Enhancement of Cathepsin G-Induced Platelet Activation by Leukocyte Elastase: Consequence for the Neutrophil-Mediated Platelet Activation

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We have focused our interest on the platelet-activating properties of two polymorphonuclear neutrophil (PMN)-derived proteinases, namely elastase (HLE) and cathepsin G (Cat.G). First of all, we observed that whereas HLE was unable to trigger platelet activation by itself, it enhanced platelet activation induced by Cat.G when both proteinases were added simultaneously. It has been recently described that, upon stimulation, PMN released Cat.G, which in turn activated surrounding platelets. Thus, we looked for a combined effect of Cat.G and HLE during this cell-to-cell interaction. When PMN (5 × 10⁶/mL) were stimulated by 0.5 μmol/L N-formyl-Met-Leu-Phe, they released 237.9 ± 49.1 nmol/L Cat.G and 381.7 ± 28.0 nmol/L HLE. Such a concentration of purified Cat.G (240 nmol/L) induced only a moderate platelet activation when added to a PMN-platelet mixture. However, when Cat.G (240 nmol/L) and HLE (380 nmol/L) were added together, the resulting platelet activation was strictly comparable to that corresponding to the addition of N-formyl-Met-Leu-Phe (P > .05) in terms of aggregation, dense and α granule secretion, and thromboxane B₂ production. In fact, Elafin, a specific HLE inhibitor, when added to the PMN-platelet cooperation system triggered by N-formyl-Met-Leu-Phe, prevented platelet activation within the same range of concentrations as for inhibition of HLE activity. In conclusion, we now show that not only Cat.G, but also HLE is involved in the PMN-mediated platelet activation.

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MATERIALS AND METHODS

Materials and reagents. Blood was obtained from the Centre National de Transfusion Sanguine (Paris, France). Bovine serum albumin (BSA) was from Euromedex (Strasbourg, France). Aprotinin, HEPES, prostacyclin, dextran, N-succinyl-alal-alapro-phe-p-nitroanilide, N-succinyl-alal-alal-p-nitroanilide, cytochalasin B, Met-Leu-Phe (FMLP), TxB₂, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Corp (St Louis, MO). Hanks’ Balanced Salt Solution (HBSS) was from Gibco (Paisley, UK). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). ACS II and [³⁵S]-serotonin were from Amersham International (Amersham, UK). Polystyreneglycol 6000 was from Merck (Darmstadt, Germany) and heparin was from Choay (Paris, France). Fibrinogen (grade L) was purchased from Kabo (Stockholm, Sweden) and was treated with diisopropyl fluorophosphate to inactivate coagulant contaminants. The enzyme immunoassay kit for WF determination (Asserachrom WF) was from Diagnostica Stago (Paris, France). The antibody and radiolabeled ligand for radioimmunoassays of TxB₂ were from URIA, Institut Pasteur, INSERM U207 (Paris, France). Recombinant Elafin (European Patent No. 402 068) was provided by Dr. J.E. Fitton from ICI Pharmaceuticals (Macclesfield, UK). Elafin is a potent specific inhibitor for HLE (Kᵢ = 6 × 10⁻¹⁰ mol/L) forming 1:1 molecular complexes with the enzyme. Preparation of human washed platelets. Platelets were purified from blood of human volunteers collected in SAG-M solution (composition: 187.6 mmol/L NaCl, 71 mmol/L dextrose, 1,950 mmol/L adenine, 10.7 mmol/L mannitol, pH 5.1). After a first centrifugation (180g for 20 minutes), the platelet-rich plasma was centrifuged for 20 minutes, then loaded platelets supplemented with 10⁻⁸ mol/L prostacyclin were centrifuged at 1,600g for 10 minutes and gently resuspended in Tyrode’s buffer (137.0 mmol/L NaCl, 2.68 mmol/L KCl, 11.9 mmol/L NaHCO₃, 0.42 mmol/L NaH₂PO₄, 0.20 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, 5.5 mmol/L glucose, 5.0 mmol/L HEPES, and 0.35% BSA; pH 7.4) supplemented with 10⁻⁸ mol/L prostacyclin.

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and 50 IU/mL heparin. The suspension was centrifuged (1,600g for 10 minutes) and cells were washed a second time with the same mixture without heparin. The last pellet was resuspended in a volume of Tyrode’s buffer such that the final platelet concentration was 4 x 10^5 cells/mL.

