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Positive Correlation Between Chymase-Like Angiotensin Ⅱ-Forming Activity in Mononuclear Cells and Serum Cholesterol Level

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Abstract

Objectives. The local renin-angiotensin system is important in cardiovascular diseases. The present study examined the association between angiotensin (Ang) Ⅱ-forming activity in fractionated peripheral leukocytes and atherosclerotic risks such as blood pressure, smoking, age and serum cholesterol level, and used a new analytical approach for the measurement of chymase-like activity in peripheral blood to assess the relationship between the chymase-like activities in leukocytes and atherosclerotic risks.

Methods. Peripheral blood samples were obtained from normal and high blood pressure patients in the presence or absence of ischemic heart disease. Mononuclear cell or polymorphonuclear cell fraction of leukocyte was isolated by centrifugation with either Lymphoprep or Polymorphprep, respectively. Chymase-like, angiotensin converting enzyme, and cathepsin G-dependent Ang Ⅱ-forming activities in the homogenates of mononuclear cell or polymorphonuclear cell fraction were measured using Ang Ⅰ as a substrate.

Results. The chymase-like Ang Ⅱ-forming activity in the mononuclear cell fraction slightly or significantly increased in non-smoker patients with high blood pressure (systolic and diastolic blood pressure, $p = 0.11$; mean blood pressure, $p < 0.05$). Chymase-like Ang Ⅱ-forming activity in the mononuclear cell fraction positively correlated with serum total cholesterol ($p < 0.05$) level.

Conclusions. Our data indicates that chymase in mononuclear cells from peripheral blood is activated by high blood pressure or hypercholesterolemia.

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Key Words
■Angiotensin Ⅱ ■Cholesterol ■Atherosclerosis ■Hypertension
■Leukocyte

INTRODUCTION

The activated renin-angiotensin system (RAS) is crucial in the structural and functional remodeling of cardiovascular diseases including hypertensive heart or renal diseases, myocardial infarction, congestive heart failure and atherosclerosis.[1] The final effector hormone of the RAS, angiotensin (Ang) Ⅱ,

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causes hypertrophy of cardiac myocytes or vascular smooth muscle cells, stimulates proliferation and collagen synthesis of fibroblasts[2] and increases oxidative stress by superoxide and H_2O_2 production in vascular smooth muscle cells via activation of p22 phox-based NADP(H) oxidase.[3-6] These effects of Ang Ⅱ directly promote the progression of many cardiovascular diseases, and recent clinical studies have confirmed that treatment with angiotensin converting enzyme (ACE) inhibitors or Ang Ⅱ type 1 (AT_1) receptor antagonists improved morbidity and mortality in most cardiovascular diseases.

Recent studies have also demonstrated the existence of an alternative Ang Ⅱ-forming pathway, independent of ACE. Several serine proteinases, such as chymase, kallikrein, and cathepsin G, are candidates for ACE-independent Ang Ⅱ-forming activities in human tissues.[7,8] Ang Ⅱ produced by both ACE and other serine proteinases is involved in the progression of many cardiovascular diseases. However, the levels of Ang Ⅱ-forming activity and the enzymes responsible differ markedly among species and organs.[8,9] For this reason there is little clinical data regarding the Ang Ⅱ-forming pathways in cardiovascular diseases. Previous clinical data have generally been obtained from pathological specimens from autopsy or operation, but not from peripheral blood. Such inconvenient methodological problems have prevented any large scale clinical data collection for clinical research of chymase. A recent clinical study has shown that human atherosclerotic aortas contain significantly higher levels of chymase-dependent Ang Ⅱ-forming activity compared to non-atherosclerotic aortas.[10] In addition, the chymase-dependent Ang Ⅱ-forming activity of internal thoracic arteries obtained from coronary bypass operations correlated positively with serum low-density lipoprotein (LDL) cholesterol levels.[11]

The present study developed a new analytical approach for the measurement of chymase-like activity using peripheral blood and found a significant correlation between chymase-like activity in circulating leukocyte fraction and serum cholesterol levels.

