Characterization of Stromelysin 1 (MMP-3), Matrilysin (MMP-7), and Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Derived Fibrin(ogen) Fragments D-Dimer and D-like Monomer: NH_2-Terminal Sequences of Late-Stage Digest Fragments

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Received May 13, 1999; Revised Manuscript Received July 27, 1999

ABSTRACT: Matrix metalloproteinases (MMPs) participate in physiological remodeling of the extracellular matrix. Recently we determined that both fibrinogen (Fg) and cross-linked fibrin (XL-Fb) are substrates for selected MMPs. Specifically, XL-Fb clots were solubilized by MMP-3 (stromelysin 1) by cleavage at γ Gly 404−Ala 405, resulting in a D-like monomer fragment. Similarly, MMP-7 (matrilysin) and MT1-MMP (membrane type 1 matrix metalloproteinase) solubilized XL-Fb clots. However, the molecular mass of fragment D-dimer, obtained after MMP-7 and MT1-MMP degradation of XL-Fb, is similar to that of fragment D-dimer from plasmin degradation (∼186 kDa). In contrast, fragment D-like monomer, from MMP-3 degradation of both fibrinogen (Fg) and XL-Fb, is similar to fragment D from plasmin degradation of Fg (∼94 kDa). Reduced chains from MMP-3, MMP-7, and MT1-MMP digests of Fg and XL-Fb were subjected to direct sequence analyses and D/D-dimer α-chain showed cleavage at both α Asp 97−Phe 98 and α Asn 102−Asn 103. Degradation of the β-chain resulted in microheterogeneity of cleavage sites at β Asp 123−Leu 124, β Asn 137−Val 138, and β Glu 141−Tyr 142, whereas all three enzymes cleaved the γ-chain at γ Thr 83−Leu 84. In both Fg and XL-Fb, several cleavage sites obtained by proteolysis with MMP-3, MMP-7, and MT1-MMP were found to be in very close proximity to those obtained by plasmin on these same substrates. That does not occur with other MMPs such as MMP-1, -2, and -9 and MT2-MMP. The degradation of XL-Fb by MMPs suggests both plasmin-dependent and independent mechanisms of fibrinolysis that might be relevant in inflammation, angiogenesis, arthritis, and atherosclerosis.

Matrix metalloproteinases (MMPs)[1] are a family of proteolytic enzymes involved in remodeling of the extracellular matrix (ECM) by degrading a number of proteins that constitute the frame of connective tissue and basement membrane such as collagen, fibronectin, vitronectin, and laminin (*1*−*5*). MMPs are also implicated in angiogenesis (*6*, *7*) and destruction of the ECM in the pathophysiology of metastatic spread of cancer (*8*−*11*), in the rupture of atherosclerotic plaques (*12*−*14*), and in the breakdown of bone and cartilage in rheumatoid arthritis (*15*−*19*).

Recently we found the both fibrinogen (Fg) and cross-linked fibrin (XL-Fb) are substrates for matrix metalloproteinases (MMPs) (*20*). However, different classes of MMPs seem to have selected avidity for both Fg and XL-Fb. MMP-3 (stromelysin 1) can completely solubilize cross-linked fibrin. One of the main cleavage sites on both Fg and XL-Fb γ chain is Gly 404−Ala 405 (*20*). Proteolysis at this site in XL-Fb leads to formation of a D-like monomer fragment. In contrast, plasmin degradation of cross-linked

† Parts of this work were presented at the Thirty-Ninth Annual Meeting of the American Society of Hematology, San Diego, CA, December 5−9, 1997, and published in abstract form [(1997) *Blood* (Suppl I) *90*, 2066]. This work was supported in part by a grant from the Retirement Research Foundation, Chicago, IL.

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[1] Abbreviations: APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent 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) of fibrinogen or fibrin 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-fibrinopeptides A and B) prepared by clotting fibrinogen with thrombin in the presence of factor XIIIa; 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); MMP-7, matrix metalloproteinase 7, matrilysin (EC 3.4.24.23); MMP-9, matrix metalloproteinase 9, gelatinase B (EC 3.4.24.35). MT1-MMP, membrane type 1 matrix metalloproteinase (MMP-14); MT2-MMP, membrane type 2 matrix metalloproteinase (MMP-15); MoAb, monoclonal antibody; PTH, phenylthiohydantoin; RAM, rabbit anti-mouse immunoglobulin; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNE buffer, 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.1 M NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4.

10.1021/bi991096g CCC: $18.00
Published on Web 09/30/1999

fibrin lead to formation of fragment D-dimer (*21*, *22*). Previously, we also showed that MMP-2 (gelatinase A) was able to partially degraded Fg and XL-Fb, whereas MMP-1 (collagenase) displayed only a limited capacity to cleave either substrate (*20*).

Evidence accumulated in recent studies has shown that MMPs represent potential therapeutic targets in a number of diseases, some of which include fibrin deposition as part of the pathophysiological process. Therefore, we investigated the ability of other MMPs, such as MMP-7 (matrilysin) (*23*), membrane type 1 MMP (MT1-MMP) (*24*, *25*), and MT2-MMP to degrade fibrin(ogen). Recent work has also shown degradation of fibrin by MMP-3 and MT1-MMP (*7*). Moreover, very recently we detected MMP-degraded fibrin in human synovial fluid with new monoclonal antibodies (*26*).

The present work reports a detailed study on the proteolysis of Fg and XL-Fb with MMP-3, -7, and -9, MT1-MMP, and MT2-MMP. Digest products of either substrate prepared with any of these enzymes were characterized by SDS−PAGE and by immunoblot analysis with specific monoclonal antibodies (MoAbs). NH_2-terminal sequence studies on separated chain fragments of late-stage degradation products were performed to identify enzyme-sensitive cleavage sites.

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents. Lyophilized human Fg (Fg ≥95% clottable, according to the manufacturer), was purchased (catalog no. 437, lots F187-1 and F194-1, American Diagnostica Inc., Greenwich, CT). Plasminogen and fibronectin were removed by affinity chromatography on lysine−Sepharose and gelatin−Sepharose, essentially as described (*27*−*29*). The amount of factor XIII in these preparations was 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 with extinction coefficient (1%, 1 cm) = 16.5 at 282 nm. Clottability of the Fg preparations used in the study was ≥94%, after removal of plasminogen and fibronectin. Human Glu-plasminogen (1 unit/0.5 mg) was from Imco (Stockholm, Sweden). Streptokinase (4500 units/mg solid), bovine serum albumin (BSA, fraction V, radioimmunoassay grade), 4-aminophenylmercuric acetate (APMA), and EDTA were from Sigma Chemical Co. (St. Louis, MO). Aprotinin (Trasylol) was from Mobay Chemical Corp. (New York). The following reagents were generous gifts: human α-thrombin (2300 units/mg) from Dr. J. Fenton (Albany, NY); pro-MMP-3 and recombinant MMP-3 from Dr. H. Nagase (University of Kansas Medical Center, Kansas City, KS) and Dr. R. Palermo (Roche, Nutley, NJ); proMMP-7 from R. Martin (Roche Bioscience, Palo Alto, CA); proMMP-9 from Dr. W. Stetler-Stevenson (NIH, Bethesda, MD); and MT1- and MT2-MMPs from InVitek (Berlin, Germany). All other reagents were of analytical grade and were purchased from Fisher Scientific (Springfield, NJ). Activity of MMPs was determined at the source and by a fluorometric assay, with an HTS7000 BioAssay Reader (Perkin-Elmer) (*25*).

