Table 3 Associations of the Presence of Coronary Artery Disease (CAD) With Risk Factors and Plasma Matrix Metalloproteinase-8 (MMP) Concentration*

Variables	*Odds ratio (95%confidence interval)*	*p value*
Age (per 10 years increase)	*2.01 (1.39–2.93)*	*<0.001*
Male	*2.50 (1.25–5.00)*	*<0.01*
Hyperlipidemia	*1.90 (1.00–3.63)*	*<0.05*
Low HDL-cholesterol (<40 mg/dl)	*3.54 (1.57–7.98)*	*<0.001*
MMP-8 (per 1 ng/ml increase)	*1.22 (1.07–1.39)*	*<0.005*

**Multiple logistic regression analysis in the 250 study patients.*
HDL, high density lipoprotein; hsCRP, high-specificity C-reactive protein.
The dependent variable was the presence of CAD. This analysis included age, gender, hypertension, hyperlipidemia, low HDL-cholesterol, diabetes mellitus, smoking and plasma concentration hsCRP and MMP-8.

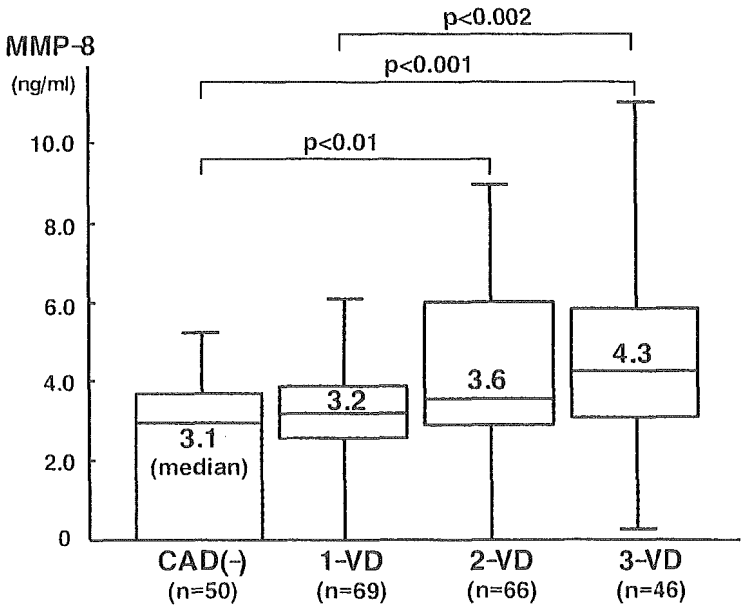

Fig 1. Plasma MMP-8 concentrations in patients with and without CAD showing the stepwise increase in concentration according to the number of >50% stenotic vessels. Compared with patients without CAD, those with 2-vessel disease (VD) and 3-VD had significantly higher MMP-8 concentrations. The MMP-8 concentration in 3-VD was also higher than that in 1-VD. The central line represents the median, the boxes span the 25th to 75th percentiles, and the error bars extend from the 10th to 90th percentiles. 1-VD, 2-VD, 3-VD: 1-, 2- and 3-vessel disease, respectively.

Results

Of the 250 patients, 181 (72%) had CAD. Compared with the 69 patients without CAD, the 181 patients were older, predominantly male, had a higher rate of diabetes and also had lower high density lipoprotein (HDL)-cholesterol and higher hsCRP concentrations (Table 1). Plasma MMP-8 concentrations also were higher in patients with CAD than in those without CAD (median 3.5 vs 3.0 ng/ml, p<0.001). After the adjustment for age and gender in patients without CAD, MMP-8 concentrations remained higher in patients with CAD (3.5 vs 3.1 ng/ml, p<0.01) (Table 2). There was a correlation between MMP-8 concentration and plasma hsCRP concentration (rs=0.28, p<0.001), but not age, HDL-cholesterol, hemoglobin (Hb) A1c, or fasting glucose concentration. In the multiple logistic regression analysis, MMP-8 concentration was found to be significantly associated with CAD, but hsCRP concentration was not (Table 3). The odds ratio for the presence of CAD was 1.22 (95%confidence interval (CI)=1.07–1.39) for a 1.0 ng/ml increase in MMP-8 concentration.

Of the 181 patients with CAD, 69 had 1-vessel, 66 had 2-vessel, and 46 had 3-vessel disease. There was a stepwise increase in MMP-8 concentration depending on the number of vessels with >50% stenosis: 3.1 in CAD(–), 3.2 in 1-vessel, 3.6 in 2-vessel, and 4.3 ng/ml in 3-vessel disease (p<0.001) (Fig 1). Patients with 3-vessel disease had higher MMP-8 concentrations and more often showed a MMP-8 concentration >4.0 ng/ml than patients without CAD or with 1- or 2-vessel disease (61% vs 20%, 23%, and 39%, p<0.05). In the multiple logistic regression analysis, high MMP-8 concentration was an independent factor for 3-vessel disease (Table 4). The odds ratio for 3-vessel disease was 4.18 (95%CI=2.08–8.40) for a MMP-8 concentration >4.0 ng/ml.

Because statin treatment may affect the plasma MMP-8 concentration, the 250 patients were divided into 2 groups: 65 on statin therapy and 185 without. There was no difference between these 2 groups in any risk factors other than hyperlipidemia. Although patients on statin therapy had a higher prevalence of CAD (82% vs 69%), but lower MMP-8 concentrations (3.1 vs 3.4 ng/ml) than those not on statins, these differences did not reach statistical significance. MMP-8 concentration correlated with the number of segments with >50% stenosis (rs=0.31) and with the severity score of stenosis (rs=0.32) (p<0.001). However, after excluding the 65 patients on statins, the MMP-8 concentration correlated better with the number of segments with >50% stenosis (rs=0.37) and the severity score (rs=0.40) (p<0.001) (Fig 2).

Regarding the lesion morphology, complex lesions were found in 41 (23%) of the 181 patients with CAD and the hsCRP concentrations were similar between CAD patients with and without complex lesions (0.70 vs 0.70 mg/L).

Table 4 Association of the Presence of 3-Vessel Disease With Risk Factors and Plasma Matrix Metalloproteinase (MMP)-8 Concentration*

Variables	*Odds ratio (95% confidence interval)*	*p value*
Age (per 10 years increase)	*1.74 (1.09–2.79)*	*<0.025*
Diabetes mellitus	*2.85 (1.42–5.71)*	*<0.005*
High MMP-8 (>4.0 ng/ml)	*4.18 (2.08–8.40)*	*<0.001*

**Multiple logistic regression analysis in the 250 study patients.*
The dependent variable was the presence of 3-vessel disease. This analysis included age, gender, hypertension, hyperlipidemia, low high density lipoprotein-cholesterol, diabetes mellitus, smoking, high-specificity C-reactive protein and high MMP-8 (>4.0 ng/ml).

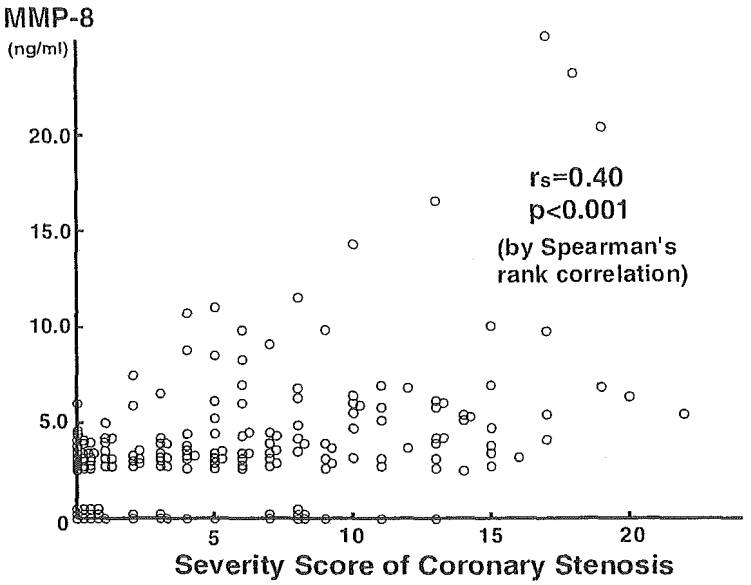

Fig 2. Correlation of plasma MMP-8 concentration with the severity score of coronary stenosis in 185 patients not on statin therapy. After the exclusion of the 65 patients on statins, MMP-8 concentrations correlated better with the severity score (rs=0.40).

MMP-8 concentrations tended to be higher in CAD patients with complex lesions than in those without (3.7 vs 3.5 ng/ml), but this did not reach statistical significance. Even after the exclusion of the patients on statins, the MMP-8 concentrations were not higher in CAD patients with complex lesions than in those without such lesions (3.9 vs 3.7 ng/ml, p=NS).

Discussion

The present study results show that plasma MMP-8 concentrations are higher in patients with CAD than in those without CAD, and that they increase as the severity of CAD increases. In multivariate analysis, MMP-8 concentration was an independent factor associated with CAD. Plasma MMP-8 concentration was found to be associated with both the presence and the severity of CAD, but not with the presence of complex coronary lesions.

