Neutrophil-Derived Matrix Metalloproteinase 9 Triggers

Acute Aortic Dissection

Running title: Kurihara et al.; Neutrophil MMP9 induces acute aortic dissection

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Abstract:

**Background**—Acute aortic dissection (AAD) is a life-threatening vascular disease without effective pharmaceutical therapy. Matrix metalloproteinases (MMPs) are implicated in the development of chronic vascular diseases including aneurysm, but the key effectors and mechanism of action remain unknown. To further define the role of MMPs in AAD, we screened circulating MMPs in AAD patients, and then generated a novel mouse model for AAD to characterize the mechanism of action.

**Methods and Results**—MMP9 and angiotensin II (AngII) were significantly elevated in blood samples from the AAD patients than in those from the patients with non-ruptured chronic aortic aneurysm or healthy volunteers. Based on the findings, we established a novel AAD model by infusing AngII to immature mice that had been received a lysyl oxidase inhibitor, β-aminopropionitrile monofumarate (BAPN). AAD was successfully developed in the thoracic aorta by AngII administration to BAPN-treated wild-type mice, with an incidence of 20, 80 and 100% after 6, 12 and 24 hours, respectively. Neutrophil infiltrations were observed in the intima of the thoracic aorta and the overexpression of MMP9 in the aorta was demonstrated by RT-PCR, gelatin zymography and immunohistochemistry. The incidence of AAD was significantly reduced by 40% following the administration of an MMP inhibitor and almost completely blocked in MMP9−/− mice without any influence on neutrophil infiltration. Neutrophil depletion by injection of anti-Gr-1 antibody also significantly decreased the incidence of AAD.

**Conclusions**—These data suggest that AAD is initiated by neutrophils that have infiltrated the aortic intima and released MMP9 in response to AngII.

**Key words:** animal model; aortic dissection; leukocytes; matrix; matrix metalloproteinase
Introduction

Acute aortic dissection (AAD) is a medical emergency that is associated with high mortality.\(^1\) Although imaging by computed tomography (CT) and elective surgical repair represent an effective approach, there are neither specific biomarkers for prompt diagnosis nor alternative therapeutic strategies for treating the disease. The acute dangers associated with the active disease in humans do not lend itself to randomized controlled trials, and the paucity of animal models complicates efforts to design detailed studies of the disease process. Thus, the underlying pathological mechanisms responsible for triggering the disease remain elusive.\(^2\) Medial degeneration including cystic medial necrosis is a common histological finding in chronically damaged aortae associated with aging, hypertension, and aortic aneurysm,\(^3,4\) and is widely accepted as an important risk factor for the development of AAD. However, the direct cellular and molecular mechanism which links the medial degeneration and the onset of AAD has not been elucidated. \(Fibrillin1\)-deficient \((Fibrillin1^{-/-})\) mice are often used as a model of Marfan syndrome, which display spontaneous development of cystic medial degeneration and ascending aortic aneurysm leading to spontaneous rupture and/or dissection, commonly emerging at 2 to 4 months of age.\(^5\) \(Lysyl oxidase-null\) mice spontaneously develop aortic rupture,\(^6\) and administration of \(\beta\)-aminopropionitrile monofumarate (BAPN), a lysyl oxidase inhibitor, can induce cystic medial degeneration in rats.\(^7,8\) These models have been classically used for aneurysm studies, and AAD is observed only by chance.\(^5,7,8\) Therefore, they are not suitable for AAD models with regard to predicting the actual onset of the dissection which is critical for understanding the pathogenesis of the disease.

Recent studies have demonstrated that matrix metalloproteinases (MMPs), including MMP1, 2, 3 and 9 are overproduced in a wide range of vascular diseases.\(^9,10\) Among these
MMPs, the importance of MMP9 has been documented in the development of chronic aortic aneurysm formation as a function of its ability to directly degrade extracellular matrix components such as elastin.11 The increased expression of MMP9 - predominantly from macrophages - has also been implicated in acute vascular crises such as atherosclerotic plaque rupture and abdominal aortic aneurysm.12-14 However, little or no information is available for the involvement of MMP9 in the development of AAD.

In the present study, we found significant elevation of MMP9 and angiotensin II (AngII) in human blood samples from AAD patients. Based on these findings, we established a novel mouse model of AAD by AngII infusion following sustained administration of BAPN. Upregulation of MMP9 from neutrophils was noted at the onset of the disease in this model. Importantly, genetic and pharmaceutical depletion of MMP9 dramatically attenuated the occurrence rate of AAD without impairing neutrophil infiltration. Furthermore, AngII per se induced neutrophil infiltration into aortic lesions, and neutrophil depletion by neutralizing antibody also attenuated the AAD incidence. Taken together, our study provides the first evidence that MMP9 released from AngII-stimulated neutrophils initiates AAD in preconditioned aorta.

