Adventitial Mast Cells Contribute to Pathogenesis in the Progression of Abdominal Aortic Aneurysm

Toshihiro Tsuruda, Johji Kato, Kinta Hatakeyama, Kazushi Kojima, Mitsuhiro Yano, Yoshikazu Yano, Kunihide Nakamura, Fukumi Nakamura-Uchiyama, Yoshibumi Matsushima, Takuroh Imamura, Toshio Onitsuka, Yuijiro Asada, Yukifumi Nawa, Tanenao Eto, Kazuo Kitamura

Abstract—Abdominal aortic aneurysm (AAA) is histologically characterized by medial degeneration and various degrees of chronic adventitial inflammation, although the mechanisms for progression of aneurysm are poorly understood. In the present study, we carried out histological study of AAA tissues of patients, and interventional animal and cell culture experiments to investigate a role of mast cells in the pathogenesis of AAA. The number of mast cells was found to increase in the outer media or adventitia of human AAA, showing a positive correlation between the cell number and the AAA diameter. Aneurysmal dilatation of the aorta was seen in the control (+/+ ) rats following periaortic application of calcium chloride (CaCl₂) treatment but not in the mast cell–deficient mutant Ws/ Ws rats. The AAA formation was accompanied by accumulation of mast cells, T lymphocytes and by activated matrix metalloproteinase 9, reduced elastin levels and augmented angiogenesis in the aortic tissue, but these changes were much less in the Ws/ Ws rats than in the controls. Similarly, mast cells were accumulated and activated at the adventitia of aneurysmal aorta in the apolipoprotein E–deficient mice. The pharmacological intervention with the tranilast, an inhibitor of mast cell degranulation, attenuated AAA development in these rodent models. In the cell culture experiment, a mast cell directly augmented matrix metalloproteinase 9 activity produced by the monocyte/macrophage. Collectively, these data suggest that adventitial mast cells play a critical role in the progression of AAA. (Circ Res. 2008;102:1368-1377.)

Key Words: adventitia ■ inflammation ■ mast cell ■ matrix metalloproteinase ■ aneurysm

Abdominal aortic aneurysm (AAA), a relatively common disorder among elderly people, is pathologically characterized by atherosclerosis of the intima and disruption or attenuation of the elastic media with various degrees of adventitial inflammatory infiltration.1,2 Because approximately 4% of adults older than 65 years harbor AAA, it is among the leading 15 causes of death in elderly persons in the United States.3 Although substantial efforts have been made to clarify the mechanism of development of AAA, there is currently no effective method to inhibit enlargement of AAA. Repair surgery is necessary to prevent rupture in patients with progressively enlarging AAA, whereas the operative risk is often relatively high because of the other complications resulting from aging.

Recent reports suggest that chronic inflammation of the aortic wall and progressive degradation of extracellular matrix proteins are involved in the development, progression, or rupture of AAA.4,5,8 As a component of the immune system, mast cells play a critical role in defending hosts against pathogens by releasing a number of immunoregulatory mediators.9 These cells have also been shown to initiate the inflammatory response by releasing proinflammatory cytokines, growth factors, angiogenic mediators, and proteases,10 as well as by recruiting other inflammatory cells, such as neutrophils, macrophages, and T lymphocytes.11–13 Mast cells are present in the outer media or adventitia of the atherosclerotic aorta,14 where enzymes in the mast cell granules are assumed to induce apoptosis in vascular smooth muscle cells and to activate matrix metalloproteinases (MMPs).15,16

Based on these findings, we hypothesized that adventitial mast cells play a pivotal role in aortic aneurysmal dilatation by destroying medial elastic tissue and by inducing the adventitial inflammation. The present study was conducted first to characterize mast cell infiltrates in human AAA tissues and then extended to clarify the role of this type of immune cell in AAA development with rodent models of AAA and with cultured mast cells.
Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournal.org. This study was approved by the Human Investigation Review Committee of the University of Miyazaki (No. 99) and conformed with the principles outlined in the Declaration of Helsinki (Cardiovasc Res. 1997;35:2–4). The animal study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2004-096-4). This investigation also conformed with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Statistical Analysis

All data were analyzed with SPSS software version 11.0 (SPSS Inc). Differences between 2 groups were analyzed by Student’s t test, and those between aortic diameters before and after periaortic application of CaCl2 were analyzed by paired t test. Multiple comparisons were done with the χ2 test or 1-way ANOVA, followed by Scheffé’s test, and the Pearson’s correlation coefficient test was used to assess the relationship between the diameter and cell density. To accurately analyze zymogram and Western blot, standard curves were made by serial dilution of the samples, and the bands on gels were quantified based on the optical densities. The data are expressed as the means±SEM or as the median with the 10% to 90% range and outlying value. Statistical significance was accepted at P<0.05.

Results

Patient Characteristics

Table I in the online data supplement shows the basal clinical parameters of the patients enrolled in this study. Both the AAA patients and those with advanced atherosclerosis were significantly (P<0.01) older than the control patients, but no difference was noted between the AAA and advanced atherosclerosis groups. The AAA group showed significantly higher rates of hypertension and cigarette smoking, but a lower rate of diabetes mellitus, compared with the advanced atherosclerosis group.

