The Fibrin(ogen)olytic Properties of Cathepsin D†

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ABSTRACT: Fibrin(ogen) is important for hemostasis and is cleared from sites of vascular injury primarily by the plasminogen activator system. However, there is emerging evidence in plasminogen activator-deficient transgenic mice that non-plasmin pathways may also be important for endogenous fibrinolysis. We have recently described an alternative, plasmin-independent fibrinolytic pathway in activated human monocytes that utilizes the integrin Mac-1 (CD11b/CD18), which directly binds and internalizes fibrin, resulting in its lysosomal degradation. The identity of the lysosomal fibrinolytic enzyme(s) responsible for monocyte/macrophage-mediated fibrinolysis is unknown. Protease inhibitor studies now suggest that an aspartyl protease is responsible for this fibrinolytic activity. We, therefore, examined the fibrinolytic properties of cathepsin D, a lysosomal aspartyl protease, and report that cathepsin D possesses both fibrinogenolytic and fibrinolytic activity. Cathepsin D cleavage of fibrinogen follows Michaelis–Menten kinetics with a Michaelis constant, K_m, of 1.5 μM; catalytic rate constant, k_{cat}, of 1.4×10^{-3} s^{-1}; and catalytic efficiency, k_{cat}/K_m, of 9.3×10^{-4} μM^{-1} s^{-1}. A pH–activity profile of fibrinogen digestion by cathepsin D demonstrates a pH optimum of 3.5 with 50% residual activity at pH 5.0. Fibrinolysis was assessed by fibrin plate and fibrin clot lysis assays. Cathepsin D possesses significant fibrinolytic activity over a dose range of 100 nM to 10 μM and is able to lyse fibrin, as well as albumin-enriched and albumin/red cell-enriched fibrin clots. Cathepsin D cleaves the α-, β-, and γ-chains of FGN, generating multiple low-molecular-weight fragments. We characterized three of the proteolytic cleavage products of fibrinogen by HPLC separation and NH_2-terminal sequence analysis to locate three unique proline-rich cleavage sites at residues 219–220, 394–395, and 521–522 on the α-chain. We conclude that cathepsin D possesses fibrin(ogen)olytic activity with a pH profile ideal for lysosomal action. These data support the existence of a plasmin-independent fibrinolytic pathway in monocytes and also suggest that proteolytic enzymes such as cathepsin D may have important implications in the pathophysiology and treatment of thrombotic disorders.

The fibrinolytic system, which dissolves fibrin thrombi and maintains blood flow at sites of vascular injury, is an important component of the normal hemostatic response. The major protease of the fibrinolytic system is plasmin (Robbins et al., 1981). Plasmin is derived from Glu- or Lys-plasminogen by the cleavage of a single Arg–Val bond; this cleavage is produced by the endogenous plasminogen activators, tissue-type plasminogen activator (t-PA) (Pennica et al., 1983) and urokinase-type plasminogen activators (u-PAs) (Steffens et al., 1982).

Neutrophils and monocytes accumulate within thrombi and may contribute to fibrinolysis by way of a variety of intracellular proteases capable of degrading matrix and adhesion proteins. The clearance of fibrin by leukocytes occurs via two distinct mechanisms: (1) the phagocytosis of soluble and insoluble fibrin (Lee & McCluskey, 1962; Riddle & Barnhart, 1964; Sherman et al., 1975) and (2) the extracellular release of fibrinolytic proteases (Plow & Edgington, 1975). Using a skin window technique, Riddle and Barnhart demonstrated fibrin phagocytosis by leukocytes in inflammatory exudates (Riddle & Barnhart, 1964) in a process later termed "leukofibrinolysis" (Lewis et al., 1972). One mechanism of "leukofibrinolysis" involves the rapid lysosomal uptake of fibrin monomer by macrophages via a receptor-mediated process involving the aminoterminus of the fibrin α-chain (Gonda & Shainoff, 1982). Several groups have independently shown that leukocyte lysates digest fibrin in a pattern distinct from plasmin digestion (Plow & Edgington, 1975; Grames et al., 1978; Bilezikian & Nossel, 1977; Francis & Marder, 1986). The primary fibrinolytic enzymes of neutrophils are elastase and cathepsin G (Plow & Edgington, 1975), which are secreted as a result of the leukocyte release reaction or cell lysis. Secreted neutrophil elastase also participates in the degradation of fibrin(ogen) within a "protected pocket" on the membrane surface of neutrophils (Wright et al., 1988; Weitz et al., 1987; Gustafson et al., 1989). There is emerging evidence that these plasmin-independent fibrinolytic mechanisms may be important for endogenous fibrinolysis. Recent work by Carmeliet and colleagues in plasminogen activator-deficient transgenic mice provides compelling evidence for the physiologic importance of alternative fibrinolytic pathways (Carmeliet et al., 1994).

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[1] Abbreviations: AAPVCK, *N*-methoxysuccinyl-L-alanyl-L-prolyl-L-valanyl chloromethyl ketone; BSA, bovine serum albumin; FGN, fibrinogen; FM, fibrin monomer; FN, fibrin; GPRP, L-glycl-L-prolyl-L-arginyl-L-proline; I-CAM-1, intercellular cell adhesion molecule 1; PBMC, peripheral blood mononuclear cells; PPACK, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone; RBC, red blood cell; SBTI, soybean trypsin inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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We have recently reported that the integrin Mac-1 (CD11b/CD18) on activated human monocytes specifically binds and internalizes fibrin leading to its lysosomal degradation (Simon et al., 1993). Mac-1 is a leukocyte integrin that binds the heterogeneous ligands C3bi (Beller et al., 1982; Talle et al., 1983), factor X (Altieri et al., 1988), intercellular adhesion molecule-1 (Diamond et al., 1991), and fibrin(ogen) (Wright et al., 1993; Altieri et al., 1986) after activation with a variety of agonists including adenosine 5′-diphosphate (ADP). The identity of the lysosomal enzyme(s) responsible for monocyte-mediated fibrinolysis via Mac-1 is presently unknown. Observations using protease inhibitors suggested to us that one possible candidate enzyme is cathepsin D, an aspartyl protease. We now report that this aspartyl protease possesses significant fibrin(ogen)olytic activity and contributes to monocyte-mediated fibrinolysis *in vitro*.

MATERIALS AND METHODS

Special Reagents. Human fibrinogen (grade L) and Glu-plasminogen were purchased from Kabi/Pharmacia, Franklin, OH. Human plasminogen-free fibrinogen was purchased from Enzyme Research Laboratories, South Bend, IN. Aprotinin, bovine spleen cathepsin D, E64D, L-glycl-L-prolyl-L-arginyl-L-proline (GPRP), leupeptin, *N*-methoxysuccinyl-L-alanyl-L-prolyl-L-valanyl chloromethyl ketone (AAPVCK), pepstatin A, and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co., St. Louis, MO. Tissue-type plasminogen activator (t-PA) was kindly provided by Genentech, Inc., South San Francisco, CA. Bovine thrombin was purchased from ICN BioMedicals, Irvine, CA. D-Phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) was purchased from Calbiochem Corp., La Jolla, CA. D-Valanyl-L-leucinyl-L-lysyl chloromethyl ketone (VLLCK) was purchased from Enzyme Systems Products, Livermore, CA. Sepracell-MN was purchased from Sepratech Corp., Oklahoma City, OK.

