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Decreased Tissue Inhibitor of Metalloproteinase-2/Matrix Metalloproteinase Ratio in the Acute Phase of Aortic Dissection

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Abstract

Purpose. Aortic dissection is characterized by fragility of the tunica media, and matrix metalloproteinases (MMPs) are enzymes that degrade the extracellular matrix of the aorta. This study examines MMPs in patients with acute aortic dissection (AAD) in an attempt to elucidate the mechanisms of their actions.

Methods. Enzyme-linked immunosorbent assays were used to measure the quantification of MMP-2, MMP-9, and the tissue inhibitor of metalloproteinase (TIMP)-2 in 30 patients with AAD, 12 patients with abdominal aortic aneurysm (AAA), and 16 control (CON) patients who underwent coronary artery bypass grafting.

Results. MMP-2 and TIMP-2 were significantly lower in the AAD group than in the CON group, at 36 ± 19 *vs* 58 ± 30 ($P < 0.01$) and at 21 ± 25 *vs* 216 ± 130 ($P < 0.001$), respectively. The TIMP-2/MMP-2 ratio was 3.7 ± 1.7 in the CON group and 0.9 ± 0.8 in the AAD group ($P < 0.001$ *vs* CON), and the TIMP-2/MMP-9 ratio was 200 ± 170 in the CON group and 37 ± 80 in the AAD group ($P < 0.001$ *vs* CON).

Conclusion. Low TIMP-2/MMP-2 and TIMP-2/MMP-9 ratios might play an important role in the onset of aortic dissection, when the tunica media becomes fragile with chronic breakage and degradation of the extracellular matrix.

Key words Matrix metalloproteinase-2 · Matrix metalloproteinase-9 · Tissue inhibitor of metalloproteinase-2 · TIMP/MMP ratio · Aortic dissection

Introduction

Improvements in diagnostic modalities and operative techniques for acute aortic dissection (AAD) have resulted in improved hospital mortality to around 20%–30%.[1,2] However, serious consequences such as acute rupture, acute myocardial infarction, or neurological complications are still associated with high hospital mortality.[3]

Although Marfan syndrome, hypertension, and arteriosclerosis have been implicated in the etiology of aortic dissection, no definite consensus has been reached.[4–7] Pathologically, cystic medial necrosis and lamellar medial necrosis are found in the arterial wall of the dissected aorta,[7] and electron microscopy shows helicoid hypertrophy of the collagenous fibers and rarefaction and fragmentation of the elastic fibers in the tunica media.[7] Biochemically, there are abnormalities in collagen and elastin in the tunica media of the dissected aorta.[8,9] The extracellular matrix, which is made of the same elastic fibers and collagenous fibers that exist in the tunica media, plays a role in maintaining the shape of the aortic wall.[10] The enzymes that degrade the extracellular matrix are matrix metalloproteinases (MMPs), which are classified into 17 types based on their matrix specificity and structural characteristics.[11,12]

MMP-2 and MMP-9 have been found in patients with an abdominal aortic aneurysm (AAA).[13–15] MMP-2 and MMP-9 generated in the macrophages cut the type IV collagen in the elastic fibers and basement membrane, and break the tunica media of the aortic wall, resulting in a fragile and enlarged aorta. The diameter of the aorta increases, and an aneurysm is formed.

According to previous studies, by using the in situ hybridization method, only mRNA expression of MMP-9 was confirmed in the tunica media in the subacute phase of aortic dissection;[16] however, by using the immunohistochemistry method, MMP-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-2 were

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also observed in the acute phase of aortic dissection.[10] This suggested that MMPs and TIMPs play important roles in the onset of aortic dissection, but the precise mechanisms have not yet been determined. The purpose of this study was to quantify those enzymes in patients with AAD and to demonstrate the roles they play in the onset of this disease.

Patients and Methods

Thirty patients who underwent surgery for AAD within 3 days of onset at the First Department of Surgery of Yokohama City University and its associated institutions between December 1999 and February 2001 were the primary subjects of this study. There were 14 men and 16 women aged from 28 to 80 years (mean age 64 ± 10 years). In addition, 12 patients who underwent surgery for AAA, being 11 men and 1 woman aged from 62 to 79 years (mean age 70 ± 5 years) and 16 patients who underwent coronary artery bypass grafting (CABG), being 9 men and 7 women aged from 59 to 81 years, were also studied, as the AAA group and the control (CON) group, respectively. The sample was taken from the anterior wall of the ascending aorta in the AAD group and the abdominal aorta in the AAA group, and from the ascending aorta at the site of central anastomosis of the CABG. In the CON group, patients with a highly calcified aortic wall or Marfan syndrome were excluded from this study. The surgical data for the AAD group are shown in Table 1. The collected specimen was immediately frozen with liquid nitrogen and stored at −80°C. Written informed consent for participation in this study was obtained from all of the subjects.

