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Critical Role of Mast Cell Chymase in Mouse Abdominal Aortic Aneurysm Formation

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Background—Mast cell chymase may participate in the pathogenesis of human abdominal aortic aneurysm (AAA), yet a direct contribution of this serine protease to AAA formation remains unknown.

Methods and Results—Human AAA lesions had high numbers of chymase-immunoreactive mast cells. Serum chymase level correlated with AAA growth rate ($P=0.009$) in a prospective clinical study. In experimental AAA produced by aortic elastase perfusion in wild-type (WT) mice or those deficient in the chymase ortholog mouse mast cell protease-4 (mMCP-4) or deficient in mMCP-5 ($Mcpt4^{-/-}$, $Mcpt5^{-/-}$), $Mcpt4^{-/-}$ but not $Mcpt5^{-/-}$ had reduced AAA formation 14 days after elastase perfusion. Even 8 weeks after perfusion, aortic expansion in $Mcpt4^{-/-}$ mice fell by 50% compared with that of the WT mice ($P=0.0003$). AAA lesions in $Mcpt4^{-/-}$ mice had fewer inflammatory cells and less apoptosis, angiogenesis, and elastin fragmentation than those of WT mice. Although $Kit^{W-sh/W-sh}$ mice had protection from AAA formation, reconstitution with mast cells from WT mice, but not those from $Mcpt4^{-/-}$ mice, partially restored the AAA phenotype. Mechanistic studies suggested that mMCP-4 regulates expression and activation of cysteine protease cathepsins, elastin degradation, angiogenesis, and vascular cell apoptosis.

Conclusions—High chymase-positive mast cell content in human AAA lesions, greatly reduced AAA formation in $Mcpt4^{-/-}$ mice, and significant correlation of serum chymase levels with human AAA expansion rate suggests participation of mast cell chymase in the progression of human and mouse AAA. (*Circulation*. 2009;120:973-982.)

Key Words: aneurysm ■ animal model ■ chymase ■ mast cells

Human chymases, which are mast cell-restricted serine proteases, can activate matrix metalloproteinases (MMPs),¹ produce angiotensin II,² induce endothelial cell (EC) and smooth muscle cell (SMC) apoptosis,^{3,4} and degrade constituents of high-density lipoprotein particles, thereby impairing their cholesterol efflux ability.⁵ Therefore, mast cell chymases may contribute to arterial remodeling in atherosclerosis and abdominal aortic aneurysm (AAA) formation. AAA involves extensive arterial extracellular matrix degradation, aortic cell apoptosis, and microvessel accumulation.⁶ To date, we lack a clinically useful soluble biomarker for AAA, and invasive repair is the only treatment for this life-threatening human disease.⁷ Although a direct link between chymase and AAA remains conjectural, extracts from human aneurysmal aortas have significantly higher mast cell chymase-associated

angiotensin II-forming activity than those from control aortas,⁸ consistent with the observation of high numbers of chymase-positive mast cells in the adventitia and media of aneurysmal aortas.⁹ We recently established an important role of mast cells in AAA in 2 mouse preparations.¹⁰ Although the role of mast cell-derived chymases in the pathogenesis of AAA remains untested, mast cell-deficient $Kit^{W-sh/W-sh}$ mice had greatly attenuated AAA formation after aortic elastase perfusion and periaortic $CaCl_2$ injury compared with wild-type (WT) control mice. Inhibition of mast cell chymase activities with a small-molecule inhibitor, NK3201, reduced elastase perfusion-induced AAA formation in dogs, hamsters, and mice.¹¹⁻¹³ Reduced AAA formation occurs with lower MMP-9 activity and mast cell numbers in the aortas. These data suggested that mast cell chymases contribute

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importantly to AAA formation, although detailed mechanisms remain unclear, and this inhibitor could have pertinent nonspecific effects.

Clinical Perspective on p 982

Protease-deficient animals permit direct testing of the functions of individual enzymes in pathological processes, including AAA.^{14,15} Unlike human chymase, the product of a solitary gene belonging to the α -chymase family, mice have a chymase family consisting of mouse mast cell proteases (mMCP)-1, -2, -4, -5, and -9. The α -chymases mMCP-1 and -2 are expressed mainly in mucosal mast cells, whereas the α -chymase mMCP-9 is expressed mainly in the uterus. In contrast, the connective tissue mast cells in most sites express the β -chymase mMCP-4 and the α -chymase mMCP-5. mMCP-4 appears to be the predominant mast cell chymase within mouse connective tissues.¹⁶ Therefore, mMCP-4 may be the most relevant human chymase ortholog in arterial remodeling. This study used mMCP-4-deficient (*Mcpt4*^{-/-}) and mMCP-5-deficient (*Mcpt5*^{-/-}) mice to test the hypothesis that these connective tissue chymases impair AAA formation directly in mice with AAA induced by aortic elastase perfusion.

Methods

Human AAA Lesion Sections and Serum Samples

Paraffin-embedded human aortic sections were prepared from 10 AAA donors (5 females and 5 males; mean age, 78.80±2.05 years) and 10 non-AAA heart transplant patients (5 females and 5 males; mean age, 41.90±4.19 years) without detectable vascular diseases from the Department of Surgery, Washington University, St Louis, Mo. These sections helped to detect chymase expression with the use of mouse anti-human chymase monoclonal antibody (Abcam). Human aortic tissue extracts were prepared from 3 female AAA patients and 3 female heart transplant donors with no detectable vascular disease from the Department of Medicine, Brigham and Women's Hospital, Boston, Mass. Tissue lysates were used for immunoblot analysis (30 μ g per lane) with the same antibody. The same blot was reprobed for β -actin to affirm equal protein loading. Separate human protocols were preapproved by the Human Investigation Review Committees at Washington University and at Brigham and Women's Hospital.

To establish the correlation of AAA expansion rates to serum chymase levels, we developed a human chymase enzyme-linked immunosorbent assay system. Briefly, mouse anti-human chymase antibody (AbD, Serotec) was used to coat a 96-well plate. Serum samples were diluted (1:100) and incubated on a precoated plate for 2 hours at room temperature. Biotinylated mouse anti-human chymase monoclonal antibody (Millipore) was used as detecting antibody. Detailed antibody information is listed in Table I in the online-only Data Supplement.

