Activation of Mac-1 (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation

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Leucocyte initiation of coagulation preserves the haemostatic balance and may aberrantly contribute to vascular injury. In addition to the extrinsic activation mediated by tissue factor: factor VIIa, monocytes express an alternative procoagulant response after binding of the zymogen factor X to the integrin Mac-1 (CD11b/CD18). Here, factor X-activating activity was found in purified monocyte granules, and coincided with size-chromatographed fractions containing cathepsin G. In contrast, elastase-containing granule fractions did not activate factor X. In the presence of Ca²⁺ ions, purified cathepsin G, but not elastase, cleaved factor X to a ~ 54 kDa catalytically active derivative, structurally indistinguishable from the procoagulant C54 kDa product, in a sequential three-step cascade, including (i) binding of factor X to Mac-1, (ii) discharge of azurophil granules, and (iii) limited proteolytic activation of membrane-bound factor X by cathepsin G. By rapidly forming thrombin and factor Xa in a protected membrane microenvironment, this pathway may contribute a ‘priming’ signal for clotting, anticoagulation and vascular cell signal transduction, in vivo.

INTRODUCTION

Among vascular cells, leucocytes, and monocytes in particular, have evolved a formidable machinery to initiate, regulate and amplify coagulation in a receptor-mediated fashion [1]. While this mechanism contributes to the haemostatic balance [2], and participates in inflammatory responses such as hyperacutetransplant rejection [3] and delayed-type hypersensitivity [4], compelling experimental evidence underscores its pathogenetic role in consumption coagulopathy [5,6] and atherosclerotic disease [7]. In this context, leucocyte generation and assembly of coagulation proteases promotes deposition of insoluble fibrin in the vasculature [8] and modulates vascular cell signal transduction, with generation of second messengers [9], transcription of activation early genes [10], and release of inflammatory cytokines [11].

For its prominent implication in vascular diseases [7], considerable effort has been devoted recently to elucidating the molecular requirements of coagulation assembly on leucocytes [1]. This is especially relevant because the main extrinsic activator of coagulation, tissue factor (TF) [12], is undetectable on normal monocytes or unperturbed endothelium in vivo [13], and its transcriptional activation requires prolonged cellular exposure to non-physiological agonists, i.e. lipopolysaccharide [12].

In an attempt to delineate potential alternative pathways of leucocyte initiation of coagulation, previous studies have demonstrated that monocytes and related cell lines bound the coagulation zymogen factor X through a high-affinity (Kd ~ 20–40 nM) recognition of the integrin Mac-1 (CD11b/CD18) [14]. In the absence of detectable TF, monocyte-bound factor X was proteolytically converted into a ~ 54 kDa product, in a reaction associated with activated factor X (factor Xa) procoagulant activity and unaffected by neutralizing anti-TF monoclonal antibodies (mAbs) [15]. The importance of the Mac-1 pathway of factor X activation has been highlighted recently in vivo, with respect to host defence mechanisms during bacterial infections [16].

In this study, we sought to re-investigate the molecular mechanism of Mac-1-dependent activation of factor X, and the alternative initiation of coagulation on monocytes. We found that inflammatory stimuli or ligand binding to Mac-1 releases leucocyte granule proteases, and that cathepsin G, but not elastase, cleaves and activates factor X at a site structurally distinct from that recognized by TF:factor VIIa.

MATERIALS AND METHODS

Cells and cell culture
The monocytic cell line THP-1 (American Type Culture Collection, A.T.C.C.; Rockville, MD, U.S.A.) was grown in complete RPMI 1640 tissue culture medium (BioWhittaker, Walkersville, MD, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY, U.S.A.), 2 mM L-glutamine (Life Technologies), and 10⁻³ M 2-mercaptoethanol (Eastman Kodak, Rochester, NY, U.S.A.). Phenotypical expression of the leucocyte integrin Mac-1

Abbreviations used: EGR-CMK, Glu-Gly-Arg-chloromethyl ketone; EPR-1, effector cell protease receptor-1; IMLP, formyl-methionyl-leucylphenylalanine; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; RVV, Russell viper venom; SBTI, soybean trypsin inhibitor; TBS, Tris-buffered saline; TF, tissue factor.

