Forum Minireview

New Approaches to Blockade of the Renin–Angiotensin–Aldosterone System:
Chymase as an Important Target to Prevent Organ Damage

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Abstract.   Chymase plays a crucial role in angiotensin II formation in various tissues. Angiotensin II induces gene expression of transforming growth factor (TGF)-β and matrix metalloproteinase (MMP)-9 precursors, and chymase can convert precursors of TGF-β and MMP-9 to their active forms. In cultured fibroblasts, significant increases in cell growth and TGF-β levels were observed after chymase injection; these increases were inhibited by a chymase inhibitor, but not by an angiotensin II–receptor blocker. In apolipoprotein E–deficient mice, abdominal aortic aneurysm (AAA) development depends on an increase in MMP-9 activities induced by angiotensin II infusion, but the inhibition of MMP-9 activation by a chymase inhibitor resulted in attenuation of the angiotensin II–induced AAA development. The upregulation of MMP-9 and TGF-β levels is involved in damage to various organs, but these gene expressions are not completely induced by angiotensin II alone. Therefore, chymase inhibition may be useful for attenuating MMP-9 and TGF-β levels, in addition to reducing angiotensin II formation, and this function may provide powerful organ protection. In this review, we propose the possible use of chymase inhibitors as agents to prevent organ damage.

Keywords: angiotensin II, angiotensin-converting enzyme, chymase, matrix metalloproteinase-9, transforming-growth factor-β
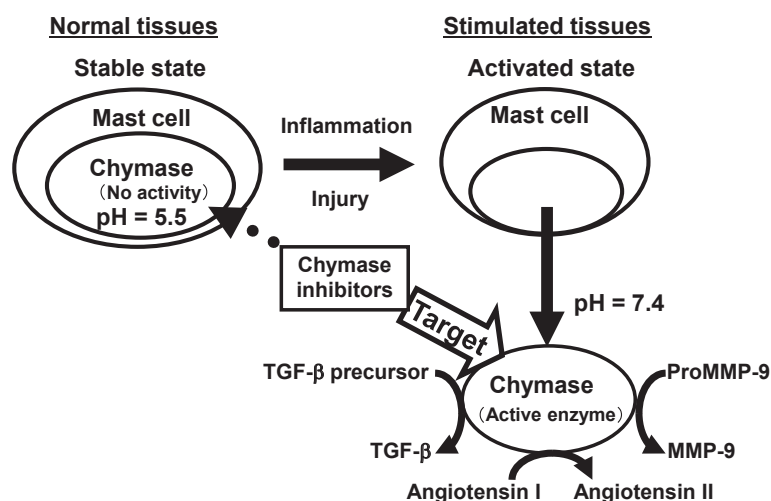
1. Introduction

Chymase (EC 3.4.21.39) is a chymotrypsin-like enzyme that is expressed in the secretory granule of mast cells. Chymase is synthesized as an inactive prochymase, and dipeptidylpeptidase I (DPPI) is necessary for the activation of chymase within the secretory granules. DPPI is a thiol proteinase with an optimal pH of 6.0. Such an optimal pH is consistent with the proposed function of DPPI as a prochymase-activating enzyme because the pH within secretory granules is regulated at pH 5.5 (1, 2). In contrast, the optimal pH of chymase is between 7 and 9, and chymase has no enzymatic activity at pH 5.5, so that it is inactive within mast cells (3, 4). Chymase exhibits enzymatic activity immediately following its release into the interstitial tissues at pH 7.4 following

various stimuli in tissues (Fig. 1). Therefore, chymase has no enzymatic activity in normal tissues, and its activity is only observed in mast cell–stimulated tissues, where it is observed with inflammation. In other words, chymase inhibitors may be expected to have high safety, because there may be no chymase inhibitor target in normal tissues.