Serum samples from 103 male AAA patients were identified by a previously described population-based screening of 65- to 73-year-old men at the Department of Vascular Surgery, Viborg Hospital, Viborg, Denmark.¹⁷ A total of 4404 men were invited to participate in this follow-up study. Of 3344 participants, 141 (4.2%) had AAA, defined as an infrarenal aortic diameter of ≥ 30 mm. Of those with AAA, 19 had diameters >50 mm who were referred for surgery, and 10 were lost to follow-up. A total of 112 were followed for >1 year (on average, for 2.9 years). Of 112 cases, 103 had serum chymase measured blindly and data presented. All participants gave informed consent. Detailed patient information was reported previously.¹⁷

Mouse AAA Model and Lesion Characterization

C57BL/6 WT mice were purchased from The Jackson Laboratory (Bar Harbor, Me). *Mcpt4*^{-/-} and *Mcpt5*^{-/-} C57BL/6 mice have been backcrossed to the same background for >10 and 4 generations,

respectively.^{18,19} Ten-week-old mice from each type were used for aortic elastase perfusion-induced AAA.¹⁴ Aneurysmal lesions were collected 7, 14, and 56 days after elastase perfusion. Mouse aortic diameters were measured before and after elastase perfusion and at the end of each time point. Aortic diameter expansion $\geq 100\%$ of that before perfusion defined AAA, according to Pyo et al.¹⁴ Each mouse aorta was isolated for both frozen section preparation and tissue protein extraction in a pH 5.5 buffer.¹⁷ Frozen sections were used for immunostaining for macrophages (Mac-3), SMCs (α -actin), T cells (CD3), apoptotic cells (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]), microvessels (CD31), chemokine (monocyte chemoattractant protein-1 [MCP-1]), mast cells (c-Kit, CD117), and elastin degradation (Verhoeff-van Gieson) (Table I in the online-only Data Supplement). SMC content and elastin fragmentation were graded as described previously.¹⁷ T cells, apoptotic cells, mast cells, MCP-1-positive cells, and microvessel numbers were counted blindly. Macrophage-positive areas were measured with the use of computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, Md).

Bone Marrow-Derived Mast Cell Preparation and *KitW-sh/W-sh* Reconstitution

Bone marrow-derived mast cells (BMMCs) were prepared and verified as described previously.²⁰ For *KitW-sh/W-sh* mice reconstitution, recipient mice at 5 weeks of age were given 1×10^7 of BMMCs from WT or *Mcpt4*^{-/-} mice via the tail vein. Five weeks after BMMC reconstitution, mice were introduced to the AAA model. Mouse abdominal aortas were harvested 14 and 56 days after elastase perfusion.

BMMC In Vitro Activity Assays

An aortic ring angiogenesis assay was performed by incubating Matrigel-embedded WT mouse aortic rings in a 96-well plate with live BMMCs from WT and *Mcpt4*^{-/-} mice (3×10^5 cells per 150 μ L RPMI-1640 with 10% fetal bovine serum per well) for 7 to 10 days. EC growth areas were determined with the use of Image-Pro Plus software and presented as square millimeters. Vascular endothelial growth factor (VEGF) (10 ng/mL; PeproTech) was used as a positive control.

Real-time polymerase chain reaction (RT-PCR) was used to determine protease mRNA levels in BMMCs. Total cellular RNA was extracted from BMMCs with the use of TRIzol reagent (GIBCO) and treated with RNase-free DNase (Ambion) to remove genomic DNA contaminants. Equal amounts of RNA were reverse-transcribed, and quantitative PCR was assessed in a single-color RT-PCR detection system (Stratagene). The level of each protease transcript was normalized to that of the β -actin transcript.

BMMC and aortic tissue extract cysteinyl cathepsin activities were examined with biotin-conjugated JPM, an affinity probe that labels active cathepsins specifically and irreversibly.¹⁷ Briefly, BMMC and pulverized aortic tissues were lysed into a pH 5.5 buffer. Five micrograms of protein from each sample was incubated with 12 mmol/L dithiothreitol and 1 μ L of biotin-conjugated JPM in 30 μ L of a pH 5.5 buffer for 1 hour at 37°C. Protein samples were then separated on a 12% SDS-PAGE followed by immunoblot detection with horseradish peroxidase-conjugated avidin (Table I in the online-only Data Supplement). BMMC lysate MMP activity was detected with a gelatin gel zymogram, essentially the same as reported previously.²¹

BMMC activity in promoting vascular SMC apoptosis was performed with the use of primary cultured mouse aortic SMC on an 8-well chamber slide.¹⁰ Confluent SMCs were stimulated to apoptosis overnight with 80 μ mol/L pyrrolidinedithiocarbamate with and without 300 μ L of degranulated BMMC supernatant in Dulbecco's modified Eagle's medium (1×10^6 BMMC supernatant/mL) from WT or *Mcpt4*^{-/-} mice as described.²² Apoptotic cells were detected with an In Situ Cell Death Detection Kit according to the instructions (Roche Diagnostics Co).

BMMC elastase activities were determined by mixing 100 μ g of BMMC extract with 20 μ g of fluorogenic elastin (DQ-elastin, Molecular Probes, Carlsbad, Calif) in 100 μ L of a pH 5.5 buffer on

