Increased Chymase Activity in Internal Thoracic Artery of Patients With Hypercholesterolemia

Yoshinari Uehara, Hidenori Urata, Manabu Sasaguri, Munehito Ideishi, Noriyuki Sakata, Tadashi Tashiro, Michio Kimura, Kikuo Arakawa

Abstract—Apart from ACE, various angiotensin II (Ang II)–forming serine proteinases (eg, chymase, kallikrein, and cathepsin G) are known to exist in human tissues, but their clinical significance or the regulatory mechanisms that control their activities are not well established. A recent clinical study has shown that chymase activity was significantly increased in human atherosclerotic or aneurysmal aorta. The association between vascular Ang II–forming activities (AIIFAs) in the human internal thoracic artery (ITA) and various clinical parameters was studied with the use of ITAs obtained from 32 patients who underwent coronary artery bypass graft surgery. Total and ACE- and chymase-dependent AIIFAs in homogenates of ITAs were determined. Total AIIFA was 8.67±0.86 (nmol Ang II formed · min⁻¹ · mg protein⁻¹ [U]), and ≈95% of the activities were due to chymase. Serum total cholesterol level, but no other risk factors, significantly correlated with chymase- (r=0.60, P<0.001) and ACE- (r=0.35, P<0.05) dependent AIIFAs, respectively. LDL cholesterol level was also correlated with chymase-dependent AIIFAs (r=0.47, P<0.05). Mast cells identified through the use of toluidine blue or immunohistochemical staining appeared in the adventitia but not in the intima or media of ITAs. Our results suggest that an increased plasma LDL cholesterol level may induce increased arterial chymase and ACE activity. (Hypertension. 2000;35:55-60.)

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

In addition to the circulating renin-angiotensin system (RAS), RAS appears to be present in various tissues and plays some role in cardiovascular homeostasis and remodeling. Locally formed angiotensin II (Ang II) may be involved in the initiation and development of atherosclerosis. Ang II stimulates growth,1 migration,2 and matrix production in smooth muscle cells3; increases the expression of adhesion molecules; and activates monocytes and promotes their adhesion to endothelial cells. Recent studies have shown that Ang II induces superoxide overproduction, which promotes the proliferation of vascular smooth muscle cells, lipid peroxidation, inactivation of nitric oxide, and stimulation of adhesion molecule expression.4,5 These direct and indirect effects of Ang II appear to contribute to the formation of atherosclerotic lesions.

Recent studies have demonstrated the existence of alternative Ang II–forming pathways, independent of ACE. Several serine proteinases, such as chymase, kallikrein, and cathepsin G, are probably responsible for ACE-independent Ang II–forming activity (AIIFA) in human tissues.6 Levels of AIIFA and the responsible enzymes differ markedly among species and organs.7,8 In humans, the tissue content of chymase is much higher than that of ACE in several organs, and in the heart homogenate, it contributes to >80% of Ang II–forming capacity in vitro9,10 and ex vivo.10 Although the pathophysiological role of human chymase is still unclear, we recently demonstrated that human atherosclerotic or aneurysmal aorta contained significantly higher levels of chymase-dependent AIIFA (dAIIFA) compared with nonatherosclerotic aorta.11 However, these clinical and experimental studies did not provide the mechanisms or the timing of Ang II involvement in the atherosclerotic process. In the present study, we examined human internal thoracic arteries (ITAs) to determine the clinical factors that affect AIIFA in ITAs.

Methods

Patients

Thirty-two patients (29 men and 3 women; mean age 63.9±1.4 years; age range 47 to 78 years) who had severe coronary stenosis underwent coronary arteriography. As listed in Table 1, 7 of 32 patients were treated with ACE inhibitors, 9 were treated with pravastatin, 6 were treated with a β-adrenoreceptor–selective antagonist, and 13 and 12 were treated with dihydropyridine or nondihydropyridine calcium antagonists, respectively. A coronary vasodilator (isosorbide dinitrate) was prescribed for all patients.

All patients underwent coronary artery bypass graft surgery at Fukuoka University Hospital between November 1995 and June 1997. The use of human ITAs was approved by the Internal Review Committee of Fukuoka University, and handling was performed in...
Patient Characteristics

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ACE-I indicates ACE inhibitor; β1-A, β1-adrenergic receptor–selective antagonist; Ca-A, calcium channel antagonist; D, dihydropyridine; Non-D, nondihydropyridine; HT, hypertension; DM, diabetes; SBP, systolic blood pressure (mm Hg); DBP, diastolic blood pressure (mm Hg); T-Cho, serum total cholesterol concentration (mmol/L); HDL, serum HDL cholesterol concentration (mmol/L); and LDL, serum LDL cholesterol concentration (mmol/L).

accordance with institutional guidelines. Informed consent was obtained from each patient before surgery.

