Identification of Matrix Metalloproteinases 3 (Stromelysin-1) and 9 (Gelatinase B) in Abdominal Aortic Aneurysm

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Abstract A prominent metalloproteinase activity with an apparent molecular mass of 80 kD and additional activities at 67 through 70, 50, and 32 kD have been observed on casein, gelatin, and elastin gel zymography in extracts from abdominal aortic aneurysms (AAAs). The forms at 80, 50, and 32 kD were isolated by affinity to recombinant tissue inhibitor of metalloproteinases, and the 80-kD and 50-kD components were shown to be derived from matrix metalloproteinase-9 (MMP-9). The relative electrophoretic mobility of these forms under reducing and nonreducing conditions corresponds to those of MMP-9 generated by MMP-3 (stromelysin-1) cleavage, and the active forms of MMP-3 at 45 and 35 kD were detected in aneurysmal extracts under reducing conditions by using specific antibody. Confirmation that the major proteolytic activity observed at 80 kD is MMP-9 was also demonstrated by immunoprecipitation of the activity with specific antibody. Comparative immunoblots of tissue extracts from 10 typical AAA patients, using specific antibody against MMP-9, revealed bands at 92, 82, 67, 51 through 53, 27, 23, and 20 kD under reducing conditions; six aortic control specimens displayed negligible immunoreactivity. This report is the first to show that known activated forms of MMP-3 and MMP-9 are present in the aneurysmal aortic wall and that they may play a role in the destruction of aortic matrix in AAA disease.

Key Words • aneurysm • matrix metalloproteinases • stromelysin-1 • gelatinase B

The extracellular matrix (ECM) is a complex network of various proteins and proteoglycans maintained by an intricate balance between the synthesis and degradation of its structural components. The maintenance of tissue integrity through remodeling and repair of tissue damage involves the interaction of numerous enzymes and the inhibitors that keep their activity in check. Disturbances in the balance between proteinases and antiproteinases have been implicated in numerous enzymes and the inhibitors that keep their activity in check. The accelerated degradation of ECM associated with the development of various pathological states, including emphysema and rheumatoid arthritis, 1-5 Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes with a broad specificity for degrading various ECM components. 6 After secretion in latent form(s), these enzymes are activated; their activation is accompanied by a loss in molecular mass. Their activity is inhibited by specific protein inhibitors called the tissue inhibitors of metalloproteinases (TIMPs). MMP-3, or stromelysin-1, degrades a number of ECM components including proteoglycan, fibronectin, laminin, and gelatin (denatured collagen). MMP-9 (92-kD type IV collagenase) has elastolytic activity in addition to its ability to degrade types IV and V collagen and gelatin. 7

Loss of elastic fibers and disruption of normal matrix structure in the vascular wall are hallmarks of abdominal aortic aneurysm (AAA) disease. Increased elastase, collagenase, and gelatinase activities in the aneurysmal aortic wall have been extensively reported and proposed as mediators of the tissue damage observed. 8-15 We have described a prominent metalloproteinase activity with an apparent molecular weight of 80 kD present in aneurysmal tissue, 16 and we have developed techniques for isolating it and several other activities (at 50 and 32 kD) for further characterization. 17 Specific identification of the observed proteinases is an important first step in determining their involvement in AAA development. In this article we demonstrate an increase in forms of MMP-3 and MMP-9 in extracts of AAA tissue and show that the major component of the previously described 80-kD activity is MMP-9.

Methods

Human Subjects

Studies were performed with the approval of the Institutional Review Board on Human Studies. Specimens from the infrarenal aorta were obtained with consent from patients undergoing aneurysm resection (n=21). After consent was obtained, control infrarenal aortic specimens were obtained from cadaveric transplant patients (n=4) or from patients undergoing aortic reconstruction for aortic occlusive disease (n=3).

Enzyme Preparation

All tissue samples were frozen and stored at −120°C until use. The aortic tissues were processed 17 by being homogenized in 50 mmol/L tris(hydroxymethyl)aminomethane (Tris)-HCl...
and 2 mol/L NaCl, pH 7.5, and centrifuged at 10 000g for 1 hour. The pellet was homogenized in 50 mmol/L Tris-HCl, 2 mol/L NaCl, and 0.5 g/L Brij 35, pH 7.5, and then centrifuged as above; the resulting pellet was rehomogenized in 50 mmol/L Tris-HCl, 1 mol/L NaCl, and 2 mol/L urea, pH 7.5, and the extract was centrifuged as above. All supernatants were dia-
lyzed against 50 mmol/L Tris-HCl, pH 7.9, and are referred to as the salt, Brij, and urea preparations, respectively.