**Methods**

**Human blood and affected aortic samples**

Between April 2004 and August 2006, 16 patients diagnosed with AAD, 11 patients with acute myocardial infarction (AMI), 12 patients with chronic non-ruptured aortic aneurysm and 16 healthy volunteers were registered in the study. All the AAD patients were free from connective tissue disorders such as Marfan syndrome, Ehlers-Danlos syndrome and aortitis diagnosed...
according to the clinical history and physical examinations. They were composed of Stanford type A (6 cases) and type B (10 cases). The blood samples of AAD and AMI were collected within 1 hour after arrival in the hospital emergency room. AAD and AMI cases that had arrived at the hospital more than 10 hours after onset of clinical symptoms were eliminated from the study. Diagnosis of AAD and AMI was confirmed by CT and electrocardiogram, respectively. Affected aortic specimens were obtained at surgery from 10 non-ruptured aortic aneurysm and 10 AAD patients, who underwent operation for aortic grafts. The human samples were collected in the Keio University Hospital and signed informed consent for the usage of the samples for the experiments was obtained from all subjects. This study was approved by the Ethical Committee of School of Medicine, Keio University.

Development of AAD model in mice

Wild-type (WT) and MMP9−/− mice on FVB background were purchased from Jackson laboratory (Bar Harbor, ME). Three-week-old male mice were fed on a regular diet and administered BAPN (Sigma-Aldrich, St. Louis, MO) dissolved in drinking water (1 g/kg/day) for 4 weeks. At 7 weeks of age, osmotic mini pumps (Alzet, Cupertino, CA) filled with 1 μg/kg/min AngII (Sigma-Aldrich) or 1.3 μg/kg/min norepinephrine (NE) (kindly provided by Daiichi-Sankyo Co.Ltd., Tokyo, Japan) were subcutaneously implanted as previously described, and the mice were sacrificed 24 hours after implantation. Blood pressure was measured by tail-cuff method at pre- and post-implantation and prior to sacrifice. Mice were scanned by micro-CT system (GE Healthcare, Tokyo, Japan) for imaging of aortae. Mouse aortae were enhanced by in situ infusion of the contrast agent and the 3-dimensional images were reconstructed. For pharmacological depletion of MMP9, BAPN-fed mice were administered by gastric lavage with a broad-spectrum MMP inhibitor, ONO-4817 (300 mg/kg/day), which was kindly provided by Ono
pharmaceutical Co.Ltd (Tokyo, Japan),\textsuperscript{17-19} daily for 2 days before AngII administration until sacrifice. Dose of ONO-4817 (300 mg/kg/day) was determined according to the information of the previous studies on the pharmacokinetics and in vivo experiments.\textsuperscript{17, 18} For neutrophil depletion experiments, BAPN-fed mice received daily intraperitoneal injections of 200 $\mu$g of anti-Gr-1 neutralizing antibody (R&D systems, Minneapolis, MN) or control IgG from 2 days before the AngII infusion until sacrifice.\textsuperscript{20} The depletion of neutrophils was confirmed by Giemsa stain of peripheral blood smears. All studies in mice were approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University.

**Additional methods**

The expanded Methods section in the online-only Data Supplement contains information on ELISA; histology and immunohistochemistry; reverse transcription-polymerase chain reaction (RT-PCR); gelatin zymography; film in situ zymography; in situ detection of superoxide.

**Statistics**

Human blood sample data were analyzed with two-sample t’s test and the occurrence rate of mouse AAD was analyzed with Fisher’s exact test. $P$ value less than 0.05 was regarded as significant. $P$ value was adjusted with Bonferroni method for pairwise comparisons in some experiments.

**Results**

**Elevated levels of MMP9 and AngII in blood samples from AAD patients**

We first screened the circulating levels of MMP1, MMP2, MMP3, MMP9 and TIMP1 in blood samples from control healthy volunteers and patients with non-ruptured chronic aortic aneurysm, AMI or AAD. There were no significant differences in average age, ratio of male to female or
prevalence of major risk factors among these groups (Table 1). As shown in Figure 1, the AAD group exhibited significantly higher level of MMP9 than the control, non-ruptured aneurysm or AMI groups, while the levels of MMP1, MMP2, MMP3 and TIMP1 did not differ between the groups.

AngII is one of the representative vasopressors implicated in the pathogenesis of many vascular diseases, including aneurysm formation. In addition, AngII is known to promote neutrophil infiltration into vascular walls, and to induce MMP9 expression in cell types such as vascular smooth muscle cells. Thus, we also measured the AngII levels in serum samples of the control, non-ruptured aneurysm, AMI and AAD groups, and found that the circulating AngII level is significantly higher in the AAD and AMI groups relative to the other groups studied (Figure 1).

