Published in final edited form as:
Circulation. 2009 September 15; 120(11): 973–982. doi:10.1161/CIRCULATIONAHA.109.849679.

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

Jiusong Sun, PhD[*], **Jie Zhang, PhD**[*], **Jes S. Lindholt, MD**, **Galina K. Sukhova, PhD**, **Jian Liu, PhD**, **Aina He, MD**, **Magnus Åbrink, PhD**, **Gunnar Pejler, PhD**, **Richard L. Stevens, PhD**, **Robert W. Thompson, MD**, **Terri L. Ennis, BS**, **Michael F. Gurish, PhD**, **Peter Libby, MD**, and **Guo-Ping Shi, DSc**

From the Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115 (J.S., J.Z., G.K.S., J.L., A.H., R.L.S., M.F.G., P.L., G-P.S.); Department of Vascular Surgery, Viborg Hospital, Viborg, Denmark (J.L.); Department of Medical Biochemistry and Microbiology, Uppsala University (M.A.) and Department of Molecular Biosciences, Swedish University of Agricultural Sciences (G.P), Uppsala, Sweden; Department of Surgery, Washington University, St. Louis, MO 63110 (R.W.T., T.L.E.).

Abstract

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 mMCP-4 (mouse mast cell protease-4) or mMCP-5-deficient (*Mcpt4*$^{-/-}$, *Mcpt5*$^{-/-}$), *Mcpt4*$^{-/-}$ but not *Mcpt5*$^{-/-}$ had reduced AAA formation 14 days after elastase perfusion. Even 8 weeks post-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 less inflammatory cells, apoptosis, angiogenesis, and elastin fragmentation than those of WT mice. While *Kit*$^{W\text{-}sh/W\text{-}sh}$ 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 suggest participation of mast cell chymase in the progression of human and mouse AAA.

Keywords

abdominal aortic aneurysm; mast cell; chymase; mMCP-4; mMCP-5

Corresponding author: Guo-Ping Shi, D.Sc., Cardiovascular Medicine, Brigham and Women's Hospital, NRB-7, 77 Avenue Louis Pasteur, Boston, MA 02115, Tel.: 617-525-4358, Fax: 617-525-4380, Email: gshi@rics.bwh.harvard.edu.
[*]These authors contributed equally to this study.

DISCLOSURES
None

INTRODUCTION

Human chymases, mast cell serine proteases, can activate matrix metalloproteinases (MMP), [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 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 aortae have significantly higher mast cell chymase-associated angiotensin II-forming activity than those from control aortae,[8] consistent with the observation of high numbers of chymase-positive mast cells in the adventitia and media of aneurysmal aortae.[9] We recently established an important role of mast cells in AAA in two mouse preparations.[10] Although the role of mast cell-derived chymases in the pathogenesis of AAA remains untested, mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice had greatly attenuated AAA formation after either aortic elastase perfusion and periaortic $CaCl_2$ injury compared with those of 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 occurs with lower MMP-9 activity and mast cell numbers in the aortae. These data suggested that mast cell chymases contribute importantly to AAA formation, although detailed mechanisms remain unclear and this inhibitor could have pertinent non-specific effects.

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 mainly expressed in mucosal mast cells and mMCP-9 in the uterus, while connective tissue mast cells express the β-chymase mMCP-4 and the α-chymase mMCP-5, and 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 in St. Louis. These sections helped detect chymase expression using 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. Tissue lysates were used for immunoblot analysis (30 μg/lane) with the same antibody. The same blot was reprobed for β-actin to affirm equal protein loading. Separate human protocols were pre-approved by the Human Investigation Review Committees at Washington University in St. Louis and at Brigham and Women's Hospital.

To establish the correlation of AAA expansion rates to serum chymase levels, we developed a human chymase ELISA 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 pre-coated plate 2 hours at room temperature. Biotinylated mouse anti-human chymase monoclonal antibody (Millipore) was used as detecting antibody. Detailed antibody information is listed in Supplementary Table 1.

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 4,404 men were invited to participate in this follow-up study. Of 3,344 participants, 141 (4.2%) had AAA, defined as an infrarenal aortic diameter of 30 mm or more. 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 Laboratories (Bar Harbor, ME). *Mcpt4*$^{-/-}$ and *Mcpt5*$^{-/-}$ 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 ≥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 pH5.5 buffer.[17] Frozen sections were used for immunostaining for macrophages (Mac-3), SMC (α-actin), T cells (CD3), apoptotic cells (TUNEL), microvessels (CD31), chemokine (monocyte chemotactic protein-1, MCP-1), mast cells (c-Kit, CD117), and elastin degradation (Verhoeff-van Gieson) (Supplementary Table 1). 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 using computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD).

