Matrix Metalloproteinases Collagenase-2, Macrophage Elastase, Collagenase-3, and Membrane Type 1-Matrix Metalloproteinase Impair Clotting by Degradation of Fibrinogen and Factor XII*

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The effects of plasma proteins on controlling the activity of matrix metalloproteinases (MMPs, matrixins) have been the focus of numerous studies, although only a few have examined the influence of matrixins on plasma proteins. Recently, it has been shown that MMPs may play a role in the degradation of fibrin. We have now investigated the role of collagenase-2 (MMP-8), macrophage elastase (MMP-12), collagenase-3 (MMP-13), and membrane type 1-matrix metalloproteinase (MT1-MMP, MMP-14) in the degradation of fibrinogen and Factor XII of the plasma clotting system. Our data demonstrate that the catalytic domains of MMP-8, MMP-12, MMP-13, and MMP-14 can proteolytically process fibrinogen and, with the exception of MMP-8, also inactivate Factor XII (Hageman factor). We have identified the amino termini of the major protein fragments. Cleavage of fibrinogen occurred in all chains and resulted in significantly impaired clotting. Moreover, rapid proteolytic inactivation of Factor XII (Hageman factor) by MMP-12, MMP-13, and MMP-14 was noted. These results support the hypothesis of an impaired thrombolytic potential of MMP-degraded Factor XII in vivo. MMP-induced degradation of fibrinogen supports a plasmin-independent fibrinolysis mechanism. Consequently, degradation of these proteins may be important in inflammation, atherosclerosis, and angiogenesis, all of which are known to be influenced by MMP activity.

The matrix metalloproteinases, MMPs¹ and matrixins, form a family of structurally and functionally related zinc-containing endopeptidases. Together they are able to degrade most of the constituents of the extracellular matrix such as basement membrane, collagens, proteoglycans, fibronectin, and laminin (1). Thus, they are implicated in connective tissue remodeling processes associated with embryonic development, pregnancy, growth, and wound repair (2). The deleterious potential of the MMPs is normally controlled by the endogenous and specific tissue inhibitors of metalloproteinases or the more general nonspecific α₂-macroglobulin (3). Disturbance of the well balanced equilibrium of MMPs and tissue inhibitors of metalloproteinases results in pathological situations such as rheumatoid and osteoarthritis, atherosclerosis, tumor growth, metastasis, and fibrosis (4–8). In addition to degradation of extracellular matrix constituents, plasma proteins such as serpins (9) or fibrinogen and cross-linked fibrin (10–12) are also cleaved.

Fibrinogen is a 340-kDa dimeric glycoprotein consisting of a pair of three polypeptide chains Aα, Bβ, and γ that are interconnected by 29 disulfide bonds. The amino termini of these chains are joined together in a central domain that can be isolated as a single fragment from a plasmin digestion of fibrinogen (13). During blood coagulation, fibrinogen participates in both the cellular phase and the fluid phase of blood clot formation (14, 15). Fibrinogen can be converted into an insoluble fibrin clot as a consequence of thrombin-catalyzed removal of fibrinopeptides A (FpA, Aα-(20–35))² and B (FpB, Bβ-(31–44)) from the Aα and Bβ chains (16).

In addition to the ordinary route of thrombin generation (17) via the tissue factor pathway, an alternative route exists that is initiated by the activation of Factor XII (Hageman factor) (18). The activation of Hageman factor to yield active α-Factor XIIa takes place by a single cleavage at ³⁷⁵R ↓ V³⁷³ (numbering includes signal peptide) (19). Eventually, cleavage at ³⁵⁸R ↓ N³⁵⁴ and ³⁶⁰R ↓ L³⁶⁲ leads to the β-Factor XII (20), which still exhibits full catalytic activity. After several more steps of zymogen activation, this alternative route leads into the ordinary pathway of blood coagulation, terminating in the proteolytic conversion of fibrinogen into fibrin.