Cells. The monoblast cell line U937 and monocytic cell line THP-1 were maintained in culture in RPMI containing 20% fetal calf serum, 20 mM (hydroxyethyl)piperazineethanesulfonic acid, and 2 mM L-glutamine. Human monocytes were prepared from whole blood by continuous density gradient separation using Sepracell-MN. Cells were counted with a Coulter counter, Model ZM.

Protein Labeling. Plasminogen-free human FGN was radiolabeled with ^{125}I using Iodobeads as previously described (Loscalzo et al., 1986). The specific activity of [^{125}I]FGN ranged (3–5) × 10^4 cpm/μg; total radioactivity was greater than 95% trichloroacetic acid (TCA)-precipitable. [^{125}I]Fibrin monomer (FM) was obtained by the addition of 1.0 unit/mL bovine thrombin to [^{125}I]FGN in the presence of 3 mM GPRP. After 15 min, 5 units/mL hirudin was added to inhibit residual thrombin activity.

Fibrinogen and Fibrin Degradation Assays. The degradation of [^{125}I]FGN and [^{125}I]FM by U937 cells, THP-1 cells, and monocyte-enriched peripheral blood mononuclear cells was examined as previously described (Simon et al., 1993). Briefly, 0.9 μM [$^{12}15I$]FGN or [^{125}I]FM was added to (8–10) × 10^6/mL monocytoid cells at 37 °C suspended in Tris-buffered saline (TBS), pH 7.4, consisting of 10 mM tris-(hydroxymethyl)aminomethane and 150 mM NaCl in the presence of 2.5 mM $CaCl_2$ and 10 μM ADP in order to stimulate the binding of FGN or FM to the integrin Mac-1. After 60–90 min, ice-cold TCA was added, and the incubation mixture was centrifuged at 12000*g* for 4 min in an Eppendorf microfuge. An aliquot of the supernatant was then counted to determine the amount of acid-soluble, radioactive material generated by the cells, i.e., ^{125}I-labeled iodotyrosine-containing peptides. Nonspecific degradation was determined by incubating 0.9 μM [^{125}I]FGN or [^{125}I]FM with monocytoid cells as above in the presence of a 20–50-fold molar excess of unlabeled FGN or FM. Nonspecific degradation accounted for approximately 30–40% of total degradation. Specific degradation (total − nonspecific) is expressed as micrograms of FGN or FM protein degraded/10^6 cells per hour.

In order to elucidate the mechanism of FGN/FM degradation, incubations were performed in the presence of the serine protease inhibitors, 40 μM D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) and 10 μg/mL soy bean trypsin inhibitor (SBTI); the granulocyte elastase inhibitor, 50 μM *N*-methoxysuccinyl-L-alanyl-L-prolyl-L-valanyl chloromethyl ketone (AAPVCK); 100 μM chloroquine, an inhibitor of lysosomal degradation; the cysteinyl protease inhibitors, 0.5 mM leupeptin, 50 μM E64D, and 10 μM Z-L-phenylalanyl-L-alanyl-CHN_2; and the aspartyl protease inhibitor, 0.9 mM pepstatin A. Relative degradation was calculated as the ratio of the rate of degradation in the presence of inhibitor to the rate of degradation in the absence of inhibitor.

Fibrinolytic Assays. The ability of cathepsin D or plasmin to cleave FGN was investigated initially in a solution-phase assay. Bovine spleen cathepsin D, purified with pepstatin-Sepharose as previously described (Ferguson et al., 1973) with modification (Cunningham & Tang, 1976), was used in these assays. This preparation results in approximately equal mixtures of single- (molecular weight = 46 000 Da) and two-chain (molecular weight 12 000 and 34 000 Da) forms of cathepsin D. Plasmin was obtained by the addition of t-PA to 9.5 μM Glu-plasminogen for 1 h at 37 °C. Varying concentrations of [^{125}I]FGN (0.03–11.8 μM) were added to 70 nM cathepsin D in 100 mM NaCl, 50 mL of sodium acetate, pH 4.0, or 40 nM plasmin in TBS, pH 7.4, and incubated at 37 °C. Aliquots, to which cold TCA was added, were removed at 0, 60, and 120 min and then centrifuged at 12000*g* for 4 min in an Eppendorf microfuge. An aliquot of this supernatant was then counted to determine the amount of ^{125}I-labeled iodotyrosine-containing peptides generated by cathepsin D or by plasmin cleavage of FGN. The rates of proteolysis as a function of [^{125}I]FGN concentration were determined in order to derive the double reciprocal plot, $1/v_i$ (min/μM) vs 1/[FGN] (μM^{-1}).

To investigate the effect of pH on the rate of FGN proteolysis, incubations with cathepsin D were also performed in 100 mM NaCl, 50 mM sodium acetate, pH 3.0–6.0. For cathepsin D, relative activity was calculated from the ratio of the rate of FGN cleavage at a specific pH (3.0, 3.5, 4.0, 4.5, 5.0, 6.0) to the rate of cleavage at pH 3.5; for plasmin, the relative activity was calculated from the ratio of the rate of FGN cleavage in 100 mM NaCl sodium acetate, pH 4.0, 5.0, and 6.0, to the rate of cleavage in TBS, pH 7.4.

Fibrin Plate Assay. The mechanism of fibrin degradation by activated THP-1 cells was also examined using the fibrin plate method as described by Astrup and Mullertz (Astrup & Mullertz, 1952) with modification. A uniform fibrin layer (approximately 2 mm thick) coating the bottom of an 8.5-cm Petri dish was produced by adding 0.5 unit/mL bovine thrombin to 10.45 mL of 1.0 mg/mL plasminogen-free FGN, 4.5 mM $CaCl_2$, in HEPES-buffered saline (HBS), pH 7.35, consisting of 140 mM NaCl, 4 mM KCl, 10 mM (hydroxyethyl)piperazineethanesulfonic acid (HEPES), 2 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.1% dextrose, and 0.4% BSA. The solution was briefly mixed, and uniform polymerization was

allowed to occur at 25 °C on a level surface. After 30 min, 500 000 ADP-activated THP-1 cells in 40 μL of HBS, pH 7.35, were carefully applied to the fibrin layer, and the Petri dish was then placed a water-jacketed incubator at 37 °C. After 24–36 h, the zone of lysis (mm^2) was determined from the product of two perpendicular diameters measured with calipers to the nearest 1 mm. Incubations were also performed in the presence and absence of the protease inhibitors listed above. Relative fibrinolysis was calculated as the ratio of the zone of lysis in the presence of the inhibitor to the zone of lysis in the absence of the inhibitor.