Immunolocalization of MMP9 in aortic lesions from AAD patients

Immunohistochemistry of patient samples indicated that the AAD tissues contain abundant MMP9-positive cells located mainly in the medial layer of dissected aorta with smaller numbers of MMP9-positive cells scattered in the aortic tissues of the non-ruptured aneurysm, predominantly localized to areas of the media displaying severe atherosclerotic changes (Figure 2A and B). Of note, neutrophils accumulated to much higher levels in the dissected aorta from the AAD patients relative to the non-ruptured aneurysm (Figure 2A and B). Morphometric analysis of MMP9-immunoreactive cells in the aortic tissues showed a statistically significant increase in the aorta from the AAD patients (171.8±97.2 cells/mm²) as compared with that from non-ruptured aneurysm patients (47.2±40.6 cells/mm²) (P<0.05). As MMP9 staining pattern in the AAD aorta was similar to that of anti-neutrophil elastase (Figure 2A), these data suggested infiltrating neutrophils as the most likely source of MMP9 in the AAD aortae. Thus, we carried
out double immunostaining of MMP9 and neutrophil elastase in the AAD aortic tissues and demonstrated that both proteinases co-localize in the cells (Figure 2C).

**Establishment of an AAD model by AngII infusion to BAPN-treated mice**

Based on the finding that both MMP9 and AngII are upregulated in the human AAD, we sought to define their roles in AAD *in vivo* by developing a relevant mouse model. As previous studies have shown that sustained administration of a lysyl oxidase inhibitor, BAPN, to premature rodents induces medial degeneration of aorta and results in aneurysm formation by disrupting the structural integrity of aortic wall as a consequence of inhibiting collagen and elastin crosslinking, we administered BAPN to 3-week-old WT mice for 4 weeks. Under these conditions, apparent aneurysm formation was observed secondary to the medial degeneration (Figure 3A). However, BAPN alone was not sufficient to trigger AAD (Figure 3A and B). Since blood pressure elevation is considered to be a major inducer of AAD, we modified our protocol by infusing vasopressive-equivalent doses of AngII or NE to BAPN-treated WT mice for up to 48 hours. Blood pressure elevated equivalently from 96.3±4.6 mmHg to 119.4±13.7 mmHg (*P*<0.05) and 111.9±5.13 mmHg (*P*<0.05) 1 hour after the infusion of AngII or NE, respectively (data not shown). Importantly, 24 hours after the infusion was initiated, AngII led to AAD in all the mice examined. Further, 30% of the mice died due to aortic rupture and subsequent hemothorax, whereas AAD was obtained in only 10% of the mice treated with NE infusion or BAPN treatment alone (Figure 3B). Enhanced CT scan and histological examination demonstrated AAD in the descending thoracic aorta of the BAPN/AngII-treated mice (Figure 3A). Time course examination of AAD formation in the BAPN/AngII-treated mice demonstrated that AAD is initiated as early as 6 hours after AngII infusion with 100% of the mice developing AAD by 24 hours (Figure 3C). Since NE infusion to BAPN-treated mice failed to induce AAD despite
changes in blood pressure similar to those observed with AngII, the singular effects of AngII on triggering AAD onset are independent of blood pressure change alone.

**Involvement of MMP9 in an AAD model**

When MMP9 expression levels were examined by RT-PCR in the mouse aortic tissues 24 hours after the infusion of AngII, high levels were detected in the aortae from the BAPN/AngII-treated mice while aortae from the other groups showed weak or negligible expression (**Figure 4A**). Gelatin zymography showed gelatinolytic bands of 92 and 87 kDa, which correspond to the latent and active forms of MMP9 respectively, but only in aortae from the BAPN/AngII-induced AAD mice, and not in aortae from the other groups (**Figure 4B**). Histological and immunohistochemical study showed that the accumulation of MMP9-positive cells, which also immunostained with anti-neutrophil antibody, localized to the media of the dissected aortae of BAPN/AngII-treated mice (**Figure 4C**). Film *in situ* zymography on the aortic tissues demonstrated that gelatinolytic activity was generated in the dissected area of the aortae from BAPN/AngII-treated mice, whereas the digestion was abrogated in gelatin film treated with 1,10-phenanthroline, and the non-dissected aortae from control mice showed negligible activity (**Figure 4D**). These data indicate that metalloproteinase(s) exist within the dissected aortic tissue. Although the experimental methods used were not specific to detect MMP9 activity, our findings including the zymographical data on the presence of active MMP9 form suggest the possibility that proMMP9 derived from neutrophils infiltrated in the aortic media is activated within the tissue. To study the location of superoxide production within the aortic tissues, we used staining with dihydroethidium (DHE), which is specific for superoxide. As shown in **Figure 4D**, strong fluorescence was detected in the dissected aortic tissue from the AAD mice, whereas control aortic tissue showed only a low-intensity fluorescence. Importantly, the infiltration of MMP9-
positive neutrophils was demonstrated not only in the dissected media, but also in the intima of non-dissected lesions of BAPN/AngII-induced AAD thoracic aortae (Figure 5). In control mice treated with BAPN alone or BAPN and NE, immunostaining of MMP9 and neutrophils was only rarely observed in the tissues (Figure 5). These findings suggest that the increased levels of MMP9 detected in the AAD aortae are derived primarily from neutrophils that infiltrate both the aortic intima and media.

