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High expression level of Toll-like receptor 2 on monocytes is an important risk factor for arteriosclerotic disease

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ABSTRACT

Background: Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns to initiate an innate immune response. We previously reported upregulation of TLR2 expression level on monocytes of stable angina pectoris patients with significant coronary artery disease (CAD) relative to control patients without significant CAD. In this study we aimed to determine whether high level of Toll-like receptor 2 (TLR2) is a risk factor for atherogenesis, independent of established risk factors including smoking, diabetes mellitus (DM), hypertension (HT), and hyperlipidemia (HL).

Methods: TLR2 expression level on circulating monocyte surfaces was measured by using our developed flow cytometry assay. Patients were classified into two groups: "Arteriosclerotic disease" group ($n = 108$) and "Control" group ($n = 70$). Patients of the first group had arteriosclerotic disease such as CAD, aortic aneurysm, or peripheral arterial disease (PAD). The "Control" group was sex- and age-matched to the "Arteriosclerotic disease" group.

Results: TLR2 expression was significantly higher in the "Arteriosclerotic disease" group than in the "Control" group ($p < 0.001$). Multivariate ordinal logistic regression analysis was performed; other known risk factors, which were represented to two nominal score points, 0 or 1, for patients with and without it, respectively, and TLR2 level, which was treated as a metric variable. DM ($p = 0.002$), HT ($p = 0.001$), HL ($p < 0.001$), and TLR2 level ($p < 0.001$) were identified as significant contributors for arteriosclerotic disease.

Conclusions: High TLR2 expression level on monocytes may be an independent risk factor for atherogenesis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease in which multiple factors contribute to arterial wall degeneration. Innate immunocompetent cells, such as monocyte/macrophage lineage cells, are responsible for the major pathology [1]. Infectious agents including *Chlamydia pneumoniae* [2], cytomegalovirus [3], and *Helicobacter pylori* [4] have been reported as atherosclerotic pathogens, although this evidence is controversial. Hagiwara et al. [5] suggested that an inflammatory mechanism might not correlate with the pathogenesis of carotid atherosclerosis among Japanese patients, based on the low pathogen-detection rate in atherosclerotic plaques. Considering the lack of association between pathogen burden and plaque development, it could be speculated that infectious agents in the arterial wall might be not absolutely required for the development of arteriosclerotic disease, but that chronic or prolonged immune responses might be essential. Here, two terms of "arteriosclerosis" and "atherosclerosis" were properly used as the clinical and pathogenic features, respectively.

C-reactive protein (CRP) is a nonspecific biomarker of inflammation, thus it was expected that high-sensitivity CRP (hsCRP) would be useful to assess the severity of arteriosclerotic disease. However, an unequivocal correlation has not been demonstrated between hsCRP and the morphological features of atherosclerotic progression [6–8]. Some large-scale clinical studies demonstrated that hsCRP seems to be a predictor of cardiovascular events [9,10], and a marker of coronary calcification [11,12].

Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns, are crucial for microbe recognition by the

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innate immune system; these molecules thus bridge the functions of innate and acquired immunity [13]. We recently showed that TLR2 expression on circulating monocytes was increased in patients with bacterial and viral infections, but not in patients with noninfectious inflammatory disease [14,15]. We also found that TLR2 level in stable angina pectoris (AP) patients with significant coronary artery disease (CAD) was more highly upregulated than in control patients with no significant CAD [16]. Mullick et al. [17] reported that complete deficiency of TLR2 led to a reduction in atherosclerosis in atherosclerosis-susceptible low-density lipoprotein receptor-deficient mice, indicating that host-derived endogenous TLR2 agonists might play a crucial role in treating atherosclerotic disease. It has also been implicated that *Porphyromonas gingivalis* accelerates the progression of atherosclerosis [18], while the critical role of TLR2 in the progression of atherosclerosis seems to be associated with monocyte chemoattractant protein-1 (MCP-1) level and macrophage recruitment to atherosclerotic lesion [19]. On a related article, Epstein-Barr virus can specifically activate human monocytes via TLR2-dependent signaling, a process that may contribute to the secretion of MCP-1 [20]. From these studies, a possibility will be arisen that high TLR2 level on circulating monocytes observed in our previous study [16] might not be an effect of atherosclerosis, but a cause/risk factor for it.

Many risk factors are known to be involved in atherogenesis. These include advanced age, male gender, familial history, hyperlipidemia (HL), hypertension (HT), diabetes mellitus (DM), and smoking habit. Of these, HL, HT, DM, and smoking are treatable and avoidable risk factors. However, patients with arteriosclerotic disease, but without these risk factors, are infrequently encountered in clinical practice. To address this anomaly, in the present study we examine TLR2 levels on monocytes from patients with arteriosclerotic disease such as AP, aortic aneurysm, or peripheral arterial disease (PAD), compared to control patients without arteriosclerotic disease. Subsequently, we statistically analyze the independency of TLR2 level on monocytes as a risk factor for arteriosclerotic disease.

2. Methods

2.1. Patient population

Consecutive patients ($n = 121$) who underwent coronary angiography for suspected arteriosclerotic diseases were enrolled in this study. Of these, 96 patients suffered from arteriosclerotic disease including AP, old myocardial infarction (OMI), thoracic/abdominal aortic aneurysm (TAA/AAA), chronic cerebral infarction (CCI), and PAD, and 12 patients had multi-atherosclerotic diseases (Table 1). AP was determined by the presence of significant stenosis (angiographic diameter ≥75%) within the coronary arteries regardless of the stable/unstable. OMI was defined as a clinical state without any more progression of myocardial damage at least one month after the event of occlusion of a coronary artery. These 108 patients formed the "Arteriosclerotic disease" group. The remaining patients ($n = 13$) had no arteriosclerotic disease; these subjects together with 57 volunteers without symptoms of arteriosclerotic disease formed the "Control" group ($n = 70$) for this study. Some volunteers were under medical treatment for HT, HL, or DM. The control subjects were age- and sex-matched to the patients with arteriosclerotic disease. Patients and volunteers were apparently infection-free for at least one month prior to blood sampling for this study and had no fever within a week either side of blood sampling. To solely assess arteriosclerotic degeneration, some patients were excluded in the present study, who suffered from disorders possibly associated with inflammatory disease such as cancer, collagen diseases, allergic diseases, acute myocardial infarction, congestive heart failure (New York Heart Association functional class II or greater), severe valvular disorders, lone atrial fibrillation, and life-threatening ventricular arrythmia. All blood samples were taken in the fasting state, and in the "Arteriosclerotic disease" group they were performed before catheter angiography; 10 ml of peripheral blood was collected from each patient into a heparinized blood collecting tube. Informed consent was obtained from all patients and volunteers, in accordance with the Kagoshima University Ethics Committee.

Table 1
Characteristics of patients with and without AD.

	Control ($n = 70$)	AD ($n = 108$)	p value
Diseases			
AP and/or OMI	–	87	–
TAA and/or AAA	–	6	–
PAD	–	3	–
CCI	–	0	–
Combinations	–	12	–
AP and/or OMI + CCI	–	2	–
AP and/or OMI + PAD	–	6	–
AP and/or OMI + AAA	–	2	–
AP + AAA + TAA	–	1	–
AAA + PAD	–	1	–
Conventional risk factors			
Age (years)	66 ± 14	69 ± 10	0.12
Gender (M/F)	51/19	79/29	1.00
Hypertension, n (%)	26 (37%)	88 (81%)	<0.001
Hyperlipidemia, n (%)	10 (14%)	70 (65%)	<0.001
Diabetes mellitus, n (%)	6 (9%)	48 (44%)	<0.001
Smoking habit, n (%)	15 (21%)	35 (32%)	0.13
Medications			
ACE inhibitors, n (%)	1 (1%)	24 (22%)	<0.001
ARB, n (%)	2 (3%)	49 (45%)	<0.001
Statins, n (%)	4 (6%)	58 (54%)	<0.001
Aspirin, n (%)	0 (0%)	90 (83%)	<0.001
Insulin, n (%)	0 (0%)	11 (10%)	<0.001
Diabetic drugs, n (%)	4 (6%)	33 (31%)	<0.001

Numbers in parentheses represent the percentage of disease prevalence and ratio of patients taking the medication. AD, "Arteriosclerotic disease" group; Control, "Control" group; AP, angina pectoris; OMI, old myocardial infarction; TAA, thoracic aortic aneurysm; AAA, abdominal aortic aneurysm; PAD, peripheral artery disease; CCI, chronic cerebral infarction; Combinations, multi-atherosclerotic diseases; ACE inhibitors, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

2.2. Classical well-known risk factors

HT was defined as systolic blood pressure (BP) over 140 mmHg and/or diastolic BP over 90 mmHg. In this study, patients with untreated HT and those on medication for HT but with normal normotension were categorized as HT. DM was characterized by recurrent or persistent hyperglycemia, diagnosed by the presence of any one of the following: fasting plasma glucose level ≥126 mg/dl; plasma glucose ≥200 mg/dl 2 h after a 75-g oral glucose load as in a glucose tolerance test; and, symptoms of hyperglycemia and casual plasma glucose ≥200 mg/dl. All patients with DM were diagnosed as type 2. HL was defined as fasting total cholesterol ≥220 mg/dl, fasting triglycerides ≥150 mg/dl, or use of antilipidemic therapy for HL. In this study, patients with a smoking habit were those classified without employing the Brinkman index.

2.3. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by density gradient centrifugation using Ficoll-Paque plus liquid (Amersham Bioscience, GE Healthcare, UK). To assess the expression level of TLR2 on monocytes,

PBMCs were divided into three tubes and stained in parallel with PE-labeled anti-TLR2 monoclonal antibody (mAb) (clone T2.1; eBioscience, San Diego, CA), anti-CD14 mAb (clone M5E2; eBioscience), or control mouse IgG2a (eBM2a; eBioscience). In this study, we employed a single-color flow cytometric analysis to avoid problems of interference among fluorescence dyes. The stained cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA). For each donor, monocytes were first gated according to the forward/side scatter properties and CD14 staining. Subsequently, the same gate setting used for the monocytes was applied to the analysis of TLR2- and control-stained PBMC samples. The mean fluorescence intensity (MFI) value for just TLR2 was obtained by subtracting the control staining MFI value from the TLR2 staining MFI value.

