Degradation of Cross-Linked Fibrin by Matrix Metalloproteinase 3 (Stromelysin 1): Hydrolysis of the γ Gly 404−Ala 405 Peptide Bond†

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Received March 26, 1996; Revised Manuscript Received July 23, 1996⊗

ABSTRACT: Matrix metalloproteinases (MMPs) can degrade a number of proteins that constitute the extracellular matrix. Previous studies have shown that atherosclerotic plaques contain substantial amounts of fibrin(ogen)-related antigen, and more recently, MMPs have been identified in such lesions. The hypothesis that MMPs play a role in the degradation of fibrinogen (Fg) and cross-linked fibrin (XL-Fb) was investigated. Fibrinogen became thrombin-unclottable when treated with matrix metalloproteinase 3 (MMP-3, stromelysin 1) but not with matrix metalloproteinase 2 (MMP-2, gelatinase A). Incubation of XL-Fb clots (made with ^{125}I-Fg) with MMP-3 resulted in complete lysis after 24 h. A D monomer-like fragment was generated by MMP-3 degradation of fibrinogen, XL-Fb, and fragment DD. Immunoreactivity with monoclonal antibody (MoAb)/4-2 (anti-γ392−406) but not with MoAb/4A5 (anti-γ397−411) suggested that a major cleavage site was within the sequence participating in the cross-linking of two γ-chains. NH_2-terminal sequence analysis of the γ-chain of the D monomer-like fragment and of a dipeptide isolated from the MMP-3 digest of XL-fibrin identified the hydrolysis of the γ Gly 404-Ala 405 peptide bond. These data indicate that the degradation of Fg and XL-Fb by MMP-3 is specific and different from plasmin. This mechanism of fibrinolysis might be of relevance in wound healing, inflammation, atherosclerosis, and other pathophysiological processes.

Matrix metalloproteinases (MMPs)[1] have the capacity to degrade a number of proteins and proteoglycans that constitute the extracellular matrix (ECM) of connective tissue. They participate in the remodeling of tissues in physiological processes such as morphogenesis and embryonic development, and in the pathophysiology of wound healing, tumor invasion, and arthritis (Nagase et al., 1991; Woessner, 1991; Werb et al., 1992; Matrisian, 1992).

The expression of MMPs and of their inhibitors is regulated by hormones, cytokines, proto-oncogenes, steroids, and growth factors [reviewed in Woessner (1991) and Matrisian (1992)]. Matrix metalloproteinase 3 (MMP-3) belongs to the stromelysin class, and its known substrates include proteoglycans, type IV collagen, fibronectin, and laminin. It is expressed in mature macrophages, endothelial cells, and fibroblasts. The zymogen, pro-MMP-3, is activated by trypsin, neutrophil elastase, plasma kallikrein, plasmin, chymotrypsin, cathepsin G, and mast cell tryptase and by mercurial compounds such as 4-aminophenylmercuric acetate (APMA) [reviewed in Nagase et al. (1991, 1992) and Kleiner and Stetler-Stevenson (1993)].

In atherosclerotic plaques, there is a large amount of fibrin(ogen)-related antigen (FRA) consisting of different molecular forms (Bini et al., 1987, 1989; Smith et al., 1990; Valenzuela et al., 1992). Two very recent studies have shown the presence of MMP-3 in human atherosclerotic plaques (Henney et al., 1991; Galis et al., 1994).

In this study, we investigated the hypothesis that MMPs, particularly MMP-3, may play a role in the degradation of fibrinogen (Fg) and cross-linked fibrin (XL-Fb). We have studied the following aspects: (1) degradation of Fg; (2) effect of MMPs digestion of Fg on its subsequent clotting with thrombin; (3) lysis of XL-Fb and purified fragment D-dimer (DD); (4) NH_2-terminal analyses of chain fragments of selected digests; (5) reactivity of the cleaved fragments with a number of specific monoclonal antibodies. We show the ability of MMP-3 to degrade Fg and to lyse XL-Fb clots.

† Parts of this work were presented at the 68th Annual Meeting of the American Heart Association, Anaheim, CA, Nov 13−16, 1995 (Bini et al., 1995), and at the Joint Meeting of the ASBMB, ASIP, AAI, New Orleans, LA, June 2−6, 1996 (Bini et al., 1996). The work of Drs. Y. Itoh and H. Nagase was supported by NIH Grant AR39189 (to H.N.).

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⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; ELISA, enzyme-linked immunoabsorbant assay; DD, fragment D-dimer, the COOH-terminal fragments (186 kDa) of cross-linked fibrin obtained by plasmin digestion in buffers containing $CaCl_2$; fragment E, the NH_2-terminal fragments (50 kDa) obtained by plasmin digestion, the formula for the predominant species is (Aα 20−78, Bβ 54−122, γ 1−53)$_2$; ECL, luminol-based enhanced chemiluminescence method; ECM, extracellular matrix; Fg, fibrinogen; XL-Fb, cross-linked fibrin II (des-FPA/des-FPB) prepared by clotting fibrinogen with thrombin in the presence of factor XIII; FPA, fibrinopeptide A (Aα 1−16); FPB, fibrinopeptide B (Bβ 1−14); FRA, fibrin(ogen)-related antigen; Hi2-DSK, a hydrophilic disulfide containing CNBr fragment (Aα 241−476) derived from the Aα-chain of fibrinogen; HPLC, high-performance liquid chromatography; HRPO, horseradish peroxidase; MMP-1, matrix metalloproteinase 1, interstitial collagenase (EC 3.4.24.7); MMP-2, matrix metalloproteinase 2, gelatinase A (EC 3.4.24.24); MMP-3, matrix metalloproteinase 3, stromelysin 1 (EC 3.4.24.17); MoAb, monoclonal antibody; PTH, phenylthiohydantoin; RAM, rabbit anti-mouse immunoglobulin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNE, 0.05 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4.

S0006-2960(96)00730-1 CCC: $12.00

Both matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 2 (MMP-2) can only partially degrade Fg, and MMP-2 has only a very limited capacity to degrade XL-Fb. We have determined that, in XL-Fb, the γ Gly 404−Ala 405 bond is a major MMP-3 cleavage site leading to the formation of a fragment D-like monomer. This indicates a specific mechanism of fibrin degradation, different from plasmin, by an endogenous enzyme.