The purpose of this work is to examine the role of MMPs in the degradation of fibrinogen and Factor XII. We therefore examined the clotting of MMP-digested fibrinogen to support the idea that some of the biological functions of fibrinogen might be hampered (11). In addition to this, the previously unreported degradation (and inactivation) of Factor XII is shown here. All digestions were subjected to SDS-PAGE followed by automated sequencing to characterize the generated fragments and identify the cleavage sites.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized human fibrinogen (F8883, >95% clottable and essentially plasminogen-free according to the manufacturer) was purchased from Sigma and dissolved to a final concentration of 2 mg/ml. Thrombin and Owren’s Veronal buffer (28.4 mM sodium barbital in 125 mM NaCl, pH 7.35) were supplied by Dade (Aguada). EDTA and the synthetic serine proteinase inhibitor Pefabloc SC (4-2-aminoethylbenzenesulfonylfluoride hydrochloride) were delivered from Merck. The chromogenic thrombin substrate S-2302 was supplied by Chromogenix (Moedlndal, Sweden). Active recombinant human cdMT1-MMP-(Ile¹¹⁴–Ile¹³⁵) and cdMMP-8-(Met³⁰⁶-Gly³⁴⁵) were prepared as described previously (21, 22). Active human neutrophil gelatinase B (MMP-9) was purified as described previously (23). Active human recombinant cdMT2-MMP was a generous gift from Dr. Horst Will of INVIVEK, GmbH.

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¹The abbreviations used are: MMP, matrix metalloproteinase; BB-94, batimastat; cd, catalytic domain; FpA, fibrinopeptide A; HPLC, high performance liquid chromatography; MT-MMP, membrane-type MMP; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

²Numbering of amino acids includes signal peptide sequences.

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The clotting time of cdMT1-MMP-treated fibrinogen was 0.6 min. Collected fractions were lyophilized, diluted in denaturing buffer, and subjected to SDS-PAGE on 10% gels. The molecular weights of apparent bands were estimated (30).

**Determination of Thrombin-induced Clotting of cdMT1-MMP-treated Fibrinogen**—The clotting time of cdMT1-MMP-treated fibrinogen was determined with a semiautomatic coagulometer by the functional method of Claus (31). 1.03 mg of fibrinogen (3.97 mmol) in Owren’s Veronal buffer was preincubated for different time intervals at 37 °C with the corresponding amount of MMP-8, MMP-12, MMP-13, and MT1-MMP, respectively. Substrate cleavage was stopped by adding a 10X molar excess of BB-94. Clotting was initiated by adding 200 μl of thrombin (90 NIH units/ml) to 200 μl of the pre-warmed (37 °C) fibrinogen sample. A timer was started with addition of the thrombin solution and halted automatically at the point of clotting by a metallic oscillator. Under the conditions used, clotting time depended mainly on the concentration of the fibrinogen. The concentration of the remaining clotable fibrinogen was calculated from the clotting time according to a calibration curve that was established with known amounts of untreated fibrinogen.

**Activity Assays**—MMP activity was determined by gelatin zymography (27) and by a continuous assay using MCA peptide (7-methoxy coumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2, 4-dinitrophenyl]-1–2-3-diaminopropionyl)-Ala-Arg-NH2) as a synthetic substrate (28).

**Digestion of Fibrinogen by MMPs**—Fibrinogen was incubated with cdMMP-8 in a 1:50 enzyme/substrate molar ratio at 37 °C for different time intervals. In the case of cdMMP-12, MMP-13, and MT1-MMP, a ratio of 1:10 was employed. All reactions were performed in 20 mM Veronal buffer alone, the Factor XII solution without active MMP were taken as measurement of the buffer alone, the batimastat solution, the active MMP and ZnCl2 for 45 and 90 min at 37 °C. After the incubation time was over, 1 μl of this solution was mixed with 100 μl of S-2302 solution (4 mM) and the absorbance at 405 nm (reference: 490 nm) was measured with an interval of 2 min for 32 min overall. Measurements of the buffer alone, the batimastat solution, the active MMP alone, and the Factor XII solution without active MMP were taken as controls.