2.4. Quantification of TLR2 levels

TLR2 expression level on monocytes was numerically presented as described previously [14,15]. In brief, a mixture of our developed TLR2-coupled standard beads, which comprises four bead groups carrying four different numbers of recombinant TLR2 molecules, was stained in parallel with PBMCs under the same experimental conditions in each assay. A calibration curve was then obtained by plotting MFI values of the standard beads. Using the calibration curve, the TLR2 expression level on monocytes was converted to the number of antibody-binding sites per cell.

2.5. Statistical analysis

Given two data sets, we assessed whether two samples of observations came from the same distribution. The Mann–Whitney *U* test was performed to identify significant difference between two groups for variables with skewed and/or homoscedastic distribution. These data were expressed as median values and 25–75% values of the distribution. The prevalence of conventional risk factors, the proportions of patients taking particular medications, and gender were compared between the "Control" and the "Arteriosclerotic disease" groups with the chi-square test/Fisher's exact probability test. Multivariate ordinal logistic regression analysis was performed to test whether TLR2 expression level on monocytes is a significant contributor for atherogenesis. All differences were considered significant at $p < 0.05$. All statistical analyses were performed with Excel Statistics 2006 for Windows® (Social Survey Research Information Co., Japan).

3. Results

3.1. Patient characteristics

The percentages of patients with each conventional risk factor are listed in Table 1, and compared between "Control" group ($n = 70$) and "Arteriosclerotic disease" group ($n = 108$). The prevalence of all conventional risk factors was higher in the "Arteriosclerotic disease" group than in the "Control" group, and chi-square test analysis showed significant differences between the groups in HT ($p < 0.001$), HL ($p < 0.001$), and DM ($p < 0.001$), but not in smoking habits ($p = 0.13$). The number of patients without any conventional risk factors was 4 in "Arteriosclerotic disease" group.

3.2. Comparison of TLR2 and CRP levels

As shown in Fig. 1A, comparison of the TLR2 expression levels on monocytes between the "Control" and "Arteriosclerotic disease" groups demonstrated that patients with arteriosclerotic disease had higher TLR2 levels than the age- and sex-matched controls (median [25–75th percentile], 5154 [4513–6109] vs. 4650 [3930–5129] sites/cell, $p < 0.001$). Next, as shown in Fig. 1B, serum CRP level was also higher in patients with arteriosclerotic disease than in control patients (median [25–75th percentile], 0.12 [0.05–0.35] vs. 0.06 [0.02–0.11] mg/dl, $p < 0.001$). In this study, the lower limit of assaying CRP was 0.02 mg/dl.

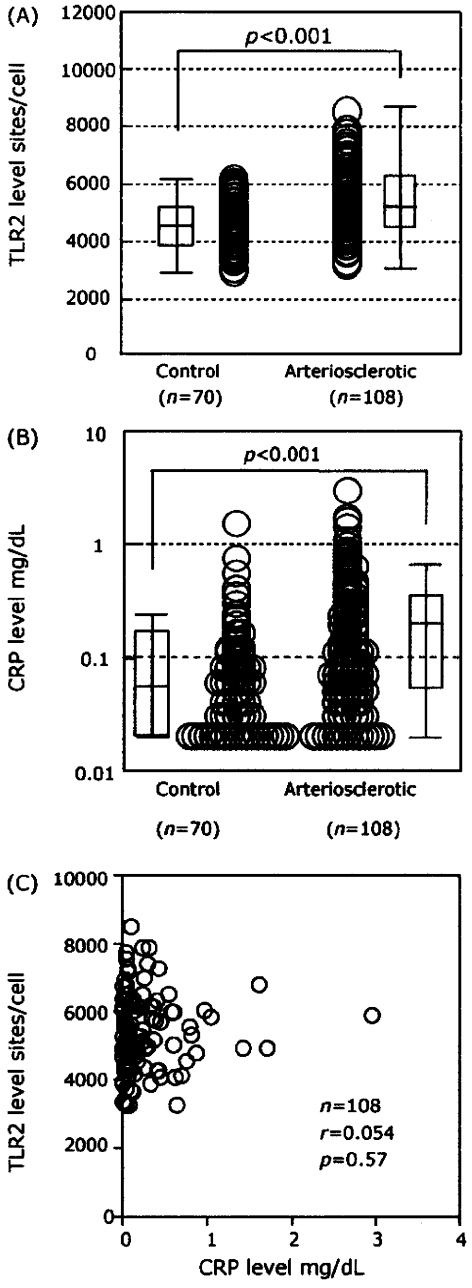

Fig. 1. (A) Toll-like receptor 2 (TLR2) levels were compared between the two groups. Circles represent individual TLR2 expression levels. (B) CRP levels were compared between the two groups. Circles represent individual CRP levels. The box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. Mann–Whitney *U* test was applied to this statistical analysis. (C) Correlation between TLR2 and CRP levels. Circles represent individual cases in "Arteriosclerotic disease" group. Number of patients (n), index of correlation (r), and significant probability (p) are indicated.

3.3. Comparison of TLR2 and CRP levels between with and without a medication

Based on reports that angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARB), statins, and insulin might repress the TLR2 expression level on monocytes [21–24], the prevalence of these medications was also listed in Table 1 and found to be significantly more abundant among the "Arteriosclerotic disease" group than the "Control" group ($p < 0.001$). Statistical comparisons among "Arteriosclerotic disease" group showed that any medications, ACE inhibitors, ARB, statins, aspirin, insulin, and anti-diabetic drugs, did not produce significant differences in TLR2 level ($p = 0.73$, $p = 0.52$, $p = 0.47$, $p = 0.98$, $p = 0.57$, and $p = 0.46$, respectively) and in CRP level ($p = 0.76$, $p = 0.89$, $p = 0.60$, $p = 0.46$, $p = 0.06$, and $p = 0.24$, respectively) between patients with and without taking each medication.

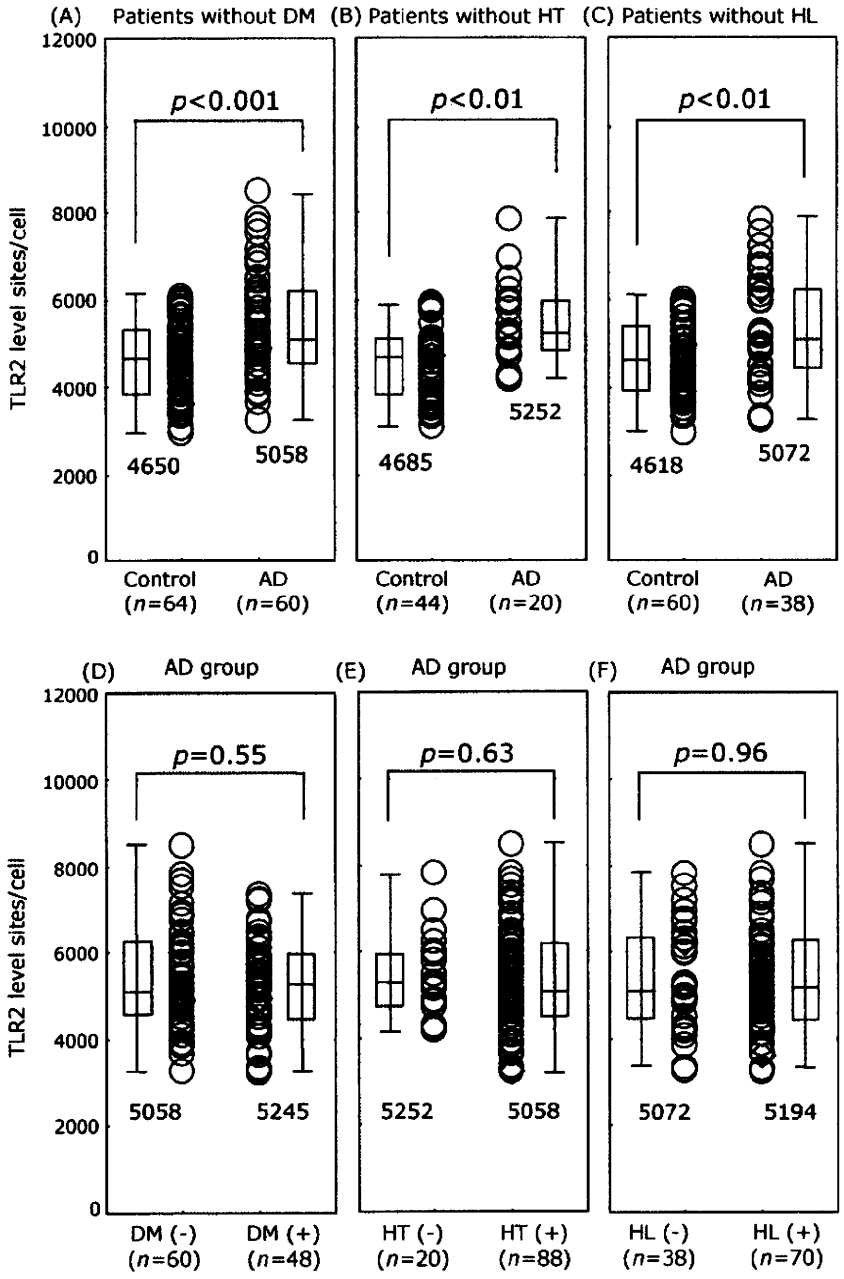

Fig. 2. Comparison in TLR2 level between two subgroups in which patients with each well-known risk factor were removed from "AD" and "Control" groups, respectively. (A) TLR2 levels were compared between the respective two subgroups in which type 2 DM patients were removed from "AD" group and "Control" group. (B) TLR2 levels were compared between the respective two subgroups in which HT patients were removed from "AD" group and "Control" group. (C) TLR2 levels were compared between the respective two subgroups in which HL patients were removed from "AD" group and "Control" group. (D) Among AD group, TLR2 levels were compared between two subgroups with and without type 2 DM. (E) Among "AD" group, TLR2 levels were compared between two subgroups with and without HT. (F) Among "AD" group, TLR2 levels were compared between two subgroups with and without HL. Circles represent individual TLR2 expression levels. The box plot and the horizontal bar show the interquartile range and median value, respectively. The whiskers extend to at most 1.5 times the box width (the interquartile range) from either or both ends of the box. Mann–Whitney *U* test was applied to this statistical analysis. AD; Arteriosclerotic disease.

