil surface to enhance fibrin formation. The clotting of recalcified platelet-free plasma (PFP) or platelet-rich plasma (PRP) supplemented with corn trypsin inhibitor (to shut down contact activation) was studied in well-plates or flow assays. Inhibitors of cathepsin G or elastase significantly delayed the burst time (t_{50}) of thrombin generation in neutrophil-supplemented PRP from 49 min to 59 and 77 min, respectively, in well-plate assays as well as reduced neutrophil-promoted fibrin deposition on fibrinogen-adherent platelets under flow conditions. In flow assays, purified cathepsin G was a far more potent activator of platelet-dependent coagulation than elastase. Anti-tissue factor had no effect on neutrophil protease-enhanced thrombin formation in PRP. The addition of cathepsin G (425 nM) or convulxin (10 nM) to PRP dramatically reduced the t_{50} of thrombin generation from 53 min to 17 or 23 min, respectively. In contrast, the addition of elastase to PRP left the t_{50} unaltered. Whereas perfusion of PFP ($\gamma_w = 62.5\ s^{-1}$) over fibrinogen-adherent platelets did not result in fibrin formation until 50 min, massive fibrin could be observed on cathepsin G-treated platelets even at 35 min. Cathepsin G addition to corn trypsin inhibitor-treated PFP produced little thrombin unless anionic phospholipid was present. However, further activation inhibition studies indicated that cathepsin G enhances fibrin deposition under flow conditions by elevating the activation state of fibrinogen-adherent platelets rather than by cleaving coagulation factors.

The triggered display of platelet procoagulant activity is the major requirement for effective blood coagulation. In the absence of tissue factor or collagen (*i.e.* biomaterial thrombosis or dysfunctional endothelium), the neutrophil and its released proteases human neutrophil elastase and cathepsin G (Cat G)[1] may help initiate platelet-dependent thrombin generation by various potential mechanisms. Both Cat G and human neutrophil elastase are serine proteinases of the chymotrypsin family.

Cat G can cleave the plasma zymogens factor V and factor X as well as activate spread human platelets via protease-activated receptor 4 with consequent calcium mobilization (1–3). Cat G treatment of platelets increases surface presentation of GPIV, P-selectin, and active GPIIb/IIIa (4). Elastase can cleave factor V (1) and potentially facilitate de-encryption of tissue factor in blood (5) by cleaving tissue-factor pathway inhibitor (6). De-encrypted tissue factor may function on the platelet surface to initiate coagulation (7). Additionally, Cat G can degrade neutrophil P-selectin glycoprotein ligand-1 (8) and platelet GPIb without an effect on platelet P-selectin (9, 10).

The sequence of platelet capture, translocation, and arrest to a surface through interactions mediated by glycoproteins GPIb-V-IX, GPIIb/IIIa, and GPIa/IIa (11–13) is followed by spreading and activation. GPVI, which is associated with the Fc receptor γ chain (14), mediates the signaling response to collagen (15–17). Once activated, platelets participate in the assembly of the tenase (IXa/VIIIa) and prothrombinase (Xa/Va) complexes by providing anionic phospholipid binding sites for prothrombin, factor X, factor V, and factor XI to help accelerate coagulation (18, 19).

Neutrophil-platelet interactions during adhesion and heterotypic aggregation have been associated with the enhancement of thrombosis. Using a Dacron graft thrombosis model, Palabrica *et al.* (20) observe that diminished neutrophil accumulation in the presence of anti-P-selectin was also accompanied by reduced fibrin deposition. Circulating levels of neutrophil-platelet complexes have been found to increase in hip arthroplasty patients (21), a group prone to deep vein thrombosis. In a different study, recombinant P-selectin glycoprotein ligand immunoglobulin reduced experimentally induced venous thrombosis (22).

However, the relative roles of human neutrophil elastase and Cat G and the prioritization of various mechanisms through which these proteases promote coagulation under flow conditions has not been well studied. Although neutrophil elastase and cathepsin G can activate platelets and/or participate in coagulation cascade reactions directly, their activity may be limited by the presence of antiproteases present in plasma (23). Our previous studies (24) have focused on the effects of neutrophil-platelet interactions on subsequent fibrin formations under venous flow conditions. We demonstrated that individ-

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[1] The abbreviations used are: Cat G, cathepsin G; CTI, corn trypsin inhibitor; fMLP, *N*-formyl-Met-Leu-Phe; GP, glycoprotein; PFP, platelet-free plasma; PRP, platelet-rich plasma; PS, phosphatidylserine; PC, phosphatidylcholine; PSPC, phosphatidylserine/phosphatidylcholine; Va, factor Va; VIIIa, factor VIIIa; IXa, factor IXa; Xa, factor Xa; XIa, factor XIa; CMK, chloromethyl ketone.

ual neutrophils alone can accelerate fibrin deposition if factor XIIa is present due to contact activation by a CD18-dependent mechanism attenuated with inhibitors of elastase or cathepsin G. In the presence of corn trypsin inhibitor (CTI) to block factor XIIa, neutrophils promote fibrin formation on fibrinogen-adherent platelets through pathways attenuated by inhibitors of human neutrophil elastase and Cat G.