Clinical parameters, including serum total, LDL, and HDL cholesterol concentrations, were determined immediately before bypass surgery. LDL cholesterol was calculated according to the method of Friedewald et al. The coronary score, a severity score of coronary stenosis, was calculated according to the method of Gensini. The table provides a summary of patient characteristics, drugs prescribed, risk factors, and serum lipid profiles.

**Histological Studies**

Each ITA was obtained during bypass surgery. The ITA was immediately placed in ice-cold saline, frozen at −80°C within 30 minutes of removal, and stored separately for histological or biochemical study. ITAs were cut into 3- to 5-mm-thick transverse segments; 4-μm-thick frozen sections were cut after embedding with TissueTek OCT Compound (Miles Inc) and then placed on glass slides. For histochemical staining, sections were fixed in 10% formalin and either directly stained with hematoxylin-eosin or treated with 6% BSA. Mouse monoclonal antibodies to human tryptase and chymase (Chemicon International Inc) and a rabbit polyclonal antibody to human ACE (a kind gift from Prof Kunio Hiwada and Dr Katsuhiko Kohara, Ehime University, Department of Internal Medicine, Ehime, Japan) and cathepsin G (Calbiochem-Novabiochem International) were diluted in PBS (pH 7.2) to adjust the antibody concentration to 0.32, 50, 0.6, and 18 μg/mL, respectively. After overnight exposure to these primary antibody solutions at 4°C, sections were treated with the corresponding second antibody against the primary antibody. Then, alkaline phosphatase-conjugated streptavidin (DAKO) diluted 1:100 in the washing buffer containing human serum (1:20 dilution) and was applied for 30 minutes at room temperature. After washing, alkaline phosphatase was developed with the use of New Fuchsin substrate (DAKO).
Biochemical Enzyme Assay

After confirmation that ITAs lacked marked atherosclerotic lesions on the basis of histological examination, chymase, ACE, and cathepsin G dAIIFAs were analyzed according to the same procedure described elsewhere. Briefly, the ITAs were placed in 50 mmol/L NaH2PO4 buffer (0.5 g/5 mL), pH 7.4, at 4°C and homogenized with a Polytron homogenizer (Kinematica GMBH) at 9000 rpm for 15 seconds at 4°C. The homogenates were centrifuged at 30 000 g for 20 minutes at 4°C. The supernatants were discarded, the pellets were resuspended in the same buffer, and homogenization and centrifugation were repeated once. With the use of a hand-driven glass/glass homogenizer, the final tissue pellets were resuspended in 50 mmol/L NaH2PO4 buffer, pH 7.4, containing 100 mmol/L NaCl and 10 mmol/L MgCl2. The protein concentration of the particulate fraction was measured with the use of Protein Assay Reagent (Pierce).

The particulate fraction prepared as described was incubated with synthetic Ang I (200 μmol/L) with or without inhibitors at 37°C for 15 minutes. The formed Ang II was analyzed through the use of HPLC (Shimazu) with a C18 reversed phase HPLC column (2.2 μm synthetic Ang I (200 (Pierce). The particulate fraction was measured with the use of Protein Assay Reagent (Pierce). ACE and chymase dAIIFAs were analyzed according to the same procedure on the basis of histological examination, chymase, ACE, and cathepsin G dAIIFAs were analyzed according to the same procedure described elsewhere. Briefly, the ITAs were placed in 50 mmol/L NaH2PO4 buffer (0.5 g/5 mL), pH 7.4, at 4°C and homogenized with a Polytron homogenizer (Kinematica GMBH) at 9000 rpm for 15 seconds at 4°C. The homogenates were centrifuged at 30 000 g for 20 minutes at 4°C. The supernatants were discarded, the pellets were resuspended in the same buffer, and homogenization and centrifugation were repeated once. With the use of a hand-driven glass/glass homogenizer, the final tissue pellets were resuspended in 50 mmol/L NaH2PO4 buffer, pH 7.4, containing 100 mmol/L NaCl and 10 mmol/L MgCl2. The protein concentration of the particulate fraction was measured with the use of Protein Assay Reagent (Pierce).