Mast Cell Density in Aortas With or Without AAA

Figure 1A illustrates the mast cell numbers positive for tryptase, a major neutral protease of mast cell secretory granules, in the controls, atherosclerotic aortas, and AAA tissues. The number of mast cells in the advance atherosclerotic aorta increased (P<0.05) as compared with the control, and a further increase (P<0.01) was observed in the AAA tissue, showing a positive correlation between the cell number and the maximal diameter of AAA (Figure 1B). Moreover, degranulated mast cells were rarely seen in the control and atherosclerotic aorta; however, the ratio of degranulated mast cells to the total mast cell number in AAA was significantly (P<0.01) higher than in the other groups (Figure 1C). Meanwhile, no significant difference was noted in the number of macrophages positive for CD68 among the groups (Figure 1D).
Protein Expressions and Gelatinase Abundance in Human AAA

The protein expressions of tryptase, stem cell factor (SCF), a ligand for the protooncogene c-kit involved in maturation and differentiation of mast cells, and phosphorylation of c-kit were significantly ($P<0.01$) increased in the AAA tissues compared with control or atherosclerotic aorta without dilation (Figure 2A and 2B). In the serial sections, the immunoreactivity for SCF was specifically localized in the cytoplasm of mast cells positive for tryptase in the adventitia of AAA specimens (Figure 2C through 2E). The zymographic gelatinase abundance corresponding to both forms of latent and active MMP-9 ($P<0.01$) and the ratio of active/latent zymographic MMP-2 ($P<0.05$) abundance were significantly increased in the AAA tissues (Figure 2A and 2B). As shown in Figure 3A and 3B, the immunoreactivity of MMP-9 was similarly distributed in the plaque area of advanced atherosclerotic aorta and AAA, although it significantly increased in the outer media and adventitia of AAA. Figure 3C shows that gelatinolytic activity corresponding to MMP-9 was distributed in the outer media or adventitia of AAA. The region where gelatinolysis occurred was observed as white or pale pink, whereas other areas with no gelatinolysis were red (Figure 3Ca and 3Cb). The specificity of this gelatinolysis was confirmed by the inhibition by the broad MMP inhibitor, 1,10-phenanthroline (Figure 3Cc and 3Cd). The MMP-9 immunoreactivity was mainly (>98%) colocalized with macrophages and some (<2%) with T lymphocytes, whereas there was no colocalization with mast cells or smooth muscle cells (supplemental Figure I).

Aneurysm Induction in Control and Mast Cell–Deficient Ws/Ws Rats

Figure 4A shows the representative immunohistological pictures of abdominal aortas of the control (+/+) rats and those of mast cell–deficient Ws/Ws at 14 days after the periaortic application of calcium chloride (CaCl$_2$). Figure 4B illustrate that the periaortic application of CaCl$_2$ led to destruction of the architecture of aortic walls, increasing aortic diameter by up to 55% in the control (+/+) rats at day 14 as compared with that before the CaCl$_2$ application, whereas the aorta of the Ws/Ws rats was found to be resistant to CaCl$_2$, showing only a 13% increase at day 14. The time-dependent aortic dilatation was accompanied with progressive adventitial inflammation characterized by infiltrations of mast cells and T lymphocytes and by increased rate of degranulated mast cells and numbers of microvessels in the control (+/+) rats, but such changes were smaller in the Ws/Ws rats. In addition, the elastin areas of the aortic media were time-dependently reduced in the control (+/+) rats but not in the Ws/Ws rats.
Zymographic gelatinase abundance of total MMP-2 of aortic tissue of the Ws/Ws rats was significantly less at day 7, and those of MMP-9 were so at days 7 and 14, compared with the control (\(H_1\)) rats. Meanwhile, no changes were noted in the number of macrophages between the control (\(H_1\)) and Ws/Ws rats with AAA induction. Furthermore, blood pressure measured with the tail-cuff method, and the number of neutrophils and apoptotic cells showed no difference between the 2 groups of rats with AAA induction (data not shown).

**Effects of Tranilast on Aneurysm Formation of Control Rats**

Figure 5A and 5B illustrates the effect of tranilast \([N-(3,4-dimethoxycinnamoyl) anthranilic acid]\) on the morphological changes in the CaCl\(_2\)-induced aneurysm formation of the control (+/+) rats. The administration of tranilast significantly attenuated the dilatation of aorta, the inflammatory infiltrations of mast cells and T lymphocytes, and the microvessel formation in the adventitia of the aortas treated with CaCl\(_2\). In addition, elastin area was preserved in aortas of the treated group. Figure 5C shows that zymographic abundance of total MMP-2 in aortic tissue was slightly reduced following treatment with tranilast, and a significant reduction \((P<0.01)\) was noted in that of MMP-9. Meanwhile, no significant changes were found in the number of neutrophils and apoptotic cells between the 2 groups of rats with or without the tranilast treatment. Furthermore, the number of neutrophils and apoptotic cells was not altered significantly between the groups (data not shown).

**Mast Cell Activation in Apolipoprotein E–Deficient Mouse Model of AAA**

Figure 6A and 6C illustrates the representative pictures and quantitative evaluations of abdominal aortas of the control and those infused with angiotensin (Ang) II subcutaneously for 28 days treated without or with tranilast in the spontaneously apolipoprotein (apo)E-deficient mice. The Ang II–induced increases in the size of aorta and plaque area were significantly \((P<0.05)\) attenuated by the tranilast treatment. Figure 6B and 6C shows the other representative pictures and quantitative evaluations of elastin formation, distribution of mast cells, T lymphocytes, macrophages, and microvessels in the apoE-deficient mice infused with Ang II. The enlarged abdominal aorta was accompanied by increasing the number...
of mast cells, T lymphocytes, and capillary vessels, as well as the proportion of degranulated mast cells in the adventitia, whereas these were significantly reduced following the tranilast treatment. In addition, a significant reduction of elastin area in aortas of these mice was preserved with the treatment \( (P < 0.01) \).