In this study, we investigated the mechanisms by which neutrophil proteases alter platelet function. Cathepsin G, more so than elastase, plays the major role in turning fibrinogen-adherent platelets procoagulant. Cathepsin G does so by elevating the activation state of fibrinogen-adherent platelets rather than by cleavage of plasma zymogens factor X and factor V in plasma. Relative rates of thrombin generation for various coagulation scenarios have been measured under static conditions to complement the videomicroscopy-imaged flow assays aimed at understanding the role of neutrophils in thrombosis under venous hemodynamic conditions.

EXPERIMENTAL PROCEDURES

Materials—Human neutrophil elastase, human cathepsin G, human thrombin, and calf skin collagen (Calbiochem), human serum albumin (Golden West Biologicals, Temecula, CA), *N*-formyl-Met-Leu-Phe (fMLP), bovine brain L-α-phosphatidyl-L-serine, and bovine brain L-α-phosphatidylcholine (Sigma), CTI, human fibrinogen, factor X, and anti-human tissue factor monoclonal antibody TFE (Enzyme Research Labs, South Bend, IN), and convulxin (Centerchem, Norwalk, CT) were stored following the manufacturers' recommendations. MeOSuc-Ala-Ala-Pro-Ala-CMK (human neutrophil elastase inhibitor) and Z-Gly-Leu-Phe-CMK (cathepsin G inhibitor) were from Enzyme Systems Products (Livermore, CA). The fluorogenic substrate for thrombin, tboc-Val-Pro-Arg-MCA (boc-VPR-MCA), was obtained from Peninsula Laboratories (San Carlos, CA).

Cell Isolation—Human blood was collected from healthy adult donors by venipuncture and anticoagulated with sodium citrate (9 parts blood to 1 part sodium citrate). Neutrophils were isolated by centrifugation over neutrophil isolation medium (Robbins Scientific) as previously described (24, 25). Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulated whole blood at 130 × *g* for 15 min. Platelet singlets were prepared by gel filtration (24). After isolation, neutrophils or platelets were diluted to final concentrations of 10^6 or 10^8 cells/ml, respectively.

Microcapillary Flow Chambers—Rectangular glass capillaries (Vitrocom, Mountain Lakes, NJ) with a cross-section of 0.2 × 2.0 mm, a length of 7 cm, and a wall thickness of 0.15 mm were used as flow chambers as previously described (24, 25). To enable adhesion of neutrophils or platelets microcapillary flow chambers were incubated with human fibrinogen solution (100 μg/ml) for 120 min at room temperature or with calf skin collagen (100 μg/ml) for 4 h at 4 °C. The chambers were rinsed, and cells were allowed to adhere under no-flow conditions as described previously (24). In selected experiments, adhesion of platelets was preceded by their treatment with human neutrophil elastase (10 μg/ml) or cathepsin G (10 μg/ml). Platelet-free plasma treated with CTI (50 μg/ml) was perfused into the flow chambers containing defined surface compositions at a controlled flow rate using a syringe pump (Harvard Apparatus). The wall shear stress (τ_w) imposed on the surface was calculated from the solution of the Navier-Stokes equation for laminar flow of a Newtonian fluid; $\tau_w = (6\ Q\mu)/(B^2W)$, where Q represents the flow rate (cm^3/s), μ represents the viscosity (0.01 poise at room temperature), B represents the total plate separation (0.02 cm), and W represents the width (0.2 cm). Consequently, the wall shear rate, γ_w (s^{-1}), can be calculated as $\gamma_w = 6Q/B^2W$. A flow rate of 50 μl/min corresponded to a shear stress of 0.625 dyne/cm^2 and a wall shear rate of 62.5 s^{-1}. To activate fibrinogen-adherent platelets, convulxin (10 nM) was perfused over surface-adherent platelets for 10 min. During flow experiments, the microcapillary flow chambers were mounted on a Zeiss Axiovert 135 microscope, and a 63× (NA 1.40) oil immersion objective lens (Plan Apochromat) was used to conduct differential interference contrast microscopy.