Histopathological Examination

A small section, 3 mm thick and containing all layers of the arterial wall, was prepared, then fixed overnight with buffered formalin and embedded in paraffin. From this section, a 4-μm slice was prepared and stained with hematoxylin–eosin and elastica van Gieson.

Table 1. Surgical data of the 30 patients with acute aortic dissection

DeBakey's classification	
Type I	21 (70%)
Type II	6 (20%)
Type IIIbR	3 (10%)
Time from onset to operation (min)	350 ± 86
Aneurysm size (mm)	56 ± 18
Thrombus in pseudolumen	15 (50%)
Operation	
Ascending aorta graft replacement	18 (60%)
Total arch graft replacement	12 (40%)

Immunohistochemistry

The slice was deparaffinated with xylene and hydrophilized with ethanol. It was then treated by blocking with 0.3% hydrogen peroxide-spiked methanol, washed with phosphate-buffered saline (PBS), and reacted with a 1% trypsin solution (Sigma Chemical, St. Louis, MO, USA) at room temperature for 30 min. Next, it was reacted with antihuman monoclonal antibodies, MMP-2, MMP-9, and TIMP-2 antibody (×100) (Fuji Chemical, Toyama, Japan) at room temperature for 1 h, then washed with PBS, and reacted with the Histofine SAB-PO (M) kit (Nichirei Biosciences, Tokyo, Japan). Finally, it was washed with PBS and reacted with 3,3′-diaminobenzidine (DAB) to induce coloration.

Extraction of Protein

The samples from the three groups were cut finely using an aseptic technique, then mixed with 50 mM-Tris-HCl buffer (pH 7.4) at five times the volume of the weight of each section, and homogenized. The mixture was centrifuged at 3000 × *g* at 4°C for 10 min, and the supernatant was collected and either examined immediately or stored at −80°C. The concentration of extracted protein was determined using the Bradford technique.

Enzyme-Linked Immunosorbent Assay (ELISA)

The value of MMP-2, MMP-9, and TIMP-2 was evaluated twice using the ELISA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the mean value was calculated. We added 100 μl of each of the standard recombinant human MMPs or TIMPs to an antihuman antibody-solidified microtiter plate, and the supernatant extracted from the sample was added and reacted at room temperature for 1 h. The residue was washed, then treated with 100 μl of horseradish peroxidase (HRP)-labeled anti-MMPs or TIMPs and reacted at room temperature for 2 h. The residue was washed, treated with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) and reacted at room temperature for 20 min. Finally, 1 M sulfuric acid was added to stop the reaction, the absorbance was measured with a microplate reader (Nihon Bio Laboratories, Tokyo, Japan) at a wavelength of 450 nm, and the concentration was analyzed from the standard curve.

Statistical Analysis

Data were analyzed using StatView 5.0 for Windows (SAS Institute, Cary, NC, USA) and values are expressed as mean ± standard deviation (SD). The study groups were compared by using a one-way analysis of

variance, and the post hoc test was done using the Fisher's Protected Least Significant Difference. Values were considered statistically significant at $P < 0.05$.

Results

Optical Microscopic Observations

Dissection of the tunica media, and partial rupture and loose density of elastic fiber were seen in the AAD group (Fig. 1A). Marked hypertrophy of the tunica intima and breakage of the elastic fibers of the tunica media were seen in the AAA group (Fig. 1B). There were no notable changes in the tunica intima or media in the CON group.

Immunohistochemistry Observations

The smooth muscle cells in the tunica media and the macrophage cells around the thrombus were positive for MMP-9 staining in the AAD group (Fig. 2A). The smooth muscle cells in the tunica media were positive for MMP-9 staining in the AAA group (Fig. 2B). No MMP-9 staining was seen in the CON group. No MMP-2 or TIMP-2 was found in any group sample.