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents. Lyophilized human Fg (Fg ≥95% clottable, according to the manufacturer) was purchased (catalog no. 437, lot no. F187-1 and lot no. F194-1; American Diagnostica Inc., Greenwich, CT). Plasminogen and fibronectin were removed by affinity chromatography on lysine−Sepharose and gelatin−Sepharose, essentially as described (Deutsch & Mertz, 1970; Engvall & Ruoslahti, 1977; Procyk et al., 1985). The amount of factor XIII in these preparations is 0.1−0.2 Loewy unit/mg of Fg according to the manufacturer. Stock solutions of Fg (12 mg/mL in TNE buffer) were stored at −70 °C until used. Fg concentration was measured spectrophotometrically in alkaline−urea using an extinction coefficient (1%, 1 cm) = 16.5 at 282 nm. The clottability of the two fibrinogen preparations used in the study was 97% and 94%, respectively, after removal of plasminogen and fibronectin. Human Glu-plasminogen (1 unit/0.5 mg) was from Imco (Stockholm, Sweden). Streptokinase (4500 unit/mg solid), bovine serum albumin (BSA, fraction V, RIA-grade), 4-aminophenylmercuric acetate (APMA), and EDTA were from Sigma Chemical Co. (St. Louis, MO). Aprotinin was from Mobay Chemical Corp. (New York, NY). Human α-thrombin (2300 units/mg) was a generous gift of Dr. J. Fenton. ^{125}I-Fg, labeled by the iodogen method (specific activity 1.5×10^6 cpm/μg of protein), was a generous gift of Drs. M. Nag and D. Banerjee, Laboratory of Membrane Biochemistry II, The New York Blood Center. Pro-MMP-1, pro-MMP-2, and pro-MMP-3 were purified as previously described (Okada et al., 1986, 1990; Suzuki et al., 1990). All other reagents were of analytical grade and were purchased from Fisher Scientific (Springfield, NJ).

Degradation of Fg. Fg (120 μg, 3.5 μM) or fibronectin (120 μg, 2.7 μM) was incubated with MMP-1, MMP-2, or MMP-3 (2−60 μg/mL, corresponding to 43 nM−1.3 μM or 1:600−1:20, E:S ratio) at 37 °C for different time intervals. Pro-MMP-1, pro-MMP-2, and pro-MMP-3 (in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Brij 35, and 0.05% NaN_3) were activated with 1 mM APMA at 37 °C for 6 h, 45 min, and 24 h, respectively, prior to addition to fibronectin and Fg solutions. All reactions were in the presence of 10 mM $CaCl_2$ at 37 °C. Digestions were terminated by addition of EDTA (25 mM final concentration). Reaction products were mixed with reducing or nonreducing buffer and subjected to SDS−PAGE.

Thrombin Clotting Times and Fibrinopeptide Release. Digests (1.5 and 3 h) of Fg with MMP-2 and MMP-3 were prepared as described above. A plasmin digest of Fg (18−20 h) (Gårdlund et al., 1972) was used as control. Intact Fg and Fg digests were clotted with thrombin as described (Bini et al., 1994). Briefly, 1.2 mg of Fg/mL or of any fibrinogen digest was clotted with thrombin (0.4 NIH unit/mL) in the presence of 20 mM $CaCl_2$. Clotting time was determined as the increase in turbidity and read at 350 nm (Blombäck & Okada, 1982). Clotting time was determined from the plot of turbidity versus time. A tangent was drawn to the steepest part of the curve; its intersection with the time axis is defined as clotting time (Blombäck et al., 1994). In all cases, the gels were formed without any observed precipitation. Clot supernatants were run on HPLC (Kudryk et al., 1989) in order to determine the release of fibrinopeptides A (FPA) and B (FPB).

Table 1: Specificity of Monoclonal Antibodies

antibody	cross-reacts with	binds native fibrinogen	reference
1D4	Aα 349−406	yes	Procyk et al., 1991
T2G1	Bβ 15−42	no	Kudryk et al., 1984
4−2	γ 392−406	no	Kudryk et al., 1991
4A5	γ 397−411	yes	Matsueda & Bernatowicz, 1988

Degradation of XL-Fb Clots. Radiolabeled clots were made with 0.1 mL of purified Fg (1.2 mg/mL in TNE buffer) containing ^{125}I-Fg (20 000 cpm) in the presence of 20 mM $CaCl_2$. Thrombin (1.5 NIH units/mL, final concentration) was added, and samples were incubated at 37 °C for 18−20 h (Bini et al., 1994). Active MMP-1, MMP-2, and MMP-3 were added in different amounts (2−60 μg/mL, corresponding to 1:600−1:20 E:S ratio) in the presence of 10 mM $CaCl_2$. Plasmin was generated by adding plasminogen (50 μg/mL) and streptokinase (1080 units/mL) to the fibrin clots (approximately 0.02−0.5 unit/mL of plasmin, final concentration, corresponding to 1:1200−1:48 E:S). Clots were gently dislodged from the wall of the test tube with a wooden stick and the contents lightly vortexed after addition of each enzyme. Incubation times were from 1 to 48 h at 37 °C. Digests with MMPs were terminated by addition of EDTA (25 mM final concentration) and those with plasmin with 5000 KIU/mL aprotinin. Clots were separated from supernatants by centrifuging at 13 000 rpm for 20 min in a Sorvall RCL-B centrifuge (SS-34 rotor). Samples were counted in a Packard Auto-γ-5000 Series gamma counter. Control fibrin clots, with and without digestion with the different enzymes, were made at the same time to be used for SDS−PAGE. The extent of cross-linking was judged by the disappearance of α- and γ-chains and the formation of $\gamma-\gamma$ dimers and α polymers as in previous work (Bini et al., 1994).

Monoclonal Antibodies (MoAbs). The specificity of each antibody (all are of the IgG1, κ isotype) used in this study is summarized in Table 1.

