are independent of those which regulate vascular reactivity. It would then be possible to have lower vascular resistance in the context of impaired vascular responsiveness. Thus, whilst we have shown that vascular reactivity is greatly influenced by the NO–cGMP pathway, perhaps vascular tone is mediated by a different NO-dependent pathway. This could be the case if the mechanism of action of NO were different in resistance arterioles from that in conduit vessels. This is suggested by the redundancy of vasoreactive mechanisms previously described in resistance vessels which are not mediators in conduit vessels, including endothelium-derived hyperpolarization factor, products of cyclo-oxygenase and neuronal NOS-derived NO (Popp *et al.* 1998; Chataigneau *et al.* 1999; Sun *et al.* 1999; Ding *et al.* 2000; Huang *et al.* 2001; Scotland *et al.* 2001). An alternative explanation is that endothelial NO production may mediate effects on chronic blood pressure independantly of vascular smooth muscle by effecting renal regulation of endovascular salt and volume.

The results of this study have implications for our understanding of the role of NO signalling in vascular function and haemodynamic regulation. Our data show discordance between systemic blood pressure and vasomotor function by aortic wire myography. This suggests that vasorelaxation responses in models of altered NO signalling should not be interpreted as direct measures of NOS enzymatic activity. Our data support the hypothesis that endothelial NO signalling is an important regulator of systemic blood pressure but suggest that therapeutic strategies targeting upregulation of eNOS should also consider the potential impact of chronic elevation of NO on desensitization of downstream signalling pathways. Furthermore, even correction of eNOS uncoupling and BH4 supplementation *in vivo* seems unlikely to alter desensitization of downstream NO signalling. This may have clinical implications for patients receiving therapeutic exogenous nitrate donors, since chronic administration in the context of disease states associated with increased oxidative stress in the vascular wall might lead to desensitization of the NO–cGMP pathway and exacerbate impairment of vascular reactivity and endothelial function.

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Toll-like receptor 4 expressions on peripheral blood monocytes were enhanced in coronary artery disease even in patients with low C-reactive protein

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Received 2 April 2006; accepted 17 August 2006

Abstract

Toll-like receptors (TLRs) play important roles in the pathogenesis of atherosclerosis. On the other hand, serum high sensitivity C-reactive protein (hsCRP) is known as an independent coronary risk factor, but cardiovascular events do occur even in low hsCRP levels. We investigated whether the TLR4 expression levels on human peripheral blood monocytes were associated with serum hsCRP levels or the occurrence of coronary artery diseases (CAD). One hundred CAD patients and 100 non-CAD subjects were enrolled. There were 72 non-CAD subjects and 53 CAD patients with low serum hsCRP levels. Among the low-hsCRP subjects, the TLR4 expression levels were higher in CAD patients than in non-CAD subjects ($P<0.05$, after being adjusted for other risk factors). Moreover, TLR4 expression levels in stable angina pectoris (SAP) patients were elevated compared with those in non-CAD subjects ($P<0.05$), and those in acute coronary syndrome patients were higher than SAP patients even in low-hsCRP subjects ($P<0.01$). In conclusion, the TLR4 expression levels on peripheral blood monocytes in CAD patients were higher than those in non-CAD subjects and correlated with disease activity, even in low-hsCRP subjects. The combined measurement of serum hsCRP and the TLR4 expression on peripheral blood monocytes, especially among low-hsCRP subjects, may become a new coronary risk marker.

Keywords: Inflammation; Immune system; Coronary artery disease

Introduction

Atherosclerosis is a chronic inflammatory disease (Ross, 1993). Vascular inflammation occurs in response to injury induced by various stimuli, such as oxidative stress (Madamanchi et al., 2005), shear stress (Shaaban and Duerinckx, 2000; Traub and Berk, 1998), and infection (Espinola-Klein et al., 2002; Danesh et al., 1997; Shor et al., 1992). The precise mechanisms whereby chronic infection causes cardiovascular disease, however, remain to be elucidated.