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(CD11b/CD18) [17] on THP-1 cells, and its role as a factor X receptor on these cells have been characterized previously [14,15]. Peripheral blood mononuclear cells (PBMCs) were isolated from acid citrate-dextrose anticoagulated blood drawn from normal informed healthy volunteers by differential centrifugation on a Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, U.S.A.) gradient (density 1.077 g/ml) at 400 g for 18 min at 22 °C. Cells were washed three times in PBS, pH 7.4, supplemented with 5 mM EDTA and suspended in complete RPMI 1640 medium.

Protein purification and labelling

The experimental procedures for the isolation and characterization of human plasma factor X have been reported [14]. Factor X was labelled with Na$I$ to a specific radioactivity of 0.5–1 µCi/µg of protein by the IODO-GEN (Pierce Chemical Co., Rockford, IL, U.S.A.) method [18], with separation of free from protein-bound radioactivity by chromatography on a PD-10 Sephadex G-25 column (Pharmacia, Piscataway, NJ, U.S.A.) pre-equilibrated with PBS, pH 7.4, and elution of 0.5 ml fractions.

Subcellular fractionation

Leucocyte granule or membrane preparations were purified as basically described by Baugh and Travis [19]. Briefly, aliquots of THP-1 cells (2.5 × 10⁶) were washed three times in PBS, pH 7.4, at 250 g for 15 min at 4 °C and once in 0.34 M sucrose (Sigma). Cells were suspended in 0.34 M sucrose/1 mg/ml heparin (Sigma), and stirred for 16 h at 4 °C. At the end of the incubation, samples were centrifuged at 30000 g for 30 min at 4 °C to pellet the granule fraction. Granules were then lysed in PBS, pH 7.4, 1 M NaCl, by five sequential cycles of freezing and thawing, followed by two rounds of centrifugation at 30000 g for 30 min at 4 °C, and collection of the supernatant. Purified granule or membrane extracts, or unfraccionated THP-1 cell extracts, were diluted 1:50 or 1:100, respectively, and normalized for protein content by absorbance at 280 nm wavelength (A₄₅₀nm > 0.4). Subcellular fractions were individually analysed for their ability to support activation of factor X as follows. Aliquots (10 µl) of the various normalized subcellular fractions, or unfraccionated THP-1 cell extracts, were mixed with 5 µg of factor X in the presence of 2.5 mM CaCl₂ in a total volume of 100 µl of Tris-buffered saline (TBS), pH 7.4, for 5 min at 22 °C. At the end of the incubation, samples were assayed for factor Xa activity by hydrolysis of the factor Xa-sensitive chromogenic substrate S-2222 (Chromogenix, Molndal, Sweden). Absorbance was quantified with a Microplate Reader (Dynatech, Chantilly, VA, U.S.A.) at a wavelength of 405 nm, as described [19]. Cathepsin G content was quantified by an ELISA-based protocol. Briefly, 96-well plastic plates (Lincoln Scientific, Santee, CA, U.S.A.) were coated with 1 µg/ml purified cathepsin G (Calbiochem) in 10 mM NaPO₄, 0.6 M NaCl, pH 7.4, for 16 h at 4 °C. At the end of the incubation, wells were washed and post-coated with 200 µl of 1 % BSA (Sigma). 10 mM NaPO₄, 0.1 % Tween-20 for 6–8 h at 4 °C. Aliquots of Sephadex G-100 subcellular fractions were incubated with sheep anti-(cathepsin G) antibody (ICN, Costa Mesa, CA, U.S.A.) at 3.5 µg/ml in a final reaction mixture containing 0.6 M NaCl, 1 % BSA for 16 h at 4 °C. At the end of the incubation, 100 µl aliquots of anti-(cathepsin G)-treated samples were added to post-coated wells for 16 h at 4 °C, washed with BSA/Tween/phosphate buffer, and further incubated with 100 µl/well of a 1:5000 dilution of biotin-conjugated rabbit anti-(sheep) serum in BSA/Tween/phosphate buffer for 2 h at 37 °C. Wells were washed, incubated with 100 µl of a 1:1000 dilution of alkaline phosphatase–streptavidin-conjugated reagent for 30 min at 22 °C, and mixed with a 1:100 dilution of p-nitrophenyl phosphate (Zymed, San Francisco, CA, U.S.A.), before quantification of absorbance at 405 nm. A cathepsin G standard curve was prepared as described above from 12 serial 2-fold dilutions of purified cathepsin G (Calbiochem) at 1 µg/ml. In another series of experiments, serum-free suspensions of THP-1 cells at 5 × 10⁶/ml were separately incubated with 5 ng/ml phorbol myristate acetate (PMA, Sigma), 1 µM formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma), 250 µg/ml fibrinogen, 4 µg/ml factor X, or 2.5 mg/ml unactivated or serum-opsonized zymosan in the presence of 2.5 mM CaCl₂. After a 5 min interval at 22 °C, supernatants from stimulated cells were collected and assayed for elastase or cathepsin G content as described above. For these experiments, serum-opsonized zymosan was prepared by re-adding 50 mg of zymosan (Sigma) for 1 h at 100 °C in 2 ml of sterile PBS, pH 7.4. After three washes in PBS, pH 7.4, 25 µg aliquots of the incubation mixture were incubated with 5 µl of normal human serum and washed three times in sterile PBS, pH 7.4, before addition to the leucocyte suspension. Mac-1 recognition of serum-opsonized zymosan has been characterized previously [20].

