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

Janet PLESCIA and Dario C. ALTIERI*

Molecular Cardiobiology Program, The Boyer Center for Molecular Medicine, Department of Pathology, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536, U.S.A.

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 Ca^{2+} ions, purified cathepsin G, but not elastase, cleaved factor X to a ~ 54 kDa catalytically active derivative, structurally indistinguishable from the procoagulant product generated on monocytes after binding to Mac-1. Factor X activation by purified cathepsin G involved limited proteolysis of a novel Leu^{177}-Leu^{178} peptide bond in the zymogen's activation peptide. Cathepsin G activation of factor X was completely inhibited by α_1 antitrypsin, α_1 antichymotrypsin, or soybean trypsin inhibitor, or by a neutralizing antiserum to cathepsin G,

while eglin, or an anti-elastase antibody, were ineffective. Affinity chromatography on active-site-dependent inhibitors Glu-Gly-Arg-chloromethyl ketone or benzamidine completely abolished factor Xa activity generated by cathepsin G. Cathepsin G was not constitutively detected on the monocyte surface by flow cytometry. However, inflammatory stimuli, including formyl peptide or phorbol ester, or Mac-1 engagement with its ligands fibrinogen, factor X or serum-opsonized zymosan, triggered monocyte degranulation and cathepsin G activation of factor X. These findings demonstrate that monocytes can alternatively initiate coagulation 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, anti-coagulation 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 hyperacute transplant 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 ($K_d \sim$

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^{-5} M 2-mercaptoethanol (Eastman Kodak, Rochester, NY, U.S.A.). Phenotypical expression of the leucocyte integrin Mac-1

(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^{125}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 (2.5×10^8) 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 unfractionated THP-1 cell extracts, were diluted 1:50 or 1:100, respectively, and normalized for protein content by absorbance at 280 nm wavelength ($A_{280} \sim 0.4$). 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 unfractionated THP-1 cell extracts, were mixed with 5 μg of factor X in the presence of 2.5 mM CaCl$_2$ 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 on a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.) at a wavelength of 405 nm, and converted into μg/ml of factor Xa using a standard curve constructed with serial increasing concentrations of Russell viper venom (RVV)-activated factor X. In another series of experiments, purified granule extracts isolated from THP-1 cells were size-fractionated by chromatography on a Sephadex G-100 (Pharmacia) column (1.5 cm × 60 cm) with elution in 0.1 M Tris/HCl, 0.5 M NaCl, pH 7.4. Protein concentration in the eluted material was determined by absorbance at a wavelength of 280 nm. Factor Xa activity and granule protease content (see below) were determined in alternate eluted fractions.

Quantification of leucocyte granule proteases

Elastase activity in subcellular chromatography fractions, or supernatants of stimulated THP-1 cells, was determined by hydrolysis of the elastase-sensitive chromogenic substrate N-t-Boc-L-alanine-p-nitrophenyl ester (NBA, Sigma), 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 Na$_2$PO$_4$, 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 Na$_2$PO$_4$, 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 5×10^7/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 CaCl$_2$. 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-fluxing 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 mg aliquots of the incubation mixture were incubated with 5 ml 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 CaCl$_2$ and 50 μg/ml factor X for 15 min at 22 °C. At the end of each incubation, 0.1 ml 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 CaCl$_2$. 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 (1.5×10^7/ml) were incubated with 5 μg/ml of ^{125}I-factor X in the presence of 2.5 mM CaCl$_2$ 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 ^{125}I-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 CaCl$_2$ 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 CaCl$_2$ 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 1:50 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 α$_1$ antitrypsin (α$_1$ proteinase inhibitor, Calbiochem), α$_1$ antichymotrypsin (Sigma), eglin (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 CaCl$_2$ in a total volume of 100 μl of TBS, pH 7.4, and factor Xa generated under the various conditions tested was determined by S-2222 hydrolysis. To test the effect of active-site-dependent inhibitors on cathepsin G-generated factor Xa, 100 μl aliquots of Glu-Gly-Arg-chloromethyl ketone (EGR-CMK, Calbiochem) were covalently coupled to CNBr-activated Sepharose (Pharmacia) to 5 mg/ml, followed by two washes in TBS, pH 7.4. Aliquots (200 μl) of factor X (2.0 mg/ml) were incubated with 100 μg/ml cathepsin G for 20 min at 22 °C, and applied to the EGR-CMK affinity matrix for 60 min at 22 °C. Aliquots of cathepsin G-treated factor X were assayed for factor Xa activity before or after incubation with EGR-CMK by S-2222 hydrolysis. In another series of experiments, 200 μl aliquots of cathepsin G-treated factor X were chromatographed on Sepharose-conjugated p-aminobenzamidine (Pierce), followed by three washes in TBS, pH 7.4. Benzamidine-bound material was eluted with 200 μl aliquots of 1.0 M benzamidine hydrochloride/0.5 M NaCl, pH 6.0, and dialysed against TBS, pH 7.4. Factor Xa activity in unbound and eluted fractions was determined by S-2222 hydrolysis as described above. Control experiments were carried out with RVV/factor Xa under the same experimental conditions.