Fluorogenic Measurement of Thrombin Generation—Human neutrophil elastase (11.5 μg/ml; 425 nM), cathepsin G (10 μg/ml; 425 nM), factor VIIa (2 nM), anti-tissue factor (50 μg/ml), anionic phospholipid vesicles (50 μM), and/or convulxin (10 nM) were mixed with recalcified citrated platelet-rich plasma supplemented with factor X (170 nM) and CTI (50 μg/ml) in a 96-well plate. Fluorogenic substrate for thrombin (tboc-Val-Pro-Arg-MCA; 20 μM) was added to the reaction mixture (150 μl/well) for kinetic measurements of thrombin generation. In selected experiments, isolated neutrophils (10^6 cells/ml), fMLP (20 μM), and elastase inhibitor (100 μM, MeOSuc-Ala-Ala-Pro-Ala-CMK) or cathepsin G inhibitor (100 μM, Z-Gly-Leu-Phe-CMK) were added to the reaction mixture. To prepare vesicles, L-α-phosphatidyl-L-serine (PS) and L-α-phosphatidylcholine (PC) were mixed (1:1 wt %) and dried under nitrogen. The dry film was hydrated with buffer (20 mM HEPES, 150 mM NaCl) and sonicated for 15 min. Using dynamic light scattering (DynaPro 99), the PSPC vesicle diameter was determined to be 158 ± 52 nm. Fluorescence in the 96-well plate was measured using a Fluoroskan Ascent fluorometer (excitation 390 nm; emission 460 nm). All well plate experiments were performed at 37 °C.

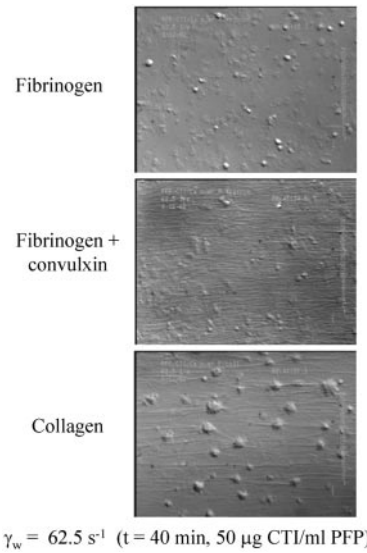

FIG. 1. **Role of platelet activation in fibrin deposition on adherent platelets.** Recalcified citrated PFP was perfused at a shear rate of 62.5 s^{-1} for 40 min over fibrinogen-adherent platelets, convulxin-treated (10 nM, 10 min) fibrinogen-adherent platelets, and collagen-adherent platelets. Massive fibrin accumulation was observed only on the latter two surfaces, demonstrating that direct stimulation of the GPVI receptor on fibrinogen-adherent platelets promotes fibrin formation to the levels supported by collagen-adherent platelets. Flow is from *right* to *left*.

RESULTS

Fibrin Formation on Fibrinogen-adherent Platelets; Role of Platelet Activation—Recalcified citrated PFP containing CTI (contact pathway inhibitor of factor XIIa (24, 26)) was perfused over three different surfaces, fibrinogen-adherent platelets, convulxin-treated fibrinogen-adherent platelets, and collagen-adherent platelets (Fig. 1) at a wall shear rate of 62.5 s^{-1} for t = 35 min. Fibrin deposition on fibrinogen-adherent platelets was essentially undetectable, but substantial fibrin deposition was observed on the latter two surfaces. Although it is known that collagen-adherent platelets are more activated and more procoagulant than fibrinogen-adherent platelets (15, 27, 28), these observations demonstrate that direct convulxin activation of platelets on a fibrinogen-coated surface can stimulate fibrin formation to levels comparable with that found on collagen-adherent platelets. Because convulxin, a C-type lectin isolated from *Crotalus durissus terrificus* venom, is a GPVI-specific platelet activator, this is the first report of activation of platelet GPVI receptor in turning platelets procoagulant under venous flow conditions for platelets maintaining adhesion via GPIIb/IIIa binding to adsorbed fibrinogen.

Enhancement of Platelet Procoagulation; Role of Neutrophil Proteases—When fibrin formation (γ_w = 62.5 s^{-1}) on a cell mixture of fibrinogen-adherent neutrophils and platelets was compared with that over surfaces coated with either neutrophils or platelets alone, extensive fibrin formation was visual-

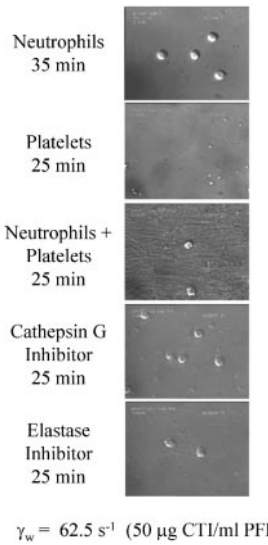

FIG. 2. **Neutrophil-augmented fibrin formation on platelets was attenuated by inhibitors of elastase or cathepsin G.** Although perfusion of recalcified citrated PFP (γ_w = 62.5 s^{-1}) over neutrophils alone or platelets alone caused no fibrin formation, dramatic levels were accumulated on the neutrophil-platelet surface. This heavy fibrin deposition was inhibited by specific peptide inhibitors of cathepsin G (100 μM Z-Gly-Leu-Phe-CMK) or elastase (100 μM MeOSuc-Ala-Ala-Pro-Ala-CMK). Flow is from *right* to *left*.