**RESULTS**

**MMP-Induced Degradation of Fibrinogen**—We examined the catabolic activities of several MMPs on fibrinogen. Samples of digestion were taken at different time intervals, heated-treated in 5 μl of 10% SDS gels. A, fibrinogen was digested by cdMT1-MMP in a 1:10 enzyme/substrate molar ratio for 3 h at 37 °C. Samples were taken at 0, 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, and 3 h. Lane 1, low molecular mass standards; lane 2, fibrinogen at time 0; lanes 3–9, samples taken at indicated time intervals; lane 10, fibrinogen after 3 h of incubation with buffer alone. B, MMP-12-induced fibrinogen degradation was performed with a 1:10 enzyme/substrate molar ratio. The reaction mixture was incubated at 37 °C for up to 2 h. Samples were taken at the indicated time intervals. Lane 1, low molecular mass standards; lanes 2–9, reaction mixture after 0, 2, 4, 8, 15, 30, 60, and 120 min of incubation; lane 10, fibrinogen after 2 h incubated with buffer alone.
MMP treatment of fibrinogen resulted in the complete disappearance of fibrinogen α-chain after a 1-min incubation. In turn, several smaller α-chain fragments with molecular masses of 15, 13, and 6 kDa became apparent and could be identified. During fibrinogen digestion, progressive decreases of the 56-kDa β-chain and the 47-kDa γ-chain were observed, leading to γ-chain fragments sized at 38 and 35 kDa, respectively. These fragments underwent further degradation to yield unidentified smaller digestion products. Thus, after 30 min of incubation no remaining β-chain could be visualized, and after 2 h the γ-chain was also completely cleaved. With MMP-12, quick disappearance of the fibrinogen α-chain could also be observed. No corresponding band was visible after a 2-min incubation. Fig. 1B shows that the bands indicating β- and γ-chains were diminished to a lesser extent than observed with MMP-14.

Consistent with the observation of fibrinogen fragmentation, the generation of lower molecular mass products was noted with time leading to a set of bands. Of these, six could be identified. During fibrinogen digestion, progressive decreases of the 56-kDa β-chain and the 47-kDa γ-chain were observed, leading to γ-chain fragments sized at 38 and 35 kDa, respectively. These fragments underwent further degradation to yield unidentified smaller digestion products. Thus, after 30 min of incubation no remaining β-chain could be visualized, and after 2 h the γ-chain was also completely cleaved. With MMP-12, quick disappearance of the fibrinogen α-chain could also be observed. No corresponding band was visible after a 2-min incubation. Fig. 1B shows that the bands indicating β- and γ-chains were diminished to a lesser extent than observed with MMP-14.

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**Characterization of Fibrinogen Fragments**—To identify the fibrinogen fragments generated by cdMMP-13 and cdMMP-14, respectively, separation by reverse-phase HPLC was performed. Fig. 2 shows a typical HPLC profile for MT1-MMP-digested fibrinogen. We analyzed the homogeneity of the various peak fractions by subjecting a sample of each fraction to SDS-PAGE under reducing conditions. Fractions containing more than one fragment were separated by SDS-PAGE and electroblotted onto a PVDF membrane.

**Effect of MMPs on the Thrombin-induced Clotting of Fibrinogen**—To further characterize the effect of fibrinogen degradation by MMPs, the clotting of digested fibrinogen was measured by the functional method of Clauss (31). Fibrinogen was incubated with cdMMP-8, cdMMP-13, cdMMP-12, and cdMMP-14, respectively, for up to 2 h, and after stopping the fibrinogen degradation by adding a 10-fold molar excess of BB-94, the clotting capability of fibrinogen was measured. Fig. 3 shows the amount of clottable fibrinogen measured at different points of time. The individual fibrinogen concentrations were calculated from standard dose-response curves of fibrinogen concentrations versus clotting time. After an 8-min incubation with cdMT1-MMP, remaining fibrinogen clotting activity was already reduced to approximately 68%. Moreover, the clotting was reduced to an even greater extent when fibrinogen was incubated with MMP-8, MMP-12, or MMP-13, respectively. These results indicate a markedly impaired coagulant activity of MMP-treated fibrinogen compared with normal fibrinogen. Similar results were obtained by measuring clotting of MMP-8, MMP-12, or MMP-13-treated fibrinogen (see Fig. 3).

**Degradation of Hageman Factor by MMPs**—To investigate the cleavage of Factor XII by MMP-13, reagents were incubated at 37 °C for up to 3 h (see Fig. 4A). The proteolytic cleavages resulted in breakdown of Factor XII into several fragments within minutes. With SDS-PAGE, after 4 min of incubation, two intermediate bands became apparent at 45 and 30 kDa, respectively. Prolonged incubation led to further degradation and disappearance of these fragments, and bands of lower molecular weight became visible (27 and 22 kDa, respectively). Amino-terminal sequence identification of these fragments showed that cleavage occurred at the beginning of the type II fibronectin-like and the epidermal growth factor-like domains. Moreover, a cleavage within the catalytic region was identified four residues downstream of the kallikrein cleavage site that leads to the activation of Factor XII. It is noteworthy that the fragment Leu177–Ser615 generated by MMP-12, MMP-13, and MT1-MMP did not show any catalytic activity against the chromogenic substrate S-2302.