SUBJECTS AND METHODS

Patients

Twenty six patients with normal and high blood pressure in the presence or absence of ischemic heart disease were included in this study. All patients stopped medication after hospitalization and peripheral blood was obtained after a five-day period during which meals were controlled to limit daily salt intake to 7 g. Standard biochemical examination was performed for each patient (Automatic Analyzer 7170S, HITACHI). The blood pressure was measured by a standard sphygmomanometer in the sitting position around 10 o'clock. Measurement was repeated at least 2 times on days 5 and 6, and the averaged values were used for data analysis. Basic patient characteristics were summarized in **Table 1**. Informed consent for this study was obtained from each patient on admission. The study protocol was approved by an institutional review committee.

Table 1 Patient characteristics

Age (yr)	64.7±11.3
Smoking (+/−)	12/14
Systolic blood pressure (mmHg)	140.3±26.8
Diastolic blood pressure (mmHg)	75.5±12.9
Mean blood pressure (mmHg)	97.1±15.9
Total cholesterol (mg/dl)	170.8±38.8
Triglyceride (mg/dl)	126.8±82.1
HDL cholesterol (mg/dl)	45.3±17.7
LDL cholesterol (mg/dl)	100.3±30.2
IHD (+/−)	19/7
1VD	5/26 (19)
2VD	6/26 (23)
3VD	8/26 (31)
Drugs	
ACE inhibitor	6/26 (23)
AT_1 receptor blocker	10/26 (38)
Spironolactone	3/26 (12)
HMG-CoA reductase inhibitor	6/26 (23)

Continuous values are mean±SD. ( ): %.
HDL=high-density lipoprotein; LDL=low-density lipoprotein; IHD=ischemic heart disease; VD=vessel disease; ACE=angiotensin converting enzyme; AT_1=angiotensin Ⅱ type 1; HMG-CoA=3-hydroxy-3-methylglutaryl coenzyme A.

White blood cell isolation

Peripheral blood was drawn from the anconeus vein. Mononuclear cells (MC) containing mainly lymphocytes, or polymorphonuclear cells (PC) containing mainly neutrophils, eosinophils and basophils were prepared using Lymphoprep or Polymorphprep, respectively.[12,13] For MC fractionation, blood was diluted by addition of an equal

volume of physiologic saline (0.9% NaCl) and layered 6 ml over 3 m*l* Lymphoprep in centrifuge tubes. Tubes were centrifuged at 800 × *g* for 30 min at 25 ℃ in a swing-out rotor. After centrifugation, the MC fraction, which was recognized as a distinct band at the sample/medium interface, was removed using a pipette. The harvested fraction was diluted with 0.9% NaCl and pelleted by centrifugation for 10 min at 250 × *g* at 25 ℃, and then stored at − 40 ℃ after resuspension in saline.

For PC fractionation, collected blood (5 m*l*) was directly layered over 5 m*l* of Polymorphprep, which was then centrifuged at 450 × *g* for more than 30 min at 25 ℃. After centrifugation, two leukocyte bands were observed. The top band consisted of mononuclear cells (discarded) and the lower band of polymorphonuclear cells, which were harvested using a pipette. To restore normal osmolality, the PC fraction was diluted by addition of the same volume of 0.45% NaCl solution. The cell suspension was further diluted to half concentration with saline, then centifuged at 400 × *g* for 10 min at 25 ℃. Cells were then resuspended in saline, and stored at − 40 ℃. Leukocyte fractions of the isolated MC and PC samples were determined by Giemza stain. The MC fraction contained 79% lymphocytes, 1% monocytes and 20% polymorphonuclear cells ($n = 4$), whereas the PC fraction contained 99% polymorphonuclear cells.

Preparation of homogenate fractions

The MC and PC fractions were frozen on dry ice and thawed three times, then centifuged at 5,000 rpm for 10 min at 4 ℃. The pellets were resuspended in 50 mmol/*l* NaH_2PO_4 buffer, pH 7.4 (containing 100 mmol/*l* NaCl, and 10 mmol/*l* $MgCl_2$), and homogenized with a glass/glass homogenizer on ice. The protein concentration of the fraction homogenate was measured by BCA Protein Assay Reagent (Pierce).

