Activated forms of MMP_2 and MMP_9 in abdominal aortic aneurysms

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Purpose: **The consistent observation of a reduction of the elastin concentration in abdominal aortic aneurysms (AAAs) has led us to investigate in AAA specimens two metalloproteinases that display elastase activity, MMP_2 (gelatinase A/72 kDa) and MMP_9 (gelatinase B/92 kDa).**

Methods: **Samples of full-thickness aortic wall, adherent thrombus, and serum were collected in 10 patients with AAAs. Samples of normal aortic wall and serum were taken from 6 age-matched control patients. Quantitative gelatin-zymography and gelatinolytic soluble assays after acetyl-phenyl mercuric acid activation were performed on serum and tissue extracts, and the results were expressed in units on a comparative wet-weight basis. Histologic analysis was performed in parallel to score the inflammatory infiltrate.**

Results: **The luminal and parietal parts of the thrombus contained, respectively, 20- and 10-fold more gelatinolytic activity than the serum. The predominate form was MMP_9. Although the total gelatinolytic activity was in the same range both in AAAs and in normal walls, a significantly higher proportion of MMP_9 was found in the aneurysmal aortic walls. Furthermore, a significant proportion of MMP_9 was under its processed active form, which was never observed in normal samples. A significantly higher proportion of MMP_2 was also present as processed active form in AAA wall. This latter parameter positively correlated with the inflammatory score.**

Conclusions: **The presence of activated MMP_9 and MMP_2 might contribute to the degradation of the extracellular matrix proteins that occurs during the development of aneurysms. (J Vasc Surg 1996;24:127-33.)**

Structural alterations of the aortic wall and degradation of matrix proteins consistently have been reported to occur in abdominal aortic aneurysms (AAAs) as compared with healthy aorta or with atherosclerotic occlusive aorta.[1-3] The analyses that we previously performed on tissue fragments collected from AAAs of increasing size demonstrated a reduction in elastin concentration that occurred during the early development of the lesion without alteration in collagen concentration, whereas an increased extractability of collagen was observed in the ruptured specimens.[4] These alterations might result from an increased proteolysis, decreased antiproteolytic activities, or both.[5,6] Increased levels of blood and tissue proteinase activities have indeed been reported in patients with AAAs; some of the previous studies described a serine-protease[7,8] as the main elastolytic activity, whereas other studies showed that the elastolytic activity presented features of the family of metalloproteinases (MMPs).[9-12]

The MMPs are connective tissue–degrading enzymes that participate in a variety of physiologic remodeling processes and in many diseases associated with excessive tissue degradation, such as arthritis, tumor invasion, periodontitis, and osteoporosis. The members of this family share a high level of gene characteristics and common features, such as a Zn-dependent catalytic site, requirement of Ca++ for activity, and secretion under a latent proenzyme form requiring a primary activation by proteolytic enzymes, organomercurials, or chaotropic agents fol-

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Table I. Material and methods

	Aneurysm diameter 66.8 ± 10.8 mm	*Control aorta <30 mm*	*Control serum*
Samples			
No.	10	6	6
Men	8	6	6
Age (yr)	70.5 ± 10.5	77.5 ± 10.0	69.0 ± 6.3

lowed by an autocatalytic activation by cleavage of the propeptide. Their activity in the tissues is regulated by specific tissue inhibitors of metalloproteinases (TIMPs).[13] MMP_2 (gelatinase A/72 kDa) and MMP_9 (gelatinase B/92 kDa) express a broad spectrum of activity and have been shown to display significant elastase activity, more potent than the stromelysins,[14] on purified substrate[15] and in organotypic culture of aortic fragments.[16] MMP_2 is produced by cells of mesenchymal lineage, whereas MMP_9 is secreted by neutrophils, macrophages, and macrophage-derived osteoclasts and likewise participate in the inflammatory response. The excessive degradation of elastin and, perhaps, other non–collagen matrix components and the presence of an inflammatory infiltrate in the wall of AAAs[2,17,18] led us to investigate the involvement of these MMPs in this disease. For this purpose, we identified and quantitated MMP_2 and MMP_9 and their extent of activation in the wall of AAAs as compared with normal aortic walls. As significant amounts of the two enzymes and their inhibitors (TIMPs) are present in the circulating blood,[19,20] they were evaluated on a comparative unit wet-weight basis in the serum and in the wall-adherent thrombus at a luminal and parietal location.