3.4. Correlation between CRP and TLR2 levels

The association between TLR2 and CRP levels in the two groups was examined by univariate regression analysis. As shown in Fig. 1C, TLR2 levels did not correlate with CRP levels ($r = 0.054$, $p = 0.57$), indicating that TLR2 expression level on monocytes might be a unique marker of cardiovascular disease, unlike CRP level.

3.5. Independency of TLR2 level from conventional risk factors and the conditional multivariate ordinal logistic regression analysis

It was recently reported that in type 1 DM patients, TLR2 level was more elevated, compared to the control group [25]. Thus, before examining the contribution of TLR2 level to arteriosclerotic disease, the independency of TLR2 level from the other risk factors, especially from DM, should be checked. For this purpose, TLR2 levels were compared between two subgroups, in which DM patients were removed from "Control" and "Arteriosclerotic disease" groups, respectively. As shown in Fig. 2A, TLR2 level was still higher in the subgroup ($n = 60$) of "Arteriosclerotic disease" than in the control ($n = 64$) subjects (median [25–75th percentile], 5058 [4631–6141] vs. 4650 [3899–5131] sites/cell, $p < 0.001$). Also, even if HT or HL patients were removed (Fig. 2B and C), these patients with arteriosclerotic disease had higher TLR2 level than the respective controls (median [25–75th percentile], 5252 [4792–5975] vs. 4685 [3867–5087] sites/cell, $p < 0.01$, 5072 [4441–6139] vs. 4618 [3975–5131] sites/cell, $p < 0.01$). Subsequently, among the "Arteriosclerotic disease" group, the statistical difference in TLR2 level was examined between patients with and without DM, HT, or HL. In any comparisons, there was no significant difference between two subgroups (Fig. 2D–F). From these findings, it was demonstrated that TLR2 level is independent from at least DM, HT and HL.

The control subjects in this study were age- and sex-matched with the patients in the "Arteriosclerotic disease" group. Therefore, conditional multivariate ordinal logistic regression analysis [26], in which two unavoidable risk factors, age and sex, were not treated as an explanatory variable, was performed to test for significant contributors to atherogenesis among TLR2 expression level and the other established risk factors. In the pretreatment of explanatory variables on this analysis, TLR2 level was represented as a metric variable to retain the information content, while HT, HL, DM, and smoking habit, were represented by two nominal score points, 0 and 1, for patients with and without it, respectively. The statistical analysis identified DM, HT, HL, and TLR2 level as explanatory variables significantly affecting on arteriosclerotic disease. However, smoking habit ($p = 0.053$) was not a significant affecting factor in this study.

Excluding smoking habit, the second logistic multivariate analysis showed that the multivariate-adjusted odds ratios of DM (Wald 9.527, $p = 0.002$), HT (Wald 10.72, $p = 0.001$), HL (Wald 19.61, $p < 0.001$), and TLR2 level (Wald 12.32, $p < 0.001$) for arteriosclerotic disease were 5.491 (95% CI, 1.862–16.19), 4.144 (95% CI, 1.770–9.706), 7.782 (95% CI, 3.139–19.30), and 1.001 (95% CI, 1.0004–1.0013), respectively (Fig. 3A). According to the logistic regression formula shown in Fig. 3A, without any other conventional risk factors, probability of arteriosclerotic disease (P) = 0.5 was corresponding to 6877 sites/cell in TLR2 expression. Therefore, TLR2 level > 6877 sites/cell was calculated as a risk factor for arteriosclerotic disease. The adjusted odds ratios are the respective coefficients of these explanatory factors. When only 1 unit of the individual dependent factor increases, the coefficient is directly added to the value of $\mathrm{Log}\{P/(1-P)\}$. In this above analysis, the established risk factors were treated as a nominal variable and only TLR2 level was treated as a metric variable. Thus, it prevents the participation of the five factors to be mutually estimated with the adjusted odds ratios.

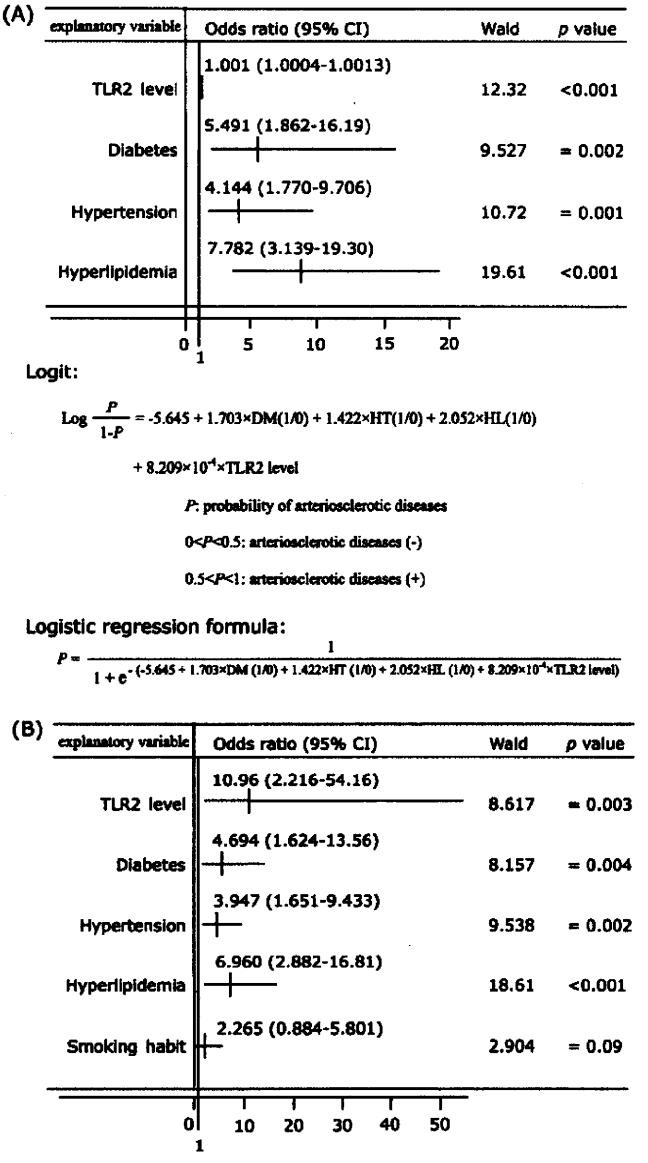

Fig. 3. Conditional multivariate ordinal logistic regression analysis. (A) Multivariate-adjusted odds ratios for DM, HT, HL, and TLR2 level for arteriosclerotic diseases, with TLR2 level treated as a metric variable. The vertical bar with horizontal line and numbers represents each multivariate odds ratio and 95% CI, respectively. The logistic regression formula is shown in the lower part of the figure. P indicates the probability of cardiovascular diseases, and $P = 0.5$ indicates a borderline presence of cardiovascular diseases. (B) Multivariate-adjusted odds ratios of smoking habit, DM, HT, HL, and TLR2 level for arteriosclerotic disease, with TLR2 level converted to a score point (1/0) at a boundary point of 6000 sites/cell. The vertical bar with horizontal line and numbers represents each multivariate odds ratio and 95% CI, respectively.

To compare the participation of all risk factors for arteriosclerotic disease with the respective adjusted odds ratios, TLR2 levels were converted to two nominal score points, 0 and 1, as for the other risk factors. For example, when TLR2 level ≥ 6000 sites/cell was defined as high and converted to score point 1, and TLR2 level < 6000 sites/cell was defined as low and converted to score point 0, the adjusted odds ratios of the respective risk factors could be estimated as shown in Fig. 3B. High TLR2 level (adjusted odds ratio = 10.96) was thus positioned as the most

important risk factor compared to the other four established influences.

4. Discussion

Both the inflammatory responses and immune system are involved in atherogenesis [1–4]. However, it is controversial whether component parts of infectious agents have participated in atherosclerosis or not [5,27]. Considering the pathogenesis of inflammatory cardiomyopathy, it could be speculated that the burden of microorganisms in atherosclerotic plaques is not critical, but the abnormally prolonged immune response against infectious agents might be crucial for the development of atherogenesis even if the microorganisms are subsequently cleared.

We previously developed a new quantitative assay system to measure TLR2 expression level on monocytes. This system enables more precise comparison of TLR2 levels assayed at different time points in individual patients. Using this technique, we showed that TLR2 levels on monocytes are possibly enhanced in bacterial and viral infections. Furthermore, we pointed out that TLR2 levels of patients with noninfectious inflammatory disease such as allergy, collagen disease, and cancer were within the normal range, even in the presence of leukocytosis and high CRP levels [14,15]. This study was designed to investigate the hypothesis that patients with arteriosclerotic disease express significantly higher TLR2 levels than patients without arteriosclerotic disease, and that high level of TLR2 implies that normal immune systems of these patients might have been consistently skewed into an infection-like inflammatory response.

ACE inhibitors, ARB, and statins, which are characterized as anti-inflammatory drugs, may suppress TLR2 expression [21–23]. Among "Arteriosclerotic disease" group, we also performed statistical comparison between patients with and without taking the medication (ACE inhibitors, ARB, statins, aspirin, insulin, and antidiabetic drugs, respectively). As a result, any kinds of medication could not influence TLR2 and CRP levels. Hence, it would seem that a medication does not underlie the significant difference in TLR2 and CRP levels between "Arteriosclerotic disease" and "Control" groups. Our different results against the above quoted reports might be produced by all different doses and by taking together some different types of drugs that show the same effect as "statin", "anti-diabetic drug", etc. Furthermore, Ghanim et al. [24] recently reported that an injection of insulin suppressed the expression of TLR2 on mononuclear cells at the transcriptional level. In our study, 11 patients among 48 type 2 DM patients were treated by insulin in "Arteriosclerotic disease" group, while in "Control" group, nobody among 6 type 2 DM patients was treated by insulin. In our study, the insulin treatments were canceled only on the day when these blood samples were taken in the fasting state, for these patients stood ready to coronary angiography and arteriography. Our different result, which insulin treatment did not constantly modulate TLR2 expression level on monocytes, might be produced by the blood sampling without an injection of insulin.