Figure 6D shows the effect of tranilast on MMP-9 abundance produced from coculture of activated HMC-1 and U937 cells: 100 and 300 \( \mu \text{mol/L} \) tranilast significantly \( (P < 0.01) \) decreased the zymographic MMP-9 abundance in the conditioned media. Next, we examined whether tranilast reduced MMP-9 abundance by acting on HMC-1 cells or on U937 cells. As shown in Figure 7C, tranilast had no direct effect on MMP-9 abundance produced from U937 incubated with conditioned media of activated HMC-1; however, it reduced MMP-9 abundance \( (P < 0.01) \) when U937 cells were cultured with the conditioned media of activated HMC-1 cells that had been pretreated with tranilast. Similar increase in MMP-9 abundance \( (P < 0.01) \) was observed when U937 cells were incubated alone with conditioned media of activated HMC-1, but this increase was attenuated \( (P < 0.05) \) in the conditioned media of HMC-1 cells that had been pretreated with 50 \( \mu \text{g/mL} \) monoclonal interferon (IFN)-\( \gamma \) antibody (Figure 8A). Figure 8B shows IFN-\( \gamma \) secretion from cultured U937 cells and control or activated HMC-1 cells. The IFN-\( \gamma \) secretion from activated HMC-1 cells significantly \( (P < 0.05) \) increased on activation, but that from U937 was minimal. Meanwhile, the IFN-\( \gamma \) secretion from activated HMC-1 cells was significantly \( (P < 0.05) \) decreased by 300 \( \mu \text{mol/L} \) tranilast (Figure 8C).

As shown in supplemental Figure IIA through IID, coculture of primary murine bone marrow–derived mast cells (BMMCs) and peritoneal macrophages also significantly \( (P < 0.05) \) increased the MMP-9 abundance, compared to that from BMMCs, activated BMMCs or peritoneal macrophages.
whereas it was significantly \( P < 0.01 \) inhibited by 100 and 300 \( \mu \text{mol/L} \) tranilast. Meanwhile, the conditioned media obtained from the activated BMMCs did not alter the MMP-9 abundance in culture media of peritoneal macrophages. The IFN-\( \gamma \) secretion from BMMCs did not increase on the activation, whereas the coculture of activated BMMCs and peritoneal macrophages stimulated to increase it, compared to that from peritoneal macrophage cultured alone.

**Discussion**

Carrying out the present study with human samples of AAA, we observed several findings that imply a role of mast cells in development or progression of AAA. First, mast cell numbers increased in the aortic adventitia and outer media with the advanced stage of atherosclerosis, but a further increase was observed in AAA, with the significant correlation between the cell number and the maximal diameter of AAA. More importantly, the proportion of degranulated mast cells was significantly higher in AAA than in controls or atherosclerotic aortas. In autopsy cases, mast cell number increased 2- to 4-fold at the aneurysmal sites of nonruptured or ruptured AAA as compared with the nondilated part of the aortas of those patients (supplemental Table II). Second, expression of tryptase and SCF, specifically localized in mast cells, and phosphorylation of c-kit (receptor for SCF) were increased in the AAA tissues compared with the controls or atherosclerotic aortas. Thus, accumulation or activation of mast cells in the outer media and adventitia appears involved in the development, progression, or rupture of AAA.

To further clarify the role of adventitial mast cells in development of AAA, we examined whether or not aneurysmal dilatation of the abdominal aortas occurred following periaortic application of \( \text{CaCl}_2 \) in mutant mast cell–deficient \( \text{Ws/Ws} \) and control \( \text{+/+} \) rats. The progressive dilatation of abdominal aortas, accompanied by mast cell accumulation and degranulation, occurred in the control \( \text{+/+} \) rats, but such dilatation was not observed in the \( \text{Ws/Ws} \) rats. In addition, spontaneously hyperlipidemic apoE-deficient mice exhibited the mast cell accumulation and activation at the aneurysmal site of abdominal aortas. Whereas, tranilast, an inhibitor of mast cell degranulation, effectively suppressed the progression of these experimental models of AAA. Collectively, our present data support the recent studies and extend our understanding of the important role for adventitial mast cells in the development of AAA.

Chronic inflammation in the aortic wall, particularly in the adventitia, is assumed to have a detrimental role in AAA. Mast cells serve as an important source of proinflammatory mediators and cytokines that can activate T lymphocytes and macrophages, whereas mast cells be-
come activated on direct contact with T lymphocytes. Accordant with these reports, T lymphocytes were increased concord with mast cells in the rodent models of AAA. In addition to proinflammatory cytokines, a number of substances or enzymes produced from mast cells are assumed to be involved in the development of AAA. Mast cells produce tryptase or chymase, which degrades extracellular matrix by activating MMPs or induce apoptosis of vascular smooth muscle cells. Indeed, mast cells double positive for tryptase and chymase were predominately observed in the adventitia of human AAA (supplemental Figure III).

It is important to note that humans and rodents share the common pathological features, showing the unchanged number of macrophages between the dilated and nondilated aortas, irrespectively of the increasing number of mast cells and MMP-9 activity. Consistent with the previous report, MMP-9 activity was mainly distributed in macrophages at the outer media and adventitia of human AAA, suggesting a possible crosstalk between mast cells and macrophages to produce MMP-9. Smoking is a risk factor for AAA, and the rate of cigarette smoking was substantially high in AAA patients in this study. Smoking is proposed to promote pathogenesis of AAA through the 5-lipoxygenase pathway, and mast cells are reported to be critical to cause an increase the enzyme in macrophages. Keeping this mind is also important that the mast cells coculturing with the monocyte/macrophage augmented zymographic activity of MMP-9. Our finding in vitro suggests an important contribution for mast cells to augment MMP-9 secretion (latent form) in macrophages by either direct cell-to-cell contact or through humoral mediators such as IFN-γ and it is thereby further activated in the extracellular compartment in vivo. On the other hand, coculturing of mast cells with the adventitial fibroblasts isolated from human AAA did not alter the zymographic MMP-2 and -9 abundance (data not shown).