MATERIALS AND METHODS

Collection of specimens. All tissues and blood samples were obtained with the approval of the Institutional Ethics Committee of the Liège University Hospital. Fragments of full-thickness aortic wall and adherent thrombus were collected 4 to 5 cm distal to the renal arteries in 10 patients undergoing elective operative repair. The mean diameter of the AAAs was 66.8 ± 10.8 mm. A sample of serum was collected from each patient. Control aortic samples were collected at autopsy within 24 hours after death from six age-matched patients without known cardiovascular disease. Age-matched control serum samples were obtained from six volunteers. The mean age was 70.5 ± 10.5 years in the AAA group, 77.5 ± 10.0 years in the control aorta group, and 69 ± 6.3 years in the control serum group (Table I). A piece of material (0.5 to 3 cm^3) was dissected from the luminal and parietal side of the thrombus and from the aortic wall, quickly frozen, weighed (wet weight), crushed in liquid nitrogen, lyophilized, and weighed again (dry weight).

Extraction of MMPs. Aliquots of powdered thrombus and aortic wall were extracted (2 ml/100 mg dry weight) overnight at 4° C in 0.05 mol/L Tris HCl pH 7.5, 1 mol/L NaCl, 2 mol/L urea. After centrifugation at 15,000 rpm in the cold, the supernatant was collected and used for measurement of gelatinase activity by a soluble assay and by zymography.

Soluble assay of gelatinase activity. The gelatinolytic activity present in tissue extracts and serum was assayed by using ^3H-gelatin as substrate. Type I collagen was purified from fetal bovine skin and radiolabeled with ^3H-acetic anhydride as previously described[21] to a specific activity of 0.79 × 106 cpm/mg collagen. Before performing the assay, the ^3H-collagen was neutralized with 1 mol/L Tris (base) and diluted with 0.05 mol/L Tris-HCl pH 7.5, 0.2 mol/L NaCl, 0.5% Triton X-100, 5 mmol/L $CaCl_2$, 3 mmol/L NaN_3 to 5,000 cpm per 50 µl of substrate solution, denatured to ^3H-gelatin by heating 10 minutes at 60° C and supplemented with 5 µl of 2.5 mmol/L solution of protease inhibitors, N-ethylmaleimide (NEM) and phenylmethane sulfonylfluoride (PMSF).

Serial dilutions (1/10 to 1/800) of the tissue extracts and of the serum, the latter being brought to 4 mol/L urea before activation, were incubated for 15 minutes at 25° C in the absence or presence of 0.3 mmol/L acetyl-phenyl mercuric acid (APMA) to activate the gelatinases. After APMA activation, some samples were supplemented with ethylenediamine tetraacetic acid (EDTA) to 10 mmol/L to inactivate the MMPs and served as blank values. Nonactivated, activated, and EDTA-inhibited samples were incubated overnight with 5,000 cpm ^3H-gelatin at 37° C, trichloroacetic acid–precipitated (12% final) in the cold and centrifuged at 2,000 rpm, and the TCA-soluble radioactivity (fragments of gelatin) was measured by liquid scintillation spectrometry. The activity was calculated from the serial dilutions fitting in the linear part of the kinetics curves. The results were expressed in units per mg wet weight (tissue) or per µl (serum), one unit being the activity able to degrade 1 µg of gelatin (=790 cpm) in 16 hours at 37° C.

Zymography assay. Serial dilutions of tissue extracts and serum were incubated for 1 hour at room temperature in 0.01 mol/L Tris HCl pH 6.8, 4% sodium dodecylsulfate (SDS) before performing polyacrylamide slab gel electrophoresis according to the technique of Laemmli[22] using a separation gel con-

Table II. Amount of gelatinolytic measured by soluble assay and by zymography and distribution between MMP_2 and MMP_9