Chymase is known to form angiotensin II from angiotensin I in cardiovascular tissues (3 – 8). In many clinical studies, blockade of angiotensin II action by angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs) has been shown to be more useful for cardiovascular protection than other anti-hypertensive drugs. However, in some cases, a dissociation between ACE inhibitors and ARBs has been observed in organ protection. In clinical studies, an ARB was successful in preventing restenosis after percutaneous coronary intervention, but an ACE inhibitor was not (9, 10). In a dog model, an ARB or a chymase inhibitor prevented vascular proliferation after balloon catheter injury, but an

Normal tissues

Stable state

Mast cell

Chymase
（No activity）
pH = 5.5

Inflammation

Injury

**Chymase
inhibitors**

Target

Stimulated tissues

Activated state

Mast cell

pH = 7.4

TGF-β precursor

Chymase
（Active enzyme）

ProMMP-9

TGF-β

MMP-9

Angiotensin I Angiotensin II

Fig. 1. Under the stable state, chymase is stored as an inactive enzyme in secretory granules because the pH within the granule is regulated at pH 5.5, in which chymase has no enzymatic activity. Chymase activity is almost at the maximum level immediately upon release into the extracellular matrix (pH 7.4) in interstitial tissues, when mast cells are activated in inflammatory and injured tissues. Chymase after the degranulation functions as an enzyme for activation of TGF-*β* and MMP-9 and angiotensin II formation. Chymase inhibition may result in attenuating actions via angiotensin II, TGF-*β,* and MMP-9.

ACE inhibitor did not (11, 12). These studies suggest that chymase-dependent angiotensin II formation may play an important role in vascular proliferation after balloon catheter injury. Chymase also converts precursors of transforming growth factor (TGF)-*β* and matrix metalloproteinase (MMP)-9 to their active forms (13 – 17). Both TGF-*β* and MMP-9 are involved in tissue inflammation and fibrosis, resulting in organ damage.

Chymase inhibitors may become a useful strategy for attenuating not only angiotensin II formation but also TGF-*β* and MMP-9 activation, and these functions may be applicable to organ protection. In this review, we propose the possible application of chymase inhibitors to prevent organ damage in various diseases.

2. Vascular proliferation

Percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) is performed in patients with ischemic heart disease. Although an ACE inhibitor failed to suppress restenosis after PCI in a clinical study, the MERCATOR study (9), an ARB was successful in preventing restenosis in a clinical study, the VAL-PREST study (10). ACE inhibitors cannot suppress chymase-dependent angiotensin II formation, and chymase-dependent angiotensin II formation may play an important role in the development of vascular proliferation after PCI. In a dog model, chymase activity was significantly augmented in the injured artery 1 month after balloon catheter injury, but ACE activity was not (12). In this model, both an ARB, candesartan (6 mg/kg per day), and an ACE inhibitor, enalapril (20 mg/kg per day), reduced blood pressure equally, but the ARB significantly attenuated vascular proliferation in the injured arteries, while the ACE inhibitor did not (11). On the other hand, a chymase inhibitor, NK3201 (1 mg/kg per day), attenu-

ated the vascular proliferation without lowering blood pressure (12). As summarized in Table 1, in the placebo-treated group, the circulating renin–angiotensin system and systemic blood pressure were not changed after balloon injury, and chymase activity was augmented in the injured artery. Both the ACE inhibitor and the ARB suppressed blood pressure and increased renin activity, but the chymase inhibitor did not. The ACE inhibitor attenuated ACE activities in the plasma and injured artery, but it did not prevent the vascular proliferation. The ARB attenuated the vascular proliferation, along with the blood pressure–lowering effect. Thus, the increase of chymase-dependent angiotensin II formation in the injured artery might play an important role in the development of vascular proliferation after balloon injury, and a chymase inhibitor might specifically inhibit the augmented chymase activity in the injured artery without systemic effects.