Assessment of Ang Ⅱ formation

Ang Ⅱ-forming activity from Ang Ⅰ was determined as described elsewhere with some modification.[9] The cells prepared as above were incubated with synthetic Ang Ⅰ (0.2 mmol/*l*) at 37 ℃ for 30 min. The Ang Ⅱ formed was analyzed by high-performance liquid chromatography using a C_{18} reverse-phase column (2.2 × 25 cm; Vydac) with a 15-minute liner acetonitrile gradient (4% to 16%) in 25 mmol/*l* triethylamine-phosphate buffer, pH 3, at a flow rate of 2 m*l*/min. Ang Ⅱ-forming activity was expressed as nanomoles or picomoles of Ang Ⅱ formed per minute per milligram of protein. Captopril (1 mmol/*l*)- or chymostatin (0.1 mmol/*l*)-inhibitable (both from Sigma Chemical Co) and aprotinin (0.24 mmol/*l*) (Bayer)-insensitive Ang Ⅱ formations were expressed as ACE- and chymase-like Ang Ⅱ-forming activity, and the aprotinin-inhibited Ang Ⅱ-forming activity was presented as cathepsin G-like activity. Ang Ⅱ-forming activity analyses for each sample were performed in duplicate, and the reproducibility and quality of all data were confirmed before statistical analyses. The inter-assay and intra-assay coefficients of variation of this assay were 8.6% ($n = 12$) and 5.1% ($n = 10$), respectively.

Statistical analysis

All data are expressed as mean ± standard deviation. A backward stepwise multiple regression analysis was carried out to identify significant predictive variables for the Ang Ⅱ-forming activity in mononuclear cells and polymorphonuclear cells. In addition, the following factors were included in a backward stepwise multiple regression analysis with the Ang Ⅱ-forming activities as a dependent variable: age; total cholesterol; triglyceride; high-density lipoprotein (HDL) cholesterol; LDL cholesterol; smoking; diabetes mellitus; ischemic heart disease and systolic, diastolic, and mean blood pressure. To examine the effects of total or LDL cholesterol on the chymase-like activity, standard linear regression analysis were performed.

Chymase-like activities were compared between groups with different blood pressure by Student's *t*-test. Statistical analysis was performed using the SAS Software Package (Release 8.2, Statistical Analysis System, SAS Institute Inc.) at Fukuoka University. *p* values of less than 5% were considered to be significant.

RESULTS

Nineteen patients had mild to severe Ang Ⅱ-forming activity by coronary angiography as shown in **Table 1**. The MC fraction contained mainly lymphocytes and monocytes, and the PC fraction contained mainly neutrophils, eosinophils and basophils. Both fractions incorporated the activities necessary to form Ang Ⅱ. As shown in **Fig. 1**, more than 93% of total Ang Ⅱ formation depended on cathepsin G-like activity which is inhibited by

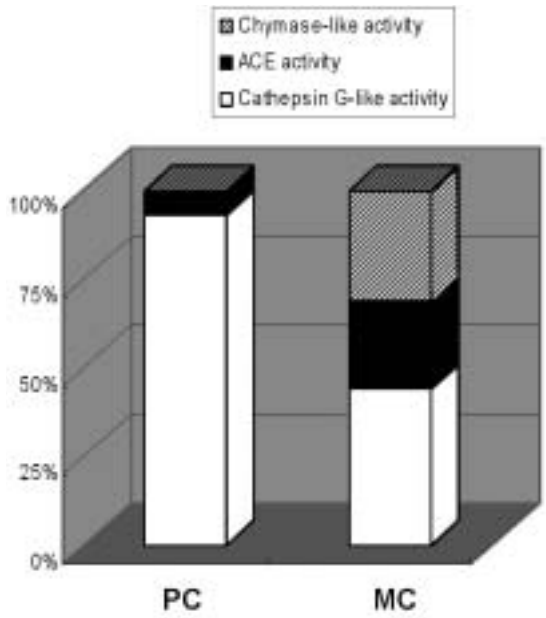

Fig. 1 Ratio of chymase-like, angiotensin converting enzyme or cathepsin G-like responsive angiotensin Ⅱ-forming activities in total angiotensin Ⅱ activities in the polymorphonuclear cell and mononuclear cells fraction
PC = polymorphonuclear cells; MC = mononuclear cells. Other abbreviation as in Table 1.