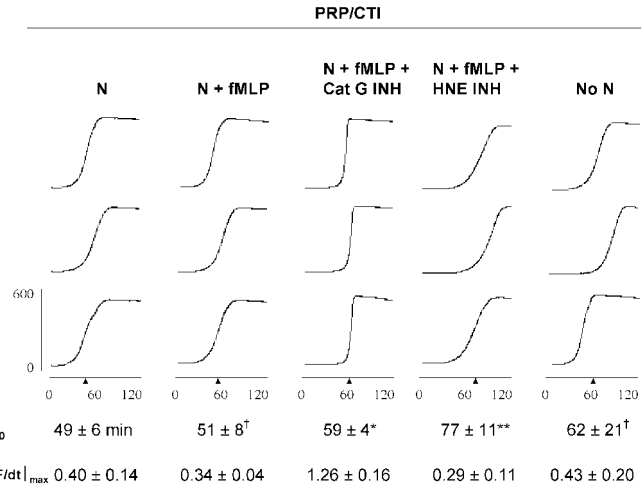

FIG. 3. **Inhibitors (*INH*) of elastase or cathepsin G attenuate neutrophil-enhanced thrombin generation in unstirred PRP.** Progress curves for thrombin production in recalcified citrated PRP supplemented with CTI (50 μg/ml) and factor X (170 nM) were obtained in the absence of neutrophils (*No N*), in the presence of neutrophils (*N*), or in the presence of fMLP-activated neutrophils. Thrombin generation was significantly delayed when a specific peptide inhibitor of cathepsin G or elastase was added to fMLP-stimulated neutrophils. All experiments were conducted in triplicate (†, not significant; *, $p < 0.05$; **, $p < 0.005$, compared with neutrophils alone). *HNE*, human neutrophil elastase; *d*F/*d*t, rate of thrombin generation.

ized on the surface coated with both neutrophils and platelets (Fig. 2), demonstrating the role of neutrophil-platelets interactions in promoting fibrin generation. In addition, this fibrin accumulation was attenuated by specific peptide inhibitors against cathepsin G (Z-Gly-Leu-Phe-CMK; 100 μM) or, to a lesser extent, elastase (MeOSuc-Ala-Ala-Pro-Ala-CMK; 100 μM), indicating the role of these neutrophil proteases in the interaction.

To quantify the effect of neutrophil-platelet interactions on platelet procoagulant activity under static conditions, thrombin (factor IIa) activity in recalcified citrated PRP (supplemented with factor X and CTI) was compared in the absence or presence of neutrophils. Also, the roles of fMLP-stimulation and inhibition of elastase or cathepsin G on the dynamics of thrombin generation were examined (Fig. 3). Consistent with the flow experiments, peak thrombin generation (t_{50}, the time to reach 50% thrombin substrate conversion) by PRP was sped up by neutrophils (13 min faster) and was significantly delayed when a specific peptide inhibitor of cathepsin G (10-min lag) or elastase (28-min lag) was added to fMLP-stimulated neutrophils in PRP. The 13-min difference in t_{50} from the columns labeled *N* and *No N* (Fig. 3) was not statistically different (at $n = 3$). However, this is due to the large standard deviation in the mean t_{50} in the absence of neutrophils, primarily induced by the t_{50} value from the last trace. To clarify the situation, we repeated the experiment and observed that introduction of more data points (not shown) along with donor variation alters the difference between mean t_{50} in columns labeled *N* and *No N* to 11 min, which is statistically significant ($p < 0.1$, $n = 6$). Thrombin generation in PRP with neutrophils present was not further influenced by fMLP. Interestingly, inhibition of cathepsin G, although delaying the time to reach maximal thrombin activity, caused a significant 3-fold increase in the maximum thrombin production rate, presumably due to protection of GPIb from degradation (9, 10).