Flow cytometry

Suspensions of PBMC or THP-1 cells at 1.5×10^7/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 CaCl$_2$ in TBS, pH 7.4, for 15 min at 22 °C. Samples were separated on a 5–20 % SDS/polyacrylamide gradient gel, transferred to Immobilon membranes (Millipore Corp., Bedford, MA, U.S.A.) at 450 mA for 1.5 h, and protein bands were visualized by Coomassie Blue staining. An approx. 54 kDa band, generated from factor X in cathepsin G-treated samples, was excised and subjected to microsequencing using an ABI 470A gas-phase sequenator with an on-line 120A ABI HPLC. Assignment of the cathepsin G cleavage site(s) in factor X was according to Fung et al. [22].

RESULTS

Previous studies demonstrated that binding of ∼ 66 kDa factor X to fMLP-stimulated THP-1 cells was followed by proteolytic processing of the zymogen with generation of a ∼ 54 kDa activation product expressing factor Xa coagulant activity, unaffected by neutralizing anti-TF mAbs [14,15]. Analysis of supernatant from stimulated THP-1 cells revealed the presence of intact, uncleaved ∼ 66 kDa factor X, indicating that the newly generated ∼ 54 kDa activation product remained entirely associated with the monocyte surface (results not shown), in agreement with previous observations [15,16]. However, chelation of bivalent cations with EDTA immediately released the membrane-bound ∼ 54 kDa band in the supernatant of stimulated THP-1 cells (results not shown).

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

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

Aliquots (10 μl) of membrane, granule, or THP-1 cell extracts normalized for protein content ($A_{280} \sim 0.4$) were incubated with 5 μg of factor X and 2.5 mM CaCl$_2$ in a total volume of 100 μl of TBS, pH 7.4, for 5 min at 22 °C. Factor Xa activity was determined by hydrolysis of the factor Xa-sensitive chromogenic substrate S-2222 at a wavelength of 405 nm, and converted into μg/ml of factor Xa using a standard curve constructed with serial increasing concentrations of RVV/factor Xa. Data are the means ± S.E.M. of two independent experiments.

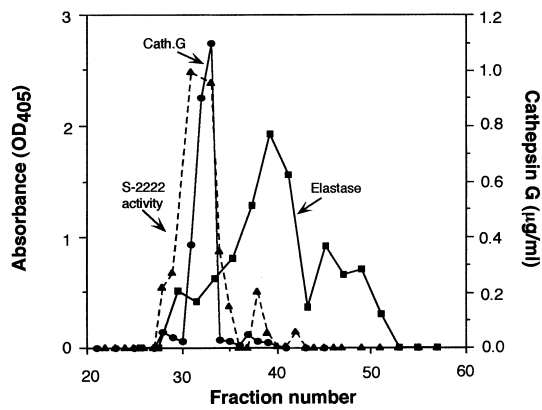| THP-1 fraction	Factor Xa generated (μg/ml)
Granules	1.77 ± 0.3
Membranes	0.29 ± 0.01
Cell extracts	1.3 ± 0.21

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-t-Boc-L-alanine-p-nitrophenyl ester hydrolysis (left-hand vertical axis).