Bone marrow-derived mast cell preparation and *Kit*$^{W\text{-}sh/W\text{-}sh}$ reconstitution

Bone marrow-derived mast cells (BMMC) were prepared and verified as described previously.[20] For *Kit* $^{W\text{-}sh/W\text{-}sh}$ *mice* reconstitution, recipient mice at 5 weeks of age were given 1×10^7 of BMMC from WT or *Mcpt4*$^{-/-}$ mice via the tail vein. Five weeks after BMMC reconstitution, mice were introduced to the AAA model. Mouse abdominal aortae 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 BMMC from WT and *Mcpt4*$^{-/-}$ mice (3×10^5 cells/150 μL RPMI-1640 with 10% fetal bovine serum/well) for 7~10 days. EC growth areas were determined using Image-Pro Plus software and presented as mm^2. 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 BMMC. Total cellular RNA was extracted from BMMC using 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-RCR 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 pH5.5 buffer. Five micrograms of protein from each sample were incubated with 12 mM dithiothreitol and 1 μl of biotin- conjugated JPM in 30 μl of a pH5.5 buffer for 1 hr at 37°C. Protein samples were then separated on a 12% SDS-PAGE followed by immunoblot detection with horseradish peroxidase-conjugated avidin (Supplementary Table 1). BMMC lysate MMP activity was detected using a gelatin gel zymogram, essentially the same as reported previously.[21]

BMMC activity in promoting vascular SMC apoptosis was performed using primary cultured mouse aortic SMC on an 8-well chamber slide.[10] Confluent SMC were stimulated to apoptosis overnight with 80 μM pyrrolidinedithiocarbamate (PDTC) with and without 300 μl of degranulated BMMC supernatant in DMEM (1×10^6 BMMC supernatant/ml) from WT or *Mcpt4*$^{-/-}$ mice as described.[22] Apoptotic cells were detected with *In Situ* Cell Death Detection Kit according to the instructions (Roche Diagnostics Co.).

BMMC elastase activities were determined by mixing 100 μg of BMMC extract with 20 μg of fluorigenic elastin (DQ-elastin, Molecular Probe) in 100 μl of a pH5.5 buffer on a 96-well plate for 5 hours. The plate was read at Excitation/Emission=505/515 nm and data were presented as relative fluorescent units.

Statistics

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's correlation test. Multivariate linear regression analysis was performed to adjust for the subjects' use of glucocortioids, smoking, low-dose aspirin, ACE-inhibitor, beta adrenergic blocker, beta adrenergic agonist, lowest ankle-brachial blood pressure index, diastolic blood pressure, and age.

Due to 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/B). 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 normalizing the protein loading for β-actin signals (ImageJ) (Figure 1D).

Correlation of serum chymase level with AAA growth rate

In this serologic prospective study of 103 AAA patients who had serum chymase measured, Pearson's 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*). Due to 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 ACE inhibitors, beta

adrenergic blocking agents, beta adrenergic agonists, or corticosteroids, ankle brachial index, diastolic blood pressure, and age. Among these confounders, serum chymase and glucocorticoid use were correlated strongly and significantly with AAA growth rate after the adjustment (P=0.009) (Table 1). Although we do not know the cause, glucocorticoid users had higher AAA growth rate (3.54±0.49 *vs.* 1.97±0.19 mm/year, P=0.01) but lower serum chymase levels (10.77±0.31 *vs.* 12.34±0.24 ng/ml, P<0.04) than non-glucocorticoid users. Therefore, chymase expression alone may not explain the glucocorticoid effects on AAA growth. Other unrecognized mechanisms may play more important roles. After excluding those receiving glucocorticoid treatments (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 level 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 post-perfusion did not show significant differences in aortic expansion between WT and *Mcpt5*$^{-/-}$ mice (data not shown). In contrast, while 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 post-perfusion still showed significant attenuation of AAA expansion (P=0.0003) and lower incidences (69% *vs.* 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 SMC than those from WT mice 7 days post-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–G), 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 two 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, as 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*$^{W\text{-}sh/W\text{-}sh}$ mice with BMMC from WT or *Mcpt4*$^{-/-}$ mice. Consistent with our earlier observation, while *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice failed to develop AAA,[10] and provision of WT BMMC restored AAA expansion at both the 14- and 56-day time points. In contrast, those that received BMMC 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 $Kit^{W\text{-}sh/W\text{-}sh}$ mice restored at least partially these variables at both time points. In contrast, most variables did not differ significantly between $Kit^{W\text{-}sh/W\text{-}sh}$ mice and those that received $Mcpt4^{-/-}$ BMMC at either time point (Figure 3B–F), 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 2G/F) or in $Kit^{W\text{-}sh/W\text{-}sh}$ mice that received $Mcpt4^{-/-}$ BMMC (Figure 3D/F) 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 using BMMC from WT and $Mcpt4^{-/-}$ mice. Relative to WT BMMC, 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 VEGF,[23] but less so by releasing inflammatory cytokines interleukin-6 (IL-6), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α).[10] To test whether mMCP-4-deficiency affects mast cell VEGF expression, we performed a VEGF ELISA on supernatants from degranulated mast cells and lysates from WT and $Mcpt4^{-/-}$ BMMC and found no significant differences (data not shown). We have previously shown that WT BMMC promote vascular SMC apoptosis.[10] $Mcpt4^{-/-}$ BMMC conferred significant protection to SMC from PDTC-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] Cysteinyl 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/F) or in $Mcpt4^{-/-}$ BMMC-reconstituted $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 3D/F), we hypothesized that absence of mMCP-4 affects cathepsin and MMP expression. $Mcpt4^{-/-}$ BMMC had significantly lower mRNA encoding all cathepsins tested, including B, S, K, and L compared with those in WT BMMC as shown by RT-PCR. Although both MMP-2 and −9 mRNAs were also higher in WT BMMC than in $Mcpt4^{-/-}$ BMMC, their RNA levels remained low in WT BMMC (Figure 4C). MMPs in $Mcpt4^{-/-}$ BMMC were undetectable by gelatin zymography (data not shown). To document further decreased cathepsin activities in $Mcpt4^{-/-}$ BMMC, we performed cysteine protease active site labeling with biotin-JPM. $Mcpt4^{-/-}$ BMMC showed reduced overall cathepsin activities relative to WT BMMC (Figure 4D). Interestingly, we detected high levels of cathepsin L mRNA but low levels of its active enzyme in WT BMMC (Figure 4C/D), 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 $Mcpt4^{-/-}$ BMMC degraded significantly less elastin than those from WT BMMC under conditions optimized for cysteine protease cathepsins[29] (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*$^{-/-}$ BMMC demonstrate impaired ability to induce vascular cell cathepsin expression. Culture of aortic SMC with^{-} BMMC showed that WT BMMC induced the activities of all major cysteinyl cathepsins to a much greater extent than *Mcpt4*$^{-/-}$ BMMC. *Mcpt4*$^{-/-}$ BMMC-treated SMC had reduced cathepsin B activity and negligible cathepsin S/K and L/C activities compared to SMC treated with WT BMMC (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 SMC by an unknown mechanism.