MMPs play an important role in the balance between collagen synthesis and breakdown, and it is this balance that determines plaque growth, plaque vulnerability and vascular remodeling.[4–6] Of the various MMPs, only MMP-1, MMP-8 and MMP-13 initiate collagen breakdown, making the collagen fragments susceptible to further degradation by other MMPs, such as MMP-2 and MMP-9.[7] In particular, MMP-8 degrades type I collagen, which is a major component of atherosclerotic plaques, 3-fold more potently than MMP-1 and MMP-13.[8,9] Increased expression of MMP-1 and MMP-13 in atherosclerotic plaques has been demonstrated,[4,5,13,14] but until recently the role of MMP-8 in atherogenesis had been neglected because it was thought that MMP-8 was produced only by neutrophils, which are not commonly present in plaques.[15]

In 2001, Herman et al first reported that endothelial cells, smooth muscle cells and macrophages in human atherosclerotic plaques express MMP-8 mRNA and protein in situ, especially in lipid-rich plaques with a thin fibrous cap.[10] They suggested that MMP-8 plays an important role in the collagen breakdown of atherosclerotic plaques, leading to plaque vulnerability or vascular remodeling. However, the association between MMP-8 and CAD has not yet been elucidated. Kai et al measured the concentrations of MMP-2 and MMP-9 in blood samples from 11 patients with unstable angina, 17 with stable angina, and 17 controls, and found higher MMP-2 concentrations in unstable or stable angina than in controls, and high concentrations of MMP-9 in unstable angina.[16] Funayama et al used a thrombectomy catheter to measure the plasma concentrations of MMP-2 and MMP-9 in the infarct-related coronary artery in 36 patients with acute myocardial infarction and found high concentrations of MMP-9, but not MMP-2.[17] Kalela et al also assessed the serum MMP-9 concentrations in 61 patients with CAD and 19 without CAD and found the MMP-9 concentrations to be high in patients with 3-vessel disease.[18] Our study has shown that the plasma concentration of MMP-8 is higher in patients with CAD than in those without CAD and is an independent factor associated with CAD. Moreover, the MMP-8 concentration is associated with the severity of CAD and is highest in patients with 3-vessel disease. In addition to age and diabetes, MMP-8 concentration is an independent factor for 3-vessel disease. Our results suggest that the plasma MMP-8 concentration

in patients with CAD reflects the severity of coronary atherosclerosis and that MMP-8 may play a role in the progression of CAD. High MMP-8 concentrations in patients with CAD may be aimed at adaptive remodeling of coronary arteries or the plaque vulnerability.

Complex coronary lesions detected by angiography are known to be associated with plaque vulnerability[12,19] and to be predictive of coronary events, such as unstable angina and myocardial infarction[12,20,21] Complex lesions are common in unstable angina and have been reported in 10–20% of patients with stable angina[12] In our study, patients with acute myocardial infarction or unstable angina were excluded, but complex lesions were found in 23% of the patients with CAD. We previously studied the serum MMP-1 concentrations in 185 patients with and without stable CAD[22] and found there was no difference between patients with and without CAD, but that the MMP-1 concentrations were characteristically high in patients with complex coronary lesions. In the present study, MMP-8 concentrations tended to be higher in patients with complex lesions than in those without complex lesions, but did not reach statistical significance. These findings suggest that the plasma MMP-8 concentration in patients with stable CAD may reflect the severity of coronary atherosclerosis rather than plaque vulnerability.

Plasma MMP-8 concentrations significantly correlated with those of hsCRP and high hsCRP concentrations are associated with an increased risk of further coronary events in patients with CAD[23,24] Although patients with acute myocardial infarction or unstable angina were excluded from our study, the plasma hsCRP and MMP-8 concentrations were higher in patients with CAD than in those without CAD. However, MMP-8 concentration, but not that of hsCRP, was an independent factor for CAD. MMP-9 activity in vascular tissues is reportedly enhanced in diabetes mellitus[25] Death et al also showed that high glucose concentration increased the activity of MMP-1, MMP-2 and MMP-9 in vascular cells in vitro[26] However, in our study, plasma MMP-8 concentrations did not correlate with HbA1c or glucose concentrations, and a high concentration was associated with CAD, especially 3-vessel disease, independent of diabetes.

Statin therapy reduces the expression of MMP-1, MMP-3 and MMP-9 in rabbit atheroma[27] and also reduced the plasma MMP-9 concentration in patients with CAD[28] In our study, patients on statins tended to have lower MMP-8 concentrations, despite a higher prevalence of CAD. After the exclusion of patients with statin, MMP-8 concentrations correlated better with the severity of CAD. These results suggest that statin therapy would affect the plasma MMP-8 concentration.

Study Limitations

First, because we did not measure the MMP-8 concentration in the coronary sinus, our study does not provide any information about the main sources of plasma MMP-8 in patients with CAD, which may be the peripheral leukocytes and macrophages. Moreover, our study was unable to establish causality, because it only showed some associations and proposed some hypotheses. Second, we studied patients undergoing angiography, who are generally considered to be a highly selected population at high-risk for CAD. These may have caused some selection bias and confounded the results. Moreover, as shown in Fig 1, there was some overlap between patients with and without CAD in the MMP-8 concentrations. Plasma MMP-8 concentration may reflect not only coronary atherosclerosis but also the degree of atherosclerosis in other vascular beds. Finally, similar to MMP-2 and -9[16,17] MMP-8 may play an important role in the development of acute coronary syndromes and a further study in patients with acute coronary syndromes is needed to elucidate this. Moreover, to confirm the predictive value of MMP-8 concentration for future coronary events in patients with CAD, our study patients should be followed

In conclusion, we found that the plasma MMP-8 concentration is associated with both the presence and the severity of CAD, which suggests that in patients with CAD the plasma MMP-8 concentration reflects the severity of coronary atherosclerosis and that MMP-8 plays a role in disease progression.

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Polymorphism of the 3'-Untranslated Region of Interleukin-12 p40 Gene is not Associated With the Presence or Severity of Coronary Artery Disease

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Background Interleukin (IL)-12 is thought to play an important role in the development of atherosclerosis and recently, polymorphism of the 3'-untranslated region of the IL-12 p40 gene (A1188C) was reported to be associated with diabetes and multiple sclerosis. However, the association between this genetic polymorphism and coronary artery disease (CAD) has not been studied.

Methods and Results The frequency of this polymorphism was investigated in 555 patients undergoing coronary angiography: 395 had CAD, of whom 161 also had a myocardial infarction (MI). With regard to the IL-12 p40 polymorphism, 125 had the A/A, 268 had the A/C, and 162 had the C/C genotype. The prevalence of CAD did not differ among the groups (71%, 73%, and 69%, respectively; p=NS). The prevalence of MI was also similar among the groups (28%, 27%, and 33%, respectively; p=NS). Moreover, the number of >50% stenotic vessels, >50% stenotic segments, and ≤50% stenotic segments did not differ among the 3 groups.

Conclusions Polymorphism of IL-12 p40 gene was not found to be associated with the presence or severity of CAD, suggesting that it does not play an important role in the development of this disease. (*Circ J* 2005; **69**: 793–797)

Key Words: Coronary artery disease; Genetics; Interleukin-12; Polymorphism

Recently, atherosclerotic diseases, such as coronary artery disease (CAD), have been recognized as chronic inflammatory diseases[1,2] In addition to macrophages, T lymphocytes, especially Th1 cells, are commonly present in the atherosclerotic lesions[3,4] and interleukin (IL)-2 and interferon-γ, which are produced by Th1 cells, also are expressed in atherosclerotic lesions[5] Together these findings suggest that the Th1-type cellular immune system is involved in the development of atherosclerosis. IL-12, which is primarily produced by macrophages, plays a key role in the induction of the Th1-type cellular immune response[6,7] and marked expression of both IL-12 mRNA and protein has been demonstrated in human atherosclerotic lesions[8] Elevated blood concentrations of IL-12 have also been reported in patients with CAD[9,10] In apolipoprotein E (apoE)-deficient mice, daily injections of IL-12 accelerated atherosclerosis[11] whereas IL-12 deficiency reduced disease progression[12] These findings suggest that IL-12 plays an important role in the development of atherosclerosis via modulation of the Th1-type cellular immune response.

IL-12 is composed of 2 subunits, p35 and p40, which are encoded by the IL12A gene on chromosome 3 and the IL12B gene on chromosome 5, respectively. Recently, a polymorphism (an A/C transition) was reported at position 1188 in the 3'-untranslated region of IL-12 p40 gene[13] and the A/A genotype was reported as associated with both type 1 diabetes mellitus[14] and multiple sclerosis[15] However, because the association between this polymorphism and atherosclerotic diseases, such as CAD, has not yet been elucidated, we investigated its frequency in 555 patients undergoing coronary angiography for suspected CAD.

Chlamydia pneumoniae (CP), one of the common human respiratory pathogens, has been often reported in seroepidemiological studies as associated with CAD;[16–18] however, its contribution to CAD remains controversial[19,20] The inflammatory response to CP infection may vary from person to person, and only certain individuals may develop CAD. We previously investigated CP seropositivity and IL-1β (a C/T transition at –511) and IL-1 receptor antagonist (IL-1Ra) (a variable number repeat in intron 2) gene polymorphisms in patients undergoing coronary angiography[21] We showed a stepwise increase in the prevalence of CAD depending on a positive interaction between CP seropositivity and IL-1 gene variants (IL-1β C/C genotype and/or IL-1Ra 2- or 3-repeat allele). To examine whether or not IL-12 p40 polymorphism may have some effect on the development of CAD associated with CP infection, we also evaluated the serum CP-specific IgG titer as an indicator of the history of CP infection.