In separate experiments, the addition of either cathepsin G inhibitor or elastase inhibitor to neutrophils, which were not treated with fMLP, in PRP delayed thrombin production by 14 min ($p < 0.05$) and 18 min ($p < 0.025$), respectively. The mean t_{50} in the presence of neutrophils alone, neutrophils with cathepsin G inhibitor, and neutrophils with elastase inhibitor (all in PRP, no fMLP) were 57 ± 9 ($n = 6$), 71 ± 9 ($n = 3$), and 75 ± 8 ($n = 3$), respectively. Preincubation of neutrophils with adherent platelets for 45 min in the absence of plasma (which contains α_1-antitrypsin) before the start of the flow experiment (Fig. 2) may explain the different "clotting times" between Figs. 2 and 3. Also, the close adhesion (and surface spreading) of neutrophils and platelets in Fig. 2, which favors delivery of Cat G from neutrophils to platelets (and adhesion-potentiated signaling), is not achieved in static assay where mass transfer- and gravity-induced heterotypic aggregation in PRP is insufficient.

Effect of Cathepsin G and Elastase on Fibrin Deposition under Flow Conditions—To test which of the neutrophil proteases (elastase or cathepsin G) played the most critical role in turning platelets procoagulant, fibrin formation on fibrinogen-adherent platelets (γ_w = 62.5 s^{-1}) was compared with that over platelets treated with either 10 μg/ml elastase or cathepsin G. We also tested if both proteases can act in a combined manner to amplify coagulation. Fibrin fibers started appearing on untreated fibrinogen-adherent platelets (control) only after 50 min (Fig. 4*A*). At 35 min of perfusion of CTI-treated PFP, untreated fibrinogen-adherent platelets showed no fibrin formation, and elastase-treated platelets showed a trace of fibrin deposition, whereas the platelets treated with either cathepsin G alone or both proteases demonstrated massive fibrin formation (Fig. 4*B*). These observations indicate that cathepsin G is more potent than elastase in turning platelets into coagulating structures. Moreover, a coupled activity by both proteases is not a prerequisite to achieve full activation of fibrinogen-adherent platelets.

To examine if cathepsin G turns platelets procoagulant through cell activation or through zymogen cleavage of plasma factors on the platelet surface, platelets were treated with different concentrations of cathepsin G (10, 1, or 0.1 μg/ml) in separate flow chambers for a short duration of 5 min. Cathepsin G treatment of platelets was then followed immediately by 5 min of buffer perfusion with the peptide inhibitor of cathepsin

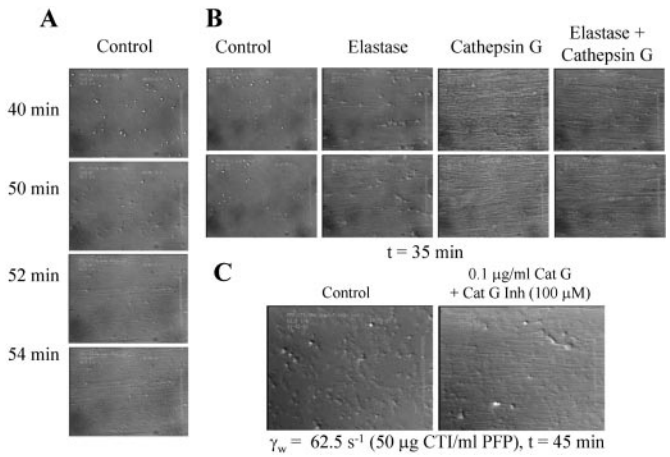

FIG. 4. **Elastase or cathepsin G promotes fibrin deposition on fibrinogen-adherent platelets.** When recalcified citrated PFP was perfused ($\gamma_w = 62.5\ s^{-1}$) over fibrinogen-adherent platelets, fibrin fibers started appearing on platelets only after 50 min (*A*). In contrast, when platelets were pretreated for 40 min with neutrophil elastase (10 μg/ml) or neutrophil cathepsin G (10 μg/ml), fibrin formation on platelets could be observed even by 35 min; this fibrin deposition was considerably greater on cathepsin G-stimulated platelets (*B*). Fibrin formation on platelets treated with both proteases was not substantially different from fibrin on platelets treated with cathepsin G alone. When PFP containing a peptide inhibitor of cathepsin G (100 μM) was perfused over platelets that had been treated with a low concentration of cathepsin G (0.1 μg/ml) and rinsed with the cathepsin G inhibitor (*Inh*, 100 μM), fibrin formation on the cells could still be observed (*C*). Flow was from *right* to *left*.

