Increased Chymase-Dependent Angiotensin II Formation in Human Atherosclerotic Aorta

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Abstract—Locally formed angiotensin II (Ang II) and mast cells may participate in the development of atherosclerosis. Chymase, which originates from mast cells, is the major Ang II–forming enzyme in the human heart and aorta in vitro. The aim of the present study was to investigate aortic Ang II–forming activity (AIIFA) and the histochemical localization of each Ang II–forming enzyme in the atheromatous human aorta. Specimens of normal (n=9), atherosclerotic (n=8), and aneurysmal (n=6) human aortas were obtained at autopsy or cardiovascular surgery from 23 subjects (16 men, 7 women). The total, angiotensin-converting enzyme (ACE)-dependent, and chymase-dependent AIIFAs in aortic specimens were determined. The histologic and cellular localization of chymase and ACE were determined by immunocytochemistry. Total AIIFA was significantly higher in atherosclerotic and aneurysmal lesions than in normal aortas. Most of AIIFA in the human aorta in vitro was chymase-dependent in both normal (82%) and atherosclerotic aortas (90%). Immunocytochemical staining of the corresponding aortic sections with antichymase, antitryptase or anti-ACE antibodies showed that chymase-positive mast cells were located in the tunica adventitia of normal and atheromatous aortas, whereas ACE-positive cells were localized in endothelial cells of normal aorta and in macrophages of atheromatous neointima. The density of chymase- and tryptase-positive mast cells in the atherosclerotic lesions was slightly but not significantly higher than that in the normal aortas, and the number of activated mast cells in the aneurysmal lesions (18%) was significantly higher than in atherosclerotic (5%) and normal (1%) aortas. Our results suggest that local Ang II formation is increased in atherosclerotic lesions and that chymase is primarily responsible for this increase. The histologic localization and potential roles of chymase in the development of atherosclerotic lesions appear to be different from those of ACE. (Hypertension. 1999;33:1399-1405.)

Key Words: renin-angiotensin system ■ atherosclerosis ■ angiotensin-converting enzyme ■ immunohistochemistry

Angiotensin II (Ang II), the final effector of the renin-angiotensin system, is thought to be involved in the initiation and development of atherosclerosis, because Ang II has numerous biological actions, including cell proliferation, migration, induction of adhesion molecules, activation of monocytes, matrix production, and induction of NADH and NADPH oxidase activity. Angiotensin-converting enzyme (ACE) inhibitors, which specifically block the renin-angiotensin system, are effective for reducing mortality and morbidity in patients with various cardiovascular diseases, including hypertension, congestive heart failure, and ischemic heart disease. The cardiovascular protective effects of ACE inhibitors could be attributed to their antiatherogenic effects. Increased ACE expression has been recognized in atherosclerotic lesions of the coronary artery. These results imply that activation of the tissue renin-angiotensin system, especially ACE, is associated with the development of atherosclerosis. ACE is generally considered to be the main Ang II–forming enzyme in the systemic circulation. In tissues, however, many serine proteinases, such as kallikrein, cathepsin G, and chymase, appear to be capable of forming Ang II. In addition, marked organ and species differences have been observed with regard to the pathways of tissue Ang II formation. Non–ACE-dependent Ang II formation in the human heart in vitro was found to be mainly due to chymase, a more potent and specific Ang II–forming serine proteinase than known Ang II–forming enzymes. Chymase immunoreactivity has been found in the secretory granules of mast, endothelial, and mesenchymal cells, and, on its release, chymase colocalized with the extracellular matrix.

A recent study demonstrated that mast cells might be involved in the development of atheromatous lesions because they accumulate in such lesions of the human arterial wall. Coronary arteries from patients who died of coronary heart disease contain significantly higher concentrations of histamine than those of control subjects. Antihistamine agents have been shown to have antiatherogenic effects. Mast-cell granules promote LDL uptake by macrophages, and stimula-
tion of mast cells may lead to the accumulation of cholesteryl esters in macrophages. Although these reports have suggested that mast cells are associated with the development of atherosclerosis, the pathological role of mast-cell chymase has not yet been clarified.

In the present study, we focused on the association between aortic Ang II–forming activity (AIIFA) and atherosclerosis. Total, ACE-dependent, and chymase-dependent AIIFA were determined in normal, atherosclerotic, and aneurysmal human aorta. The histologic localization of these Ang II–forming enzymes was also identified in normal and atherosclerotic aorta.