Degradation of Fg and XL-Fb. Briefly, Fg or XL-Fb (1.2 mg/mL Fg or 3.5 μM, 0.4 NIH unit of thrombin/mL, 20 mM $CaCl_2$) was incubated with MMP-3, -7, and -9 and MT1- and MT2-MMP (50 μg/mL corresponding to 0.5−2.5 μM or 1:24 w/w E:S ratio) at 37 °C for 24 h. ProMMP-3 and proMMP-7 (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 24 and 1 h, respectively. MMP-9 was similarly activated at 40 °C for 1 h. MT1- and MT2-MMP were active forms from the manufacturer. All reactions were in the presence of 10 mM $CaCl_2$ at 37 °C. Digestions were stopped with EDTA (25 mM). Reaction products were mixed with reducing or nonreducing buffer and subjected to SDS−PAGE.

Monoclonal Antibodies. MoAb/T54 was recently developed in our laboratory (*35*). MoAb/T54 reacts with D-like core fragments found in MMP-3 digests of Fg and XL-Fb and corresponding fragments present in MMP-7 digests of these same substrates. Antibody T54 does not react with intact fibrinogen but binds two peptides (Bβ 123−127 and Bβ 123−128) found in late-stage plasmin digests of Fg and XL-Fb. MoAb/4A5 (anti-γ 397−411) was a kind gift of Dr. G. Matsueda (Bristol-Myers Squibb, Princeton, NJ). This antibody reacts with intact fibrinogen and plasmin-generated fragments D and DD, but its immunoreactivity is lost upon degradation of fibrin(ogen) by MMP-3 (*20*).

Gel Electrophoresis/Immunoblotting. Samples of Fg and XL-Fb degraded with plasmin or MMPs were subjected to SDS−PAGE under 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 1% bromophenol blue. Nonreduced samples were made in the same buffer without DTT. SDS−PAGE was performed on 5−15% gradient or 10% and 12.5% polyacrylamide gels, as indicated, in Tris−glycine buffer (*30*) 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 (*31*) with few modifications (*32*). Membranes were stained with colloidal gold (colloidal gold total protein stain, Bio-Rad, Hercules, CA) prior to immunoblotting. Membranes were blocked with 5% dry milk (Carnation, Nestle, Glendale, CA), incubated overnight with a selected primary antibody and then probed with RAM-HRPO prepared as described (*33*) with RAM purchased from Dako (Carpinteria, CA) and HRPO (type VI) from Sigma. Bound peroxidase complexes were detected with 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 C., Rochester, NY) in 10−30 s. Molecular mass was calculated from 5−15% gradient SDS−polyacrylamide gels after scanning (ScanJet 6100C/T, Hewlett-Packard) and analysis with Gel-Pro Analyzer Software 2.0 (Media Cybernetics, Silver Spring, MD).

NH_2-Terminal Sequence Analyses. Fg and XL-Fb digested with MMP-3, -7, and -9 and MT1-MMP were separated under reducing conditions on an SDS−12.5% polyacrylamide gel (*30*) and electroblotted to a polyvinylidene difluoride membrane (PVDF) (*34*). Digests for sequencing were also made from Fg devoid of plasminogen and fibronectin and with different preparation of MMPs. The portion of the membranes corresponding to each protein band were excised

Table 1: NH_2-Terminal Sequence Analysis of Cross-Linked Fibrin Degraded with MMP-3 (Enzyme/Substrate Ratio 1/24) for 24 h at 37 °C

fragment (kDa)	fragment NH_2 terminus	amino acid recovery (pmol) in cycle							
		1	2	3	4	5	6	7	8
88	γ-84	Leu (1)	Lys (1)	Ser (1)	Arg (0.5)	Lys (1)	Met (1)	Leu (0.5)	Glu (0.2)
40	γ-84	Leu (10)	Lys (10)	Ser (10)	Arg (5)	Lys (4)	Met (5)	Leu (4)	Glu (−)[a]
	β-121	Leu (5)	Lys (4)	Asp (4)	Leu (2)	Trp (1)	Gln (5)	Lys (2)	Arg (1)
	γ-405	Ala (7)	Lys (*)[b]	Gln(4)	Ala (3)	Gly (1)	Asp (−)	Val (−)	
38	β-127	Lys (4)	Arg (4)	Gln(2)	Lys (3)	Gln (7)	Val (2)	Lys (1)	Asp (2)
	γ-84	Leu (4)	Lys (5)	Ser (2)	Arg (2)	Lys (2)	Met (2)	Leu (2)	Glu (5)
36	β-142	Tyr (5)	Ser (1)	Ser (1)	Glu (1)	Leu (3)	Glu (1)	Lys (2)	His (1)
14	γ-1	Tyr (4)	Val (6)	Ala (3)	Thr (3)	Arg (3)	Asp (2)	Asn (−)	Cys (−)
	α-103	Asn (11)	Arg (15)	Asp (7)	Asn (9)	Thr (3)	Tyr (8)	Asn (4)	Arg (6)
12.5	γ-1	Tyr (12)	Val (17)	Ala (10)	Thr (6)	Arg (8)	Asp (3)	Asn (5)	Cys (−)
	α-103	Asn (1)	Arg (1)	Asp (1)	Asn (1)	Thr (−)	Tyr (0.5)	Asn (−)	Arg (0.5)
	α-414	Leu (3)	Val (−)	Thr (0.2)	Ser (0.2)	Lys (1)	Gly (1)	Asp (2)	Lys (1)
12	α-17	Gly (12)	Pro (8)	Arg (6)	Val (8)	Val (15)	Glu (12)	Arg (9)	His (4)
	β-51	Thr (5.5)	Gln (9)	Lys (10)	Lys (6)	Val (−)	Glu (?)	Arg (−)	Lys (−)
	α-20	Val (6)	Val (7)	Glu (3)	Arg (5)	His (2.5)	Gln (2)	Ser (3)	Ala (5)
	β-52	Gln (4)	Lys (5)	Lys (−)	Val (−)	Glu (1)	Arg (1)	Lys (1)	Ala (−)

[a] (−) Recovery undetectable. [b] (*) Present as cross-link.

Table 2: NH_2-Terminal Sequence Analysis of Cross-Linked Fibrin Degraded with MMP-7 (Enzyme/Substrate Ratio 1/24) for 24 h at 37 °C

fragment (kDa)	fragment NH_2 terminus	amino acid recovery (pmol) in cycle							
		1	2	3	4	5	6	7	8
90	γ−84	Leu (3)	Lys (2)	Ser (1)	Arg (0.5)	Lys (1)	Met (1)	Leu (1)	Glu (0.5)
82	γ-84	Leu (15)	Lys (13)	Ser (1)	Arg (8)	Lys (9)	Met (6)	Leu (8)	Glu (1)
38	β-124	Leu (15)	Trp (8)	Gln (7)	Lys (11)	Arg (7)	Gln(6)	Lys (10)	Gln(6)
36	β-142	Tyr (2)	Ser (1)	Ser (1)	Glu (1)	Leu (1)	Glu (1)	Lys (0.5)	His (0.5)
14	α-98	Phe (16)	Ser (2)	Ser (2)	Ala (8)	Asn (5)	Asn (6)	Arg (7)	Asp (1)
12.5	γ-1	Tyr (20)	Val (18)	Ala (17)	Thr (5)	Arg (10)	Asp (6)	Asn (5)	Cys (−)[a]
	α-423	Leu (9)	Arg (5)	Thr (1)	Gly (3)	Lys (3)	Glu ()	Lys (4)	Val (3)
12	α-17	Gly (8)	Pro (10)	Arg (11)	Val (6)	Val (10)	Glu (5)	Arg (6)	His (6)
	α-20	Val (4)	Val (5)	Glu (4)	Arg (−)	His (1)	Gln(2)	Ser (3)	Ala (3)

[a] (−) Recovery undetectable.

and subjected to automated sequencing on a 494/HT Procise sequencing system controlled by Procise control software and a 610/A data analysis system.

