Macrophage Metalloelastase, MMP-12, Cleaves Human Apolipoprotein(a) in the Linker Region between Kringles IV-4 and IV-5

POTENTIAL RELEVANCE TO LIPOPROTEIN(a) BIOLOGY*

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Celina Edelstein‡§, Steven D. Shapiro†, Olga Klezovitch‡, and Angelo M. Scanu‡‡

From the Departments of †Medicine and of ‡Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637 and the ‡Department of Medicine, Washington University, St. Louis, Missouri 63110

In this study we found that macrophage metalloelastase, MMP-12, cleaves, in vitro, apolipoprotein(a) (apo(a)) in the Asn3518-Val3519 bond located in the linker region between kringles IV-4 and IV-5, a bond immediately upstream of the Il3520-Leu3521 bond, shown previously to be the site of action by neutrophil elastase (NE). We have also shown that human apo(a) injected into the tail vein of control mice undergoes degradation as reflected by the appearance of immunoreactive fragments in the plasma and in the urine of these animals. To define whether either or both of these enzymes may be responsible for the in vivo apo(a) cleavage, we injected intravenously MMP-12−/−, NE−/− mice and litter mates, all of the same strain, with either lipoprotein(a) (Lp(a)), full-length free apo(a), or its N-terminal fragment, F1, obtained by the in vitro cleavage of apo(a) by NE. In the plasma of Lp(a)/apo(a)-injected mice, F1 was detected in control and NE−/− mice but was virtually absent in the MMP-12−/− mice. Moreover, fragments of the F1 type were present in the urine of the animals except for the MMP-12−/− mice. These fragments were significantly smaller in size than those observed in the plasma. All of the animals injected with F1 exhibited small sized fragments in their urine. These observations provide evidence that, in the mouse strain used, MMP-12 plays an important role in the generation of F1 from injected human Lp(a)/apo(a) and that this fragment undergoes further cleavage during renal transit via a mechanism that is neither NE- nor MMP-12-dependent. Thus, factors influencing the expression of MMP-12 may have a modulating action on the biology of Lp(a).

Following the original observation by Oida et al. (1), several reports have demonstrated the presence of immunoreactive apo(a)2 in human urine. Particularly significant were the studies by Mooser et al. (2, 3) who by combining chemical and immunochromical techniques provided evidence that urinary apo(a) was represented by fragments derived from its N-terminal region. When, following purification, these urinary fragments were injected intravenously into mice, they were excreted in the urine without an apparent change in size. The same authors also reported the presence in the plasma of apo(a) fragments unattached to apoB100. Upon their intravenous injection into mice, these fragments were rapidly excreted in the urine as smaller sized components (3). Taken together, the results were interpreted to indicate that the apo(a) fragments in human plasma are derived from Lp(a)/apo(a) and are in turn the source of the apo(a) fragments in the urine. Unanswered in those studies was the underlying cause of the apo(a) fragment formation as well as the site or sites for its occurrence. We reported previously that limited in vitro proteolysis of apo(a), by either pancreatic or leukocyte elastase, is attended by the cleavage at the Ile3520-Leu3521 bond located in the linker domain between kringles IV-4 and IV-5 (4, 5). This cleavage generates two main fragments, one representing the N-terminal domain, F1, and the other the C-terminal domain, F2. When these two fragments were separately injected intravenously into mice, F1 but not F2 was rapidly excreted in the urine (4). This observation prompted us to examine whether the cleavage site on apo(a) was limited to serine proteases such as pancreatic and leukocyte or neutrophil elastase (NE) or could also apply to other inflammatory enzymes. Among them we considered matrix metalloproteinases (MMPs), which are metallo-dependent enzymes involved in the homeostasis of the extracellular matrix and also shown to play an important role in the atherosclerotic process (6–13). In this work, we directed our attention to macrophage metalloelastase, also referred to as MMP-12, which was first identified as an elastolytic metalloproteinase secreted by inflammatory macrophages (14) and structurally defined (15, 16). This enzyme has a broad substrate specificity for matrix macromolecules such as fibronectin, laminin, vitronectin, and proteoglycans (17). In the current study, we now show that MMP-12 cleaves apo(a) in vitro and also provide evidence that, in the mouse strain used, it is involved in the generation of F1 in vivo.