Preparation of human purified neutrophils. Blood was collected as for platelet preparation, mixed with dextran at a 1% final concentration, and erythrocytes were allowed to sediment for 30 minutes. The supernatant was then layered over Ficoll-Paque (1 vol for 2 vol of cell suspension) and then centrifuged (350g for 45 minutes). The pellet was resuspended in a lysis buffer (composition: 155 mmol/L NH4Cl, 2.96 mmol/L KHCO3, 3.72 mmol/L EDTA[Na2],) and very gently inverted for 5 minutes to lyse resting erythrocytes. Cell suspension was then centrifuged (350g for 10 minutes) and washed twice with HBSS without CaCl2 and MgCl2. Finally, the PMN pel-let obtained was resuspended in a final volume of HBSS such that the cell concentration was 10^6 PMN/mL. The viability of recovered PMN was 98.7% ± 0.5%, as measured by the Trypan blue dye exclusion method, and their purity, evaluated using Türk’s stain, was 96.2% ± 2.1%.

Purification of Cat.G and HLE. Cat.G and HLE were purified as previously described from human PMN, according to the method of Baugh and Travis modified by Martodam et al.

Sodium dodecyl sulfate (SDS) gel electrophoresis indicated that enzyme preparations were free of contaminants and had an apparent molecular weight of 28,000. Enzymatic activities of Cat.G and HLE were determined by following the hydrolysis of their specific synthetic substrates in the presence of increasing amounts of tritiated α1-antitrypsin. The linear regression curve obtained allowed us to extrapolate the active site concentration of both proteinases as previously described. Concentrations of proteinases indicated in the present report were deduced from these active site titration curves (specific activities, 100%). By spectrophotometric studies, it was also verified that no HLE was present in the Cat.G batch, and that there was no Cat.G in the HLE batch. In addition, by enzyme immunoassays it was shown that HLE and Cat.G were devoid of proteinase 3 contamination.

Preparation of PMSF-treated HLE. HLE was treated with PMSF to block its catalytic site. Thus, the proteinase (2 mg/mL), purified as described above, was incubated for 60 minutes at ambient temperature in presence of 1.25 mmol/L PMSF and the mixture was then dialysed using a microconcentrator to remove the inhibitor. HLE so treated had effectively lost its proteolytic activity, as assessed by measuring the hydrolysis of its synthetic specific substrate. Protein concentration was determined by the method of Lowry et al.

Aggregation and serotonin release determination. Aggregation and serotonin release were performed using a Dual Aggro-meter (Chrono-Log Corp, Haverford, PA). Two hundred fifty microliters of the platelet suspension and 250 µL of HBSS were placed in a siliconized glass cuvette in the presence of fibrinogen (0.7 mg/mL). Before each experiment, Cat.G and MgCl2 concentrations were adjusted to 1.3 and 1.0 µmol/L, respectively. After 1 minute at 37°C under stirring, cells were challenged by HLE, Cat.G, or both proteinases added simultaneously. Resulting aggregations were monitored by changes in light transmission. The reactions were stopped 3 minutes later by centrifugation (14,000g for 2 minutes at 4°C), and the release of [3H]-serotonin by platelets, measured on 400 µL of supernatant, was determined by scintillation counting (Counter 1212 Rackbeta; LKB, Wallac, Stockholm, Sweden) for the PMN platelet cooperation system study. HBSS was replaced by PMN and cells were stirred for 5 minutes at 37°C in the presence of fibrinogen (0.7 mg/mL) and cytochalasin B (5 µg/mL) before stimulation by FMLP (0.5 µmol/L) or proteinases. Aggregations were expressed in percent of the maximal light transmission, and serotonin releases in percent of the total [3H]-serotonin content of the platelets.

Enzymatic activity determinations. Enzymatic activities of HLE and Cat.G were performed by measuring hydrolysis of their specific synthetic substrates, ie, N-succinyl-ala-ala-ala-p-nitroani-lide and N-succinyl-ala-ala-pro-phe-p-nitroanilide, respectively. Supernatants of cells stimulated as for the aggregation procedure (150 µL) or a same volume of a fixed concentration of purified enzymes in HBSS/Tyrode (1:1) were mixed in the test cuvette with 345 µL of 0.1 mol/L Tris/HCl buffer, pH 8, and placed at 37°C. One minute later, 5 µL of substrate in N-methyl pyrrolidone (1 mmol/L final concentration) was added. Hydrolysis of the substrate was monitored spectrophotometrically by observing the release of p-nitroaniline at 410 nm.