Other characteristic features of human AAA are angiogenesis and apoptosis of smooth muscle cells. A number of biological active substances contained in mast cell granules and the activation of the MMP-9 coincides with angiogenic process. Consistent with this notion, the experimental models of AAA were accompanied by augmented angiogenesis in the aorta, but these changes were reduced by the tranilast. On the other hand, this study indicates the apoptosis might play a minor role in the mechanism. Taken together, this study suggests that mast cells contribute to the pathogenesis of AAA by activating MMP-9 and angiogenesis with the other inflammatory cells in the adventitia of aortic wall.

Lastly, we should mention limitations of the present study. First, the phenotype of Ws/Ws rats is not specific to mast cell deletion but also shows hypopigmentation of the skin and...
hypoplastic anemia at an early age. Because mast cells were not reconstituted to the aortas of Ws/Ws rats, we could not verify the roles of mast cells by their reconstitution. The second, tranilast, was initially identified as an inhibitor of mast cell degranulation. However, this compound has been shown the antiproliferative and antiinflammatory actions in other cell types, such as vascular smooth muscle cells and neutrophils. We demonstrated the specific action of

Figure 7. A, Zymographic MMP-9 abundance in conditioned media following coculture of the activated mast cell line HMC-1 (aHMC-1) and monocyte/macrophage line U937. **P<0.01 vs HMC-1; ##P<0.01 vs aHMC-1; ##P<0.01 vs U937. B, Effect of tranilast on zymographic MMP-9 abundance in the conditioned media of coculture of HMC-1 and U937 cells. **P<0.01 vs untreated cells; ##P<0.01 vs 100 μmol/L tranilast. C, Effect of tranilast on zymographic MMP-9 abundance before or after transferring the conditioned medium of activated HMC-1 cells to U937 cells. **P<0.01 vs untreated cells; ##P<0.01 vs 100 μmol/L tranilast. Protein loading was evaluated by the silver stain of the media. INT indicates optical intensity of the lytic bands on the zymogram, and data are presented as the means±SEM of 4 to 6 samples examined.

Figure 8. A, Zymographic MMP-9 abundance culturing U937 cells with the conditioned media of activated HMC-1 with or without pretreatment of anti-IFN-γ antibody for 24 hour. SF, serum-free media. **P<0.01 vs incubated without conditioned media; #P<0.05 vs incubated with conditioned media without anti-IFN-γ antibody. B, Secretion of IFN-γ from U937, HMC-1, and activated mast cell line HMC-1 (aHMC-1). **P<0.01 vs U937; #P<0.05 vs HMC-1. C, Secretion of IFN-γ from the activated HMC-1 cells with or without tranilast treatment. *P<0.05 vs activated HMC-1 cells without tranilast. Protein loading was evaluated by the silver stain of the media. INT indicates optical intensity of the lytic bands on the zymogram, and data are presented as the means±SEM of 4 to 6 samples examined.
tralast on mast cells but not macrophages in vitro; however, we cannot deny the possibility that this compound would affect the other vascular components. In this study, tranilast inhibited the accumulation of mast cells in both experimental models of AAA but did not change the proportion of the cells degranulated in the rat model. Transforming growth factor-β is reported to be increased in human AAA and is also an important chemotaxin for recruiting the mast cells. Although the inhibition of mast cell degranulation by the tranilast may likely depend on species specific or temporal during the aneurysmal formation, we postulate that tranilast may have a potential to attenuate the aneurysmal formation by inhibiting the migration of mast cells through transforming growth factor-β signaling, as well as by attenuating the activation of the cells (supplemental Figure IV). Further studies would be aimed to explore the detailed mechanisms by which tranilast prevent AAA, but this study illustrates the feasibility for tranilast as a therapeutic tool in preventing AAA from enlarging.

In summary, this study provides the evidence for the involvement of adventitial mast cells in concert with other inflammatory cells in AAA development, offering a novel therapeutic strategy of pharmacologically suppressing mast cell activity in treating patients with AAA.

Acknowledgments
We are greatly indebted to Dr Akira Ishihara (Prefectural Nobeoka Hospital, Miyazaki, Japan) for preparation of ruptured aneurysm tissues and to Ritsuko Sotomura and Yoko Sekita for expert technical assistance.

Sources of Funding
This study was supported by grants-in-aid from the 21st Century Centers of Excellence Program (Life Science) and Scientific Research from the Ministry of Education, Culture, Sport, Science and Technology, Japan; grants-in-aid from the Suzuken Memorial Foundation; the Mochida Memorial Foundation for Medical and Pharmaceutical Research; and a Kimura Memorial Heart Foundation research grant.

Disclosures
None.