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Table 1 Clinical Characteristics and Frequency of IL-12 p40 Gene Polymorphism in Patients With and Without Coronary Artery Disease (CAD)

	CAD (−) (n=160)	CAD (−) vs (+)	CAD (+) (n=395)	MI (−) (n=234)	MI (−) vs (+)	MI (+) (n=161)
Age (years)	61±10	<0.001	65±9	65±9	<0.05	63±9
Gender (male)	103 (64%)	<0.001	324 (82%)	181 (77%)	<0.01	143 (89%)
Hypertension	84 (53%)	<0.05	227 (63%)	158 (68%)	<0.05	89 (55%)
Systolic BP (mmHg)	131±17	NS	134±20	137±19	<0.001	130±22
Hyperlipidemia	47 (29%)	<0.001	181 (46%)	123 (53%)	<0.005	58 (36%)
TC (mg/dl)	202±35	NS	201±36	205±35	<0.01	195±36
HDL-C (mg/dl)	56±16	<0.001	48±14	50±15	<0.02	46±11
Diabetes	27 (17%)	<0.001	133 (34%)	85 (36%)	NS	48 (30%)
Smoking	87 (54%)	<0.001	282 (71%)	161 (69%)	NS	121 (75%)
IL-12 p40 polymorphism						
A/A genotype	36 (23%)	NS	89 (23%)	54 (23%)	NS	35 (22%)
A/C genotype	73 (46%)	NS	195 (49%)	123 (53%)	NS	72 (45%)
C/C genotype	51 (32%)	NS	111 (28%)	57 (24%)	NS	54 (34%)
CP IgG seropositivity	85 (59%)	NS	248 (63%)	145 (62%)	NS	103 (64%)

Data are mean value±SD or number (%) of patients.
BP, blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol.

Table 2 Factors Associated With the Presence of Coronary Artery Disease (CAD) and Myocardial Infarction (MI)

Variables	Odds ratio (95%CI)	p value
Coronary artery disease		
Age (per 10 year increase)	1.8 (1.4–2.3)	<0.001
Gender (male)	2.2 (1.3–3.8)	<0.01
Hyperlipidemia	2.5 (1.6–3.8)	<0.001
Diabetes	2.2 (1.3–3.6)	<0.005
HDL-C (per 10 mg/dl increase)	0.7 (0.6–0.8)	<0.001
Myocardial infarction		
Gender (male)	2.3 (1.2–4.2)	<0.01
HDL-C (per 10 mg/dl increase)	0.8 (0.7–0.9)	<0.001

The dependent variables were the presence of CAD and MI.
This analysis included age, gender, hypertension, hyperlipidemia, HDL-C, smoking, diabetes mellitus, chlamydia pneumoniae seropositivity, and A/A genotype.
CI, confidence interval.

Methods

Study Patients

The study group comprised 555 consecutive Japanese patients (427 males; mean age 63±9 years) who underwent coronary angiography for suspected CAD at the National Defense Medical College Hospital. Patients with a history of coronary artery bypass surgery were excluded. Of the 555 patients, 331 (60%) had hypertension (blood pressure ≥140/90 mmHg or medication), 228 (41%) had hyperlipidemia (total cholesterol concentration >240 mg/dl or medication), 160 (29%) had diabetes mellitus (fasting glucose concentration ≥126 mg/dl or on hypoglycemic drugs or insulin), and 369 (66%) were smokers (≥5 cigarettes/day). Our study, in which genetic analysis was performed, was approved by the ethics committee of the hospital. After written informed consent was obtained, fasting blood samples were taken on the morning of the day that coronary angiography was performed.

Coronary Angiography

Coronary angiograms were recorded by a femoral approach using the Judkins technique and a cineangiogram system (Toshiba, Tokyo, Japan). All angiograms were evaluated by one of our team (Y.M.) who was unaware of the genotype data. CAD was defined as at least 1 coronary artery with >50% luminal diameter stenosis. Myocardial infarction (MI) was confirmed by documented coronary artery stenosis plus either elevated cardiac enzymes or diagnostic changes on electrocardiogram. The severity of CAD was represented as the number of >50% stenotic vessels and the number of >50% stenotic segments. Moreover, the number of mildly stenotic segments (≤50%) and the number of diffusely stenotic segments (>10 mm) were also evaluated. The coronary artery segments were defined according to the Coronary Artery Surgery Study classification.

Genotyping and CP Serology

We used the Invader assay to analyze IL-12 p40 polymorphism because it combines structure-specific cleavage enzymes and a universal fluorescent resonance energy transfer (FRET) system[22] After genomic DNA was extracted from the blood samples, the region (233 bp) containing the polymorphic site was amplified by polymerase chain reaction (PCR), as reported by Hall et al[13] and the PCR products were used for the assay according to our protocol[23] The primary probes (probe 1 for the A allele, cgcgccgaggAGATGCTAAATGCTCATTGA, and probe 2 for the C allele, acggacgcggagCGATGCTAAA TGCTCATTG) and the Invader probe (CTTTAAAA CGTTTTT TTAGGATCACAATGATATCTTTGCTGTA TTTGTATAGTTT) were designed using the Invader Creator software package to obtain a theoretical annealing temperature of 63°C and 77°C, respectively. After putting the probes, PCR products and $MgCl_2$ into the reaction wells of the FRET detection plates, they were incubated at 63°C for 0.5–2 h in a thermal cycler. The fluorescent intensities of FAM dye (A allele) and RED dye (C allele) in each well were measured using Cytoflour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA, USA) for genotyping. The concordance rate of genotyping between the Invader assay and the PCR-restriction fragment length polymorphism analysis was 100% for 980 samples in our previous reports[23,24]

The serum CP-specific IgG titer was measured using an enzyme-linked immunosorbent assay with a commercially

Table 3 Clinical Characteristics and Angiographic Findings of Patients With the A/A, A/C or C/C Genotype

	A/A (n=125)	A/A vs A/C	A/C (n=268)	A/C vs C/C	C/C (n=162)	C/C vs A/A
Age (years)	62±9	NS	63±9	NS	64±9	NS
Gender (male)	107 (86%)	<0.05	197 (74%)	NS	123 (76%)	NS
Hypertension	68 (54%)	NS	166 (62%)	NS	97 (60%)	NS
Systolic BP (mmHg)	133±19	NS	133±18	NS	133±22	NS
Hyperlipidemia	42 (34%)	NS	114 (43%)	NS	72 (44%)	NS
TC (mg/dl)	200±37	NS	202±34	NS	201±36	NS
HDL-C (mg/dl)	51±16	NS	50±14	NS	50±16	NS
Diabetes	33 (26%)	NS	78 (29%)	NS	49 (30%)	NS
Smoking	85 (68%)	NS	182 (68%)	NS	102 (63%)	NS
CAD	89 (71%)	NS	195 (73%)	NS	111 (69%)	NS
MI	35 (28%)	NS	72 (27%)	NS	54 (33%)	NS
Unstable angina	8 (6%)	NS	10 (4%)	NS	6 (4%)	NS
Angiographic findings						
>50% stenotic vessels	1.3±1.0	NS	1.3±1.0	NS	1.3±1.1	NS
3-vessel disease	17 (14%)	NS	44 (16%)	NS	25 (15%)	NS
>50% stenotic segments	1.8±1.7	NS	1.8±1.6	NS	1.7±1.6	NS
≤50% stenotic segments	1.3±1.2	NS	1.3±1.2	NS	1.4±1.3	NS
Diffuse lesion (+)	58 (46%)	NS	121 (45%)	NS	77 (48%)	NS

See Table 1 and 2 for abbreviations.

Table 4 Interaction Between IL-12 p40 Gene Polymorphism and Chlamydia Pneumoniae (CP) Seropositivity

CP Seropositivity A/A genotype	Neither (n=157)	CP seropositivity alone (n=273)	A/A genotype alone (n=55)	Combined (n=70)
Age (years)	65±9	63±9	61±10	63±9
Gender (male)	102 (65%)*	218 (80%)	47 (85%)	60 (86%)
Hypertension	93 (59%)	170 (62%)	32 (58%)	36 (51%)
Systolic BP (mmHg)	130±20	135±19	132±14	134±23
Hyperlipidemia	79 (50%)**	107 (39%)	19 (35%)	23 (33%)
TC (mg/dl)	201±35	202±35	200±37	201±37
HDL-C (mg/dl)	54±16	48±14	51±18	51±14
Diabetes	45 (29%)	82 (30%)	8 (15%)	25 (36%)
Smoking	90 (57%)†	194 (71%)	35 (64%)	50 (71%)
CAD	107 (68%)	199 (73%)	40 (73%)	49 (70%)
MI	42 (27%)	84 (31%)	16 (29%)	19 (27%)
Unstable angina	5 (3%)	11 (4%)	3 (5%)	5 (7%)
Angiographic findings				
>50% stenotic vessels	1.2±1.0	1.3±1.1	1.2±1.0	1.3±1.1
3-vessel disease	19 (12%)	50 (18%)	5 (9%)	12 (17%)
>50% stenotic segments	1.7±1.6	1.8±1.6	1.7±1.5	1.9±1.9
≤50% stenotic segments	1.3±1.2	1.3±1.2	1.1±1.0	1.4±1.3
Diffuse lesion (+)	73 (46%)	125 (46%)	21 (38%)	37 (53%)

**p<0.01 vs patients with CP seropositivity alone and those with A/A genotype alone; **p<0.05 vs patients with seropositivity alone and those with combined A/A and seropositivity; †p<0.01 vs patients with seropositivity alone.*

available kit (HITAZYME CP TM, Hitachi Chemical, Japan). This assay used the CP outer membrane complex as a CP-specific antigen. The cut off index of ≥1.10 is considered to be seropositive.

Statistical Analysis

Differences between 2 groups were evaluated by unpaired t-test for continuous variables and by chi-square test for categorical variables. Differences among 3 or more groups were evaluated by ANOVA with Scheffe's test for continuous variables and by chi-square test for categorical variables. Multiple logistic regression analysis was used to elucidate any association between the polymorphism and CAD. A p-value <0.05 was considered to be statistically significant. Results are presented as the mean value±SD.