RESULTS

Chain Composition of Fibrinogen and Cross-Linked Fibrin Degraded with MMP-3, -7, and -9. Fg and XL-Fb were incubated with MMP-3, -7, and -9 (1:24 w/w) at different time intervals. MMP-7 degradation of Fg leads to degradation of all chains at about 24 h with formation of two chain fragments of about 38 and 36 kDa (Figure 1, lanes 3−5) that were identified as originating from γ- and β-chains, respectively, by NH_2-terminal sequence (see Table 2). Identification of the γ-chain remnant was also made by immunoblot analysis (Figure 2). Degradation of XL-Fb with MMP-7 was extensive and led to formation of a degraded γ-dimer of about 82 kDa and a main β-chain remnant of about 38 kDa and a fainter one of about 36 kDa (Figure 1, lanes 7−9, and Table 2). Two lower molecular mass bands of 16 and 14 kDa appeared at 8−48 h in both Fg and XL-Fb degraded with MMP-7 (Figure 1, lanes 3−5 and 7−9) and, from sequence analysis, both originate from different regions of the α-chain (Table 2). Degradation of Fg and XL-Fb by MMP-3 (Figure 1, lanes 10−12) was as previously determined and shows, in the case of XL-Fb, degradation of γ dimer to a γ-like monomer (Figure 1, lanes 11 and 12, and Table 1). Degradation of XL-Fb by MMP-9 was minimal, showing only a 20 kDa fragment that was not present in the control cross-linked fibrin samples (Figure 1, compare lanes 13 and 14 with the control in lane 15).

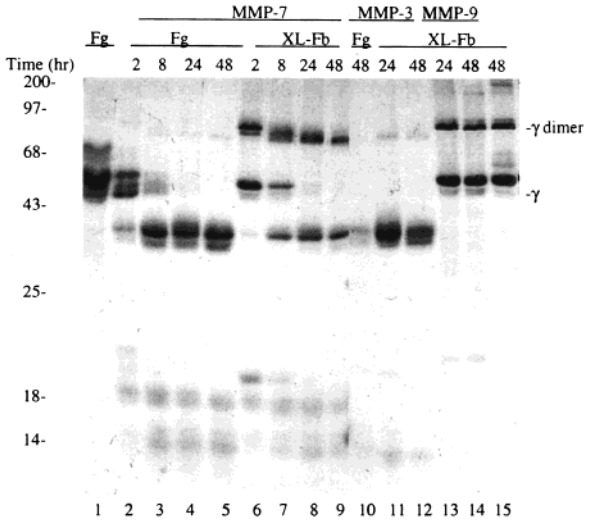

FIGURE 1: Chain composition of Fg and XL-Fb degraded with MMP-7, -3, and -9. All enzymes were used at E:S = 1:24 (w/w), incubated for the indicated time intervals at 37 °C, and stopped with EDTA (25 mM). The digests were reduced and separated by SDS−PAGE (12.5%). Samples of Fg digested with the indicated enzymes were: MMP-7 (lanes 2−5) and MMP-3 (lane 10). Samples of digested XL-Fb were MMP-7 (lanes 6−9), MMP-3 (lanes 11 and 12), and MMP-9 (lanes 13 and 14). Intact Fg is in lane 1 and nondegraded XL-Fb, incubated for 48 h as the treated samples, is in lane 15. The gel was stained for protein with Coomassie Blue.

Immunochemical Identification of the γ-Chain Remnants in the MMP-3, -7, and -9 Cleavage Products of Fg and XL-Fb. The same samples shown in Figure 1 were immuno-

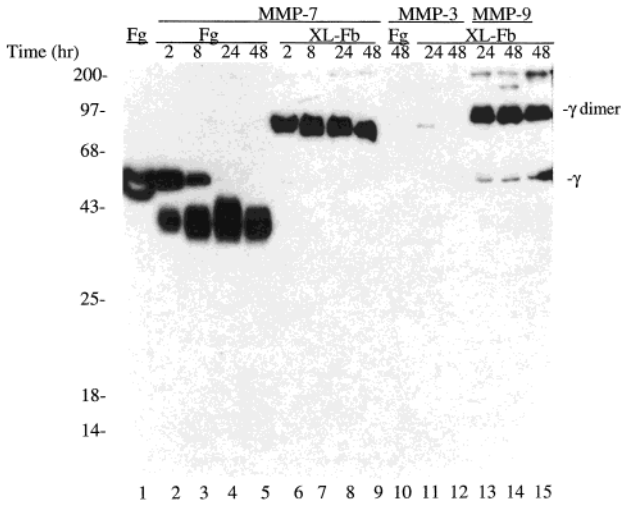

FIGURE 2: Identification of γ-chain remnants in MMP-3, -7, and -9 digests of Fg and XL-Fb. A nitrocellulose membrane containing the same reduced samples (in the same order) as in Figure 1 was immunoblotted with MoAb/4A5 (anti-γ 397−411). Specific antibody-bound fibrin(ogen) chain remnants were detected by use of RAM-HRPO and the chemiluminescent substrate.

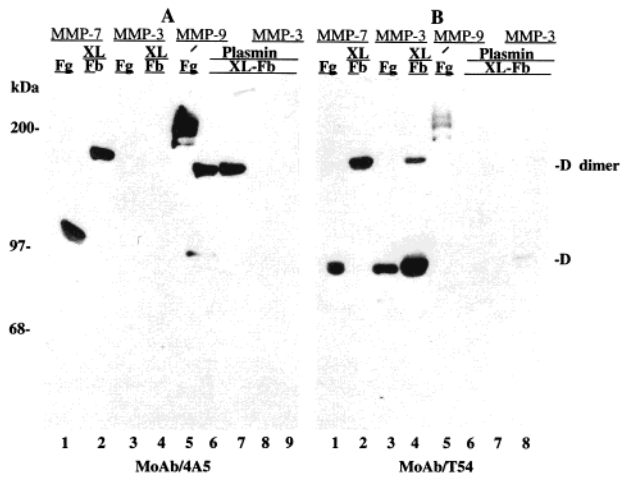

FIGURE 3: Immunoblot analysis of high molecular weight degradation products of Fg and XL-Fb generated by MMP-3, -7, and -9. Samples were subjected to SDS−PAGE (7.5%) under nonreducing conditions. The following samples were electrophoresed: MMP-7- (1:24 w/w) digested Fg (lanes 1) and XL-Fb (lanes 2); MMP-3- (1:24 w/w) digested Fg (lanes 3) and XL-Fb (lanes 4); MMP-9- (1:24 w/w) digested Fg (lanes 5); plasmin (1:20 w/w) digest of XL-Fb (lanes 6); plasmin (1:200 w/w) digest of XL-Fb (lane 7 in panel A only); plasmin (1:200 w/w) and MMP-3 (1:24) digest of XL-Fb (lane 8 in panel A and lane 7 in panel B); plasmin (1:200 w/w) and MMP-3 (1:24) digest of XL-Fb in the presence of aprotinin (lane 9 in panel A and lane 8 in panel B). Aliquots of the same samples were applied to two different gels. After electrophoresis, samples were transferred to nitrocellulose, and membranes were blotted with either MoAb/4A5 (panel A) or MoAb/T54 (panel B). Specific antibody-bound fibrin(ogen) chains were detected by use of RAM-HRPO and the chemiluminescent substrate.