Methods

Reagents
An antichymase rabbit IgG was obtained as described previously. Peptide-based antibody for chymase was a kind gift from Suntory Peptide Institute Inc, respectively. An antitryptase monoclonal antibody was purchased from Chemicon, and a mouse monoclonal antibody (KP-1) came from DAKO. An antitryptase antibody was purchased from Chemicon, and a mouse monoclonal antibody (KP-1) came from DAKO. Naphthol AS-D chloroacetate, a histochemical substrate; captorpril; chymostatin; and other chemicals were purchased from Sigma Chemical Co. Aprotinin and angiotensin I were purchased from Bayer and Peptide Institute Inc, respectively.

Human Samples
Samples taken at autopsy included 4 of 9 normal, 3 of 8 atherosclerotic, and 0 of 6 aneurysmal aorta. The other samples were obtained during cardiovascular surgery at Fukuoka University Hospital. Most autopsy samples were obtained within 4 hours after death; 2 of 7 aortas were obtained 12 hours after death, but the cadavers were kept at 4°C before autopsy. None of the donor subjects (16 men, 7 women), aged 46 to 83 years, had been treated with ACE inhibitors. All aortic specimens contained intima, media, and adventitia, as confirmed histologically. The use of human aorta was approved by the Internal Review Committee of Fukuoka University and handling was performed in accordance with institutional guidelines.

Preservation of Tissues and Preparation of the Aortic Particular Fraction
The handling and preparation of the aortic particulate fraction were performed according to the method described previously by our laboratory.

Assessment of AIIFA in Particulate Fractions
Assessment of AIIFA was performed according to the method described previously by our laboratory.

Histopathologic and Immunohistochemical Studies
For pathological analysis, tissues were fixed with 10% formalin and embedded in paraffin wax. Sections were cut 3-μm thick and stained with hematoxylin and cosin and elastica–van Gieson stain. To identify mast cells, sections were stained by toluidine blue and antitryptase antibody. Immunohistochemistry of the aortic tissues was gathered according to the methods described by Hsu et al. Sections were stained by the avidin–biotin alkaline phosphatase method. Because preliminary results obtained with both protein and peptide antibodies for chymase immunohistochemistry were similar, all further immunohistochemical analyses were performed using the peptide antibody. The paraffin-embedded sections were deparaffinized in xylene and dehydrated in a graded series of ethanol solutions. Sections were then rinsed 3 times with a washing solution (0.05 mol/L Tris-HCl buffer containing 0.145 mol/L NaCl, pH 7.4) for 5 minutes at room temperature. Preliminary experiments indicated that protease treatment for antichymase antibody and autolysis treatment for anti-ACE antibody were necessary to unmask antigenicity and to obtain optimal staining similar to that seen with frozen sections. Pretreatment using antitryptase or cathepsin G antibody was not necessary for the study. Immunohistochemical staining using frozen sections was also performed to clarify the appropriateness of pretreatment for the paraffin-embedded sections (data not shown). Because the pattern of immunohistochemical staining was similar in both frozen and paraffin-embedded sections after adjustment, as described above, paraffin-embedded sections were used throughout the study. After sections were incubated for 5 minutes in a blocking solution containing 10% normal nonimmune serum (horse or goat) from species from which the secondary antibody was obtained, sections were incubated for 1 hour with the primary antibody (anti-human chymase polyclonal antibody 50 μg/mL, anti-human ACE polyclonal antibody 0.6 μg/mL, antihuman trypstyle monoclonal antibody 0.32 μg/mL, or anti–cathepsin G antibody 18 μg/mL) at room temperature. After being rinsed with the washing solution, sections were incubated with biotin-labeled secondary antibody (horse anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin) for 30 minutes at room temperature and washed 3 times in the washing solution. Sections were applied to peroxidase-conjugated avidin for 30 minutes at room temperature and then rinsed 3 times with the washing solution. Sections were incubated in a freshly prepared solution of 0.5 mmol/L naphthol AS-BI–phosphoric acid, 0.27 mmol/L azoized new fuchsin, and 1 mmol/L levamisole in 0.2 mol/L Tris buffer, pH 8.5, until staining was observed to be adequate (≈15 to 30 minutes). Sections were then rinsed 3 times with the washing solution, postfixed in 10% buffered formalin for 10 minutes, counterstained with hematoxylin, and mounted. First, we checked the specificity of each antibody against chymase and ACE, as described below. Negative controls were prepared by replacing the primary antibodies with preimmune rabbit IgG. A positive control for immunohistochemical staining was obtained using sections from the intact small intestine and the spleen of a mastocytoma patient for chymase and from normal aorta for ACE. This test staining was repeated several times until stable optimal staining was achieved for each protein.