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Statistical Analysis

Values are expressed as mean±SEM. Univariate analysis of the effect of each potential factor on each AIIFA was performed with linear regression for continuous variables (age; body mass index; systolic and diastolic blood pressures; total, LDL, and HDL cholesterol levels; uric acid and fasting blood glucose levels; and coronary severity score) and with 1-way ANOVA for categorical variables (smoking, history of hypertension or diabetes mellitus, treatment with ACE inhibitor or β-adrenergic blocker). A value of P<0.05 was considered significant.

Results

To confirm that the ITAs used in the present study were not atherosclerotic, histopathological analyses were performed for 9 representative ITAs. No remarkable intimal lesion was observed in any ITA (Figure 1A), and only focal intimal thickening (arrows in B) was observed in 2 of 9 ITAs.

All ITA samples contained significant amounts of AIIFAs. Total AIIFA and chymase dAIIFA levels were 8.67±2.05 and 8.29±0.82 U (95% versus total AIIFA), whereas ACE dAIIFA was 0.45±0.13 U (5% versus total AIIFA).

Serum total and LDL cholesterol concentrations significantly correlated with chymase and ACE dAIIFAs (Figures 2 and 3, respectively). Because the majority of total AIIFAs were due to chymase dAIIFAs, total or LDL cholesterol levels also significantly correlated with total AIIFAs (n=32, r=0.59, P<0.001; n=21, r=0.44, P<0.05, respectively).

When the analysis was performed only in 9 patients who were treated with an HMG-CoA reductase inhibitor, pravastatin, chymase dAIIFA correlated significantly with total cholesterol concentration (n=9, r=0.88, P<0.01), similar to untreated patients (n=23, r=0.48, P<0.05).

Except for serum total and LDL cholesterol levels, no other significant correlation in an univariate analysis was found between chymase dAIIFAs in ITAs and other clinical parameters, such as age (r=0.22), systolic (r=0.06) and diastolic (r=0.21) blood pressures, body mass index (r=0.02), triglycerides (r=0.17), HDL cholesterol (r=0.22), uric acid (r=0.31), fasting blood glucose (r=0.01), and Gensini’s score (r=0.19). There was no significant difference between the groups with and those without a history of hypertension (n=22 and 10, chymase dAIIFA 8.2±1.0 and 8.4±1.3 U, P=0.78), diabetes (n=18 and 14, chymase dAIIFA 8.3±1.2 and 8.2±1.2 U, P=0.94), or smoking (n=22 and 10, chymase dAIIFA 8.4±0.9 and 8.0±1.8 U, P=0.81), respectively.
There were no significant differences in chymase and ACE dAIIFA of ITAs between the patients treated with or without an ACE inhibitor (n=7 and 25, chymase dAIIFA 9.3±1.8 and 8.0±0.9 U, NS; ACE dAIIFA 0.66±0.24 and 0.40±0.16 U, NS) or a β1-adrenoreceptor blocker (n=6 and 26, chymase dAIIFA 8.7±1.4 and 8.2±1.0 U, NS; ACE dAIIFA 0.58±0.26 and 0.42±0.16 U, NS), respectively, or among the patients treated with dihydropyridine, a nondihydropyridine calcium channel antagonist, or neither (n=7 without either treatment, 13 for dihydropyridine, and 12 for nondihydropyridine; chymase dAIIFA 8.5±1.5, 9.8±1.5, and 6.5±1.2 U, NS; ACE dAIIFA 0.81±0.33, 0.46±0.25, and 0.24±0.11 U, NS, respectively). Because most of the patients (29 of 32) in the present study were male, a gender effect could not be appropriately evaluated.

Mast cells positive for toluidine blue, anti-tryptase, or chymase antibody appeared only in the adventitia of the ITAs, not in the intima or media (Figure 1, C and D). The number of chymase- and tryptase-immunopositive cells correlated with vascular chymase activity (n=9, r=0.69, P<0.05; n=9, r=0.68, P<0.05, respectively). Anti–cathepsin G antibody–positive cells were not detected in ITA sections (data not shown).

**Discussion**

Our results provide evidence that increased AIIFA in the intact ITAs was related to increased serum LDL cholesterol levels. In addition, chymase-containing mast cells are present only in the adventitia, not in the intima or media, suggesting that increased adventitial mast cells appear to be the source of increased vascular chymase activity.