Gel Electrophoresis/Immunoblotting. Samples of Fg and XL-Fb degraded with plasmin or MMPs were subjected to SDS−PAGE using both reducing and nonreducing conditions. Reduced samples were prepared in 62.5 mM Tris buffer, pH 6.8, containing 4% SDS, 8 M urea, 5% DTT, 10% glycerol, and 0.1% Bromophenol Blue. Nonreduced samples were made in the same buffer without DTT. SDS−PAGE was performed using 5−15% gradient or 12.5% polyacrylamide gels in Tris−glycine buffer (Laemmli, 1970) or with 5% and 7.5% mini gels in phosphate buffer (McDonagh et al., 1972) following general procedures. Prestained molecular mass standards used were myosin (200 kDa), phosphorylase B (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa) (Bethesda Research Laboratories, Gaithersburg, MD). Transfer to nitrocellulose membranes for immunoblot analyses was as described (Towbin et al., 1979) with few modifications (Kudryk et al., 1989). In some experiments, membranes

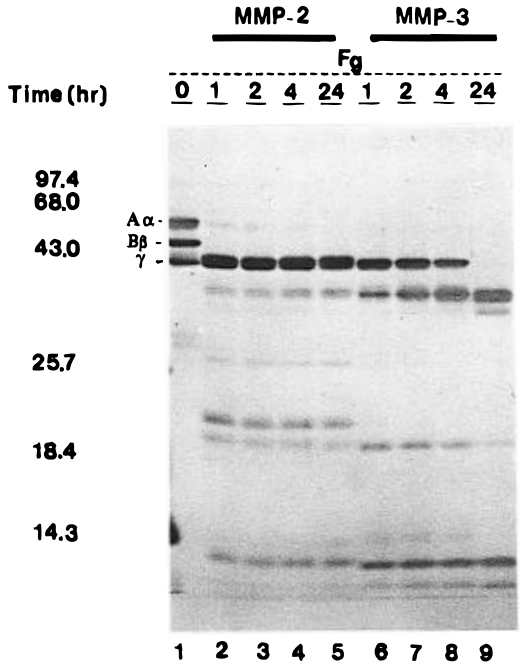

FIGURE 1: Degradation of Fg with MMP-2 and MMP-3. Both enzymes were used at E:S = 1:20 (w/w), and incubation was for 1, 2, 4, and 24 h at 37 °C. Digests were terminated by the addition of EDTA (25 mM final concentration). The following samples were reduced and separated by SDS−PAGE (12.5%): nondigested Fg (lane 1); Fg digested with MMP-2, lanes 2−5; Fg digested with MMP-3, lanes 6−9. The gel was stained for protein with Coomassie Blue.

were stained with colloidal gold prior to immunoblotting (Colloidal Gold Total Protein Stain; BioRad, Hercules, CA). Membranes were blocked with 5% dry milk (Carnation; Nestlé, Glendale, CA) or with 5% BSA, incubated overnight with a selected primary antibody (Table 1), and then probed with a second antibody. Rabbit anti-mouse horseradish peroxidase (RAM-HRPO) was prepared as described (Goding, 1986) using RAM purchased from Dako (Carpinteria, CA) and HRPO (type VI) from Sigma. Bound peroxidase complexes were detected using the chemilumiscent substrate (ECL Western blotting detection system; Amersham Life Science, Arlington Heights, IL). Light emitted from the hydrolysis of the added Luminol substrate exposed the provided film (Kodak X-Omat RP; Eastman Kodak Co., Rochester, NY) in 10−30 s.

Amino Acid Sequence Analyses. Fg and XL-Fb digested with MMP-3 were separated under reducing conditions on a 12.5% SDS−PAGE (Laemmli, 1970) and electroblotted to a poly(vinylidene difluoride) membrane (PVDF) (Matsudaira, 1987). Digests for sequencing were also made from Fg devoid of plasminogen, fibronectin, and factor XIII and with different preparations of MMP-3. The portion of the membrane that contained the γ-chain fragment was excised and subjected to automated sequencing on a Model 477A Applied Biosystems Inc. pulsed liquid-phase sequencer with a Model 120A on-line phenylthiohydantoin amino acid analyzer.

CNBr Degradation of MMP-3-Digested XL-Fb. To identify the MMP-3 cleavage site in the γ-chain cross-link domain, a 24 h MMP-3 digest of XL-Fb was degraded with CNBr (Blombäck et al., 1968). Resulting fragments were purified by reverse-phase HPLC using a Vydac C-4 column (1.0 × 25 cm; The Sep/a/ra/tions Group, Hesperia, CA). The column was developed at room temperature using the following gradient constructed with 0.05% trifluoroacetic acid (TFA, solvent A) and 50% acetonitrile in A (solvent B): at 5% B/0.5 min; 5−50% B/at 50 min; 50−100% B/at 70 min; 100% B/at 75 min. Column flow rate was 1.0 mL/min, and fractions (1 mL) were monitored for reactivity with MoAb/4-2 (anti-γ392−406). The antibody-reactive fraction was further purified by FPLC using the Superdex Peptide HR 10/30 column [1.0 × (30−31) cm; Pharmacia Biotech, Piscataway, NJ]. The column was developed at room temperature using 20 mM phosphate buffer (pH 7.2), additionally containing 0.25 M NaCl. The fraction reacting with MoAb/4-2 was pooled, desalted by passage over Sep-Pak (Millipore Corp., Milford, MA), and subjected to sequence analysis.

Table 2

sample	E:S ratio, w/w	incubation time (h)	thrombin time	turbidity (same day)	turbidity (next day)
Fg control[a]			68 s	0.841	0.973[b]
enzymes					
MMP-2	1:200	1.5	>10 min	0.433	0.963
		1.5	>10 min	0.377	0.823
		3	>10 min	0.681	1.039
		3	>10 min	0.758	1.125
MMP-3	1:600	1.5	>10 min[c]		0.025
		1.5	>10 min		0.035
		3	>10 min		0.004
		3	>10 min		0.029
MMP-3	1:200	1.5	>10 min[d]		0.001
		1.5	>10 min		0.000
		3	>10 min		0.008
		3	>10 min		0.012
plasmin	1:1200	1.5	107.5 s	0.396	
		1.5	103.9 s	0.399	
		3	139.1 s	0.502	
		3			
plasmin	1:240	1.5	unclottable		
		1.5	unclottable		
		3	unclottable		

[a] Fibrinogen (Fg) concentration in all samples: 12 mg/mL. [b] $n = 5$. [c] Soft clot the next day at both 1.5 and 3 h. [d] No clot the next day.

RESULTS

Fg Degradation. Using MMP-2 and MMP-3 in comparable amounts (6 μg/120 μg of Fg), both the Aα- and Bβ-chains of Fg were extensively degraded in 1 h (Figure 1, lanes 2 and 6). A longer (24 h) incubation resulted in further cleavage of both chains (lanes 5 and 9). Degradation of Fg γ-chains with MMP-3 was extensive at 24 h (Figure 1, lane 9) and different from that with MMP-2 (lane 5). Significant degradation with MMP-2 and MMP-3 was also obtained at lower concentration of enzyme (0.2−0.6 μg/120 μg of Fg, data not shown). MMP-1 at the highest concentration (6 μg/120 μg of Fg) showed apparently intact Bβ- and γ-chains and only partial degradation of the Aα-chains (data not shown).

