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Mast Cell Chymase and Trypsin in Abdominal Aortic Aneurysm Formation

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Abstract

Mast cells (MCs) are implicated in the pathogenesis of atherosclerosis and abdominal aortic aneurysm (AAA). MC-specific chymase and trypsin play important roles in inducing endothelial cell expression of adhesion molecules and chemokines to promote leukocyte recruitment, degrading matrix proteins and activating protease-activated receptors to trigger smooth-muscle cell apoptosis, and activating other proteases to degrade medial elastin and to enhance angiogenesis. In experimental AAA, absence or pharmacological inhibition of chymase or trypsin reduced AAA formation and associated arterial pathologies, proving that these MC proteases participate directly in AAA formation. Increased levels of these proteases in human AAA lesions and in plasma from AAA patients suggest that these proteases are also essential to human AAA pathogenesis. Development of chymase or trypsin inhibitors or their antibodies may have therapeutic potential among affected human subjects.

Abdominal aortic aneurysm (AAA) is an inflammatory arterial disease that affects up to 9% of males over age 65. Lack of routine monitoring may jeopardize AAA patients, particularly those who have asymptomatic rupture-prone AAAs. Although endovascular surgical repair remains the only clinical treatment for human AAA, recent discoveries in the molecular and cellular mechanisms of AAA may lead to the development of novel preventive strategies and medications. The pathogenesis of AAA involves enhanced expression of endothelial adhesion molecules and chemokines, inflammatory cell adhesion and trans-subendothelium migration, aortic media inflammation and matrix protein destruction, smooth-muscle cell (SMC) loss and arterial wall thinning, luminal endothelial cell (EC) death and desquamation, and ultimately aortic thrombus formation and rupture. Macrophages, T cells, and neutrophils are well-known inflammatory cells in human AAA lesions. Recent studies demonstrated that mast cells (MCs) are also present in human and experimental AAAs, and participate directly in experimental AAA (Sun et al. 2007b, Tsuruda et al. 2008, Mäyränpää et al. 2009).

In normal human aortas, very few MCs are located at the adventitia and intima (Mäyränpää et al. 2009). In human atherosclerotic lesions, abundant MCs are present in the intima,

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media, and adventitia (Jeziorska et al. 1997). In contrast, MCs increase mainly in the media and adventitia in human AAA lesions, together with T cells and macrophages, but are negligible in the intima (Mäyränpää et al. 2009). The numbers of total and degranulated (activated) MCs in the outer media and adventitia of human AAA are significantly higher than those in early and advanced human atherosclerotic lesions and normal aortas (Tsuruda et al. 2008). MC numbers in human AAA lesions correlate with maximal aortic diameters. AAA tissue extract preparation showed increased expression and activities of matrix metalloproteinase (MMP)-2 and MMP-9 (by gelatin zymography) and increased expression of MC-specific tryptase and stem cell factor (SCF) (Tsuruda et al. 2008). Although the exact molecule that drives MCs accumulation in human AAA lesions remains unknown, increased mRNA and protein levels of leukotriene C4 and its three key enzymes (5-lipoxygenase [5-LO], 5-LO-activating protein, and LTC4 synthase) were detected at the site of enriched inflammatory cells (Di Gennaro et al. 2010), suggesting that leukotriene participates in MC recruitment in human AAA. Several of our studies established a direct role of MC cytokines, chymase, and tryptase in AAA formation (Sun et al. 2007b, Sun et al. 2009, Zhang et al. 2011). In this brief review, we summarize our current understanding of how MC chymase and tryptase participate in the pathogenesis of AAA.