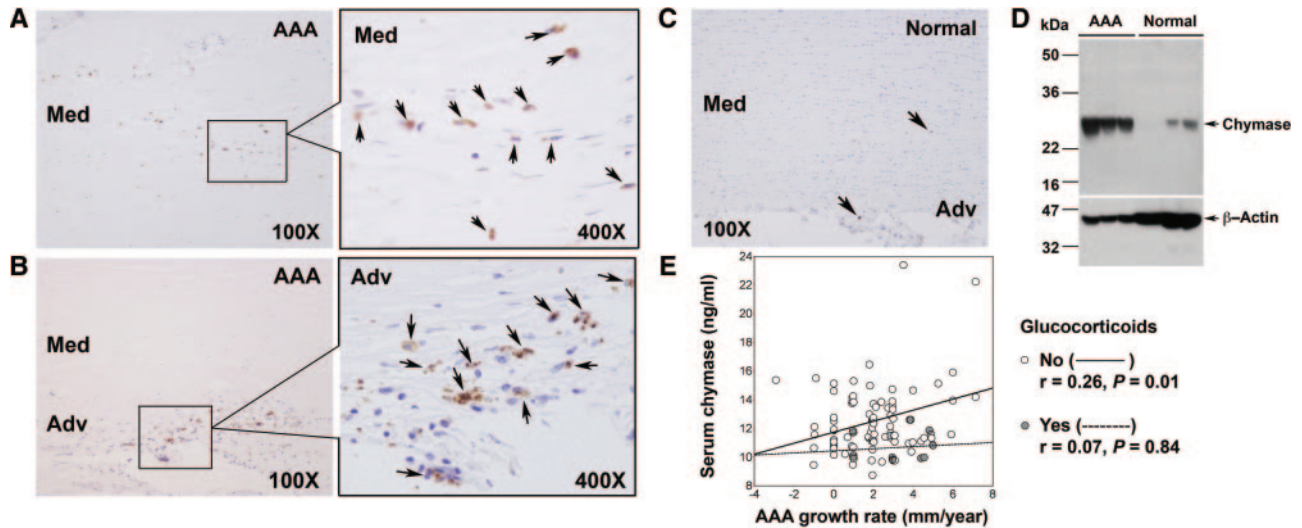


Figure 1. Chymase expression in human AAA lesions. A, Human AAA lesion chymase immunostaining demonstrated chymase-positive mast cells in the media (Med). B, Human AAA lesion chymase immunostaining demonstrated chymase-positive mast cells in the adventitia (Adv). C, Many fewer chymase-positive mast cells were seen in media or adventitia from aortas of heart transplant donors who had no detectable vascular diseases. Arrows indicate chymase-positive mast cells. Purified mouse IgG was used as immunostaining negative controls (not shown). D, Human aortic tissue chymase Western blot analysis. β -Actin immunoblot was used for protein loading control. E, Correlation of serum chymase and AAA growth rate among patients with (filled circles) or without (empty circles) glucocorticoid treatments (Pearson correlation test). $P < 0.05$ was considered statistically significant.

a 96-well plate for 5 hours. The plate was read at excitation/emission=505/515 nm, and data were presented as relative fluorescence units.

Statistical Analysis

The annual growth rate of human AAA during the first year after serum sampling was calculated as the difference between maximal anterior-posterior AAA diameter divided by the observation time. The crude correlation between serum chymase, AAA size, and growth rate was calculated with Pearson correlation test. Multivariate linear regression analysis was performed to adjust for the subjects' use of glucocorticoids, smoking, low-dose aspirin, angiotensin-converting enzyme inhibitor, β -adrenergic blocker, β -adrenergic agonist, lowest ankle-brachial blood pressure index, diastolic blood pressure, and age.

Because of small sample size and abnormal data distribution, a Mann-Whitney U test was used for data from mouse experiments throughout the study. P values < 0.05 were considered statistically significant.

Results

Increased Chymase Expression in Human AAA Lesions

Human AAA lesions had many more chymase-immunoreactive mast cells than did healthy aortas. Chymase-positive mast cells localized throughout the AAA lesions from media to adventitia (Figure 1A and 1B). In contrast, the media and adventitia of human aortas without AAA had many fewer chymase-positive mast cells (Figure 1C). Consistent with this observation, immunoblot analysis of aortic tissue extracts showed 11-fold higher levels of chymase proteins by densitometry in AAA than in normal aortas after the protein loading for β -actin signals was normalized (ImageJ) (Figure 1D).

Correlation of Serum Chymase Level With AAA Growth Rate

In this serological prospective study of 103 AAA patients who had serum chymase measured, Pearson correlation test

did not reveal significant associations between chymase and initial AAA size ($r=0.02$, $P=0.833$). However, serum chymase levels correlated modestly with AAA growth rate ($r=0.19$, $P=0.06$). Because of the various medications used by these patients, we performed a multivariate linear regression analysis by adjusting for potentially known confounders of AAA, including smoking, low-dose aspirin, use of angiotensin-converting enzyme inhibitors, β -adrenergic blocking agents, β -adrenergic agonists, or corticosteroids, ankle-brachial index, diastolic blood pressure, and age. Among these confounders, serum chymase and glucocorticoid use correlated strongly and significantly with AAA growth rate after the adjustment ($P=0.009$) (Table). Although we do not know the cause, glucocorticoid users had higher AAA growth rate (3.54 ± 0.49 versus 1.97 ± 0.19 mm/y; $P=0.01$) but lower serum chymase levels (10.77 ± 0.31 versus 12.34 ± 0.24 ng/mL; $P < 0.04$) than nonglucocorticoid users. Therefore, chymase expression alone may not explain the glucocorticoid effects on AAA growth. Other unrecognized mechanisms may play more important roles. After those receiving glucocorticoid treatments were excluded ($n=10$), the modest correlation between serum chymase and AAA growth rate became significant ($r=0.26$, $P=0.01$), although we did not see any correlation among the glucocorticoid users ($r=0.07$, $P=0.84$) (Figure 1E).

Reduced AAA Formation in mMCP-4-Deficient Mice

Increased chymase-positive mast cells in human AAA lesions and significant association of serum chymase levels with AAA growth rate suggested the involvement of chymases in AAA development. To test this hypothesis directly, we performed elastase aortic perfusion to induce AAA in *Mcpt4*^{-/-} and *Mcpt5*^{-/-} mice.¹⁴ Analysis at 7 and 14 days after perfusion did not show significant differences in aortic expansion between WT and *Mcpt5*^{-/-} mice (data not shown).

Table. Correlation Coefficient Between AAA Growth Rate and Different Confounders*

Variables	Mean±SEM (Range) or No. of Patients	Standardized Coefficient (β)†	<i>t</i>	<i>P</i> ‡
Chymase, ng/mL	12.18±2.26 (8.76-23.40)	0.267	2.658	0.009
Initial AAA size, mm	33.69±4.14 (30.00-44.00)	0.198	1.957	0.054
Current smoking	62/103	0.121	1.187	0.238
Low-dose aspirin	46/103	-0.058	-0.565	0.574
ACE inhibitor	11/103	0.050	0.480	0.632
β -Blockage	16/103	-0.105	-1.017	0.312
β -Agonist	13/103	-0.091	-0.822	0.413
Steroid	10/103	0.299	2.656	0.009
Ankle-brachial index	1.00±0.22 (0.38-1.61)	-0.048	-0.474	0.636
Diastolic blood pressure, mm Hg	92.70±13.27 (65.00-130.00)	0.048	0.487	0.627
Age, y	68.00±2.95 (64.30-73.70)	0.080	0.818	0.416

ACE indicates angiotensin-converting enzyme.

*All 103 patients are male.

†Multivariate linear regression analysis.

‡ $P < 0.05$ was considered statistically significant.

In contrast, whereas all WT mice developed AAA 14 days after elastase perfusion (100% incidence), none of the *Mcpt4*^{-/-} mice did (0% incidence) ($P < 0.0001$). Analysis 56 days after perfusion still showed significant attenuation of AAA expansion ($P = 0.0003$) and lower incidences (69% versus 100%) in *Mcpt4*^{-/-} mice compared with WT mice (Figure 2A). Inflammatory cell accumulation, including Mac-3⁺ macrophages (Figure 2B) and CD3⁺ T cells (Figure 2C), also diminished in *Mcpt4*^{-/-} mice relative to WT control mice at the 14-day or 56-day time point. In contrast, aortas from *Mcpt4*^{-/-} mice had more SMCs than those from WT mice 7 days after perfusion (Figure 2D), although such differences diminished at later time points.