Reduction of AAD incidence by pharmaceutical and genetic depletion of MMP9 or by neutrophil depletion

To determine whether MMP9 plays a direct role in the AAD mouse model, BAPN/AngII-treated mice were treated with synthetic MMP inhibitor, ONO-4817. As shown in Figure 6A, when ONO-4817 was orally administered on daily basis to BAPN-treated WT mice from 48 hours prior to AngII infusion until sacrifice, the incidence of AAD was significantly decreased from 100% to 60% and spontaneous death caused by aortic rupture was completely blocked. Consistent with these observations, when MMP9^−/− mice were treated with BAPN/AngII, AAD incidence was remarkably attenuated to basal levels (10% of the MMP9^−/− mice) whereas BAPN alone induced thoracic aneurysm formation at a frequency comparable to that observed in WT mice (Figure 6A and B). Hence, these findings support a direct role for MMP9 in the development of AAD.

While multiple cell populations are capable of expressing MMP9 (e.g., macrophages, endothelial cells, and vascular smooth muscle cells), neutrophil infiltrates dominated the affected tissues. As such, we depleted neutrophils during aneurysm formation by treating the mice with anti-Gr-1 neutralizing antibody. The number of circulating neutrophils was reduced to 20% of control levels by intraperitoneal injection of the antibody for 48 hours prior to AngII
infusion (data not shown), and as expected, neutrophil depletion significantly attenuated the AAD incidence (Figure 6A).

**AngII-induced neutrophil infiltration to aortic intima independently from MMP9 expression**

Infiltration of neutrophils into the aortic intima was observed in BAPN/AngII-treated WT mice with or without ONO-4817 administration as well as in BAPN/AngII-treated *MMP9* / mice (Figure 7A). Hence, these data demonstrate that AngII promotes neutrophil infiltration independently of MMP9 expression in our AAD model, but these results do not exclude the possibility that normal rates of neutrophil infiltration are maintained only within the non-crosslinked, fragile vessel wall caused by chronic BAPN treatment. Thus, we further assessed the effect of AngII on neutrophil infiltration by infusing AngII in BAPN-untreated WT or *MMP9* / mice wherein vascular structural integrity is intact. As shown in Figure 7B, neutrophil infiltration into the aortic intima was unaffected between the two groups, indicating that AngII infusion evokes neutrophil infiltration to the intact aortic wall independently of MMP9 expression.

**Discussion**

In the present study, we have demonstrated that serum levels of MMP9 and AngII are elevated in AAD patients, but not in patient populations with chronic, non-ruptured aneurysms. Furthermore, increased circulating levels of MMP9 correlated with the presence of MMP9-positive neutrophils that accumulated in the aortic tissues of AAD patients. Given these findings from studies of human AAD specimens, we established a novel mouse model that unfailingly develops AAD within 24 hours of AngII infusion. This model was dependent on preconditioning...
mouse aortae with the lysyl oxidase inhibitor, BAPN, in order to create an aneurysmal, pre-AAD status in immature mice. Collagen and elastin crosslinks, which are critical for maintaining vessel wall integrity, are disrupted by BAPN administration, leading to the generation of mechanically fragile aortae that both display medial degeneration and develop aortic aneurysms. This type of aortic aneurysm is typically seen in human connective tissue diseases such as Marfan syndrome but the histology of cystic medial degeneration is commonly seen in aneurysms that arise secondary to aging and atherosclerosis as well. It is not clearly elucidated whether aortic matrix crosslinks are different between normal and aneurysmal aorta, however, several studies from human pathological samples indicate that the composition of the aortic extracellular matrix changes as medial degeneration proceeds with enhanced deposition of proteoglycans and decreased collagen content, coupled with apoptosis of vascular smooth muscle cells. These findings suggest that the aortic media and its matrix components are disorganized as a function of disease progression, thus leading to the generation of an aneurysmal aorta with weakened mechanical properties. As such, our mouse model would appear to recapitulate a similar state wherein suitable triggers, such as AngII, precipitate the transition from a preconditioned, chronic aortic aneurysm to AAD.