EXPERIMENTAL PROCEDURES

Reagents—Human leukocyte elastase (EC 3.4.21.37) was purchased from Sigma. Antiserum to purified preparations of apo(a), Lp(a), and LDL were raised in the rabbit and affinity purified as described previously (18). Anti-Lp(a) were shown to be devoid of immunoreactivity to LDL and plasminogen; anti-LDL was unreactive to apo(a).

Studies in Mice—Mice deficient in NE (NE−/−) or MMP-12 (MMP−/−) in a pure 129SvJ background were generated by targeted disruption of either the NE or MMP-12 gene in RW4 embryonic stem cells as described previously (19, 20). All mice were housed in individual

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10019
cages under normal light. The evening before the experiment, the animals were given a 10% sucrose solution to drink ad libitum in place of water. The following morning the mice were anesthetized with Metapane prior to the injection into the tail vein of 25–250 μg of either Lp(a), apo(a), or fragment F1 in a volume of 100 μl. The mice were then placed in metabolism cages to standard test chamber and 10% sucrose solution to drink. Blood samples were withdrawn from the orbital vein into heparinized hematoctit tubes at the specified time points and immediately iced. Urine was collected at 0–3, 3–5, and 5–24 h. ELISA quantitation, sensitive to < 0.0015 mg/dl of apo(a), was performed on the urine samples to determine the levels of apo(a)-reacting material. The results indicated that to what degree the sample was concentrated for electrophoretic detection. The latter was estimated to be > 0.03 mg/dl. The urine was concentrated in Amicon Centriprep filters.

Human Subjects—The subjects used for the preparation of Lp(a) and LDL were healthy donors with plasma Lp(a) protein levels in the range of 15–43 mg/dl determined as described previously (4). Plasmapheresis was performed in the Blood Bank of the University of Chicago. All of the subjects used in the study gave a written informed consent. The steps for Lp(a) isolation were carried out immediately after blood drawing using the procedure outlined below. We used an additional five subjects for studying the apo(a) fragments in their plasma and urine. These were also healthy subjects with a known phenotype and genotype. Their plasma Lp(a) protein levels varied between 1 and 12 mg/dl, and the apo(a) isoforms varied between 350 and 550 kDa. In each study, 24–36 h urine samples were collected, centrifuged for 15 min at 3000 rpm, and either used fresh or frozen immediately at –80 °C. Before use, they were concentrated 200-fold in microconcentrators (Amicon Corp. Beverly, MA). All of the subjects used in the study gave a written informed consent.

Preparation of Human Lp(a) and LDL—To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% NaN3, 10,000 units/liter aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lp(a) were isolated by sequential ultracentrifugation and lysine-Sepharose chromatography as described previously (21). The purity of the product was assessed by mobility on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA) and Western blots of SDS-PAGE, utilizing anti-Lp(a) and anti-apoB100. The LDL preparations used in this study were isolated at density of 1.003–1.050 g/ml by sequential flotation as described previously (22). To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% NaN3, 10,000 units/liter aprotinin, and 1 mM phenylmethylsulfonyl fluoride.

Phenotyping and Genotyping of Apo(a) and apo(a) Phenotyping was performed on isolated apo(a) samples by SDS-PAGE followed by immuno blotting using anti-Lp(a) (23). The mobility of the individual apo(a) bands was compared with isolated apo(a) isoforms of known molecular weights (21). For apo(a) genotyping, DNA plugs were prepared from blood mononuclear cells and subsequently fractionated by pulsed field gel electrophoresis, the gels were electroblotted onto Immobilon PSQ sheets (Millipore) according to the instructions of the manufacturer.