Results

Clinical Characteristics and IL-12 p40 Polymorphism

Of the 555 study patients, 395 (71%) had CAD, of whom 161 also had a MI. Compared with the 160 patients without CAD, those with CAD were older, predominantly male, had higher rates of hypertension, hyperlipidemia, diabetes and smoking, and also had lower high-density lipoprotein-cholesterol concentrations (Table 1). As for the IL-12 p40 polymorphism, the percentages of patients having A/A, A/C, and C/C genotypes were 23%, 48%, and 29%, respectively, a genotype distribution that did not deviate from the Hardy-Weinberg equilibrium. The percentage of patients who had the A/A genotype did not differ between patients with and without CAD (23% vs 23%, p=NS) (Table 1). The frequency of the A allele was also similar in patients with and without CAD (47% vs 45%, p=NS). Moreover, there was no substantial difference in the frequencies of the A/A genotype (22% vs 23%)

and the A allele (44% vs 49%) (p=NS) between CAD patients with and without MI. To test the independent association between the A/A genotype and CAD or MI, clinical variables were entered into a multivariate logistic regression model, but the A/A genotype was not an independent factor associated with either CAD or MI (Table 2).

IL-12 p40 Polymorphism and the Severity of CAD

To test the association between IL-12 p40 polymorphism and the severity of CAD, the 555 study patients were divided into 3 groups according to their genotypes: 125 with the A/A genotype, 268 with A/C genotype, and 162 with C/C genotype. Although the rate of males with the A/A genotype was high, the prevalence of CAD or MI was not higher in patients with the A/A genotype than in those with the A/C or C/C genotype (Table 3). The percentage of patients who had class II or III unstable angina, as defined by Braunwald's classification,[25] was also similar among the 3 groups. Regarding the severity of CAD, the numbers of >50% stenotic vessels and >50% stenotic segments did not differ among the 3 groups. Moreover, the number of mildly stenotic segments (≤50%) and the prevalence of diffuse lesion (>10 mm) were also similar.

Interaction Between IL-12 p40 Polymorphism and CP Seropositivity

The prevalence of CP IgG seropositivity did not significantly differ between patients with and without CAD (63% vs 59%) (Table 1). To clarify the effect of IL-12 p40 polymorphism on CAD associated with CP infection, the 555 study patients were divided into 4 groups according to the presence or absence of CP seropositivity and/or A/A genotype: 157 with neither, 273 with CP seropositivity alone, 55 with A/A genotype alone, and 70 with combined A/A genotype and CP seropositivity. The prevalence of CAD did not differ among the 4 groups: 68%, 73%, 73% and 70% respectively (p=NS) (Table 4). The prevalence of MI was also similar in the 4 groups: 27%, 31%, 29% and 27%, respectively (p=NS). The numbers of >50% stenotic vessels and >50% stenotic segments did not differ among the 4 groups. In a multivariate analysis, the A/A genotype was not an independent factor for CAD or MI in either group of patients with or without CP seropositivity.

Discussion

In 2001, polymorphism of the 3'-untranslated region of IL-12 p40 gene (the A/A genotype) was reported to be associated with type 1 diabetes[14] and multiple sclerosis.[15] The A/A genotype was shown to be associated with increased IL-12 expression in vitro,[14] but recent studies failed to show such an association between this polymorphism and diabetes[26,27] or multiple sclerosis.[28] However, the functional effect of this polymorphism on IL-12 expression remains contradictory.[26,29] The polymorphism has also been reported as associated with psoriasis vulgaris and atopic dermatitis.[30] In type 2 diabetes, patients with the A/A genotype have been shown to have higher plasma concentrations of oxidized low-density lipoprotein than those with the A/C or C/C genotype.[31] In our investigation of the association between the polymorphism and CAD in 555 patients who underwent coronary angiography the frequency did not differ between patients with and without CAD, nor was an association found between this polymorphism and the severity of CAD.

Seroepidemiological studies have reported that CP infection is associated with CAD[16–18] and CP organisms have been detected within atheroma.[32,33] CP infection was demonstrated to accelerate atherosclerosis in a rabbit model;[34] however, the potential contribution of CP infection to CAD remains controversial. Two prospective studies failed to show any association between CP seropositivity and CAD,[19,20] nor did we find any significant association between CP seropositivity and CAD in the present study. The inflammatory response to CP infection may vary from person to person, and only certain individuals may develop CAD. We previously investigated CP seropositivity and IL-1β (C/T at −511) and IL-1Ra (a variable number repeat) gene polymorphisms in 292 patients undergoing coronary angiography.[21] We demonstrated a stepwise increase in the prevalence of CAD depending on the positive interaction between CP seropositivity and IL-1 gene variants (IL-1β C/C genotype and/or IL-1Ra 2- or 3-repeat allele), and the highest CAD prevalence was seen in patients with combined variants and seropositivity. Moreover, IL-1 gene variants were found to be associated with MI only in patients with CP seropositivity. However, in the present study, no such interaction between CP seropositivity and IL-12 p40 polymorphism was found, and the prevalence of CAD or MI did not differ among the 4 groups divided by the presence or absence of CP seropositivity and/or A/A genotype. Our results suggest that IL-12 p40 polymorphism is unlikely to influence the susceptibility to CAD associated with CP infection.

Study Limitations

First, our study is cross-sectional and as such cannot establish causality. It only shows some association and is hypothesis-generating. Second, we did not have healthy controls. Patients undergoing angiography are generally considered to be a highly selected population at high-risk for CAD. This may have caused some selection bias and confounded the results. Third, our study was in the Japanese population and so the results may not be applicable to other ethnic populations.

Conclusion

IL-12 p40 polymorphism was not found to be associated with the presence or severity of CAD, which suggests that it does not play an important role in the development of CAD. However, to fully elucidate its effect on the development of CAD, a further prospective study is needed in a large population.

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Letter to the Editor

Lack of any association between persistent hepatitis B or C virus infection and coronary artery disease

Inflammation is recognized to be involved in the pathogenesis of atherosclerosis. Infectious agents may play a role in the development of atherosclerosis. Recently, Ishizaka et al. [1,2] reported the positivity for hepatitis B virus (HBV) surface antigen (HBsAg) and hepatitis C (HCV) antibody (Ab) to be associated with carotid atherosclerosis. Fukui et al. [3] reported carotid plaque to be more prevalent in diabetic patients with HCV Ab positivity than in those without it. However, two other studies failed to show any association between HBV or HCV infection and carotid atherosclerosis [4,5].

To test associations between HBV or HCV infection and coronary artery disease (CAD), we investigated HBsAg, HCV Ab, and HCV core antigen in 524 patients with CAD. They had coronary angiography and were found to have CAD (>50% luminar diameter stenosis). Results were compared with those of 106 age- and gender-matched controls who were angiographically found to have normal coronary arteries. Our study was approved by the ethics committee of the hospital. After informed consent was obtained, blood samples were taken. Serum HBsAg and HCV Ab levels were measured by chemiluminescent enzyme immunoassay (Lumipulse II HBsAg and HCV, Fujirebio, Japan). The cutoff index $\geq$1.0 was considered positive. Serum HCV core antigen was measured by the recently developed immuno-radiometric assay (IRMA) (Ortho HCV Antigen IRMA, Ortho-Clinical Diagnostics, Japan) [6]. This level of $\geq$20 fmol/l was considered positive. Differences between two groups were evaluated by unpaired *t*-test for continuous variables and by chi-square test for categorical variables. Differences among $\geq$3 groups were evaluated by ANOVA for continuous variables and by chi-square test for categorical variables. A *P*-value of <0.05 was considered statistically significant.

HBsAg positivity was not more prevalent in CAD patients than in controls (0.8% versus 1.9%, P=NS) (Table 1). The prevalence of HCV Ab positivity tended to be higher in CAD patients than in controls (3.4% versus 2.8%) and to increase with the number of >50% stenotic vessels: 2.9% in 1-vessel, 3.6% in 2-vessel, and 4.1% in 3-vessel disease (Table 2). However, these differences did not reach statistical significance, and Spearman's rank test showed no correlation between the positivity and the number of stenotic vessels. Moreover, the prevalence of HCV core antigen positivity was low even in CAD patients and did not differ between CAD patients and controls (1.0% versus 0.9%). It was similar in patients with 1-vessel, 2-vessel, and 3-vessel disease (0.5, 1.5, and 0.8%). Of the 524 CAD patients, 211 had myocardial infarction (MI). However, neither CAD patients, with or without MI, showed higher prevalence of HBsAg, HCV Ab or core antigen positivity than controls (Table 1). To test independent associations between HBV or HCV positivity and CAD, clinical variables were entered into a multivariate logistic regression model. Neither HBV nor HCV positivity was an independent factor for CAD or MI.

Ishizaka et al. [1] reported the prevalence of HCV Ab positivity in 4784 Japanese subjects undergoing health-screening tests to be higher in subjects with carotid plaque (3.7%) than in those without it (1.7%). During the preparation of our manuscript, Vassalle et al. [7] reported the prevalence of HCV Ab positivity in 491 Italian patients with CAD and 195 with normal coronary arteries to be higher in CAD patients (6.3% versus 2%) and to increase with the number of stenotic vessels. However, they did not consider any history of MI and did not assess HCV core antigen, which is a better marker for viremia and persistent infection than HCV Ab [6,8]. We evaluated both HCV Ab and core antigen in 524 Japanese patients with CAD and 106 with normal coronary arteries. In our study, HCV Ab positivity tended to be more prevalent in CAD patients than in controls (3.4% versus 2.8%), but this difference did not reach statistical significance. Notably, the prevalence of HCV core antigen positivity was low even in CAD patients and did not differ between CAD patients and controls (1.0% versus 0.9%). Our results suggest that persistent HCV infection is uncommon in CAD patients and that HCV infection most likely does not play an important role in the development of CAD.