Gelation of Fg Degraded with MMP-3. Fg digested with MMP-2 (1.5 and 3 h) did not clot within 10 min (arbitrarily taken as maximum time), but was still capable of forming a fibrin gel after overnight incubation with thrombin as shown by turbidity data (Table 2). Differently, both MMP-3 and plasmin digests, at comparable time and concentration, were unclottable even after overnight incubation. Turbidity data indicated that gelation was obtained only from reaction mixtures of Fg digested with MMP-2 and with the lowest plasmin concentration. Furthermore, turbidity values of the same preparations measured the next day showed that

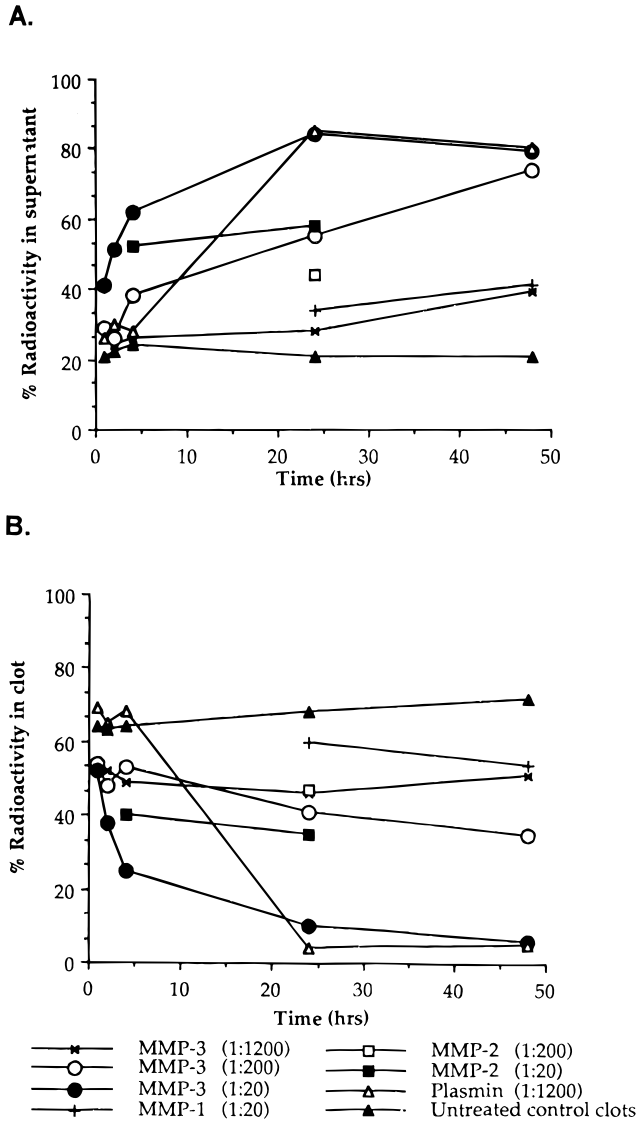

FIGURE 2: Degradation of XL-Fb. XL-Fb clots were prepared with Fg (120 μg), ^{125}I-Fg (20 000 cpm), and thrombin (1.5 NIH units/mL) in buffer containing 20 mM $CaCl_2$. MMPs were added in the indicated amounts (E:S, w/w) for different time intervals. Fibrinolysis was measured both by the release of radioactivity into the supernatant (A) and by the residual radioactivity in each clot (B). Controls, with no addition of enzyme as well as a plasmin digest of XL-Fb, are also shown. Data represent mean values of 2−4 separate experiments.

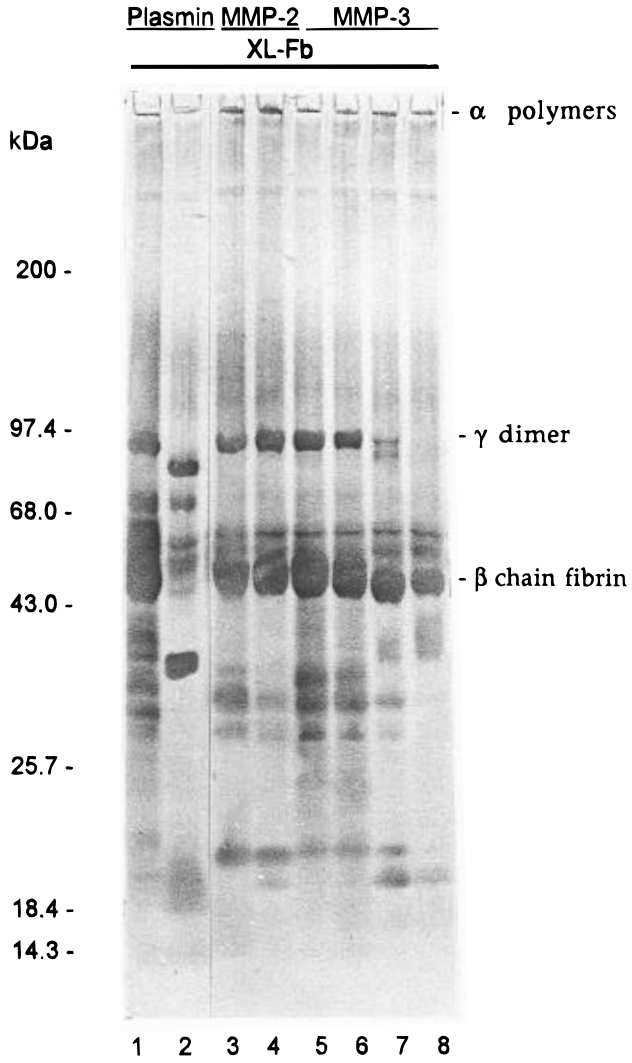

FIGURE 3: Chain composition of XL-Fb digested with different MMPs and plasmin. XL-Fb was digested (24 h) at different E:S ratios. Digests with MMPs were terminated with EDTA (25 mM final concentration) and those with plasmin with aprotinin (5000 KIU/mL). The digests were reduced and separated by SDS−PAGE (5−15% gradient). Samples of XL-Fb digested with the indicated enzymes were as follows: plasmin, 1:1200 and 1:240 (lanes 1 and 2); MMP-2, 1:200 and 1:20 (lanes 3 and 4); MMP-3, 1:600, 1:200, 1:20, and 1:10 (lanes 5−8). After transfer to nitrocellulose, the membrane was stained for protein with colloidal gold.

reaction mixtures of Fg and MMP-2 were similar to control while Fg digests generated by MMP-3, or plasmin, had low or no turbidity (Table 2). HPLC profiles of supernatants from all digests showed normal release of fibrinopeptides A and B with thrombin (not shown).