blotted with MoAbs/4A5 (anti-γ 397−411) and T54 (anti-β 123−127) to evaluate the size and/or epitope integrity of the Bβ/β and γ/γ-dimer chains in either Fg or XL-Fb degraded with MMP-3, -7, and -9. Immunoblotting with MoAb/4A5 shows that Fg and XL-Fb degraded with MMP-7 still maintained the γ-chain 397−411 epitope reactive with MoAb/4A5 (Figure 2, lanes 2−9). XL-Fb degraded with MMP-9 (Figure 2, lanes 13 and 14) reacted similarly to intact XL-Fb with this same antibody (Figure 2, lane 15). MMP-3 digests of both Fg and XL-Fb did not react with MoAb/4A5, as previously described (*20*), since both γ/γ-dimer chains are cleaved by MMP-3 at the γ 404−405 peptide bond (Figure 2, lanes 10−12).

Immunoblotting of some of the same samples in Figures 1 and 2 under nonreducing conditions is shown in Figure 3. MoAb/4A5 reacts with fragment D and D-dimer from Fg and XL-Fb degraded with MMP-7 (Figure 3A, lanes 1 and 2) and plasmin (Figure 3A, lanes 6 and 7) but not with Fg and XL-Fb degraded with MMP-3 (Figure 3A, lanes 3 and 4). An MMP-9 digest of Fg (Figure 3A, lane 5) shows a fragment Y-like degradation product that is reactive with MoAb/4A5. When XL-Fb was first digested with plasmin (24 h) and second with MMP-3 (24 h), immunoreactivity with MoAb/4A5 was lost because of cleavage at the γ Gly 404−Ala 405 peptide bond as discussed above (Figure 3A, lane 8). Addition of aprotinin did not inhibit degradation by MMP-3 with previously plasmin-digested XL-Fb (Figure 3A, lane 9). Newly developed MoAb/T54 (anti-Bβ 123−127) was also utilized for immunoblot analysis of the same degradation products under nonreducing conditions. Late-stage plasmin degradation products of both Fg and XL-Fb do not react with MoAb/T54 since β Asp 134 was identified as a new NH_2-terminus (*36*). As shown in Figure 3B, MoAb/T54 reacts with fragment D/D-dimer obtained after degradation of Fg and XL-Fb with MMP-3 and MMP-7 (lanes 1−4) but not with plasmin-degraded XL-Fb (lanes 6−8). Little reactivity was obtained with this same antibody and the fragment Y-like degradation product generated from Fg incubated with MMP-9 (Figure 3B, lane 5).

Degradation of XL-Fb with Plasmin, MMP-3, or Plasmin/MMP-3 Mixtures for Different Time Intervals. XL-Fb clots were incubated at different time intervals in the presence of plasmin, plasmin and MMP-3, and MMP-3 alone in the presence/absence of aprotinin. Samples under reduced conditions were subjected to SDS−PAGE (12.5%). As shown in Figure 4, plasmin degradation of XL-Fb produced the expected degradation pattern with a γ-dimer of about 90 kDa and a β-chain of about 43 kDa at both higher (1:24 E:S ratio) and lower (1:200) enzyme concentrations (lanes 3−6). Addition of MMP-3 to XL-Fb with plasmin (Figure 4, lanes 7−9) exhibited virtually the same pattern as MMP-3 alone (Figure 4, lanes 13 and 14). Addition of aprotinin, in the presence or absence of plasmin, did not affect MMP-3 degradation of XL-Fb (Figure 4, lanes 10−12).

Size and NH_2-Terminal Sequence of Chain Remnants of Late-Stage Digests of Fg and XL-Fb by MMP-3, -7, and -9. Fg and XL-Fb were degraded with MMP-3, -7, and -9 for 24 h. Digests similar to those shown in Figures 1−3 were electroblotted onto PVDF membranes, and protein bands were sequenced. The γ-chain sequence for Fg and XL-Fb digests gave Leu-Lys-Ser-Arg-Lys, indicating that MMP-3 cleaved both substrates at the γ Thr 83−Leu 84 bond (Figure 5). 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 as previously described (*20*). The NH_2-terminal sequences of the other α-, β-, and γ-chain fragments excised from the PVDF membrane are as indicated in Figure 5. (Intact Fg chains are α 610 amino acids, 68 kDa, with Ala as the major NH_2-terminus; Bβ 461 amino acids, 57 kDa

Table 3: NH_2-Terminal Sequence Analysis of Cross-Linked Fibrin Degraded with MT1-MMP (Enzyme/Substrate Ratio 1/24) for 24 h at 37 °C

fragment (kDa)	fragment NH_2 terminus	amino acid recovery (pmol) in cycle							
		1	2	3	4	5	6	7	8
88	γ-84	Leu (4)	Lys (4)	Ser (0.8)	Arg (3)	Lys (2)	Met (2)	Leu (2)	Glu (1)
	γ-79	Ile (2)	Asp (3)	Ala (1)	Ala (1)	Thr (1)	Leu (1)	Lys (1)	Ser (0.2)
	γ-1	Tyr (2)	Val (1)	Ala (1)	Thr (0.5)	Arg (2)	Asp (2)	Asn (1)	Cys (−)
81	γ-84	Leu (10)	Lys (10)	Ser (3)	Arg (6)	Lys (5)	Met (6)	Leu (4)	Glu (2)
	γ-79	Ile (3)	Asp (5)	Ala (1)	Ala (1)	Thr (2)	Leu (2)	Lys (1)	Ser (0.3)
52	β-15	Gly (2)	His (1)	Arg (2)	Pro (1)	Leu (1)	Asp (1)	Lys (0.4)	Lys (1)
49	β-51	Thr (2)	Gln (2)	Lys (1)	Lys (1)	Val (1)	Glu (1)	Arg (1)	Lys (0.7)
38	β-124	Leu (7)	Trp (3)	Gln (5)	Lys (5)	Arg (6)	Gln (6)	Lys (3)	Gln (2)
36	β-138	Val (4)	Val (4)	Asn (2)	Glu (3)	Tyr (3)	Ser (4)	Ser (4)	Glu (1)
	β-142	Tyr (1)	Ser (0.3)	Ser (0.4)	Glu (0.5)	Leu (1)	Glu (1)	Lys (1)	His (0.5)
14	β-15	Gly (2)	His (0.7)	Arg (1)	Pro (0.5)	Leu (1)	Asp (2)	Lys (0.5)	Lys (1)
12.5	α-98	Phe (19)	Ser (1)	Ser (2)	Ala (8)	Asn (7)	Asn (9)	Arg (9)	Asp (3)
	α-103	Asn (6)	Arg (5)	Asp (4)	Asn (2)	Thr (0.6)	Tyr (2)	Asn (1)	Arg (4)
	α-414	Leu (6)	Val (3)	Thr (0.8)	Ser (1)	Lys (1)	Gly (2)	Asp (3)	Lys (2)
	β-51	Thr (3)	Gln (3)	Lys (1)	Lys (1)	Val (1)	Glu (2)	Arg (−)	Lys (2)
	γ-1	Tyr (2)	Val (1)	Ala (1)	Thr (−)	Arg (0.4)	Asp (0.6)	Asn (0.5)	Cys (−)
12	α-17	Gly (36)	Pro (12)	Arg (18)	Val (12)	Val (18)	Glu (7)	Arg (12)	His (10)

[a] (−) Recovery undetectable.