Structure–function characterization of factor X cleavage by leucocyte granule proteases

Activated factor X (factor Xa) activity generated in fractionated THP-1 cell extracts or purified leucocyte granules was quantified by a one-stage sensitive clotting assay, as described [15]. Briefly, aliquots of granule chromatography fractions were incubated with 2.5 mM CaCl₂ and 50 µg/ml factor X for 15 min at 22 °C. At the end of each incubation, 0.1 µl aliquots from each reaction mixture were added to 0.1 ml of factor VII- and factor X-deficient plasma (Sigma) in duplicate borosilicate glass tubes at 37 °C, and the clotting time was recorded after addition of 0.1 ml of 50 mM CaCl₂. Clotting times were converted into ng/ml of factor Xa activity using a standard curve constructed with increasing concentrations of RVV/factor Xa [15]. Alternatively, factor Xa activity was monitored by hydrolysis of the chromogenic substrate S-2222, and quantified as described above. Analysis of factor X proteolytic processing after binding to Mac-1 was carried out as described previously [15]. Briefly, aliquots of fMLP (10 µM)-stimulated THP-1 cells (1.5 × 10⁷/ml) were incubated with 5 µg/ml of 10¹⁵I-factor X in the presence of 2.5 mM CaCl₂ for 15 min at 22 °C. After washes in PBS, pH 7.4, or PBS plus 5 mM EDTA, aliquots of the cell suspension, supernatant, or supernatant from EDTA-treated cells were separated by
electrophoresis on a 5–20% SDS/polyacrylamide gradient gel under non-reducing conditions. Radioactive bands were visualized by autoradiography using a Kodak X-Omat AR X-ray film and intensifying screens. As characterized previously, EDTA treatment immediately dissociates Mac-1-bound factor X from the monocyte surface [15]. In another series of experiments, 0.5–2 µg/ml aliquots of 125I-labelled factor X were separately incubated with increasing concentrations (0.1–100 µg/ml) of purified elastase (Calbiochem, La Jolla, CA, U.S.A.) or cathepsin G (Calbiochem) in TBS, pH 7.4, in the presence of 2.5 mM CaCl₂ for 5–30 min at 22 °C. At the end of each incubation, samples were electrophoresed on a 5–20% SDS/polyacrylamide gradient gel followed by autoradiography. In other experiments, 100 µg/ml aliquots of cathepsin G were incubated with 2-fold serial increasing concentrations of factor X (0.15–50 µg/ml) in the presence of 2.5 mM CaCl₂ in a total volume of 100 µl of TBS, pH 7.4, for 5 min at 22 °C, before quantification of factor Xa activity by S-2222 hydrolysis.