In CABG, the internal thoracic artery and saphenous vein have been frequently used as coronary artery bypass conduits. However, the poor performance of the saphenous vein compared with the internal thoracic artery is called "vein graft disease" (18). In a dog grafted model, the external jugular vein was grafted to the carotid artery (19). The ACE activity in the grafted vein was significantly decreased up to 7 days after grafting, and particularly after 1 and 3 days, it was suppressed in the grafted veins to less than 10% of the control value (20). The reason why the ACE activity was decreased in the acute period after the operation is thought to be dependent on the loss of the endothelium; the endothelium in grafted veins is put under arterial pressure just after grafting, resulting in loss of the endothelium where ACE is located. On the other hand, chymase is located in the media and adventitia in veins (21), and chymase activity was maintained in the grafted veins just after the operation

Table 1. Effects of ARB, ACE inhibitor, and chymase inhibitor on vascular proliferation after balloon injury in dogs

Parameters	Dog balloon-injury model			
	Vehicle	ARB	ACE inhibitor	Chymase inhibitor
Blood pressure	No change	Lowering	Lowering	No effect
Plasma				
Renin	No change	Increase	Increase	No effect
ACE	No change	No effect	Inhibit	No effect
Iinjured artery				
ACE	No change	No effect	Inhibit	No effect
Chymase	Increase	No effect	No effect	Inhibit
Vascular proliferation	Increase	Decrease	No effect	Decrease

and was significantly augmented at 7 days. Considering these findings, up to 7 days after grafting, angiotensin II formation in the grafted veins is thought to depend mainly on the chymase-dependent angiotensin II–forming pathway. Moreover, the angiotensin II concentration and the mRNA levels of fibronectin, collagen I, and collagen III, all of which are induced by increased angiotensin II action, were significantly increased in the grafted veins 7 days after grafting. These findings suggest that chymase-dependent angiotensin II formation may play an important role in increasing extracellular matrix components such as fibronectin, collagen I, and collagen III in vascular tissues. In this model, the vein was infiltrated for 20 min in the solution with a chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)$_2$ (10 μM), before grafting and then grafted to the artery. Seven days after the operation, the angiotensin II concentration and the mRNA levels of fibronectin, collagen I, and collagen III were strongly attenuated in the chymase inhibitor–treated vein. Therefore, the activation of chymase in the grafted veins in the acute phase after grafting may play an important role in the development of vascular proliferation. In fact, a single treatment with Suc-Val-Pro-Phe^P(OPh)$_2$ into grafted veins maintained its attenuating effect on vascular proliferation even 3 months after the operation (22).

Hemodialysis vascular access dysfunction is an important cause of morbidity and hospitalization among hemodialysis patients. This access dysfunction is usually caused by stenosis of the venous side in either native arteriovenous fistulae or polytetrafluoroethylene (PTFE) grafts (23). In a dog model, an arteriovenous fistula was created between the brachial artery and vein. Four weeks after arteriovenous fistula creation, eccentric neointimal formation was most evident in the venous side compared with the arterial side wall in the arteriovenous anastomosis (23). Chymase-positive mast cells were markedly accumulated in the proliferating neointima and media of

the venous side. In association with the reduction in chymase expression, a marked decrease in angiotensin II–positive areas was achieved by a chymase inhibitor (NK3201), and the neointima formation was also significantly suppressed (23). Although an ACE inhibitor (lisinopril) also provided some beneficial effects with regard to the prevention of neointimal formation, it was less than that seen with chymase inhibition (23). The stenosis after the implantation of PTFE grafts was also significantly attenuated by NK3201 (24). These findings indicate that mast cell-derived chymase plays an essential role in the stenosis of arteriovenous fistula or PTFE grafts and that chymase inhibition may be a therapeutic target for the treatment of hemodialysis vascular access dysfunction in clinical settings.

Thus, chymase-dependent angiotensin II formation is deeply involved in the development of vascular proliferation, such as that occurring after PCI, CABG, and arteriovenous fistula creation, and such vascular proliferation may become an important target of chymase inhibitors.