A different procedure was used for testing the enzymatic inhibitory effect of Elafin. Thus, substrate of HLE or Cat.G (1 mmol/L final concentration) and increasing amounts of Elafin were placed at 37°C in a test cuvette containing HBSS/Tyrode’s buffer (1:1). One minute later, 380 nmol/L HLE or 240 nmol/L Cat.G were added and the hydrolysis of the substrate observed spectrophotometrically. Results were expressed in percent of enzymatic activity in comparison with controls performed without inhibitor.

vWF and TxB2 release determinations. The amount of released vWF was determined from supernatants of PMN-platelet suspensions stimulated by 0.5 µmol/L FMLP, 240 nmol/L Cat.G, 380 nmol/L HLE, or the combination of both proteinases. After centrifugation of stimulated samples (14,000g for 2 minutes), supernatants were collected. Corresponding pellets were resuspended in 0.1% Triton X-100 overnight to lyse cells and to extract the remaining vWF. An enzyme immunoassay was used to quantify vWF according to the test protocol recommended by the manufacturer. Results were expressed as the percentage of release as compared with the total platelet vWF content.

Concentrations of TxB2 produced from platelets and recovered in cell-free supernatants were measured by radioimmunoassay as previously described.

Statistics. Results were expressed as mean ± SD of at least three distinct experiments. Statistical analysis was performed by Student’s t-test. Results were significant in the case where P < .05 (*).

RESULTS

Enhancement by HLE of platelet activation induced by Cat.G. Results presented in Fig 1 show that, when platelets were challenged simultaneously by the combination of a threshold concentration of Cat.G and increasing concentrations of HLE, both inactive by themselves, it resulted in a strong platelet activation. The concentration of Cat.G used in these experiments depended on platelet reactivity and was between 83 and 108 nmol/L. The chosen concentration induced only a shape change, but neither aggregation nor serotonin release (1.9% ± 3.6% and 1.4% ± 2.3%, respectively; n = 8; P > .05). Used in combination with HLE, Cat.G provoked a strong platelet activation that was significant when HLE concentrations ranged from 100 nmol/L for aggregation and from 200 nmol/L for serotonin release up to 1 µmol/L (P < .05). This potentiating effect of HLE was prevented by blocking its catalytic site. Indeed, PMSF-treated HLE up to 1 µmol/L failed to enhance the platelet-activating effect of Cat.G (Fig 1).

Evaluation of concentrations of Cat.G and HLE released from FMLP-activated PMN. By spectrophotometric studies we estimated the concentrations of Cat.G and HLE re-
HLE, CAT.G, AND PMN-MEDIATED PLATELET ACTIVATION

A

![Graph A](image)

B

![Graph B](image)

**Fig 1.** Potentiation by HLE of platelet activation induced by Cat.G. Platelets (2 \times 10^6 cells/mL) were incubated at 37°C in Tyrode/HBSS buffer (1:1) and challenged 1 minute later by the simultaneous addition of 83 to 108 nmol/L of Cat.G and increasing concentrations of HLE pretreated (PMSF-HLE) or not pretreated with PMSF. Parameters of platelet activation measured were aggregation (A) and serotonin release (B) expressed as percentages of light transmission (L.T.) and total cell content, respectively. Each value is the mean ± SD of five to eight experiments.

Effects of HLE, Cat.G, and the combination of both proteases on the PMN-platelet mixed suspension. The above calculated concentrations of proteases were considered as the total amount released from FMLP-activated PMN and susceptible to encounter nearby platelets. As shown in Fig 2, 240 nmol/L of purified Cat.G induced only a moderate platelet aggregation that depended on platelet batch sensitivity and never exceeded 25%. This effect was accompanied by weak releases of serotonin from dense granules (10.4% ± 6.4%; n = 5) and of vWF from α granules (11.2% ± 4.9%; n = 4), whereas TxB2 production was too small to be detected (Fig 3). When the estimated concentration of HLE released from FMLP-activated PMN was added to the cell suspension, ie, 380 nmol/L, we confirmed that no activation occurred. However, stimulation of the mixed cell suspension with both proteases added together induced a platelet activation strictly comparable with that obtained with 0.5 μmol/L FMLP (P > .05; n = 4). Thus, aggregation was of 40.2% ± 4.7% versus 42.9% ± 4.0%, serotonin release of 48.4% ± 13.6% versus 63.2% ± 13.3%, vWF release of 44.4% ± 11.0% versus 41.2% ± 11.2%, and TxB2 production of 12.4 ± 13.9 ng/mL versus 13.9 ± 3.1 ng/mL for the combination HLE-Cat.G and FMLP, respectively (Fig 3).