Previous studies have suggested possible roles for MMP9 in the development of chronic atherosclerosis-derived aneurysms as well as connective tissue disease-related aortic aneurysm. By contrast, our findings demonstrate that AAD formation itself proceeds in an MMP9-dependent fashion that is inhibited significantly by either pharmacologic or genetic targeting of MMP9. Previous study by Gough et al. has shown that macrophages overexpressing autoactivating MMP9 induce atherosclerotic plaque rupture by disruptions of fibrous cap in apoE−/− mice, suggesting the ability of MMP9 to destroy aortic tissue. Altogether,
these data support the notion that MMP9 is responsible for triggering aortic dissection from the preconditioned aneurysmal aorta. We have further demonstrated that neutrophil infiltration is observed in the intima of the pre-dissecting aorta as well as in the dissected media and that neutrophil depletion attenuates AAD incidence significantly. The importance of inflammation in the pathogenesis of vascular diseases is well documented, but until now, it has been difficult to determine whether neutrophil accumulation triggers dissection or occurs as a consequence of the massive vascular damage that develops during dissection. As MMP9-positive neutrophils were confined to the intima of non-dissected lesions while the dissected lesions displayed strong staining for MMP9-positive neutrophils primarily within the media, we posit that neutrophils infiltrate the intima at the initiation of dissection. Despite the importance of neutrophils, we cannot rule out the contribution of other immune effector cells that might be recruited subsequent to the inflammatory responses initiated by infiltrating neutrophils, as depleting neutrophils attenuated AAD, but not to the degree observed with MMP9 targeting. Indeed, the up-regulated MMP9 mRNA levels detected in AAD aortic samples 24 hours after AngII infusion could result from non-neutrophil effector cells, as neutrophil MMP9 synthesis is mostly completed at earlier stages of differentiation, with mature neutrophils primarily storing MMP9 in granule compartments.\textsuperscript{34} Nevertheless, the rapid induction of aortic dissection in our model as early as 6 hours after AngII infusion in tandem with the accumulation of MMP9-positive neutrophils supports the conclusion that infiltrating neutrophils directly or indirectly trigger the initiation of dissection.

MMP9 is a multifunctional proteinase endowed with the ability to degrade multiple extracellular matrix macromolecules, including types III, IV and V collagens, denatured collagens (i.e., gelatin) and elastin.\textsuperscript{11,35} Furthermore, MMP-9 can modulate inflammatory
responses by hydrolyzing a variety of cytokines and chemokines.\textsuperscript{36} For example, MMP9 can activate pro-interleukin-1 $\beta$ (pro-IL-1$\beta$),\textsuperscript{37} increase the bioactivity of IL-8\textsuperscript{38} and promote IL-13-induced pulmonary inflammation,\textsuperscript{39} while inactivating neutrophil chemokines such as GRO$\alpha$ and mature IL-1$\beta$.\textsuperscript{37, 38} A recent clinical trial has shown that the treatment of aortic abdominal aneurysms with doxycycline, a non-specific MMP inhibitor, reduced neutrophil and cytotoxic T cell infiltration into the aneurysmal wall in association with decreased production of MMP9 and inflammatory cytokines.\textsuperscript{40} MMP9 has also been reported to affect neutrophil chemotactic activity in lung injury models.\textsuperscript{41, 42} In the present study, however, we detected no differences in neutrophil infiltration within the aortic tissues of OND-4817-treated mice or between WT and \textit{MMP9$^{+/}$} mice in our AAD model. Furthermore, in contrast to malignant cells whose MMP-dependent invasive activity is affected by collagen crosslinks,\textsuperscript{43} our study showed that neutrophils similarly infiltrate aortic tissues in both BAPN-treated and -untreated mice. Together, these data suggest that neutrophil infiltration proceeds independently of MMP9 activity or collagen crosslinks in this model system.

A detailed mechanism as to how MMP9 contributes to the initiation of AAD remains to be defined. In recent studies using \textit{Fibrillin1$^{-/}$} mice, which spontaneously develop AAD secondary to connective tissue defect, the elevation of transforming growth factor- $\beta$ (TGF-$\beta$) levels and its downstream signaling cascade contribute to the pathogenesis of aortic aneurysm and dissection.\textsuperscript{44} In this model, MMP2 and MMP9 were both upregulated and doxycycline attenuated disease progression.\textsuperscript{31, 32} As MMP9 is a potent activator of latent TGF-$\beta$,\textsuperscript{45} it is interesting to speculate that upregulated MMP9 activity may trigger AAD by \textit{in situ} activation of TGF-$\beta$ in the affected aortic media of the mice.