Enzymohistochemistry
The frozen sections adjacent to those used for immunohistochemical staining were used for enzyme histochemistry. The enzymatic activity of the chymase (chymostatin-sensitive enzyme) was visualized by the method of Seppä using naphthol AS-D chloroacetate as a substrate. The reaction medium consisted of (1) 0.1 mol/L potassium phosphate buffer, pH 6.0; (2) 2 mg/mL freshly prepared and filtered Fast Garnet GBC salt in buffer (step 1); and (3) 5 mg/mL naphthol AS-D chloroacetate in DMSO. All 3 reagents were mixed at a proportion of 60:20:1, respectively, and filtered before use. Sections were incubated in the reaction medium overnight at 4°C, rinsed in water, and mounted. Positive cells were identified by their brown color.

Morphometric Analysis
Various magnifications were used for the morphometric analysis: ×200 for counting the immunopositive mast cells (chymase and trypstyle antibody–positive cells) and toluidine blue–positive cells, ×400 for counting degranulated mast cells, and ×100 for counting ACE antibody–positive cells. We counted the number of cells in 4 and 16 visual fields, which corresponded to 1 mm² at magnifications of ×200 and ×400, respectively. We present the average value for each section. Such counting was performed in normal, atherosclerotic, and aneurysmal lesions of human aortas. Extracellular localization of >5 isolated secondary granules scattered around mast cell was considered to be a sign of degranulated and activated mast cells.
mast cells. In stable mast cells, the secretary granules were packed in the cytoplasm and were not identified as isolated granules by light microscopic examination.

Statistical Analysis
All numerical data shown in the text represent the mean ± SEM. Statistical analyses were performed using Scheffe’s F test following 1-way ANOVA to compare the levels of total, ACE-dependent, and chymase-dependent AIIFA in normal, atherosclerotic, and aneurysmal aortas. The number of positive or degranulated mast cells under different pathological conditions was also counted as described above. A value of \( P < 0.05 \) was considered statistically significant.

Results
AIIFA in Normal and Atherosclerotic Aorta
A representative high-performance liquid chromatographic analysis of AIIFA in normal and atherosclerotic aortas is presented in Figure 1. Total AIIFA in atherosclerotic lesions was higher than in normal aortas (Figure 1). This activity was minimally inhibited in the presence of captopril but was significantly inhibited in the presence of chymostatin in both normal and atherosclerotic aortas (Figure 1). Figure 2 shows that total AIIFA in atherosclerotic lesions and aortic aneurysmal lesions was significantly higher than in the normal aorta. Significant increases in chymase-dependent AIIFA in atherosclerotic (93.5% versus total AIIFA) and aneurysmal lesions (94.8% versus total AIIFA) were observed compared with the normal aorta (85.6% versus total AIIFA) (Figure 2). On the other hand, ACE-dependent AIIFA did not show any significant change in atherosclerotic (4.9% versus total AIIFA) or aneurysmal lesions (13.8% versus total AIIFA) compared with those in normal aorta (4.5% versus total AIIFA) (Figure 2). Similarly, no significant difference was noted in non–ACE-dependent, nonchymase-dependent AIIFA among normal, atherosclerotic, and aneurysmal aorta.

Localization of Chymase and ACE in Human Intact and Atherosclerotic Aorta
Figure 3 shows representative immunohistochemical negative and positive staining for chymase (Figure 3A and 3B) and ACE (Figure 3C and 3D). As positive controls, chymaselike (Figure 3B) and tryptaselike (Figure 3E) immunoreactivities were clearly found in the cytosolic region of mast cells in the small intestine, and ACE-like immunoreactivity was seen in...
endothelial cells of the vasa vasorum located in the adventitia of the aorta (Figure 3D). The analysis of adjacent sections using preimmune serum (Figure 3A and 3C) did not show any positive signal. Sections obtained from normal and diseased aortas were processed using these staining conditions.