Effects of Elafin, a specific HLE inhibitor, on PMN-induced platelet activation. The specificity of Elafin was tested on enzymatic activities of HLE and Cat.G under experimental conditions corresponding to cell stimulation. The enzymatic activity of 380 nmol/L HLE was significantly inhibited by 100 nmol/L Elafin (P < .05) and was totally blocked by 500 nmol/L, whereas such a concentration was ineffective against 240 nmol/L Cat.G (data not shown). When PMN and platelets were preincubated for 1 minute with Elafin before their stimulation by 0.5 μmol/L FMLP, it resulted in an inhibition of platelet activation in a concentration-dependent manner between 0.25 and 4 μmol/L (Fig 4). Within the same range of concentrations, the enzymatic activity of released HLE was also inhibited in a concentration-dependent manner (Fig 4). By contrast, Elafin, up to 4 μmol/L, failed to affect the enzymatic activity of released Cat.G. The fact that Cat.G activity recovered from the supernatant was not affected by preincubation of the mixed cell population with Elafin proved that PMN activation by FMLP was not modified by the presence of the

1.1149 x (r = .92). Thus, the concentration of Cat.G effectively released, calculated by taking in account this equation, was of 237.9 ± 49.1 nmol/L (n = 11).

Following the same experimental procedure, the concentration of HLE released in the PMN-platelet cooperation system was calculated. Projection of the values of the optical density variations obtained with stimulated supernatants on the titration curve indicated that 181.7 ± 16.0 nmol/L HLE were present at 3 minutes (y = −2.857 + 0.0526 x; r = .98). However, contrary to what was observed for Cat.G, there was a significant shift between the added concentrations of purified HLE to the cell suspension and the concentrations recovered after 3 minutes of stirring and centrifugation (y = −37.233 + 0.5736 x; r = .91). Thus, the effective concentration of HLE released was 381.7 ± 28.0 nmol/L (n = 6).

Releasing from FMLP-activated PMN. First of all, the enzymatic activity of increasing concentrations of purified Cat.G was evaluated by observing the hydrolysis of its specific synthetic substrate. From the equation corresponding to this titration curve (y = −0.6109 + 0.0323 x; r = .97), the concentration of Cat.G released from PMN was estimated in the experimental system in which PMN and platelets were mixed together and challenged by 0.5 μmol/L FMLP. The concentration of Cat.G so deduced was 264.6 ± 53.3 nmol/L (n = 11). It was then verified that, when purified Cat.G was added to PMN-platelet suspensions, stirred at 37°C for 3 minutes, and centrifuged (as in the case of the FMLP challenge), the concentrations of the protease recovered in supernatants were correlated with the concentrations added. In fact, it was almost the case because the equation corresponding to measured concentrations as a function of added concentrations was y = −10.7189 +
HLE inhibitor. This was confirmed by measuring β-glucuronidase release from activated PMN. In no instance was there a significant difference between controls and Elafin-treated cells, ie, 40.2% ± 3.2% versus 39.8% ± 2.2% (n = 3; P > .05). A direct effect of Elafin on platelets was also discarded because platelet activation by purified Cat.G or collagen was similar regardless of whether Elafin was added (data not shown). Thus, inhibition of the PMN-mediated platelet activation by Elafin resulted from an inhibition of HLE enzymatic activity.

**DISCUSSION**

It is well known that PMN play a key role in the pathogenesis of inflammation by releasing reactive oxygen metabolites and their granule content. In fact, PMN can create an environment in which proteinases are the most active participants because they are able to exert a more efficient and specific effect than oxidants.18 HLE and Cat.G are the two major neutral proteinases contained in PMN granules and both enzymes have caused interest in recent years due to their possible involvement in diseases causing tissue destruction.19,20 In this report we have focused our interest on platelet activation induced by these two proteinases.