3. Aortic aneurysm

Abdominal aortic aneurysms (AAAs) are generally characterized by widespread destruction of elastic lamellae in the media and by an inflammatory response in the vascular wall. The pathophysiology of AAAs includes aortic atherosclerosis, chronic inflammation within the outer aortic wall, and an imbalance between the production and degradation of structural extracellular matrix proteins (25, 26). MMP-9 is the member of the MMP family that has the highest affinity for elastin as a substrate; it is thus considered to play an important role in the pathology of AAAs (25, 26). In human aorta, chymase-positive mast cells are barely detected in normal vessels, and only in the adventitial area (27). However, in human AAAs, chymase-positive mast cells are de-

tected in the medial area, in addition to the adventitial area, and the number of mast cells is obviously increased in comparison with the normal aorta (27). Using an extract of vascular tissues from human AAAs, we incubated the extract with a chymase inhibitor or purified human chymase for 3 h at 37°C (17). MMP-9 activity was significantly higher in extracts of human AAAs 3 h after incubation than in the extracts that were not incubated; this increase was significantly attenuated by a chymase inhibitor. In contrast, MMP-9 activity was further increased by adding purified human chymase. Furthermore, purified human proMMP-9 was also activated by purified human chymase in vitro, and the cleavage site was determined (17). Thus, chymase can directly activate proMMP-9 to MMP-9 in vitro.

In hamsters and dogs, chymase and total angiotensin II–forming activities were significantly augmented in AAAs induced by elastase (28, 29). Furthermore, the MMP-9 activity was also augmented in the AAAs (29). However, not only chymase but also total angiotensin II–forming and MMP-9 activities in the AAAs were significantly attenuated by a chymase inhibitor, resulted in preventing AAA development (29). Angiotensin II induces MMP-9 expression in vascular tissues, and it has been unclear whether the inhibition of MMP-9 activation by chymase inhibitors might be involved in the prevention of AAA development in these animal models. To clarify the contribution of MMP-9 inactivation by chymase inhibition in vivo, we investigated whether a chymase inhibitor suppresses angiotensin II–induced AAA development in apolipoprotein E–deficient mice (30). AAAs developed at the suprarenal region of the abdominal aorta when angiotensin II (1000 ng/kg per min) was continually infused for 4 weeks and chymase or placebo was given for the same period as the angiotensin II infusion. In this model, a significant expansion of aortic diameter was observed, along with significant augmentation of chymase and MMP-9 activities in the aorta from the placebo-treated group. On the other hand, not only chymase activity but also MMP-9 activity was significantly attenuated in the chymase inhibitor–treated group, and AAA development was prevented. In this model, MMP-9 expression was induced by angiotensin II, and the target of chymase inhibitors might be downstream of angiotensin II action, such as inhibition of MMP-9 activation. In fact, using an extract of AAA, proMMP-9 was mainly activated by chymase in vitro (30). More recently, in patients with AAA, a significant correlation of serum chymase levels and AAA expansion rate was observed (31). Furthermore, in chymase-deficient mice, the development of AAA was significantly attenuated along with reduction of MMP-9 (31). Thus, the mechanism of the chymase inhibitor for preventing AAA development might include not only the inhibition of angiotensin II formation but also the inactivation of proMMP-9.

4. Diabetic retinopathy

In general, chymase is expressed in mast cells, but an increase in chymase has been shown to be associated with deposition of advanced glycation end products (AGEs), and chymase expression was induced by AGEs via its receptor-ERK1/2 MAP kinase pathway in cultured vascular smooth muscle cells (VSMCs) (32). In cultured VSMCs, high glucose levels also upregulated chymase-dependent angiotensin II formation, but not ACE-dependent angiotensin II formation, via ERK1/2 MAP kinase activation (33). Therefore, chymase-dependent angiotensin II formation may be increased by high glucose levels or AGEs in vascular tissues of patients with diabetes, and it may play a crucial role in diabetic-induced tissue remodeling.