Table 2 Correlation between ischemic heart disease risks and chymase-like activities in MC fractions

	Chymase-like activity	
	r	*p* value
Age	0.0797	0.69
Systolic blood pressure	0.0165	0.94
Diastolic blood pressure	0.0145	0.95
Mean blood pressure	0.0772	0.72
Total cholesterol	0.5000	0.01*
Triglyceride	0.2833	0.19
HDL cholesterol	0.3102	0.15
LDL cholesterol	0.3649	0.10

*Statistically significant.
Abbreviations as in Table 1, Fig. 1.

aprotinin, and the PC fraction had little chymase-like Ang Ⅱ-forming activity. However, chymostatin inhibited, chymase-like Ang Ⅱ-forming activity that was not inhibited by aprotinin was observed in the MC fraction, in addition to chymase-like, ACE and cathepsin G-like Ang Ⅱ-forming activity of 31.2%, 24.5% and 44.3%, respectively.

The RAS is important in the regulation of blood pressure, and the blockade of this system with ACE inhibitor or AT_1 receptor antagonist is effective for the treatment of hypertension. Therefore, ACE- or chymase-dependent Ang Ⅱ formation in leukocytes were analyzed to examine any association with blood pressure levels. In both the MC and PC fractions, there were no significant correlations between chymase-like activities and systolic, diastolic or mean blood pressures (**Table 2**).

Smoking is one of the important risk factors for cardiovascular disease, and also affects the level of blood pressure. In the present study, the diastolic blood pressure, but not systolic blood pressure, was significantly higher in smoking than in non-smoking patients ($p < 0.05$). All patients were divided into two groups depending on the average value of systolic, diastolic or mean blood pressure in patient of smoking or non-smoking patients. We found that chymase-like activity slightly (systolic and diastolic blood pressures; $p = 0.11$) or significantly (mean blood pressure; $p < 0.05$) increased in each group with higher blood pressure group in non-smokers (**Fig. 2**), but not in smokers (data not shown).

Leukocyte numbers in total and each fraction of the hospitalized patients were compared between the groups with or without high blood pressure, but there was no significant difference. Interestingly, multiple regression analysis showed serum total and LDL cholesterol levels were correlated with chymase-like Ang Ⅱ formation in the MC fraction. There was a positive correlation between chymase-like activity in the MC fraction and serum total cholesterol level ($r = 0.5000$, $p < 0.05$; **Fig. 3−upper**) or serum LDL cholesterol level ($r = 0.4649$, $p = 0.095$; **Fig. 3−lower**), although there was no relationship between chymase-like activity and serum HDL cholesterol level. Moreover, there was no significant correlation between serum total cholesterol level and other Ang Ⅱ-forming activity such as ACE and cathepsin G-like activities (**Table 3**). On the other hand, chymase-like activity in the PC fraction was not positively correlated with serum cholesterol levels such as total and LDL cholesterol (**Table 3**).