Finally, our study has validated AngII as a potent inducer of mouse AAD, a result that
complements the higher levels of AngII detected in our AAD patient population. Long-term
AngII infusion is known to lead to spontaneous aortic dissection in atherosclerosis models using
apoE−/− mice.46 Recent studies have also demonstrated that Losartan, an antagonist of the AngII
receptor, AT1, prevents aortic aneurysm development and aortic root dilation in Marfanoid mice
and humans.44,47 It is clear that the role of AngII in the induction of AAD is not due to
vasopressor effects alone as NE failed to trigger AAD in our model despite similar levels of
hypertension. One of the significant differences between the aortic lesions observed in
BAPN/AngII-treated versus BAPN/NE-treated WT mice was the presence of neutrophil
infiltrates in the BAPN/AngII-treated mice, suggesting that AngII acts as a potent stimulus for
neutrophil infiltration into the aorta intima. Indeed, recent studies indicate that AngII can induce
neutrophil infiltration.22,23 In addition, AngII is also able to activate neutrophils and stimulate
the NADPH oxidase-dependent production of reactive oxygen metabolites which may promote
the oxidative autoactivation of proMMP9.48,49 In the present study, we could show the
gelatinolytic metalloproteinase activity and superoxide production in the neutrophil-infiltrated
dissecting aortic tissue from the BAPN/AngII-treated mice. Thus, AngII likely plays a key role
in triggering the AAD onset not only by attracting neutrophils to the affected sites, but also by
stimulating the release and activation of proMMP9.

This study has a few limitations. First, we used a mouse model to demonstrate the role of
neutrophil-derived MMP9 activity in the AAD formation. Because mouse models do not
stringently recapitulate human disease progression, the results of our AAD model may be
different in humans. In the mouse model, AAD was induced in the descending thoracic aorta by
AngII infusion to the young mice treated with BAPN. Preconditioning for the AAD induction,
i.e. aneurism formation, by BAPN treatment is artificial, and may be applicable for AAD in the
patients with connective tissue disorders but not for commonly observed AAD in humans such as our cases in the present study. Another limitation of this study is that the activation mechanism of proMMP9 within aortic tissues has not been examined in mouse or human AAD. Although our study on the mouse model has suggested possible involvement of reactive oxygen species (ROS) in the activation, detailed studies as to whether proMMP9 activation and AAD incidence are suppressed by antioxidant therapy and which ROS are required for proMMP9 activation in co-culture system of neutrophils and smooth muscle cells will be necessary. Moreover, ROS-mediated proMMP9 activation in the human aortic tissues from the AAD patients needs to be investigated by future work.

AAD is a potentially fatal disease whose prompt diagnosis and treatment are required for successful intervention. Although the fibrin product, D-dimer, is the only established biomarker for AAD, the differential diagnosis of AMI versus pulmonary embolism, which displays similar symptoms to that of AAD, can be difficult. In this context, MMP9 could serve as a potential biomarker for the diagnosis of AAD. Furthermore, while the dominant treatment for AAD relies on surgical reinforcement of the affected aorta, our data raise the possibility that the preventive administration of AngII receptor blockers and/or MMP9-specific inhibitors to patients at risk with non-ruptured atherosclerotic aneurysm could prove useful as effective therapeutics to reduce AAD incidence.

**Conflict of Interest Disclosures:** None.

**References:**


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**Table 1.** Background of human peripheral blood and aortic samples.

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N/A, not applicable.

**Figure Legends:**

**Figure 1.** Circulating levels of MMP9 and AngII are elevated in the blood samples from the AAD patients. MMP1, MMP2, MMP3, MMP9, TIMP-1 and AngII were assayed by the ELISA systems for each marker in the human peripheral blood samples from control healthy volunteers (n=16), patients with non-ruptured chronic aortic aneurysm (n=12), AMI (n=11) or AAD (n=18). Values are mean ± SEM. *P* value was adjusted with Bonferroni method for pairwise comparisons. *, *P*<0.05 vs control.

**Figure 2.** Dissected aortic media from AAD patients is infiltrated by numerous MMP9-positive neutrophils. **A and B,** Aortic tissues obtained from the patients with AAD (A) or non-ruptured aneurysm (B) were subjected to histological and immunohistochemical studies for MMP9 and neutrophil elastase (Neutrophil) on serial paraffin sections. High-power view of the rectangular
areas is shown in the lower rows in each panel. Asterisks indicate false lumen of the dissected aorta. Adv, adventitia; Med, media; Int, intima. Red arrowheads; positively immunostained cells. Note that, in AAD samples, MMP9 is expressed by the cells mainly in the media and the distribution of MMP9-positive cells and neutrophil elastase-positive cells is closely related. Scale bars on the upper and lower panels, 200 μm and 50 μm, respectively. H&E, hematoxylin and eosin stain. C, Double immunostaining of MMP9 and neutrophil elastase on paraffin sections of the AAD aortic tissue. Inset shows high-power view of a double immunostained cells. Red arrowheads, MMP9 and neutrophil elastase-double positive cells; Non-immune, non-immune IgG. Scale bar, 50μm.