	Serum		*Tissue samples*			
			Thrombus		*Wall*	
	Control (n = 6)	*AAA (n = 10)*	*Luminal (n = 10)*	*Parietal (n = 10)*	*AAA (n = 10)*	*Control (n = 6)*
Total activity						
Soluble assay*	3.0 ± 0.9	2.8 ± 0.8	41.9 ± 51.3	13.1 ± 15.8	11.1 ± 7.8	5.5 ± 4.8
Zymography†	2.7 ± 0.5	5.6 ± 2.5‡	125.4 ± 93.0	68.4 ± 56.4	71.4 ± 39.4	53.6 ± 44.6
Distribution (in%)						
MMP_2	55 ± 13	56 ± 11	19 ± 16	33 ± 17	49 ± 16	71 ± 8
MMP_9	45 ± 13	44 ± 11	81 ± 16	67 ± 17	51 ± 16‡	29 ± 8

Results are expressed in units per µl of serum or per mg of wet weight of tissue, *one unit in soluble assay corresponding to enzyme activity degrading 1 µg of gelatin in 16 hours at 37° C and †one unit in zymography assay corresponding to bleaching of 1 arbitrary volume of gelatin containing acrylamide gel.
‡Significantly different from control values, $p < 0.02$.

taining 1% gelatin. After migration, the gels were washed two times in 2% Triton at 30° C and incubated at 37° C overnight in 0.05 mol/L Tris HCl pH 7.6, 10 mmol/L $CaCl_2$ to allow for gelatin degradation. The gels were stained with Coomassie Blue, the gelatinase bands appearing as white on a blue background. The intensity of the bands was recorded with a LKB Ultrascan XL laser scanning densitometer. The enzyme activities were calculated from the linear part of the regression curves relating the intensity of the bands and serial dilutions of the tissue extract or serum.

Histologic analysis. The specimens of aortic tissue and thrombus, luminal and parietal, were fixed in 3.5% saline-buffered formaldehyde and processed with standard techniques for paraffin embedding. Hematoxylin and eosin–stained sections were used to evaluate the density, localization, and nature of the inflammatory cells. The intensity of the inflammatory reaction was scored as mild (+), moderate (++), or severe (+++).

Statistical analysis. Statistical analysis was performed by Student's *t* test, and the distribution of each variable was characterized by the mean and standard deviation. Results were considered to be significant at the 5% critical level ($p < 0.05$).

RESULTS

The patterns of distribution of MMP_2 and MMP_9 display obvious differences in the various samples (Fig. 1). They are representative of the quantitative results in Tables II and III.

Table II illustrates the gelatinolytic activity measured by soluble assay and by zymography, expressed in units per mg wet weight of extracted tissue or per µl (serum), in the serum, extracts of luminal and parietal fragments of the thrombus and of the aortic wall from the 10 patients with AAAs compared with six normal age-matched serum samples and six samples of normal aortic wall from age-matched individuals. The distribution of gelatinolytic activity between MMP_2 and MMP_9 is recorded from zymograms and reported as the sum of the zymogen and of the processed forms of each of the two enzymes.

All reported activities were inhibited by EDTA, a property characteristic of the Ca-dependent MMPs and resistant to the thiol-proteinases inhibitor, NEM, and to the serine proteinases inhibitor, PMSF.

Gelatinase activity in the serum. By using the soluble assay after APMA activation, the total gelatinase activity measured in the serum was similar in AAAs and normal controls whereas two times more activity was found in AAA serum by zymography (Table II). The gelatinolytic activity was almost equally distributed between the MMP_2 and MMP_9 in the serum of control and in AAAs. It must be noted that in five of the 10 samples of AAAs, around 10% of the MMP_9 was found under an activated processed form (Table III), whereas none of the control samples displayed such processing.

Gelatinase activity in the thrombus. A high level of gelatinolytic activity was observed in the luminal thrombus in contact with the blood and, to a lesser extent, in the parietal segment of the thrombus (Table II). As compared with the activity in the serum and expressed on the same basis (1 µl of serum = 1 mg wet weight of thrombus), the luminal part of the thrombus contained 15 (by soluble assay) to 25 (by zymography) times more gelatinolytic activity than the serum. These levels dropped in the parietal segment of the thrombus. This high gelatinolytic activity was mainly a result of MMP_9, representing

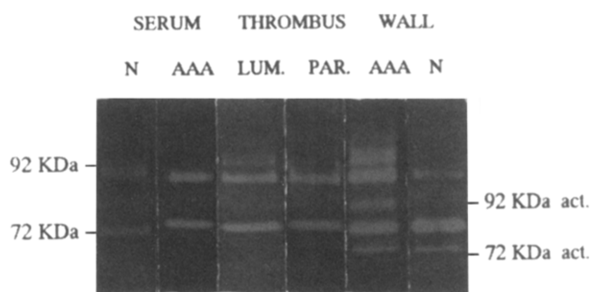

Fig. 1. Representative example of MMP_2 and MMP_9 activity under latent (92 and 72 kDa) or processed (92 and 72 kDa act) forms measured by zymography in serum and vessel wall *(WALL)* extracts of normal aorta *(N)* and AAAs. Extracts of samples from luminal *(LUM)* and parietal *(PAR)* parts of adhering thrombus are also shown.