Nineteen patients had ischemic heart disease. Chymase-like activities in both MC and PC fractions did not show any elevation compared to those in non-ischemic heart disease patients. Associations of other risk factors such as age, diabetes mellitus, uric acid and smoking with Ang Ⅱ-forming activi-

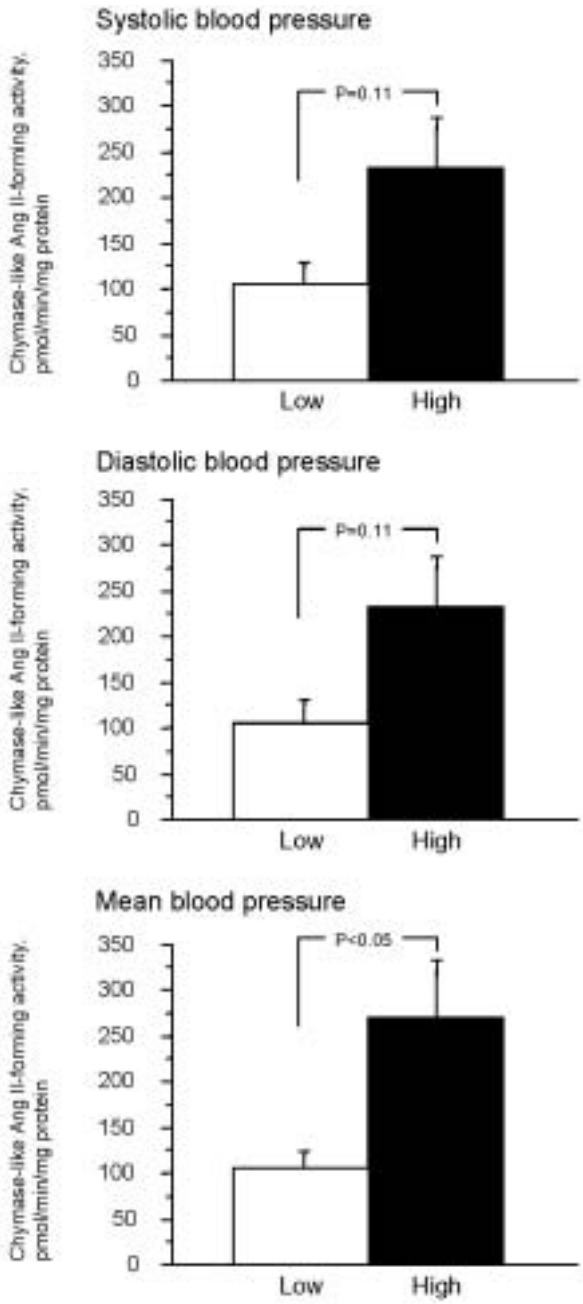

Fig. 2 Patients were divided into two groups (Low: open bar; High: closed bar) based on average value of systolic (*upper*), diastolic (*middle*) or mean blood (*lower*) pressure in non-smoking patients

The chymase-like activity was increased in groups with high blood pressure (systolic blood pressure, $p = 0.11$; diastolic blood pressure, $p = 0.11$; mean blood pressure, $p < 0.05$) in non-smokers.

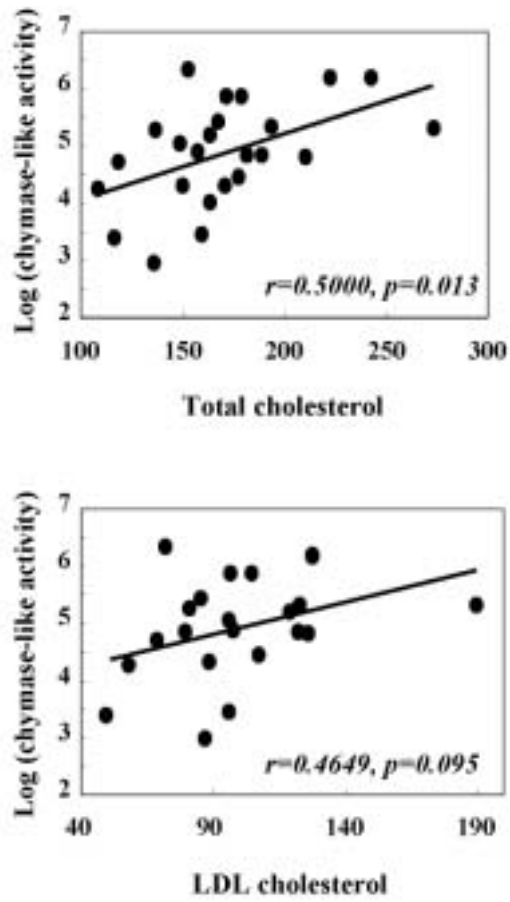

Fig. 3 Positive correlation between logarithm of chymase-like Ang Ⅱ-forming activity and serum total cholesterol (*upper*) or low-density lipoprotein cholesterol level (*lower*)

Abbreviation as in Table 1.