HCV Ab positivity appeared to be less prevalent in our patients with CAD (3.4%) than in Ishizaka's subjects with carotid plaque (3.7%) [1]. Ishizaka et al. [8] further reported HCV core antigen positivity to be more prevalent in subjects with carotid plaque (3.2%) than in those without it (0.6%). We measured HCV core antigen by the recently developed

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Table 1
Clinical characteristics and the prevalence of HBsAg, HCV Ab. and HCV core antigen positivity in controls and CAD patients

	Controls		CAD				
	$n=106$	Controls vs. CAD	$n=524$	Controls vs. MI (−)	MI (−) ($n=313$)	Controls vs. MI (+)	MI (+) ($n=211$)
Age (years)	64 ± 8	NS	64 ± 9	NS	65 ± 9	NS	63 ± 10
Gender (male)	87 (82%)	NS	429 (82%)	NS	246 (79%)	NS	183 (87%)
Hypertension	56 (53%)	<0.025	342 (65%)	<0.005	219 (70%)	NS	123 (58%)
Systolic BP (mmHg)	132 ± 18	NS	134 ± 21	<0.05	137 ± 20	NS	130 ± 21
Hyperlipidemia	36 (34%)	<0.01	258 (49%)	<0.001	173 (55%)	NS	85 (40%)
TC (mg/dl)	204 ± 36	NS	199 ± 35	NS	202 ± 34	<0.05	195 ± 36
HDL-C (mg/dl)	57 ± 17	<0.001	49 ± 13	<0.001	50 ± 14	<0.001	47 ± 13
Diabetes mellitus	22 (21%)	<0.025	172 (33%)	<0.025	107 (34%)	NS	65 (31%)
Smoking	64 (60%)	NS	364 (69%)	NS	207 (66%)	<0.025	157 (74%)
HBsAg	2 (1.9%)	NS	4 (0.8%)	NS	2 (0.6%)	NS	2 (0.9%)
HCV Ab	3 (2.8%)	NS	18 (3.4%)	NS	11 (3.5%)	NS	7 (3.3%)
HCV core antigen	1 (0.9%)	NS	5 (1.0%)	NS	3 (1.0%)	NS	2 (0.9%)

Data are presented as the mean value ± S.D. or the number (%) of patients. BP, blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol. Hypertension was defined as BP ≥ 140/90 mmHg or on drugs, hyperlipidemia was defined as TC > 240 mg/dl or on drugs, and diabetes was defined as fasting glucose level ≥ 126 mg/dl or on treatment.

Table 2
Associations between the prevalence of HBsAg, HCV Ab. and HCV core antigen positivity and the number of >50% stenotic vessels

	Controls ($n=106$)	1-vessel disease ($n=205$)	2-vessel disease ($n=197$)	3-vessel disease ($n=122$)	P-value
Age (years)	64 ± 8	63 ± 10	65 ± 9	65 ± 9	NS
Gender (male)	87 (82%)	163 (80%)	165 (84%)	101 (83%)	NS
Hypertension	56 (53%)	129 (63%)	128 (65%)	85 (70%)	NS
Systolic BP (mmHg)	132 ± 18	134 ± 22	134 ± 20	134 ± 19	NS
Hyperlipidemia	36 (34%)	95 (46%)	97 (49%)	66 (54%)	<0.025
TC (mg/dl)	204 ± 36	199 ± 36	198 ± 32	201 ± 36	NS
HDL-C (mg/dl)	57 ± 17	50 ± 15	48 ± 12	48 ± 12	<0.001
Diabetes mellitus	22 (21%)	56 (27%)	62 (31%)	54 (44%)	<0.001
Smoking	64 (60%)	140 (68%)	137 (70%)	87 (71%)	NS
HBsAg	2 (1.9%)	2 (1.0%)	1 (0.5%)	1 (0.8%)	NS
HCV Ab	3 (2.8%)	6 (2.9%)	7 (3.6%)	5 (4.1%)	NS
HCV core antigen	1 (0.9%)	1 (0.5%)	3 (1.5%)	1 (0.8%)	NS

IRMA, which is more sensitive than the fluorescent enzyme immunoassay as used in Ishizaka's study [9]. The prevalence of HCV core antigen positivity was similar in our controls (0.9%) and Ishizaka's subjects without carotid plaque (0.6%), but it appeared to be much lower in our CAD patients (1.0%) than in Ishizaka's subjects with carotid plaque (3.2%). The potential role of persistent HCV infection may be much less in the development of CAD than in carotid atherosclerosis.

Ishizaka et al. [2] reported HBsAg positivity to be more prevalent in subjects with carotid plaque (1.3%) than without it (0.7%). Tomiyama et al. [10] evaluated pulse wave velocity and HBsAg in 7514 subjects undergoing health-screening tests, but they showed HBsAg positivity to be unassociated with increased pulse wave velocity. Our study showed HBsAg positivity to be uncommon in CAD patients (0.8%) and to be unassociated with CAD, suggesting that HBV infection most likely does not play an important role in the development of CAD.

In conclusion, persistent HBV and HCV infection was uncommon in patients with CAD, while it was also not associated with the presence and severity of CAD. Our results suggest that either HBV or HCV infection most likely does not play an important role in the development of CAD.

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Levels of Matrix Metalloproteinase-1 in Patients With and Without Coronary Artery Disease and Relation to Complex and Noncomplex Coronary Plaques

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We investigated the association between serum levels of matrix metalloproteinase-1 (MMP-1) and coronary artery disease (CAD) in 185 patients who underwent elective coronary angiography. MMP-1 levels did not differ between patients who had CAD and those who did not and did not correlate with the number of >50% stenotic vessels or segments, but MMP-1 levels were significantly higher in patients who had CAD and complex coronary lesions than in those who did not have such lesions and those who did not have CAD. High serum levels of MMP-1 were associated with the presence of complex lesions in patients who had CAD. ©2005 by Excerpta Medica Inc.

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Interstitial collagen is a major component of atherosclerotic plaques.[1–3] Collagen synthesis is generally upregulated in plaques.[3] Matrix metalloproteinases (MMPs) play an important role in collagen breakdown that leads to plaque instability and vascular remodeling.[4–6] Among the various MMPs, only interstitial collagenases can initiate collagen breakdown, thus making collagen fragments susceptible to further degradation by other MMPs, such as MMP-2 and MMP-9.[7] The major human interstitial collagenase, MMP-1, was shown to be expressed and produced by macrophages, smooth muscles cells, and endothelial cells in atherosclerotic plaques, especially in vulnerable plaques.[5,6,8] However, the association between serum MMP-1 levels and coronary artery disease (CAD) has not been fully clarified. Inoue et al[9] studied MMP-1 levels in 20 patients who had unstable angina, 20 who had stable angina, and 20 controls. They found MMP-1 levels in the coronary sinus to be high in unstable angina, but there was no difference in the aorta across 3 groups. Tziakas et al[10] also reported no difference in serum MMP-1 levels between 20 patients who had unstable angina and 16 controls. However, these studies investigated only a small number of patients and did not consider any coronary artery lesion morphology. We investigated the association between serum MMP-1 levels and the presence, severity, and lesion morphology of coronary artery stenosis in patients who underwent coronary angiography.

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We measured serum levels of MMP-1 in 185 consecutive patients who had elective coronary angiography for suspected CAD at the National Defense Medical College Hospital (Saitama, Japan). Patients who had a history of myocardial infarction ≤6 months, those who had unstable angina at rest ≤48 hours (class III unstable angina according to Braunwald's classification[11]), or those who had a history of percutaneous coronary intervention or coronary artery bypass surgery were excluded. Patients who had heart failure, cardiomyopathy, or valvular heart diseases were also excluded. Because statin therapy can affect MMP levels[12,13] and cause plaque regression or stabilization,[14,15] patients taking statins were excluded. Our study was approved by the hospital ethics committee. After written informed consent was obtained, blood samples were taken in a fasting state on the morning of the day when angiography was performed. Serum was stored at −80°C until analyzed.

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TABLE 1 Clinical Characteristics of Patients Who Had Coronary Artery Disease and Those Who Did Not

Variables	Without CAD (n = 57)	p Value*	With CAD (n = 128)	With CAD: Without Complex Lesions (n = 96)	With CAD: p Value†	With CAD: With Complex Lesions (n = 32)
Age (yrs)	63 ± 8	<0.02	66 ± 8	66 ± 8	NS	66 ± 8
Men	35 (61%)	<0.001	110 (86%)	83 (86%)	NS	27 (84%)
Systemic hypertension*	33 (58%)	NS	82 (64%)	63 (66%)	NS	19 (59%)
Systolic blood pressure (mm Hg)	133 ± 15	NS	136 ± 18	136 ± 17	NS	136 ± 21
Hyperlipidemia	11 (19%)	NS	26 (20%)	19 (20%)	NS	7 (22%)
Total cholesterol (mg/dl)	207 ± 28	NS	205 ± 33	203 ± 34	NS	210 ± 30
HDL cholesterol (mg/dl)	58 ± 15	<0.001	49 ± 15	49 ± 15	NS	49 ± 15
Diabetes mellitus	12 (21%)	NS	48 (38%)	34 (35%)	NS	14 (44%)
Smoker	31 (54%)	NS	84 (66%)	64 (67%)	NS	20 (63%)
Unstable angina pectoris			20 (16%)	14 (15%)	NS	6 (19%)
Class I			15 (12%)	11 (11%)	NS	4 (13%)
Class II			5 (4%)	3 (3%)	NS	2 (6%)
Class III			0 (0%)	0 (0%)		0 (0%)
C-reactive protein (mg/l)	0.50	<0.05	0.73	0.73	NS	0.75

Data are presented as mean ± SD or numbers of patients (percentages). C-reactive protein levels are presented as medians. Systemic hypertension was defined as blood pressures ≥140/90 mm Hg or therapy to decrease blood pressure. Hyperlipidemia was defined as a total cholesterol level >240 mg/dl. Diabetes mellitus was defined as a fasting glucose level ≥126 mg/dl or use of insulin or hypoglycemic drugs. Unstable angina pectoris was classified according to Braunwald's classification.[11]

*Patients who had CAD versus those who did not.

†Patients who had CAD and complex lesions versus those who had CAD but no complex lesions.

HDL = high-density lipoprotein.