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 Ca^{2+} ions, purified cathepsin G cleaved factor X in a concentration-dependent reaction. At 100 μg/ml of added cathepsin G, the $\sim$ 66 kDa factor X zymogen band was completely converted into a $\sim$ 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/gel electrophoresis followed by silver staining of the activated product (results not shown). Indistinguishable results were obtained when cathepsin G was substituted with purified monocyte granules (results not shown). Comparable concentrations of elastase produced a different cleavage pattern of factor X, generating variable amounts of a $\sim$ 54 kDa product plus additional proteolytic fragments of $\sim$ 32, 30 and 22 kDa (Figure 2A). Factor X proteolysis by leucocyte
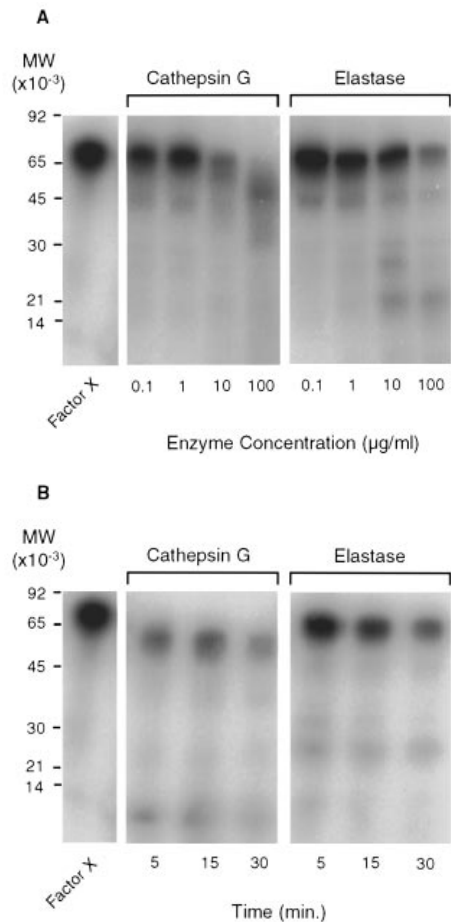

Figure 2   Factor X proteolysis by leucocyte granule proteases

(**A**) ^{125}I-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 CaCl$_2$ for 15 min at 22 °C. Samples were separated by electrophoresis on a 5–20 % SDS/polyacrylamide gradient gel and visualized by autoradiography. Abbreviation: MW, molecular mass (Da). (**B**) The experimental conditions are as in (**A**), except that ^{125}I-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

Aliquots (200 μl) of factor X (2.0 mg/ml) were incubated with 100 μg/ml cathepsin G for 20 min at 22 °C, assayed for factor Xa activity by S-2222 hydrolysis, and applied to (**A**) immobilized EGR-CMK–Sepharose (5 mg/ml) or (**B**) benzamidine–Sepharose. After washes, factor Xa activity was quantified in the unbound material or in the fractions eluted with 1.0 M benzamidine, 0.5 M NaCl (eluate), by S-2222 hydrolysis and converted into μg/ml of factor Xa, as described in Table 1.

Additions	Factor Xa generated (μg/ml)
A	
Factor X	0.12 ± 0.12
Cathepsin G	0.10 ± 0.02
Factor X + cathepsin G	0.75 ± 0.18
Factor X + cathepsin G after EGR-CMK	0.15 ± 0.1
B	
Factor X + cathepsin G	0.63 ± 0.1
Factor X + cathepsin G after benzamidine	0.005 ± 0.004
Benzamidine eluate	0.40 ± 0.12

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

(**A**) Purified cathepsin G or elastase (100 μg/ml) was separately preincubated with 100 μg/ml concentrations of the indicated protease inhibitors for 15 min at 22 °C. Samples were mixed with 5 μg of factor X (F.X) in a total volume of 100 μl of TBS, pH 7.4, in the presence of 2.5 mM CaCl$_2$, before quantification of factor Xa activity as described in Table 1. (**B**) Cathepsin G (100 μg/ml) was preincubated with 1:50 dilution of non-immune sheep serum (control), sheep anti-(human cathepsin G) serum, or 1:200 ascites dilution of anti-elastase mAb 1478 for 60 min at 22 °C, before addition of 5 μg/ml aliquots of factor X for 15 min at 22 °C, and determination of factor Xa activity. Factor Xa activity in the presence of control anti-EPR-1 mAb 12H1 was 0.6 μg/ml. Data are the means $\pm$ S.E.M. of at least two independent experiments.

| Additions	Factor Xa generated (μg/ml)
(A)	
F.X	0.15 $\pm$ 0.05
RVV/F.Xa	1.00 $\pm$ 0.05
Cathepsin G	0.12 $\pm$ 0.11
F.X + cathepsin G	2.20 $\pm$ 0.10
F.X + cathepsin G + α_1 antitrypsin	0.11 $\pm$ 0.00
F.X + cathepsin G + α_1 antichymotrypsin	0.07 $\pm$ 0.04
F.X + cathepsin G + SBTI	0.05 $\pm$ 0.00
F.X + cathepsin G + eglin	1.50 $\pm$ 0.075
Elastase	0.05 $\pm$ 0.00
F.X + elastase	0.18 $\pm$ 0.00
(B)	
F.X + cathepsin G	0.55 $\pm$ 0.027
F.X + cathepsin G + control	0.65 $\pm$ 0.06
F.X + cathepsin G + anti-cathepsin G	0.07 $\pm$ 0.06
F.X + cathepsin G + anti-elastase	0.47 $\pm$ 0.03

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/factor 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[178]-Asp[179]-Phe[180]-Asn[181]-Gln[182]-Thr[183]-Gln[184]-Pro[185]-Glu[186] in the zymogen's activation peptide [22]. A second N-terminal sequence was also derived from the same band beginning at residues Asp[176]-Leu[177]-Leu[178].