Alternatively, in clinical practice it is generally hard to keep good control of their plasma glucose levels, even though DM patients have had the medical treatment. Also in this clinical study, the fasting plasma glucose levels of some DM (type 2) patients were still high. Nonetheless, there was no significant difference in TLR2 level between patients with and without DM among "Arteriosclerotic disease" group (Fig. 2D). Hence, it seems that plasma high glucose level does not contribute to the enhancement of TLR2 expression, and the independency of TLR2 level from DM was clinically ensured. Likewise, TLR2 level seems to be independent from either HT or HL (Fig. 2E and F).

In apolipoprotein E-null mice, inactivation of TLR2 reduced lipid accumulation and decreased macrophage recruitment to the aortic sinus, as well as down-regulating MCP-1 levels [19]. TLR2 deficiency has also been associated with reduced progression of atherosclerosis [18,19]. These findings implicate that TLR2 expression on monocytes might participate in atherogenesis as a risk factor. In clinical practice, we infrequently encounter patients with abnormally rapid development of atherosclerosis in the absence of known conventional risk factors. According to the logistic regression formula used here, the probability of arteriosclerotic disease (P) in a patient, who has 8000 sites/cell in TLR2 expression, without any conventional risk factors was calculated as 0.72 (>0.5).

The mechanism underlying the modulation of TLR2 expression on monocytes remains unknown. We observed in an *ex vivo* study that interferon-γ and tumor necrosis factor-α (TNF-α) potently upregulate TLR2 levels on human monocytes, while interleukin-4 downregulates TLR2 (data not shown). TLR2 level is probably modulated *in vivo* by not only its ligands but also by the integration of cytokines [28]. It also remains unclear what effect the enhanced TLR2 level has on the monocytes. Hadley et al. [29] showed that the reciprocal upregulation of TLR2 and TLR4 monocyte-surface expression could increase the cell's sensitivity to other ligands, leading to enhanced intracellular signaling and proinflammatory-cytokine release, including TNF-α. On the other hand, proinflammatory cytokines are the established participants in atherosclerosis [30], and therefore, the association between TLR2 upregulation and some cytokines might synergize the atheroma development.

In order to propose scientifically the use of TLR2 as an important clinical marker, the prospective interventional trial through a medication against the atherosclerotic progression will be required in future. Prior to it, it will be needed to assess the sufficient dose of a medication that could reduce TLR2 level. So, another clinical research is ongoing to test whether a planning medication, such as taking 20 mg simvastatin/pravastatin during six months, downregulates TLR2 level on monocytes in the individual patient, and to investigate whether it consequently leads to atherosclerotic regression. And now, in this study any relationships between the specific forms of arteriopathy and TLR2 levels could not be elucidated. For, the patients' population was composed mainly of AP and/or OMI patients with significant coronary artery stenosis, and patients with TAA/AAA, CCI and PAD were too small to be statistically examined.

5. Conclusions

We demonstrated that TLR2 level on monocytes in parallel with well-known conventional risk factors, HL, HT, DM, is an independent risk factor. This finding suggested that TLR2 level on monocytes might play a critical role in plaque formation of atherosclerosis and thus provide a new therapeutic target for arteriosclerotic diseases.

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Original Article

Effect of Uric Acid on Coronary Microvascular Endothelial Function in Women: Association with eGFR and ADMA

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Aim: The aim of this study was to investigate the role of uric acid (UA) in coronary endothelial function via its effects on renal function, other coronary risk factors and asymmetric dimethylarginine (ADMA) in men and women.

Methods: The study population consisted of 194 consecutive patients (119 men and 75 women) without coronary artery disease. The relationships between UA and coronary endothelial function, estimated glomerular filtration rate (eGFR), ADMA or other biochemical or anthropometric parameters were investigated.

Results: Monovariate analysis of female participants demonstrated that % change in coronary blood flow (CBF) induced by acetylcholine (ACh) was inversely correlated with UA, ADMA and age ($r=-0.32$, $p<0.01$; $r=-0.31$, $p<0.05$; $r=-0.23$, $p<0.05$, respectively), and positively correlated with eGFR ($r=0.27$, $p<0.05$). Stepwise regression analysis showed that UA was the only independent predictor of % change in CBF induced by ACh (F value 4.969, $p<0.05$). Similar analysis of male participants failed to show significant correlations of these variables except for age in monovariate analysis ($r=-0.19$, $p<0.05$). Meanwhile, UA was inversely correlated with eGFR in both men and in women ($r=-0.25$, $p<0.01$; $r=-0.59$, $p<0.0001$, respectively), and ADMA was positively correlated with UA and inversely correlated with eGFR ($r=0.36$, $p<0.05$; $r=-0.42$, $p<0.01$, respectively) in women but not in men.

Conclusion: High concentrations of UA correlate with coronary endothelial microvascular dysfunction in women. Further, serum UA concentration is related to eGFR and ADMA only in women, which may result in impaired endothelial function in resistance coronary arteries in women but not in men.

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Key words; Uric acid, Endothelial function, Estimated glomerular filtration rate (eGFR), Asymmetric dimethylarginine (ADMA)

Introduction

While epidemiologic studies suggest that hyperuricemia is strongly correlated with cardiovascular disease[1], it is unclear whether uric acid (UA) levels are an independent risk factor for cardiovascular disease. Multivariate analysis of the Framingham Heart study[2] cohorts failed to demonstrate a relationship between UA and cardiovascular disease. However, analysis of the First National Health and Nutrition Examination Survey (NHANES-1) study cohort suggested that increased serum UA levels are independently and significantly associated with an increased risk of car-

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diovascular mortality[3]. Furthermore, the correlation between UA levels and cardiovascular risk was stronger in women than in men in the NHANES-1 study. While the reported findings have been contradictory in male populations, it has been largely reported that cardiovascular disease is related to serum UA concentration levels in female populations[4, 5].

Recent evidence suggests that endothelial dysfunction is a fundamental mechanism whereby UA may affect cardiovascular function. For example, Zoccali and colleagues demonstrated an inverse and significant relationship between UA and acetylcholine-stimulated vasodilation in patients with untreated essential hypertension, even after adjusting for differences in traditional cardiovascular risk factors[6]. However, gender-based differences between elevations in UA and endothelial dysfunction have not been definitively investigated.

Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide (NO) synthase inhibitor. Vallance and colleagues reported that the accumulation of endogenous ADMA can impair NO synthesis and thereby contribute to hypertension and immune dysfunction associated with chronic renal failure[7]. Further, another study suggested that ADMA levels increase in response to oxidative stress and that elevated ADMA levels lead to endothelial dysfunction and atherosclerosis. Thus, ADMA may be a marker of atherosclerosis[8]. Plasma ADMA concentration was markedly higher in patients with renal disease than in control subjects[9]. UA level was also increased in patients with renal dysfunction[10]. Although these prior studies suggest that UA and ADMA levels may correlate with cardiovascular risk or renal disease, the relationship between UA and ADMA has not yet been characterized.

UA levels can vary within humans secondary to inducing factors (e.g., high purine or protein diets, alcohol consumption) or changes in excretion. Hyperuricemia can lead to the development of renal disease[11] and is associated with factors that contribute to metabolic syndrome[12]. Further, UA levels increase with menopause[13], although other mechanisms related to gender-based differences in UA levels remain unclear.

Based on these observations, the goal of this study was to investigate the gender-specific relationship between UA and coronary endothelial function.

Subjects and Methods

Study Population

From December 1999 to December 2005, 194 consecutive patients (119 men, 75 women), who had been referred to Kagoshima University Hospital for cardiac catheterization to exclude coronary artery disease, were considered for enrollment in this study.

Angiographic inclusion criteria were: 1) angiographically smooth arteries; 2) mild irregularities, less than 30% lumen diameter stenosis by visual assessment in any major conduit vessel; and 3) proximal coronary arteries greater than 2.0 mm in diameter. Patients with a history of variant angina, previous myocardial infarction, previous coronary revascularization, valvular heart disease, cardiomyopathy, or myocarditis were excluded[14].

Long-acting nitrates and calcium channel-blocking agents were withheld for 48 hours before the study to allow for the assessment of baseline coronary physiology.

Written informed consent was obtained from all patients before catheterization in accordance with guidelines established by the Committee for the Protection of Human Subjects at our institution.

Study Protocol

Diagnostic coronary angiography was performed using a 6F Judkins catheter with a standard femoral percutaneous approach. Five thousand units of heparin were administered at the beginning of the procedure. Non-ionic contrast material was used in all patients. No nitroglycerin was given prior to the diagnostic procedure. Coronary blood flow (CBF) response to papaverine, acetylcholine (ACh), and nitroglycerin was studied according to previous reports[15, 16]. After control coronary angiograms, interventions were performed as follows: 1) a 0.014-inch Doppler guidewire (Cardiometrics, Santa Anna, CA) was introduced into the left anterior descending coronary artery; 2) after obtaining a stable Doppler signal, a bolus of papaverine (an endothelium-independent vasodilator in resistance coronary arteries) (12.5 mg/5 mL) was injected through a catheter; 3) infusion of ACh (an endothelium-dependent vasodilator in resistance and conduit coronary arteries) (0.5 mL/min) at a dose of 3 μg/min for 2 min was performed via the catheter; and 4) a bolus of nitroglycerin (an endothelium-independent vasodilator in conduit coronary arteries) (200 μg/5 mL) was administered[17, 18]. There was a minimum 5-min interval between drug infusions. Coronary arteriography was performed before and 2 min after each dose of ACh and after administration of nitroglycerin. Phasic coronary blood flow velocities, arterial blood pressure, and heart rate were monitored continuously and recorded. Measurements obtained during steady state conditions were used as control values for later analysis.