Apoptosis, angiogenesis, and elastin loss characterize human AAA.⁶ To examine whether reduced AAA in *Mcpt4*^{-/-} mice also impaired these variables, we performed TUNEL (apoptosis), CD31 (angiogenesis), and Verhoeff-van Gieson (elastin) staining with frozen aortic sections prepared from WT and *Mcpt4*^{-/-} mice from different time points. As we anticipated, the numbers of both apoptotic cells and CD31⁺ microvessels as well as the degree of elastin fragmentation fell significantly in AAA lesions from *Mcpt4*^{-/-} mice compared with those from WT control mice at most of the time points examined, although not at all time points (Figure 2E through 2G), suggesting that multiple variables contributed to reduced AAA in *Mcpt4*^{-/-} mice and that chymase expression affected these variables differently at assorted time points. Reduced AAA in *Mcpt4*^{-/-} mice did not result from altered accumulation of mast cells in AAA lesions. Lesion MCP-1⁺ or CD117⁺ cell content did not differ between the 2 groups (data not shown).

Mast Cell Reconstitution and AAA Formation in Mast Cell-Deficient Mice

Reduced AAA in *Mcpt4*^{-/-} mice might not result solely from the lack of mMCP-4 because aside from mast cells, mMCP-4 deficiency may affect other cell types, which could contribute indirectly to AAA formation. To examine this hypothesis, we reconstituted mast cell-deficient *Kit*^{W-sh/W-sh} mice with BM-

MCs from WT or *Mcpt4*^{-/-} mice. Consistent with our earlier observation, *Kit*^{W-sh/W-sh} mice failed to develop AAA,¹⁰ and provision of WT BMMCs restored AAA expansion at both the 14- and 56-day time points. In contrast, those that received BMMCs from *Mcpt4*^{-/-} mice remained protected from aortic expansion at both time points (Figure 3A).

In addition to measuring aortic diameters for those BMMC-reconstituted mice, we also determined their lesion macrophage and T cell content, CD31⁺ microvessel number, total apoptotic cell number, and elastin fragmentation. WT BMMC-reconstituted *Kit*^{W-sh/W-sh} mice restored at least partially these variables at both time points. In contrast, most variables did not differ significantly between *Kit*^{W-sh/W-sh} mice and those that received *Mcpt4*^{-/-} BMMCs at either time point (Figure 3B through 3F), supporting the hypothesis that reduced AAA in *Mcpt4*^{-/-} mice resulted from the absence of mMCP-4 from mast cells.

Chymase mMCP-4 Stimulates

Microvascularization and Vascular Cell Apoptosis

Microvascularization and cell death occur in human AAA. Impaired angiogenesis and apoptosis in *Mcpt4*^{-/-} mice (Figure 2F and 2G) or in *Kit*^{W-sh/W-sh} mice that received *Mcpt4*^{-/-} BMMCs (Figure 3D and 3F) could result from reduced AAA formation in these mice. To examine whether mMCP-4 participated directly in angiogenesis and vascular cell apoptosis, we performed aortic ring microvessel outgrowth and SMC apoptosis assays with the use of BMMCs from WT and *Mcpt4*^{-/-} mice. Relative to WT BMMCs, those lacking mMCP-4 showed greatly reduced activity in promoting aortic ring microvessel outgrowth (Figure 4A). Earlier studies suggested that mast cells contribute to microvessel growth via VEGF²³ but less so by releasing inflammatory cytokines interleukin-6 (IL-6), interferon- γ , and tumor necrosis factor- α .¹⁰ To test whether mMCP-4 deficiency affects mast cell VEGF expression, we performed a VEGF enzyme-linked immunosorbent assay on supernatants from degranulated mast cells and lysates from WT and *Mcpt4*^{-/-} BMMCs and found no significant differences (data not shown). We have previously