The ability of cathepsin D or plasmin to cleave FGN was investigated using this fibrin plate method. In these experiments, the uniform fibrin layer was produced with plasminogen-free FGN or Kabi grade L human FGN in TBS, pH 7.4, containing 45 mM $CaCl_2$ (Kabi grade L FGN contains sufficient plasminogen for plasmin generation by the plasminogen activator, t-PA). To this layer, 20-μL samples containing varying concentrations of cathepsin D (16 nM to 10 μM) in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, or t-PA (16 nM to 2 μM) in TBS, pH 7.4, were carefully applied to the fibrin layer and allowed to incubate 37 °C. After 18 h, the zone of lysis was determined and then plotted as a function of cathepsin D or t-PA concentration. In this fibrin plate assay, there is no spontaneous lysis after 24 h with Kabi grade L FGN or plasminogen-free FGN.

Fibrin Clot Lysis Assay. The ability of cathepsin D to lyse fibrin clots was investigated as follows. Fibrin clots were formed by the addition of bovine thrombin (1 unit/mL) to 0.65 mL of 2.0 mg/mL plasminogen-free FGN in TBS, pH 7.4, to which [^{125}I]FGN (≈500 000 total counts) was added in the presence of 5 mM $CaCl_2$. Bovine serum albumin (BSA)-enriched, BSA/red blood cell (RBC)-enriched, and RBC-enriched fibrin clots were also formed by the addition of 11.5 mg/mL BSA and/or 10^7 RBC/mL to the fibrinogen solution. The clots were formed in 4-mL test tubes and, after 30 min, the tubes were centrifuged at 3000 rpm for 10 min. The residual supernatant was removed, and the tubes were then counted to assess total [^{125}I]FGN incorporation. To each fibrin clot were added 1.0 mL of 100 nM NaCl, 50 nM sodium acetate, pH 4.0–6.0, containing 5 units/mL hirudin to inhibit residual thrombin activity, 40 μM PPACK to inhibit serine protease activity, and 400 nM cathepsin D or sodium acetate buffer as control. The final pH after the addition of acetate buffer, pH 6.0, to the neutral fibrin clot was determined. The addition of 1.0 mL of 100 mM of NaCl, 50 mM sodium acetate, pH 4.0 and 6.0, to a fibrin clot formed in 0.65 mL of TBS, pH 7.4, resulted in a final pH equal to 4.0 and 6.1, respectively. The test tubes were subsequently placed on a rocker, and 50-μL aliquots of supernatant were removed over time to assay released [^{125}I]FGN counts. Percent clot lysis [(counts released/total counts in each tube) × 100] was calculated over time. Counts released secondary to contaminant proteases or to rocking alone represented less than 10% of total counts in each tube and were subtracted from each sample.

Cleavage Patterns of FGN Proteolysis. The cleavage pattern of FGN proteolysis by cathepsin D was investigated using sodium dodecyl sulfate–polyacylamide gel electrophoresis (SDS–PAGE) and HPLC. Plasminogen-free FGN was incubated with cathepsin D (molar ratio, substrate-to-enzyme of 5–2000:1) at 37 °C. Aliquots were removed over time, and the reaction was stopped by boiling for 5 min in sample buffer for electrophoresis containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue, and 2% 2-mercaptoethanol. FGN cleavage fragments were separated and analyzed by SDS–PAGE performed on 7.5–12.5% linear gradient slab gels using a discontinuous buffer system (Laemmli, 1970), and the gels were stained with Coomassie Brilliant Blue.

Table 1: Inhibitors of Fibrinogen Degradation

inhibitor	relative degradation[a]	inhibitor	relative degradation[a]
aprotinin	0.85 ± 0.16	factor X	0.10 ± 0.15*
SBTI	1.21 ± 0.10	Z-Phe-Ala-CHN_2	1.17 ± 0.03
PPACK	0.90 ± 0.26	E64D	1.13 ± 0.24
AAPVCK	1.00 ± 0.06	leupeptin	1.48 ± 0.12
chloroquine	0.25 ± 0.12*	pepstatin A	0.68 ± 0.17*

[a] The degradation of FGN by THP-1 cells was investigated in the presence of potential inhibitors, as described in Materials and Methods. These included 280 KIU/mL aprotinin, 10 μg/mL SBTI, 40 μM PPACK, 50 μM AAPVCK, 100 μM chloroquine, 400 nM factor X, 10 μM Z-Phe-Ala-CHN_2, 50 μM E64D, 0.5 mM leupeptin, and 0.9 mM pepstatin A. Relative degradation was calculated from the ratio of the rate of degradation in the presence of inhibitor to the rate of degradation in the absence of inhibitor. Each value represents the mean ± standard deviation (n = 3–5). *Indicates significant inhibition, $p < 0.01$.

The generation of fibrinogen degradation products by cathepsin D and plasmin was also compared using autoradiography. [^{125}I]FGN (1.5 μM) was incubated with 100 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, and 100 nM plasmin in TBS, pH 7.4, at 37 °C. Aliquots were removed over time, and [^{125}I]FGN cleavage fragments were separated and analyzed by SDS–PAGE performed under nonreducing conditions on an 8–20% linear gradient slab gel. The dried gel was then exposed to Kodak X-Omat AR film.

FGN (3.0 μM) was also incubated with 100 nM cathepsin D, in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, and 25–100 nM plasmin TBS, pH 7.4, and the cleavage fragments were separated and analyzed by SDS–PAGE performed under nonreducing conditions on an 8% uniform slab gel loading 10 μg of protein per lane. The amount of FGN degraded over time was assessed by determining the relative intensity of the 340 000-Da band, corresponding to intact FGN, at a given time compared to time zero using Image software (National Institutes of Health, Bethesda, MD). The $t_{1/2}$-linear was calculated from the plot of log relative intensity versus time.

To isolate FGN fragments for amino-terminal sequence analysis, a terminal cathepsin D digest of FGN was reduced with 45 mM dithioetheritol, alkylated with 100 mM iodoacetamide, and then subjected to HPLC using a reverse-phase column developed with an acetonitrile gradient in 0.06% trifluoroacetic acid (0–100% over 70 min). Amino-terminal sequence analysis of the isolated proteolytic fragments was performed on an Applied Biosystems 477A Protein Sequencer with an on-line phenylthiohydantoin-amino acid analyzer.