**Figure 3.** AngII infusion to BAPN-treated WT mice induces AAD. A, Demonstration of AAD in BAPN/AngII-treated mice (BAPN+AngII) by 3-dimensional images of enhanced CT scan and histology of mouse aortae. WT mice were treated with vehicle for 4 weeks (control), BAPN alone for 4 weeks (BAPN) or BAPN for 4 weeks and then AngII for 24 hours (BAPN+AngII). The inlet in the BAPN+AngII mouse aorta (upper panel) shows the horizontal cross-section image of the dissected lesion. Arrow and asterisk indicate true and false lumens, respectively. Lower panels show histology of the aortae from the control, BAPN-treated or BAPN/AngII-treated mice stained by elastica Van Gieson (EVG) or H&E. Bottom panel is the high-power view of the rectangular areas of the middle panels. Note the aortic dissection (asterisk, false lumen) in the BAPN/AngII-treated mouse. Scale bars, 200 μm and 100 μm for the bottom panel. B, AAD incidence in WT mice treated with control, BAPN alone, BAPN+AngII or BAPN and NE (BAPN+NE) (n=10 for each group). Shaded area in BAPN/AngII-treated group denotes aortic rupture with dissection. P value was adjusted with Bonferroni method for pairwise comparisons. *, P<0.05 vs control. C, AAD incidence in BAPN-treated WT mice at different
time points after AngII infusion (n=10 for each time point).

**Figure 4.** AAD in BAPN/AngII-treated WT mice is accompanied by enhanced MMP9 expression, neutrophil infiltration and superoxide production. **A,** Analysis of *MMP9* mRNA expression by RT-PCR in aortic tissues from the WT mice treated with vehicle (control), BAPN, BAPN/AngII (BAPN+AngII) or BAPN/NE (BAPN+NE). PCR products of three independent samples from each group are shown. **B,** Gelatinolytic activity of the aortic tissue homogenates was assessed by gelatin zymography. BAPN/AngII-treated WT aorta shows gelatinolytic bands of 92 and 87 kDa, corresponding to proMMP9 and active MMP9, respectively. **C,** Serial paraffin sections of dissected thoracic aorta of BAPN/AngII-treated WT mice were stained with H&E and immunostained with anti-MMP9 antibody, anti-neutrophil antibody or control IgG. Note the infiltration of MMP9-positive neutrophils in the media of dissected lesion. **D,** Film *in situ* zymography and DHE staining of aortic tissues from BAPN/AngII-treated or control vehicle-treated WT mice. Serial frozen sections were made and stained with H&E or subjected to film *in situ* zymography using gelatin films coated with or without 1,10-phenanthroline. Frozen sections were also stained with DHE. Scale bars, 200 μm.

**Figure 5.** Infiltration of MMP9-positive neutrophils in the intima of non-dissected lesions of BAPN/AngII-induced AAD thoracic aortae. MMP9 expression and neutrophil infiltration were examined by histology and immunohistochemistry in the aortae of untreated WT mice (control) or WT mice treated with BAPN alone, BAPN+AngII, and BAPN+NE. Note the accumulated MMP9-positive neutrophils in the intima of non-dissected aorta from BAPN/AngII-treated mouse. Inlets in the BAPN+AngII group show higher-power view of the rectangular areas. Scale bar, 200 μm.
Figure 6. Pharmaceutical and genetic depletion of MMP9 or immunological depletion of neutrophils attenuates AAD incidence in mice. A, AAD incidence in BAPN/AngII-treated WT mice that were treated with ONO-4817 (BAPN+AngII+ONO-4817) (left panel) or anti-Gr-1 antibody (BAPN+AngII+Gr-1) (middle panel) and the incidence in BAPN/AngII-treated MMP9−/− mice (right panel) were compared with that in BAPN/AngII-treated WT mice (n=10 for each group). P value was adjusted with Bonferroni method for pairwise comparisons. *, P<0.05 vs BAPN/AngII-treated WT mice. B, Three-dimentional images of enhanced CT scan (upper panel) and histology (middle and bottom panels) of the aortae from MMP9−/− mice that were treated with vehicle for 4 weeks (control), BAPN alone for 4 weeks (BAPN) or BAPN for 4 weeks and then AngII for 24 hours (BAPN+AngII). Note that AAD formation is blocked in BAPN/AngII-treated MMP9−/− mouse. Bottom panel shows high-power view of the rectangular areas in the middle panel. Scale bars, 200 μm and 100 μm for the middle and bottom panels, respectively.

Figure 7. AngII-induced neutrophil infiltration in aortic intima is independent of MMP9 expression or BAPN treatment. A, Histological and immunohistochemical analyses of MMP9 expression and neutrophil infiltration in the aortic intima of the WT mice treated with BAPN and AngII (BAPN+AngII), BAPN, AngII and ONO-4817 (BAPN+AngII+ONO-4817) or BAPN, AngII and anti-Gr-1 antibody (BAPN+AngII+Gr-1) and those in the intima of the MMP9−/− mice treated with BAPN and AngII (BAPN+AngII) (n=10 for each group). Note that neutrophil infiltration is independent of MMP9 expression. Scale bar, 200 μm. B, Histological and immunohistochemical analyses of MMP9 expression and neutrophil infiltration in the aortic tissues of BAPN-untreated WT and MMP9−/− mice 24 hours after AngII infusion. Note that neutrophil infiltration is independent of BAPN treatment. Scale bar, 200 μm.
Neutrophil-Derived Matrix Metalloproteinase 9 Triggers Acute Aortic Dissection
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SUPPLEMENTAL MATERIAL