XL-Fb Degradation. Clots prepared with Fg/^{125}I-Fg were incubated with MMP-1, MMP-2, and MMP-3, or plasmin, at various concentrations for different time intervals. After a 24 h incubation, both MMP-3 (6 μg/120 μg of Fg) and plasmin (0.02 IU/mL) released over 80% of the radioactivity into the supernatant (Figure 2A). Residual radioactivity in the clot at 24 h was ≤10% (Figure 2B). The concentration of plasmin used in the lysis experiments was chosen on the basis of obtaining a slow lysis (Liu et al., 1986). In preliminary experiments, similar release of radioactivity was measured in the supernatants using plasmin at 1:240 and 1:1200 ratio (E:S, w/w), which was used throughout the study. Degradation with MMP-2 at the highest concentration, at 24 h, was similar to that obtained with MMP-3 at 1:200. Degradation with MMP-1 at the highest concentration, after 48 h, was similar to MMP-3 at 1:1200. The clottability of both cold fibrinogen preparations used in these experiments was ≥94%, as described under Experimental Procedures. However, the decrease in clottability, seen in the experiments shown in Figure 2, might be due to instability caused by the radioiodination procedure combined with the efficiency of the γ-counter.

Chain Composition of XL-Fb Degraded by MMPs and Plasmin. XL-Fb digested with MMP-2, MMP-3, or plasmin was analyzed by SDS−PAGE (Figure 3). The colloidal gold stained nitrocellulose membrane shows intact cross-linked fibrin γ-dimer chain (94 kDa) degraded by plasmin into DD γ-dimer chain (76 kDa) at a higher E:S ratio (lane 2). Previously described early degradation of the α-chain is shown (50 kDa) (Pizzo et al., 1973; Takagi & Doolittle, 1975), which is further degraded in lane 2. No degradation of cross-linked fibrin γ-dimer was observed with MMP-2,

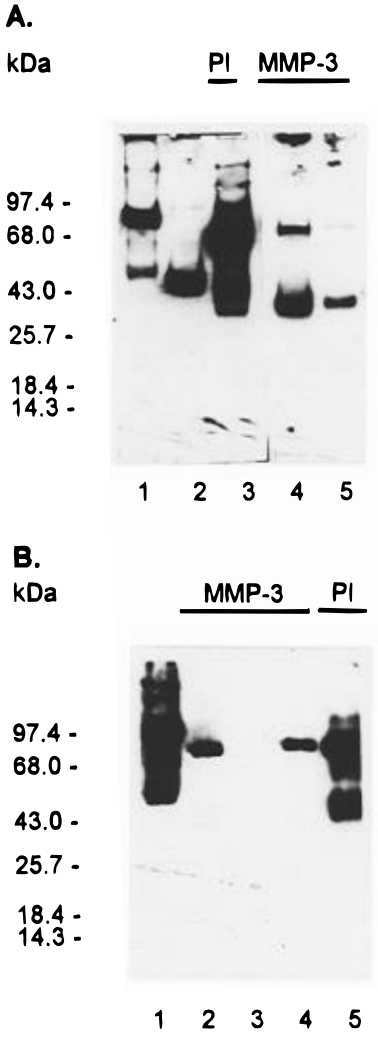

FIGURE 4: MMP-3-induced cleavage of the γ-chain cross-link domain. Samples were subjected to SDS−PAGE (7% gels) under reducing conditions. The following samples were electrophoresed: (A) XL-Fb clot incubated at 37 °C/24 h without enzyme (lane 1); Fg (lane 2); plasmin digest (24 h) of XL-Fb (lane 3); MMP-3 (1:20, w/w) digest (24 h) of XL-Fb, residual clot (lane 4); MMP-3 (1:20, w/w) digest (48 h) of XL-Fb, total digest (lane 5). (B) XL-Fb clot incubated at 37 °C/48 h without enzyme (lane 1); MMP-3 (1:20, w/w) digest (24 h) of XL-Fb, residual clot (lane 2); clot supernatant (lane 3); total digest (lane 4); plasmin digest (24 h) of XL-Fb (lane 5). After electrophoresis, samples were transferred to nitrocellulose, and membranes were blotted with either MoAb/4-2 (anti-γ392−406) or MoAb/4A5 (anti-γ397−411), (A) and (B), respectively. Specific antibody-bound fibrin(ogen) chains were detected using RAM-HRPO and the chemiluminescent substrate.

at the highest E:S ratio (1:20) (lane 4). Significant degradation of this chain was obtained with MMP-3 (E:S = 1:20) at 24 h (lane 7), and complete degradation was obtained by increasing E:S 2-fold (lane 8). The pattern of degradation of the cross-linked fibrin γ-dimer chain by MMP-3 is different from that obtained with plasmin.

To further identify chain remnants of XL-Fb digested with MMPs and plasmin, immunoblot analysis was performed using MoAbs specific for defined-sequence epitopes present on the three chains (Table 1). As shown in Figure 4A, MoAb/4-2 (anti-γ392−406) reacted with both XL-Fb γ-dimer (94 kDa, lane 1) and Fg γ-chain (47 kDa, lane 2). Residual γ-chain monomer in the cross-linked fibrin preparation also reacted with MoAb/4-2 (lane 1). DD γ-dimer (76 kDa) and fragment D γ-chain monomer [resulting from both Fg and XL-Fb plasmin digests (Siebenlist & Mosesson, 1992)] also