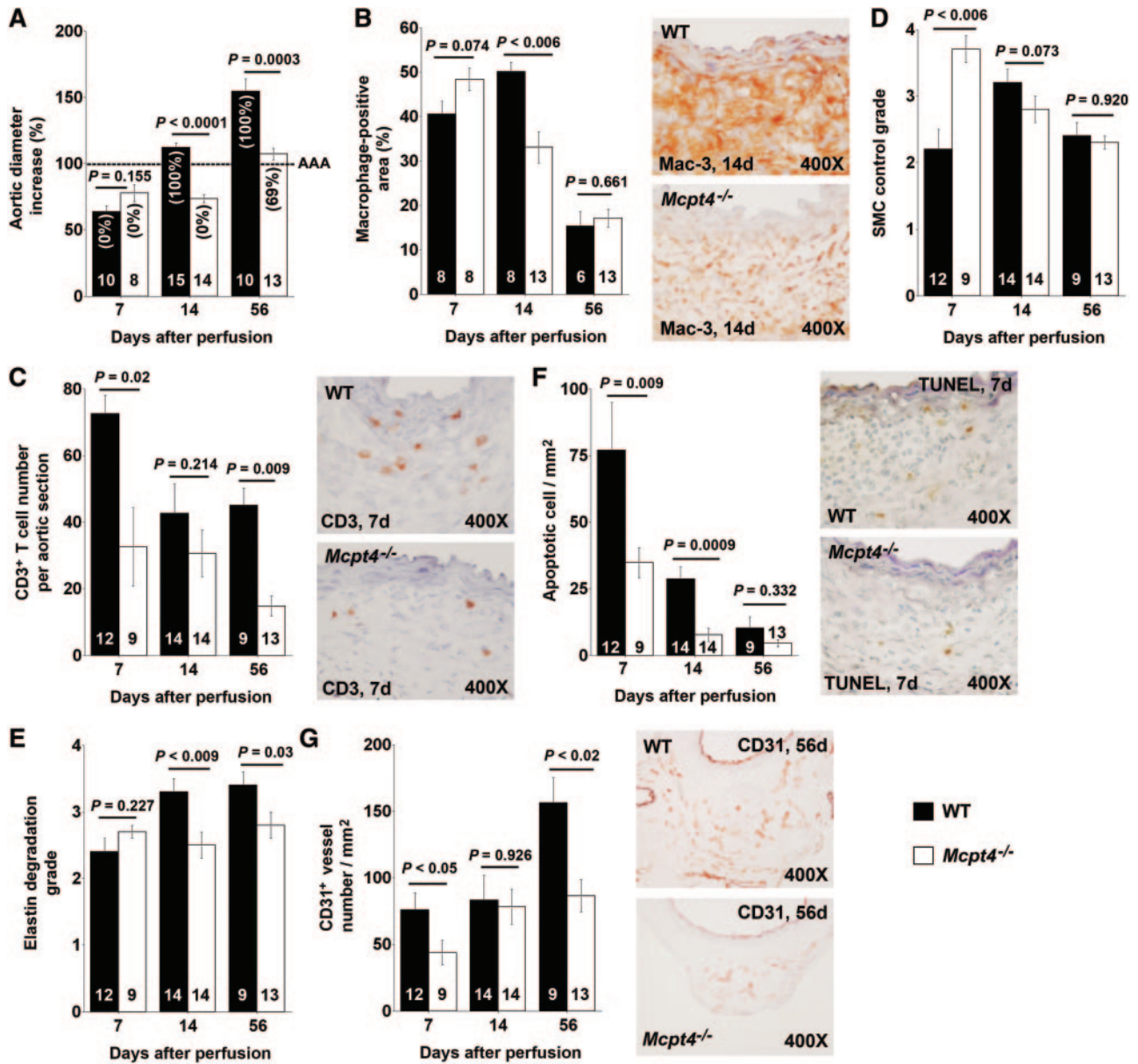


Figure 2. WT and *Mcpt4*^{-/-} mouse AAA lesion characterizations. Lesion aortic diameter changes (A), Mac-3⁺ macrophage area percentage (B), CD3⁺ T cell number (C), SMC content in grades (D), elastin degradation in grades (E), apoptotic cells (F), and CD31⁺ microvessel numbers (G) between WT and *Mcpt4*^{-/-} mice at 3 time points are shown. Representative photographs for panels B, C, F, and G are presented to the right. Number of animals used for each experiment is indicated in each bar. Purified rat IgG was used as negative control (not shown). All data are mean ± SEM. *P* < 0.05 was considered statistically significant (nonparametric Mann-Whitney test).

shown that WT BMMCs promote vascular SMC apoptosis.¹⁰ *Mcpt4*^{-/-} BMMCs conferred significant protection to SMCs from pyrrolidinedithiocarbamate-induced apoptosis (Figure 4B), suggesting a direct role of mMCP-4 in both microvascularization and apoptosis during the development of experimental mouse AAA.

Deficiency of mMCP-4 Reduces Cysteine Protease Cathepsin Expression in Mast Cells

Angiogenesis and apoptosis both require protease activities. We have shown that the cysteine protease cathepsin S can modulate microvessel growth.^{24,25} MMPs promote angiogenesis by triggering the release of VEGF.²⁶ Cysteiny cathepsins also participate in apoptosis by cleaving the Bcl-2 family

member Bid followed by mitochondria cytochrome *c* release.²⁷ Likewise, MMPs participate in cell death although via different mechanisms.²⁸ Considering reduced angiogenesis and apoptosis in *Mcpt4*^{-/-} mice (Figure 2C and 2F) or in *Mcpt4*^{-/-} BMMC-reconstituted *Kit*^{W-sh/W-sh} mice (Figure 3D and 3F), we hypothesized that absence of mMCP-4 affects cathepsin and/or MMP expression. *Mcpt4*^{-/-} BMMCs had significantly lower mRNA encoding all cathepsins tested, including B, S, K, and L, compared with those in WT BMMCs, as shown by RT-PCR. Although both MMP-2 and -9 mRNAs were also higher in WT BMMCs than in *Mcpt4*^{-/-} BMMCs, their RNA levels remained low in WT BMMCs (Figure 4C). MMPs in *Mcpt4*^{-/-} BMMCs were undetectable by gelatin zymography (data not shown). To document