Serum MMP-1 levels were measured with a 1-step sandwich enzyme immunoassay using a commercially available kit (Daiichi Pharmaceutical Co., Toyama, Japan). This kit was developed based on the method by Zhang et al,[16] and it measures the total concentration of the precursor form, the active form, and the tissue inhibitor of metalloproteinase (TIMP-1) or TIMP-2 complex forms of MMP-1 in serum. This assay mainly detects the precursor form, and the recognition rate decreases to 50%, 10%, and <3% for the active form, the TIMP-1 complex form, and the TIMP-2 complex form, respectively. However, it is highly specific and does not cross-react with other collagenases. The lower detection limit of this assay was 0.1 ng/ml. Plasma high-sensitivity C-reactive protein levels were also measured with a BNII nephelometer (Dade Behring, Tokyo, Japan).

Coronary angiograms were recorded by a femoral approach using Judkins' technique and a cineangiographic system (Toshiba, Tokyo, Japan). All angiograms were evaluated by one of the investigators (YM), who was blinded to MMP-1 data. CAD was defined as ≥1 coronary artery with >50% luminal diameter stenosis. Severity of CAD was defined as the number of >50% stenotic vessels and >50% and >25% stenotic segments. Coronary artery segments were defined according to the Coronary Artery Surgery Study. According to the classification of Ambrose and Israel,[17] coronary artery lesions were classified as simple if they had concentric or eccentric smooth borders and as complex if they had sharp overhanging edges, irregular borders, or intraluminal lucency.

Differences between groups were evaluated by unpaired *t* test for parametric variables, by Mann-Whitney U statistical test for nonparametric variables, and by chi-square test for categorical variables. Differences across ≥3 groups were evaluated by analysis of variance with Scheffé's test for parametric variables, by the Kruskal-Wallis test for nonparametric variables, and by chi-square test for categorical variables. Correlations between serum levels of MMP-1 and severity of CAD or other factors were evaluated by Spearman's rank correlation test. Forward stepwise multiple logistic regression analysis was used to elucidate the association between MMP-1 levels and complex lesions. A p value <0.05 was considered statistically significant. Results are expressed as mean ± SD or medians.

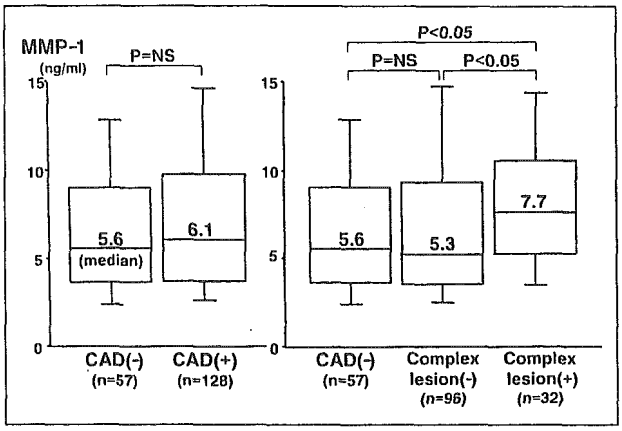

FIGURE 1. *Left,* **there was no significant difference in MMP-1 levels between patients who had CAD and those who did not.** *Right,* **however, MMP-1 levels were significantly higher in patients who had CAD and complex lesions than in those without complex lesions and CAD. Values are medians** *(central lines),* **25th to 75th percentiles** *(box spans),* **and 10th to 90th percentiles** *(error bars).* **− = absent; + = present.**

Of 185 patients, 128 had CAD. Compared with 57 patients who did not have CAD, those who did were older, were predominantly men, had a higher rate of diabetes, and had lower levels of high-density lipoprotein cholesterol and higher levels of C-reactive protein (Table 1). However, MMP-1 levels did not differ between patients who had CAD and those who did not (median 6.1 vs 5.6 ng/ml; Figure 1). Of the 128 patients who had CAD, 46 had 1-vessel disease, 48 had 2-vessel disease, and 34 had 3-vessel disease. MMP-1 levels were 5.6 ng/ml in patients who did not have CAD, 6.3 ng/ml in those who had 1-vessel disease, 5.4 ng/ml in those who had 2-vessel disease, and 7.0 ng/ml in those who had 3-vessel disease. There was no significant difference in MMP-1 levels across the 4 groups. In addition, MMP-1 levels showed no significant correlations with the number of stenotic segments. Of the 128 patients who had CAD, 20 (16%) had class I or II unstable angina (Table 1). MMP-1 levels were 4.2 ng/ml in 15 patients who had class I unstable angina, 6.8 ng/ml in 5 who had class II, 6.4 ng/ml in 108 who had no symptoms of unstable angina, and 5.6 ng/ml in 57 who did not have CAD. There was no significant difference in MMP-1 levels across the 4 groups.

Complex lesions were found in 32 of 128 patients (25%) who had CAD, 9 of whom had ≥ 2 lesions. C-reactive protein levels did not differ between patients who had CAD and complex lesions and those who had CAD and no such lesions (Table 1), but MMP-1 levels were higher in patients who had CAD and complex lesions than in those who did not have complex lesions and those who did not have CAD (7.7 vs 5.3 and 5.6 ng/ml, $p < 0.05$; Figure 1). MMP-1 levels in patients who had ≥ 2 complex lesions and those who had 1 lesion were 10.2 and 7.1 ng/ml, respectively. Patients who had CAD and complex lesions had MMP-1 levels >6.0 ng/ml more often than did those who did not have complex lesions and those who did not have CAD (69% vs 44% and 44%, $p < 0.05$). To elucidate the independent association between MMP-1 levels and complex lesions, clinical variables (age, gender, hypertension, hyperlipidemia, smoking, diabetes, and levels of high-density lipoprotein cholesterol, C-reactive protein, and MMP-1) were entered into a multivariate logistic regression model. Multivariate analysis showed that a high level of MMP-1 was the only factor associated with complex lesions, independent of risk factors. The odds ratio for complex lesions was 2.4 (95% confidence interval 1.2 to 6.3) for a MMP-1 level >6.0 ng/ml ($p < 0.02$).

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In our study, serum MMP-1 levels did not differ between patients who had CAD and those who did not and did not show any association with severity of CAD. However, high MMP-1 levels were associated with the presence of complex coronary lesions in patients who had CAD. Angiographic complex lesions are recognized as associated with plaque instability[17,18] and are predictive of coronary events, such as myocardial infarction.[17,19,20] Our results suggest that high MMP-1 levels in patients who have CAD may reflect plaque instability in coronary arteries or may be aimed at destabilization of coronary plaques. However, there was some overlapping in MMP-1 levels between patients who had complex lesions and those who did not (Figure 1). Therefore, MMP-1 levels in patients who have CAD may reflect plaque instability in not only coronary arteries but also other vascular beds. Patients who have CAD and high MMP-1 levels may be at high risk for further cardiovascular events. To elucidate the predictive value of MMP-1 levels for such events, a follow-up study of our patients is needed.

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Original Articles

Characterization of the Expression of TLR2 (Toll-like Receptor 2) and TLR4 on Circulating Monocytes in Coronary Artery Disease

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TLRs are receptors involved in the recognition of pathogens by the innate immune system, and TLR2 and TLR4 play important roles in the activation of monocytes. A total of 105 consecutive patients who underwent coronary angiography comprised of 46 with stable effort angina (SA), 41 with unstable angina (UA), and 18 with no significant CAD (CNT) were enrolled. The baseline expression levels of TLR2 and TLR4 on monocytes in peripheral blood mononuclear cells (PBMCs) were determined by flow-cytometric analysis. Since TLR2 expression has been reported to be regulated by TLR4 signaling, we cultured PBMCs with or without lipopolysaccharide (LPS, 1 μg/ml). At baseline, TLR4 levels (mean of fluorescence intensity) in SA (145 ± 58, $p < 0.05$) and UA (164 ± 65, $p < 0.01$) were higher than those in CNT (107 ± 37). As for TLR2, levels were higher in UA (108 ± 36, $p < 0.05$) than in SA (94 ± 18) and CNT (87 ± 22). After stimulation with LPS, TLR2 levels increased in SA but decreased in UA. In conclusions, TLR4 levels increased in both SA and UA. Monocytes in UA were characterized by elevated TLR2 levels and unresponsiveness of the TLR2 levels to TLR4 stimulation. ***J Atheroscler Thromb, 2005; 12: 53–60.***

Key words: Unstable angina, Stable effort angina, Monocyte, Lipopolysaccharide

Introduction

Atherosclerosis is now considered to be a chronic inflammatory process due to arterial injury (1–5). Pathological studies have shown that there is invasion of macrophages and T-lymphocytes into atherosclerotic plaques, which suggests that both innate and acquired immunity are involved in the pathogenesis of atherosclerosis (5, 6). Since acquired immunity is induced by innate immunity, it is important to clarify the mechanism for the activation of innate immunity in the pathogenesis of atherosclerosis.

Toll-like receptors (TLRs) have been identified as receptors involved in microbial recognition by the innate immune system (7). TLRs belong to the pattern recognition receptors that recognize the pathogen-associated molecular pattern, common molecular features of target microorganisms. To date, 10 TLRs have been identified (7). When TLRs on macrophages are activated, this leads to activation of the NF-κ B pathway which brings about the production of pro-inflammatory cytokines and expression of co-stimulatory molecules, resulting in the induction of acquired immunity (8–10). In the context of atherosclerosis, evidence suggests that TLRs are involved in human atherosclerosis (11, 12). Pathological examination of human atherosclerotic plaques have shown that TLR4, a receptor for the lipopolysaccharide (LPS) of gram negative-bacteria, and TLR2, a receptor for the pepti-

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doglycan of gram-positive bacteria, are expressed on macrophages invading atherosclerotic plaques. In this case, the immunoreactivity of these receptors was co-localized with the nuclear translocation of NF-κ B, a marker of the activation of this pathway, suggesting that these TLRs could play an important role in macrophage activation in such plaques (11).