References


Adventitial Mast Cells Contribute to Pathogenesis in the Progression of Abdominal Aortic Aneurysm

Toshihiro Tsuruda, Johji Kato, Kinta Hatakeyama, Kazushi Kojima, Mitsuhiro Yano, Yoshikazu Yano, Kunihide Nakamura, Fukumi Nakamura-Uchiyama, Yoshibumi Matsushima, Takuroh Imamura, Toshio Onitsuka, Yujiro Asada, Yukifumi Nawa, Tanenao Eto and Kazuo Kitamura

Circ Res. 2008;102:1368-1377; originally published online May 1, 2008;
doi: 10.1161/CIRCRESAHA.108.173682

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/11/1368

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/05/05/CIRCRESAHA.108.173682.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Expanded Materials and Methods

Study Patients with Atherosclerosis or AAA

Aneurysm tissues were obtained from 57 Japanese patients with atherosclerosis-associated AAA during elective repair surgery with written informed consent. Maximal diameters of the AAAs were measured during the surgery, and the AAA tissues were collected from the anterior part of abdominal aortas, where dilatation was judged to be maximal. Fifty-seven aortic tissues looking mostly normal or showing various degrees of atherosclerosis without aneurysmal dilatation were collected from the anterior part of descending thoracic aortas at autopsy performed within 6 h postmortem. This was done because there were reportedly no difference in mast cell number between thoracic and abdominal aorta and because mast cells began to disappear from vascular tissues at 6 h postmortem. We classified 57 aortic tissues without AAA into three groups: 22 cases of controls without atherosclerosis lesions such as accumulation of foamy macrophages, extracellular lipid pool or excess amount of collagen or necrosis, 12 cases of early-stage atherosclerosis with fatty streaks and 23 cases of advanced atherosclerosis with extracellular lipid core formation. The aortic tissues were fixed in 10% formalin or frozen in liquid nitrogen immediately after resection. Hypertension was defined as systolic blood pressure $\geq$ 140 mm Hg or
diastolic blood pressure $\geq 90$ mmHg or taking any antihypertensive medicine, and diabetes mellitus as fasting plasma glucose level $\geq 126$ mg/dL, two-hour postprandial glucose $\geq 200$ mg/dL, or taking any medicine for diabetes mellitus.

**Animal Experiments**

Aneurysm induction.

Rat model. Twelve-week-old male mast cell-deficient Ws/Ws $^{3,4}$ and control (+/+) rats (Japan SLC, Hamamatsu, Shizuoka, Japan) were anesthetized by 50 mg/kg pentobarbital sodium and laparotomy was performed under aseptic conditions. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal tissue. Aortic diameters were measured three times at the middle portion between the renal artery and iliac artery bifurcation. After baseline measurements, peri-aortic application of 0.4 mol/L CaCl$_2$ was performed to the outside of infrarenal abdominal aorta by rubbing with a sterile cotton swab for 15 min. The CaCl$_2$ concentration was determined according to the previous studies $^{5,6}$ and our preliminary experiments, where doses of 0.25 to 0.5 mol/L were tested. The aorta was rinsed with 0.9% sterile saline five times and the incision was then closed. At the indicated time point in the experimental period, the rats underwent laparotomy.
again after anesthetizing with pentobarbital sodium. Aortic diameter was measured at
the same portion of the aorta and thereafter the rats were sacrificed by pentobarbital
overdose. Aortic tissues were frozen in liquid nitrogen, or fixed in 4%
paraformaldehyde or the Carnoy’s solution for histological analysis. Either 400
mg/kg/day tranilast (Kissei Pharmaceutical Co., Ltd.) dissolved in 1%NaHCO₃ or
vehicle solution was orally given to the control (+/+ ) rats twice a day. The treatment
with tranilast was started 7 days before the CaCl₂ application and continued for 21 days.
The dosage of tranilast was determined by the previous reports ⁷, ⁸ and personal
communication to Dr. Yasuo Takehana, Kissei Pharmaceutical Co., Ltd. The dose used
in this study was supposed to be comparable to 600-900 mg/day in humans ⁹. The
aortic diameters were measured and tissues were collected as described above.

Mouse model. Seven to eleven-month-old male spontaneously hyperlipidemic
apolipoprotein E deficient mice ¹⁰,¹¹ (C. KOR/STm-ApoE⁻/-, Japan SLC, BALB/c
background) fed a standard rodent chow were randomly assigned to 3 groups: untreated
control (n=6), infusion of Ang II (1000 ng/kg/min) subcutaneously by implanted
mini-osmotic pumps (Alzet, Model 1004; DURECT Co.) (n=10), or Ang II plus 0.5%
tranilast containing compounded diet ¹² (n=7) for 28 days. In the preliminary
experiment, both backgrounds (C57Bl6 and BALB/c) exhibited the similar extent and
frequency (approximately 60%) of aneurysmal formation, but the variation of atherosclerotic features in BALB/c background was relatively constant, compared to that of C57Bl6. Tranilast was started 7 days before the Ang II infusion and continued for 28 days. Aortic tissues were fixed in 4% paraformaldehyde, and some of them were also frozen in liquid nitrogen for MMP assay.

**Histology and Immunohistochemistry**

Human aorta. Aortic tissues fixed in 10% formalin were embedded in paraffin wax. Tissue sections of 3 µm in thickness were microwaved at 95 °C for 1 h in 10 mmol/L citrate buffer (pH 6.0) for staining of mast cell tryptase, covered with proteinase K at room temperature for 15 min for CD68, autoclaved at 121 °C for 15 min for MMP-9, or with 0.05% pronase at 37 °C for 10 min for SCF, prior to incubation with the primary antibodies. The sections were incubated at 4 °C overnight with the monoclonal antibodies against SCF (16 µg/mL, Clone hKL 12, BMA Biomedicals AG), tryptase (80 µg/mL, Clone AA1, DakoCytomation), MMP-9 (5 µg/mL, Clone F-69, Daiichi Fine chemical) or CD68 (Clone PG-M1, DakoCytomation), as previously described 13. The cellular localization of MMP-9 was evaluated by double immunofluorescence staining with the rabbit anti-human MMP-9 polyclonal antibody (Cell signaling) and
monoclonal antibodies against CD68, CD3 (Clone F7.2.38, DakoCytomation), tryptase or α-smooth muscle actin (Clone 1A4, DakoCytomation) overnight at 4 °C, followed by staining with fluorescein isothiocyanate-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch) for 20 min.