Electrophoretic Methods—SDS-PAGE (4 and 4–12% polyacrylamide) was performed on a Nova system (Novex, San Diego, CA) for 1.5 h at constant voltage (120 V) at 22 °C. The samples were prepared by heating at 95 °C for 5 min in sample buffer which consisted of 94 mM phosphate buffer, pH 7.0, 1% SDS and 2 μl urea with or without 3% β-ME. Immediately after electrophoresis, the gels were placed onto Immobilon-P sheets (Millipore Corp.) that were previously wetted with a buffer containing 48 mM Tris, 39 mM glycine, pH 8.9. Blotting was performed on a horizontal semi-dry electrolot apparatus (Amersham Pharmacia Biotech, Inc.) at 0.8–1 mA/cm2 for 45 min at 22 °C.

Immunoblotting—After electrophoresis, the Immobilon-P sheets were blocked in PBS containing 5% non-fat dry powdered milk and 0.3% Tween 20 followed by incubation with anti-apo(a) or anti-apoB100 antibody. The blots were washed and incubated with anti-rabbit horse radish peroxidase-labeled IgG. Subsequently, the blots were developed with the ECL Western Detection Reagent (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. All sequencing procedures were carried out in the core laboratory of the Macromolecular and Structural Analysis Facility at the University of Kentucky.

Lipoprotein and Apolipoprotein Analyses—Lp(a) and LDL protein were quantitated by a sandwich ELISA essentially as described previously (18) except that anti-Lp(a) IgG was used as the capture antibody and anti-apoB100 IgG conjugated to alkaline phosphatase as the detection antibody. For the ELISA quantitation of apo(a), anti-apo(a) IgG conjugated to alkaline phosphatase was used as the detection antibody. Subsequently, an extinction coefficient (ε595 = 3.13 ml·mg⁻¹·cm⁻¹) was established for apo(a) in the 30% sucrose solution. Protein determinations were performed by the Bio-Rad DC Protein Assay.

Studies on the in Vitro Proteolytic Cleave of Lp(a)/apo(a) by a Purified Recombinant MMP-12—Mice recombinant MMP-12 was expressed in Escherichia coli and purified as described previously (15). The active enzyme was incubated with Lp(a)/apo(a) (final concentration, 0.1 μg apo(a)) at varying weight ratios of protein to enzyme (25, 50, and 100:1, P:E) at 37 °C in 50 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 10 mM CaCl2, 0.05% Brij 35, 0.02% NaN3. After 18 h of incubation, samples were removed, EDTA (final concentration 50 mM) was added to stop the reaction, and SDS-PAGE was performed under un-reduced and reduced conditions.

RESULTS

Studies on the in Vitro Proteolytic Cleave of Lp(a)/apo(a) by a Purified Recombinant MMP-12—The ability of MMP-12 to hydrolyze Lp(a) was assessed at varying weight ratios of apo(a) to enzyme using anti-apo(a) and anti-apoB100 immunoblotting of 4% SDS-PAGE. Fig. 1A shows the digestion patterns of Lp(a) after an 18-h incubation at 37 °C, under un-reduced conditions, probed with anti-apo(a) (left) or anti-apoB100 antibodies (right). The level of digestion of apo(a) and apoB100 increased as a function of enzyme concentration (Fig. 1A, lanes 3 to 5, left and right panels). Notably, in the left panel, bands marked with an arrow decreased in intensity as the enzyme concentration increased. Three other bands of major intensity were also present (left panel), one of which exhibited a mobility corresponding to F1. Immunostaining of the same gels with anti-apoB100 (right panel) showed that a large proportion of the products of apoB100 hydrolysis in the Lp(a) particle co-migrated with bands corresponding to the apo(a) immunostained ones in the left panel. On the reduced gels, there were two major bands that migrated at an apparent mass of 220 and 170 kDa (Fig. 1B, lanes 2–4, left panel). The mobilities of these bands corresponded to fragments F1 and F2 present in a reported (4) way. The bands corresponding to F1 co-migrated with a slower mobility on reduced gels than under un-reduced conditions, likely because of the effect of the reducing agent on the conformation of the fragment. Immunostaining of the reduced gels with anti-apoB100 (Fig. 1B, right panel) showed that the gel pattern was essentially the same as observed in the unreduced gel (Fig. 1A, right panel). When apo(a) was digested with...
MMP-12, the pattern was the same as shown in Fig. 1B left panel for Lp(a) under reduced conditions. The results suggest that apo(a) in Lp(a) was cleaved at a single site producing two major fragments, F1 and F2, the latter covalently linked to truncated forms of apoB100.