Figures 4, 5, and 6 show representative immunohistochemical staining for ACE (A), chymase (B), and tryptase (C), and enzymohistochemical visualization of chymase-like enzymatic activity (D) in normal (Figure 4), atherosclerotic (Figure 5), and aneurysmal (Figure 6) aortas. ACE immunoreactivity in the normal aorta was seen only in endothelial cells (Figure 4A), a finding similar to the results of a previous study and our preliminary study (Figure 3D). Positive immunoreactivity for chymase or tryptase appeared in mast cells, which were located mainly in the adventitia of the normal aorta (Figure 4B and 4C). These results in normal aortas indicated that mast cells that were immunohistochemically and enzymohistochemically positive for chymase and tryptase were detected mainly in the adventitia but rarely in the intima and media.

The intensity of ACE immunoreactivity in the luminal side endothelial cells of the atherosclerotic or aneurysmal aorta was almost equal to that of the normal aorta (data not shown). In addition to endothelial cells, ACE-positive cells were detected in the neointima of the atherosclerotic (Figure 5A) and aneurysmal (Figure 6A) lesions but not in the media or adventitia except in the endothelial cells of the vasa vasorum. These ACE-positive cells in the neointima appeared to be macrophages because they were also positive for KP-1 antibody, a specific marker for macrophage (data not shown). On the other hand, tryptasellike and chymaselike immunoreactive cells seemed to be increased in atherosclerotic aorta. These immunoreactive cells were mainly located in the tunica adventitia (Figures 5B and 5C, 6B, and 6C) and were negative for KP-1 staining (data not shown). These results indicate that tryptasellike and chymaselike immunoreactive cells are not macrophages but mast cells.

Unlike ACE, the histologic localization of tryptasellike or chymaselike immunoreactive cells in the atherosclerotic aorta did not differ from those of normal aorta. Anti–cathepsin G antibody–positive cells were scarcely observed in both intact and atherosclerotic aortas (data not shown), indicating that active neutrophil invasion was not apparent. This finding supported our biochemical data, which showed no significant difference in non–ACE-dependent, nonchymase-dependent, namely, cathepsin G–dependent or kallikrein-dependent AIIFA, between normal and atherosclerotic aorta.

To determine the number of tryptase-containing or both tryptase- and chymase-containing mast cells, the number of mast cells was counted after staining for chymase or tryptase in normal and atherosclerotic aortas. The density of mast cells immunoreactive for chymase versus tryptase in normal, atherosclerotic, and aneurysmal lesions was 3.8±0.8 versus 5.3±1.3/mm², 5.7±1.8 versus 8.8±2.6/mm², and 6.0±1.7 versus 12.2±3.0/mm², respectively. Although the average values of chymase or tryptase antibody–positive mast-cell
densities were relatively higher in atherosclerotic and aneurysmal aortas than those in normal aortas, the difference was not statistically significant.

In aneurysmal aorta, which indicates an advanced form of atherosclerosis, mast-cell granules were scattered around mast cells and located in the interstitium (Figure 6B, 6C, and 6D), which indicates that chymase and trypsin were secreted from mast-cell granules, a sign of activated mast cells (Figure 3F). Therefore, the density of degranulated mast cells was analyzed as a marker for activated mast cells. Figure 7 shows the percentage of degranulated mast cells in normal, atherosclerotic, and aneurysmal aortas. The proportion of degranulated mast cells in the aneurysmal lesions was significantly higher than in the atherosclerotic lesions and normal aortas.

### Discussion

Indirect, but not direct, evidence supports the notion that activation of the tissue renin-angiotensin system, especially ACE, is associated with the development of atherosclerosis in humans. ACE expression is augmented in atherosclerotic lesions of the coronary artery and is found mainly in macrophages and the endothelium of neovascularization. Furthermore, ACE expression in cultured monocytes is enhanced 3-fold after differentiation of the cells into macrophages and increases even more after stimulation with acetylated LDL. Using an experimental model, Nickenberg et al demonstrated a significantly higher density of AT1 receptor and its mRNA in hypercholesterolemic rabbit aortas versus normocholesterolemic rabbits. ACE inhibitors prevent the development of atherosclerosis in Watanabe rabbits. These results support the concept that increased angiotensinergic input at the tissue level is associated with the development of atherosclerotic changes.