RESULTS

Fibrinolysis via Mac-1. We have previously shown that monocytes possess an alternative fibrinolytic pathway that utilizes the integrin Mac-1, which directly binds and internalizes FGN resulting in its lysosomal degradation (Simon et al., 1993). At 37 °C, FGN and fibrin monomer (FM) are internalized and degraded at rates of 0.37 ± 0.13 and 0.55 ± 0.03 (mean ± SD) μg/10^6 cells per h by U-937 cells, 1.38 ± 0.02 and 1.20 ± 0.30 μg/10^6 cells per h by THP-1 cells, and 2.10 ± 0.20 and 2.52 ± 0.18 μg/10^6 cells per h by monocyte-enriched human peripheral blood mononuclear cells, respectively. In order to elucidate the mechanism of FGN degradation, incubations were performed in the presence of potential inhibitors (Table 1). The serine protease inhibitors,

Table 2: Inhibitors of Fibrinolysis[a]

inhibitor	relative fibrinolysis	inhibitor	relative fibrinolysis
PPACK	1.33 ± 0.06	Factor X	0.24 ● 0.12*
VLLCK	1.28 ± 0.18	EDTA	0.32 ± 0.24*
AAPVCK	1.66 ± 0.07	Pepstatin A	0.18 ± 0.13*

[a] The mechanism of fibrin degradation by activated THP-1 cells was examined using fibrin plate method: 500 000 ADP-activated THP-1 cells in 40 μL of HBS, pH 7.35, were carefully applied to a fibrin layer and allowed to incubate at 37 °C as described in Materials and Methods. After 24 h, the zone of lysis (mm^2) was determined from the product of two perpendicular diameters measured with calipers to the nearest 1 mm. Incubations were also performed in the presence and absence of the protease inhibitors, 40 μM PPACK, 50 μM VLLCK, 50 μM AAPVCK, and 0.9 mM pepstatin A, and in the presence and absence of 400 nM factor X and 20 mM EDTA. Relative fibrinolysis was calculated as the ratio of the zone of lysis in the presence of inhibitor to the zone of lysis in the absence of inhibitor. Each value represents the mean ± standard deviation (n = 3–9). *Indicates significant inhibition, $p < 0.01$.

aprotinin, SBTI, and PPACK, and the specific elastase inhibitors, AAPVCK, did not significantly inhibit degradation. FGN degradation, however, was inhibited by chloroquine, suggesting that a lysosomal pathway is involved. Factor X, a competitive ligand with FGN for the Mac-1 receptor (Altieri et al., 1988), also significantly blocked degradation by 90 ± 15%. Leupeptin, E64D, and Z-Phe-Ala-CHN_2—all lysosomal cysteinyl protease inhibitors—failed to block degradation. The aspartyl protease inhibitor, pepstatin A, inhibited FGN degradation by 32%.

The mechanism of fibrin degradation by activated THP-1 cells was also examined using the fibrin plate method (Table 2). Fibrinolysis by ADP-activated THP-1 cells results in a zone of lysis after 24 h. Similar to FGN degradation, fibrinolysis by THP-1 cells was not inhibited by the plasmin inhibitor, VLLCK, or the elastase inhibitor, AAPVCK. A role for Mac-1 in this fibrinolytic pathway is supported by significant inhibition with factor X, a competitive ligand with FGN for the Mac-1 receptor (Altieri et al., 1988), and with the calcium chelator, EDTA. Pepstatin A inhibited THP-1 cell-mediated fibrinolysis by 82%. Given the pepstatin results demonstrating that aspartyl protease activity is at least partially responsible for this fibrin(ogen)olytic activity, we examined the fibrin(ogen)olytic properties of cathepsin D.

Fibrin(ogen)olysis by Cathepsin D. We initially investigated the ability of cathepsin D to cleave [^{125}I]fibrinogen and generate non-TCA-precipitable ^{125}I-labeled iodotyrosine peptides. Figure 1a,b demonstrates that cathepsin D cleavage of FGN follows Michaelis–Menten kinetics with a Michaelis constant, K_m, of 1.5 μM; a catalytic rate constant, k_{cat}, of 1.4 × 10^{-3} s^{-1}; and a catalytic efficiency, k_{cat}/K_m, of 9.3 × 10^{-4} s^{-1} μM^{-1}. Pepstatin A inhibited greater than 99% of this fibrinogenolytic activity while the serine protease inhibitor, PPACK, and cysteinyl protease inhibitor, leupeptin, did not inhibit the fibrinogenolytic activity of this cathepsin D preparation, ruling out any significant contribution by alternate contaminant proteases.

Table 3 compares the fibrin(ogen)olytic potential of cathepsin D with that of plasmin. Plasmin cleavage of FGN also follows Michaelis–Menten kinetics with a K_m of 7.3 μM; a k_{cat} of 0.10 s^{-1}; and catalytic efficiency, k_{cat}/K_m, of 1.4 × 10^{-3} μM^{-1} s^{-1}. These data indicate that at their respective pH optima (see below), plasmin is 15-fold more efficient than cathepsin D in the cleavage of FGN. The kinetic analysis, which relies on the generation of TCA-soluble fragments, must be interpreted cautiously given the fact that plasmin-derived fibrinogen degradation products (FDPs) are larger than cathepsin D-derived FDPs (Figure 2a). In order to compare directly the rates of FGN cleavage by cathepsin D and plasmin via a method that is independent of TCA solubility, we examined the initial cleavage of intact FGN by determining the relative intensity of the 340 000-Da band over time (Figure 2b). The $t_{1/2}$ for FGN cleavage by 100 nM cathepsin D and 25 nM plasmin are 25 and 15 min, respectively. Under these conditions, approximatley 6.5-fold more cathepsin D than plasmin is required to degrade an equivalent amount of FGN.

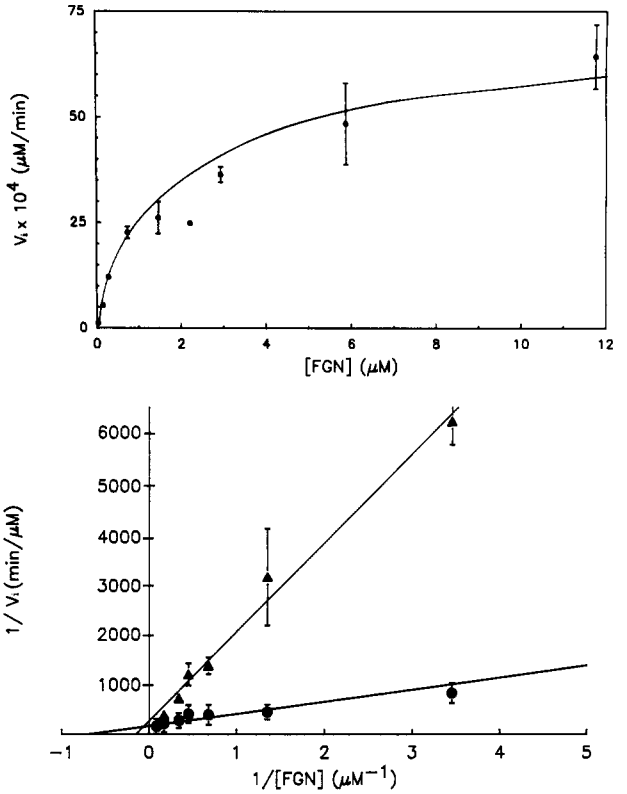


FIGURE 1: Kinetics of the cleavage of FGN by cathepsin D. The ability of cathepsin D to cleave FGN was investigated as described in Materials and Methods. Varying concentrations of [^{125}I]FGN (0.03–11.8 μM) were added to 70 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0. The initial rates of proteolysis (v, μM/min) as a function of FGN concentration ([FGN], μM) were plotted (a, top), and the double reciprocal plot $1/v$ (min/μM) vs $1/[FGN]$ (μM^{-1}) was derived (b, bottom). The double-reciprocal plot for the cleavage of FGN by 40 nM plasmin in TBS, pH 7.4 (▲), is included for comparison with cathepsin D (●). Each point represents the mean ● standard deviation of three experiments.