Immunofluorescent images were analyzed with a confocal scanning system (Olympus IX71).

Rat aorta. Abdominal aortae were fixed in 4% paraformaldehyde or the Carnoy’s solution for staining with the monoclonal antibodies against T-lymphocytes CD3 (1:40, Clone 1F4, Serotec) and monocyte/macrophage CD68 (1:400, Clone ED1, Chemicon), the polyclonal antibodies against von Willebrand factor (DakoCytomation) and alcian blue-safranin O, or Victoria blue. The number of neutrophils was determined by counting the granular leukocytes having a nucleus with 3 to 5 lobes connected by threads of chromatin and cytoplasm containing very fine granules. The tissue sections of 3 µm in thickness fixed in 4% paraformaldehyde were pretreated before incubation with the primary antibodies: CD3, microwaved at 95 °C for 1 h in 10 mmol/L citrate buffer (pH 6.0); CD68, covered with 0.05% pronase at 37 °C for 10 min; von Willebrand factor, covered with proteinase K at 37 °C for 15 min. For alcian blue-safranin O staining, the tissue sections fixed in the Carnoy’s solution were stained.
with 0.1% alcian blue dissolved in 0.7N HCl overnight, followed by incubation with 0.5% safranin O dissolved in 0.125N HCl for 30 min. Apoptotic cells were detected in the tissue sections fixed in 4% paraformaldehyde with *in situ* detection of terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling (Medical & Biological Laboratories Co., Ltd). Negative control staining was carried out by omitting the first antibody or by using a non-immune isotype-matched IgG. Positive controls were prepared as follows: rat intestine infected with strongyloides for alcian blue-safranin O; rat thymus for CD3 and apoptotic cells; rat spleen for CD68 (Supplemental Figure V).

Mouse aorta. Three µm-section of aortae fixed in 4% paraformaldehyde was carried out to stain with the polyclonal antibodies against T-lymphocytes CD3 (1:100, Abcam) and CD31 (abcam), the monoclonal antibody against macrophage F4/80 (clone Cl:A3-1, 1:10,000, Cedarlane) and toluidine blue (pH 2.5), or Victoria blue. The tissue sections were autoclaved at 121 °C for 15 min in 10 mmol/L citrate buffer (pH 6.0) before incubation with the primary antibodies for CD31 and F4/80. A catalyzed signal amplification system (CSA-DakoCytomation) was used for detecting F4/80 antigen. The numbers of neutrophils and apoptotic cells were detected as described above.


**Histological Assessment**

The number of mast cells and macrophages in the human aortic specimens were determined by counting the cells at magnification of x400 regardless of presence or absence of those cells throughout the whole area of outer-media and adventitia on the one section per each patient in a blind manner, and the average was analyzed as an individual value. Outer-media was defined as outer two-thirds of the media. For the rodent aortic specimens stained by the above-mentioned methods, the morphological analysis was performed as previously described \(^{14}\). The numbers of mast cells, T-lymphocytes, macrophages, neutrophils, capillary endothelial and apoptotic cells in the adventitia or media were determined by counting cells in a blind manner at x400. The average number for these types of rodent cell was used as an individual value or normalized by the adventitial area.

**Cell Culture**

HMC-1 cells, a human mast cell leukemia line, were kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN, USA) and U937 cells, a monocytic cell line, were obtained from American Type Culture Collection. Both types of cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.
Bone marrow derived mast cells (BMMC) was obtained from 4 week-old BALB/c mice. Bone marrow cells were cultured in 50% WEHI-3 cell (Riken Cell Bank, Tsukuba, Japan) conditioned medium/50% RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 0.1 mmol/L non-essential amino acid and antibiotics, according to the previous report 15. After 5 weeks of culture, mast cell number and purity were assessed by alcian blue-safranin O, and cell viability was determined by trypan blue dye exclusion. BMMC used in the experiments consisted of more than 95% mast cell and were of 98% or greater viability. Peritoneal macrophages derived from BALB/c mice were obtained by harvest from peritoneal lavage with 10 mL RPMI-1640 supplemented with 10% fetal bovine serum, according to the previous report 16. Peritoneal cells were centrifuged at 1500 rpm, 4 °C for 10 min and washed with RPMI-1640 medium. They were plated onto 24-well culture dishes at a concentration of 1 × 10⁶ cells/well, and allowed to adhere on the culture dishes for 1 h at 37 °C. The non-adherent cells were removed and the adherent cells were used for the experiment a day after isolation. Macrophages isolated by this procedure were more than 95% pure as measured by staining for the F4/80 antigen.