Assessment of Coronary Blood Flow

Doppler flow velocity spectra were analyzed on-line to determine time-averaged peak velocity. Volumetric CBF was determined from the formula: CBF = cross-sectional area × average peak velocity × 0.5[19]. Coronary flow reserve to papaverine was calculated as the ratio of maximal CBF induced by papaverine to basal CBF, which was equivalent to the endothelium-independent function of the resistance coronary artery. Endothelium-dependent function was calculated as the percent increase in CBF in response to ACh[15, 20, 21].

Quantitative Coronary Angiographic Images

Technically suitable single-plane angiograms were selected for computer analysis. Quantitative coronary angiographic images (DBAC-1000; MID Corporation, Fukuoka, Japan) were recorded using validated densitometric analysis, as previously reported[22]. An end-diastolic still frame at each infusion (baseline, ACh, nitroglycerin) was selected from the angiographic sequence. Endothelium-dependent and -independent vasodilation of the conduit coronary artery was estimated by measuring the luminal diameter at the tip of the Doppler guidewire positioning at the proximal site of left anterior descending coronary artery. These measurements were performed by experienced observers who were unaware of the coronary vascular reactivity tests.

Baseline Measurements and Biochemical Analysis

Diagnosis of hypertension was based on systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg, or current treatment with antihypertensive drugs. Diabetes was defined on the basis of a fasting plasma glucose level ≥126 mg/dL, or HbA1c level ≥6.5%, or the use of oral hypoglycemic agents or insulin. Hyperlipidemia was defined on the basis of a fasting plasma low density lipoprotein (LDL) -cholesterol ≥140 mg/dL, or triglycerides ≥150 mg/dL, or active use of lipid-lowering medication. Cigarette smoking and menstruation status were determined by self-report. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2).

Blood samples were obtained from subjects in the fasting state. White blood cell counts were measured using commercially available kits. Serum UA was measured in local laboratories using an automated technique based on the uricase/pod method, and serum creatinine, triglycerides, high density lipoprotein (HDL) -cholesterol and LDL-cholesterol values were measured by enzymatic methods (Roche Diagnostics Co., Ltd., Basel, Switzerland), using an autoanalyzer (Modular Analytics, Roche Diagnostics Co., Ltd.). High sensitivity C-reactive protein (CRP) was measured by latex-enhanced nephelometry (Denka Seiken Co., Ltd., Tokyo, Japan). Immunoreactive insulin was determined by a specific enzyme immunoassay with various reagents (TOSOH Co., Ltd., Yamaguchi, Japan). Insulin resistance was evaluated by the homeostasis model assessment ratio (HOMA-R) index calculated as follows: fasting plasma glucose (mg/dL) × fasting plasma insulin ($\mu U/mL$)/405[23]. Glomerular filtration rate (GFR) was estimated from the Modification of Diet in Renal Disease (MDRD) equation for Japanese patients, recently proposed by a working group of the Japanese Chronic Kidney Disease Initiative: $eGFR = 0.741 \times 175 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times (0.742, \text{if female})$ mL/min/1.73 m^2 [24].

ADMA Measurements

After April 2002, 113 (64 men and 49 women) of 194 consecutive patients enrolled in the coronary flow study had their serum ADMA concentrations measured. The relationship between UA and eGFR or ADMA was also assessed.

Fasting blood samples for ADMA measurement were obtained at the time of coronary flow study and stored at −80℃ until analysis. Serum ADMA concentrations were measured by high-performance liquid chromatography (HPLC) with precolumn derivatization with o-phthalaldehyde, as described previously[25].

Statistical Analysis

Statistical analysis was performed using Stat View Version 5.0 software. Values are expressed as the mean ± SD. The two groups were compared using Student's regression analysis unpaired t test. Risk factors and drugs were compared between groups using Pearson's chi-square test. Predictive factors for percent change in CBF induced by ACh were determined by stepwise regression analysis. Differences between the number of risk factors and the three grades of percent change in CBF induced by ACh and serum UA level were analyzed using Spearman's rank correlation coefficient. Statistical analysis of parallelism in linear regression was employed to compare the slopes of the simple regression[26]. Statistical significance was accepted when the p value was < 0.05.

Results

Patient Characteristics

One hundered ninety-four patients were evaluated. Patient characteristics are summarized in **Table 1**. Mean age was not significantly different when com-

Table 1. Patient background 1

	Men (n=119)	Women (n=75)	p
Age (years)	63±12	63±13	NS
Post-menopausal		62 (83%)	
Risk Factor			
Current Smoking	44 (37%)	2 (3%)	<0.001
Obesity (BMI ≥25)	36 (30%)	23 (31%)	NS
Hypertension	72 (61%)	42 (56%)	NS
Hyperlipidemia	34 (29%)	32 (43%)	NS
Diabetes mellitus	15 (13%)	13 (17%)	NS
Drugs			
ACE inhibitor	25 (21%)	14 (19%)	NS
ARB	45 (38%)	28 (37%)	NS
Calcium channel blocker	54 (45%)	33 (44%)	NS
Statin	7 (6%)	19 (25%)	<0.001
Aspirin	45 (38%)	27 (36%)	NS
Furosemide	39 (33%)	26 (35%)	NS
Spironolactone	20 (17%)	14 (19%)	NS
Allopurinol	7 (6%)	7 (9%)	NS

Values are the means ± SD or numbers of patients (percentages).
ACE, Angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; NS: not significant

paring the two groups. Sixty-two post-menopausal women were included in the 75 women in the study population. Coronary risk factors were also similar when comparing the two groups, except for active smoking status, which was observed more frequently in men than in women ($p<0.001$). There was no significant difference between groups in the frequency of using cardiac medications, with the exception of statin use, which was more frequent in women than in men ($p<0.001$). Serum UA levels were significantly higher in men than in women (6.7±1.5 mg/dL vs. 5.6±2.0 mg/dL, $p<0.0001$). Serum creatinine levels were also higher in men than in women (0.88±0.23 mg/dL vs. 0.70±0.28 mg/dL, $p<0.0001$), while HDL-cholesterol level was significantly higher in women than in men (53±15 mg/dL versus 59±14 mg/dL, $p<0.01$) (**Table 2**).

Coronary hemodynamic characteristics are summarized in **Table 3**. Baseline coronary artery diameter (CAD) and CBF were similar when comparing the two groups. There was no significant difference in the percent change in CBF induced by papaverine, coronary vascular resistance, percent change in CBF and CAD induced by ACh, or percent change in CAD induced by nitroglycerin when comparing men and women (**Table 3**).

Table 2. Patient background 2

	Men (n=119)	Women (n=75)	p
Mean BP (mmHg)	92±16	90±14	NS
Uric Acid (mg/dL)	6.7±1.5	5.6±2.0	<0.0001
WBC (/μL)	5,505±1,349	5,346±1,963	NS
hsCRP (mg/dL)	0.25±0.44	0.17±0.31	NS
Body mass index (kg/m^2)	23.3±3.6	23.4±3.8	NS
LDL-Cholesterol (mg/dL)	111±30	119±33	NS
HDL-Cholesterol (mg/dL)	53±15	59±14	<0.01
Triglycerides (mg/dL)	122±85	127±85	NS
HOMA-R	2.0±2.5	1.8±1.6	NS
Creatinine (mg/dL)	0.88±0.23	0.70±0.28	<0.0001
eGFR (mL/min/1.73 m^2)	70.2±19.3	72.3±25.2	NS

Values are the means ± SD.
BP, blood pressure; eGFR, estimated glomerular filtration rate; HDL-Cholesterol, high density lipoprotein cholesterol; HOMA-R, homeostasis assessment of insulin resistance index; hsCRP, highly sensitive C-reactive protein; LDL-Cholesterol, low density lipoprotein cholesterol; WBC, white blood cell; NS, not significant

Table 3. Coronary hemodynamic characteristics

	Men (n=119)	Women (n=75)	p
CAD at baseline (mm)	2.9±0.7	2.8±0.6	NS
CBF at baseline (mL/min)	73.5±46.1	72.8±45.3	NS
% change in CBF induced by papaverine (%)	210.4±97.4	183.7±96.0	NS
Coronary vascular resistance (mmHg min/mL)	1.8±1.2	1.9±1.3	NS
% change in CBF induced by acetylcholine (%)	38.9±58.8	32.0±54.6	NS
% change in CAD induced by acetylcholine (%)	3.2±17.4	1.2±10.0	NS
% change in CAD induced by nitroglycerin (%)	15.4±21.2	14.1±17.3	NS

Values are the mean ± SD.
CAD, coronary artery diameter; CBF, coronary blood flow; NS: not significant

Gender Difference in Coronary Endothelial Function

In univariate analysis of the female study population, serum UA, ADMA level and age inversely correlated with the percent change in CBF induced by ACh ($r=-0.32$, $p<0.01$; $r=-0.31$, $p<0.05$; $r=-0.23$, $p<0.05$, respectively) (**Fig. 1, 2**). Further, eGFR positively correlated with the percent change in CBF induced by ACh ($r=0.27$, $p<0.05$) (**Fig. 3**). Stepwise regression analysis showed that serum UA level was the only independent predictor of percent change in CBF induced by ACh in women (**Table 4**). In con-

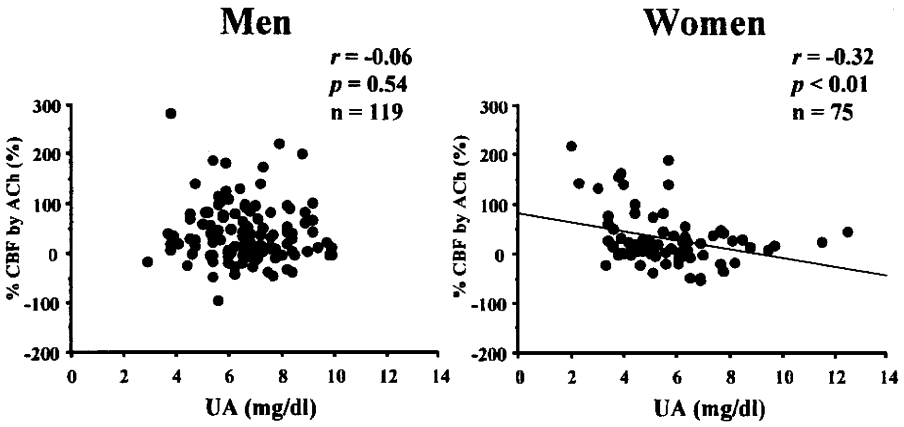

Fig. 1. Relationship between serum UA level and percent change in CBF induced by ACh.
ACh, acetylcholine; CBF, coronary blood flow; UA, uric acid