### Effect of inhibitors on factor X activation

For these experiments, 100 µg/ml aliquots of cathepsin G were separately incubated with 1:5 dilutions of control sheep serum (Sigma), sheep anti-(human cathepsin G) serum (ICN, Costa Mesa, CA, U.S.A.), 1:200 ascites dilution of anti-elastase mAb 1478 (Chemicon International, Temecula, CA, U.S.A.), or isotype-matched (IgM) control anti-(effector cell protease receptor-1) (EPR-1) mAb 12H1 [21]. After a 60 min incubation at 22 °C, samples were incubated with 5 µg/ml factor X for an additional 15 min at 22 °C, and factor Xa activity generated under the various conditions was determined by S-2222 hydrolysis. In another series of experiments, the effect of protease inhibitors on cathepsin G activation of factor X was investigated. For these experiments, 100 µg/ml solutions of elastase or cathepsin G were separately incubated with 100–250 µg/ml of a1 antitrypsin (a1 proteinase inhibitor, Calbiochem), a1 antichymotrypsin (Sigma), egin (Sigma), or soybean trypsin inhibitor (SBTI, Sigma) for 15 min at 22 °C. Samples were mixed with 5 µg of factor X in the presence of 2.5 mM CaCl₂ in a total volume of 100 µl of TBS, pH 7.4, and factor Xa generated under the various conditions was determined by S-2222 hydrolysis.

### Subcellular localization of monocyte factor X-activating activity

In order to identify the source of monocyte factor X-activating activity [15,16], membrane or granule extracts were purified from monocyte THP-1 cells, normalized for protein content, and tested for factor X-activating activity by S-2222 hydrolysis. As shown in Table 1, purified THP-1 cell granules generated factor Xa activity, while membrane fractions were 7- to 9-fold less efficient, under the same experimental conditions (Table 1). Unfractionated THP-1 cell extracts generated comparable amounts of factor Xa activity to that observed with purified monocyte THP-1 cells at 1.5 x 10⁶/ml in RPMI 1640 were blocked in 20% (v/v) normal human serum for 30 min at 4 °C to prevent non-specific Fc-mediated antibody binding, and incubated with control serum, sheep anti-(cathepsin G) serum, or anti-EPR-1 mAb 2E1 for 30 min at 4 °C. Cells were washed, stained with a 1:20 dilution of fluorescein-conjugated goat anti-(sheep) or anti-(mouse) serum (Tago Inc., Burlingame, CA, U.S.A.), washed, and immediately analysed on a Becton-Dickinson FacStar fluorescence-activated cell sorter.

### Microsequencing

Aliquots of factor X were incubated with 100 µg/ml of cathepsin G in the presence of 2.5 mM CaCl₂ in TBS, pH 7.4, for 15 min at 22 °C. Samples were separated on a 5–20% SDS/polyacrylamide gel followed by autoradiography. In other experiments, 200 µg/ml aliquots of cathepsin G were incubated with 2-fold serial increasing concentrations of factor X for 15 min at 22 °C, and factor Xa activity generated under the various conditions was determined by S-2222 hydrolysis. In another series of experiments, the effect of protease inhibitors on cathepsin G activation of factor X was investigated. For these experiments, 100 µg/ml solutions of elastase or cathepsin G were separately incubated with 100–250 µg/ml of a1 antitrypsin (a1 proteinase inhibitor, Calbiochem), a1 antichymotrypsin (Sigma), egin (Sigma), or soybean trypsin inhibitor (SBTI, Sigma) for 15 min at 22 °C. Samples were mixed with 5 µg of factor X in the presence of 2.5 mM CaCl₂ in a total volume of 100 µl of TBS, pH 7.4, and factor Xa generated under the various conditions was determined by S-2222 hydrolysis.