In diabetic patients, vascular endothelial growth factor (VEGF) plays a major role in the initiation and development of diabetic retinopathy. VEGF has been shown to be a major regulator of vascular permeability, angiogenesis, and endothelial cell proliferation. Angiotensin II promotes the expression of VEGF on the pericytes surrounding endothelial cells (34). In retina, NADPH oxidase induced by angiotensin II might be involved in VEGF expression in diabetic rats (35). In the vitreous of eyes with diabetic retinopathy, ACE activity, VEGF levels, and MMP-9 levels were significantly augmented; and significant correlations between ACE activity and VEGF level, ACE activity and MMP-9 level, and VEGF and MMP-9 levels were observed (36). VEGF also stimulates MMP-9 expression in human vascular smooth muscle cells (37). On the other hand, AGEs are the ultimate result of non-enzymatic glycation and the oxidation of proteins and lipids, and their accumulation in the microvasculature is known to be a key factor in the development of diabetic retinopathy (38, 39). AGEs increase NADPH oxidase and induce retinal VEGF expression (40). Sugiyama et al. (41) demonstrated that chronic ARB treatment inhibited AGEs and reduced VEGF gene expression in diabetic rats. This finding shows that an inhibitory mechanism related to ARB treatment might be involved in the inhibition of AGEs, suggesting that angiotensin II may increase AGE formation. In contrast, an antisense oligodeoxynucleotide against VEGF has been shown in mouse and nonhuman primate models to prevent neovascularization in diabetic retinopathy (42, 43). MMP inhibitors may also prevent retinal neovascularization in a mouse model (44) Therefore, chymase inhibition may be useful for prevention of diabetic retinopathy via reduction of AGEs and MMP-9 levels.

Chymase inhibition may prevent pancreatic islet degradation. In hamsters with streptozotocin (STZ)-induced diabetes, chymase- and angiotensin II–forming activities were significantly augmented in the pancreas (45). A chymase inhibitor, TY-51469, significantly attenuated blood glucose levels and reduced chymase and total angiotensin II–forming activities, as well as the malondialdehyde level, which indicates oxidative stress. After STZ injection, the number of pancreatic islets was dramatically decreased, but the number was significantly maintained in hamsters treated with TY-51469 (45). In type 2 diabetes in human and animal models, angiotensin II may also contribute to islet disorganization (46), and chymase inhibition may be useful for preventing the progression of type 2 diabetes, in addition to preventing diabetic retinopathy.

5. Cardiac dysfunction and fibrosis

Chymase is involved in cardiac dysfunction after myocardial infarction (47 – 49). In hamsters, chymase- and angiotensin II–positive cells in the cardiac tissues were significantly increased 1 day after myocardial infarction (47). Chymase inhibitors attenuated the chymase activity and the cardiac dysfunction, which extended survival (48, 49). The attenuation of cardiac dysfunction and the extension of survival were also observed with ARB treatment (47). Therefore, chymase-dependent angiotensin II formation may accelerate cardiac dysfunction after myocardial infarction. However, in these studies, it has been unclear whether chymase-dependent angiotensin II is directly involved in the cardiac fibrosis after myocardial infarction (47 – 49).

On the other hand, chymase also contributes to the activation of TGF-β, which is released from a latent TGF-β–binding protein in fibroblasts (14). The latent TGF-β–binding protein is cleaved as latent TGF-β, and the latent form of TGF-β is activated to TGF-β by extremes of pH and by plasmin (50 – 52). Taipale et al. (13) suggested that rat chymase could contribute to the release of latent TGF-β from latent TGF-β–binding proteins of the extracellular matrix of epithelial and endothelial cells. In cultured human fibroblasts, chymase was found to significantly increase cell proliferation in fibroblasts (14). This increased cell proliferation was completely suppressed by a chymase inhibitor, but not by an ARB (14). In media supernatants of cultured fibroblasts, the TGF-β level was significantly increased after the injection of chymase, but this increase in TGF-β level was inhibited by a chymase inhibitor. Anti–TGF-β neutralizing antibody completely suppressed cell proliferation induced by human chymase, indicating that chymase induced the cell proliferation through TGF-β activation

in vitro.