The partial amino-terminal sequences of F1 and F2 obtained by MMP-12 digestion of Lp(a) and apo(a) are shown in Fig. 2. By aligning these sequences with those of apo(a), using the program CLUSTAL W, we located the cleavage site at the Asn3518–Val3519 bond in the linker region between kringles IV-4 and IV-5. These fragments correspond to the N- and C-terminal domain of apo(a) containing kringles IV-1 to IV-4 (N-terminal), and IV-5 through IV-10, kringe V, and the protease region (C-terminal).

Because apoB100 was also digested by MMP-12, it was of interest to determine whether the fragments remained on the surface of the Lp(a) particle or formed separate lipoprotein species, each consisting of various truncated forms of apoB100 disulfide linked to F2. To this end, MMP-12-digested Lp(a) was electrophoretically separated on native nondenatured polyacrylamide gels that were immunostained with anti-apo(a) and anti-apoB100 (Fig. 3). Digested Lp(a) exhibited two bands (lane 4), one of which also stained with anti-apoB100 (lane 6). The band with the faster mobility in lane 4 corresponded to F1. In turn, when LDL was digested with MMP-12, the pattern was the same as for undigested LDL (compare lanes 5 and 6).

Taken together, we interpreted these results to show that 1) MMP-12 hydrolyzes both apo(a) and apoB100 components of Lp(a); 2) the hydrolytic products consist of apo(a) fragment F1 and a miniLp(a) particle in which the C-terminal region of apo(a), fragment F2, is covalently linked to truncated forms of apoB100; and 3) the apoB100 fragments remain on the surface of the particle, whereas the N-terminal fragment of apo(a), F1, is released.

Studies in Mouse Plasma—We examined, on anti-apo(a) immunostained blots of reduced 4% SDS-PAGE, plasma samples from mice bled 3 h after intravenous injection of either Lp(a), apo(a), or F1. The electrophoretic pattern of the plasma samples after injection of apo(a) into NE−/− and control mice (Fig. 4A, lanes 2 and 3) contained bands corresponding to full-length apo(a) and fragments thereof, one migrating in the position of F1, indicating that a fragmentation process had occurred in vivo. In contrast, the 3-h plasma sample of MMP-12−/− mice exhibited a major band corresponding to full-length apo(a) along with some minor bands of low immunostaining intensity but none with the mobility of F1 (lane 4). The electrophoretic patterns, under reduced conditions, of the plasma from mice injected with Lp(a) (data not shown) were comparable with those shown in Fig. 4A, lanes 2 to 4.