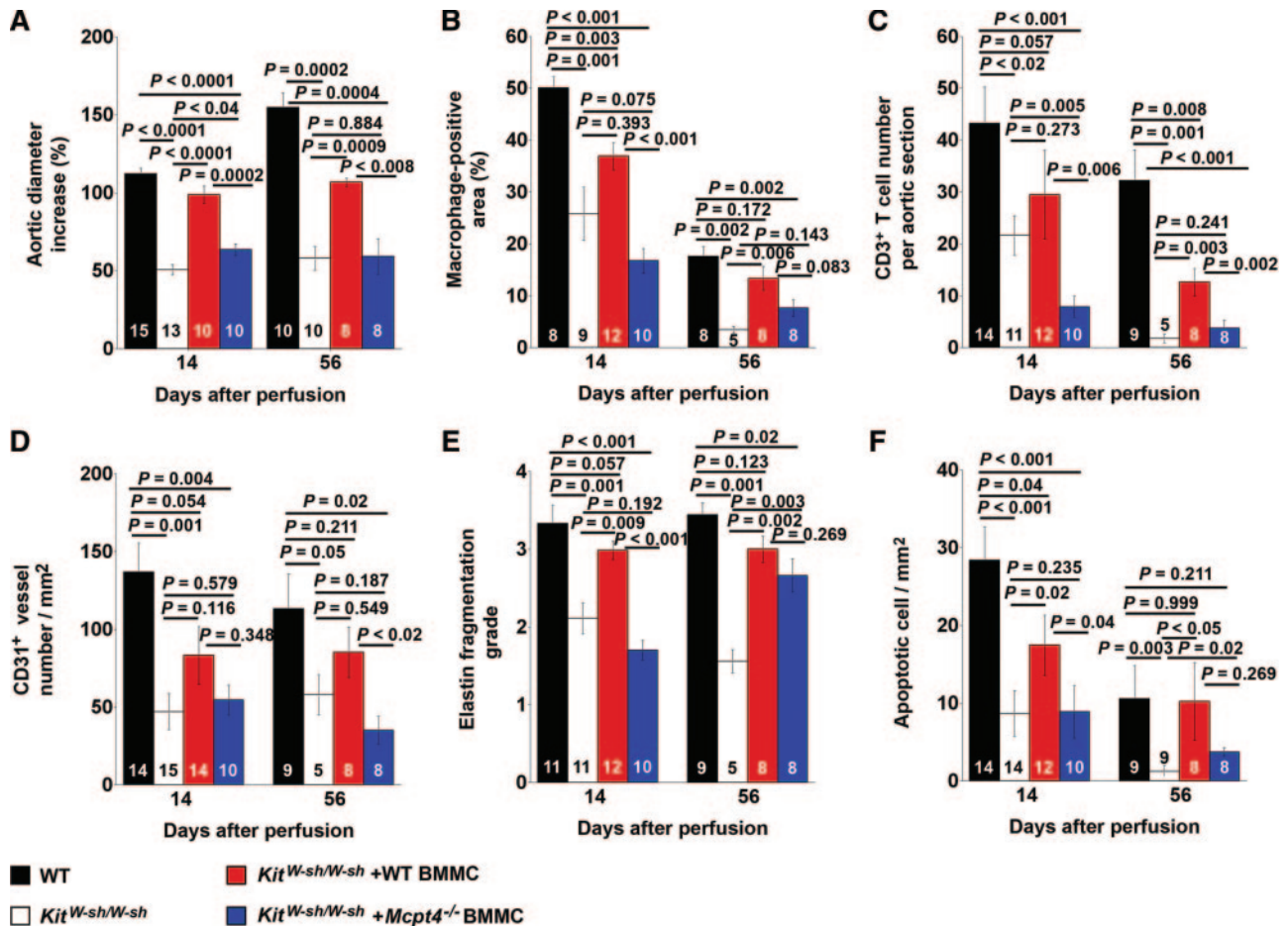


Figure 3. *Kit^{W-sh/W-sh}* mice reconstitution and lesion characterization. Reconstitution of *Kit^{W-sh/W-sh}* mice with WT but not *Mcpt4*^{-/-} BMMCs restored partially the aortic expansion (A), lesion content of macrophages (B), CD3⁺ T cell number (C), CD31⁺ microvessel number (D), level of aortic wall elastin degradation (E), and number of apoptotic cells (F) at both 14-day and 56-day time points. The number of mice for each experimental group is indicated in the bar. All data are mean±SEM. *P*<0.05 was considered statistically significant (nonparametric Mann–Whitney test).

further decreased cathepsin activities in *Mcpt4*^{-/-} BMMCs, we performed cysteine protease active site labeling with biotin-JPM. *Mcpt4*^{-/-} BMMCs showed reduced overall cathepsin activities relative to WT BMMCs (Figure 4D). Interestingly, we detected high levels of cathepsin L mRNA but low levels of its active enzyme in WT BMMCs (Figure 4C and 4D), suggesting substantial regulation at the translation and/or activation levels. Assay of elastin degradation in vitro supported this observation. An equal amount of cell lysate from *Mcpt4*^{-/-} BMMCs degraded significantly less elastin than those from WT BMMCs under conditions optimized for cysteine protease cathepsins²⁹ (Figure 4E), suggesting that absence of mMCP-4 not only affects mast cell cathepsin mRNAs but also their activities.

mMCP-4 Induces Aortic SMC Cathepsin Activities

Mast cells induce vascular cell cathepsin expression via inflammatory cytokines, and these cathepsins may in turn participate in aortic wall remodeling.²⁰ We tested whether *Mcpt4*^{-/-} BMMCs demonstrate impaired ability to induce vascular cell cathepsin expression. Culture of aortic SMCs with BMMCs showed that WT BMMCs induced the activi-

ties of all major cysteinyl cathepsins to a much greater extent than *Mcpt4*^{-/-} BMMCs. *Mcpt4*^{-/-} BMMC-treated SMCs had reduced cathepsin B activity and negligible cathepsin S/K and L/C activities compared with SMCs treated with WT BMMCs (Figure 4F). Thus, the absence of mMCP-4 in mast cells not only altered mast cell cathepsin expression and activities but also decreased the ability of mast cells to induce cathepsin activity in vascular SMCs by an unknown mechanism.

Deficiency of mMCP-4 Reduces Aortic Wall Cathepsin Activities

Reduced cathepsin activities in *Mcpt4*^{-/-} BMMCs and in *Mcpt4*^{-/-} BMMC-treated SMCs suggest that reduced development of AAA in *Mcpt4*^{-/-} mice resulted at least in part from reduced cathepsin activities in the vessel wall. We performed cathepsin active site labeling by incubating biotin-JPM with aortic tissue extracts from WT and *Mcpt4*^{-/-} mice. Consistent with our hypothesis, aortic tissues from *Mcpt4*^{-/-} mice contained much less cathepsin activity, especially cathepsins S/K, than those of WT mice (Figure 5A). In situ elastin zymography yielded similar results. Under conditions optimized for cathepsin activities (pH 5.5 with ethylenediaminetetraacetic acid),²⁹ frozen aortic sections from WT mice