80% and 70% of the total activity in the luminal and the parietal parts of the thrombus, respectively. In the luminal thrombus, no activated form was observed, whereas in the parietal thrombus 10% of the MMP_9 and 30% of the MMP_2 were present as activated processed enzyme (Table III).

Gelatinase activity in the aortic wall. The total gelatinase activity measured in the AAA wall was similar to that found in the parietal thrombus and did not significantly differ from the activity present in the normal aortic walls (Table II). Whereas the preponderant form of gelatinase in the normal aorta was MMP_2, a significantly higher proportion of MMP_9 was detected in AAA ($p < 0.05$). Moreover, a significant proportion of the MMP_9 appeared in the AAA wall as fully processed activated enzyme, which was never observed in the control aortic wall. The activated processed form of MMP_2 was also significantly increased, more than doubled in the wall of AAA as compared with controls (Fig. 1 and Table III). A gelatinolytic activity migrating above the latent pro-MMP_9 was also observed in most of the AAA wall samples (Fig. 1). It was not taken into account in the calculation of MMP_9 activity.

Histologic examination. Microscopic examination of the AAA wall showed medial and intimal fibrosis often associated with atherosclerosis, focal calcifications, perivascular sclerosis, and thickening of the vasa vasorum. The thrombi consisted of a fibrinous material infiltrated diffusely or locally by degenerated red cells and rare leukocytes. No obvious morphologic difference was found between the luminal and the parietal thrombus. Fig. 2 is a representative example of our series of specimens, showing the low density of the inflammatory infiltrate in the luminal (Fig. 2, *A*) and parietal (Fig. 2, *B*) side of the

Table III. Activated forms of MMP_2 and MMP_9 gelatinases

	MMP_2	*MMP_9*
Serum control	0	0
Serum AAA		12 ± 12%*†
Thrombus luminal	0	0
Thrombus parietal	27 ± 24%	9 ± 10%
Wall AAA	35 ± 12%*	21 ± 15%*
Wall control	17 ± 5%	0

*Significantly different from control walls, $p < 0.05$.
†Results are expressed in percent of total MMP_2 or MMP_9 activity.

adherent thrombus and the preferential localization of inflammatory cells in the adventitia and the media of the aortic wall (Fig. 2, *C*). They consisted predominantly in mononuclear cells (lymphoplasmacytic cells and macrophages) beside some polymorphonuclear neutrophils. A linear regression relationship was tentatively established between the extent of the inflammatory cells infiltrate in the aortic wall and the level of expression and activation of the two gelatinases (Table IV). The only significant positive correlation ($r = 0.46$) was between the level of activated MMP_2 and the density of the infiltrate.

DISCUSSION

The enzyme activities measured in the serum and in the tissue extracts display an array of features specific of the MMPs. They are inhibited by EDTA, resistant to PMSF, and activated by NEM, organomercurials, and SDS. The latent forms and processed forms of these enzymes display adequate molecular size as compared with the enzyme secreted by A2058 cell line for the MMP_2[23] and 12-0-tetradecanoylphorbol-13-acetate–induced transformed endothelial cells ECV-304 for the MMP_9[24] (data not shown).

The two assays used for measuring gelatinase activity provide somewhat different information. In the soluble assay, the activation of the gelatinases is performed by an organomercurial reagent that does not dissociate the enzymes from their complex with the TIMPs. This technique therefore measures the total free MMP_2 and MMP_9 activities. By zymography, the gelatinases are activated by SDS and dissociated from their inhibitors by electrophoresis. This technique allows discrimination of the MMP_2 and MMP_9 as well as their processed activated forms by their molecular size and definition of a distribution pattern of each form. This distribution was used to calculate the gelatinolytic activity, measured by the soluble assay, attributable to each form. The difference between the values measured by zymography and by the soluble assay provides an indirect estimation of the TIMPs.