To study the effect of mast cells on release of MMP-9 from macrophages, HMC-1 or BMMC cells were first activated by incubation with 50 ng/mL phorbol myristate acetate
and 5x10^{-7} \text{ mol/L calcium ionophore A23187} for 2 h at 37 \degree \text{C} by referring to the previous reports\textsuperscript{17,18}, followed by extensive washing with phosphate buffer three times. U937 cells or peritoneal macrophages (1x10^6 cells/cm\textsuperscript{2}) were then co-cultured with the activated mast cells at a cell number ratio of 10:1 for 24 h in serum-free RPMI 1640 supplemented with 5 \mu g/mL insulin, 5 \mu g/mL transferrin and 5 ng/mL sodium selenite in a 24-well culture plate (Figure VI in online supplement). To examine whether or not a cell-to-cell contact is essential for augmented MMP-9 activity, the conditioned serum-free medium collected after culturing activated mast cells for 24 h were transferred to macrophages, and were then cultured for another 24 h in a 24-well culture plate. (Figure VII in online supplement). Some of the conditioned serum-free media obtained from the activated HMC-1 cells were pretreated with monoclonal interferon (IFN)-\gamma antibody (DakoCytomation) for 24 h, and they were centrifuged at 2500 rpm, 10 min, following to transfer to the U937 cells. To see the effect of tranilast, the indicated concentration of tranilast was added to HMC-1 cells or BMMC for 1 h prior to the activation described above. After washing the cells extensively, they were co-cultured with U937 cells or peritoneal macrophages for 24 h with tranilast (Figure VIII in online supplement). Lastly, to identify which cell types of mast cells or monocyte/macrophages the tranilast affects, the conditioned media obtained from the
activated HMC-1 cells pretreated with tranilast for 24 h was transferred to U937 cells, and they were further cultured for 24 h in the absence or presence of tranilast. In other setting experiment, tranilast was added to U937 cells after transferring the conditioned medium of activated HMC-1 without tranilast treatment (Figure IX in online supplement). The dose of tranilast was determined based upon the previous studies, where 100 µmol/L of tranilast produced approximately a half reduction in histamine release, while plasma level reached and maintained above 280 µmol/L for 4 h following to a single oral administration of 200 mg/kg tranilast 19, 20.

**Western Blot**

Denatured protein samples (10-15 µg) from whole tissue lysates of the control, atherosclerotic aorta and AAA of human or rodent aorta were subjected to sodium dodecyl sulfate-polyacrylamide gel as previously described 21. The separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (BIO-RAD). After blocking the non-specific background with 5% skim milk, PVDF membranes were incubated with the primary antibodies against mast cell tryptase (1:1000, clone AA1, DakoCytomation), SCF (10 µg/mL, clone hkL-12, BMA Biochemicals), c-kit (0.5 µg/mL, phosphor Y730, abcam), transforming growth
factor-β1 (0.1 μg/mL, Santa Cruz) or β-actin (clone AC-15, Sigma), followed by incubation with horseradish peroxidase-coupled second antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and intensities of the bands were analyzed densitometrically (Chemi Doc™ Documentation System, BIO-RAD). β-actin was used to normalize the expression level in homogenized aortas.

**In vitro Zymographic Gelatinase Abundance**

Equal protein concentration of aortic tissues in humans (15 μg) and rodents (10 μg) frozen by liquid nitrogen were pulverized and incubated for 1 h on ice with 100 μL extraction buffer containing 1% Triton X-100, 50 mmol/L HEPES, 50 mmol/L NaCl and the EDTA-free protease inhibitor cocktail (Roche). The tissue samples or conditioned media were centrifuged at 13,000 rpm for 5 min and subjected to 10% gelatin zymogram (Invitrogen) as previously described 21. After washing the gels with renaturing buffer for 30 min three times, they were incubated with the developing buffer at room temperature, followed by further incubation with the fresh developing buffer at 37 °C for 48 h. Culture medium obtained from human fibrosarcoma HT1080 was used as a positive control. After destaining Coomassie brilliant blue, the molecular sizes of lytic bands were assessed by the positive controls and by molecular weight standards.
Either $\beta$-actin or silver staining was used as a protein-loading control.

**In Situ Zymography**

Gelatinolytic activity in the human AAA was analyzed with gelatin-coated film (gifted by Dr. Ryoichi Nemori, Fuji Film Co.), according to the method reported previously 22. Frozen section of tissue samples (3 $\mu$m) was placed on this film and incubated in a humidified chamber at 37 °C for 12 h. Then, the film was stained with Biebrich Scarlet Stain Solution for 4 min and counterstained with Meyer hematoxylin. Negative controls were prepared by including an MMP inhibitor, 1,10-phenanthroline on the gelatin-coated film. Gelatinolysis was detected as the disappearance (white or pale pink color) of Biebrich Scarlet Stain (red color). MMP-9 staining was done with the serial sections as described above.

**IFN-$\gamma$**

The concentration of IFN-$\gamma$ in the conditioned medium cultured for human or murine cells in the serum-free medium for 24 h was determined by an immunoenzymometric assay using a commercially available kit (Biosource).
References


Figure legends for Supplemental Figures

Supplemental Figure I

Localization of immunoreactive MMP-9 (upper panel, red), CD68, tryptase, CD3 or α-smooth muscle actin (middle panel, green) and merged images (lower panel, yellow) in the human AAA specimen. Bar, 100 µm.

Supplemental Figure II

Representative pictures of gelatin zymogram, quantitative evaluations of MMP-9 and IFN-γ secretion in conditioned media of primary mast cells and/or monocyte/macrophage.  

A: Zymographic MMP-9 abundance in conditioned media following co-culture of the activated bone marrow-derived mast cells (aBMMC) and peritoneal macrophages (PM).  **p<0.01 vs. BMMC; ##p<0.01 vs. aBMMC; +p<0.05 vs. PM.  

B: Effect of tranilast on zymographic MMP-9 abundance in the conditioned media of co-culture of aBMMC and PM.  **p<0.01 vs. untreated cells.  

C: Zymographic MMP-9 abundance culturing PM with the conditioned media of aBMMC.  

D: Secretion of IFN-γ from BMMC, aBMMC, PM and co-culture of aBMMC and PM.  *p<0.05 vs. BMMC; #p<0.05 vs. aBMMC.  SF, serum free media.  Protein loading was evaluated by the silver stain of the media.  INT indicates optical intensity of the lytic bands on the zymogram, and data are presented as the
means±SEM of four to six samples examined.