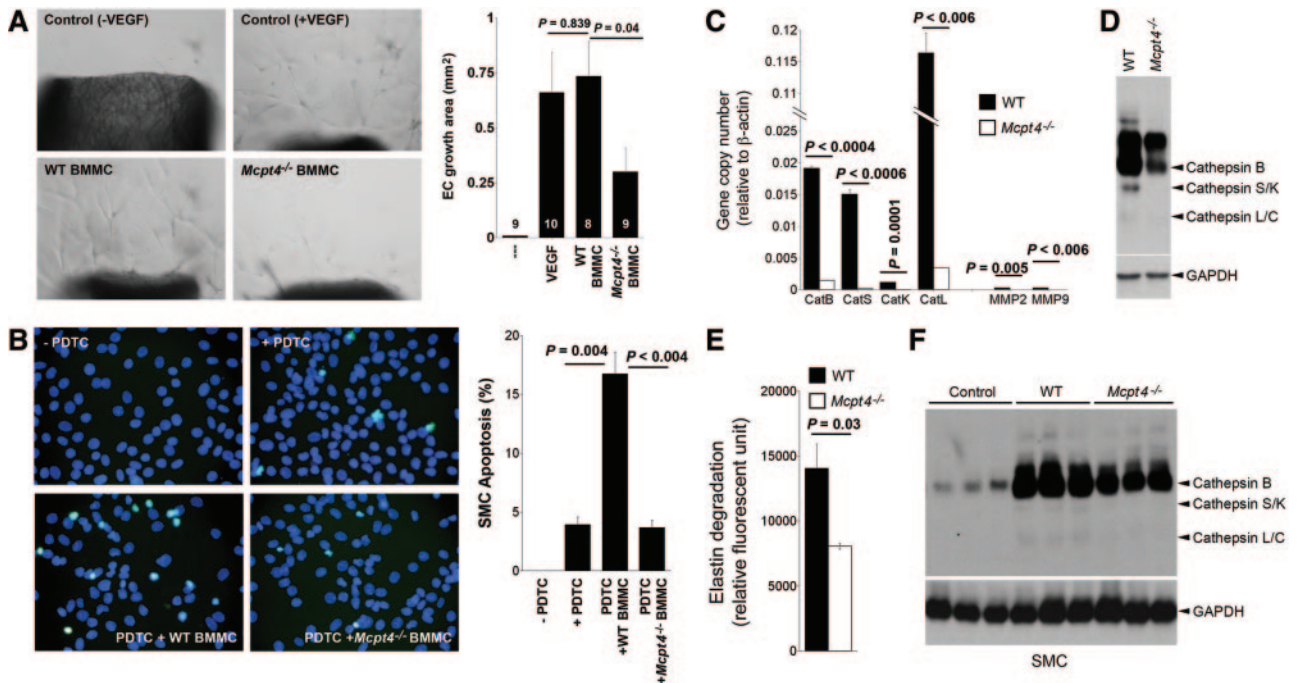


Figure 4. Mast cell activities. A, Mouse aortic ring assay. VEGF was used as positive control. The number of rings for each experiment is shown in the bar. Representative aortic rings are shown in the left panels. B, BMMC-mediated SMC apoptosis. Representative photographs are presented in the left panels. Green fluorescent cells are apoptotic SMCs. PDTC indicates pyrrolidinedithiocarbamate. C, RT-PCR shows reduced cathepsins and MMP expression in *Mcpt4*^{-/-} BMMCs (mean±SEM of 6 independent experiments). D, BMMC cell lysate biotin-JPM labeling (representative of 3 independent experiments). E, Fluorogenic elastin degradation with BMMC cell lysates. Data are mean±SEM of 4 experiments. F, Biotin-JPM labeling of different live BMMC-treated mouse aortic SMC lysates. GAPDH immunoblots were used for protein loading controls in D and F.

(Figure 5B) had greater activity in digesting fluorogenic elastin than those from *Mcpt4*^{-/-} mice (Figure 5C).

Discussion

We have previously shown that mast cells play an important role in mouse AAA formation.¹⁰ These cells produce cytokines, chemokines, and proteases, all of which may participate in the pathogenesis of AAA.⁷⁻⁹ Like macrophages, mast cells produce large quantities of cathepsins B, S, K, L, and C.³⁰ In contrast with macrophages, however, mast cells express specialized proteases, including the serine protease chymases. These proteases can activate MMPs and process angiotensinogen to produce angiotensin II, mediators proven critical in mouse AAA formation.^{14,31} Human AAA lesions contain chymases and their substrates; whether these proteases participate causally in the pathogenesis remains uncer-

tain. This study supports the hypothesis that mast cell chymase participates directly in the pathogenesis of AAA formation by assisting microvessel growth, vascular cell apoptosis, and cysteinyl cathepsin expression and activation. Yet these findings raise unanswered questions.

Preoperative glucocorticoid therapy has been associated with benefit in isolated cases of inflammatory aneurysms.³² Such treatment in patients with AAA increases IL-10 (a generally anti-inflammatory cytokine) and decreases IL-6 (a proinflammatory cytokine) and levels of the inflammatory marker C-reactive protein in serum.^{33,34} In contrast to these beneficial effects, our data showed that patients who received glucocorticoid therapy had a nearly doubled rate of AAA growth (mean±SEM, 3.54±0.49 versus 1.97±0.19 mm/y; P=0.01) compared with patients who did not receive the therapy, although this treatment also significantly reduced the

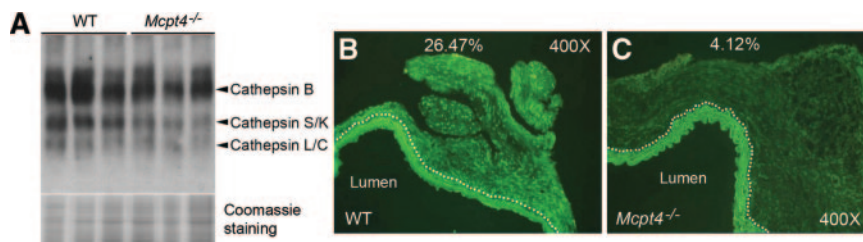


Figure 5. Reduced cathepsin activities in AAA lesions from *Mcpt4*^{-/-} mice. A, JPM labeling of mouse aortic tissue extracts. SDS-PAGE Coomassie staining was used for protein loading control. Arrows indicate active cathepsins. B, WT mouse frozen AAA cross section in situ elastase activity zymograph. C, *Mcpt4*^{-/-} mouse frozen AAA cross section in situ elastase activity zymograph. Lumen and percentage of fluorescence intensity are indicated. Images were obtained with the same magnification and shutter speed, and all data are from the 14-day time point experiments.

serum chymase levels (mean±SEM, 10.77±0.31 versus 12.34±0.24 ng/mL; $P<0.04$). Although this population included only 10 patients receiving glucocorticoids, the data indicate the potential of glucocorticoids as a strong confounder in the analysis of chymase activity and AAA progression and therefore the multivariate analysis included these patients. The positive association between AAA growth and use of glucocorticoids could be confounded by indication. For instance, patients often use glucocorticoids because they have severe pulmonary obstructive disease, which independently is associated with increased aortic expansion rate and rupture risk.³⁵ Alternatively, glucocorticoids can inhibit vascular SMC proliferation³⁶ and extracellular matrix protein synthesis.³⁷ Thus, in glucocorticoid-treated AAA, an unfavorable balance between extracellular matrix degradation versus synthesis may prevail despite the steroid-induced muting of aspects of inflammation, a hypothesis that requires further investigation.

We found that mast cell chymase expression affected the expression of other proteases. Reduced cysteinyl cathepsin expression in *Mcpt4*^{-/-} BMMCs leads to the hypothesis that the mechanism for the reduced AAA in *Mcpt4*^{-/-} mice might result not only from the absence of mMCP-4 but also reduced mast cell cathepsin expression. Although mMCP-4 can activate procollagenase, mMCP-4 itself is not a collagenase or an elastase.³⁸ Chymase mMCP-5 has elastinolytic activity, but lack of mMCP-5 did not impair AAA formation (data not shown). Therefore, neither mMCP-4 nor mMCP-5 alone likely contributes to the massive aortic wall elastinolysis during AAA formation. In contrast, cathepsins S, K, L, and C are potent elastases and collagenases, and these secreted cysteine proteases contributed to AAA formation¹⁵ (G. Shi, DSc, unpublished data, 2009). Absence of mMCP-4 reduced an array of elastinolytic and collagenolytic cathepsins in mast cells, which may account in part for reduced elastinolysis in *Mcpt4*^{-/-} mice (Figures 2E and 3E). Besides these cysteine proteases, mMCP-4 deficiency also affected the expression of cathepsin G, a serine protease present in human AAA capable of elastin degradation and angiotensin II generation.^{39–41} BMMCs from *Mcpt4*^{-/-} mice had significantly lower levels of cathepsin G transcripts than those in WT BMMCs after normalizing to β -actin transcripts (mean±SEM, 0.013±0.0005 versus 0.0006±0.0003; $P<0.00005$), as shown by RT-PCR. Thus, reduced mast cell cathepsin G expression may also contribute to the observed attenuation in AAA formation in *Mcpt4*^{-/-} mice.