We observed that, although HLE did not activate platelets by itself, in confirmation of previous works,6,9,11 it did enhance the platelet-activating property of Cat.G. This effect of HLE is most probably not due to a specific modification of Cat.G receptors. Indeed, this effect was also observed with other platelet agonists such as collagen and the endoperoxide analog U46619 (data not shown). By contrast, it can be assumed that it is a consequence of the proteolytic activity of HLE because the blockade of its catalytic site by PMSF treatment suppressed the potentiation. As mentioned in the introduction, HLE cleaves different membrane GP. It thus can be hypothesized that the observed potentiating phenomenon is possibly related to such proteolysis, although an inhibitory effect has usually been reported.9,10,12,13 In fact, under the same experimental conditions, we also found an inhibitory activity for a concentration as low as 0.4 μmol/L but for a 6-minute preincubation time of platelets with HLE before challenge by Cat.G (Renesto et al, to be published). Thus, the key factor is apparently the delay time between additions of HLE and Cat.G. Nonetheless, it should be mentioned that a previous work has reported on platelet aggregation upon addition of fibrinogen to HLE-pretreated platelets,11 an effect due to the exposure of fibrinogen receptor sites. Apart from the fact that this was observed after a long preincubation time (60 minutes), there is an important discrepancy with our data. Indeed, fibrinogen addition never triggered a true cell activation, as evaluated by the absence of adenosine triphosphate release.11 In contrast, we observed serotonin and vWF release, as well as TxB, formation, three recognized parameters of an intracellular activation process. To our knowledge, this is the first report on an enhancing effect of platelet activation by HLE. It remains now to determine by which underlying mechanism this phenomenon occurs.

We are now in the process of studying the modifications of platelet membrane GP by HLE as a function of time and concentrations.

Recently, the capacity of FMLP-stimulated PMN to activate surrounding platelets has been described.2,3,5,7 The role of the mediator responsible for this cell-to-cell cooperation has been attributed to Cat.G, for which a specific binding site on platelets has been described.22 This conclusion resulted from experiments showing that proteinase inhibitors such as eglin C and, more specifically, α1-antichymotrypsin suppressed the cooperation.6,7 Because, under these experimental conditions, Cat.G was able to activate platelets,23 whereas HLE was unable to do it,4 a participation of the latter was discarded. However, the above-mentioned results showing that HLE exhibits the capacity to potentiate platelet activation induced by Cat.G have suggested a new scheme of the PMN-mediated platelet activation involving both proteinases.

To show the involvement of HLE in this cooperation system, we first evaluated the total amount of proteinases released from FMLP-activated PMN, ie, 237.9 ± 49.1 nmol/L and 381.7 ± 28 nmol/L for Cat.G and HLE, respectively. Interestingly, the addition of 240 nmol/L of Cat.G to the PMN-platelet mixture was not sufficient to induce a strong platelet aggregation and in most of the cases only a shape change was observed. In confirmation of previous work,5,9,11 380 nmol/L HLE failed to trigger platelet activation. In contrast, the challenge of the mixed cell population with 240 nmol/L Cat.G and 380 nmol/L HLE added to-
gether induced an activation of platelets that was comparable to that obtained with 0.5 μmol/L FMLP. These results led us to postulate that not only Cat.G but also HLE participates in the PMN-mediated platelet activation. A confirmation of this new scheme was obtained using Elafin, a specific inhibitor directed against HLE. Indeed, Elafin suppressed platelet activation induced by 0.5 μmol/L FMLP in the PMN-platelet cooperation system. Because Elafin by itself had no direct inhibitory effect either on FMLP-mediated PMN degranulation or on Cat.G-mediated platelet activation, it was deduced that inhibition was effective through specific binding to HLE. This was confirmed by measuring the enzymatic activities of Cat.G and HLE and serotonin release were determined from corresponding supernatants by spectrophotometric measurement and scintillation counting, respectively. These data from a single experiment are representative of four other experiments.

In summary, our present results show that, when PMN are stimulated by FMLP, they release Cat.G and HLE, which are both responsible for activation of surrounding platelets. These results showed an important new pathway by which both enzymes are susceptible to intervene in the pathogenesis of diseases in which participation of PMN and platelets is likely to occur. Among the different concerned pathologic states, glomerulonephritis and, more particularly, the adult respiratory distress syndrome (ARDS) can be mentioned. Indeed, it has been reported that, in the latter pathology, not only PMNs but also platelets were involved, and consequently a cell-to-cell cooperation between both cell populations may have a preponderant importance. Moreover, patients with ARDS have an increased concentration of GMP-140 and thrombospondin on the surface of their platelets, showing in vivo platelet secretion and proteins from platelet origin have been found in their alveolar lining fluids.

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Enhancement of cathepsin G-induced platelet activation by leukocyte elastase: consequence for the neutrophil-mediated platelet activation

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