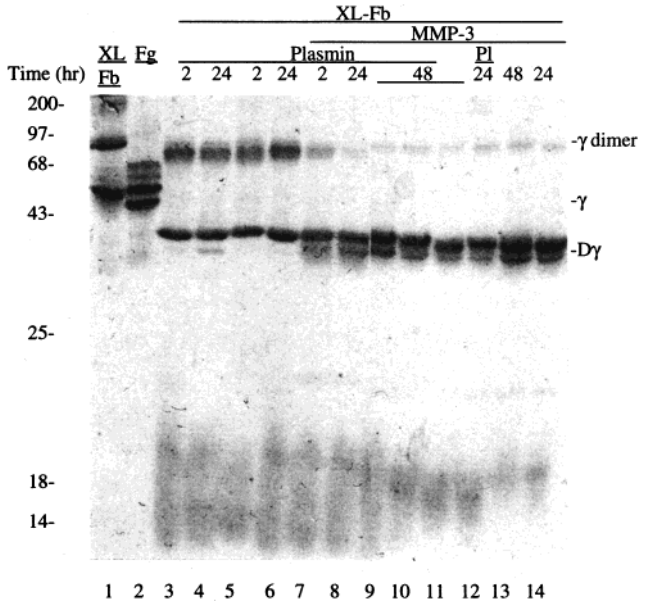

FIGURE 4: Degradation of XL-Fb with plasmin (Pl), MMP-3, or plasmin/MMP-3 mixtures for different time intervals. The following samples were reduced and separated by SDS−PAGE (12.5%): XL-Fb (lane 1); Fg (lane 2); XL-Fb degraded with plasmin (1:20 w/w) for 2 h (lane 3) and 24 h (lane 4); XL-Fb degraded with plasmin (1:200 w/w) for 2 h (lane 5) and 24 h (lane 6); XL-Fb degraded with plasmin (1:200 w/w) and MMP-3 (1:24 w/w) for 2 h (lane 7), 24 h (lane 8), and 48 h (lane 9); XL-Fb degraded with plasmin (1:200 w/w) and MMP-3 (1:24 w/w) in the presence of aprotinin for 48 h (lane 10); XL-Fb degraded with MMP-3 (1:240 w/w) in the presence of aprotinin for 48 h (lane 11); XL-Fb degraded with plasmin (1:200 w/w) and MMP-3 (1:24 w/w) in the presence of aprotinin for 24 h (lane 12); and XL-Fb degraded with MMP-3 (1:24 w/w) for 48 h (lane 13) and 24 h (lane 14). Protein bands were stained with Coomassie Blue.

with a blocked NH_2-terminus; and γ 411 amino acids, 47 kDa, with Tyr as NH_2-terminus.)

The NH_2-terminal sequences of chain remnants of XL-Fb degraded with MMP-3 and MMP-7 are shown in Tables 1 and 2. Leu 84 is the NH_2-terminal residue of the γ-chain in MMP-7 digests of both Fg and XL-Fb (Figure 5 and Table 2). This indicates that MMP-7 has identical cleavage site in this region of the γ-chain as MMP-3. The β-chain remnant in fragments D/D-dimer in MMP-7 digests of both substrates

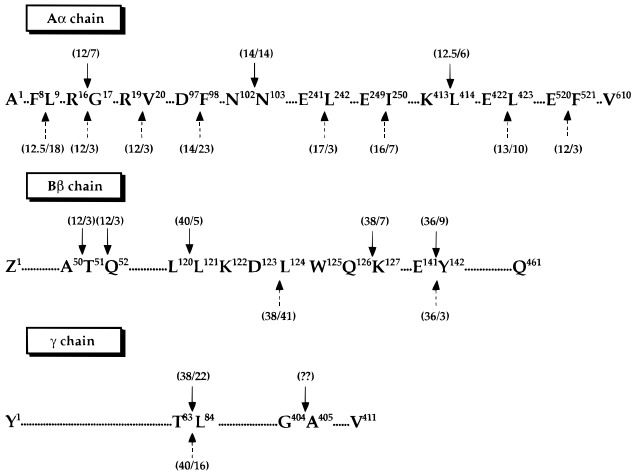

FIGURE 5: Schematic identifying major cleavage sites on human fibrinogen generated by either MMP-3 (solid downward-pointing arrows) or MMP-7 (broken upward-pointing arrows). Corresponding data with both enzymes and fully cross-linked fibrin as substrate are presented in Tables 1 and 2, respectively. Digests of fibrinogen with each enzyme were subjected to SDS−PAGE (12.5% gels) under reducing conditions and each band was subjected to sequence analysis (8 cycles). The size of fibrinogen chain remnants is indicated in kilodaltons (first value within parentheses) and the yield of the NH_2-terminal residue in picomoles (second value within parentheses). Although a γ-chain peptide encompassing the sequence A405−V411 was not identified in the present study, MMP-3 most probably cleaves fibrinogen at G404−A405 as previously reported for this same enzyme and XL-Fb as substrate (*20*). This conclusion is based on the fact that D-like fragments resulting from MMP-3 cleavage of fibrinogen fail to react with MoAb/4A5 (Figure 3A).

gave several sequences, the major starting at Bβ-124. This finding explains the reactivity of MoAb/T54 with both MMP-3 and -7 digests of Fg and XL-Fb (Figure 3B). As already mentioned, the epitope reactive with this antibody is contained within the sequence Bβ 123−127. At least three β-chain remnants in fragment D-like monomers are obtained in MMP-3 digests of both Fg and XL-Fb (Figure 5, Table 1) and two in MMP-7 digests (Figure 5 and Table 2). Therefore, this region of the β-chain is particularly susceptible to multiple cleavages by both MMPs. Due to the proximity of all these cleavage sites, it is to be expected that bands with nearly identical size contain a mixture of

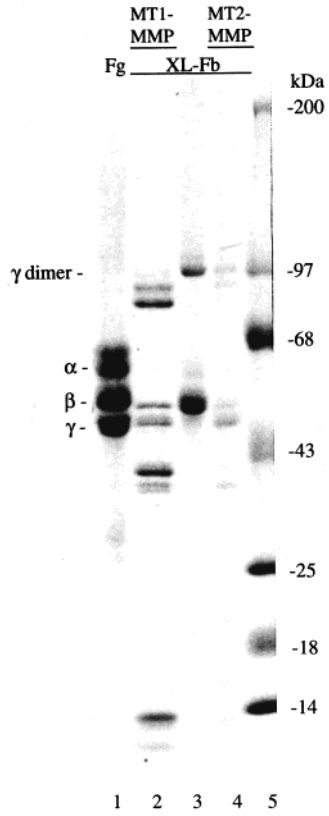

FIGURE 6: Degradation of XL-Fb with MT1-MMP and MT2-MMP. The following samples were reduced and separated by SDS−PAGE (5−15%): Fg (lane 1); XL-Fb degraded with MT1-MMP (1:24 w/w) for 24 h (lane 2); XL-Fb (lane 3); XL-Fb degraded with MT2-MMP (1:24 w/w) for 24 h (lane 4); marker proteins (lane 5). Protein bands were stained with Coomassie Blue.