Table 3: Kinetic Constants for the Cleavage of Fibrinogen by Cathepsin D and Plasmin[a]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
cathepsin D	1.5	1.4×10^{-3}	9.3×10^{-4}
plasmin	7.3	0.10	1.4×10^{-2}

[a] Varying concentrations of [^{125}I]FGN were added to 70 nM cathepsin D in 100 nM NaCl, 50 mM sodium acetate, pH 4.0, or 40 nM plasmin in TBS, pH 7.4. Kinetic constants for the cleavage of fibrinogen by cathepsin D and plasmin were determined from double reciprocal plots, $1/v$ (min/μM) vs $1/[FGN]$ (μM^{-1}), as depicted in Figure 1b.

pH Dependence. Cathepsin D is an aspartyl protease with a pH optimum of 3.5 for hydrolysis of human hemoglobin (Ikeda et al., 1989; Takaahashi & Tang, 1981). Figure 3 shows the pH–activity profiles of cathepsin D and of plasmin for FGN. The pH optimum for cathepsin D is approximately 3.5 with 50% activity at pH 5.0 and 10% at pH 6.0. In contrast,

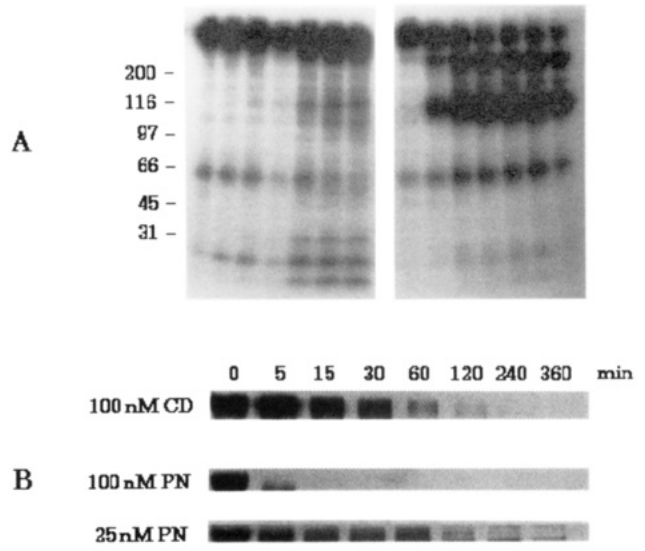


FIGURE 2: Fibrinogen degradation by plasmin and cathepsin D. The generation of fibrinogen degradation products by cathepsin D and plasmin was investigated using autoradiography. [^{125}I]FGN (1.5 μM) was incubated with 100 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0 (a, left panel), and 100 nM plasmin TBS, pH 7.4 (a, right panel), at 37 °C. Aliquots were removed over time (0, 5, 15, 30, 60, 120, and 240 min), and [^{125}I]FGN cleavage fragments were separated and analyzed by SDS–PAGE performed under nonreducing conditions on an 8–20% linear gradient slab gel. The gel was intentionally placed on film for a prolonged exposure in order to accentuate low-molecular-weight fragments. FGN (3.0 μM) was also incubated with 100 nM cathepsin D, in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, and 25–100-fold nM plasmin TBS, pH 7.4, and the cleavage fragments were separated and analyzed by SDS-PAGE performed under nonreducing conditions on an 8% uniform slab gel (b). The amount of FGN degraded over time was assessed by determining the relative intensity of the 340 000-Da band at the indicated time compared to time zero using Image software (National Institutes of Health, Bethesda, MD), and the $t_{1/2}$ was calculated from the plot of log relative versus time. Relative densitometric values at 0, 5, 15, and 30 min—100 nM cathepsin D, 1.00, 1.05, 0.71, and 0.40, respectively; 25 nM plasmin, 1.00, 0.68, 0.48, and 0.42, respectively.

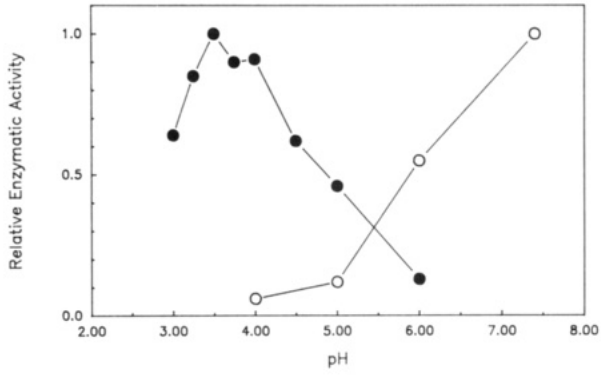


FIGURE 3: pH profile of cathepsin D and plasmin fibrinogenolytic activity. The effect of pH on the rate of FGN proteolysis by cathepsin D and plasmin was investigated as described in Materials and Methods. Incubations were performed in 100 mM NaCl, 50 mM sodium acetate, pH 3.0–6.0. Incubations at pH 7.4 were performed in TBS. For cathepsin D (●), relative activity was calculated from the ratio of the rate of FGN cleavage at the specified pH to the rate of cleavage at pH 3.5. For plasmin (○), relative activity was calculated from the ratio of rate of FGN cleavage at the specified pH to the rate of cleavage at pH 7.4. Each value represents the mean of two experiments, each performed in duplicate.

plasmin has diminished proteolytic activity in the acidic pH range with 10% residual activity at pH 5.0 and 6% at pH 4.0.

Fibrinolysis by Cathepsin D. The ability of cathepsin D to lyse fibrin was investigated utilizing the fibrin plate assay

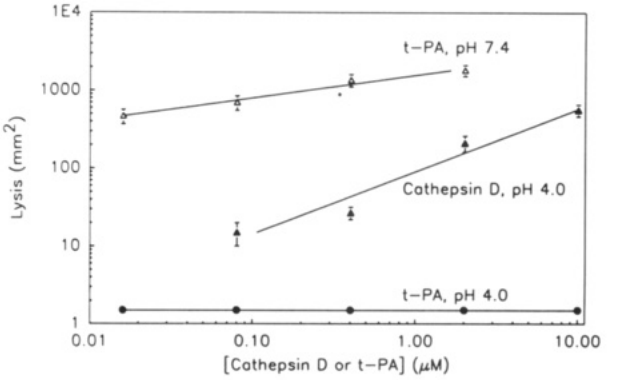


FIGURE 4: Fibrin plate assay. The ability of cathepsin D to lyse fibrin was examined using the fibrin plate method with modification. To a uniform fibrin layer were applied 20-μL samples containing varying concentrations of cathepsin D (▲) in 100 mM NaCl, 50 mM sodium acetate, pH 4.0; t-PA in TBS, pH 7.4 (△); or t-PA in 100 mM NaCl, 50 mM sodium acetate, pH 4.0 (●), and these were allowed to incubate at 37 °C. After 18 h, the zone of lysis (mm^2) was determined from the product of two perpendicular diameters. The zone of lysis was then plotted as a function of cathepsin D or t-PA concentration. Each value represents the mean ± standard deviation, $n = 3$.