Deficiency of mMCP-4 reduces aortic wall cathepsin activities

Reduced cathepsin activities in *Mcpt4*$^{-/-}$ BMMC and in *Mcpt4*$^{-/-}$ BMMC-treated SMC 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, pH5.5 with ethylenediaminetetraacetic acid (EDTA),[29] frozen aortic sections from WT mice (Figure 5B) had greater activity in digesting fluorigenic 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 uncertain. This study supports the hypothesis that mast cell chymase participates directly in the pathogenesis in 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 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 pro-inflammatory 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 (3.54±0.49 *vs.* 1.97±0.19 mm/year, mean±SEM, P=0.01) compared with patients who did not receive the therapy, although this treatment also significantly reduced the serum chymase levels (10.77±0.31 *vs.* 12.34±0.24, ng/ml, mean±SEM, P<0.04). While 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 associates with increased aortic expansion rate and rupture risk.[35] Alternatively, glucocorticoids can inhibit vascular SMC proliferation[36] 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, an hypothesis that requires further investigation.

We found that mast cell chymase expression affected the expression of other proteases. Reduced cysteinyl cathepsin expression in *Mcpt4*$^{-/-}$ BMMC 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 to reduced mast cell cathepsin expression. Although mMCP-4 can activate pro-collagenase, 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 elastinolyis during AAA formation. In contrast, cathepsins S, K, L, and C are potent elastases and collagenases, and these secreted cysteine proteases contributed to AAA formation (ref 15, and Shi unpublished data). 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 (Figure 2E/Figure 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 angiogensin II generation.[39–41] BMMC from *Mcpt4*$^{-/-}$ mice had significantly lower levels of cathepsin G transcripts than those in WT BMMC after normalizing to β-actin transcripts (0.013±0.0005 *vs.* 0.0006±0.0003, mean±SEM, $P<0.00005$), 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 angiogenesis[42] and apoptosis.[43] However, these in vitro studies employed purified chymases in isolated cells. Direct involvement of chymase in thee processes in vivo remains unknown. This study showed results similar to those of prior studies of angiogenesis and apoptosis both in vitro (Figure 4A/B) and in vivo (Figure 2F/G). Reduced cysteinyl cathepsin expression and activities in *Mcpt4*$^{-/-}$ BMMC (Figure 4C/D/E) and impaired ability of *Mcpt4*$^{-/-}$ BMMC to induce SMC cathepsin expression raised the possibility that mast cell chymases participate in arterial remodeling in part by regulating cathepsin activities, an 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 EC indicated that lack of cathepsin S did not change the expression or activities of other cathepsins, the cathepsin inhibitor cystatin C, MMP, or of tissue inhibitors of MMPs.[24] Chymase might participate in cathepsin activation as it does in the case of pro-MMPs,[1] an 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). Yet 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 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-l|3 expression by EC.[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 BMMC from *Mcpt4*$^{-/-}$ mice through collagen I-precoated filters compared to BMMC 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 pathologic 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Mrs. Eugenia Shvartz for technical assistance and Ms. Joan Perry for editorial assistance.

SOURCES OF FUNDING

This study is supported by NIH grants HL60942, HL81090, HL88547 (GPS); HL56985 (PL); HL36110 (RLS); HL083762, HL56701 (RWT); EIA award (0840118N) from AHA (GPS), and EU-FP7: 200647; FAD project (JSL).