Neutrophil-Derived Matrix Metalloproteinase 9 Triggers Acute Aortic Dissection

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7Correspondence should be addressed to Ryoko Shimizu-Hirota (phone: 81-3-3353-1211 ext 62312, email: ryoko.shimizuhirota@gmail.com; Department of Internal Medicine, Division of Endocrinology, Metabolism and Nephrology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan) or Yasunori Okada (email: okada@z6.keio.jp; Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan)
Supplemental Methods

ELISA

Levels of matrix metalloproteinase1, 2, 3 and 9 (MMP1, MMP2, MMP3 and MMP9), tissue inhibitor of metalloproteinases-1 (TIMP1) and AngII were measured by sandwich ELISA from the blood samples of all the registered patients as previously described.1, 2

Histology and immunohistochemistry

Sections (4 μm thick) were cut from the paraffin-embedded aortic specimens obtained at surgery from the AAD patients or non-ruptured aneurysm patients. The sections were stained with hematoxylin and eosin (H&E) or by elastica Van Gieson (EVG) stain, and immunostained with mouse monoclonal antibodies against human MMP9 (56-2A4; Daiichi Fine Chemicals, Ltd., Takaoka, Japan) 3 and neutrophil elastase (Santa Cruz Biotechnology, Santa Cruz, CA) or control IgG (Santa Cruz Biotechnology). The degree of MMP9-immunoreactive cell density was evaluated by morphometric analysis of the immunostained tissue sections. In each section, the aortic tissues from the AAD or non-ruptured aneurysm patients was photographed, and the number of immunoreactive cells was calculated by two independent pathologists (M.S. and Y.O.) by observing 5 different fields at magnification of 200×. The average number of positive cells per square millimeter was calculated as MMP9-immunoreactive cell density. For double immunostaining for neutrophil elastase and MMP9, the paraffin sections from the AAD patients were immunostained with anti-neutrophil elastase antibody (Santa Cruz Biotechnology) and then anti-MMP9 antibody (56-2A4;
Daiichi Fine Chemicals, Ltd.) by using the Envision System (Vector Laboratories) and additionally HistoGreen Substrate Kit (AbCys SA, Paris, France) according to the manufacturer’s protocols. The serial sections were stained with H&E or immunostained with control IgG.

Mouse aortic tissues were also stained with H&E and EVG or immunostained with anti-mouse MMP9 antibody (R&D systems), anti-neutrophil antibody as an allotypic mouse neutrophil marker (7/4 clone; Serotec, Oxford, UK) or control IgG. Histology and immunohistochemistry were evaluated by pathologists (M.S. and Y.O.) who were not informed of the results of the experimental treatments.

RT-PCR
Total RNA was extracted from mouse aortae, and then RT-PCR was performed as previously described.4 The primer sequences were as follows; MMP9 (sense, 5'-TGTACCGCTATGGTTACACCCG-3'; antisense, 5'-CGCGACACCAACTGGATGAC-3') and GAPDH (sense, 5'-GCCAAGGTCATCCATGACAAAC-3'; antisense, 5'-GTCCACCACCCTTGTTGCTGTA-3').

Gelatin zymography, film in situ zymography and in situ detection of superoxide
Mouse aortae were snap-frozen in liquid nitrogen and gelatin zymography was performed with homogenate supernatants prepared from the tissue samples according to the methods previously described.4,5 For film in situ zymography,
fresh mouse aortae were embedded without fixation in Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN), and subjected to in situ zymography.\textsuperscript{1}

Frozen sections were made by a cryostat (Miles, Inc.), and mounted onto gelatin films which were prepared by coating a cross-linked gelatin on a polyester base.\textsuperscript{1}

Some frozen sections were also placed on the gelatin films coated with 1,10-phenanthroline, a metalloproteinase inhibitor. The films with sections were incubated for 24 h at 37°C and then stained with 1.0% Amido Black 10B (Sigma Aldrich, Saint Louis, MO).\textsuperscript{1} Serial sections were stained with H&E. In situ detection of superoxide in the aortic tissues from the AAD mice and control mice was performed according to the methods previously described.\textsuperscript{6} Briefly, frozen sections of the aortic tissues were incubated with 10 mM dihydroethidium (DHE) in PBS for 30 min at 37°C in a humidified chamber protected from light. Ethidium bromide generated from DHE by oxidation with superoxide within the tissue was detected by confocal microscope using a 543-nm He-Ne laser combined with a 560-nm long-pass filter (Carl Zeiss MicroImaging Inc., Tokyo, Japan).

References