Toll-like receptors (TLRs) are pathogen-associated molecular pattern recognition molecules that play a crucial role in innate immunity as the first defense system against microbial infection (Akira and Hemmi, 2003). Eleven TLRs have been identified, and TLR1, TLR2, and TLR4 are intensely expressed in human atherosclerotic vessels (Edfeldt et al., 2002), and deletion of the TLR4 gene was shown to reduce atherosclerosis in apoprotein E knockout mice (Michelsen et al., 2004). We recently demonstrated that TLRs are expressed on human platelets and coronary thrombi of patients with acute coronary syndrome (Shiraki et al., 2004). These findings strongly suggest that the TLRs yield an important role in the pathogenesis of atherosclerotic vascular diseases. The association between TLRs and cardiovascular diseases is supported by the recent observations that a single nucleoside polymorphism of the TLR4 gene is closely related to a decreased

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0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.08.027

risk of acute coronary events (Kiechl et al., 2002). Furthermore, very recently Methe et al. reported the enhanced expression of TLR4 in circulating monocytes in patients with unstable angina and acute myocardial infarction (Methe et al., 2005).

It is well recognized that there is an increase in serum C-reactive protein (CRP) levels in acute coronary syndrome, reflecting the presence of systemic inflammation in this pathological state. In addition, serum levels of CRP have been revealed as an independent predictor of cardiovascular events in patients with stable and unstable angina, high-risk individuals, and apparently healthy individuals. The Center for Disease Control and Prevention and the American Heart Association issued the first set of clinical guidelines for CRP as a part of global risk prediction and suggested that serum levels of high-sensitivity CRP (hsCRP) under 1 mg/L, 1 to 3 mg/L, and 3 or more mg/L represent low, moderate, and high vascular risk, respectively (Smith et al., 2004). Individuals with hsCRP under 1 mg/L are not, however, necessarily free of cardiovascular disease. Serum hsCRP levels are largely influenced by various factors including systemic inflammatory status. Further, hsCRP, which is a marker of systemic inflammation, may not always be associated with inflammatory changes occurring locally at the coronary vasculature.

In the present study, we investigated TLR4 expression levels on human peripheral blood monocytes in patients with coronary artery disease. Then we assessed whether TLR4 expression levels were related to serum hsCRP levels to develop a future coronary risk factor from the standpoint of inflammatory aspects of atherosclerotic vascular diseases.

Materials and methods

Subjects

The study protocol was approved by the Human Research Committee of Kobe University of Medicine and Kobe Steel Hospital, and written informed consent was obtained from all participants. CAD group included patients admitted to Kobe University Hospital for a diagnosis of stable angina pectoris (SAP) and acute coronary syndrome (ACS), which includes unstable angina (UAP) or acute myocardial infarction (AMI). UAP was defined according to the Braunwald's classification. SAP patients had clinical evidence of Canadian Cardiovascular Society class II and III, and coronary artery stenosis angiographically documented greater than 75% and perfusion scintigraphy showed myocardial ischemia.

The non-CAD subjects (non-CAD group) were inpatients and outpatients of Kobe University Hospital and Kobe Steel Hospital with diagnosis of hypertension, hyperlipidemia, diabetes, chest pain syndrome, or valvular heart disease. These subjects had neither symptoms of angina pectoris nor electrocardiographical abnormalities after exercise-loading test.

Patients with coronary interventions with 7 days, previous myocardial infarction within 6 months, heart failure (ejection fraction<40% or fractional shortening<20%), renal failure (serum creatinin levels>2.0 mg/dl), cancer, autoimmune disease, and infectious disease were excluded from the study. None of the patients were taking any anti-inflammatory agents other than aspirin (up to 100 mg daily).