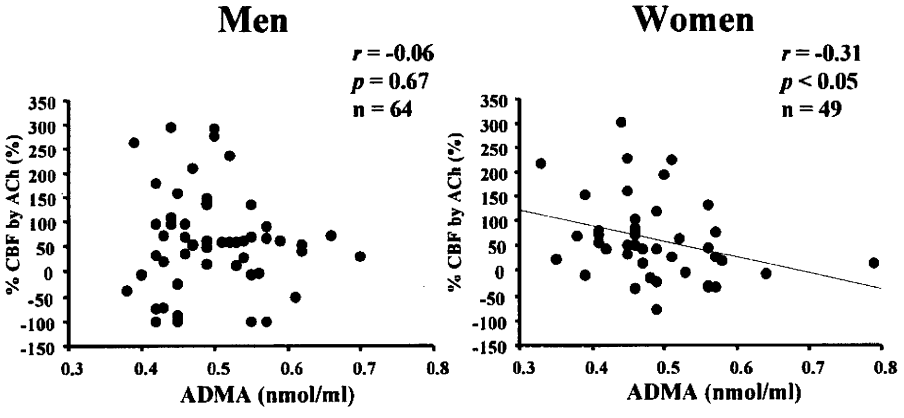

Fig. 2. Relationship between serum ADMA level and percent change in CBF induced by ACh.
ACh, acetylcholine; ADMA, asymmetric dimethylarginine; CBF, coronary blood flow

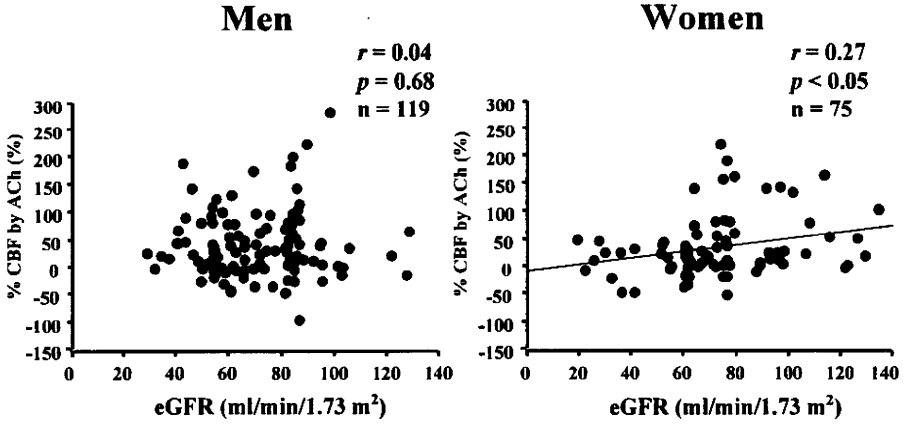

Fig. 3. Relationship between eGFR and percent change in CBF induced by ACh.
ACh, acetylcholine; CBF, coronary blood flow; eGFR, estimated glomerular filtration rate

Table 4. Percent change in CBF induced by acetylcholine: Potential predictive factors

Parameter	Men				Women			
	Univariate Analysis		Stepwise Multiple Regression analysis		Univariate Analysis		Stepwise Multiple Regression Analysis	
	r	*p*	*F*	*p*	*r*	*p*	*F*	*p*
Age	−0.19	<0.05	0.569	NS	−0.23	<0.05	0.139	NS
Mean BP	0.04	NS	0.070		0.08	NS	0.001	
Body mass index	0.05	NS	0.130		−0.05	NS	0.368	
Uric acid	−0.06	NS	0.246		−0.32	<0.01	4.969	<0.05
WBC	0.06	NS	0.026		0.04	NS	0.138	
hsCRP	−0.02	NS	0.024		−0.21	NS	0.791	
LDL-cholesterol	−0.07	NS	0.960		−0.06	NS	0.119	
HDL-cholesterol	0.10	NS	0.402		0.08	NS	0.108	
Triglycerides	−0.08	NS	0.620		−0.17	NS	1.001	
HbA1c	−0.14	NS	3.987		−0.07	NS	3.355	
HOMA-R	−0.15	NS	1.153		−0.11	NS	0.464	
Creatinine	−0.01	NS	0.433		−0.21	NS	0.032	
eGFR	0.04	NS	1.079		0.27	<0.05	0.356	NS

ADMA, asymmetric dimethylarginine; BP, blood pressure; CBF, coronary blood flow; eGFR, estimated glomerular filtration rate; HDL-cholesterol, high density lipoprotein cholesterol; HOMA-R, homeostasis assessment of insulin resistance index; hsCRP, highly sensitive C-reactive protein; LDL-cholesterol, low density lipoprotein cholesterol; WBC, white blood cell; NS, not significant

trast, age inversely correlated with the percent change in CBF by ACh ($r=-0.19$, $p<0.05$) in univariate analysis in the male study population. However, serum UA and ADMA levels did not correlate with the percent change in CBF by ACh in univariate analysis in this population ($r=-0.06$, $p=0.54$; $r=-0.06$, $p=0.67$, respectively). Stepwise regression analysis showed no independent predictor of percent change in CBF in response to ACh (**Table 4**).

As previously described, coronary endothelial function, as reflected by ACh-induced percent change in CBF, was classified into three different grades: poor (<0%), fair (0%–50%), and good (>50%)[14]; therefore, the mean UA concentration was also analyzed after stratifying according to CBF grade. Mean UA level increased as CBF grade worsened in women (good: UA 4.2±1.3 mg/dL, fair: UA 5.6±2.0 mg/dL, poor: UA 5.8±1.4 mg/dL, $p<0.001$) but not in men (good: UA 6.6±1.5 mg/dL, fair: UA 6.7±1.6 mg/dL, poor: UA 6.6±1.5 mg/dL, $p=0.83$).

Relationship between UA Concentration and eGFR or ADMA

The correlation between UA and eGFR is shown in **Fig. 4**. eGFR significantly decreased with increasing UA in both groups (eGFR decreased 3.1 and 7.7 mL/min/1.73 m^2 for every 1 mg/day increase in UA, respectively), suggesting that renal function decreased in response to increasing UA concentrations ($r=-0.25$, $p<0.01$; $r=-0.59$, $p<0.0001$, respectively). The slope for women was significantly ($p<0.01$) steeper than for men, suggesting that renal dysfunction progressed more rapidly in response to elevated UA concentration in women than in men. Serum UA levels positively correlated with ADMA only in women ($r=0.36$, $p<0.05$) (**Fig. 5**). Moreover, serum ADMA levels inversely correlated with eGFR in women ($r=-0.42$, $p<0.01$) but not in men (**Fig. 6**).

Relationship between BMI and UA Concentrations

Serum UA levels were measured in obese (BMI ≥25) and non-obese (BMI <25) subjects. The mean UA concentration was significantly higher in obese men than in non-obese men (7.2±1.4 mg/dL vs. 6.5 ±1.6 mg/dL, $p<0.05$), but there was no difference in UA concentrations when comparing obese women and non-obese women (5.4±1.5 mg/dL vs. 5.7±2.1 mg/dL). Moreover, serum UA levels positively correlated with BMI in men ($r=0.24$, $p<0.05$) but not in women.

Relationship between Number of Coronary Risk Factor and UA Concentrations

Coronary risk factors include hypertension, diabetes mellitus, hyperlipidemia and obesity. Smoking status was excluded from analysis because there were

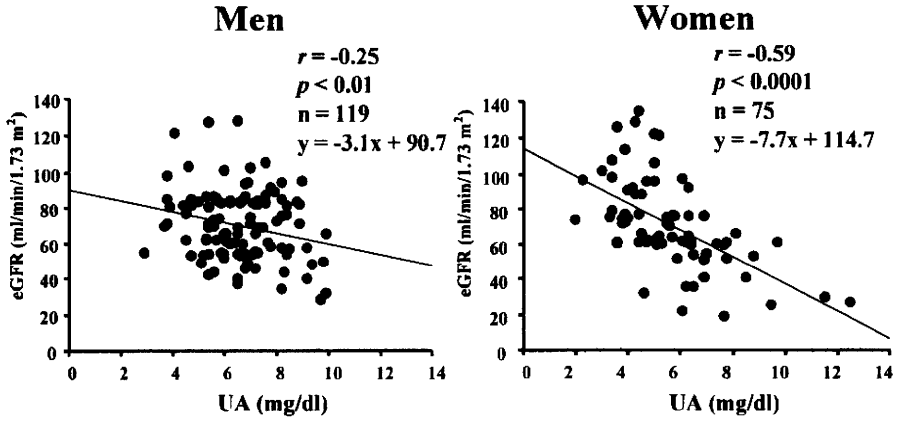

Fig. 4. Relationship between serum UA level and eGFR.
eGFR, estimated glomerular filtration rate; UA, uric acid