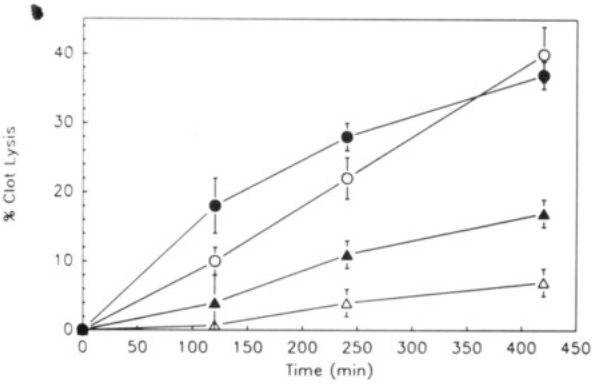


FIGURE 5: Fibrin clot lysis assay. The ability of cathepsin D to lyse fibrin clots was investigated as described in detail in Materials and Methods. Fibrin clots were also enriched with either 11.5 mg/mL BSA, 10^7 RBC/mL, or 11.5 mg/mL BSA, 10^7 RBC/mL. To each fibrin clot was added 400 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, containing 5 units/mL hirudin to inhibit residual thrombin activity and 40 μM PPACK to inhibit serine protease activity. Percent clot lysis was calculated over time for clots composed of fibrin (○), BSA-enriched fibrin (●), BSA/RBC-enriched fibrin (▲), or RBC-enriched fibrin (△). Each value represents the mean ± standard deviation of three experiments.

as described by Astrup and Mullertz with modification (Astrup & Mullertz, 1952). The extent of lysis as a function of cathepsin D concentration is depicted in Figure 4. Increasing fibrinolysis is evident over a dose range of 100 nM to 10 μM cathepsin D. For comparison to a known fibrinolytic agent, the responses to t-PA at pH 7.4 and pH 4.0 are also included. It is important to note that the fibrin layer was formed at pH 7.4 in all cases, and the fibrinolytic agent was added in 20 μL of buffer at the specified pH. At pH 7.4, t-PA is approximately 100-fold more potent than cathepsin D; however, when layered onto the fibrin plate in a sample buffer at pH 4.0, t-PA manifests negligible plasmin-mediated fibrinolytic activity compared to cathepsin D.

In addition to the fibrin platelet assay, we also investigated the ability of cathepsin D to lyse fibrin clots, BSA-enriched fibrin clots, BSA/RBC-enriched fibrin clots, and RBC-enriched fibrin clots. Clots were specifically enriched with red blood cells and BSA because both are present in *in vivo* thrombi and, in addition, hemoglobin and albumin are known substrates for cathepsin D (Ikeda et al., 1989; Takaahashi &

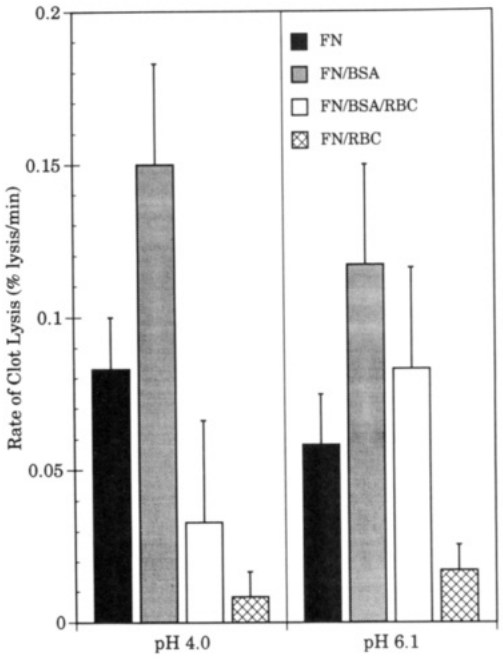
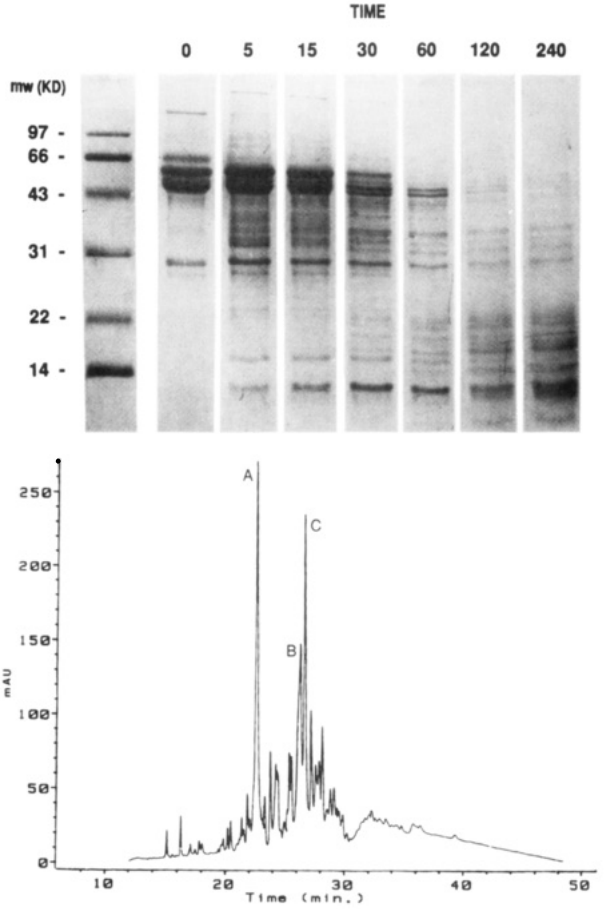


FIGURE 6: Rates of fibrin clot lysis affected by clot composition. The initial rates of clot lysis of fibrin clots (closed bars), BSA-enriched fibrin clots (shaded bars), BSA/RBC-enriched fibrin clots (open bars), and RBC-enriched fibrin clots (hatched bars) were calculated from the initial slope of a plot of percent clot lysis versus time, as shown in Figure 5 and as described in Materials and Methods. The effect of pH on the rates of fibrin clot lysis was also investigated by adding cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0 and 6.0, to the fibrin clot formed in TBS at pH 7.4. The addition of acetate buffer, pH 4.0 and 6.0, to a fibrin clot formed in TBS, pH 7.4, resulted in a final pH equal to 4.0 and 6.1, respectively. Each value represents the mean ± standard deviation of three experiments.