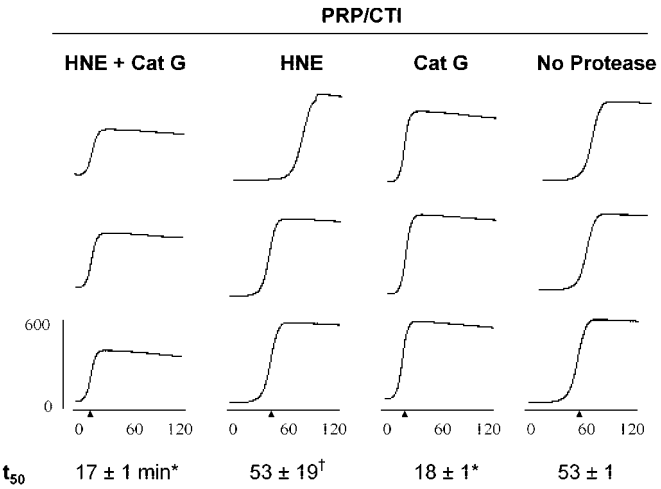

FIG. 5. **Neutrophil cathepsin G enhances thrombin generation in PRP.** Progress curves of fluorescence intensity for fluorogenic thrombin substrate (boc-VPR-MCA) conversion in recalcified citrated PRP supplemented with CTI (50 μg/ml) and factor X (170 nM) were obtained in the presence of elastase, presence of cathepsin G, presence of both proteases, or in the absence of both elastase and cathepsin G. Although the addition of cathepsin G to PRP dramatically reduced the t_{50} of thrombin generation, the addition of elastase had no effect. All experiments were conducted in triplicate (†, not significant; *, $p < 0.05$; **, $p < 0.005$, compared with no protease). *HNE*, human neutrophil elastase.

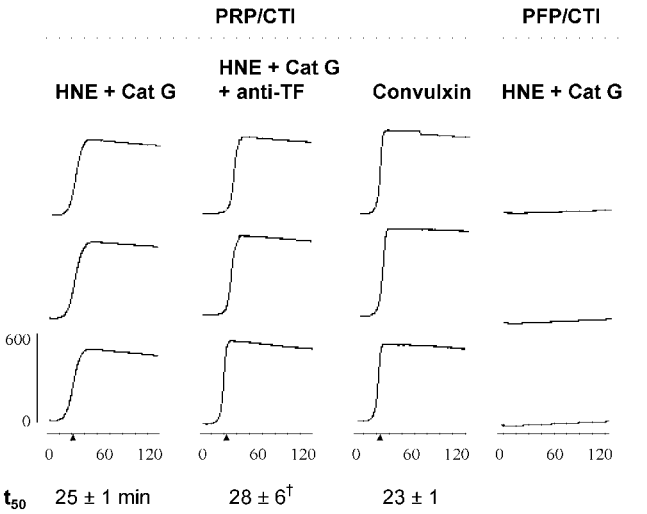

FIG. 6. **GPVI activation enhances thrombin generation on platelets to levels supported by protease-mediated activation.** Measurements of thrombin generation in recalcified citrated PFP supplemented with CTI and factor X were made in the presence of both cathepsin G and elastase, in the presence of both proteases and a tissue factor antibody (*anti-TF*; 50 μg/ml), and in the presence of convulxin alone. Although the tissue factor antibody did not appear to play a role, a similarity in kinetic behavior of thrombin production on convulxin-stimulated platelets and protease-activated platelets was observed. Negligible thrombin production was observed when neutrophil proteases were added in PFP (no platelets). All experiments were conducted in triplicate. *HNE*, human neutrophil elastase.

G (100 μM) and subsequent perfusion of recalcified PFP/CTI supplemented with the peptide inhibitor (100 μM). Although no fibrin formation could be observed on fibrinogen-adherent platelets without cathepsin G treatment (control) at 45 min, the cathepsin G-treated platelets supported abundant fibrin deposition. Fig. 4*C* shows fibrin formation on platelets treated with the lowest cathepsin G concentration (0.1 μg/ml). Identical results were seen for 1.0 and 10 μg/ml Cat G pretreatment of platelets (not shown). Formation of fibrin on platelets treated with a low concentration of cathepsin G and then rinsed with a cathepsin G inhibitor indicated that platelet activation is the dominant mechanism through which cathepsin G turns fibrinogen-adherent platelets procoagulant.

Effect of Cathepsin G and Elastase on Thrombin Production—To verify that cathepsin G is a more potent activator of platelet procoagulant activity, thrombin generation in recalcified citrated PRP/CTI was compared with that in the presence of cathepsin G, elastase, or both proteases. Although the addition of cathepsin G to PRP reduced the t_{50} of thrombin generation from 53 to 18 min, the addition of elastase had no effect (Fig. 5). Treatment of PRP with both neutrophil proteases resulted in a t_{50} of 17 min, indicating that this promptness in thrombin production was mainly due to cathepsin G activity on platelets with no cooperativity with human neutrophil elastase.