Role of mast cells in experimental AAA

The direct participation of MCs in AAA formation recently has been tested in several experimental animals. In both aortic elastase perfusion-induced and peri-aortic CaCl_2 injury-induced AAA in mice, the absence of MCs in *Kit^{W-sh/W-sh}* mice protected against AAA formation. AAA lesion characterization demonstrated that MC deficiency significantly reduced media elastin degradation, media SMC loss, lesion macrophage and T-cell contents, adventitial angiogenesis, and lesion activities of cysteinyl cathepsins, MMP-2, and MMP-9. Reduced AAA formation, lesion macrophage contents, inflammatory cytokines (e.g., interferon- γ [IFN- γ]), and protease activities (cathepsins and MMPs) in *Kit^{W-sh/W-sh}* mice are fully or partially restored after mice received exogenous MCs from wild-type (WT) or tumor necrosis factor (TNF)- α -deficient mice, but not those from interleukin (IL)-6-deficient or IFN- γ -deficient mice. Compared with AAA formation in WT mice, MC activation with compound 48/80 enlarged AAA sizes, increased lesion inflammation, protease expression, and elastin degradation, and reduced lesion MC number. In contrast, all these phenotypes were diminished or partially suppressed after mice received the MC stabilizer cromolyn (Sun et al. 2007b). In peri-aortic CaCl_2 injury-induced AAA in rats, both total and activated MCs were increased in AAA lesions at 3, 7, and 14 days after injury. Absence of MCs in Ws/Ws rats or stabilization of MCs with tranilast (N-[3,4-dimethoxycinnamonyl]-anthranilic acid, a mast cell blocker) preserved elastin degradation, reduced MMP-2 and MMP-9 activities, and prevented AAA formation. Although aortic wall tissue macrophage numbers did not change, T-cell content and capillary density were significantly blunted. In angiotensin-II (Ang-II) infusion-induced AAA in apolipoprotein E-deficient (*ApoE^{-/-}*) mice, tranilast also reduced AAA lesion area, lesion total and degranulated MC contents, T-cell content, capillary density, and MMP-9 expression and activity (Tsuruda et al. 2008). These observations from several experimental AAA models prove a direct participation of MCs in experimental AAA.

Interactions between mast cells and AAA-pertinent cells

SMCs, ECs, and inflammatory cells, such as macrophages and T cells, are major cell types in human AAA lesions. MCs interact with these cells and change their pathological functions. MCs, for example, use TNF- α to induce MMP-9 expression in macrophages and T cells (Baram et al. 2001, Tchougounova et al. 2005). Treatment of human macrophages with anti-TNF- α antibody suppresses MC-induced MMP-9 expression (Tsuruda et al. 2008).

Conversely, MCs are rich in MMP-9, and T cells induce MC release of MMP-9 (Baram et al. 2001), another important protease in AAA formation.

MCs interact with SMCs and induce SMC apoptosis via either pro-inflammatory cytokines or proteases. MC-derived TNF- α binds to SMC surface death receptor, and initiates caspase-mediated apoptosis (Kovanen 2007). MCs also induce SMC apoptosis via the anoikis mechanism. MC-derived chymase triggers SMC apoptosis by inhibiting SMC collagen synthesis (Wang et al. 2001) or degrading SMC fibronectin and vitronectin, leading to SMC focal adhesion complex disruption (Leskinen et al. 2006, Heikkilä et al. 2008). MCs also release granzyme B to induce SMC apoptosis and EC disorganization (Pardo et al. 2007). We have shown that MCs release IL-6 and IFN- γ to induce SMC and EC cysteinyl cathepsin expression (Sun et al. 2007a), which may indirectly affect SMC or EC apoptosis (Stoka et al. 2007).

When MC releasate was added to rat or human ECs, it induced EC apoptosis by inactivating the focal adhesion kinase (FAK) and Akt-dependent signaling pathways and activating caspase-8 and caspase-9 (Heikkilä et al. 2008). Chymase in the releasate degrades EC extracellular matrix protein vitronectin and fibronectin, providing additional mechanisms of MCs in inducing EC apoptosis. Antibodies against chymase or TNF- α improved EC survival (Heikkilä et al. 2008). Neovascularization is an important signature of human AAA — particularly prominent in thrombus-covered AAA or in thick aneurysmal wall — and correlates with medial MC density. Both MCs and neovascularization proportion in the media are significantly higher in human AAA lesions than in normal aortas. While human AAA tissue extracts have increased expression of proangiogenic factors (e.g., VE-cadherin, vascular endothelial growth factor [VEGF], and its receptor, FLT1) and MC proteases (tryptase, chymase, and cathepsin G), AAA lumens contain CD42b⁺ platelet deposits and lack CD31⁺CD34⁺ ECs (Mäyränpää et al. 2009). Therefore, MC–EC interactions may promote angiogenesis in the microvessels, but may induce luminal EC death, endothelial erosion, and aortic rupture. Indeed, neovessels are more prevalent at the site of rupture in human AAA than in specimens from non-ruptured AAA (Choke et al. 2006). Essential roles of MCs in experimental AAA are summarized in Figure 1.