REFERENCES

1. Furubayashi K, Takai S, Jin D, Miyazaki M, Katsumata T, Inagaki S, Kimura M, Tanaka K, Nishimoto M, Fukumoto H. Chymase activates promatrix metalloproteinase-9 in human abdominal aortic aneurysm. Clin Chim Acta 2008;388:214–216. [PubMed: 17964292]
2. Miyazaki M, Takai S. Tissue angiotensin II generating system by angiotensin-converting enzyme and chymase. J Pharmacol Sci 2006;100:391–397. [PubMed: 16799256]
3. Leskinen MJ, Heikkilä HM, Speer MY, Hakala JK, Laine M, Kovanen PT, Lindstedt KA. Mast cell chymase induces smooth muscle cell apoptosis by disrupting NF-kappaB-mediated survival signaling. Exp Cell Res 2006;312:1289–1298. [PubMed: 16460729]
4. Heikkilä HM, Lätti S, Leskinen MJ, Hakala JK, Kovanen PT, Lindstedt KA. Activated mast cells induce endothelial cell apoptosis by a combined action of chymase and tumor necrosis factor-alpha. Arterioscler Thromb Vasc Biol 2008;28:309–314. [PubMed: 18079408]
5. Lee M, Calabresi L, Chiesa G, Franceschini G, Kovanen PT. Mast cell chymase degrades apoE and apoA-II in apoA-I-knockout mouse plasma and reduces its ability to promote cellular cholesterol efflux. Arterioscler Thromb Vasc Biol 2002;22:1475–1481. [PubMed: 12231569]
6. Daugherty A, Cassis LA. Mechanisms of abdominal aortic aneurysm formation. Curr Atheroscler Rep 2002;4:222–227. [PubMed: 11931720]
7. Wolf YG, Fogarty TJ, Olcott C IV, Hill BB, Harris EJ, Mitchell RS, Miller DC, Dalman RL, Zarins CK. Endovascular repair of abdominal aortic aneurysms: eligibility rate and impact on the rate of open repair. J Vasc Surg 2000;32:519–523. [PubMed: 10957658]
8. Tsunemi K, Takai S, Nishimoto M, Yuda A, Hasegawa S, Sawada Y, Fukumoto H, Sasaki S, Miyazaki M. Possible roles of angiotensin II-forming enzymes, angiotensin converting enzyme and chymase-like enzyme, in the human aneurysmal aorta. Hypertens Res 2002;25:817–822. [PubMed: 12484503]
9. Nishimoto M, Takai S, Fukumoto H, Tsunemi K, Yuda A, Sawada Y, Yamada M, Jin D, Sakaguchi M, Nishimoto Y, Sasaki S, Miyazaki M. Increased local angiotensin II formation in aneurysmal aorta. Life Sci 2002;71:2195–2205. [PubMed: 12204777]
10. Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P, Sun C, Zhang Y, Liu J, Ennis TL, Knispel R, Xiong W, Thompson RW, Baxter BT, Shi GP. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. J Clin Invest 2007;117:3359–3368. [PubMed: 17932568]
11. Furubayashi K, Takai S, Jin D, Muramatsu M, Ibaraki T, Nishimoto M, Fukumoto H, Katsumata T, Miyazaki M. The significance of chymase in the progression of abdominal aortic aneurysms in dogs. Hypertens Res 2007;30:349–357. [PubMed: 17541214]
12. Tsunemi K, Takai S, Nishimoto M, Jin D, Sakaguchi M, Muramatsu M, Yuda A, Sasaki S, Miyazaki M. A specific 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), suppresses development of abdominal aortic aneurysm in hamsters. J Pharmacol Exp Ther 2004;309:879–883. [PubMed: 14960660]

13. Inoue N, Muramatsu M, Jin D, Takai S, Hayashi T, Katayama H, Kitaura Y, Tamai H, Miyazaki M. Effects of chymase inhibitor on angiotensin II-induced abdominal aortic aneurysm development in apolipoprotein E-deficient mice. Atherosclerosis. 2008in press.
14. Pyo R, Lee JK, Shipley JM, Curci JA, Mao D, Ziporin SJ, Ennis TL, Shapiro SD, Senior RM, Thompson RW. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. J Clin Invest 2000;105:1641–1649. [PubMed: 10841523]
15. Pagano MB, Bartoli MA, Ennis TL, Mao D, Simmons PM, Thompson RW, Pham CT. Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms. Proc Natl Acad Sci U S A 2007;104:2855–2860. [PubMed: 17301245]
16. Pejler G, Abrink M, Ringvall M, Wernersson S. Mast cell proteases. Adv Immunol 2007;95:167–255. [PubMed: 17869614]
17. Pan JH, Lindholt JS, Sukhova GK, Baugh JA, Henneberg EW, Bucala R, Donnelly SC, Libby P, Metz C, Shi GP. Macrophage migration inhibitory factor is associated with aneurysmal expansion. J Vasc Surg 2003;37:628–635. [PubMed: 12618703]
18. Tchougounova E, Pejler G, Åbrink M. The Chymase, Mouse Mast Cell Protease 4, Constitutes the Major Chymotrypsin-like Activity in Peritoneum and Ear Tissue. A Role for Mouse Mast Cell Protease 4 in Thrombin Regulation and Fibronectin Turnover. J Exp Med 2003;198:423–431. [PubMed: 12900518]
19. Abonia JP, Friend DS, Austen WG Jr, Moore FD Jr, Carroll MC, Chan R, Afnan J, Humbles A, Gerard C, Knight P, Kanaoka Y, Yasuda S, Morokawa N, Austen KF, Stevens RL, Gurish MF. Mast cell protease 5 mediates ischemia-reperfusion injury of mouse skeletal muscle. J Immunol 2005;174:7285–7291. [PubMed: 15905575]
20. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, MacFarlane LA, Mallen-St Clair J, Shi GP. Mast cells promote atherosclerosis by releasing proinflammatory cytokines. Nat Med 2007;13:719–724. [PubMed: 17546038]
21. Fang KC, Raymond WW, Lazarus SC, Caughey GH. Dog mastocytoma cells secrete a 92-kD gelatinase activated extracellularly by mast cell chymase. J Clin Invest 97:1589–1596. [PubMed: 8601622]19096
22. Tsai JC, Jain M, Hsieh CM, Lee WS, Yoshizumi M, Patterson C, Perrella MA, Cooke C, Wang H, Haber E, Schlegel R, Lee ME. Induction of apoptosis by pyrrolidinedithiocarbamate and N-acetylcysteine in vascular smooth muscle cells. J Biol Chem 1996;271:3667–3670. [PubMed: 8631978]
23. Heissig B, Rafii S, Akiyama H, Ohki Y, Sato Y, Rafael T, Zhu Z, Hicklin DJ, Okumura K, Ogawa H, Werb Z, Hattori K. Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. J Exp Med 2005;202:739–750. [PubMed: 16157686]
24. Shi GP, Sukhova GK, Kuzuya M, Ye Q, Du J, Zhang Y, Pan JH, Lu ML, Cheng XW, Iguchi A, Perrey S, Lee AM, Chapman HA, Libby P. Deficiency of the cysteine protease cathepsin S impairs microvessel growth. Circ Res 2003;92:493–500. [PubMed: 12600886]
25. Wang B, Sun J, Kitamoto S, Yang M, Grubb A, Chapman HA, Kalluri R, Shi GP. Cathepsin S controls angiogenesis and tumor growth via matrix-derived angiogenic factors. J Biol Chem 2006;281:6020–6029. [PubMed: 16365041]
26. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737–744. [PubMed: 11025665]
27. Stoka V, Turk V, Turk B. Lysosomal cysteine cathepsins: signaling pathways in apoptosis. Biol Chem 2007;388:555–560. [PubMed: 17552902]
28. Newby AC. Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. Cardiovasc Res 2006;69:614–624. [PubMed: 16266693]

29. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest 1998;102:576–583. [PubMed: 9691094]
30. Mallen-St Clair J, Shi GP, Sutherland RE, Chapman HA, Caughey GH, Wolters PJ. Cathepsins L and S are not required for activation of dipeptidyl peptidase I (cathepsin C) in mice. Biol Chem 2006;387:1143–1146. [PubMed: 16895486]
31. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 2000;105:1605–1612. [PubMed: 10841519]
32. Sumino H, Kanda T, Nakamura T, Sakamaki T, Sakamoto H, Sato K, Tange S, Ichikawa S, Nagai R. Steroid therapy is effective in a young patient with an inflammatory abdominal aortic aneurysm. J Med 1999;30:67–74. [PubMed: 10515242]
33. Turner S, Derham C, Orsi NM, Bosomworth M, Bellamy MC, Howell SJ. Randomized clinical trial of the effects of methylprednisolone on renal function after major vascular surgery. Br J Surg 2008;95:50–56. [PubMed: 18027383]
34. Komori K, Ishida M, Matsumoto T, Kume M, Ohta S, Takeuchi K, Onohara T, Sugimachi K. Cytokine patterns and the effects of a preoperative steroid treatment in the patients with abdominal aortic aneurysms. Int Angiol 1999;18:193–197. [PubMed: 10688417]
35. Axelrod DA, Henke PK, Wakefield TW, Stanley JC, Jacobs LA, Graham LM, Greenfield LJ, Upchurch GR Jr. Impact of chronic obstructive pulmonary disease on elective and emergency abdominal aortic aneurysm repair. J Vasc Surg 2001;33:72–76. [PubMed: 11137926]
36. Longenecker JP, Kilty LA, Johnson LK. Glucocorticoid inhibition of vascular smooth muscle cell proliferation: influence of homologous extracellular matrix and serum mitogens. J Cell Biol 1984;98:534–540. [PubMed: 6693494]
37. Autio P, Oikarinen A, Melkko J, Risteli J, Risteli L. Systemic glucocorticoids decrease the synthesis of type I and type III collagen in human skin in vivo, whereas isotretinoin treatment has little effect. Br J Dermatol 1994;131:660–663. [PubMed: 7999597]
38. Saarinen J, Kalkkinen N, Welgus HG, Kovanen PT. Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase. J Biol Chem 1994;269:18134–18140. [PubMed: 8027075]
39. Gacko M, Chyczewski L. Activity and localization of cathepsin B, D and G in aortic aneurysm. Int Surg 1997;82:398–402. [PubMed: 9412840]
40. Boudier C, Godeau G, Hornebeck W, Robert L, Bieth JG. The elastolytic activity of cathepsin G: an ex vivo study with dermal elastin. Am J Respir Cell Mol Biol 1991;4:497–503. [PubMed: 1711351]
41. Whitman SC. All of the components required for angiotensin II formation are expressed locally in human atherosclerotic lesions, including a long suspected player cathepsin G. J Hypertens 2004;22:39–42. [PubMed: 15106791]
42. Muramatsu M, Katada J, Hayashi I, Majima M. Chymase as a proangiogenic factor. A possible involvement of chymase-angiotensin-dependent pathway in the hamster sponge angiogenesis model. J Biol Chem 2000;275:5545–5552. [PubMed: 10681534]
43. Leskinen MJ, Heikkilä HM, Speer MY, Hakala JK, Laine M, Kovanen PT, Lindstedt KA. Mast cell chymase induces smooth muscle cell apoptosis by disrupting NF-kappaB-mediated survival signaling. Exp Cell Res 2006;312:1289–1298. [PubMed: 16460729]
44. Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. J Immunol 1998;161:1939–1946. [PubMed: 9712064]