Tang, 1981). Figure 5 demonstrates a typical time course for cathepsin D lysis of a fibrin clot. Forty percent lysis occurred by 420 min. The initial rates of clot lysis by cathepsin D as a function of clot composition and pH are depicted in Figure 6. Fibrinolysis by cathepsin D is accelerated 2–4-fold in BSA-enriched fibrin clots, perhaps secondary to the stabilization of cathepsin D by BSA or the fact that albumin has been shown to stimulate cathepsin D cleavage of hemoglobin as a substrate (Kalski et al., 1987). RBC-enriched fibrin clots are comparatively resistant to lysis by cathepsin D; however, in the presence of albumin, lysis of RBC-enriched fibrin clots occurs efficiently. The mechanism of this resistance of RBC-enriched fibrin clots in the absence of albumin is unclear, especially since the only known endogenous inhibitor of cathepsin D activity is α-2 macroglobulin (Thomas et al., 1989) but may be secondary to the known ability of RBCs to promote α-chain cross-linking of fibrin (Murthy & Lorand, 1990).

Figure 6 also demonstrates that cathepsin D expresses significant fibrinolytic activity at pH 6.1 in BSA-enriched and BSA/RBC-enriched clots. This finding stands in direct contrast to the pH–activity profile of cathepsin D for fibrinogen in solution illustrated in Figure 3, which demonstrates only 10% residual activity at pH 6.0. One possible explanation for this pH shift may relate to the demonstration by Jupp and co-workers (Jupp et al., 1988) that membrane-bound forms of the closely related aspartyl protease, cathepsin E (*vide infra*), retain activity at a higher pH than cytosolic forms of cathepsin E. Interactions of cathepsin D with the fibrin surface may induce similar changes in activity, perhaps by inducing conformational changes in the enzyme.



A 211 PNLVPGNFK*SQLQKVPPEWKALTDMP 236 (α-chain)

B 386 PNDPNWGTF*EEVSGNVSPGTRREV 411 (α-chain)

C 511 PGFFSPMLGEF*VSETESRGSESGIFT 536 (α-chain)

FIGURE 7: FGN and its proteolytic fragments generated by cathepsin D. Cleavage of FGN by cathepsin D was performed by incubating plasminogen-free FGN with cathepsin D (molar ratio, substrate-to-enzyme, 1000:1) at 37 °C. A time course (0–240 min) of proteolysis by cathepsin D was first examined by performing SDS–PAGE uniform 12.5% gels under reducing conditions (a, top). A terminal digestion of FGN cleavage by cathepsin D was obtained by incubating FGN with cathepsin D at 37 °C overnight. To isolate FGN fragments for amino-terminal sequence analysis, this terminal digest was reduced, alkylated, and then subjected to HPLC using a reverse-phase column (b, middle). Three major peaks were identified (A, B, C), and amino-terminal sequence analysis (8–15 cycles) was subsequently performed (c, bottom). The actual sequence obtained is underlined, and cleavage sites were localized according to the published amino acid sequence of human fibrinogen (Doolittle et al., 1979). Cleavage sites are indicated by an asterisk, and proline residues appear in bold-face type (**P**).

Characterization of Fibrinogen Cleavage Products. To identify the cleavage products of FGN generated by cathepsin D and to locate their cleavage sites, we separated digests with SDS–PAGE or HPLC and performed amino-terminal sequence analysis as described in Materials and Methods. A time course of proteolysis by cathepsin D is shown in Figure 7a. Cleavage of the α-, β-, and γ-chains of FGN occurs rapidly with the generation of multiple lower-molecular-weight bands. To characterize representative cleavage sites, a reverse-phase HPLC separation of a terminal digest of FGN was performed, yielding three major peaks (Figure 7b: A, B, C). These predominant peaks were subjected to amino-terminal sequence

analysis identifying cleavage sites at residues 219–220 (A), 394–395 (B), and 521–522 (C) on the α-chain of FGN (Figure 7c). Residue 219–220 is contained within the interzonal region of the α-chain (residues 195–239) as originally described by Doolittle and colleagues (Doolittle et al., 1979). This region is susceptible to cleavage by several proteases including plasmin, trypsin, and thermolysin, and is felt to be relatively exposed and readily accessible to proteolytic attack (Takagi & Doolittle, 1975). The cleavage site at residue 394–395 is contained within zone ZM (middle section) of the α-chain (Doolittle et al., 1979). This region contains seven of the α-chain's 10 tryptophan residues and is relatively nonpolar with more than half of the amino acids in this region comprised of glycine, serine, proline, and threonine. This section of the α-chain is felt to be in a random coil conformation with regularly interspersed turns. The cleavage site at residue 521–522 is contained within the carboxy-terminal zone (residues 425–610). Cathepsin D cleaves FGN at these three sites in proline-rich domains (Figure 7c), an observation also noted in the cleavage of apoB-100 by cathepsin D (Van Der Westhuyzen et al., 1980). Interestingly, cathepsin D cleavage of FGN at these three sites does not conform to the common sequence pattern of "hydrophobic-charged-x-x-charged" reported for cathepsin D cleavage of other cathepsin D substrates (van Noort & van der Drift, 1989).

DISCUSSION

Coupled with our earlier results (Simon et al., 1993), this study provides evidence for an alternative fibrinolytic pathway that uses the integrin Mac-1 and cathepsin D. This pathway involves a two-step mechanism in which Mac-1 on activated monocytoid cells first binds fibrin(ogen), which is followed by its internalization and lysosomal degradation. Data presented here suggest that aspartyl protease activity contributes to Mac-1-mediated fibrinolysis *in vitro* and delineates a specific mechanism for plasmin-independent fibrinolysis involving the lysosomal aspartyl protease, cathepsin D.

The clearance of fibrin by leukocytes has been shown to occur via two distinct mechanisms involving the phagocytosis of soluble and insoluble fibrin (Lee & McCluskey, 1962; Riddle & Barnhart, 1964; Sherman et al., 1975) termed "leukofibrinolysis" (Lewis et al., 1972) or the extracellular release of fibrinolytic proteases (Plow & Edgington, 1975). Gonda and Shainoff demonstrated that one such mechanism of "leukofibrinolysis" in macrophages involves the aminoterminus of the fibrin α-chain (Gonda & Shainoff, 1982). This particular pathway in macrophages is blocked by the tripeptide, Gly-Pro-Arg. Importantly, the mechanism of fibrinogen/fibrin monomer binding and uptake by Mac-1 differs from that described by Gonda and Shainoff because this Mac-1 pathway is not inhibited by Gly-Pro-Arg-(Pro) (Simon et al., 1993). While our data suggest that Mac-1 mediates the clearance of fibrin via direct binding and internalization, previous investigators have shown that Mac-1 may also be involved in the release of an extracellular fibrinolytic protease. Neutrophils have been shown to secrete elastase within a "protected pocket" on the membrane surface in response to Mac-1–fibrin binding (Wright et al., 1988; Weitz et al., 1987; Gustafson et al., 1989).