Mast cell protease functions related to AAA

Mast cell-specific neutral proteases include chymase, tryptase, and carboxypeptidase A (Pejler et al. 2010), and account for more than 25% of total cellular proteins (Schwartz et al. 1987, Pejler et al. 2010) — among which, tryptase is the predominant member and accounts for about 20% of total cellular proteins (Schwartz 1990), 3~50-fold greater mass than chymase (Schwartz et al. 1987). Chymase and tryptase are common antigens used to detect MCs. As in AAA lesions, chymase-positive and tryptase-positive MCs are next to von Willebrand factor-positive microvessels and correlate with each other in human atherosclerotic lesions (Kaartinen et al. 1996, Mäyränpää et al. 2006), suggesting a role of MC proteases in EC biology. Indeed, MCs induce EC death and chemokine expression. For example, treatment of fresh human coronary arteries intraluminally with recombinant human chymase or tryptase induced EC retraction, disruption, and desquamation, due to degradation of the EC adhesion molecules VE-cadherin, vitronectin, and fibronectin (Heikkilä et al. 2008, Mäyränpää et al. 2006). Although there is no study to detect MCs in the aortic wall from early AAA lesions, we have shown MC attachment in early human atherosclerotic lesions — i.e., fatty streaks (Xu and Shi 2012). Such attachment may induce EC chemokine expression, thereby recruiting other inflammatory cells, such as macrophages (Liu et al. 2009). When human umbilical cord ECs were treated with human leukemic MC granules for 24 hours, MC granules significantly increased EC expression of monocyte chemotactic protein-1 (MCP-1) and IL-8 at protein and RNA levels. This MC granule

activity can be blocked by anti-tryptase antibody, but not by chymase inhibitor SF-8257. Recombinant tryptase, but not recombinant chymase, also induces MCP-1 and IL-8 protein expression in ECs (Kinoshita et al. 2005). Tryptase-mediated chemoattractant molecule expression on ECs may also be mediated through protease-activated receptor (PAR) activation. Tryptase activates PAR-2 on ECs (Corvera et al. 1997), leading to increased vascular permeability, smooth muscle relaxation, systemic hypotension, bronchoconstriction, and leukocyte migration and infiltration (Vergnolle et al. 2001). As an alternative mechanism, tryptase activation of PAR-2 consequently activates membrane-associated iPLA2 (calcium-independent phospholipase A2) and enhances leukocyte adhesion to the EC monolayers (White and McHowat. 2007).

Tryptase and chymase share many proteolytic activities that are pertinent to AAA pathogenesis. Although MMP-9 activation in human AAA or in mice may depend on chymase (Tchougounova et al. 2005), chymase and tryptase both activate MMP-1 and MMP-3, which subsequently activate MMP-9 (Gruber et al. 1989, Saarinen et al. 1994). As in patients with coronary heart diseases, AAA patients have higher levels of serum low-density lipoprotein (LDL) cholesterol, but lower levels of serum high-density lipoprotein (HDL) cholesterol (Takagi et al. 2010). MC chymase and tryptase modify HDL by depleting pre β -HDL and other lipid-poor HDL particles, and reduce foam cell cholesterol efflux facilitated by ATP-binding cassette transporter A1 (ABCA1) (Lee-Rueckert and Kovanen 2006). In human macrophages and possibly in SMCs, tryptase promotes intracellular lipid accumulation and foam-cell formation by reducing the expression of nuclear receptor LXR α (regulates lipid homeostasis), ABCA1, ABCG1 (involved in the cholesterol efflux pathway and macrophage foam-cell formation), and sterol regulatory element binding protein-1c (SREBP-1c) (regulates gene for de novo lipogenesis). Tryptase inhibitor APC366 can inhibit all of these activities (Yeong et al. 2010). These tryptase and chymase activities may explain why human AAA lesions also contain foam cells derived from macrophages and SMCs (Tanaskovi et al. 2010).

Chymase and tryptase also have their own unique activities. While chymase cleaves vitronectin and pro-collagen, tryptase degrades type IV collagen, although all of these contribute to diminished arterial wall structural integrity and enhanced propensity for AAA formation. Chymase, together with angiotensin-converting enzymes (ACEs), is essential in converting Ang-I into Ang-II (Miyazaki et al. 2006). We often consider Ang-II-forming activity as one of the readouts of chymase and ACE activities from AAA lesions. Indeed, both degranulated MC numbers and Ang-II-forming activities are higher in AAA lesions than in atherosclerotic lesions (Ihara et al. 1999). In addition to vasodilation activity, Ang-II also activates macrophages, leading to MCP-1 expression, which causes more monocyte/macrophage and neutrophil recruitment to the site of inflammation (Schieffer et al. 2000, Yusof et al. 2007).