Table 1

	Total population			Low CRP subjects		
	Non-CAD group (n=100)	CAD group (n=100)	P	Non-CAD group (n=72)	CAD group (n=49)	P
Age, y	63.6±12.4	68.0±8.4	0.03	62.9±12.6	67.8±7.9	n.s.
Male sex, %	59	77	0.01	56.9	77.6	0.032
BMI, kg/m^2	24.2±3.0	24.3±3.0	n.s	23.9±2.9	23.8±3.0	n.s
Hypertension, %	67	84		68	88	
Systolic blood pressure, mm Hg	131.5±16.6	132.3±20.0	n.s.	130.9±16.2	133.6±20.4	n.s.
Diastolic blood pressure, mm Hg	76.1±10.0	71.1±9.6	<0.001	76.3±10.6	72.2±10.6	n.s.
Hyperlipidemia, %	41	53		43	55	
Total cholesterol, mg/dl	203.3±30.1	184.8±29.3	<0.0001	199.9±29.5	183.4±28.6	0.003
HDL cholesterol, mg/dl	58.3±14.5	47.4±11.7	<0.0001	61.0±15.0	48.5±12.1	<0.0001
Triglycerides, mg/dl	144.2±72.0	133.3±55.1	n.s.	143.0±66.9	136.0±62.8	n.s.
Diabetes mellitus, %	14	52		10	53	
HbAlc, %	5.5±0.9	6.1±1.2	<0.0001	5.5±0.9	6.1±1.1	<0.0001
CRP, mg/L	0.51 (0.31–1.16)	0.93 (0.37–2.0)	<0.01	0.39 (0.22–0.61)	0.39 (0.24–0.63)	n.s.
Medications, %						
Aspirin	19	79	<0.0001	17	89	<0.0001
Renin–angiotensin inhibitors	36	56	0.001	39	64	0.013
β-blockers	16	48	<0.0001	17	56	<0.0001
Ca-blockers	50	36	n.s.	51	38	n.s.
Diuretics	7	15	n.s.	9	15	n.s.
Nitrates	5	59	<0.0001	7	63	<0.0001
Statins	29	50	<0.001	33	56	0.023
Oral antidiabetics and insulin	5	25	<0.0001	7	35	<0.001

Data are shown as mean±SD if normally distributed or otherwise by median with 25% and 75% percentiles.
Differences in baseline parameters were analyzed with the Mann–Whitney U test or the Pearson's correlation coefficiency where appropriate.
CAD = coronary artery disease. BMI = body mass index. HDL = high-density lipoprotein. CRP = C-reactive protein. n.s. = not significant.

Laboratory methods

Blood samples were drawn from each subject under standardized conditions and before coronary angiography if performed. Samples were centrifuged at 1700 g for 10 min, immediately divided into aliquots, and frozen until analysis. HsCRP was measured by the enzyme-linked immunosorbent assay.

Flow cytometry

Red blood cells in whole blood (1 mL) were lysed, and then centrifuged at 750 g for 10 min. The pellet was washed twice with phosphate-buffered saline, and fixed in 2% paraformaldehyde. The cells were then incubated with phycoerythrin (PE)-conjugated mouse anti-human TLR4 antibody (eBioscience, San Diego, CA). Isotype PE-conjugated IgG was used as a negative control. After washing with PBS, the cells were analyzed using the FACScan flow cytometer and CELLQuest^R software (BD Biosciences Clontech., Palo Alto, CA). To evaluate the relative surface expressions of TLR4 on peripheral blood monocytes, the mean fluorescent intensity (MFI) was determined, and then the ratio of TLR4 MFI to negative control MFI was calculated. As previously reported (Okumura et al., 2003), the relative surface expressions of TLR4 on peripheral blood monocytes were evaluated by use of the ratio of TLR4 MFI to negative control MFI.