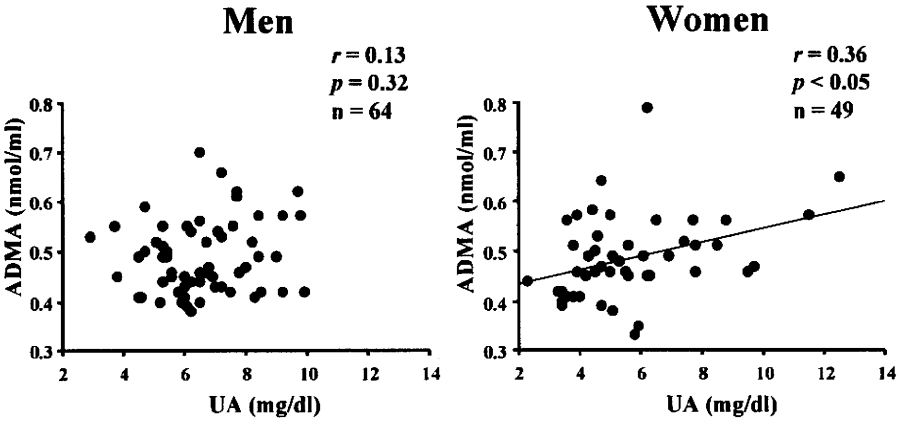

Fig. 5. Relationship between serum UA level and ADMA.
ADMA, asymmetric dimethylarginine; UA, uric acid

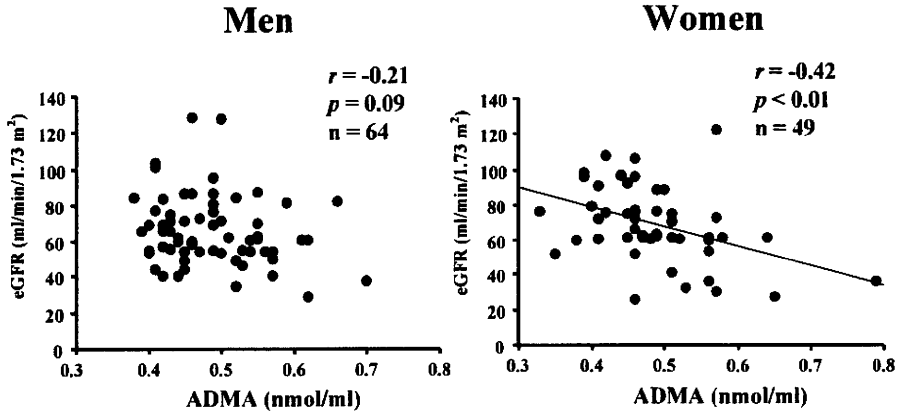

Fig. 6. Relationship between serum ADMA level and eGFR.
ADMA, asymmetric dimethylarginine; eGFR, estimated glomerular filtration rate

relatively few women smokers in the study population. In men, the mean UA concentration increased along with an increasing number of coronary risk factors. (0 risk factors, 6.3 ± 1.6 mg/dL; 1 risk factor, 6.5 ± 1.6; 2 risk factors, 6.9 ± 1.5 mg/dL; 3 or 4 risk factors, 7.4 ± 1.3 mg/dL, $p<0.05$). By contrast, there was no correlation between the number of risk factors and the mean UA level in women (0 risk factors, 5.4 ± 2.2 mg/dL; 1 risk factor, 5.7 ± 1.7 mg/dL; 2 risk factors, 5.9 ± 2.5 mg/dL; 3 or 4 risk factors, 5.4 ± 1.4 mg/dL). Even after considering other risk factors (**Table 4**), UA remained the only independent predictor of ACh-induced percent change in CBF.

Discussion

Relationship between Coronary Endothelial Function and Serum UA Level in Women

The present study demonstrated that serum UA level inversely correlated with endothelium-dependent vasodilation of the resistance coronary arteries in women but not in men. In the Worksite Study, serum UA level was independently and specifically associated with cardiovascular events in hypertensive patients. Despite blood pressure control, serum UA level increased during treatment and was significantly and directly associated with cardiovascular disease. In fact, that study predicted that a serum UA level greater than 7.5 mg/dL in men and greater than 6.2 mg/dL in women was associated with an elevated risk of cardiovascular disease[27]. In another study, coronary endothelial function was classified according to ACh-induced percent change in CBF into one of three different grades: poor (<0%), fair (0%–50%), and good (>50%)[14]. Using this classification system to evaluate the present study population revealed that 10 women with normal UA (<6.0 mg/dL) had a poor CBF response to ACh (**Fig. 1**). This is consistent with the observations from the Progetto Ipertensione Umbria Monitoraggio Ambulatoriale (PIUMA) study, in which patients in the highest quartile of serum UA (>6.2 mg/dL in men; >4.6 mg/dL in women) had an increased risk of subsequent cardiovascular events and death from all causes. Based on these data, the PIUMA investigators proposed that "healthy" UA levels were 4.5–6.2 mg/dL in men and 3.2–4.6 mg/dL in women[28]. Based upon the solubility limit of urate in serum, hyperuricemia is generally defined as a serum UA level above 7 mg/dL in both genders[29]. Even in patients with gout, reduction of the serum UA level to below 6 mg/dL is recommended to prevent recurrence and assist in resolution of tophi[30]. The PIUMA investigators, as well as the results from the present study, suggest that reduction of UA to even lower levels is associated with additional increments of endothelial function in women.

Kielstein *et al.*[9] demonstrated that eGFR correlated with ADMA in patients with renal dysfunction. Comparing men without renal dysfunction (eGFR ≥60, $n=37$) and men with renal dysfunction (eGFR <60, $n=27$) in the present study, ADMA inversely correlated with eGFR in men with renal dysfunction ($r=-0.43$, $p=0.026$), but not in men without renal dysfunction ($r=-0.18$, $p=0.275$). Thus, the inclusion of men without renal dysfunction likely resulted in the absence of a significant correlation between ADMA and eGFR when considering the whole study population of men.

Several previous studies have demonstrated that UA elevation in men may be related to urate overproduction associated with obesity rather than decreased urate excretion[31-34]. These reports are consistent with the results from the present study that the UA level did not correlate with ADMA. Several studies also showed that hyperuricemia lead to cardiovascular events in men as well as women[27, 28]. However, these studies had different set values for UA relative to the elevated risk of cardiovascular disease between men and women (men: 6.2–7.5 mg/dL; women: 4.6–6.2 mg/dL). We investigated the relationship between the UA level and endothelial dysfunction for the consequences in all patients, so our study population included many men with a normal range of UA, which may explain why we did not observe a significant correlation between UA and endothelial dysfunction in men.

Relationship between UA and Renal Function Include of eGFR and ADMA Level in Women

Measurement of eGFR is the gold standard for the assessment of renal function[35]. In our study, there was a correlation between UA and eGFR in both men and women, as illustrated in **Fig. 4**. The correlation between these variables was stronger in women than in men, which suggests that renal dysfunction and UA levels were more strongly related in women than in men. Recent epidemiologic data suggest that hyperuricemia (≥6.0 mg/dL) is an independent predictor of end-stage renal disease in women[36]. Meanwhile, a decrease in eGFR may result in hyperuricemia. As estrogen stimulates urinary urate excretion, premenopausal women have lower serum UA levels than men or postmenopausal women[13]. Generally, hyperuricemia results from factors that increase urate generation or decrease urate excretion[1], and decreased urate excretion may be a more predominant cause of hyperuricemia in women. Indeed, the present study demon-

strated that renal dysfunction and the UA level were more closely related in women than men. Further, most of the women enrolled in this study were postmenopausal and hence, can be assumed to have decreased urate excretion or hypoestrogenemia.

Endothelial dysfunction is characterized by reduced endogenous NO activity, which may be attributed to elevated ADMA[37]. ADMA is an endogenous competitive inhibitor of NO synthase and an independent marker of cardiovascular risk[38]. Elevated endogenous ADMA levels are associated with systemic manifestations of endothelial dysfunction in patients with cardiovascular risk factors[39]. The present study demonstrated that the serum ADMA level inversely correlated with ACh-induced percent change in CBF and eGFR levels and positively correlated with the serum UA level only in women. These data suggest that hyperuricemia-induced renal damage in women may be mediated via the accumulation of ADMA and secondary reductions in NO-dependent coronary endothelial function.

Relationship between Coronary Risk Factors and Hyperuricemia

The reason for the absence of correlation between UA and endothelial function in men remains unclear. Several epidemiological studies have reported a close relationship between hyperuricemia and hypertension, obesity, hyperlipidemia and diabetes[40-42]. The present study demonstrated that the mean UA concentration was significantly higher in obese than non-obese men. Moreover, serum UA levels positively correlated with BMI only in men.

Visceral fat obesity represents a greater risk for various diseases than subcutaneous fat obesity. The pathogenesis of hyperuricemia in patients with visceral fat is related to low urinary urate excretion and overproduction of UA[31]. Indeed, several studies have suggested that an excess flow of free fatty acid from accumulated visceral fat to the liver may increase the production of UA via de novo purine synthesis and the pentose phosphate pathway[32, 33]. Meanwhile, hyperinsulinemia associated with visceral fat obesity may reduce urinary urate excretion in parallel with a decrease in urinary sodium excretion[34]. However, in the present study, there was no correlation between serum UA levels and HOMA-R or the insulin level (data not shown). Matsuura and colleagues reported that hyperuricemia may not be caused by the low urinary urate excretion associated with hyperinsulinemia in Japanese patients due to genetic susceptibility to impaired insulin-secreting ability[31]. Therefore, UA elevation in men may be related to urate overproduction associated with obesity rather than decreased urate excretion. In this study, serum UA levels increased along with an increasing number of coronary risk factors in men. As several risk factors in men are multifactorially related to endothelial dysfunction and are suspected to overlap, UA alone is not a risk factor of endothelial dysfunction. Serum UA levels in women did not correlate with either the number of coronary risk factors or obesity but did correlate closely with renal dysfunction.

Study Limitations

Several limitations of this study must be considered when interpreting the results. First, this study was a retrospective analysis of coronary flow research; nevertheless, the present study does provide a preliminary framework for planning future prospective studies. Second, smoking was observed much more frequently in men than in women, which suggests that smoking may have influenced our results. Although smoking would be expected to have an unfavorable effect on endothelial function, there was no difference in coronary endothelial function between men and women in this study. Moreover, UA levels and the percent change in coronary blood flow induced by ACh were comparable in men regardless of smoking status (data not shown). Therefore, differences in smoking status should have no bearing on the interpretation of the present data. Third, many reports suggest that microalbuminuria is a predictor of cardiac events. Indeed, Cosson and colleagues reported that microalbuminuria correlated with coronary endothelial dysfunction[43]; however, microalbuminuria was not measured in the evaluation of renal function in the present study. Fourth, all study patients had angiographically normal or mildly diseased coronary arteries, limiting the generalization of these data to patients with advanced coronary artery disease. Finally, the study population was relatively small, and the resulting statistical power may have been insufficient to demonstrate differences in some parameters.