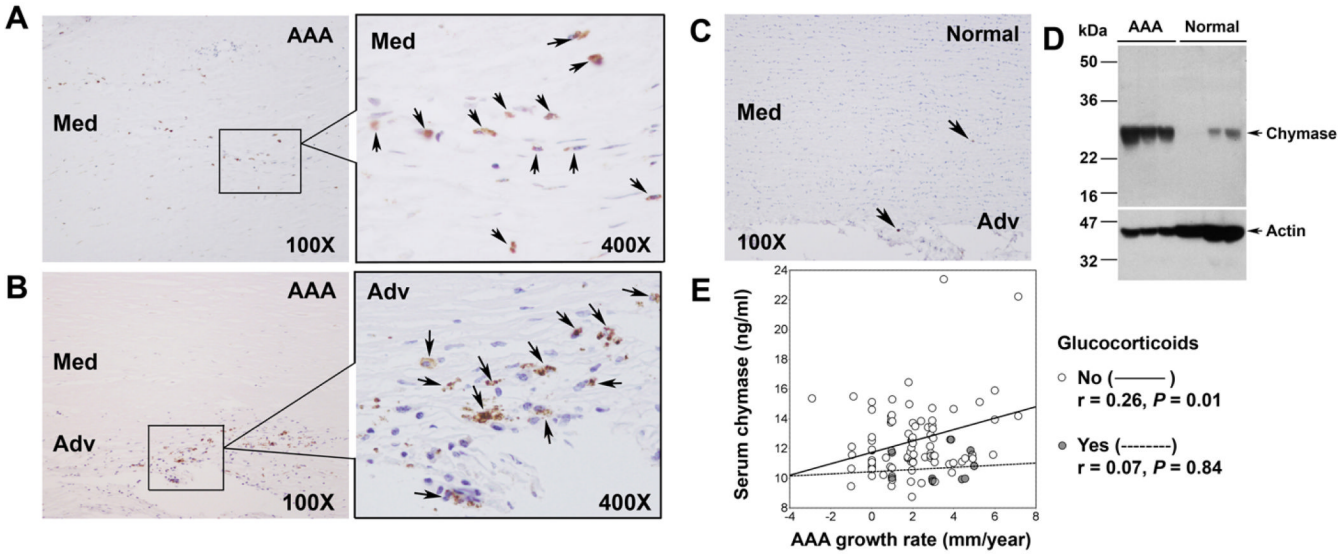

Figure 1.

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 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. Beta-actin immunoblot was used for protein loading control. **E**. Correlation of serum chymase and AAA growth rate among patients with (dark dots) or without (empty dots) glucocorticoids treatments (Pearson's correlation test). P<0.05 was considered statistical significant.