### Subcellular localization of factor X-activating activity

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### Table 1 Subcellular localization of factor X-activating activity in THP-1 cells

<table>
<thead>
<tr>
<th>THP-1 fraction</th>
<th>Factor Xa generated (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granules</td>
<td>1.77 ± 0.3</td>
</tr>
<tr>
<td>Membranes</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Cell extracts</td>
<td>1.3 ± 0.21</td>
</tr>
</tbody>
</table>
granule fractions (Table 1). No hydrolysis of S-2222 was observed in granule or membrane THP-1 cell fractions in the absence of factor X (results not shown). A potential granule localization of factor X-activating activity in monocytes was further investigated. Purified granule extracts from THP-1 cells were size-chromatographed on a Sephadex G-100 column and alternate fractions were monitored for factor X-activating activity and granule protease content. Monocyte granule fractions containing cathepsin G-immunoreactive material generated maximal factor Xa activity, while elastase-containing fractions were not associated with factor Xa activity (Figure 1). Individual granule fractions containing cathepsin G progressively shortened the clotting time of a factor VII- and X-deficient plasma, with generation of 175 ng/ml of factor Xa for fraction 31, 295 ng/ml of factor Xa for fraction 32, and 410 ng/ml of factor Xa for the peak fraction 33 (Figure 1). The lack of a precise superimposition of the peaks of factor Xa activity and cathepsin G antigen may reflect the ability of elastase, present in cathepsin G-containing fractions, to inactivate factor X (Figure 1, and see below).

Structure–function characterization of factor X cleavage by leucocyte granule proteases

The putative role of cathepsin G in factor X activation was investigated directly, in a cell-free system. In the presence of Ca²⁺ ions, purified cathepsin G cleaved factor X in a concentration-dependent reaction. At 100 µg/ml of added cathepsin G, the ~66 kDa factor X zymogen band was completely converted into a ~54 kDa product (Figure 2A), structurally indistinguishable from the procoagulant material generated on monocytes after binding of factor X to Mac-1 [15,16]. At factor X concentrations of 0.15–0.25 µg/ml, 100% of the substrate was converted by cathepsin G into catalytically active factor Xa, as judged by both S-2222 hydrolysis and SDS/polyacrylamide gradient gel and visualized by autoradiography. Abbreviation: MW, molecular mass (Da). The experimental conditions are as in (A), except that [125I]factor X (0.5 µg/ml) was incubated with 100 µg/ml cathepsin G or elastase for the indicated time intervals at 22 °C, before separation by electrophoresis and autoradiography. Molecular mass markers are shown on the left.

Table 2 Generation of bona fide catalytically active factor Xa by cathepsin G cleavage

<table>
<thead>
<tr>
<th>Additions</th>
<th>Factor Xa generated (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Factor X</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Factor X + cathepsin G</td>
<td>0.15 ± 0.15</td>
</tr>
<tr>
<td>Factor X + cathepsin G after EGR-CMK</td>
<td>0.15 ± 0.15</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Factor X + cathepsin G</td>
<td>0.63 ± 0.1</td>
</tr>
<tr>
<td>Factor X + cathepsin G after benzamidine</td>
<td>0.005 ± 0.004</td>
</tr>
<tr>
<td>Benzamidine eluate</td>
<td>0.40 ± 0.12</td>
</tr>
</tbody>
</table>

Figure 1 Factor Xa activity in fractionated THP-1 granule extracts
Purified granules isolated from THP-1 cells were size-fractionated by chromatography on a Sephadex G-100 column. Alternate fractions (horizontal axis) were monitored for (i) factor Xa activity by S-2222 hydrolysis (left-hand vertical axis), (ii) cathepsin G antigen (right-hand vertical axis), and (iii) elastase activity by N-Boc-L-alanine-p-nitrophenyl ester hydrolysis (left-hand vertical axis).

Figure 2 Factor X proteolysis by leucocyte granule proteases
(A) [125I] factor X (0.5 µg/ml) was incubated with the indicated increasing concentrations of purified elastase or cathepsin G in TBS, pH 7.4, containing 2.5 mM CaCl₂ for 15 min at 22 °C. The experimental conditions are as in (A), except that [125I]factor X (0.5 µg/ml) was incubated with 100 µg/ml cathepsin G or elastase for the indicated time intervals at 22 °C, before separation by electrophoresis and autoradiography. Molecular mass markers are shown on the left.
granule proteases occurred rapidly, and appeared complete at the earliest time interval analysed, after a 5 min incubation at 22 °C (Figure 2B).