The banding pattern of plasma after injection of F1 (Fig. 4A, lanes 6–8) was similar among all of the mice and resembled the injected material (lane 5). All the patterns contained a major band at 220 kDa and other bands of minor immunostaining intensity in the range of 200–135 kDa. The electrophoretic pattern of the plasma of all the mice remained unaltered at each time point after injection, although the intensity of these

FIG. 1. Proteolytic cleavage of Lp(a) by MMP-12. A, pattern of anti-apo(a) and anti-apoB100 immunostained 4% SDS-PAGE (unreduced) of Lp(a) before and after incubation with MMP-12 for 18 h at 37 °C at various apo(a) to enzyme weight ratios. Left panel, lanes 1 and 2, undigested Lp(a) and purified F1, respectively; lanes 3–5, digests at protein:enzyme weight ratios of 100, 50, and 25:1, respectively. Right panel, lanes 1 and 2, Lp(a) and LDL; lanes 3–5, same as in left panel. B, electrophoretic pattern under reduced conditions of Lp(a) before and after incubation with MMP-12. Left panel, lane 1, Lp(a); lanes 2–4, digests at protein to enzyme weight ratios of 100, 50, and 25:1. Right panel, lane 1, LDL; lanes 2–4, same as in left panel.

FIG. 2. Amino acid sequence alignments of fragments F1 and F2 obtained from MMP-12 digests of apo(a). The alignment shown was constructed according to the program ClustalW using the apo(a) sequence from McLean et al. (25). Apo(a) is composed of repeats of KIV numbered 1–10, one KIV, and a protease domain (P). The first 10 amino acids obtained by sequencing F1 were aligned to the amino acid sequence of the mature apo(a). The amino acid sequence of F2 began with valine at position 3519. The MMP-12 cleavage site was determined to be in the linker region between KIV-4 and KIV-5. The dashed lines refer to undetermined amino acids, and the dotted lines are continuation of sequences upstream and downstream of the protein sequences.

FIG. 3. Native electrophoretic gels of Lp(a) before and after digestion with MMP-12. Lp(a) was treated with MMP-12 for 18 h at 37 °C at an apo(a) to enzyme weight ratio of 100:1. The digest was then separated on a 4% native polyacrylamide gel and immunostained with anti-apo(a) and anti-apoB100. Lanes 1 and 2, apo(a) and F1; lanes 3 and 4, Lp(a) before and after digestion; lanes 5 and 6, LDL before and after digestion; lanes 7 and 8, Lp(a) before and after digestion.
Studies in Mouse Urine—We examined the urine of mice, collected 24 h after injection of either Lp(a), apo(a) or F1, by anti-apo(a) immunostaining of reduced 4–12% gradient SDS-PAGE. The urinary patterns of apo(a) after injection into NE−/− and control mice were comparable (Fig. 4B, lane 2 and 3) and showed a broad spectrum of bands including those migrating at 110, 81, 57, and 33 kDa. In contrast, the urine from MMP-12−/− mice was devoid of bands (lane 4), even after concentrating the urine 3-fold more than that of either the NE−/− or control mice. In turn, the electrophoretic pattern of the urine from all of the mice injected with F1, contained fragments (lanes 6–8) that were smaller in size than the injected material (lane 5) and similar to those observed in NE−/− and control mice injected with full-length apo(a) (lanes 2 and 3).

To rule out artifactual contributions to the formation of fragments, the collected urine samples from injected mice were incubated at 37°C overnight and compared with freshly collected urine kept on ice. Western blot analysis showed no formation of new fragments. In addition, no new fragments were observed when the urine of a control un.injected mouse was incubated with intact Lp(a) or its derivatives. These results provide evidence that F1 is the component of apo(a) targeted for renal excretion and that in our mouse model, this fragment is generated by the action of MMP-12 on apo(a).

**Cleavage of Apo(a) by MMP-12**

Our studies have shown that, in vitro, MMP-12 cleaves human apo(a) at the Asn3518-Val3519 bond located in the linker between kringles IV-4 and IV-5. This bond is immediately upstream of the Ile3520-Leu3521 bond that we previously showed to be the site of cleavage by either pancreatic or leukocyte elastase (4, 5). The reason for the high substrate specificity by the elastases and MMP-12 is unclear. One plausible explanation is that the linker between kringles IV-4 and IV-5, contrary to the other linkers of apo(a), is unglycosylated as predicted on the basis of sequence analysis (25). It is of note that the proximity of the bond sites cleaved by NE and MMP-12, results in the generation of two main fragments of a nearly equal size.