**Supplemental Figure III.** Phenotypes of mast cells distributed in the outer-media and adventitia of human AAA. Mast cells double positive for tryptase and chymase were shown by single arrow (▲), tryptase positive/chymase negative by double arrows (▼▼) and tryptase negative/chymase positive by triangle (△). Scale bar, 20 µm. To identify the phenotype of the human mast cells distributed in AAA, slide sections (n=26) were double stained with the monoclonal antibodies against tryptase and chymase using a Histamine kit (Nichirei, Co.), according to the manufacturer’s instruction. In double-labeling immunostaining for tryptase and chymase (1:100, Clone CC1, abcam), the activity of alkaline phosphatase for tryptase was visualized with new fuchsin in red (Nichirei, Co.) and for chymase with 3, 3’, 5’, 5’-tetramethylbenzidine in blue (Vector laboratories). +, positive; -, negative.

**Supplemental Figure IV.** Effect of tranilast on TGF-β1 expression in control (+/+). Homogenized aorta obtained from control (+/+) rats injured by calcium chloride with (n=5) or without (n=5) tranilast treatment were applied for western blot as described in the Methods section. Data are presented as the means±SEM. **p<0.01 vs. untreated rats. β-actin was used for protein loading control.

**Supplemental Figure V.** Negative controls for mouse IgG of human (A) or rat (B)
aorta and those for rabbit IgG of rat aorta (C). Positive controls for alcian blue-safranin O of rat intestine infected by strongyloides (D), CD3 of rat thymus (E), CD68 of rat spleen (F) or apoptotic cells of rat thymus (G). Arrows indicate the TUNEL-positive cells in rat thymus. Scale bar, 50 μm.

**Supplemental Figures VI to IX.**

Protocols of cell culture experiment to examine the effect of tranilast are illustrated.
Supplemental Figure I. Tsuruda, et al.

MMP-9

Macrophage

Mast cell

T-lymphocyte

Smooth muscle

Merge

Merge

Merge

Merge
Supplemental Figure III. Tsuruda, et al.
Supplemental Figure IV. Tsuruda, et al.

TGF-β1
β-actin
Tranilast
(-) (+)

TGF-β1 (INT/mm²)

Tranilast
(-) (+)

**
Supplemental Figure V. Tsuruda, et al.
Supplemental Figure VI. Tsuruda, et al.

PMA A23187 for 2h

Transfer the activated HMC-1 cells or BMMC

Co-culture for 24 h

Extensive wash

HMC-1 cells or BMMC

U937 cells or Peritoneal macrophages

Co-culture
U937 cells or Peritoneal macrophages

PMA A23187 for 2h

Serum-free media with or without anti-IFN-γ antibody for 24 h

HMC-1 cells or BMMC

Extensive wash

Spin

Transfer the conditioned medium, and culture for 24 h

Supplemental Figure VII. Tsuruda, et al.
Supplemental Figure VIII. Tsuruda, et al.

Tranilast

PMA A23187 for 2 h

HMC-1 cells or BMMC

Transfer the activated HMC-1 cells or BMMC

Extensive wash

U937 cells or Peritoneal macrophages

Tranilast

Co-culture for 24 h

Co-culture
1) Tranilast treatment to HMC-1 cells

Tranilast

PMA A23187 for 2 h

Extensive wash

HMC-1 cells

Transfer the conditioned medium, culture the U937 for 24 h

HMC-1 cells

U937 cells

2) Tranilast treatment to U937 cells

PMA A23187 for 2 h

Extensive wash

HMC-1 cells

Transfer the conditioned medium, culture the U937 for 24 h

HMC-1 cells

U937 cells
Supplemental Table I. Tsuruda et al.

<table>
<thead>
<tr>
<th>Patients’ Characteristics</th>
<th>Control</th>
<th>Atherosclerosis</th>
<th>AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Early</td>
<td>Advanced</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Ages (years)</td>
<td>44±5</td>
<td>49±5</td>
<td>71±3**</td>
</tr>
<tr>
<td>Male (%)</td>
<td>82</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>14</td>
<td>8</td>
<td>44*†</td>
</tr>
<tr>
<td>Diabetes Mellitus (%)</td>
<td>18</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>36</td>
<td>17</td>
<td>52</td>
</tr>
</tbody>
</table>

Patients’ Characteristics
Data are expressed as means ± SEM or percentages. *p<0.05, **p<0.01 vs. control, †p<0.05, ††p<0.01 vs. early stage atherosclerosis and ##p<0.01 vs. advanced atherosclerosis.
<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Gender</th>
<th>Non-aneurysmal site</th>
<th>Aneurysmal site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mast cell density (per mm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-ruptured cases</td>
<td>Ruptured cases</td>
</tr>
<tr>
<td>1</td>
<td>79</td>
<td>M</td>
<td>1.9</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>F</td>
<td>1.3</td>
<td>2.1</td>
</tr>
<tr>
<td>1</td>
<td>73</td>
<td>M</td>
<td>2.3</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>M</td>
<td>1.3</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>M</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>M</td>
<td>N.D.</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>M</td>
<td>N.D.</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>76</td>
<td>M</td>
<td>1.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**Mast Cell Density in Non-Aneurysmal or Aneurysmal Aortas from Autopsy Cases with AAA**

Mast cells were stained with monoclonal anti-human tryptase antibody as described in the Method section, and the cell numbers of at least 10 fields were counted in the non-aneurysmal atherosclerotic aorta and in the aneurysm site under the microscope (x400 magnification). The mean density of mast cells at the aneurysmal sites was 4.3 per mm², a number two- to four- fold greater than that of the non-dilated sites in six cases. N.D. indicates “not determined”.