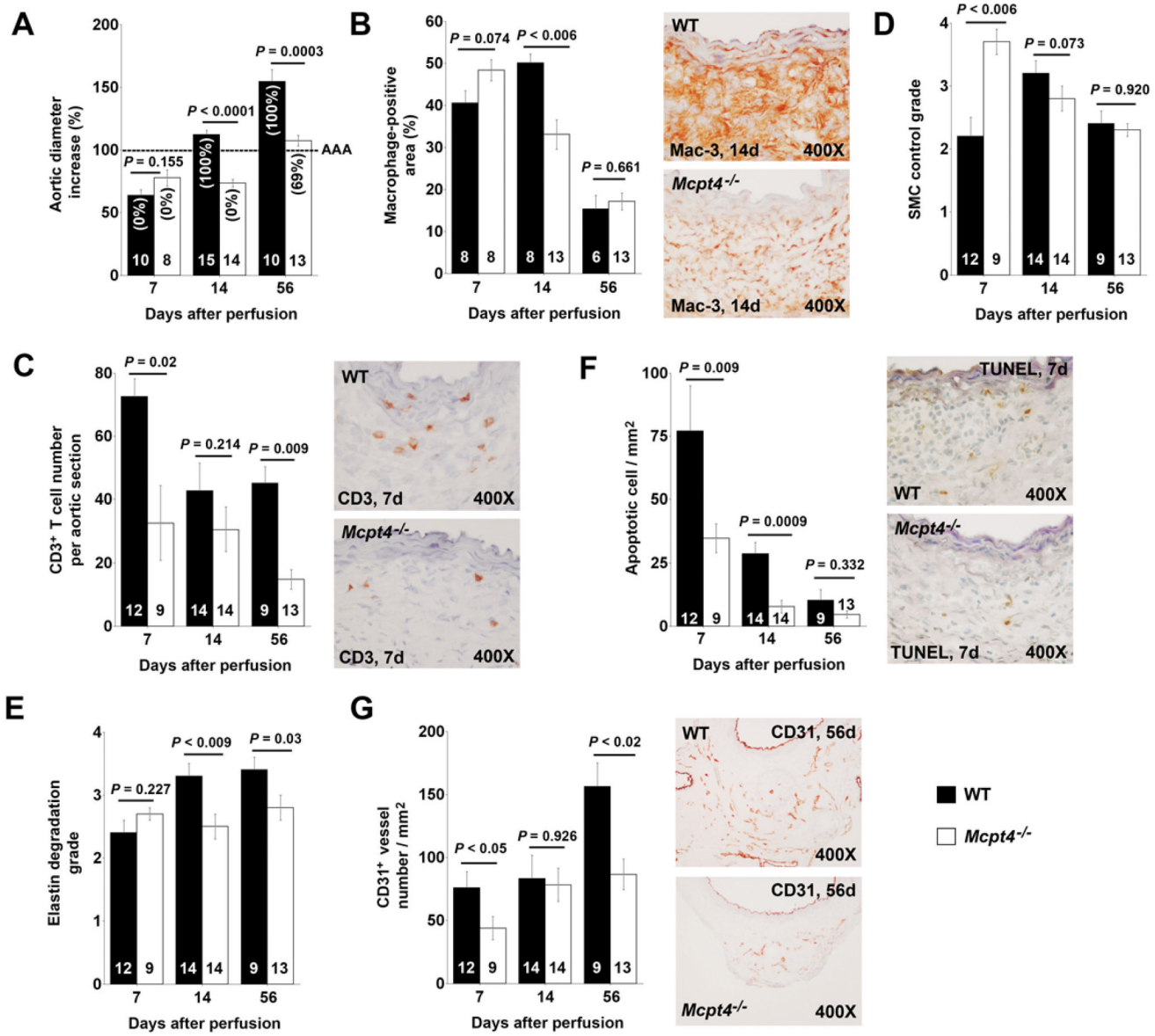

Figure 2.

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 grade (**E**), apoptotic cells (**F**), and CD31^{+} microvessel numbers (**G**) between WT and *Mcpt4*$^{-/-}$ mice at three time points. 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 controls (not shown). All data were mean±SEM. P<0.05 was considered statistical significant, non-parametric Mann-Whitney test.

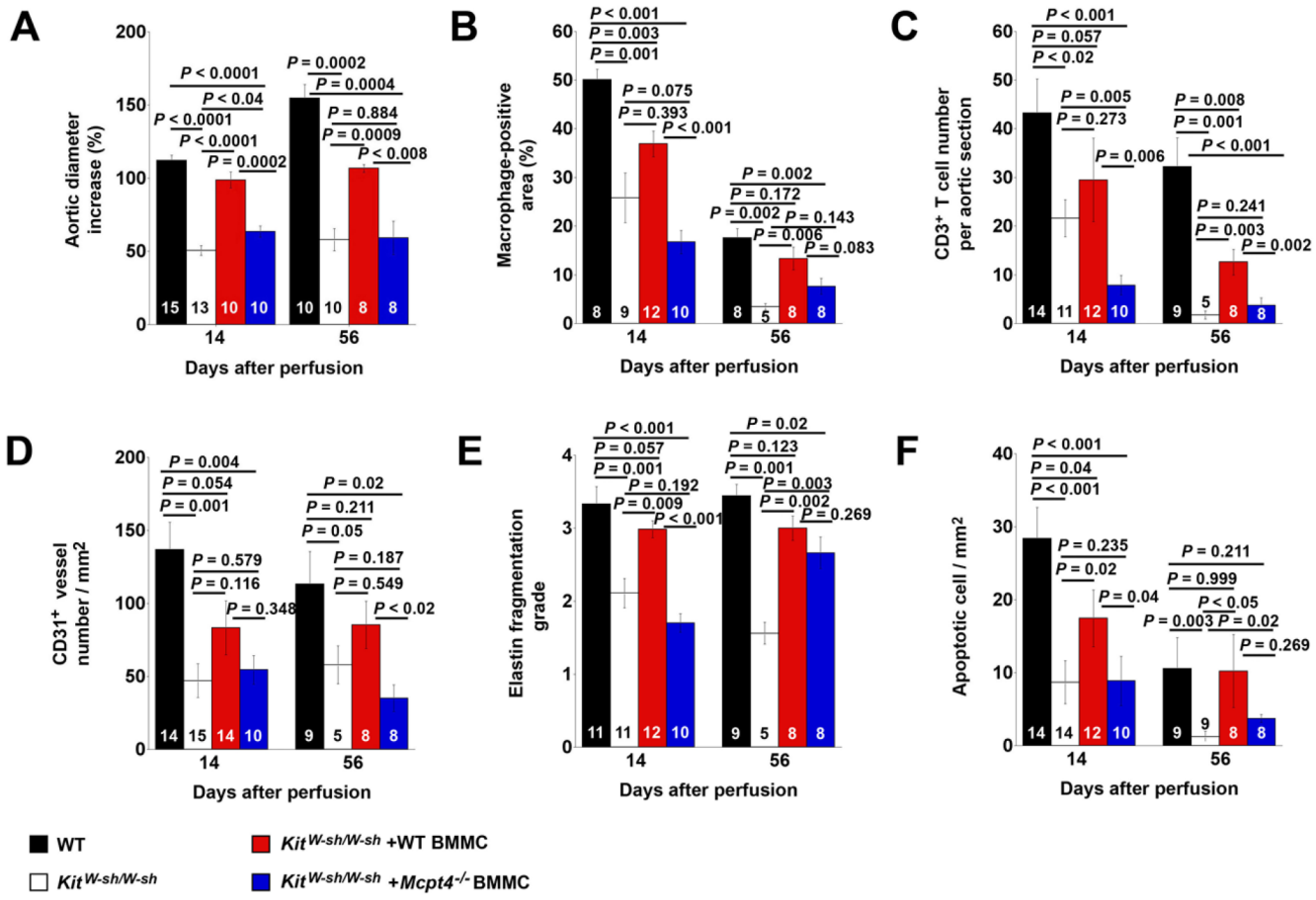

Figure 3.

Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstitution and lesion characterization. Reconstitution of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with WT but not *Mcpt4*$^{-/-}$ BMMC 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 experiment group is indicated in the bar. All data were mean±SEM. P<0.05 was considered statistical significant, non-parametric Mann-Whitney test.