Quantification of MMP-2, MMP-9, and TIMP-2 by the ELISA Method

MMP-2 was significantly lower in the AAD and AAA group than in the CON group (CON, 58 ± 30; AAA, 19 ± 17; $P < 0.001$ *vs* CON); AAD, 36 ± 19 ng/mg of soluble proteins ($P < 0.01$ *vs* CON and $P < 0.05$ *vs* AAA) (Fig. 3).

MMP-9 did not differ significantly among the groups (CON, 2.5 ± 3.4; AAA, 7.4 ± 10.0; AAD, 11.2 ± 18.4) (Fig. 4). TIMP-2 was significantly lower in the AAD and AAA group than in the CON group (CON, 216 ± 130;

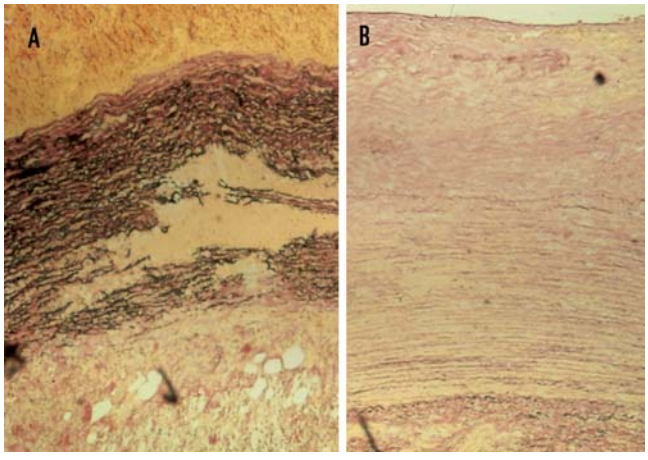

Fig. 1. A Aortic dissection of the tunica media showing partial rupture and loose density of the elastic fibers (elastica van Gieson stain, ×40). **B** The tunica media in an abdominal aortic aneurysm showing rupture of the elastic fibers (elastica van Gieson stain, ×40)

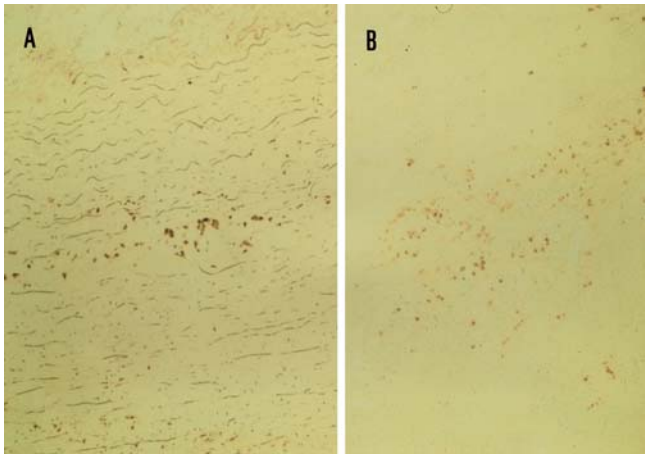

Fig. 2. A Immunohistochemical localization of matrix metalloproteinase (MMP)-9 in aortic dissection. The smooth muscle cells in the tunica media and macrophage cells around the thrombus were positive for MMP-9 staining (×400). **B** Immunohistochemical localization of MMP-9 in an abdominal aortic aneurysm. The smooth muscle cells in the tunica media were positive for MMP-9 staining (×400)