Direct role of chymase and tryptase in AAA formation

MC accumulation in human AAA lesions has been confirmed using either chymase antibody-mediated or tryptase antibody-mediated immunohistological analysis. In a prospective clinical follow-up study of 103 Danish men, we found that plasma chymase levels correlated with AAA annual growth rate, but did not associate with initial AAA size (Sun et al. 2009). In a related Danish population-based randomized screening trial of 100 men with defined AAA and 35 age-matched male controls, plasma tryptase levels (after log transformation) were significantly higher in AAA patients than in controls, although many patients in the control group were smokers (42.9%) and had other cardiovascular complications, such as acute myocardial infarction, angina pectoris, stroke, or hypertension (total 25.7%). Initial AAA size and tryptase levels correlated weakly and insignificantly

($P=0.106$), but the mean annual AAA growth rate correlated significantly with plasma trypsin levels before ($P=0.003$) and after ($P=0.005$) adjustment for more than 10 potential AAA risk factors. Of note, Cox regression analysis demonstrated that high plasma trypsin levels in AAA patients increased the risk of later surgical repair before (hazard ratio [HR]: 1.74) and after (HR: 2.15) adjustment for AAA risk factors. Plasma trypsin levels also associated with overall mortality before (HR: 1.43) and after (HR: 3.17) adjustment (Zhang et al. 2011). These clinical observations suggest an important role of chymase and trypsin in human AAA.

The roles of chymase and trypsin in AAA have been tested in several experimental AAA models. In Ang-II infusion-induced AAA in *Apoe*^{-/-} mice, oral administration of the chymase inhibitor NK3201 (30 mg/kg/day) significantly suppressed AAA lesion area, and reduced lesion MOMA-2⁺ monocyte/macrophage cells and MMP-9 expression and activity (Inoue et al. 2009). In elastase perfusion-induced AAA in dogs, while aortic diameter increased consistently at 2, 4, 6, and 8 weeks post-perfusion, NK3201 (1 mg/kg/day) treatment prevented post-perfusion aortic diameter increase. NK3201 suppressed lumen area, aortic wall chymase levels, neutrophil contents, chymase and Ang-II forming activities, and MMP-9 activities (Furubayashi et al. 2007). In the same model in hamsters, NK3201 also attenuated AAA formation (Tsunemi et al. 2004). We recently proved a direct role of chymase in AAA. Using mice deficient in mouse MC protease-4 (MMCP-4; a human chymase ortholog) with aortic elastase perfusion-induced AAA, we demonstrated that MCs lacking chymase expression greatly reduced their ability to promote AAA formation. At 14 days post-perfusion, MMCP-4-deficient (*Mcpt4*^{-/-}) mice were fully protected from AAA formation. At 8 weeks after elastase perfusion, *Mcpt4*^{-/-} mice remained protected. Reduced AAA in *Mcpt4*^{-/-} mice associated with reduced lesion adventitia cell apoptosis, media elastin degradation, and adventitia angiogenesis. All such improvements in *Mcpt4*^{-/-} mice are partially reversed after mice receive adoptive transfer of MCs from WT mice, but not those from *Mcpt4*^{-/-} mice (Sun et al. 2009). By unknown mechanisms, the absence of MMCP-4 reduced MMP and cysteinyl cathepsin expression in MCs, and reduced cathepsin activities in SMCs and the aortic wall. Reduced protease expression and activities may explain impaired media elastin degradation and adventitia angiogenesis. As discussed, chymase cleaves vitronectin (Heikkilä et al. 2008) and fibronectin (Leskinen et al. 2006), which may lead to SMC focal adhesion complex disruption. Chymase also inhibits SMC collagen synthesis (Wang et al. 2001). All these chymase activities contribute to SMC apoptosis, although we did not test whether matrix protein expression and deposition were altered in *Mcpt4*^{-/-} mice. Unlike trypsin, chymase does not affect EC chemokine expression (Kinoshita et al. 2005), but it may still activate PAR-2 (Sharma et al. 2007). These activities may impair chymase-mediated leukocyte adhesion, migration, and accumulation in AAA lesions. Indeed, AAA lesion macrophage and T-cell contents in *Mcpt4*^{-/-} mice were not always reduced at all three post-perfusion time points (Sun et al. 2009).