In fact, the immunohistochemical data in the present study indicate that ACE antibody–positive cells, mostly macrophages, were increased in the neointimal lesion of the atherosclerotic or aneurysmal aorta (Figures 5A and 6A). These findings were in agreement with those of previous studies. However, our biochemical analysis showed that ACE-dependent AIIFAs in atherosclerotic or aneurysmal lesions were not significantly higher than in the normal aorta. The average value of the diseased aorta appeared to be higher but did not reach a statistically significant level, probably due to a considerable data variation. It is likely that the discrepancy between biochemical and immunohistochemical data was due to the heterogeneous distribution of ACE antibody–positive cells because the location of these ACE positive macrophages was restricted to the neointimal plaque lesion. ACE-positive macrophages were not found in the media or adventitia. On the other hand, in biochemical analysis, the whole aorta was used to measure ACE-dependent AIIFA, and the results showed that the change in ACE activity per mg protein was small and did not reach a statistically significant level.

No detailed information is available regarding non–ACE-dependent, especially chymase-dependent, Ang II formation in the human atherosclerotic aorta. The present results are the first to demonstrate that AIIFA in human atherosclerotic aorta in vitro is significantly higher than in normal aorta and that the main enzyme responsible is chymase, not ACE nor cathepsin G. These results indicate that the increased aortic Ang II formation caused by chymase, in addition to that caused by ACE, could be associated with the progression of vascular diseases such as atherosclerosis. This hypothesis is supported by the fact that, in monkeys, high-cholesterol diet induced aortic atherosclerosis with increased vascular chymase expression. Several reports have indicated that chymase could be involved in tissue degradation by activating procollagenase or by directly degrading extracellular matrix proteins such as fibronectin and fibrinogen. These direct actions of chymase are independent of its AIIFA. Because the main actions reported for Ang II are cell proliferation and migration but not tissue degradation, higher tissue Ang II formation is unlikely to be the mechanism for the development of aneurysmal lesions, which are characterized by thinning of the medial layer of the aorta. A previous study reported many degradative enzymes are involved in the development of an aneurysm. Therefore, the degradative actions of chymase are more likely to be involved in the progression of aneurysmal changes. Our study showed a significant increase in the density of degranulated mast cells in aneurysmal aortas but not in normal or atherosclerotic aortas, which indicates that the secreted degradative enzymes, including chymase from activated mast cells, could be involved in the aneurysmal changes in the human aorta. The results of the present study imply that chymase could be associated with atheromatous or aneurysmal changes via different mechanisms.

Studies have reported that chronic adventitial stimulation with affinity-gel resins containing interleukin-1β (IL-1β), a cytokine known to be involved in the development of atherosclerosis, induced coronary intimal lesions and vasospastic responses in pigs in vivo. In this regard, human chymase is involved in the conversion of IL-1β precursor to active IL-1β. In addition, recent studies have shown that the adventitia may be involved in the development of vascular lesions after balloon injury or by atherosclerosis. Although the detailed mechanisms are not clear, the results of the present and other studies suggest that adventitial chymase
may be involved in the formation of atherosclerotic plaques. Because ACE in atherosclerotic artery is located in the neointimal lesion, the histologic distribution of ACE and chymase is different not only in normal arteries but also in atherosclerotic arteries, which indicates that the pathological contribution of chymase in the development of atherosclerosis differs from that of ACE.

We have recently demonstrated the presence of considerable species and organ differences in the tissue AIIFA and its component. For example, the cardiac AIIFA in humans was higher than that in other species, and the majority of the cardiac AIIFA in humans, dogs, monkeys, and hamsters was due to chymase, whereas in rats, rabbits and pigs, it was due to ACE. Furthermore, rat chymase is an Ang II–degrading enzyme, whereas chymases in humans, monkeys, and dogs are Ang II–generating enzymes. A recent clinical megatrial, Evaluation of Losartan In The Elderly, showed that the AT1 receptor antagonist losartan was superior to the ACE inhibitor captopril for reducing mortality in elderly patients with congestive heart failure (about half of the deaths were due to ischemic heart disease). One possible explanation for the difference in the beneficial outcome between ACE inhibitor and AT1 receptor antagonist is that all Ang II derived from both ACE and non-ACE enzymes could be blocked by AT1 receptor antagonist but not by ACE inhibitor. These clinical data suggest that non–ACE-dependent Ang II formation in humans, which probably results from chymase, may play some role in the pathological processes of atherosclerotic cardiovascular diseases.

In summary, increased chymase-dependent AIIFA in vitro was found in atherosclerotic and aneurysmal lesions in the human aorta. The histologic distribution of chymase is distinct from that of ACE, which suggests that chymase and ACE may have different pathological roles in the development of atherosclerosis. A further study using a specific chymase inhibitor is necessary to identify the pathophysiologic role of chymase in development of cardiovascular disease.

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