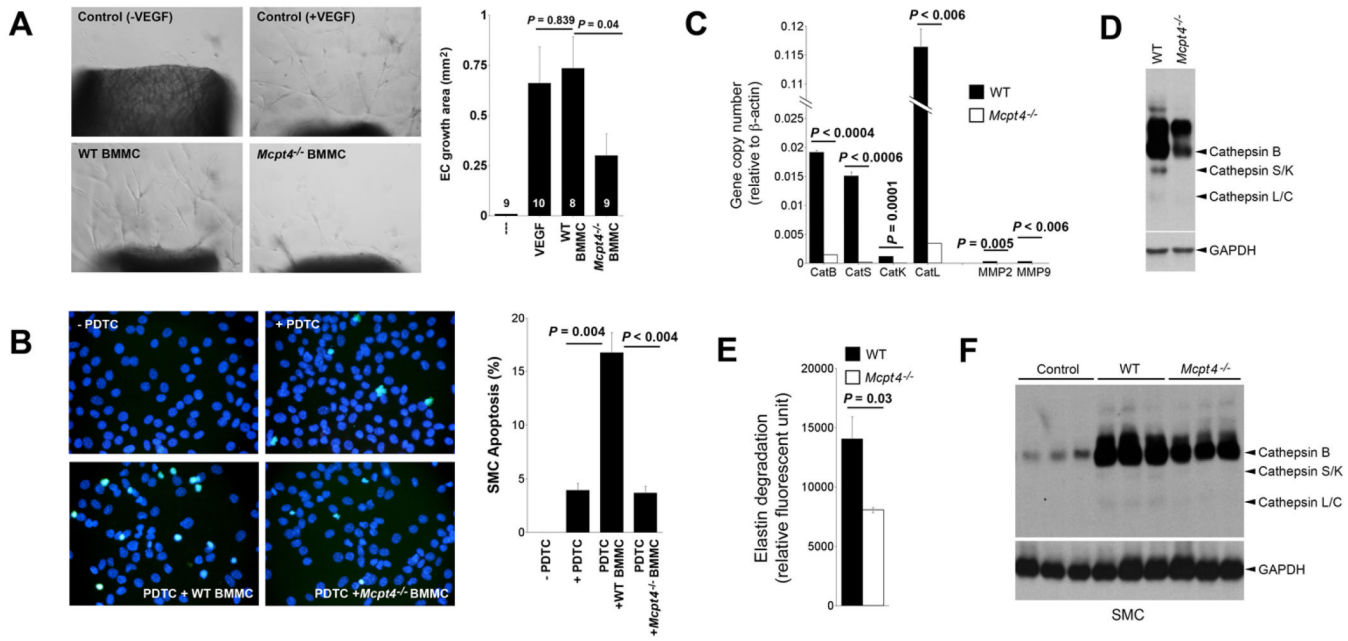

Figure 4.

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

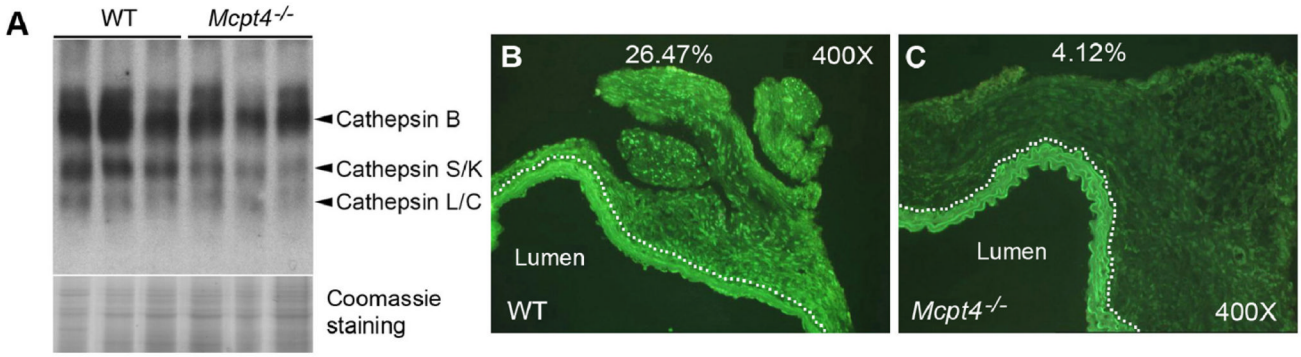

Figure 5.

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 crosssection in situ elastase activity zymograph. Lumen and percentage of fluorescence intensity were indicated. Images were obtained using the same magnification and shutter speed and all data were from the 14-day time point experiments.

Table 1

Correlation coefficient between AAA growth rate and different confounders.[a]

Variables	Mean ± SEM or patient # [Range]	Standardized Coefficient (Beta)[b]	t value	*P* value[c]
Chymase (ng/ml)	12.18 ± 2.26 [8.76~23.40]	0.267	2.658	0.009
Initial AAA size	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
Beta blockage	16/103	−0.105	−1.017	0.312
Beta 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	92.70 ± 13.27 [65.00~130.00]	0.048	0.487	0.627
Age	68.00 ± 2.95 [64.30~73.70]	0.080	0.818	0.416

[a] All 103 patients are male;

[b] Multivariate linear regression analysis;

[c] $P<0.05$ was considered statistically significant.