Previously studied fibrinolytic enzymes from leukocytes include elastase and cathepsin D (Plow & Edgington, 1975). We have found that the aspartyl protease inhibitor, pepstatin A, blocks monocyte-mediated fibrinolysis by 82%, suggesting that the lysosomal aspartyl protease, cathepsin D, needs to be considered along with elastase and cathepsin G as a potentially relevant leukocyte-derived fibrinolytic enzyme. The contribution and significance of cathepsin D in monocyte-mediated fibrinolysis *in vivo* remains to be determined and is the focus of on-going studies.

Cathepsin D is a lysosomal aspartyl protease that functions within an acidic pH range (Ikeda et al., 1989). Similar to the aspartyl proteases, renin and pepsin, cathepsin D is synthesized as a preproenzyme and cleaved to a proenzyme, procathepsin D, that is then further processed to single-chain and two-chain forms (Richo & Connor, 1991). A partial list of known substrates for cathepsin D includes hemoglobin (Ikeda et al., 1989; Takaahashi & Tang, 1981), serum albumin (Ikeda et al., 1989; Takaahashi & Tang, 1981), apolipoprotein B-100 (Van Der Westhuyzen et al., 1981), myelin basic protein (Whitaker & Sayer, 1979), collagen (Helseth & Veis, 1984), somatostatin (Benuck et al., 1977), angiotensin (Hackenthal et al., 1978), and big endothelin-1 (Takaoka et al., 1990). Widely distributed in human tissues, cathepsin D plays important physiologic roles in the lysosomal catabolism of proteins and in the processing and activation of proteolytic enzymes (procathepsin B) (Samarel et al., 1989), hormones (somatostatin) (Benuck et al., 1977), and growth factors (transforming growth factor-β) (Rochefort et al., 1990). Pathophysiologically, cathepsin D has been implicated in tumor invasion and metastasis in breast (Spyratos et al., 1989; Tandom et al., 1990) and ovarian (Montcourrier et al., 1989) carcinomas and in melanoma (Leto et al., 1992) owing to its ability to degrade extracellular matrix (Briozzo et al., 1988) and, perhaps, to its autocrine mitogenic activity through interaction with the mannose 6-phosphate/IGF-II receptor (Mathieu et al., 1990).

The data presented here now demonstrate that cathepsin D also possesses significant fibrin(ogen)olytic activity. The cleavage of FGN by cathepsin D follows Michaelis–Menten kinetics with a Michaelis constant, K_m, of 1.5 μM; a catalytic rate constant, k_{cat}, of 1.4×10^{-3} s^{-1}; and a corresponding catalytic efficiency, k_{cat}/K_m, of 9.3×10^{-4} μM^{-1} s^{-1}. Table 3 compares the fibrin(ogen)olytic potential of cathepsin D with plasmin at their respective pH optima and demonstrates that plasmin is 15-fold catalytically more efficient than cathepsin D in the cleavage of FGN. Figure 4 directly compares the fibrinolytic potential of cathepsin D and t-PA in the fibrin plate assay at their respective pH optima. Under these conditions, t-PA is approximately 100-fold more potent than cathepsin D.

In addition to the predominant intracellular localization of cathepsin D in lysosomes and endosomes, there are membrane-associated and secreted pools of cathepsin D. Cathepsin D binds to the monocyte/macrophage mannose-6-phosphate receptor (Haslik & Von Figura, 1981; Varki & Kornfeld, 1983), which determines the sorting of cathepsin D between intracellular and plasma membrane compartments. Furthermore, Young and colleagues (Young et al., 1991) have noted a 40-fold increase in secreted cathepsin D activity by incubation of rat peritoneal macrophages with mannose, methyl α-glucopyranoside, and *N*-acetylglycosamine, but not mannose 6-phosphate. Binding to this receptor was of high affinity with a K_D of 27 nM and approximately 200 000 cathepsin D bound/cell. In addition, cathepsin D has been recently found to associate with HepG2 cells in a mannose-6-phosphate-independent manner (Rijnboutt et al., 1991).

The physiologic role of receptor-bound cathepsin D on the cell surface is unknown. Interestingly, membrane-bound cathepsin D has the potential for enhanced activity; Kolski and colleagues have shown that phospholipids stimulate

cathepsin D activity (Kolski et al., 1987). Binding to the mannose receptor may also provide a mechanism for the release of cathepsin D in areas of inflammation or ischemia. Local regions of hyperacidity can form during inflammation as macrophages switch to glycolysis and secrete lactic acid, resulting in pH 3.5–5.0 (Young & Zygas, 1987; von Arden & Krouger, 1979; Etherington et al., 1981). Low pH is capable of resulting in ligand–mannose receptor dissociation (Young et al., 1991). Rossman and colleagues have shown that inflammatory mediators, such as γ-interferon, markedly increase secreted cathepsin D activity in human monocytes/macrophages (Rossman et al., 1990). Cathepsin D is, thus, well poised to clear FGN/fibrin in areas of inflammation and ischemia where plasminogen activator/plasmin activity is limited by acidic pH (see Figures 3 and 4). The use of pharmacologic doses of cathepsin D as a therapeutic thrombolytic agent is currently being investigated in our laboratory. Indeed, preliminary data using the rabbit jugular model of thrombolysis (Collen et al., 1983) suggest that cathepsin D possesses significant *in vivo* thrombolytic activity when administered via intravenous infusion (data not shown).

It is also important to consider a closely related aspartyl protease, cathepsin E (Yamamoto et al., 1978; Wiederanders et al., 1989; Yonezawa et al., 1987). Cathepsin E is similar to cathepsin D with respect to size, substrate specificity (both are able to cleave hemoglobin and albumin), inhibition by pepstatin A, resistance to urea denaturation, and dependence of enzymatic activity on acid pH (Yonezawa et al., 1987). Distinct from cathepsin D, however, cathepsin E is a nonlysosomal aspartyl protease (Yonezawa et al., 1988). Yonezawa and colleagues (Yonezawa et al., 1988), employing density centrifugation on discontinuous Percoll gradients, found a striking difference in the subcellular localization between cathepsin E and cathepsin D: cathepsin E was associated with the membrane and soluble (P3) fractions and cathepsin D with the azurophilic granule-rich (P1) and specific granule-rich (P2) fractions. The distribution of cathepsin D and cathepsin E is also species-specific (Yonezawa et al., 1991): cathepsin D is found in human leukocytes; cathepsin E is present in rat leukocytes, but absent in human leukocytes and human leukemic cell lines. Therefore, we have focused these studies on the relevant human, leukocyte aspartyl protease, cathepsin D.

Endogenous fibrinolysis has been attributed predominantly to the plasminogen activators, t-PA and u-PA (Pennica et al., 1983; Steffens et al., 1982). The investigation of non-plasmin-mediated fibrinolytic mechanism(s) is particularly relevant in light of the recent report by Carmeliet and co-workers (Carmeliet et al., 1994) that plasminogen activator-deficient transgenic mice possess an alternative fibrinolytic pathway capable of clearing thrombi slowly. The data presented here support the existence of a plasmin-independent fibrinolytic pathway in activated human monocytoid cells that involves Mac-1 and cathepsin D. The existence of this fibrinolytic mechanism may have important implications in the pathophysiology and treatment of thrombotic disorders.

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