The protein concentrations of the samples were estimated by using the dye-binding technique (BioRad Laboratories). The activities of the preparations were analyzed by using modifications of methods for casein and gelatin zymography.18 Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed, and samples were run either after heating to 100°C for 5 minutes in the presence of 0.32 mol/L β-mercaptoethanol and 3.5 mmol/L SDS (reducing conditions) or without heating in the presence of 86.7 mmol/L SDS (nonreducing conditions). Molecular-weight standards were run on all gels under reducing conditions. Prestained low-range standards (BioRad Laboratories) were used as general markers; the molecular weight of each protein in the preparation (indicated on the figures in kilodaltons) was determined in our system by using low-range and high-range molecular-weight standards from Pharmacia-LKB.

The dialyzed Brij and urea preparations were partially purified on a recombinant (r) TIMP-1–Sepharose column. The rTIMP-1–purified preparations were pooled and concen-
trated. The proteins were separated on a nonreduced SDS–polyacrylamide gel (10%). The individual activities at 80, 50, and 32 kD were electroeluted from the areas of the gel corresponding to the appropriate molecular weight.17 In addition, the protein from the high-molecular-weight region of the gel (>97 kD) was electroeluted. The electroeluted proteins were concentrated by using Centricon-10 concentration units (Amicon). rTIMP-1 was a generous gift from Synergen, Inc.

Immunoblotting Analysis

The Brij preparations for the comparative patient studies were equalized for protein concentration prior to separation on SDS–polyacrylamide gels under reducing conditions. The electroeluted protein preparations at 80 and 50 kD were run on SDS–polyacrylamide gels under reducing and nonreducing conditions as described above. The proteins were transferred electrophoretically to nitrocellulose filters (65 V for 2 hours) for immunoblotting analysis.11 Sheep antiserum specific to MMP-924 and proMMP-925 were prepared. The nitrocellulose filter was washed in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1 g/L Tween-20, pH 7.5 (TTBS), and then incubated for 18 hours at 4°C with either antiserum to MMP-3 (1:3000) or purified immunoglobulin (Ig) G to proMMP-9 (0.5 g/L) di-
luted in TTBS. The nitrocellulose filter was processed by using an alkaline phosphatase–labeled Vectastain ABC kit for de-
tecting sheep IgG (Vector Laboratories) according to the manufacturer’s protocol. The immunoblots were visualized by using the alkaline phosphatase substrate nitro blue tetra-
zolium/5-bromo-4-chloro-3-indolyl phosphate with theVectastain substrate kit IV (Vector Laboratories) according to the manufacturer’s protocol.

Immunoprecipitation

The rTIMP-1 affinity-purified enzyme was stirred in the presence or absence of sheep anti-human proMMP-9 IgG (final concentration, 0.5 g/L) for 18 hours at 4°C. The enzyme samples were then treated for 4 hours at 4°C with a 1:1 slurry of protein G–agarose (Sigma Chemical Co) in 50 mmol/L Tris-HCl, 0.005 mmol/L phenylmethylsulfonyl fluoride, 3.5 mmol/L SDS, and 3.1 mmol/L NaN3, pH 7.9. The agarose beads were pelleted, and the activities remaining in the resultant supernatants were assessed on casein and gelatin zymography.

![Fig 1. Immunoblot analysis detecting the presence of matrix metalloproteinase (MMP)-9 in control and abdominal aneurysm aortic tissue extracts. The Brij preparations from control and abdominal aneurysmal aortic (AAA) tissue (equalized for total protein) were run on a sodium dodecyl sulfate–polyacrylamide gel (10%), blotted on nitrocellulose, and detected by using sheep anti-
human MMP-9 immunoglobulin G (see "Methods"). Control extracts are shown in lanes a through c; AAA extracts are shown in lanes d through h. Standards in kilodaltons are indicated on the left.](image-url)
purified on the rTIMP-1 affinity column were further isolated by electroelution from an SDS–polyacrylamide gel under nonreducing conditions. These activities on cassin zymography are shown in Fig 2B.