UA has both prooxidant and antioxidant properties. Hyperuricemia is associated with endothelial dysfunction and cardiovascular diseases; in contrast, UA administration increases serum antioxidant capacity in healthy volunteers[44] and improves endothelial function in patients with type 1 diabetes and in smokers[45]. Furthermore, higher serum UA concentrations are associated with elevated total serum antioxidant capacity among individuals with atherosclerosis, which is consistent with experimental evidence suggesting that hyperuricemia may be a compensatory mechanism to counteract oxidative damage related to atherosclero-

sis[46]. Indeed, high UA concentration might have a protective as well as non-protective role associated with increased cardiovascular risk[45]. These observations suggest that the protective role of UA deserve further investigation.

Conclusions

High UA concentration strongly correlates with coronary endothelial microvascular dysfunction in women. Further, in women, serum UA concentrations correlate with eGFR and ADMA. Thus, hyperuricemia-induced coronary endothelial dysfunction may be mediated via the accumulation of ADMA and renal damage in women.

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ORIGINAL CONTRIBUTIONS

Additive Interaction of Metabolic Syndrome and Chronic Kidney Disease on Cardiac Hypertrophy, and Risk of Cardiovascular Disease in Hypertension

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BACKGROUND

Recent epidemiologic analyses have demonstrated a link between the metabolic syndrome (MetS) and chronic kidney disease (CKD). We examined the association between MetS, CKD, and left ventricular hypertrophy (LVH), and prospectively investigated the predictive value of the combination of MetS and CKD for cardiovascular disease (CVD) in essential hypertension.

METHODS

A total of 1,160 essential hypertensive patients (mean age 63 years, 53% male) underwent clinical evaluation, laboratory testing, and Doppler echocardiography, and were monitored for a mean follow-up of 4.8 years.

RESULTS

At baseline, total subjects were divided into four groups according to the presence/absence of MetS and/or CKD, and, compared to the group without MetS and CKD ($MetS^-/CKD^-$); those with MetS and CKD ($MetS^+/CKD^+$) had a multivariate-adjusted odds ratio of 2.40 (95% confidence interval (CI) 1.66–3.48) for LVH. During the follow-up period, 172 subjects developed CVD. Multiple Cox regression analysis including LV mass index (LVMI) showed that the presence of MetS as well as that of CKD were each independent predictors of CVD (hazard ratio 1.90 for MetS, 1.82 for CKD). We then divided the total subjects into four groups, and found that, compared to the $MetS^-/CKD^-$ group, multivariate-adjusted HR for the $MetS^+/CKD^+$ group was 3.58 (95% CI 2.14–5.95).

CONCLUSIONS

Our findings suggest that, in essential hypertension, the combination of MetS and CKD is a strong risk for LVH as well as a strong and independent predictor of subsequent CVD. These findings highlight the clinical importance of the concomitance of MetS and CKD in essential hypertension.

Keywords: blood pressure; cardiovascular disease; chronic kidney disease; hypertension; left ventricular hypertrophy; metabolic syndrome; risk factor

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Hypertension is a common risk factor for cardiovascular disease (CVD), and the cardiovascular prognosis in patients with hypertension depends not only on the level of blood pressure (BP), but also on the presence of associated risk factors. In the past few years, there has been growing attention to a condition known as the metabolic syndrome (MetS),[1] which is characterized by a cluster of atherosclerotic risk factors, including obesity, hypertension, insulin resistance, and dyslipidemia, as well as chronic kidney disease (CKD).[2] Individuals with MetS or CKD are at increased risk of CVD as well as death from CVD and all causes.[3–8] Furthermore, recent epidemiologic analyses have demonstrated a link between MetS and CKD.[9–11] However, whether the concomitance of MetS and CKD contributes to the development of CVD is unknown.

Echocardiography is a well-established procedure to diagnose increased left ventricular (LV) mass, and its presence is thought to increase CVD risk through a series of unfavorable metabolic, functional, and structural cardiac changes.[12–14] The assessment of LV geometry in addition to LV hypertrophy (LVH) is important for evaluation of the peculiar hemodynamic pattern such as a combination of pressure and volume stimuli, contractile efficiency, and prognosis.[15] Insulin resistance, oxidative stress, and inflammation have been implicated in the pathogenesis of MetS and CKD, which also have been shown to be associated with LVH. Increased LV mass has been shown to be associated with MetS and CKD;[16–20] however, we could not find any previous studies examining the hypothesis that the combination of MetS and CKD may be a strong risk for LVH.

The influence of increased LV mass on the association of MetS and/or CKD with CVD is also unknown. The

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association between MetS or CKD and increased CVD could be mediated through increased LV mass, and this may be one of the pathways linking MetS and CKD to CVD. Therefore, in this study, we investigated the potential interrelationship between MetS, CKD, and the risk of LVH in essential hypertensive subjects. Furthermore, we also examined prospectively whether MetS and CKD interact to substantially increase the risk of CVD in hypertension. Moreover, we additionally examined whether this association would be independent of LV mass.

METHODS

Study subjects. This study enrolled essential hypertensive patients in normal sinus rhythm, who had good-quality echocardiographic recordings, and monitored them for a mean follow-up of 4.8 ± 2.7 years. In our laboratory (the National Cardiovascular Center in Osaka, Japan), all hypertensive patients attended the echocardiography laboratory, and echocardiographic data were routinely collected consecutively. From 1,263 patients at the time of the baseline examination, we excluded patients with missing data of MetS or CKD components (n = 77) and patients receiving regular hemodialysis therapy (n = 26), leaving 1,160 patients (545 women) for this analysis. Exclusion criteria included acute coronary syndrome, congestive heart failure (CHF) (New York Heart Association class II or greater), secondary hypertension, moderate or severe aortic or mitral regurgitation, heart rate ≥100 bpm, and low ejection fraction (<45%). All procedures in this study were carried out in accordance with institutional and national ethical guidelines for human studies. All participants enrolled in this study were Japanese, and all gave informed consent to participate.

Baseline clinical characteristics. Hypertension was defined as systolic BP ≥140 mm Hg or diastolic BP ≥90 mm Hg on repeated measurements, or receiving antihypertensive treatment. Diabetes mellitus was defined according to the American Diabetes Association criteria.[21] Smoking status was determined by interview, and defined as never-smoker, past-smoker (those with a history of habitual smoking but had quit), and current-smoker. Previous CVD was defined as a history of myocardial infarction, CHF, or stroke.

After fasting overnight, BP was measured with an appropriate arm cuff and a mercury column sphygmomanometer on the left arm after a resting period of at least 10 min in the supine position. After BP measurement, venous blood and urine sampling from all subjects was performed. Height and body weight were measured, and body mass index (BMI) was calculated. The following parameters were also determined: triglycerides, high-density lipoprotein cholesterol, C-reactive protein (CRP), and creatinine.

Definition of MetS and CKD. MetS was defined according to the guidelines of the National Cholesterol Education Program Third Adult Treatment Panel with modification for body size.[1]

In this study, all patients were hypertensive and thus, participants had MetS if they fulfilled two or more of the following.

1. Elevated BMI (in lieu of waist measurement, which was not available in our database). The frequency of BMI ≥30 kg/m^2 is 2–3% in Japan and 20–30% in Western countries.[22–24] Because of the differences in BMI between Japanese and Western populations, values ≥25 kg/m^2 were considered elevated (in contrast to ≥30 kg/m^2 in Western populations) according to the criteria of the Japan Society for the Study of Obesity.[22,25]
2. Elevated triglycerides (≥150 mg/dl).
3. Low high-density lipoprotein cholesterol (<40 mg/dl in men, <50 mg/dl in women).
4. Impaired fasting glucose (fasting plasma glucose ≥110 mg/dl and/or a history of diabetes).

The estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease formula in ml/min. CKD and its stages were defined according to the guidelines of the National Kidney Foundation classification of CKD,[2] which defines from eGFR of <60 ml/min/1.73 m^2 or dipstick proteinuria (≥1+) as follows: eGFR ≥90 ml/min/1.73 m^2 without proteinuria (high BP), eGFR 60–89 ml/min/1.73 m^2 without proteinuria (high BP with reduced GFR), eGFR ≥90 ml/min/1.73 m^2 with proteinuria (stage 1), eGFR 60–89 ml/min/1.73 m^2 with proteinuria (stage 2), and stages 3–5 were classified according to the level of kidney function (eGFR 30–59, 15–29, and <15 ml/min/1.73 m^2, respectively), regardless of the presence of other markers of kidney damage.[2] Subjects were diagnosed with CKD if they were classified as CKD stage 1–5.

Echocardiographic methods and calculation of derived variables. Phased-array echocardiography with M-mode, two-dimensional, pulsed, and color-flow Doppler capabilities was performed in all study participants, as previously described.[26,27] Estimates of LV mass were calculated according to the American Society of Echocardiography criteria[28] applied to the formula of Devereux *et al.*[29] LV mass index (LVMI) was calculated by dividing LV mass by body surface area. LVH was defined as LVMI >125 g/m^2 for men and >110 g/m^2 for women.[30] Relative wall thickness (RWT) was calculated as (interventricular septal + posterior wall thickness)/LV internal diameter.[31] Concentric remodeling was defined as normal LVMI with RWT >0.45 (ref. 31). Eccentric hypertrophy was defined as LVH with RWT ≤0.45. Concentric hypertrophy was defined as LVH with RWT >0.45 (ref. 32). LV filling was assessed by recording mitral flow by a standard pulsed Doppler technique, and the following parameters were considered: the ratio of peak early-to-late diastolic filling velocity (*E*/*A* ratio) and the deceleration time of early diastolic LV filling.

Clinical end points. For survival analysis, observation began on the date of echocardiography, with verified dates updated through October 2007. All of the subjects were followed at