While chymase activity in AAA formation has been studied in several experimental AAA models using chymase inhibitor or chymase-deficient mice, trypsin inhibitors have not been tested in experimental AAA formation. Our recent study using mouse MC protease-6 (MMCP-6)-deficient (*Mcpt6*^{-/-}) mice, however, proved that trypsin participates directly in AAA. In elastase perfusion-induced AAA, while aortic diameters increased over time at 7, 14, and 56 days post-perfusion in WT mice, those in *Mcpt6*^{-/-} mice remained unchanged. Lesion macrophage and T-cell contents and major histocompatibility complex class-II (MHC-II) molecule expression were also significantly reduced in *Mcpt6*^{-/-} mice (Zhang et al. 2011). Reduced AAA formation and lesion inflammation in *Mcpt6*^{-/-} mice were largely reversed after mice receiving exogenously administered MCs obtained from WT mice, but not those from *Mcpt6*^{-/-} mice, confirming a role of MC trypsin in experimental AAA. As

discussed, tryptase induces EC expression of chemokines IL-8 and MCP-1 (Kinoshita et al. 2005). AAA lesions from *Mcpt6*^{-/-} mice had significantly reduced MCP-1 expression at both 14 days and 56 days post-perfusion. ECs from *Mcpt6*^{-/-} mice also had significantly reduced expression of (cytokines) TNF- α and IL-6 and of (chemokines) KC (an IL-8 homolog), MIP-2 (macrophage inflammatory protein-2), and LIX (lipopolysaccharide-induced CXC), which may all contribute to reduced leukocyte accumulation in AAA lesions in *Mcpt6*^{-/-} mice — although we do not know whether PAR-2 activation was also impaired in AAA lesions from *Mcpt6*^{-/-} mice. Furthermore, monocytes from *Mcpt6*^{-/-} mice showed reduced migration in response to chemoattractant challenge. ECs and monocytes do not express tryptase, and we therefore do not have an explanation for these observations. Other mechanisms may exist.

Tryptase activates MMP-1 and MMP-3, which indirectly affect MMP-9 activities (Gruber et al. 1989, Saarinen et al. 1994), but absence of MMCP-6 only affected cathepsin expression and activities in monocytes and MCs, and did not affect MMP, cathepsin G, or chymase (Zhang et al. 2011). Tryptase mediates collagen-IV proteolysis (Iddamalgoda et al. 2008), which may affect SMC survival. In cultured mouse aortic SMCs, MCs induced SMC apoptosis, but those from *Mcpt6*^{-/-} mice showed greatly reduced induction of SMC apoptosis (Zhang et al. 2011). Consistent with these observations, AAA lesion media SMC apoptosis was also reduced in *Mcpt6*^{-/-} mice, compared with those from WT mice, but tryptase deficiency did not affect adventitia cell apoptosis (Zhang et al. 2011). While chymase deficiency reduced AAA lesion angiogenesis or in vitro microvessel growth, absence of tryptase did not affect angiogenesis in AAA lesions. Tryptase therefore may not play a major role in MC-induced angiogenesis (Sun et al. 2007b, Mäyränpää et al. 2009). Figure 1 summarizes the roles of both chymase and tryptase in experimental AAA.

Summary and future perspective

Both chymase and tryptase are increased in human and experimental AAA lesions, and in blood from AAA patients. Although more mechanisms may still be revealed, available evidence suggests that these proteases contribute to leukocyte adhesion and migration, EC and SMC apoptosis, expression and activation of MMP and cathepsins, and foam-cell formation. MC protease regulation of MMP and cysteinyl cathepsin expression and activation, which have been proven essential in human and experimental AAA and atherosclerosis (Keeling et al. 2005, Cheng et al. 2011, Cheng et al. 2012), suggests that chymase and tryptase indirectly influence AAA formation. Direct evidence of chymase and tryptase participation in AAA came from experimental AAA using mice deficient in MMCP-4 and MMCP-6. These lines of studies suggest the potential of using chymase and tryptase inhibitors or antibodies in human AAA medication. Although not tested in humans, positive data in several experimental AAA experiments with chymase inhibitor NK3201 strongly support this possibility. We believe that tryptase inhibitor will have similar inhibitory activity in experimental AAA, and possibly in human AAA.

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