Fig 3 demonstrates that both the 80- and 50-kD electroeluted preparations were recognized by using the specific antibody preparation to MMP-9 under both reducing and nonreducing conditions. The 80-kD enzyme preparation showed bands of immunoreactive material at 80 and also at 67 through 70 kD under nonreducing conditions (Fig 3, lane a). When reduced, heterogeneous bands of immunoreactive material in this preparation were run at 82 through 86 and 67 through 75 kD (Fig 3, lane b). The 67- through 70-kD bands were not observed in initial blots and increased in amount over time after extraction, suggesting that they were probably produced from the 80-kD material by degradation. The electroeluted enzyme preparations are not pure, and the preparation may contain other proteinases that can degrade the 80-kD protein over time, resulting in two immunoreactive bands.

The 50-kD enzyme preparation showed a single band of immunoreactive material with the expected molecular mass under nonreducing conditions (Fig 3, lane c).

Upon reduction, this material exhibited heterogeneous bands of 50 through 70 kD (Fig 3, lane d). Stronger immunoreactivity with the reduced sample is probably due to the increased exposure of epitopes.

Immunoprecipitation

Gelatin zymography of the rTIMP-1 affinity-purified enzyme showed major proteolytic activity at approximately 80 through 90 kD in the AAA samples; the molecular weight of the forms suggested that this activity could be derived from MMP-9. To confirm that these activities were indeed MMP-9–based, the sample was subjected to immunoprecipitation with anti-MMP-9 antibody. The activity at approximately 80 through 90 kD appears as a doublet at approximately 85 and 80 kD.

Fig 4. Gelatin zymography demonstrating the immunoprecipitation of the 80-kD activity with specific antibody to matrix metalloproteinase–9. The recombinant tissue inhibitor of metalloproteinase–1 affinity-purified enzyme preparation was subjected to gelatin zymography (see "Methods") without treatment (lane a), after treatment with protein G–agarose only (control) (lane b), and after immunoprecipitation with sheep anti-human proMMP-9 immunoglobulin G and protein G–agarose (lane c). Standards in kilodaltons are indicated on the left.
MMP-3 in abdominal aneurysmal aortic tissue preparations. The recombinant tissue inhibitor of metalloproteinase-1 (rTIMP-1) affinity-purified enzyme preparation and the preparation electroeluted from the high-molecular-weight region of a sodium dodecyl sulfate (SDS)-polyacrylamide gel (see "Methods") were run on an SDS-polyacrylamide gel (10%) under reducing and nonreducing conditions and immunoblotted. The immunoreactive material was detected by using sheep anti-human MMP-3 serum. The rTIMP-1 affinity-purified preparation under nonreducing and reducing conditions is shown in lanes b and d, respectively; the high-molecular-weight preparation under nonreducing and reducing conditions is shown in lanes a and c, respectively. Standards in kilodaltons are indicated on the left.

Studies of MMP-3 Enzyme Forms

When the rTIMP-1 affinity-purified enzyme was subjected to immunoblotting analysis using antibody to human MMP-3, bands of 97 through 120 kD were detected under nonreducing conditions (Fig 5, lane b). Under reducing conditions, bands of 45 and 35 kD corresponding to known active forms of MMP-3 were observed (Fig 5, lane d). This suggests that the nonreduced high-molecular-weight species contains active forms of MMP-3. This is further supported by the demonstration that a preparation electroeluted from the high-molecular-weight region (>97 kD) of a nonreducing gel that is recognized by the anti-human MMP-3 serum (Fig 5, lane a) yields a band at 45 kD under reducing conditions (Fig 5, lane c).

The electroeluted enzyme preparation of 32 kD (Fig 2, lane c) did not show reactivity with anti-human MMP-3 serum; this may be due to the limited amount of antigen available in the purified material, or this activity is derived from a distinct enzyme. It should also be noted that no immunoreactive material was observed with either anti-MMP-3 serum or anti-MMP-9 IgG in rTIMP-1 affinity column eluates from control aortic extracts.

Discussion

MMP-9 is a member of the MMP family with elastolytic activity. This enzyme became a prime candidate in our efforts to identify the prominent 80-kD metalloproteinase observed in AAA tissue extracts due to its molecular weight correspondence with an active form of MMP-9. Its production by macrophages, which are significantly increased in aneurysmal aorta, and its expression in other pathological states associated with connective tissue degradation. Others have noted the presence of MMP-9 in AAA tissue, but its identity as a major active form has not been established.