Table 3 Correlation between serum total cholesterol level and Ang Ⅱ-forming activities in the MC and PC fractions

	Serum total cholesterol	
	r	*p* value
MC fraction		
Chymase-like activity	0.5000	0.01*
ACE activity	0.1599	0.46
Cathepsin G-like activity	−0.1487	0.49
PC fraction		
Chymase-like activity	0.2100	0.42
ACE activity	−0.3553	0.16
Cathepsin G-like activity	−0.0552	0.83

*Statistically significant.

Abbreviations as in Table 1, Fig. 1.

ties were also determined, but had no significant correlation with Ang Ⅱ-forming activity (data not shown).

DISCUSSION

The three major findings of the present study are as follows. This simple assay method for leukocyte Ang Ⅱ-forming activity can easily be used for a large scale sampling analyses. Chymase-dependent Ang Ⅱ formation in the MC fraction of patients with higher blood pressure was slightly or significantly higher than that in non-smoking patients with lower blood pressure, suggesting that chymase activity in leukocytes increased along with the severity of hypertension, and in turn, smoking might have affect the blood pressure elevation. Serum total cholesterol level was the most important contributing factor to the increase of chymase-dependent Ang Ⅱ-forming activity in the MC fraction, whereas other clinical parameters had no significant influence on the lymphocyte Ang Ⅱ-forming activity.

Chymase increases in various cardiovascular diseases, mainly based on actual tissue analyses obtained in surgery or by biopsy.[9−11,14,15] Such tissue analyses are inconvenient and the number of samples is restricted, making it difficult to conduct efficient clinical studies. The present study has confirmed the feasibility of analyzing Ang Ⅱ-forming activity in mononuclear cells (ACE, chymase-like and cathepsin G-like activities) by relatively simple isolation of the peripheral leukocytes. This will be an advantage for a future large clinical study. In the current laboratory tests, the degree of RAS activity was determined on the basis of plasma renin activity, ACE activity, aldosterone concentration and plasma Ang Ⅰ or Ang Ⅱ concentrations. No reports were available regarding tests using leukocytes. The results of the present study might be useful for estimating the effects of leukocyte Ang Ⅱ formation on tissue injury related with atherosclerotic risks.

There was no significant correlation between blood pressure and chmase-like activity in the MC fraction of all subjects. However, elevated chymase activity was observed in the MC fraction of non-smokers with relatively higher blood pressure. Since the average blood pressure in the smokers was significantly elevated compared to that of non-smokers,[16] these results suggest a potential association between chymase-dependent Ang Ⅱ-forming activity in leukocytes and blood pressure levels. The present and other basic experiments have provided an indicator to this association. Human chymase cDNA-introduced transgenic mice showed AT_1 receptor-dependent mild hypertension.[17] The ACE-independent Ang Ⅱ forming pathway contributes to regulation for blood pressure in the mast cell deficient mouse model.[18] ACE gene knockout mice also showed variations in blood pressure corresponding to the degree of ACE gene expression.[19] These results suggest that increased Ang Ⅱ-forming activity by chymase or ACE results in elevated blood pressure and cigarette smoking accelerates this process.

Recently, basic and clinical studies have suggested that MC fraction leukocytes (mainly lymphocytes) are important in tissue injuries complicated with hypertension and hypercholesterolemia. Mononuclear cells are activated by Ang Ⅱ and secrete several cytokines such as tumor necrosis factor-α and monocyte chemoattractant protein-1.[20, 21] Chymase activates a precursor of interleukin (IL)-1 β *in vitro*.[22] Human chymase transgenic mice also showed increased serum IL-1β,[17] suggesting the possibility that chymase causes tissue injuries through IL-1β. In fact, IL-1β increased in the peripheral blood of hypertensive patients.[23] These basic and clinical data suggest that Ang Ⅱ formed by chymase in the MC fraction might induce increased levels of tissue-injuring cytokines. Such cytokines including Ang Ⅱ aggravate organopathy associated with hypertension.