In addition to this, we have discovered that Factor XII cannot be activated by kallikrein after MMP-induced cleavage. This was shown by a separate experiment in which latent Factor XII was subjected to treatment by MMPs and tested for activation with the synthetic substrate S-2302. After addition of kallikrein, the control of untreated Factor XII yielded the expected cleavage of the chromogenic substrate whereas MMP-digested Hageman factor showed no activity.

**Cleavage of Hageman factor by cdMMP-12** is an as yet unknown capability of this matrix metalloproteinase. The time course of this degradation is shown in Fig. 4B. The amino termini were also identified as His29 and Leu317 after cleavage by MMP-13. The amino terminus Tyr31 at the beginning of the

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**Table I**

| Enzyme | kDa | NH₂-terminal sequence | Origin |
|--------|-----|-----------------------|--------|
| MMP-8  | 68  | 20ADSGED             | α-chain |
|        | 32  | 20ADSGED             | α-chain |
|        | 30  | 20ADSGED             | α-chain |
|        | 13  | 442LRTGKEKV          | α-chain |
|        | 12  | 442LRTGKEKV          | α-chain |
|        | 10  | 442LRTGKEKV          | α-chain |
| MMP-12 | 50  | 20ADSGED             | α-chain |
|        | 30  | 20ADSGED             | α-chain |
|        | 25  | 20ADSGED             | α-chain |
|        | 15  | 540FVSETSREG         | α-chain |
|        | 13  | 433LVTSDKGD          | α-chain |
|        | 12  | 540FVSETSREG         | α-chain |
| MMP-13 | 45  | 271YVATRDN           | γ-chain |
|        | 35  | ND                   | β-chain |
|        | 32  | 20ADSGED             | α-chain |
|        | 28  | 124RNSVDLNXN         | β-chain |
|        | 16  | ND                   | β-chain |
| MMP-14 | 45  | 442LRTGKEKV          | α-chain |
|        | 38  | 105DXYAATLKR         | γ-chain |
|        | 35  | 92LYTPNDES           | γ-chain |
|        | 13  | 433LVTSDKGD          | α-chain |
|        | 6   | 117FXSNNR            | α-chain |

a The numbering of amino acids of all proteins includes signal peptide sequence.

b Not determined.
type II fibronectin-like domain was identified as a yet unknown cleavage site generated by cdMMP-12. Moreover, the specificity for the cleavage site of human metalloelastase (HME) at residue Leu360 at the beginning of the catalytic region was also unknown. Cleavage of Hageman factor by MMP-14 generates fragments with approximate molecular masses of 50, 45, 33, 30, and 12 kDa. The identified amino terminus Leu377 was the same as that obtained with MMP-12 and MMP-13. The amino-terminal residue Leu351 was also generated by MMP-14 (Table 2).

**DISCUSSION**

Proteolytic Degradation of Fibrinogen—The data presented in this work demonstrate the fibrinogenolytic activities of the catalytic domains of MMP-8, MMP-12, MMP-13, and MT1-MMP. All enzymes degrade predominantly the αa-chain of fibrinogen and at longer incubation times the ββ- and γ-chain as well. The structural alterations in fibrinogen caused by different concentrations of the employed matrix metalloproteinases were analyzed by electrophoresis of treated fibrinogen in polyacrylamide gels under denaturing and reducing conditions. We found that the MMP-treated fibrinogen was rapidly degraded to lower molecular mass intermediates (Fig. 1). At the earliest time point (1 min), the αa-chains were almost completely cleaved whereas the ββ- and γ-chains were apparently unaffected. When the incubation time was extended to 3 h, the staining intensities of the ββ- and γ-chains were also diminished. Most of the generated fibrinogen fragments were identified by amino-terminal sequencing (see Table 1). Sequencing data revealed that these fragments resulted mainly from cleavages in the αa- and γ-chains of fibrinogen (Fig. 5). However, we were not able to determine the amino termini of all generated fragments. Amino acid sequence analysis of yet unidentified digestion products is currently being performed.

Effect of MMPs on the Thrombin-induced Clotting of Fibrinogen—To determine whether MMP treatment of fibrinogen influences thrombin-catalyzed fibrin polymerization, we measured the clotting time of fibrinogen after digestion with cdMMP-8, cdMMP-12, cdMMP-13, and cdMT1-MMP. The clotting time reflected the amount of intact fibrinogen remaining after proteolytic attack by the individual matrixin.