Two immunoreactive forms of MMP-9 purified from AAA tissue extracts at 80 and 50 kD have been identified in the present work. These correspond to activities observed on substrate gel zymography using gelatin, casein, and -elastin (not shown) as substrates, and the activity observed at 80 kD can be immunoprecipitated with MMP-9-specific antibody. In addition, the observation that recombinant MMP-9 is processed to an active form at 40 through 50 kD supports our data.

The general amounts of the material recognized by the antibody to MMP-9 were greatly increased in AAA tissue extracts compared with control specimens. Together with our previous work showing that TIMP-1, a major inhibitor of MMPs, is decreased in amount in aneurysmal aorta, the present studies suggest an overall increase in the amount of enzyme "free" to degrade the surrounding matrix. In addition, the 82- and 67-kD bands are in agreement with those described as activated forms of MMP-9. The immunoreactive bands at 27, 23, and 20 kD do not correspond to any observed activity, so they are probably fragments of MMP-9 generated during activation or by proteolytic degradation.

The 82- through 86-kD material observed on immunoblots of the 80-kD preparation under reducing conditions agrees with the molecular forms of MMP-9 activated by MMP-3. The 67-kD material produced over time in the 80-kD electroeluted preparation corresponds well with a 67- or 68-kD form generated when proMMP-9 is activated by MMP-3 in the absence of TIMP. In addition, the immunoreactive forms at 67 kD and 50 through 70 kD observed under reducing conditions are consistent with those described as activated forms of MMP-9. The immunoreactive bands at 27, 23, and 20 kD do not correspond to any observed activity, so they are probably fragments of MMP-9 generated during activation or by proteolytic degradation.

MMP-3 activates both proMMP-9 and proMMP-1, and forms consistent with the known active forms of MMP-3 at 45 and 35 kD were demonstrated by immunoblotting in preparations from AAA tissue under reducing conditions. The forms of MMP-9 observed at approximately 80 kD and 67 through 70 kD are consistent with those described activated by MMP-3 (at 82 through 86 and 67 kD), suggesting that this pathway may be important in AAA disease. In addition, MMP-3
is capable of degrading a number of ECM components.\(^4\) Thus, it is likely to be involved in the observed aortic tissue degradation as well as participating in proMMP-9 activation. The high-molecular-weight immunoreactive species of MMP-3 observed under nonreducing conditions suggests that MMP-3 exists as high-molecular-weight complexes in the tissue. The significance of these complexes and their importance in the activation cascade require further investigation.

One observation made during the purification procedure may explain some of the previous controversies over the exact nature of the major proteolytic activities in the AAA wall. A portion of the 80-kD activity (as determined by zymography) could not be bound to the rTIMP-1 affinity column even in repeated trials. This activity was inhibited by phenylmethylsulfonyl fluoride, a serine protease inhibitor, suggesting that some of the activity demonstrated in the tissue extracts on casein and gelatin gel enzymography is not MMP-9. Plasmin is a serine protease with the ability to degrade these substrates, and the approximate molecular weight of the active form of plasmin corresponds well to the residual activity after rTIMP-1-Sepharose chromatography.\(^33\) Plasmin may play a role in MMP-1 and MMP-3 activation,\(^31,32\) although its involvement in MMP-9 activation is probably indirect.

MMP-9 may possess elastinolytic activity,\(^7\) and the destruction of elastin is proposed as an initiating event leading to aneurysmal changes in the aortic wall.\(^34\) MMP-9 also facilitates the action of MMP-1 (collagenase) on native type I and type III collagens, which are major constituents of the vascular wall.\(^35\) In addition, Okada et al\(^25\) report that MMP-9 has some ability to degrade fibrillar collagens. The presence of MMP-1 in aneurysmal aorta has not been firmly established.\(^8,13,15\) We have observed forms consistent with known active forms of MMP-1 in AAA tissue extracts using specific antibodies,\(^17\) and increased amounts of immunoreactive material to MMP-1 are present in AAA compared with control specimens.\(^36\) The possible presence of active forms of MMP-3 and a serine proteinase suggests a cascade of events leading to activated forms of both MMP-1 and MMP-9 that could then lead to the concerted degradation of elastin, fibrillar collagens, and other ECM components in the vascular wall during aneurysm development and progression.

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