Chymases of humans, hamsters, dogs, and monkeys activate not only angiotensin II but also TGF-β, a major stimulator of myocardial fibrosis, while rat chymase activates TGF-β but not angiotensin II (13, 14, 53 – 55). To clarify the role of chymase-dependent TGF-β activation, we evaluated whether chymase inhibition prevents cardiac fibrosis and cardiac dysfunction after myocardial infarction in rats (56). Four weeks after myocardial infarction, echocardiography revealed that chymase inhibitor treatment reduced the akinetic area and improved cardiac function (56). Chymase activity in the non-infarcted myocardium was significantly increased in the vehicle group, but it was significantly reduced by chymase inhibitor treatment (56). The fibrotic area in the cardiac tissues and the mRNA levels of collagen I and collagen III were also significantly lower in the chymase inhibitor–treated group. Recently, we also demonstrated that cardiac fibrotic areas under intermittent hypoxia were also significantly attenuated by treatment with a chymase inhibitor in rats (57). Therefore, the pathway forming chymase-dependent TGF-β may play an important role in myocardial fibrosis and cardiac dysfunction after myocardial infarction.

Mast cells are found in increased numbers in the myocardial fibrotic area in cardiomyopathy, and this increase in the number of cardiac mast cells may contribute to the development of fibroblast proliferation in cardiac tissues of cardiomyopathic patients (58). On the other hand, increased TGF-β levels are observed in the cardiac tissue of cardiomyopathic patients and hamsters, which induce the expression of collagen I and collagen III genes (59, 60). In a hamster model of cardiomyopathy, mast cell number and chymase activities in cardiac tissues were significantly increased along with the increases of collagen I and collagen III gene expressions (14). In this model, a chymase inhibitor, BCEAB, significantly suppressed chymase activity, mRNA levels, and the fibrotic area in the heart, which prevented cardiac dysfunction (14). However, the chymase inhibitor could not reduce the cardiac hypertrophy. The administration of anti–TGF-β neutralizing antibody prevented both the expression of collagen genes and cardiac fibrosis, but not cardiac hypertrophy, in rats with hypertensive myocardial fibrosis (61). Gene expression of TGF-β is known to be induced by angiotensin II. Chymase can produce angiotensin II from angiotensin I, and this angiotensin II produced by chymase may be involved in the pathogenesis of cardiac fibrosis in cardiomyopathic hamsters. However, the ARB reduced not only the cardiac fibrosis but also the cardiac hypertrophy in the hamster model of cardiomyopathy (62, 63). The difference between ARBs and chymase inhibitors with respect to the effect on cardiac hypertro-

phy suggests different mechanisms are involved in their improvement of cardiac function. Increases in cardiac chymase activity in cardiomyopathy may contribute to TGF-β activation rather than angiotensin II formation, and this may play an important role in inducing cardiac fibrosis.

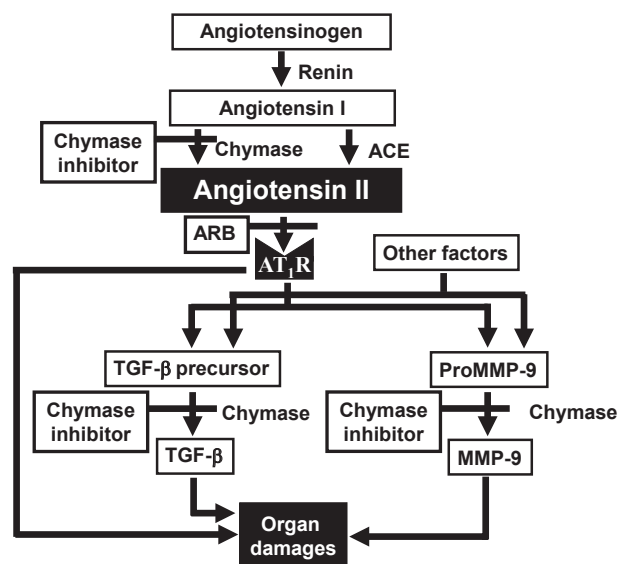## 6. Target diseases other than cardiovascular diseases