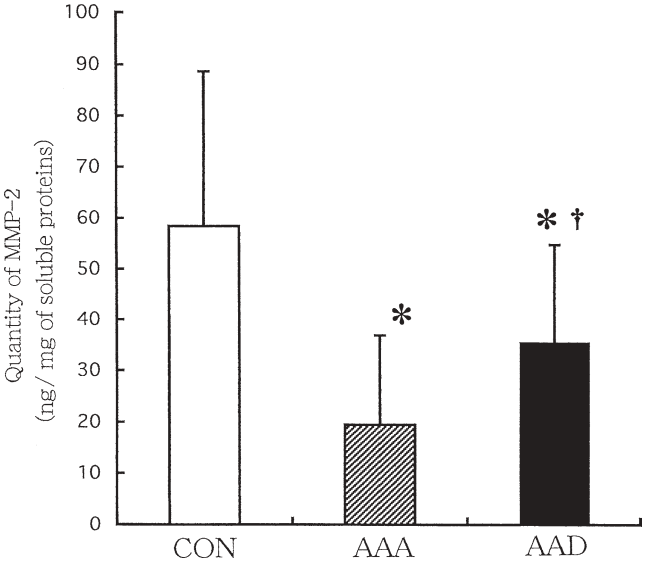

Fig. 3. Bar graph showing the MMP-2 values. *Asterisks* indicate significant differences from the control (*CON*) in the abdominal aortic aneurysm (*AAA*) and acute aortic dissection (*AAD*) groups. The *dagger* indicates a significant difference between the AAA and AAD groups. Values: CON, 58 ± 30; AAA, 19 ± 17 ($P < 0.001$ *vs* CON); AAD, 36 ± 19 ($P < 0.01$ *vs* CON and $P < 0.05$ *vs* AAA)

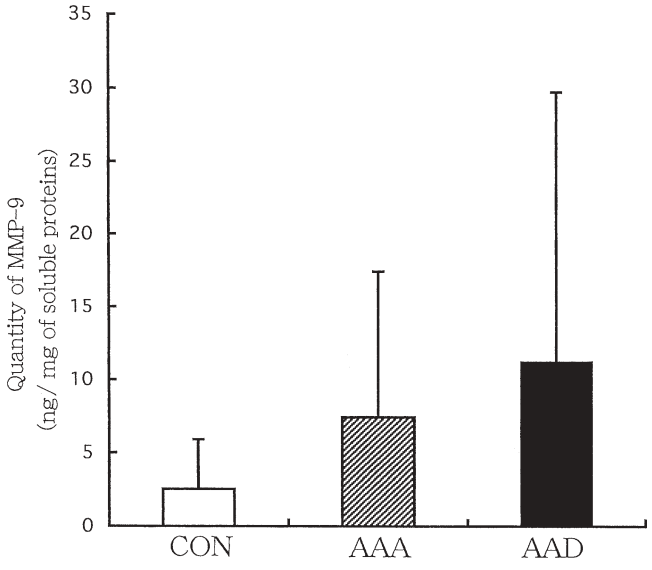

Fig. 4. Bar graph showing the MMP-9 values, which were higher in the AAD and AAA groups than in the CON group, without significance. Values: CON, 2.5 ± 3.4; AAA, 7.4 ± 10.0; AAD, 11.2 ± 18.4

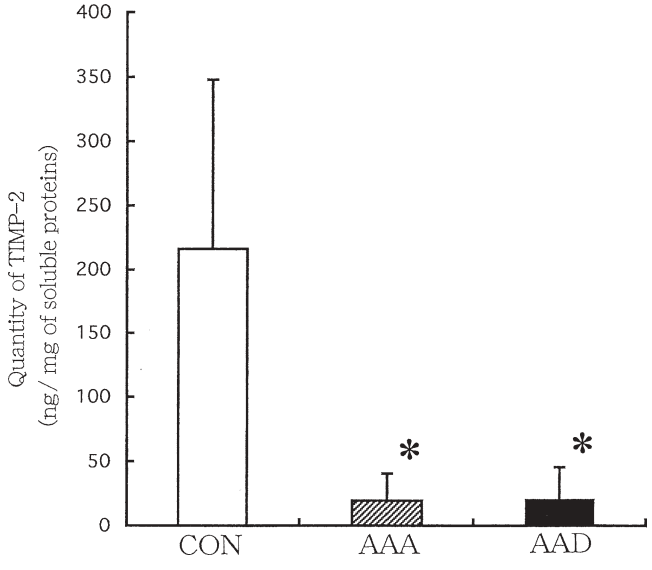

Fig. 5. Bar graph showing the tissue inhibitor of metalloproteinase (TIMP)-2 values. *Asterisks* indicate a significant difference from CON in the AAA and AAD groups. Values: CON, 216 ± 130; AAA, 20 ± 21 ($P < 0.001$ *vs* CON); AAD, 21 ± 25 ($P < 0.001$ *vs* CON)

AAA, 20 ± 21; $P < 0.001$ *vs* CON); AAD, 21 ± 25 ng/ mg of soluble proteins ($P < 0.001$ *vs* CON) (Fig. 5).

The protein ratios of TIMP-2/MMP-2 and TIMP-2/ MMP-9 were also calculated. The TIMP-2/MMP-2 ratio was 3.7 ± 1.7 in the CON group, 1.9 ± 1.3 in the AAA group ($P < 0.001$ *vs* CON), and 0.9 ± 0.8 in the AAD group ($P < 0.001$ *vs* CON and $P < 0.05$ *vs* AAA) (Fig.