In a separate set of experiments, the possible role of tissue factor in protease-mediated activation of coagulation (6, 7) was assessed by comparing thrombin generation in PRP treated with cathepsin G and elastase in the presence or absence of a tissue factor antibody. The presence of this antibody did not significantly alter thrombin production (Fig. 6). It is interesting to note that t_{50} for thrombin production due to elastase and cathepsin G activity on platelets was comparable with t_{50} for thrombin produced as a result of platelet stimulation by the potent GPVI agonist, convulxin (no proteases). As a control, progress curves were obtained to analyze thrombin generation in PFP (no platelets) in the presence of both cathepsin G and elastase. An extremely low rate of thrombin generation (Fig. 6) implied that thrombin production seen in all earlier experiments was solely platelet-dependent and that even high concentrations of neutrophil proteases do not result in significant thrombin production in plasma lacking platelets.

We investigated the role of anionic phospholipid surface as a cofactor for neutrophil-enhanced thrombin generation. The production of thrombin in PFP (no platelets) was measured in the presence or absence of PSPC vesicles (Fig. 7). PSPC addition to PFP produced little thrombin, indicating that mere

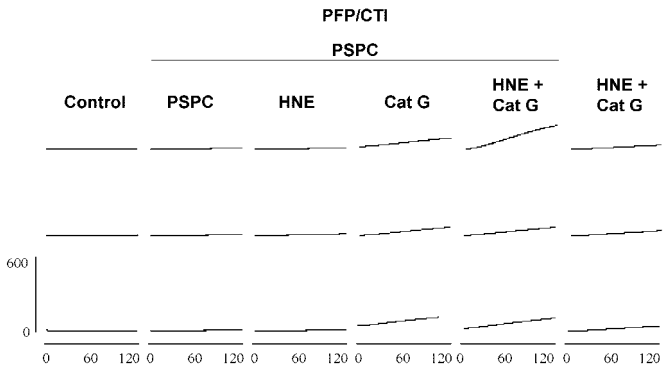

FIG. 7. **Thrombin production in plasma by cathepsin G and elastase; role of phospholipid.** Thrombin production in recalcified citrated PFP supplemented with CTI (50 μg/ml) and factor X (170 nM) was measured in the presence of anionic phospholipid vesicles (*PSPC*). The presence of PSPC did not affect thrombin generation. Although addition of neutrophil elastase to PFP containing PSPC did not enhance thrombin generation, a slight promotion was observed when either cathepsin G or both elastase and cathepsin G were added. Negligible thrombin production was observed when both neutrophil proteases were present in PFP with no PSPC. *HNE*, human neutrophil elastase.

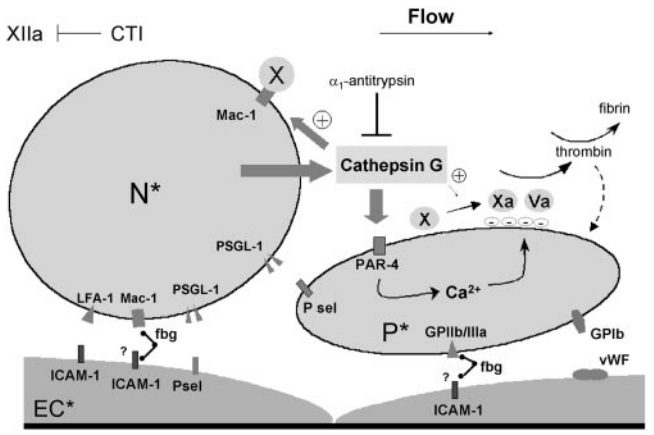

FIG. 8. **Enhancement of platelet procoagulant activity by neutrophil cathepsin G in the absence of tissue factor, collagen, and contact activation.** Shown is a schematic of pathways through which cathepsin G may promote platelet-dependent coagulation, including cleavage of Mac-1-bound factor X on neutrophils (2, 15), activation of platelets via protease-activated receptor 4, and to a lesser extent, cleavage of plasma zymogens, such as factors X and V. *fbg*, fibrinogen; *Psel*, P-selectin; *N**, activated neutrophil; *P**, activated platelet; *EC**, activated endothelial cell; *IIb/IIIa*, glycoprotein IIb/IIIa; *LFA-1*, leukocyte function-associated antigen-1; *PSGL-1*, P-selectin glycoprotein ligand-1; *ICAM-1*, intercellular adhesion molecule-1; *XIIa*, factor XIIa; *vWF*, von Willebrand factor.

phosphatidylserine exposure during platelet activation is not sufficient for coagulation of the plasma. When neutrophil elastase was added to PFP containing PSPC, thrombin generation was also minimal. However, a detectable level of slow thrombin production was observed when cathepsin G or elastase/cathepsin G was added to PFP/CTI containing PSPC. This is the first report of PSPC enhancing cathepsin G-mediated thrombin production in PFP treated with CTI, potentially due to protection of lipid bound cathepsin G from inhibition. These results indicated that platelet phospholipid exposure alone is not sufficient for the cathepsin G-enhanced coagulation seen in Figs. 2, 4, 5, and 6. As a control when a progress curve for thrombin generation in PFP containing both elastase and cathepsin G was obtained, negligible thrombin production (also seen earlier in Fig. 6) was detected.