A number of studies have shown that an increased plasma LDL cholesterol level induces atherosclerotic changes in cardiovascular tissues. In addition, it has been postulated that activated tissue RAS might be involved in the development of arteriosclerosis. Cardiac human chymase, but not rodent chymase, is a potent and specific Ang II–forming serine proteinase in vitro and ex vivo; furthermore, a recent report with the use of canine hearts indicated that cardiac canine chymase is involved in interstitial Ang II formation in vivo. Therefore, increased chymase dAIIFA associated with a high LDL cholesterol level may be one of the important components of the activated local RAS.

Many reports provide convincing experimental evidence of a direct or an indirect association between Ang II and arterial lipid deposition, including superoxide overproduction; activation of monocytes, increasing adhesion to endothelial cells; direct modification of LDL molecules; and increased oxidized LDL uptake into monocytes. In fact, increases in tissue ACE and chymase have been demonstrated in the atherosclerotic tissue of humans and animal models, as well as in animal models. Nickenig et al also reported that the AT1
receptor in rat vascular smooth muscle cells was upregulated by treatment with LDL. These results were further substantiated by the inhibitory effect of an ACE inhibitor or AT1 receptor antagonist on the progression of atherogenic changes in rabbits fed a high cholesterol diet\(^27\) and in apolipoprotein E–deficient mice,\(^28\) respectively. These results are lines of evidence that suggest Ang II plays a key role in the initiation and development of atherogenic lesion formation.

The results of the present study suggest for the first time a direct association between serum LDL cholesterol levels and human arterial chymase and ACE activities, although detailed mechanisms for augmented chymase and ACE activities were not addressed in this study. In a separate experimental study, hamsters fed a high cholesterol diet showed increased aortic chymase activity before the appearance of atherosclerotic changes.\(^1,2,3,29\) Histological examination of ITAs did not show any increased number of mast cells in neointimal lesions were observed in the monkeys fed a high cholesterol diet, although the association between serum cholesterol levels and vascular AIIFA was not determined in these studies. Therefore, the current hypothesis is that a high cholesterol circulation level increases tissue ACE or chymase expression, resulting in an increased tissue Ang II level, which triggers a sequence of events that lead to atherogenic changes.

It has been reported that oxidized LDL infusion induces leukocyte adhesion to endothelial cells, mast cell degranulation, and albumin leakage from the rat mesenteric artery and that these changes are inhibited by pretreatment with monoclonal antibodies for CD11/CD18 and P-selectin.\(^30,31\) These results indicate a potential physicochemical link between oxidized LDL cholesterol and mast cell activation, which is likely to be involved in the upregulation of vascular chymase under conditions of high LDL cholesterol. Because the expression mechanism of human chymase remains unclear, the effects of native and oxidized LDL on chymase expression must be determined.

Previous studies have shown an increased number of mast cells in advanced atherosclerotic neointimal lesions, suggesting that an increased number of mast cells is associated with neointimal formation and plaque rupture.\(^1,2,3,32\) However, an increased number of mast cells in neointimal lesions were noted only in advanced, not in early, stages of atherosclerosis.\(^1,11,32,33\) Histological examination of ITAs did not show any apparent neointimal plaque formation (only neointimal thickening), and chymase-positive mast cells appeared only in the adventitia, suggesting that at least these ITAs were not in the advanced stage of atherosclerosis. These results suggest that the increased chymase dAIIFA of ITAs was due to the increased mast cell density in the adventitia.

Recent studies have shown that the arterial adventitia is involved in the development of neointimal lesions in atherosclerosis.\(^34\) Experimental hypercholesterolemia in pigs and nonhuman primates stimulates the accumulation of inflammatory cells in arterial adventitia. Furthermore, the extent of the inflammatory process in the adventitia of atherosclerotic plaques is correlated with the severity of intimal disease.\(^34\) In addition, a recent study provided direct evidence that chronic adventitial stimulation with Spongel containing interleukin-1\(\beta\) (IL-1\(\beta\)), a cytokine known to be involved in the development of atherosclerosis,\(^35\) induced intimal lesions and vasospastic responses in the coronary artery of pigs.\(^36\) In this regard, human chymase is also known to convert IL-1\(\beta\) precursor to active IL-1\(\beta\),\(^37\) suggesting the potential involvement of the adventitial chymase in the development of neointimal lesions.

In summary, there was a significant positive correlation between serum LDL cholesterol level and chymase or ACE dAIIFA in ITAs, and it appears that higher serum LDL cholesterol levels may be associated with mast cell proliferation in the arterial adventitia, resulting in increased local chymase-dependent Ang II formation.

Acknowledgments

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References


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