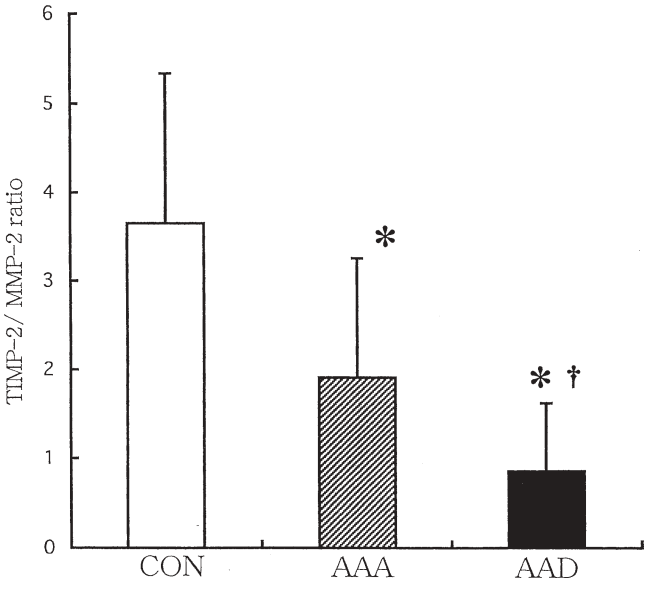

Fig. 6. Bar graph showing the TIMP-2/MMP-2 ratios. *Asterisks* indicate a significant difference from CON in the AAA and AAD groups. The *dagger* indicates a significant difference between the AAA and AAD groups. Values: CON, 3.7 ± 1.7; AAA, 1.9 ± 1.3 ($P < 0.001$ *vs* CON); AAD, 0.9 ± 0.8 ($P < 0.001$ *vs* CON and $P < 0.05$ *vs* AAA)

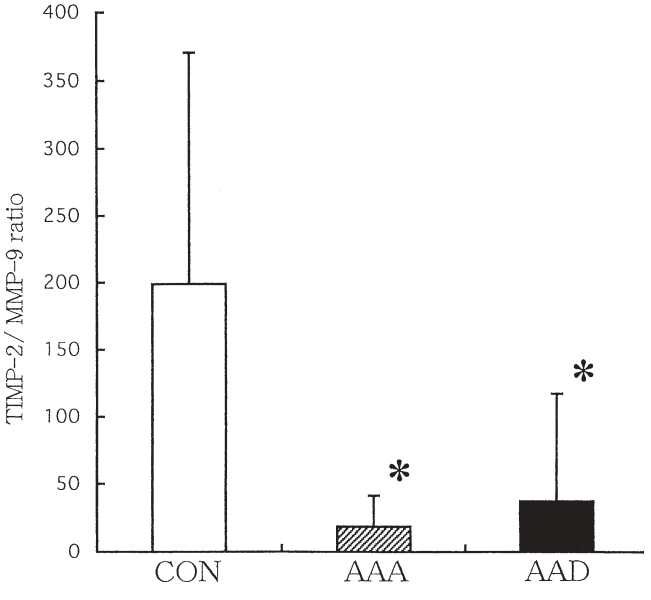

Fig. 7. Bar graph showing the TIMP-2/MMP-9 ratios. *Asterisks* indicate a significant difference from CON in the AAA and AAD groups. Values: CON, 200 ± 170; AAA, 19 ± 23 ($P < 0.001$ *vs* CON); AAD, 37 ± 80 ($P < 0.001$ *vs* CON)

6). The TIMP-2/MMP-9 ratio was 200 ± 170 in the CON group, 19 ± 23 in the AAA group ($P < 0.001$ *vs* CON), and 37 ± 80 in the AAD group ($P < 0.001$ *vs* CON) (Fig. 7).