DISCUSSION

We demonstrated that adherent neutrophils enhanced thrombin generation and fibrin formation on fibrinogen-adherent platelets through released proteases cathepsin G and elastase either in flow assays or well-plate assays. Among these two proteases, cathepsin G was far more potent than elastase in activating platelet-dependent coagulation of CTI-treated PRP. Cathepsin G does so by elevating the activation state of platelets rather than by cleaving coagulation factors in plasma or on the platelet anionic surface (Fig. 8). The increased thrombin production of neutrophil protease-treated PRP is not altered by anti-tissue factor, suggesting that Cat G activity does not necessarily result in detectable de-encryption of tissue factor. We show that direct Cat G activation of fibrinogen-adherent platelets can also turn platelet procoagulant to levels comparable with that of convulxin-stimulated platelets or collagen-adherent platelets.

The addition of PSPC alone to CTI-treated platelet free plasma demonstrated that the presence of a negatively charged phospholipid surface is not sufficient to initiate coagulation. Cathepsin G is cationic (29), and the slight increase in thrombin levels on the addition of PSPC supports the view that cathepsin G-mediated cleavage of coagulation factors on a negatively charged surface may help support, albeit in a secondary role, cathepsin G-mediated platelet activation. In comparison to cathepsin G-enhanced thrombin generation in PFP containing PSPC, a five times higher thrombin production in PRP (presence of platelets) indicates that platelets have additional means, beyond merely presenting negative phospholipid, through which they respond to cathepsin G stimulation. These results are in agreement with studies by Sumner *et al.* (30), which show that PS exposure does not correlate with factor Xa or thrombin production on platelets and suggest that surface participants other than PS are involved in coagulation (31). Fibrin accumulation under the conditions of Fig. 4*C* supports the view that cathepsin G is predominantly activating platelets rather than cleaving zymogens on the platelet surface.

Neutrophil-enhanced platelet-dependent coagulation (under factor XIIa inhibition by CTI) could not be attenuated by an antibody against tissue factor. It remains possible that after an initial production of minuscule amounts of thrombin through a platelet-supported factor VIIa pathway, factor XI was being activated to XIa on activated platelets by thrombin to trigger the intrinsic pathway in the absence of the upstream contact pathway protein factor XIIa (32–34). It also remains a possibility that surface-bound Cat G activates platelet-released factor V.

Although the addition of a cathepsin G inhibitor to neutrophils in PRP attenuated thrombin generation in plasma, it unexpectedly increased the maximum rate of thrombin generation by 3.7 times (as seen in the difference in slopes on the progress curves in Fig. 3). This may be attributed to the degradation of platelet GPIb by cathepsin G. The surface expression of GPIb, which has been found to be the counter-receptor for neutrophil Mac-1 (35), is expected to increase after the interaction of platelets with neutrophils. However, it is known that neutrophil cathepsin G can degrade GPIb (9, 10). In the light of these studies, whereas the addition of cathepsin G inhibitor to PRP containing neutrophils may not have prevented neutrophil-assisted platelet GPIb expression, it may have blocked the degradation of GPIb by neutrophil cathepsin G. Additionally, GPIb, when left intact, would allow factor XI binding to it where activation of factor XI by a minimal amount of thrombin can accelerate thrombin production via the intrinsic coagulation pathway (33). As expected, this increased rate of thrombin generation is not observed in the absence of neutrophil-platelet interactions due to a possible lack of increased GPIb expression. However, an overall delay in thrombin pro-

duction in the presence of cathepsin G inhibitor was most likely the result of a decline in platelet activation levels due to cathepsin G inhibition.

Under flow conditions, mass transfer by dispersion and convection can alter the concentration of local factors (36). The burst in thrombin production is vital to make fibrin under flow conditions, and this fibrin is critical for clot stabilization. The close contact of activated platelets and activated neutrophils mediated by P-selectin glycoprotein ligand-1 in the absence of tissue factor or collagen may allow for the function of Cat G. Transfer of Cat G to platelet anionic phospholipid may also facilitate its activating function in the presence of plasma inhibitors such as α_1-antitrypsin (37, 38).

In summary, we show that neutrophil proteases, most notably cathepsin G, can promote thrombin generation and subsequent fibrin formation on platelets under static or venous flow conditions. Cat G does so by elevating the activation state of platelets. This platelet activation and resultant procoagulant activity involves additional factors beyond anionic phospholipid exposure by activated platelets. These events may be clinically relevant in situations void of collagen or tissue factor such as those involving biomaterial thrombosis or endothelial dysfunction and neutrophil activation during deep vein thrombosis.

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