Various diseases other than cardiovascular diseases may be appropriate targets for chymase inhibitor treatment. For example, ulcerative colitis is an inflammatory bowel disease (IBD) affecting the distal colon, and MMPs are reportedly involved in the pathogenesis of IBD via degradation of extracellular matrix (64). In particular, MMP-9 may play an important role in the degradation of ECM in ulcerative colitis. Oral administration of dextran sodium sulfate (DSS) is used to induce colitis in animal models, and the resulting colonic lesions resemble those observed in patients with ulcerative colitis (65). DSS-induced colitis is significantly attenuated in MMP-9–deficient mice (66). MMP-9 inhibition might thus offer a useful strategy for attenuating the development of colitis. Using this mouse model, both chymase and MMP-9 activities were significantly increased after DSS administration (67). We recently demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs)-induced small intestinal damage was involved in chymase-dependent MMP-9 formation (68). In these models, the disease activity index and histological damage score were significantly attenuated by chymase inhibitors via inhibition of chymase and MMP-9 activities (67, 68).

Chymase may be involved in the pathogenesis of tissue fibrosis that occurs in tissues other than cardiac tissues. In patients with idiopathic pulmonary fibrosis, TGF-β is increased, and in animal models of bleomycin-induced pulmonary fibrosis, TGF-β may also play an important role in the development of pulmonary fibrosis (69). For example, administration of anti–TGF-β antibodies could reduce bleomycin-induced pulmonary fibrosis via reduction of collagen mRNA levels (70). The chemotherapeutic agent bleomycin is known to cause lung fibrosis in humans and animal experimental models. In bleomycin-induced pulmonary fibrosis in hamsters, significant increases of chymase activity and fibrotic areas in pulmonary tissues after bleomycin treatment were significantly reduced by treatment with a chymase inhibitor (71). Fun et al. (72) demonstrated the significance of chymase-dependent angiotensin II formation to the progression of tubulointerstitial fibrosis in obstructed kidneys in hamsters. In human liver fibrosis, both chymase and angiotensin II levels were significantly aug-

mented, and significant correlations between chymase and angiotensin II levels, between chymase level and fibrotic degree, and between angiotensin II level and fibrotic degree were observed (73). Furthermore, chymase inhibition significantly attenuated the liver fibrosis in experimental animal models (74, 75). Therefore, chymase inhibitors may be promising for the prevention of tissue fibrosis via attenuation of angiotensin II formation and TGF-β activation.

7. Conclusion

Chymase is known to increase tissue levels of angiotensin II formation, and its function directly induces the development of cardiovascular remodeling. Furthermore, angiotensin II also indirectly promotes cardiovascular damage via augmentation of MMP-9 and TGF-β gene expressions. Although MMP-9 and TGF-β are known to be strongly involved in the pathogenesis of tissue remodeling, these factors are not necessarily induced only by angiotensin II. Factors other than angiotensin II stimulation contribute to the augmentation of MMP-9 and TGF-

Fig. 2. Angiotensinogen is cleaved to form angiotensin I by renin. Angiotensin I is mainly converted to angiotensin II by ACE and chymase. Angiotensin II directly induces organ damage and also indirectly promotes cardiovascular damage via augmentation of MMP-9 and TGF-β gene expressions. Although MMP-9 and TGF-β are known to be strongly involved in the organ damage, these factors are not necessarily induced only by angiotensin II. Factors other than angiotensin II stimulation contribute to the augmentation of MMP-9 and TGF-β gene expressions. ARBs are not able to attenuate the MMP-9 and TGF-β actions via the induction by factors other than angiotensin II, but chymase inhibitors may attenuate their actions via inhibition of their activation, indicating that they may be able to prevent organ damage. AT$_1$R: angiotensin II type 1 receptor.

β gene expressions. In such cases, ARBs are not able to attenuate the MMP-9 and TGF-β actions, but chymase inhibitors may attenuate their actions via inhibition of their activation, indicating the possibility for preventing damage to various organs, including cardiovascular tissues (Fig. 2). Chymase may become an important target to prevent organ damage in various diseases.

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