Effect of inhibitors on cathepsin G-activation of factor X

Pre-incubation of cathepsin G with α_1 antitrypsin (α_1 proteinase inhibitor), α_1 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).
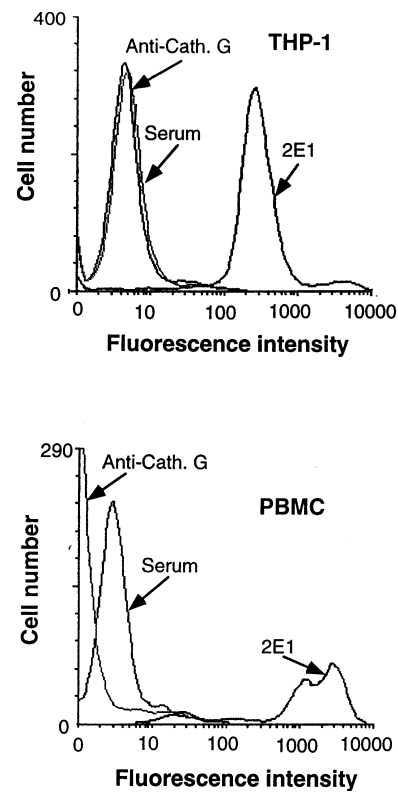
Figure 3 Reactivity of anti-(cathepsin G) serum with leucocytes

THP-1 cells or PBMCs at 1×10^7/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

THP-1 cells were incubated with fMLP (1 μM), PMA (5 ng/ml), Mac-1 ligands fibrinogen (250 μg/ml) or factor X (4 μg/ml), or unactivated or serum-opsonized (activated) zymosan (2.5 mg/ml) for 5 min at 22 °C. At the end of the incubation, aliquots of the cell supernatant were collected and assayed for cathepsin G antigen by immunoassay or elastase activity by NBA hydrolysis.

Stimulus	Cathepsin G (ng/ml)	Elastase (A_{405})
fMLP	34 $\pm$ 17	1.78 $\pm$ 0.94
PMA	55 $\pm$ 18	7.80 $\pm$ 2.1
Factor X	174 $\pm$ 50	5.14 $\pm$ 0.81
Fibrinogen	62 $\pm$ 46	1.78 $\pm$ 1.5
Unactivated zymosan	24 $\pm$ 23	0.14 $\pm$ 0.14
Opsonized zymosan	93 $\pm$ 7	4.21 $\pm$ 0.84

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 fMLP or PMA resulted in monocyte degranulation, as
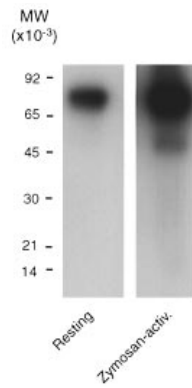
Figure 4   Factor X cleavage by released leucocyte granule proteases

Supernatants from resting or zymosan-stimulated THP-1 cells were incubated with 1 μg/ml ^{125}I-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 ^{125}I-labelled factor X and generated a $\sim$ 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, Leu^{177}-Leu^{178}, 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 Ca^{2+} 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 $\sim$ 54 kDa activation product, indistinguishable from that observed on the monocyte surface [15]. This reaction was observed in the presence of millimolar concentrations of Ca^{2+} 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. α_1 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 Leu^{177}-Leu^{178} in the zymogen's activation peptide. Although a natural substrate for cathepsin G has not yet been identified, the sequence described here appears to fulfil 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 Leu^{178} in the factor X heavy chain, instead of the canonical Ile^{195} 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 Leu^{178} in cathepsin G-cleaved factor X and Asp^{378}, 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 cysteine 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 Asp^{176}. 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 Ca^{2+} 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-

phils 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 factor Xa retention on the monocyte surface may reside in the ability of these cells to co-express a membrane factor Xa receptor, i.e. EPR-1 [21], which may function as an acceptor/binding site for the newly generated factor Xa on the cell surface. It is also noteworthy that cathepsin G may activate the prothrombinase cofactor, factor V, on monocytes [45,46], thus suggesting that Mac-1-dependent release of cathepsin G may enable these cells 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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