Clinical studies have demonstrated the effects of polymorphism of the TLR4 gene on the progression of atherosclerosis (13, 14). In this regard, it has been shown that the Asp299Gly allele of the TLR4 gene, which reduces the effectiveness of signal transduction by this receptor, is associated with slower progression of atherosclerosis in the carotid artery, suggesting that the activation of the TLR4 pathway could play an important role in the progression of atherosclerosis (14).

Atherosclerosis [including that in coronary artery disease (CAD)] has been reported to be associated not only with local inflammation in the arterial walls but also with the systemic inflammatory response (15, 16). In the case of patients with CAD, previous studies have suggested that both stable effort angina (SA) and unstable angina (UA) were associated with a systemic inflammatory response, though to different extents (17).

We speculated that TLR2 and TLR4 are involved in the activation of circulating monocytes in patients with CAD and that their expression levels may therefore change depending on monocyte activation levels. So, we measured the expression levels of TLR2 and TLR4 on circulating monocytes in patients with SA and UA as well in patients with angiographically normal coronary arteries (CNT) and found the monocytes of UA patients to be characterized by increased TLR2 levels. TLR4 levels were elevated to about the same extent in UA and SA and higher than those in CNT.

It has been reported that there is cross talk between TLR4 and TLR2 (18), through which TLR2 expression levels are up regulated by TLR4 stimulation (19–22). In view of this, we also studied changes in TLR2 expression levels in response to TLR4 stimulation induced by LPS. Though there was no difference in TLR4 expression levels between SA and UA patients, the responsiveness of TLR2 to TLR4 stimulation differed between these two patient groups. As expected, TLR2 levels were up regulated by TLR4 stimulation in the SA patients, but interestingly, they were down regulated by TLR4 stimulation in the UA patients.

Materials and Methods

Patient population

One hundred and five consecutive patients who underwent coronary angiography for suspected ischemic heart disease between January 2002 and February 2003 were enrolled in this study. Patients with advanced kidney or liver disease, heart failure, acute or recent myocardial infarction within 6 months or a history of major surgery or trauma within the previous month were excluded. The study protocol was approved by the Ethics Committee of the National Defense Medical College and written informed consent was obtained from all patients. We divided the patients into 3 groups—SA, UA and CNT-with 46, 41 and 18 patients, respectively, in each group. The SA patients had had no acute events or any worsening of symptoms during the previous 6 months. The diagnosis of UA was made according to the classification of Braunwald (19) and any patients with an elevation in either serum creatinine kinase and/or Troponin T levels at admission were excluded (Table 1).

Peripheral blood mononuclear cells

Heparinized venous blood samples (4 ml) were taken at admission. Within 30 minutes of sampling, peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using Lymphocyte Separation Medium (ICN, Biomedical Inc., Aurora, OH, USA). The expression levels of TLR4 and TLR2 on CD 14 positive PBMCs were determined by flow cytometric analysis at baseline as well as after culturing PBMCs in the presence or absence of LPS.

Table 1. Clinical Backgrounds of the Three Groups.

	CNT ($N = 18$)	SA ($N = 46$)	UA ($N = 41$)	P ns
Age (y)	58 ± 11	66 ± 9	64 ± 11	0.042
Sex (M/F)	14 /4	39 /7	29 /12	ns
Risk factors, n (%)				
Hypertension	7 (39)	20 (43)	24 (59)	ns
Smoking	8 (44)	21 (46)	22 (54)	ns
Hypercholesterolemia	4 (22)	22 (48)	21 (51)	ns
Diabetes	2 (11)	8 (17)	14 (34)	0.025
Therapy, n (%)				
Aspirin	14 (78)	42 (91)	33 (80)	ns
Beta-blockers	8 (44)	11 (24)	17 (41)	ns
ACE inhibitors	6 (33)	11 (24)	8 (20)	ns
Nitrates	6 (33)	20 (43)	11 (27)	ns
Statins	4 (22)	17 (37)	17 (41)	ns
Calcium-channel blockers	9 (50)	23 (50)	25 (61)	ns
Angiographic findings, n (%)				< 0.001
1-Vessel disease	0	26 (57)	18 (44)	
2-Vessel disease	0	13 (28)	9 (22)	
3-Vessel disease	0	7 (15)	14 (34)	

Cell cultures and LPS stimulation

PBMCs (1×10^6 cells / 2 ml / well) were cultured in RPMI-1640 medium containing 10% human serum and antibiotics/ antimycotics (Life Technologies, Ground Island, NY, USA) in the presence or absence of 1 μg/ml of LPS (*Escherichia coli* 127, B8; Sigma-Aldrich Co., St. Louis, MO, USA) for 24 hours in 24-well culture dishes under 5% CO_2 .

Flow cytometric analysis

Firstly, TLR4 expressed on PBMCs were stained with mouse anti-human TLR4 mAb (HTA 125; Medical & Biological Laboratories Co., Aichi, Japan), and then washed with staining buffer (phosphate-buffered saline (PBS) containing 5% fetal calf serum, 10 mM EDTA and 0.1% sodium azide). Next, development was conducted with Biotin-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) and PE-conjugated streptavidin (Medical & Biological Laboratories Co.) and the preparations were washed twice with staining buffer. Secondly, CD14 and TLR2 expressed on PBMCs were stained with APC-conjugated anti-human CD14 mAb (RMO 52; Medical & Biological Laboratories Co.) and FITC-conjugated anti-human TLR2 mAb (TL 2.1; CASCADE, MA, USA), and then preparations were washed with staining buffer. After these procedures, the PBMCs were re-suspended in staining buffer, and placed in a flow cytometric analyzer (FACS Calibur; Becton Dickinson, Cockeysville, MD, USA) Analysis of the fluorescence intensities (MFI) determined for TLR4 and TLR2 on monocytes expressing CD14 was conducted using Cell Quest software (Becton Dickinson).

Statistics

All results were expressed as median values (IQR). Differences in patient characteristic parameters were analyzed using Pearson's χ^2 test and any significant differences between the 2 groups were evaluated using the Mann-Whitney U test. Values in more than 3 groups were tested by a 1-way analysis of variance (ANOVA) and then applying the Kruskal-Wallis test. A value of $p < 0.05$ was regarded as significant. All statistical analyses were performed with the SigmaStat software package (SPSS) 6.1 for the Macintosh.

Results

Patient characteristics

The median age of the SA group was higher than that of the UA CNT groups. In the UA group, there were more diabetic patients than in the other groups and this group had more patients with multi-vessel disease than the SA group. Other than these findings, however, there were no significant differences between the three groups as regards their clinical background, which included coronary risk factors and medications (Table 1).

Baseline levels of TLR2 and TLR4

Baseline TLR2 levels were significantly higher in the UA group (mean fluorescence intensity; 107.5 ± 36.3) than in the SA (94.0 ± 17.7, $p = 0.043$) and CNT (86.9 ± 21.6, $p = 0.031$) groups. They were not significantly different between the SA and CNT groups (Fig. 1).

The baseline TLR4 levels were significantly higher in the SA (145.3 ± 58.2) and UA (163.8 ± 64.5) groups than in the CNT (106.9 ± 36.7) group (SA vs CNT; $p = 0.012$, UA vs CNT; $p = 0.0008$). They were not significantly different between the SA and UA groups (Fig. 2).

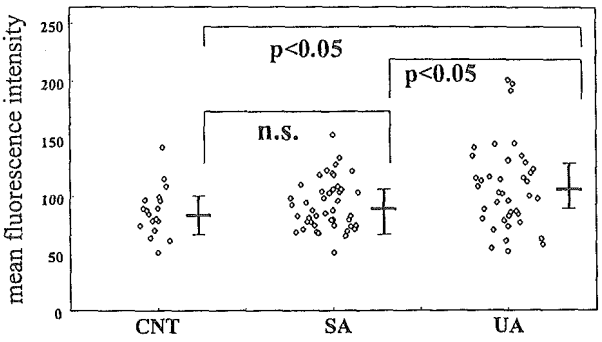

Fig. 1. Baseline TLR2 expression levels on monocytes in the three groups.
The expression levels were measured as the mean of fluorescence intensity and the bar plots show medians in the 25th and 75th percentiles.
Kruskal-Wallis test: $p = 0.04$

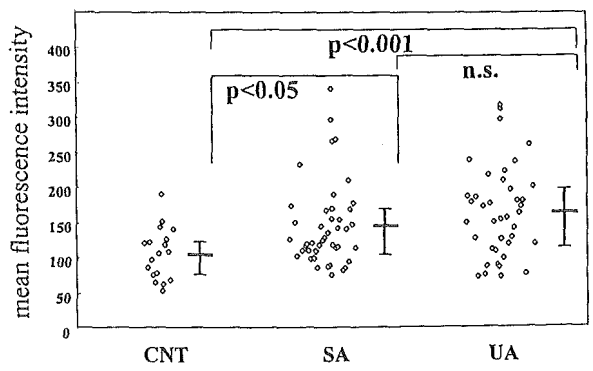

Fig. 2. Baseline TLR4 expression levels on monocytes in the three groups.
Expression levels were measured as the mean of fluorescence intensity and the bar plots show medians in the 25th and 75th percentiles.
Kruskal-Wallis test: $p = 0.0019$

Changes in TLR2 and TLR4 levels in response to LPS stimulation

To quantify the effect of LPS stimulation on the expression levels of the TLRs, the TLR levels of LPS stimulated samples were normalized to those of the corresponding control samples with no LPS stimulation, and levels were expressed as percentages. Figure 3 is a representative example showing the effect of LPS stimulation on the TLR2 and TLR4 levels on monocytes from SA and UA patients. After culturing PBMCs from SA patients with or without LPS, TLR2 levels were 189 and 166, respectively. When normalized, the value for the LPS stimulated sample was 114% meaning that LPS stimulation had increased the TLR2 level by 14%. After culturing PBMCs from UA patients with or without LPS, the TLR2 levels were 208 and 242, respectively. In this case, normalization produced a value of 86% indicating that LPS stimulation had decreased the TLR2 level by 14%. With the same SA and UA patients, LPS stimulation increased TLR4 levels 47 and 1%, respectively.