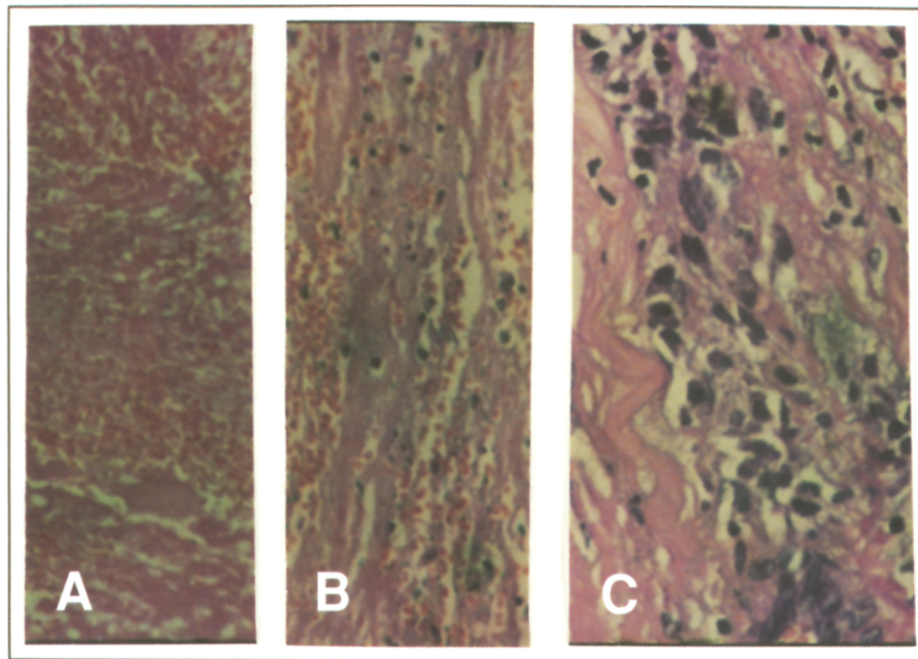

Fig. 2. Hematoxylin-eosin stained sections of luminal **(A)** and parietal **(B)** side of adherent thrombus, which consisted essentially of fibrinous material infiltrated by degenerated red cells and rare leukocytes. The aortic wall **(C)** shows medial and intimal fibrosis associated with mononuclear cell infiltrate predominating in adventitia and media.

Table IV. Relationship between inflammatory cells infiltrate in aortic wall and latent and activated forms of MMP_2 and MMP_9

		MMP_9 (units/mg)		*MMP_2 (units/mg)*	
Infiltrate	*No.*	*Latent*	*Activated*	*Latent*	*Activated*
+*	2	0.90 (0.78 to 1.17)	0.23 (0.18 to 0.27)	0.99 (0.24 to 1.75)	0.33 (0.17 to 0.50)
++	5	1.47 (0.30 to 2.95)	0.48 (0.03 to 1.47)	1.05 (0.64 to 2.50)	0.51 (0.22 to 0.99)
+++	2	2.86 (0.63 to 5.09)	0.25 (0.25 to 0.26)	1.62 (0.63 to 2.61)	0.83 (0.42 to 1.25)
r		NS	NS	NS	0.46

*Intensity of infiltrate was scored as mild (+), moderate (++), or severe (+++).
r, Correlation coefficient; *NS*, not significant.

The main conclusions that can be drawn from our investigations are (1) aneurysmal aortic walls contain a significantly higher amount of MMP_9 than control specimens, and (2) the activated processed forms of both MMP_2 and MMP_9 are significantly increased.