The ability of cathepsin G to generate catalytically active, bona fide, factor Xa was investigated. Affinity chromatography of cathepsin G-activated factor X on immobilized active-site-dependent inhibitors, e.g. EGR-CMK–Sepharose or benzamidine–Sepharose, completely removed factor Xa coagulant activity (Table 2). However, elution of benzamidine-bound material with free benzamidine resulted in nearly complete recovery of factor Xa activity (Table 2). Indistinguishable results were obtained with RVV/F.Xa (results not shown).

Microsequencing of the ~54 kDa activated factor X product generated by purified cathepsin G revealed a new N-terminus sequence beginning with Leu<sup>178</sup>-Asp<sup>179</sup>-Phe<sup>180</sup>-Asn<sup>181</sup>-Gln<sup>182</sup>-Thr<sup>183</sup>-Gln<sup>184</sup>-Pro<sup>185</sup>-Glu<sup>186</sup> in the zymogen’s activation peptide [22]. A second N-terminal sequence was also derived from the same band beginning at residues Asp<sup>176</sup>-Leu<sup>177</sup>-Leu<sup>178</sup>.

**Effect of inhibitors on cathepsin G-activation of factor X**

Pre-incubation of cathepsin G with α<sub>1</sub> antitrypsin (α<sub>1</sub> proteinase inhibitor), α<sub>1</sub> antichymotrypsin, or SBTI completely abolished factor X activation (Table 3). In contrast, an elastase-specific inhibitor, eglin, was ineffective, and, consistently with the data presented above, purified elastase did not generate factor Xa activity (Table 3). Similar results were obtained when factor X activation was mediated by purified monocyte granules (results not shown). The effect of neutralizing antibodies to granule proteases on factor X activation was also investigated. An anti-(cathepsin G) serum completely inhibited factor X activation by purified cathepsin G, while non-immune serum, a neutralizing anti-elastase mAb, or an isotype-matched control anti-EPR-1 mAb, 12H1 [21], did not diminish factor Xa activity, under the same experimental conditions (Table 3).

**Table 3 Effects of inhibitors on factor X activation by cathepsin G**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Factor Xa generated (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>F.X</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>RVV/F.Xa</td>
<td>1.00±0.05</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>0.12±0.11</td>
</tr>
<tr>
<td>F.X + cathepsin G</td>
<td>2.20±0.10</td>
</tr>
<tr>
<td>F.X + cathepsin G + α&lt;sub&gt;1&lt;/sub&gt; antitrypsin</td>
<td>0.11±0.00</td>
</tr>
<tr>
<td>F.X + cathepsin G + α&lt;sub&gt;1&lt;/sub&gt; antichymotrypsin</td>
<td>0.07±0.04</td>
</tr>
<tr>
<td>F.X + cathepsin G + SBTI</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td>F.X + cathepsin G + eglin</td>
<td>1.50±0.075</td>
</tr>
<tr>
<td>Elastase</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td>F.X + elastase</td>
<td>0.18±0.00</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
</tr>
<tr>
<td>F.X + cathepsin G</td>
<td>0.55±0.027</td>
</tr>
<tr>
<td>F.X + cathepsin G + control</td>
<td>0.65±0.06</td>
</tr>
<tr>
<td>F.X + cathepsin G + anti-cathepsin G</td>
<td>0.07±0.06</td>
</tr>
<tr>
<td>F.X + cathepsin G + anti-elastase</td>
<td>0.47±0.03</td>
</tr>
</tbody>
</table>

**Figure 3 Reactivity of anti-(cathepsin G) serum with leucocytes**

THP-1 cells or PBMCs at 1×10<sup>7</sup>/ml were blocked in 20% (v/v) human serum for 30 min at 4 °C, and incubated with a 1:20 dilution of control non-immune serum, anti-(cathepsin G) serum, or anti-EPR-1 mAb 2E1 (IgG2a) for 30 min at 4 °C. After washes, cells were incubated with a 1:20 dilution of fluorescein-conjugated goat anti-(sheep IgG) or anti-(mouse IgG), and analysed by flow cytometry. The abscissa shows fluorescence intensity on a 4-log scale, the ordinate the cell number.