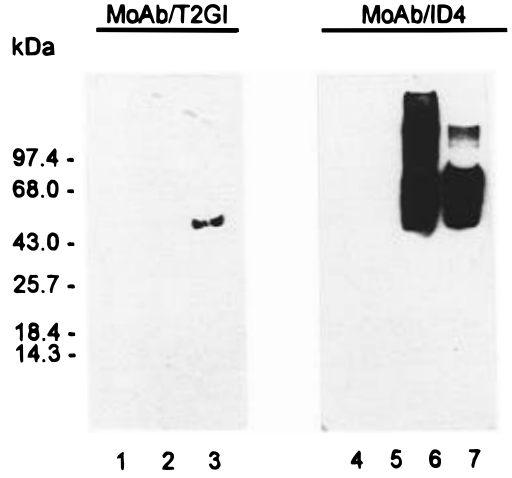

FIGURE 5: MMP-3-induced cleavage of fibrin β- and α-chains. Samples were subjected to SDS−PAGE (7% gels) under reducing conditions. The following samples were electrophoresed: MMP-3 (1:200, w/w) digest (24 h) of XL-Fb (lanes 1 and 4); MMP-3 (1:20, w/w) digest (24 h) of XL-Fb (lanes 2 and 5); nondigested XL-Fb (lane 3); XL-Fb clot incubated at 37 °C/24 h without enzyme (lane 6); Fg (lane 7). After electrophoresis, samples were transferred to nitrocellulose, and membranes were blotted with either MoAb/T2G1 (anti-Bβ15−42) or MoAb/1D4 (anti-Aα349−406), left and right panels, respectively. Specific antibody-bound fibrin(ogen) chains were detected using RAM-HRPO and the chemiluminescent substrate.

react equally with MoAb/4-2 (lane 3). A 24 h digest of XL-Fb, generated by MMP-3, showed reduced DD γ-dimer chain in the residual clot but significant amounts of fragment D monomer-like γ-chain (36 kDa, lane 4). A longer incubation (48 h) showed only Fragment D monomer-like γ-chain in the total digest (lane 5). Digests with both enzymes bound MoAb/4-2. Analysis of these same digests with MoAb/4A5 (anti-γ397−411) showed reactivity only with the γ-dimer band from both intact and plasmin-digested XL-Fb (Figure 4B, lanes 1 and 5) and with residual DD γ-dimer from digests generated by MMP-3 (lanes 2 and 4). The fragment D monomer-like γ-chain (36 kDa), fully reactive with MoAb/4-2 (Figure 4A, lanes 4 and 5), failed to bind MoAb/4A5 (Figure 4B, lanes 2 and 4). MoAb/4A5 also reacts with $\alpha\gamma$-diads as shown by a band with higher molecular mass than γ-dimer in lane 1. The gels were purposefully overloaded to show the difference in reactivity of the two antibodies. Immunoblot analysis of the same samples under nonreducing conditions showed similar loss of immunoreactivity with MoAb/4A5 (data not shown).

Immunoblot analysis of MMP-3 digests of XL-Fb using MoAb/T2G1 (anti-Bβ15−42) and MoAb/1D4 (anti-Aα349−406) is shown in Figure 5. MoAb/T2G1 immunoreactivity was lost in MMP-3 digests (lanes 1 and 2), while present on the β-chain of XL-Fb (lane 3). MoAb/1D4 immunoreactivity was also completely lost in MMP-3 digests (lanes 4 and 5).

Digestion of Fragment D-Dimer (DD) by MMP-3. As shown in Figure 6A, progressive degradation of DD (186 kDa) into a D-monomer-like fragment was observed in a time-dependent manner. The size of the resulting monomer was slightly larger than fragment D (93 kDa), obtained during plasmin degradation of Fg. Analysis of products under reducing conditions indicated that the DD γ-dimer chain (76 kDa) was converted to the γ-monomer-like chain.

Analysis of MMP Digestion Products by ELISA. Polyvinyl microtiter plates were coated with appropriate dilutions of different digests, and binding was determined as described

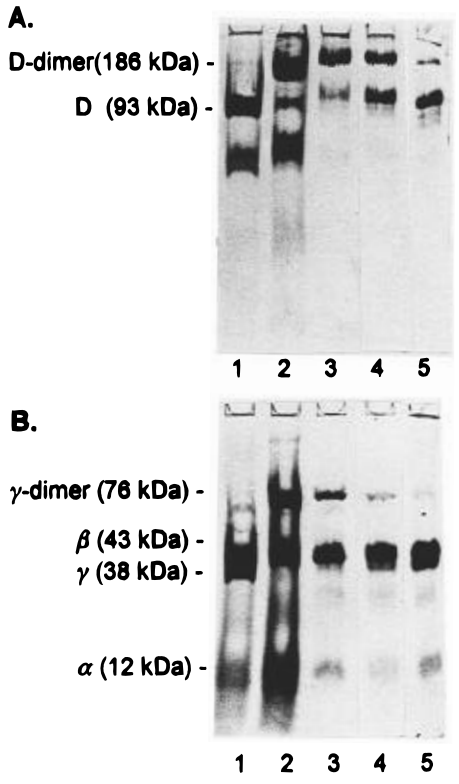

FIGURE 6: Degradation of fragment D-dimer (DD) with MMP-3. Purified DD was digested at 37 °C with MMP-3 (1:20) and for different time intervals. The reactions were terminated with 25 mM EDTA. Digest products were separated on SDS−PAGE (7% gels) under both nonreducing (panel A) and reducing (panel B) conditions. The following samples were electrophoresed: plasmin digest of Fg (lane 1); plasmin digest of XL-Fb (lane 2); MMP-3 (1:200) digest (4 h) of DD (lane 3); MMP-3 (1:20) digest (4 h) of DD (lane 4); MMP-3 (1:20) digest (24 h) of DD (lane 5). The gel was stained for protein with Coomassie Blue.

(Kudryk et al., 1984). In accord with the results shown in Figure 4A,B, digests with increasing concentrations of MMP-3 failed to bind MoAb/4A5 (anti-γ397−411) but were reactive with MoAb/4-2 (anti-γ392−406). Results from ELISA (direct binding) with MMP-3 digests and MoAb/1D4 (anti-Aα349−406) suggest that the 1D4-reactive epitope in such digests is destroyed. This epitope is not destroyed by trypsin treatment of Fg. MoAb/1D4 binds equally to plasmin digests of Fg and XL-Fb.