In the present study, a positive correlation was found between chymase-like activity in the MC fraction and serum cholesterol level. However, the details of the mechanism remain uncertain. Our previous paper showed a positive relationship between chymase-dependent Ang Ⅱ-forming activity of the internal thoracic arteries obtained from coronary bypass operations and serum LDL cholesterol levels.[11] High cholesterol loading for 14 weeks to normal hamsters increased aortic chymase-dependent Ang Ⅱ-forming activity and showed a positive correlation between aortic chymase Ang Ⅱ-forming activity and serum LDL cholesterol level. In addition, orally active chymase inhibitor suppressed the development of aortic atherosclerotic changes.[24] These results suggest that the increased chymase activities of mononuclear cells in peripheral blood may reflect the up-regulation of local vascular chymase levels, which exag-

gerate the development of atherosclerosis.

Leukocytes in the PC fraction are mainly made up of multi-nucleated cells, which increase in response to acute inflammation, whereas leukocytes in the MC fraction, mainly lymphocytes, increase in response to subacute to chronic inflammation. Increased Ang Ⅱ formation in the peripheral MC may reflect the presence of atheromatous disease, a chronic complication associated with increased serum cholesterol level. The positive correlation of chymase dependent Ang Ⅱ formation in the MC fraction with serum cholesterol level and no correlation with leukocyte number suggest that Ang Ⅱ formation in the MC fraction could be used as a marker for tissue inflammation associated with increased blood pressure and LDL cholesterol. Recent clinical studies provided evidence that the RAS blockers such as ACE inhibitor or AT_1 receptor antagonist were more effective to prevent progression of organopathy caused by high blood pressure than other antihypertensives (LIFE,[25] RENAAL,[26] MARVAL,[27] FACET,[28] ABCD[29]). Therefore, leukocyte Ang Ⅱ-forming activity, especially in mononuclear cells, may be provide a direct marker of organopathy and a useful indicator of anti-atherosclerotic treatment. Further clinical studies are necessary to prove this aspect of Ang Ⅱ formation in the MC fraction .

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要　　約

白血球単核球分画におけるキマーゼ依存性アンジオテンシンⅡ産生活性と血中コレステロール値との相関

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出石　宗仁　　朔　啓二郎　　浦田　秀則

目　的: 局所レニン・アンジオテンシン系は，心血管病の進展に重要な役割を担っていることが知られている．本研究では末梢循環白血球におけるアンジオテンシンⅡ産生活性と血圧，喫煙，年齢およびコレステロールなどの動脈硬化性リスクとの関連を検討した．

方　法: 正常血圧者および高血圧患者からの血液サンプルを用いて，Lymphoprep あるいは Polymorphprep を用いて白血球単核球分画および顆粒球分画をそれぞれ遠心抽出した．白血球単核球および顆粒球分画血球組織ホモジネートにおけるキマーゼ，アンジオテンシン変換酵素，カテプシンG依存性のアンジオテンシンⅡ産生活性を合成アンジオテンシンⅠを基質として用いて測定した．

結　果: 白血球単核球分画中のキマーゼ依存性アンジオテンシンⅡ産生活性は，非喫煙者において血圧高値群で有意に上昇していた(収縮期血圧，$p=0.11$; 拡張期血圧，$p=0.11$; 平均血圧，$p<0.05$)．また，白血球単核球分画中のキマーゼ依存性アンジオテンシンⅡ産生活性と血中総コレステロール値($p<0.05$)との間に正相関が認められた．

結　論: これらの結果は，末梢血単核球分画中のキマーゼが，血圧やコレステロールの上昇によって活性化されることを示唆している．

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