Bβ-chain related NH_2-termini as observed in Tables 1 and 2.

Pattern of Degradation of XL-Fb with Membrane Type 1 and 2 Matrix Metalloproteinases. MT1- and MT2-MMP were incubated with Fg and XL-Fb following our MMP digestion protocol. As shown in Figure 6, MT1-MMP extensively cleaved all chains in XL-Fb, maintaining the cross-linked γ-dimer chain remnant (Figure 6, lane 2), similarly to plasmin-degraded XL-Fb. In contrast, MT2-MMP leads to only partly degraded fibrin under comparable experimental conditions (Figure 6, lane 4; compare with intact cross-linked fibrin, lane 3). Similarly, MT1-MMP extensively degrades Fg while MT2-MMP leads to only limited degradation (not shown).

The NH_2-terminal sequences of XL-Fb degraded with MT1-MMP are shown in Table 3. The first residue of the γ-chain remnant is Leu 84, identical to that obtained with MMP-3 and MMP-7 digests of Fg and XL-Fb. Multiple NH_2-termini for the β-chain remnants in D-dimers obtained with MT1-MMP were identified (Table 3) similarly to those obtained with both MMP-3 and -7.

Formation of X-, Y-, D-, and E-like Fragments after Fibrinogen Degradation with MMP-2 and MMP-3 as a Function of Time. Fg was incubated for various time intervals as indicated in Figure 7 with either enzyme (nonreduced condition, SDS−7.5% PAGE). The pattern of degradation is compared to the one obtained with plasmin (Figure 7, inset). Degradation of Fg with both MMP-2 and -3 was very rapid, with formation of an X-like fragment as early as 10 min. Degradation products obtained with MMP-2 at 24 h are a mixture of X-, Y-, and D-like species. Degradation with MMP-3 was more extensive, resulting in total conversion to fragment D-like and E-like species after 24 h. The degradation of Fg with MMP-3 generates fragments that are similar in M_r to those obtained with plasmin (Figure 7, inset). In the presence of calcium, fibrinogen is progressively degraded by plasmin to transient degradation products fragment X (Fg-X, M_r 225 000−333 000) and fragment Y (Fg-Y, M_r 150 000−170 000), and terminal core products

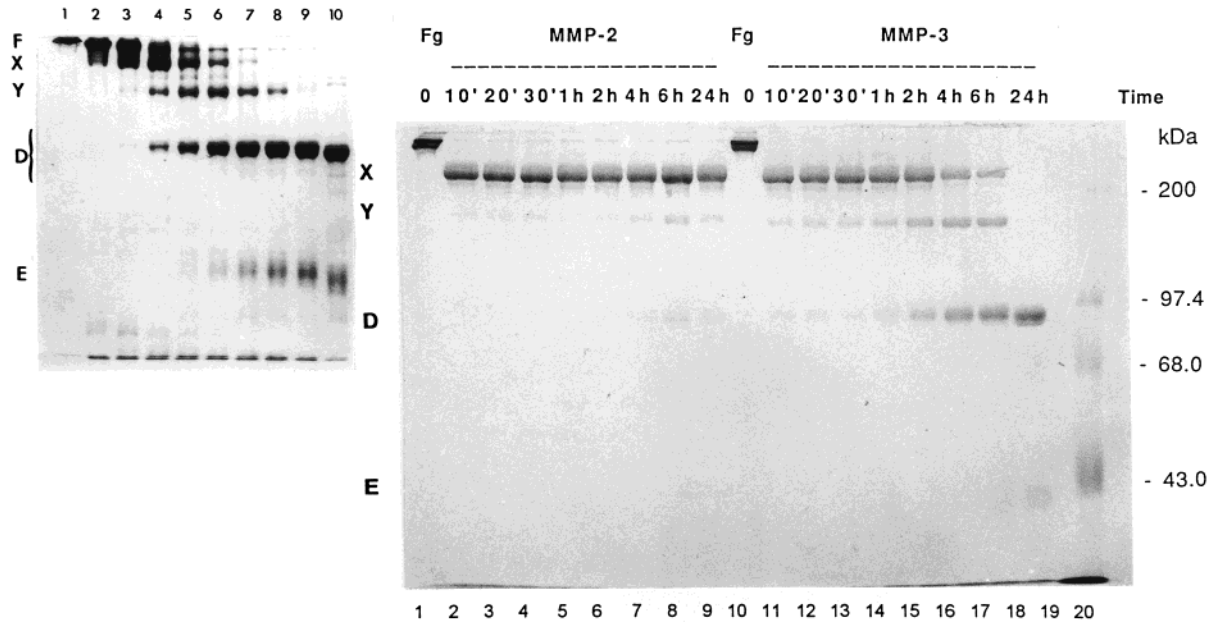

FIGURE 7: Rate of Fg degradation with MMP-2 and MMP-3. Both enzymes were used at E:S = 1:24 (w/w), and incubation time was as indicated, at 37 °C. Samples were separated by SDS−PAGE (7.5%, nonreducing conditions). Migration of intact Fg (F, lane 1 in the inset) and plasmin degradation products fragments X, Y, D, and E are indicated at the left margin in the inset (digestion times of 25 and 50 min and 1, 1.5, 2, 2.5, 3, 4, and 22 h for lanes 2−10 in the inset). Note the similarity in the migration of comparable fragments obtained after degradation with MMP-3. Control Fg is in lanes 1 and 10. Lane 20, BRL. Protein bands were stained with Coomassie Blue.

Table 4: Peptide Bonds Cleaved by MMP-3, MMP-7, and MT1-MMP in Generation of Fragments D/DD: Comparison with Plasmin

chain[a] D/DD	amino acid sequence			
	MMP-3	MMP-7	MT1-MMP	plasmin
α	Asn^{102}−Asn^{103}	Asp^{97}−Phe^{98}	Asp^{97}−Phe^{98}	Arg^{104}−Asp^{105}
β1	Leu^{120}−Leu^{121}	Asp^{123}−Leu^{124}	Asp^{123}−Leu^{124}	Lys^{133}−Asp^{134}
β2	Glu^{141}−Thr^{142}	Glu^{141}−Thr^{142}	Asn^{137}−Val^{138}	
β3	Gln^{126}−Lys^{127}	Asn^{137}−Val^{138}	Glu^{141}−Thr^{142}	
β4	Asn^{137}−Val^{138}	Leu^{120}−Leu^{121}		
γ1	Thr^{83}−Leu^{84}	Thr^{83}−Leu^{84}	Thr^{83}−Leu^{84}	Lys^{62}−Ala^{63}
γ2				Lys^{85}−Ser^{86}

[a] Chain designation is based on relative mobility on SDS−PAGE; the lower number corresponds to the larger chain remnant.

fragment D (Fg-D, M_r 94 000) and fragment E (Fg-E, M_r 50 000).