The two forms of gelatinolytic activity, MMP_2 and MMP_9, in the serum were almost equally represented, both in control patients and in patients with AAAs. Latent gelatinases are regular plasma components.[19] The MMP_9 probably originates from granulocytes. This observation is supported by the higher proportion of the MMP_9 in human serum compared with plasma,[25] as we observed in our samples, probably on its release during the in vitro clotting process. The MMP_2 originates from multiple cellular sources, as discussed by Moutsiakis et al.[20] The higher gelatinolytic activity measured in AAA serum by zymography as compared with the soluble assay suggests that circulating gelatinase activity might be partly inhibited by complexing with TIMPs. It is also noteworthy that five of the 10 patients with AAAs had a detectable amount of the activated processed MMP_9, which was never detected in the control group. By examining the clinical and biologic parameters in these five patients, results indicated that they differed by the size of the aneurysmal lesion. The mean aortic diameter of the patients who showed no processed MMP_9 form was 58.0 ± 5.7 mm (range, 50 to 65 mm), whereas that in the group of patients who showed detectable levels of

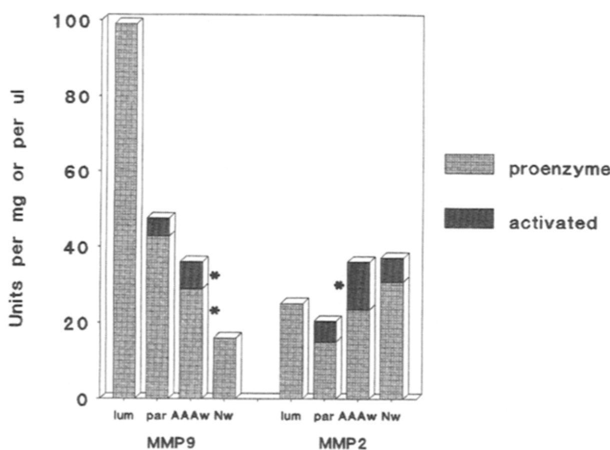

Fig. 3. Illustration of gradient of decreasing level of MMP_9 activity and increasing activation of both MMP_9 and MMP_2 from luminal thrombus toward aortic aneurysmal wall. *, Significantly different from control values.

activated MMP_9 was 75.6 ± 6.2 mm (range, 65 to 80 mm).

The large amount of MMP_9 activity in the luminal part of the thrombus could not be related to its sustained release by inflammatory cells because they were almost absent in this section of the thrombus. The enzyme might have been released during thrombus formation and sequestered by complex formation with other proteins. Both MMP_9 and MMP_2 are formed of structural domains that have been shown to participate in their binding to matrix components.[26] In addition to the propeptide, the catalytic, and the C-terminal hemopexin-like domain, the MMP_9 contains a fibronectin-like region and a type V collagen-homologous region that might participate in interactions with extracellular proteins such as fibrin. The MMP_2 was found as the predominating form in the aortic wall of the control group, whereas a significant shift toward MMP_9 was observed in the AAA specimen. MMP_2 is synthesized by the smooth muscle cells of the intima and the media of the wall as well as by the adventitial fibroblasts, as described by Herron et al.[10] It does not differ in absolute value in the control and AAA groups. The higher proportion of MMP_9 in the AAA is probably related to the presence of inflammatory cells known to produce this MMP. It was recently identified by immunohistochemical analysis[17] and in situ hybridization[18] in macrophages infiltrating the aneurysmal aorta. The MMP_9-positive cells represented, however, a subset of only 10% to 20% of the inflammatory cells.[18] This finding might explain why we were unable to find a significant correlation between the level of expression of the MMP_9 in AAA and the score of infiltrating cells. Other types of inflammatory cells might also produce the MMP_9, such as neutrophils that present the special feature to secrete the monomeric 92 kDa form and a heterodimer of 125 kDa where the MMP_9 is disulfide-linked with α2 microglobulin.[27] The gelatinolytic band migrating above the 92 kDa in our AAA wall samples might originate from polymorphonuclear leukocytes observed in the adventitia and the media of the AAA wall. Recent reports[28,29] identified in AAA, besides the MMP_9, the stromelysin 1 (MMP_3), interstitial collagenase (MMP_1) in high-molecular weight forms and the activated forms of MMP_9 and MMP_3. To our knowledge, our report is the first to demonstrate the presence of the activated form of MMP_2 in significantly higher proportion in the AAA than in normal aortic wall. The gradient of processing of both MMP_2 and MMP_9 observed in our study (Fig. 3) increasing from the luminal thrombus toward the AAA wall suggests that the activation process might originate in the wall.

Because the aneurysmal aortic wall contains a greater amount of MMP_9 with a significant proportion of activated form, unlike the enzyme in the normal aortic wall, and also a significantly larger amount of activated MMP_2, the possibility exists that these enzymes contribute to the degradation of extracellular matrix proteins observed in AAA.

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