The effect of LPS stimulation on the TLR2 levels in SA and UA patients is shown in Fig. 4. In the SA group, TLR2 was up-regulated in 42 of the 46 patients (91.3%), while in the UA group TLR2 was down-regulated in 37 of the 41 patients (90.2%). In the SA patients, the TLR2 levels of LPS stimulated samples were significantly higher than those of control samples (114 ± 11% of control values, $p < 0.0001$) (Fig. 5). In the UA patients, the TLR2 levels of LPS stimulated samples were significantly lower than those of control samples (91 ± 11% of control values, $p < 0.0001$) (Fig. 6).

The effect of LPS stimulation on the TLR4 levels in the SA and UA patients is shown in Fig. 7. In both the SA and UA group, the response of TLR4 levels to LPS stimulation varried, with a down-regulation in about half of the patients and up-regulation in the other half. TLR4 levels in the LPS stimulated samples were not significantly different from those of control samples for both SA (125 ± 57% of control values) and UA (103 ± 35% of control values) patients. Also, the normalized expression levels

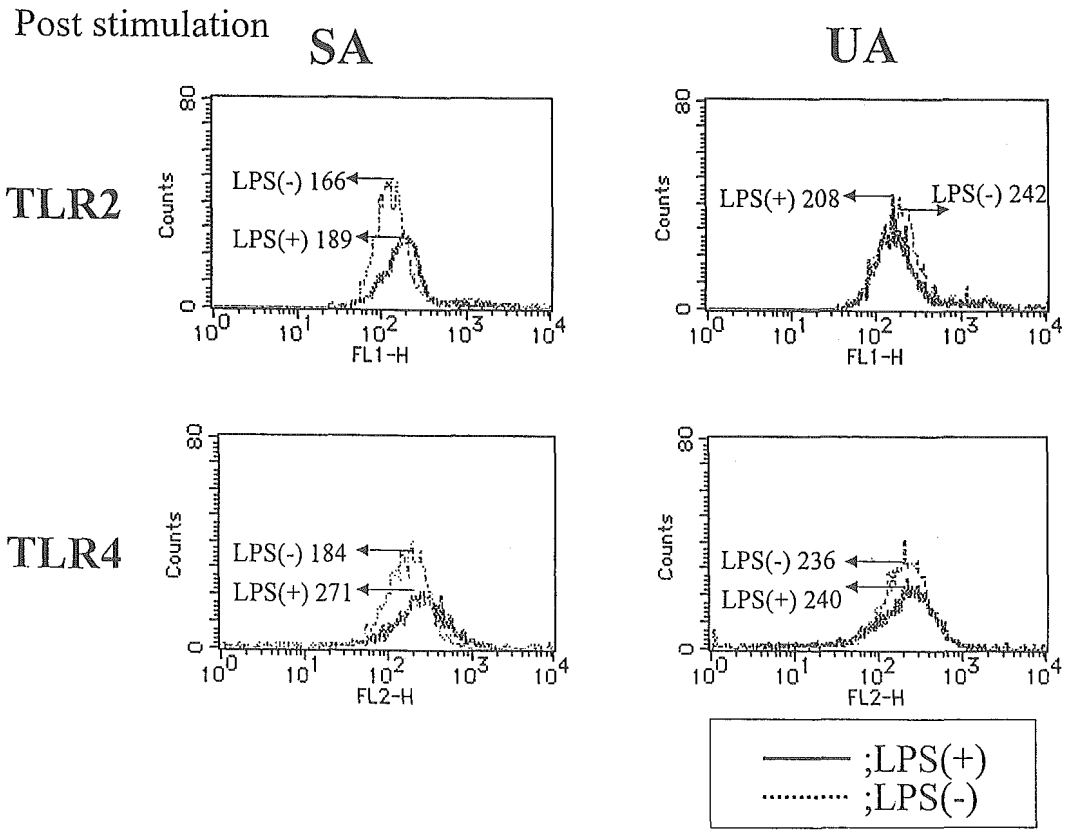

Fig. 3. Representative cases showing effect of LPS stimulation on TLR levels in SA and UA patients.
Expression levels of TLR2 and TLR4 on monocytes from patient with SA or UA after cultivation with or without LPS (1ug/ml) for 24 hours. Solid lines and dotted lines indicate the distribution of fluorescence intensity in LPS stimulated and unstimulated monocytes, respectively. The expression levels are indicated as MFI (mean of fluorescence intensity).

were not significantly different between the 2 groups (SA: 125 ± 57%, UA: 103 ± 35%).

Discussion

Monocyte-macrophages have been reported to play a key role in the pathogenesis of atherosclerosis and microorganisms such as Chlamiydia pneumoniae and cytomegalovirus have been found to aggravate atherosclerosis by activating immune systems in the host. Further, toll-like receptors have been shown to be the receptors by which innate immunity cells recognize invading microorganisms.

Among the 10 TLRs identified, TLR4 is the best characterized. Although TLR4 was identified as a receptor for the LPS of gram-negative bacteria, it also has been reported to be a receptor of endogenous ligands such as fibrinogen (23) and heat-shock protein 60 (24). Since levels of these endogenous ligands are known to be elevated in subjects with atherosclerosis (25), TLR4 is thought to play a crucial role in activating monocyte-macrophages in atherosclerosis.

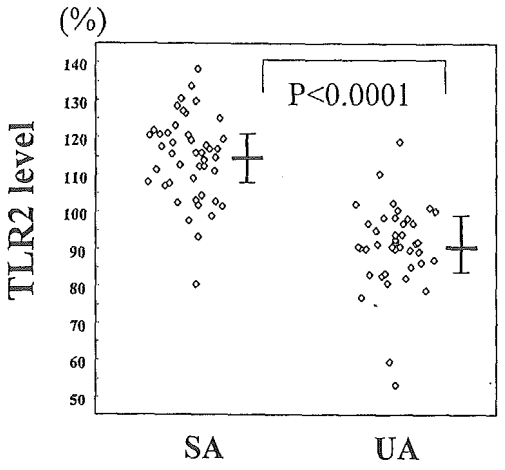

Fig. 4. Effect of LPS stimulation on TLR2 levels in SA and UA patients expression levels of TLR2 of LPS stimulated monocytes of patients with SA and UA are shown as normalized expression levels. The bar plots show medians in the 25th and 75th percentiles.

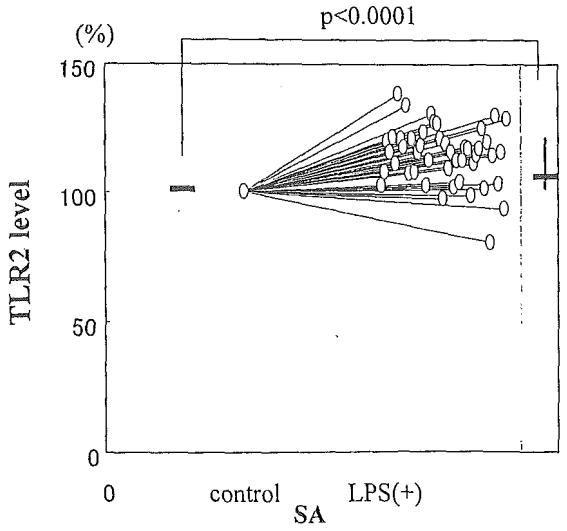

Fig. 5. Effect of LPS stimulation on TLR2 expression levels in SA patients.

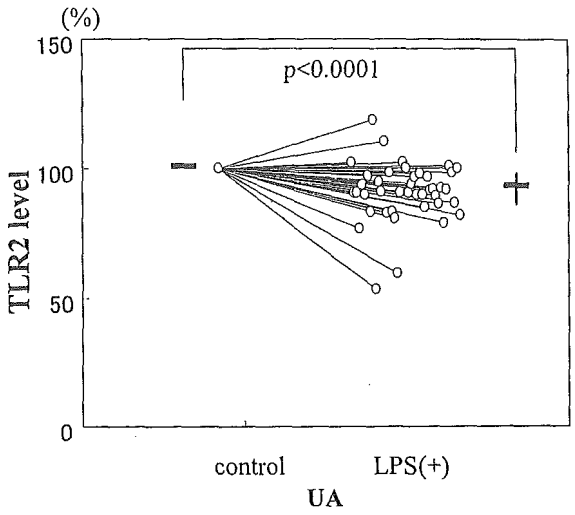

Fig. 6. Effect of LPS stimulation on TLR2 expression levels in UA patients.

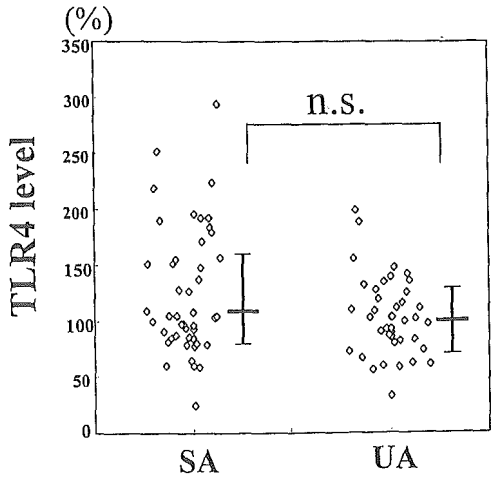

Fig. 7. Effect of LPS stimulation on TLR4 Levels in SA and UA Patients TLR4 expression levels of LPS stimulated monocytes of patients with SA and UA are shown as normalized expression levels. The bar plots show medians in the 25th and 75th percentiles.