**Table 4 Mac-1 (CD11b/CD18)-dependent leucocyte degranulation**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cathepsin G (ng/ml)</th>
<th>Elastase (A&lt;sub&gt;405&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMLP</td>
<td>34±17</td>
<td>1.78±0.94</td>
</tr>
<tr>
<td>PMA</td>
<td>55±18</td>
<td>7.80±2.1</td>
</tr>
<tr>
<td>Factor X</td>
<td>174±50</td>
<td>5.14±0.61</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>62±46</td>
<td>1.76±1.5</td>
</tr>
<tr>
<td>Unactivated zymosan</td>
<td>24±23</td>
<td>0.14±0.14</td>
</tr>
<tr>
<td>Opsonized zymosan</td>
<td>93±7</td>
<td>4.21±0.84</td>
</tr>
</tbody>
</table>

**Mechanism of leucocyte degranulation and factor X activation**

A potential constitutive expression of cathepsin G on monocytes was investigated. Consistent with the inability of purified leucocyte membranes to activate factor X (Table 1), anti-(cathepsin G) serum did not react with PBMCs or THP-1 cells by flow cytometry, while both cell types strongly bound anti-EPR-1 mAb 2E1 [21] (Figure 3). However, exposure to inflammatory stimulus IMLP or PMA resulted in monocyte degranulation, as
are shown on the left.

...factor X for 15 min at 22 °C, before electrophoresis on a 5–20% SDS/polyacrylamide gradient gel under non-reducing conditions and autoradiography. Molecular mass (MW) markers in Da are shown on the left.

judged by detectable cathepsin G and elastase activity in the supernatant of stimulated THP-1 cells (Table 4). Similarly, engagement of Mac-1 with its natural ligands, factor X, fibrinogen, or serum-opsonized zymosan, induced release of elastase and cathepsin G, while unactivated zymosan was ineffective (Table 4). Finally, aliquots of cathepsin G-containing supernatant from zymosan-activated THP-1 cells cleaved 15S1-labelled factor X and generated a ~54 kDa activation product (Figure 4), indistinguishable from that observed on the monocyte surface [15], or after factor X activation by purified cathepsin G (Figure 2). In contrast, control supernatants from resting THP-1 cells did not cleave factor X, under the same experimental conditions (Figure 4).

**DISCUSSION**

In this study, we have characterized a pathway of leucocyte initiation of coagulation independent of the extrinsic activators TF:factor VIIa [12]. This is centred on limited proteolytic activation of factor X [14,15], and is organized in three sequential steps. First, inflammatory stimuli or ligand binding to Mac-1 induce release of leucocyte granule proteases [23]. Secondly, released cathepsin G, but not elastase, cleaves and activates membrane-bound factor X at a new peptide bond, Leu177-Leu178, in the zymogen’s activation peptide. Thirdly, the newly generated factor Xa remains associated with the monocyte membrane, and promotes procoagulant activity and thrombin formation.

In addition to its role in leucocyte adherence [17], ligand binding to Mac-1 has been shown previously to trigger effector cell responses, including gene transcription [24], cytokine release [25] and leucocyte oxidative burst [26]. Consistently with our observations with serum-opsonized zymosan, leucocyte degranulation was stimulated during active leucocyte phagocytosis [27], and a direct role for Mac-1 in cytokine-induced leucocyte granule discharge has been demonstrated [28].

In this context, the potential role of leucocyte granule proteases on haemostatic mechanisms has been extensively debated. In addition to stimulating platelet aggregation and secretion [29] and down-modulating von Willebrand factor-dependent platelet adhesion to the subendothelium [30], elastase and/or cathepsin G have been shown previously to cleave several coagulation proteins [31,32], to contribute an alternative fibrinolytic pathway [33], or to generate inactive, Gla domainless factor X, in a reaction entirely prevented by physiological concentrations of Ca2+ ions [34,35].

Here, leucocyte granules containing cathepsin G expressed factor X-activating activity, as judged by hydrolysis of a small chromogenic substrate, i.e. S-2222, as well as by a conventional clotting assay of a factor VII- and factor X-deficient plasma. In a cell-free system, purified cathepsin G cleaved factor X to a ~54 kDa activation product, indistinguishable from that observed on the monocyte surface [15]. This reaction was observed in the presence of millimolar concentrations of Ca2+ ions, which prevented removal of the zymogen’s light chain, in agreement with previous observations [34,35]. Generation of factor Xa under these experimental conditions was completely abolished by inhibitors of cathepsin G (i.e. α1 proteinase inhibitor), or by an anti-(cathepsin G) serum, while elastase inhibitors (i.e. eglin) or an anti-elastase antibody were ineffective.