Size and Sequence Analysis of Chains of MMP-3-Digested Fg and XL-Fb. Fg and XL-Fb digested with MMP-3 were subjected to SDS−PAGE under reducing conditions, and the proteins were electroblotted on a PVDF membrane. The γ-chain sequence for both digests gave Leu-Lys-Ser-Arg-Lys, indicating that MMP-3 cleaves both Fg and XL-Fb at the γ Thr 83−Leu 84 bond. This same band from the XL-Fb digest also gave a second γ-chain sequence (Ala-X-Gln-Ala-Gly-Asp), indicating hydrolysis at the γ Gly 404−Ala 405 bond, in the γ-chain cross-link region.

Characterization of a CNBr-Derived γ-Chain Fragment from MMP-3 Digests of XL-Fb. To confirm the results obtained from sequencing the γ-chain of XL-Fb degraded with MMP-3, the same digest was treated with CNBr, and the fragment reactive with MoAb/4-2 (anti-γ392−406) was isolated. This same peptide did not react with MoAb/4A5. The sequenced fragment corresponded to a cross-linked dipeptide with NH_2-terminals γ Lys 385, resulting from CNBr cleavage, and γ Ala 405 (Figure 7). The two sequences were recovered in comparable molar quantities. In cycle 3, NH_2-terminal analyses showed no PTH residues (due to a machine error) but evidence for Gln and Ile in cycle 3 was seen in cycle 4 as lag. These data support our conclusion that MMP-3 cleaves at γ Gly 404−Ala 405 within the cross-link region of the fibrin γ-chain.

DISCUSSION

The first part of this paper characterizes the proteolytic activity of three members of the MMP family on soluble fibrinogen (Fg), which is not normally part of the extracellular matrix. MMP-1 partially degrades only the Fg Aα-chain at high E:S ratio. MMP-2 and -3 also degrade this chain but more extensively, more rapidly, and at a much lower E:S ratio. Fg digested with MMP-3 is thrombin-unclottable as is Fg treated with plasmin. Degradation with MMP-2, at higher E:S ratios, resulted in Fg digests which, on subsequent incubation with thrombin, produced gels with increasing turbidity.

The XL-Fb clots were gradually degraded by MMP-3 and resulted in near-complete lysis at 24 h. The amount of degradation with MMP-3 (1:20) at 24 h was comparable to plasmin (1:1200). Therefore, MMP-3 is a slower fibrinolytic enzyme than plasmin. The rates of clot solubilization with MMP-1 and MMP-2 were much slower; at the same E:S ratio (1:20), only 34% and 58%, respectively, were solubilized after 24 h, whereas 84% was degraded by MMP-3. In digests with MMP-3 and plasmin, residual clot radioactivity was $\leq$10%. These results indicated that digestion of XL-Fb with MMP-3 progressed further, possibly with a different and more specific mechanism than that obtained with either MMP-1 or MMP-2. However, the weaker activity of MMP-2 may be due to rapid autolysis of the enzyme after activation by APMA (Okada et al., 1990).

The pattern of XL-Fb degradation with MMP-3 is different from that obtained with plasmin. In digests with MMP-3 (1:20), only very small amounts of γ-dimer remain. At higher levels of this same enzyme, no dimers can be detected. MMP-2 does not seem to affect the degradation of γ-dimer significantly. Two monoclonal antibodies, MoAb/4-2 and MoAb/4A5, reactive with different epitopes in the sequence γ 392−411, were used to define the regions of cleavage of XL-Fb by MMP-3 in comparison to plasmin. This segment of the chain contains residues (γ Gln 398 and γ Lys 406) that participate in covalent cross-linking [forming ϵ-(γ-Glu)−Lys isopeptide bonds] on neighboring molecules, leading to fibrin stabilization mediated by factor XIIIa (Chen & Doolittle, 1971). MoAb/4A5 recognizes an epitope in the COOH-terminal region of this peptide (γ 397−411), while MoAb/4-2 reacts with its NH_2-terminal end (γ 392−406). Both antibodies bind to microtiter plates coated with the plasmin-derived digest products fragments D and DD. Only MoAb/4A5 competes with such fragments when each is in solution (Kudryk et al., 1991). In a 24 h MMP-3 digest of XL-Fb, both residual DD γ-dimer chain and fragment D monomer-like γ-chain were reactive with MoAb/4-2. Longer (48 h) digests resulted in fragment D monomer-like γ-chain only, which was still reactive with MoAb/4-2. Analysis of these same digests with MoAb/4A5 (anti-γ397−411) showed only the DD γ-dimer band to be reactive. The fragment D monomer-like γ-chain failed to bind MoAb/4A5. Reactivity with MoAb/4-2 was expected since this antibody also binds a synthetic peptide whose sequence corresponds to γ 392−400. MoAb/4A5 reacts with synthetic peptides correspond-

Cycle	PTH Amino Acid / yield (pmol)	
1	K (215)	A (673)
2	I (648)	–
3	I*	Q*
4	P (406)	A (438)
5	F (352)	G (565)
6	N (219)	D (202)
7	R (200)	V (115)
8	L (313)	–
9	T (179)	–
10	I (349)	–

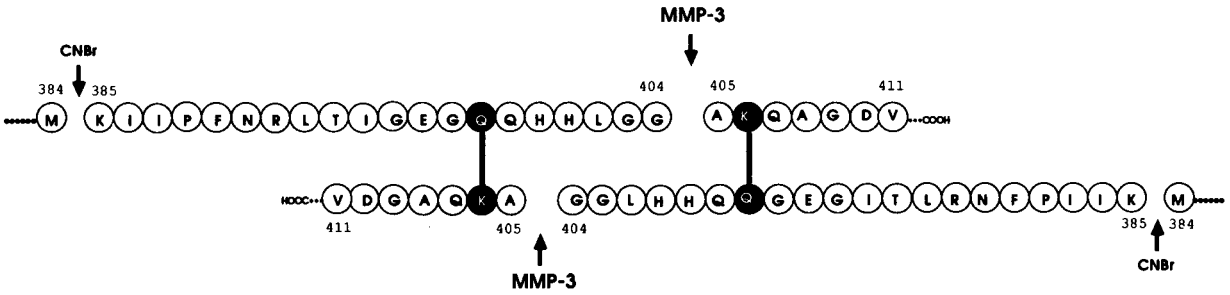

FIGURE 7: MMP-3 cleavage sites in the γ-chain of XL-Fb. The inset shows the partial sequence for the cross-linked dipeptide isolated from XL-Fb digested with MMP-3 after CNBr degradation. The dipeptide contains a single ϵ-(γ-Glu)−Lys bond, with one NH_2-terminal as γ Lys 385, resulting from CNBr cleavage at γ Met 384−Lys 385. The presence of the second NH_2-terminal, γ Ala 405, supports our conclusion that MMP-3 cleaves at γ Gly 404−Ala 405 in the cross-link region of the γ-chain.

ing to γ 392−411 and γ 397−411, but all activity is lost when either peptide is cleaved with trypsin at γ Lys 406−Gln 407. These results probably explained the lack of reactivity of MoAb/4A5 with both Fg and Fb γ-chain derived from digestion with MMP-3 and suggested that a major MMP-3 cleavage site was within the γ-chain cross-link domain resulting in the degradation of the γ-dimer. Purified fragment DD was also cleaved with MMP-3 to a D-monomer-like fragment.