Time digests of Fg with MMP-3 were reduced and separated by SDS−PAGE and electroblotted onto a PVDF membrane (not shown). Results show that the bond γ Thr 83−Leu 84 was already cleaved at 1 h. NH_2-terminal sequence of Fg degraded with MMP-2 at 24 h indicated intact γ-chain, while the other chains gave evidence of cleavage of the following peptide bonds: α Arg 16−Gly 17, α Arg 20−Val 21, β Lys 35−Val 36, and β Leu 40−Gln 41.

Fragments D and D-Dimer from MMP-3, MMP-7, MT1-MMP, and Plasmin Degradation of Fibrin(ogen). A summary of peptide bonds cleaved and resulting NH_2-terminal sequences of α-, β-, and γ-chains of fragments D and D-dimer obtained after degradation of Fg and XL-Fb with MMP-3, MMP-7, and MT1-MMP is shown in Table 4. MMP-3, MMP-7, and MT1-MMP share similarities in their cleavage of the same substrates with heterogeneity in the cleavage of the β-chain. All three enzymes cleave the γ-chain at Thr 83−Leu 84. Plasmin cleavage sites on the same substrates are in very close proximity to those obtained with MMP-3, MMP-7, and MT1-MMP as indicated in Table 4.

DISCUSSION

Fibrin provides a posthemostatic healing matrix. In that context it participates in tissue remodeling and is ultimately removed by proteolytic enzymes. A large number of studies have shown that plasmin is the principal fibrinolytic enzyme. However, recent studies in transgenic animals have suggested that matrix metalloproteinases (MMPs) might be an additional physiological system participating in fibrin removal. Our studies and those of others have shown that fibrin(ogen) is a substrate for several MMPs (*7*, *20*).

In previous work we showed that MMP-3 degrades both Fg and XL-Fb specifically and differently from plasmin (*20*). In the present study, we have investigated the ability of other MMPs (MMP-7 and -9, MT1- and MT2-MMP) to degrade Fg and lyse XL-Fb clots. Results show that MMP-7 progressively degrades all Fg and XL-Fb chains. The pattern of the reduced β-chains of Fg degraded with MMP-7 is similar, in part, to those obtained with MMP-3. However, in XL-Fb degraded with MMP-7 the γ-chains remain cross-linked. MMP-9 does not seem to degrade XL-Fb to any significant extent. Results obtained with MMP-7 digests of XL-Fb immunoblotted with MoAb/4A5 (anti-γ 397−411) extended our understanding on the ability of these enzymes to degrade Fg and XL-Fb γ-chain. In fact, Fg and XL-Fb γ-chain degraded by MMP-7 did not lose immunoreactivity with MoAb/4A5, contrary to Fg and XL-Fb degraded with MMP-3. We previously showed that MMP-3 cleaves the 4A5 antibody-reactive epitope at γ Gly 404−Ala 405 (*20*). Fg and XL-Fb exposed to MMP-9 retain full immunoreactivity with MoAb/4A5 since both substrates are largely refractory to this enzyme.

Recently, it was shown that MT1-MMP can degrade a number of substrates in the extracellular matrix (*25*). Results from a different study confirmed our previous finding on the ability of MMP-3 to fully solubilize fibrin and also showed that MT1-MMP displayed similar properties whether present in soluble form or as a pericellular enzyme on the membrane of endothelial cells (*7*). In this study we have determined that MT1-MMP can completely degrade XL-Fb while MT2-MMP only leads to partial digestion under similar experimental conditions. Moreover, the specific cleavage sites of MT1-MMP on XL-Fb are similar to those obtained with MMP-3 and -7.

Fragments generated after degradation of Fg with MMP-3, MMP-7, and MT1-MMP and plasmin are similar in size. The bonds split by MMP-3 in XL-Fb are similar to those split in Fg with specific cleavage of γ 404−405. That does not occur with plasmin. The proximity of cleavage sites with MMP-3, MMP-7, MT1-MMP, and plasmin on both Fg and XL-Fb is observed on all chains. Plasmin cleaves Fg α-chain at Arg 104−Asp 105, while MMP-3 cleaves at Asn 102−Asn 103 and MMP-7 and MT1-MMP cleave at Asp 97−Phe 98. Plasmin cleaves Fg β-chain Lys 133−Asp 134 (*36*) and MMP-3, MMP-7, and MT1-MMP show heterogeneity of cleavage at β Leu 120−Leu 121, β Asp 123−Leu 124, β Gln 126−Lys 127, β Asn 137−Val 138, and β Glu 141−Thr 142.

Studies with natural proteins and with synthetic peptides and substrates have shown that MMP-7 has a preference for hydrophobic residues on the P1′ site (*37*−*39*) while MMP-3 has a broader specificity and, in addition to hydrophobic aliphatic residues, also accommodates residues Phe, Trp, Met, Tyr, and Ser at that position (*39*−*41*). MT1-MMP also cleaves gelatinase A at a hydrophobic residue (Asn 37−Leu 38) (*42*). Amino-terminal residues of proteolytic fragments generated by MT1-MMP on different matrix proteins are not available (*25*). All three enzymes specifically cleave both Fg and XL-Fb β and γ chain with Leu at the P1′ site. MMP-3 cleaves the α chain with Asn at the P1′ site, while MMP-7 and MT1-MMP cleave with Phe at P1′ site.

Plasmin cleaves at γ Lys 62−Ala 63 and more slowly at γ Lys 85−Ser 86 (*36*). Fg digested by plasmin, in physiological conditions, results in fragment D species with both γ Ala 63−Val 411 and γ Ser 86−Val 411 sequences. MMP-3, MMP-7, and MT1-MMP all cleave 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 γ-chain comprising the sequence γ Leu 84−Gly 404. Also, both the α- and the β-chains of MMP-3-generated fragment D-like monomer are similar to plasmin fragment D. This can be explained by susceptibility of Fg to proteolysis in selected areas (*43*)

MMP-7 and MT1-MMP generated fragment D-dimers are similar, in part, to that obtained with plasmin degradation. The clot is degraded but the $\gamma-\gamma$ cross-link remains intact. Interestingly, among all human proteolytic enzymes the bond split at γ 404−405 is specific and unique to MMP-3.

MMP-7 degrades a number of matrix proteins (aggrecan, collagen type IV, fibronectin, laminin, and elastin (*44*). Like MMP-3, MMP-7 can be activated by plasmin, and also by MMP-3 itself (*23*). Recent studies have indicated the presence of MMP-7 in atherosclerotic plaques (*14*). As shown from our data, MMP-7 has a strong proteolytic action on Fg and also has the ability of solubilizing XL-Fb clots.

Previously characterized MoAbs to fibrin(ogen) were tested against both Fg and XL-Fb digested with MMP-3, -7, and -9 to evaluate their immunoreactivity. A monoclonal antibody (MoAb/T54) that reacts with D-like and D-dimer-like fragments generated by both MMP-3 and MMP-7, but not by plasmin, has recently been identified. The sequence of MT1-MMP fragments (Table 3) suggests that some of the D-dimer-like fragments generated by this enzyme should also be fully reactive with the same monoclonal. MT1-MMP can degrade Fg and XL-Fb more extensively than MT2-MMP, similarly to what was reported for fibronectin (*25*).