Structurally, factor X activation by cathepsin G involved a novel cleavage site at Leu177-Leu178 in the zymogen’s activation peptide. Although a natural substrate for cathepsin G has not yet been identified, the sequence described here appears to fulfill the requirements for a genuine cathepsin G recognition, as deduced from peptide 4-nitroanilides [36], and containing a Leu at the P1 position and another hydrophobic residue (Leu or Phe) at P3 [36]. This alternative cleavage site would originate a new N terminus Leu179 in the factor X heavy chain, instead of the canonical Ile185 generated by TF:factor VIIa cleavage [22]. Based on the degree of amino acid packing surrounding the factor Xa catalytic domain, as determined by high-resolution X-ray crystallography [37], the presence of this new N-terminus may not be incompatible with a classical mechanism of zymogen activation, involving the formation of a salt bridge between the highly conserved Leu179 in cathepsin G-cleaved factor X and Asp175, adjacent to the Ser active site. Indeed, immobilized active-site-dependent inhibitors EGR-CMK, or benzamidine, completely removed factor Xa coagulant activity generated by cathepsin G cleavage, thus demonstrating that catalytically active, *bona fide*, factor Xa was generated under these experimental conditions. It should be also pointed out that alternative cleavage sites producing catalytically active factor Xa have been reported previously. Similarly to the situation reported here, Gordon and Mourad have shown that factor X activation by a cytokine protease cancer procoagulant involved limited proteolysis of three distinct peptide bonds in the zymogen’s activation peptide, including the generation of a new N-terminal Asp in the factor X heavy chain [38], reminiscent of the additional cleavage site by cathepsin G at Asp175. Consistently with current models of zymogen activation [39], the shorter activation peptide predicted from cathepsin G cleavage of factor X was released from the cell surface, as judged by the ability of a sequence-specific antibody to the factor X activation peptide to react with the supernatant of stimulated monocytes, under these experimental conditions (H. ten Cate and K. Bauer, unpublished work).

At variance with this model of cathepsin G-dependent activation of factor X, elastase produced a different pattern of zymogen proteolysis, associated with functionally inactive fragments, even in the presence of Ca2+ ions [40]. This suggests that for their compartmentalization and simultaneous release during leucocyte degranulation, elastase may effectively down-modulate the degree of factor X activation by cathepsin G. In this context, initiation of coagulation via the Mac-1/cathepsin G pathway may occur more efficiently on monocytes compared with neutrophils, because of their higher number of cathepsin G-binding sites [41], and their 20 times lower content of elastase [42]. Consistently with this prediction, factor X cleavage on neutro-
philis appeared predominantly mediated by elastase, and, in agreement with the in vitro data presented here, was not associated with factor Xa coagulant activity (J. Plescia and D. C. Altieri, unpublished work). On the other hand, cathepsin G-generated factor Xa remained associated with the monocyte surface with full coagulant activity [15,16], thus escaping further proteolysis by elastase and/or inactivation by plasma anti-proteinase inhibitors. As anticipated previously [43,44], and reiterated here in flow cytometry studies, the molecular basis of proteinase inhibitors. As anticipated previously [43,44], and proteolysis by elastase and G-generated factor Xa remained associated with the monocyte surface-bound factor X (this study), and to accelerate the process both to initiate coagulation via limited proteolytic activation of surface-bound factor X (this study), and to accelerate the process of thrombin formation through the assembly of a functional prothrombinase complex [45,46].

In summary, these studies provide evidence for an alternative pathway of monocyte initiation of coagulation, independently of the extrinsic activators TF:factor VIIa [12]. In the absence of transcriptional activation of the TF gene, this mechanism may be important for contributing a 'first signal' in a protected vascular cell microenvironment for coagulation [47], anticoagulation [48], and protease-dependent mechanisms of vascular cell activation and signal transduction.

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REFERENCES