Discussion

Tissue reconstruction occurs in most physiological and pathological processes, including wound healing.[17] Under these conditions, the existing extracellular matrix is broken down to allow construction of a new extracellular matrix. The enzymes accountable for destroying the extracellular matrix protein have been attributed to various types of matrix proteinases.[18] Matrix proteinases are classified roughly into the enzyme groups (MMPs) and matrix serine proteinases (MSPs). Matrix serine proteinases include plasminogen activating factor, elastase, plasmin, and trypsin. Matrix metalloproteinases directly destroy the extracellular matrix, whereas MSPs act subordinately.[11,12] Among the MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) degrade type IV collagen and gelatin in the basement membrane and play a central role in blood vessel metabolism.[13–15] Furthermore, activated MMP is regulated by a protein-inhibiting factor called tissue inhibitor of metalloproteinases (TIMP). Four subtypes of TIMP have been described, among which TIMP-2 inhibits MMP-2 and MMP-9 specifically.[19]

Marfan syndrome is associated with a high incidence of aortic dissection.[20] Pathological evidence of cystic medial necrosis and genetic abnormality have been confirmed in Marfan syndrome patients with aortic dissection.[21,22] On the other hand, cystic medial necrosis and genetic abnormality are rarely found in patients with aortic dissection who do not have Marfan syndrome.[7] Thus, it is reasonable to consider that the causes of aortic dissection are different in these two groups of patients. The pathological findings of patients without Marfan syndrome show rupture and a decrease in elastic fibers in the tunica media. Thus, it is considered that fragility of the tunica media is still a cause of aortic dissection in patients without Marfan syndrome.[7]

In this series, the MMP-9 levels varied without significant difference among the groups. However, MMP-9 was positive for the staining of smooth muscle cells of the tunica media in AAD and macrophage cells in thrombus. MMP-9 is rarely expressed normally, but is released from macrophages in the acute phase of a tissue disorder[23] and disappears again in the chronic phase.[24] In AAD, mRNA of MMP-9 was expressed in the wall of the aneurysm in the subacute phase, but not in the chronic phase.[16] The expression of MMP-9 in aortic dissection is considered to be part of reaction to wound healing of the tunica media, which would have ruptured with the onset of dissection.[16] It has already been reported that the chronic release of MMP-9 contributes to extracellular matrix degradation in AAAs.[25,26]

The AAD group in this study had significantly lower MMP-2 levels than the other two groups. In the wound-healing process, MMP-2 contributed to the recovery of connective tissue.[27] The production of MMP-2 in the vascular wall was almost constant,[11] and it began to gradually increase immediately after injury, peaking about 1 week later.[24] It is considered that a decrease in the productivity of MMP-2 following acute aortic dissection is not a reaction in the acute phase, but represents a chronic decrease in the ability of the tunica media to heal.

Tissue inhibitor of metalloproteinase is expressed in plasma and most organs normally, and inhibits and controls MMPs.[19] In this study, TIMP-2 and the TIMP-2/MMP-2 and the TIMP-2/MMP-9 ratios were all low in the AAD group. Thus, the proteolytic balance of AAD is shifted toward matrix degradation by a decrease in the TIMP level. It is considered that the decrease in the TIMPs/MMPs ratio seen in AAA is involved in the formation and extension of the aneurysm.[25,26,28] TIMP-2 expresses a similar pattern to MMP-2, gradually increasing immediately after injury and peaking about 1 week later.[24] It is considered that the low value of TIMP-2 in AAD is not an acute reaction to wound healing after the onset of dissection, but represents a chronic condition before the onset of dissection. It is also considered that decreases in TIMP-2, the TIMP-2/MMP-2 ratio, and the TIMP-2/MMP-9 ratio led to fragility of the tunica media and played an important role in the onset of aortic dissection.

To our knowledge, this is the first study to describe the quantification of MMPs and TIMPs in AAD, although we could not detect these enzymes in the preonset and chronic phases. While further studies with competitive polymerase chain reaction to determine the mRNA quantification of MMPs and TIMP are needed, we present good evidence of a decreased TIMP/MMP ratio in AAD.

Fragility of the tunica media, a condition predisposing to aortic dissection, is possibly improved by increasing the concentration of TIMPs in the aortic wall. However, the reason that the concentration of TIMPs in the aortic wall decreases is still not understood, nor is its relationship with certain diseases such as hypertension and arteriosclerosis. These mechanisms must be delineated in the future.

To summarize our findings, the levels of MMP-2 and TIMP-2 in the wall of the aneurysm in the acute phase of aortic dissection were lower than those in a control group. Both the TIMP-2/MMP-2 ratio and the TIMP-2/MMP-9 ratio were also lower. Thus a TIMP-2/MMP-2 ratio and a low TIMP-2/MMP-9 ratio might play an important role in the onset of aortic dissection, when the tunica media becomes fragile with chronic breakage and degradation of the extracellular matrix.

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