In summary, our studies show that MMP-3, MMP-7, and MT1-MMP are capable of fully solubilizing XL-Fb while the other MMPs tested (MMP-1, -2, and -9 and MT2-MMP) only minimally or partly degraded Fg or XL-Fb. The NH_2-terminal sequences of the fragments obtained after full degradation of XL-Fb with MMP-3, MMP-7, and MT1-MMP were determined. The molecular masses of those fragments were calculated, showing that proteolytic cleavage of Fg and XL-Fb with MMPs results in a number of well-defined fragments, similarly to plasmin (*21*, *36*, *45*). The similarity of the cleavage sites obtained on XL-Fb with MMP-3, MMP-7, and MT1-MMP, but not with MMP-1, -2, and -9 and MT2-MMP, indicate common mechanisms of degradation of Fg and XL-Fb by selected MMPs that might participate in the removal of fibrin in the normal and pathological matrix. Very recent preliminary data indicate the presence of MMP-degraded fibrin(ogen) in synovial fluid from patients with rheumatoid arthritis and other arthritides (*26*). Further studies will elucidate the pathophysiological relevance of this mechanism in vivo.

ACKNOWLEDGMENT

We thank Dr. J. G. Farmar (Microchemistry Laboratory of the New York Blood Center) and Steven J. Smith (Protein Chemistry Laboratory, University of Texas Medical Branch) for NH_2-terminal analyses.

SUPPORTING INFORMATION AVAILABLE

Two tables showing NH_2-terminal sequence analysis of fibrinogen degraded with MMP-3 and MMP-7. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Werb, Z., Tremble, P., and Damsky, C. (1990) *Cell Diff. Dev. 32*, 299−306.
2. Matrisian, L. M. (1990) *Trends Genet. 6*, 121−125.
3. Kleiner, D. E., and Stetler-Stevenson, W. G. (1993) *Curr. Opin. Cell Biol. 5*, 891−897.
4. Birkedal-Hansen, H. (1995) *Curr. Opin. Cell Biol. 7*, 728−735.
5. Nagase, H. (1996) in *Zinc metalloproteases in health and disease* (Hooper, N. M., Ed.) pp 153−204, Taylor & Francis, London.
6. Fisher, C., Gilbertson-Beadling, S., Powers, E. A., Petzold, G., Poorman, R., and Mitchell, M. A. (1994) *Dev. Biol. 162*, 499−510.
7. Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998) *Cell 95*, 365−377.
8. Liotta, L. A., and Stetler-Stevenson, W. G. (1990) *Semin. Cancer Biol. 1*, 99−106.
9. Guedez, L., Lim, M. S., and Stetler-Stevenson, W. G. (1996) *Crit. Rev. Oncogenesis 7*, 205−225.
10. Stetler-Stevenson, W. G. (1996) *Am. J. Pathol. 148*, 1345−1350.
11. Chambers, A. F., and Matrisian, L. M. (1997) *J. Natl. Cancer Inst. 89*, 1260−1270.
12. Henney, A. M., Wakeley, P. R., Davies, M. J., Foster, K., Hembry, R., Murphy, G., and Humphries, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8154−8158.
13. Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994) *J. Clin. Invest. 94*, 2493−2503.
14. Halpert, I., Sires, U. I., Roby, J. D., Potter-Perigo, S., Wight, T. N., Shapiro, S. D., Welgus, H. G., Wickline, S. A., and Parks, W. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9748−9753.
15. Okada, Y., Nagase, H., and Harris, E. D. J. (1986) *J. Biol. Chem. 261*, 14245−14255.
16. Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M. T., Iwata, K., and Nagase, H. (1992) *Lab. Invest. 66*, 680−690.
17. Hembry, R. M., Murphy, G., Cawston, T. E., Dingle, J. T., and Reynolds, J. J. (1995) *Ann. Rheum. Dis. 54*, 25−32.
18. Murphy, G. (1995) *Acta Orthrop. Scand. 66* (Suppl. 266), 55−60.
19. Ohta, S., Imai, K., Yamashita, K., Matsumoto, T., Azumano, I., and Okada, Y. (1998) *Lab. Invest. 78*, 79−87.
20. Bini, A., Itoh, Y., Kudryk, B. J., and Nagase, H. (1996) *Biochemistry 35*, 13056−13063.
21. Pizzo, S. V., Taylor, L. M. J., Schwartz, M. L., Hill, R. L., and McKee, P. A. (1973) *J. Biol. Chem. 248*, 4584−4590.
22. Siebenlist, K. R., and Mosesson, M. W. (1992) *Biochemistry 31*, 936−941.
23. Wilson, C. L., and Matrisian, L. M. (1996) *Int. J. Biochem. Cell Biol. 28*, 123−126.
24. Ohuci, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) *J. Biol. Chem. 272*, 2446−2451.
25. D'Ortho, M.-P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith, B., Timpl, R., Zardi, L., and Murphy, G. (1997) *Eur. J. Biochem. 250*, 751−757.
26. Bini, A., Wu, D., Ivashkiv, L., Ahadi, M. Z., and Kudryk, B. J. (1999) *FASEB J. 13*, A206.
27. Deutsch, D. G., and Mertz, E. T. (1970) *Science 170*, 1095−1096.
28. Engvall, E., and Ruoslahti, E. (1977) *Int. J. Cancer 20*, 1−5.
29. Procyk, R., Adamson, L., Block, M., and Blombäck, B. (1985) *Thromb. Res. 40*, 833−852.
30. Laemmli, U. K. (1970) *Nature 227*, 680−685.
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
32. Kudryk, B., Gidlund, M., Rohoza, A., Ahadi, M., Coiffe, D., and Weitz, J. I. (1989) *Blood 74*, 1036−1044.
33. Goding, J. W. (1986) *Monoclonal Antibodies: Principles and Practice*, 2nd ed., Academic Press, New York.
34. Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035−10038.
35. Kudryk, B. J., Ahadi, M., Rohoza, A., S., C., Schnuer, J., and Bini, A. (1998) *Blood Coag. Fibrinol. 9*, 691 (abstr).
36. Collen, D., Kudryk, B., Hessel, B., and Blombäck, B. (1975) *J. Biol. Chem. 250*, 5808−5817.

37. Netzel-Arnett, S., Sang, Q.-X., Moore, W. G. I., Navre, M., Birkedal-Hansen, H., and Van Wart, H. E. (1993) *Biochemistry 32*, 6427−6432.
38. Sires, U. I., Griffin, G. L., Broekelmann, T. J., Mecham, R. P., Murphy, G., Chung, A. E., Welgus, H. G., and Senior, R. M. (1993) *J. Biol. Chem. 268*, 2069−2074.
39. Welch, A. R., Holman, C. M., Huber, M., Brenner, M. C., Browner, M. F., and Van Wart, H. E. (1996) *Biochemistry 35*, 10103−10109.
40. Nagase, H., Fields, C. G., and Fields, G. B. (1994) *J. Biol. Chem. 269*, 20952−20957.
41. Nagase, H. (1995) *Methods Enzymol. 248*, 449−470.
42. Pei, D., and Weiss, S. J. (1996) *J. Biol. Chem. 271*, 9135−9140.
43. Doolittle, R. F., Everse, S. J., and Spraggon, G. (1996) *FASEB J. 10*, 1464−1470.
44. Wilson, C. L., and Matrisian, L. M. (1996) *Int. J. Biochem. Cell Biol. 28*, 123−126.
45. Marder, V. J., Shulman, N. R., and Carroll, W. R. (1969) *J. Biol. Chem. 244*, 2111−2119.

BI991096G