One of the interesting aspects of our current studies was the observation that Lp(a)/apo(a)-injected MMP-12−/−, contrary to the NE−/− mice, had virtually no immunodetectable fragments in their plasma and none in their urine. Yet, the MMP-12−/− mice were able to excrete in the urine intravenously injected F1. These observations when coupled with the finding that MMP-12 was able to cleave in vitro, apo(a), strongly suggest that in the mouse, this enzyme was responsible for the generation of F1 from injected human apo(a). The results also suggest that the kidney has a recognition site for free F1 but not when it is a component of a full-length apo(a). The reason for the selective recognition of F1 by the kidney is unclear. Both size (about 200 kDa) and high degree of glycosylation (about 30% by weight) of F1 speak against a simple filtration process as suggested by Mooser et al. (3). A receptor mechanism is a more likely possibility. However, our knowledge about receptors that specifically recognize Lp(a)/apo(a) is not firmly established. An involvement by the VLDL receptor in the recognition of apo(a) has been recently suggested (26). Based on the current findings, it would be of interest to determine whether F1 is a ligand for this receptor. It is important to note that, in all of the animals injected with either Lp(a)/apo(a) or F1, the urinary fragments, if present, were comparable in size and smaller than those in the plasma, suggesting that in our mouse strain neither NE nor MMP-12 was involved in the second step degradation by the kidney perhaps by additional MMPs. In this regard MMP-2 and 9 have been reported in the renal tissue (27, 28).

A question that was not specifically addressed in the current studies was the site(s) of the first step of apo(a) fragmentation generating F1. The plasma appears to be an unlikely source based on the findings by Mooser et al. (2, 3) and our own (4, 5) showing that neither mouse nor human plasma has the capacity to cleave, in vitro, Lp(a)/apo(a). Moreover, any MMP-12 activity in the plasma would be neutralized by circulating tissue inhibitors of MMP, at least under physiological conditions. A more likely possibility is that the apo(a) fragments are generated at tissue sites by the action of MMPs secreted by macrophages unopposed by local tissue inhibitors of MMP. Because F1 and F2 are generated in vitro by the action of both NE (5) and MMP-12 on apo(a), one has to ask why only F1 was present in the plasma of the NE−/− and control mice injected.
intravenously with apo(a). This may be because of the fact that F2 but not F1 binds to matrix macromolecules such as fibrinogen and fibronectin (4) and as exemplified by our recent findings on the proteoglycan decorin (29). On the other hand, the cleavage of F1, for which no ligand has been identified thus far (30), once formed at tissue sites would be free to diffuse and return to the blood stream by a reverse flow process.

The current studies invite the question as to whether the results on the murine models may apply to man. It is of interest to note that mouse MMP-12 has a high degree of homology with its human counterpart (16). Moreover, the apo(a) fragments that are spontaneously present in the plasma of human subjects are of a similar size as those found in the plasma of mice injected with human apo(a) (2–4) and also as shown by the current results in Fig. 4. In addition, the urine of human subjects has been shown previously (2–4) and in this study to contain apo(a) immunoreactive fragments which are smaller in size than those in the plasma, suggesting that the human kidney is involved in the second step of degradation of apo(a). However, despite these similarities more direct documentation is needed to establish that MMP-12, or other MMPs, are involved in the fragmentation of apo(a) in human subjects.

In summary, our results have shown that MMP-12 cleaves apo(a) in the linker region between kringles IV-4 and IV-5 and subsequently degraded by the kidney. The emerging notion may account for the generation in the plasma of F1, which is also provide evidence from mouse models that such a cleavage is needed to establish that MMP-12, or other MMPs, are involved in the generation of F1 may be controlled by the activity of MMP-12, suggests that the activity of this enzyme may play an important role in the pathobiology of Lp(a).

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