We have proven that the degradation of fibrinogen by MMPs resulted in a loss of fibrinogen function. Under the applied conditions, fibrinogen showed dramatically impaired clotting ability. In this context, we would like to mention that we were not able to determine correct clotting times of fibrinogen sam-

![Image](image_url)
The carboxyl terminus of the man factor by MMP-12, MMP-13, and MT1-MMP in comparison to trypsin (37, 38). Fig. 6 displays the different cleavages of Hageman factor into its active form, Factor XIIa, respectively. The cleavage sites for the individual proteinases are indicated by the corresponding arrows.

Only one-half of the symmetrical cleavage sites for cdMT1-MMP. Nevertheless, platelet aggregation may be inhibited because the binding peptides are no longer interconnected. Fibrinogen mediates cellular adhesion of a number of different cell types including thrombocytes, endothelial cells, and tumor cells.

Fibrinogen contains RGD sequences both at the amino and carboxyl terminus of the α-chain. These sequences as well as the carboxyl terminus of the γ-chain bind to the platelet glycoprotein IIb-IIIa (integrin αIIbβ3) (34, 35). During incubation of fibrinogen with cdMT1-MMP, both α-chain RGD sequences were removed as well as the γ-chain dodecapeptide sequence (see Fig. 4). With MMP-8, MMP-12, and MMP-13, at least one of the RGD motifs is released. These cleaved fibrinogen fragments may still be recognized by and bound to glycoprotein IIb-IIIa. Nevertheless, platelet aggregation may be inhibited because the binding peptides are no longer interconnected.

The data presented in this study show for the first time the degradation of Factor XII by matrixins prevents the self-assembly of large protofibrils and fibers. The clotting of fibrinogen was studied with in vitro assays because the catalytic domain of MT1-MMP was not enzymatically active in stabilized human plasma due to the presence of citrate or EDTA. The physiological significance of our findings refers to the obviously altered coagulant properties of digested fibrinogen. This fact may influence a number of cellular events including tumor growth, wound healing, and cell-attachment. Fibrinogen mediates cellular adhesion of a number of different cell types including thrombocytes, endothelial cells, and tumor cells.

There is evidence for the successive release of MMPs in buffycoat-depleted red cell concentrates of blood donors during storage (45, 46). It is anticipated that these matrixins may also have the same mode of action in vivo. Taken together, these findings are consistent with an interaction of MMPs with blood components, especially with plasma proteins of the clotting system.

Consequences—The important role of matrixins in tumor invasion is suggested by a large number of correlation studies demonstrating a direct relationship between increased expression of proteases in tumor tissues and their invasive and metastatic behavior (47). Recently, several groups have shown the degradation of several plasma proteins by some MMPs. Our experiments clearly support the findings that proteolytic activity of matrixins is not restricted to extracellular matrix components. Moreover, the spectrum for MMPs is broader, now including proteins involved in hemostasis like fibrinogen (Fig. 5 and Refs. 10 and 11), Factor XIIa (Fig. 6), plasminogen (48), and plasmin (43). These data suggest that MMPs in interaction with plasma proteins may play a role in the control of coagulation. In this work, we demonstrate a possible down-regulation of thrombotic potential caused by the impact of MMP on proteins participating in coagulation processes. This is an important finding that goes beyond physiological and pathological
Impact of MMPs on Coagulation Proteins

33013

conditions. Moreover, with respect to the successive release of MMPs from residual leucocytes during storage (9, 45, 46, 49), degradation of plasma proteins by MMP-8, MMP-9, MMP-12, and MMP-13 may occur when administering red cell concentrates to patients.

Comments—Our experiments were conducted with carboxyl-terminal truncated enzymes. It had been suggested that the substrate specificity of matrixins is attributed to the presence of the hemopexin-like domain at the carboxyl terminus of most of the MMPs (50). However, D’Ortho et al. (51) compared the substrate specificity of the catalytic domain of MT1-MMP with that of a variant of MT1-MMP containing the catalytic domain and the carboxyl-terminal hemopexin-like domain. These authors noted that both enzyme variants degraded the same substrates with comparable efficiency, with the exception of the triple-helical collagens I and III. Cleavage of collagens required the presence of both the catalytic and the hemopexin-like domain. This matrixin domain appeared to have no role in the proteolytic specificity directed toward other substrates. We made the same observation with MMP-8, MMP-9 (52), and MMP-14 (results not shown).

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