MMP-3 degradation products of XL-Fb were also probed with two other MoAbs, T2G1 (anti-Bβ15−42) and 1D4 (anti-Aα349−406), respectively. Both epitopes are present in XL-Fb. In plasmin digests of XL-Fb, many different size bands ($\geq$20 kDa) react with MoAb/1D4, while all MoAb/T2G1 reactivity is lost. Even relatively low concentrations of MMP-3 (1:200) resulted in digests which failed to react with these antibodies on immunoblotting. No immunoreactivity with MoAb/1D4 in MMP-3 digests of XL-Fb could be detected by competition ELISA. This antibody reacts identically with Aα 349−406 and Hi2-DSK (Aα 241−476) before and after complete digestion with trypsin.

Results from ELISA (direct binding) performed on digests of Fg and XL-Fb with MMP-3 showed that the linear sequence epitope γ 392−406 (MoAb/4-2-reactive), but not γ 397−411 (MoAb/4A5-reactive), could be detected by this method. This confirmed the results obtained with immunoblotting and further suggested that MMP-3 cleaved within the sequence γ 397−411. NH_2-terminal sequence analysis of the dipeptide (isolated from CNBr degradation of the XL-fibrin digest with MMP-3) indicated γ Ala 405 as the first residue of the second sequence. As expected, the dipeptide reacted with MoAb/4-2 but not with MoAb/4A5, as in the total digests.

Data obtained from sequence analysis of MMP-3 digests of Fg and XL-Fb showed the proximity of cleavage sites with plasmin on these same substrates. Plasmin cleaves at γ Lys 62−Ala 63 and slower at γ Lys 85−Ser 86 (Collen et al., 1975). Fibrinogen digested by plasmin results in fragment D species with both γ Ala 63−Val 411 and γ Ser 86−Val 411. MMP-3 cleaves both Fg and XL-Fb at γ Thr 83−Leu 84. In addition, since MMP-3 hydrolyzes the Gly 404−Ala 405 peptide bond, the γ-chain has a very similar molecular weight to plasmin-generated fragment D γ-chain. However, in the digests of XL-Fb with MMP-3, this is not a real monomer since the sequence γ 405−411 is cross-linked to the adjacent branch of the γ-chain comprising the sequence γ Leu 84−Gly 404. It should be noted that the recovery of similar quantities of the two sequences from the γ-chains of the CNBr-generated dipeptide indicated that, albeit slow, the degradation of XL-Fb by MMP-3, resulting in formation of the D monomer-like fragment, was near-complete.

Enzymes other than plasmin can degrade fibrin(ogen) to different extents. Endogenous leukocyte proteases (Plow & Edgington, 1975; Bilezikian & Nossel, 1977), later identified as elastase and cathepsin-G [Gramse et al., 1978; Plow, 1980; reviewed in Plow and Edgington (1982)], can partially degrade fibrin(ogen). Fibrinolytic enzymes isolated from snakes can be grouped into two different classes (Guan et al., 1991). Those enzymes that preferentially degrade the Aα-chain of fibrinogen and also the α- and β-chains of fibrin are zinc metalloproteases, and all can be inhibited by EDTA. The second class of enzymes are serine proteinases and exhibit specificity for the β-chain of fibrin (Guan et al., 1991). An endopeptidase from Puff adder venom (*Bitis arietans*) can cleave at the γ-chain cross-linking region and thereby cleave fragment D-dimer into a D-like monomer (Purves et al., 1987). Similar activity has been shown for a proteinase from *Aeromonas hydrophila* (Loewy et al., 1993). The cleavage site of MMP-3 on the γ-chain seems identical to this enzyme and similar to the one from Puff adder venom. Enzymes from two different leeches (Budzynski, 1991; Zavalova et al., 1993) seem to share similar properties. However, MMP-3 is the first enzyme present in man to show this specificity. Therefore, this mechanism of degradation of cross-linked fibrin has been conserved, from bacteria to vertebrates, through different enzymes that might share a common ancestral origin. MMPs have also been identified in leukocytes (Welgus et al., 1990). It has been shown that MMP-2 and MMP-9 possess elastase activity (Senior et al.,

1991), to which some of the complex proteolytic activity, initially observed in granulocytes, could be attributed (Sterrenberg et al., 1983). MMP-3 has been shown to be expressed mainly in mature macrophages (Campbell et al., 1991), and recently in macrophage-derived foam cells from experimental atheroma (Galis et al., 1995).

In this report, we show the ability of MMP-3 to degrade Fg and to completely lyse XL-Fb clots degrading the D-dimer fragment to a D-like monomer by hydrolysis of the γ Gly 404−Ala 405 peptide bond. This novel activity seems to be specific for MMP-3 since both MMP-1 and MMP-2 can only partially degrade Fg and MMP-2 has only a limited capacity to degrade XL-Fb. Proteolysis of Fg and XL-Fb by MMP-3 might represent an additional fibrinolytic pathway of endogenous degradation. This mechanism might be of pathophysiological relevance in every process that involves fibrin formation and degradation, such as wound healing, inflammation, atherosclerosis, malignancy, and renal disease. The regulation of degradation of fibrin(ogen) in the ECM in the vessel wall might be an important factor in the development and complications of human atherosclerosis.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. G. Matsueda for his generous gift of monoclonal antibody 4A5 and Dr. J. G. Farmar (MicroChemistry Laboratory of the New York Blood Center) for NH_2-terminal analyses. We thank Norie Joson and Steve Callender for technical help, and Tellervo Huima-Byron and Ylena Oksov for excellent art work. We thank Drs. B. Blombäck, C. Redman, and C. Bianco for critically reviewing the manuscript.

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BI960730C