Mast cell chymases promote angiogenesis⁴² and apoptosis.⁴³ However, these in vitro studies employed purified chymases in isolated cells. Direct involvement of chymase in these processes in vivo remains unknown. This study showed results similar to those of prior studies of angiogenesis and apoptosis both in vitro (Figure 4A and 4B) and in vivo (Figure 2F and 2G). Reduced cysteinyl cathepsin expression and activities in *Mcpt4*^{-/-} BMMCs (Figure 4C through 4E) and impaired ability of *Mcpt4*^{-/-} BMMCs to induce SMC cathepsin expression raised the possibility that mast cell chymases participate in arterial remodeling in part by regulating cathepsin activities, a hypothesis that remains to be tested.

One important finding that we currently cannot explain is the impact of mMCP-4 on cathepsin and MMP expression. Our earlier observations from cathepsin S-deficient ECs indicated that lack of cathepsin S did not change the expression or activities of other cathepsins, the cathepsin inhibitor cystatin C, MMP, or tissue inhibitors of MMPs.²⁴ Chymase might participate in cathepsin activation, as it does in the case of pro-MMPs,¹ a hypothesis consistent with the observations of decreased cathepsin activities in the absence of mMCP-4 in both interacting with biotin-conjugated JPM (Figure 4D) and in degrading matrix elastin (Figure 4E). However, other explanations may apply. Mast cells lacking mMCP-4 contained greatly less cathepsin and MMP mRNA than did WT mast cells, suggesting that mMCP-4 regulates transcription and/or RNA-stability factors that control cathepsin expression. A protease that regulates the transcription of other proteases has not been reported, but an indirect pathway is possible. For instance, mast cell tryptase stimulates IL-8 and IL-1 β expression by ECs.⁴⁴ Chymase may act in a similar manner. The resulting inflammatory cytokines can exhibit autocrine and paracrine effects on protease expression.

Although not shown in this study, we found significantly lower transmigration of BMMCs from *Mcpt4*^{-/-} mice through collagen I–precoated filters compared with BMMCs from WT mice, suggesting impaired mast cell recruitment or accumulation to AAA lesions. However, the AAA of *Mcpt4*^{-/-} and WT mice had similar MCP-1 levels and CD117⁺ mast cell numbers (data not shown). These data suggest that mast cell transmigration in vitro requires protease activities but that other factors play more important roles in mast cell accumulation during AAA development, a hypothesis that merits further study. In conclusion, increased mast cell chymase expression in human or mouse AAA lesions directly promotes the pathological progression in part by regulating pertinent protease expression and activities. Inhibition of chymase activities limited experimental AAA formation and may assist in attenuating the progression of this irreversible human aortic disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Mast cells, the primary players in allergic immune responses, have also been implicated in the pathogenesis of many nonallergic human diseases, such as cancer, rheumatoid arthritis, and multiple sclerosis. We have recently shown that these inflammatory cells play detrimental roles in the pathogenesis of both atherosclerosis and abdominal aortic aneurysm (AAA). In mice, absence or inactivation of mast cells prevents the progression of these common vascular diseases, providing the novel concept of controlling these diseases with the use of currently available generic mast cell stabilizers in humans. Mechanistic analysis demonstrated that mast cells release proinflammatory cytokines to induce the expression and secretion of atherosclerosis- and AAA-pertinent cysteine protease cathepsins from vascular smooth muscle cells and endothelial cells. Such cysteinyl cathepsins have been suggested to participate directly in these vascular diseases mainly through their elastase and collagenase activities. Like macrophages, mast cells have reservoirs of proteases. Besides common cysteinyl cathepsins and matrix metalloproteinases, mast cells have the unique serine proteases tryptase and chymase. In this study, we proved the direct involvement of chymase in aortic elastase perfusion-induced mouse AAA. Mast cell chymases have been suggested to participate in vascular remodeling by activating matrix metalloproteinases and generating angiotensin II, critical molecules in the pathogenesis of atherosclerosis and AAA. In addition to these known pathological roles, we demonstrated in this study that mast cell chymases also control cysteinyl cathepsin expression and activation, neovascularization, and aortic smooth muscle cell apoptosis. Reduced AAA formation from mice lacking mast cell chymase and restoration of such reductions with reconstitution of mast cells from wild-type mice but not those of chymase-deficient mice strongly support a direct participation of mast cell chymases in mouse AAA formation. Observations of the AAA inhibitory effects of synthetic small-molecule orally active chymase inhibitor [2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-(3,4-dioxo-1-phenyl-7-(2-pyridyloxy)-2-heptyl)acetamide; NK3201] in several animal AAA models illustrate the possibility of controlling human AAA progression and associated complications in the near future.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Antibodies used in this study.

Antibody name	Application	Supplier	Catalog Number	Final Concentration
mouse anti-human chymase	Immunohistochemistry	AbD Serotec	MCA1930	2 µg/ml
mouse anti-human chymase	Immunoblot	AbD Serotec	MCA1930	2 µg/ml
mouse anti-human chymase	ELISA	AbD Serotec	MCA1930	0.2 µg/ml
Biotin-mouse anti-human chymase	ELISA	Millipore	MAB1254B	0.667 µg/ml
Rat anti-mouse Mac-3	Immunohistochemistry	Pharmingen	553322	0.555 µg/ml
Rat anti-mouse a-actin	Immunohistochemistry	Sigma	F3777	5.6 µg/ml
Rat anti-mouse CD3	Immunohistochemistry	Pharmingen	555273	10 µg/ml
Rat anti-mouse CD31	Immunohistochemistry	Pharmingen	553370	0.333 µg/ml
Hamster anti-mouse MCP-1	Immunohistochemistry	Pharmingen	551217	20 µg/ml
Rat anti-mouse c-Kit (CD117)	Immunohistochemistry	eBioscience	17-1171-83	4 µg/ml
Rat IgG	Immunohistochemistry	Pharmingen	553985	5 µg/ml
Mouse IgG	Immunohistochemistry	DAKO	X0931	0.5 µg/ml
HRP-Avidin	Immunoblot	Sigma	A7419	0.36 µg/ml