Statistical analysis

Continuous variables are presented as mean±SD if normally distributed or otherwise by median with 25% and 75% percentiles. Differences in baseline parameters were analyzed with the Mann–Whitney U test or the Pearson's correlation coefficiency where appropriate. The data for coronary risk factors and medications regimen were included in a multiple logistic regression analysis. To compare the TLR4 expression levels on peripheral blood monocytes among clinical stages, the data were analyzed using the Wilcoxon's rank test and Kruskal–Wallis test where appropriate. A P value of less than 0.05 was considered statistically significant. For statistical analysis, StatView version 5.0 was used (Abacus Concepts, Berkeley, CA).

Results

Characteristics of the study population and hsCRP levels

The characteristics of the study population are shown in Table 1. One hundred patients with CAD (CAD group; 79 patients with SAP and 21 patients with ACS), and 100 non-CAD subjects (non-CAD group) were enrolled. All subjects were Japanese. As expected, coronary risk factors, including low HDL-cholesterol levels, diabetes mellitus, and taking medications regimen were significantly more frequent in the CAD group than in the non-CAD group. Total cholesterol levels and diastolic blood pressure were lower in the CAD group; however, this might have been due to the medication regimens (Table 1).

The hsCRP levels of the CAD group were significantly higher than those of the non-CAD group (0.93 [0.39–2.02] vs 0.51 [0.31–1.16] mg/L, $P<0.01$, Fig. 1A). The serum hsCRP levels of 53 CAD patients, however, were under 1 mg/L, indicating that subjects with low serum hsCRP levels are not necessarily free of CAD. In low-hsCRP subjects whose hsCRP levels were under 1 mg/L ($n=125$; CAD, $n=53$, non-CAD, $n=72$), there were no significant differences in hsCRP levels between the CAD and non-CAD group (0.42 [0.25–0.63] vs 0.39 [0.22–0.61] mg, $P=0.64$, Fig. 1B).

TLR4 expression levels on PBMCs were higher in CAD patients

The TLR4 expression levels on peripheral blood monocytes were analyzed by flow cytometry and these values were compared with the serum hsCRP levels. The expression of TLR4 on peripheral blood monocytes was confirmed with double staining of CD14 and TLR4 as shown in Fig. 2A and B. The TLR4 expression levels were significantly higher in the CAD group than in the non-CAD group (1.21 [1.13–1.47] vs 1.19 [1.10–1.37]; ratio of TLR4 MFI to negative control MFI,

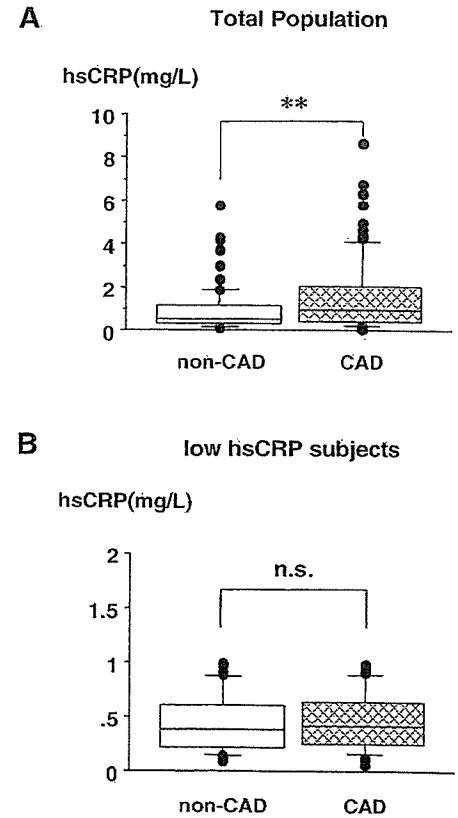

Fig. 1. Serum hsCRP levels of CAD patients and non-CAD subjects in the total population (A) ($n=200$; CAD $n=100$, non-CAD $n=100$) and in the low-hsCRP subjects (B) whose serum hsCRP levels were under 1 mg/L ($n=125$; CAD, $n=53$, non-CAD, $n=72$). Values are expressed as mean±25th percentile. **$P<0.01$, Mann–Whitney U test was used for statistical analysis.

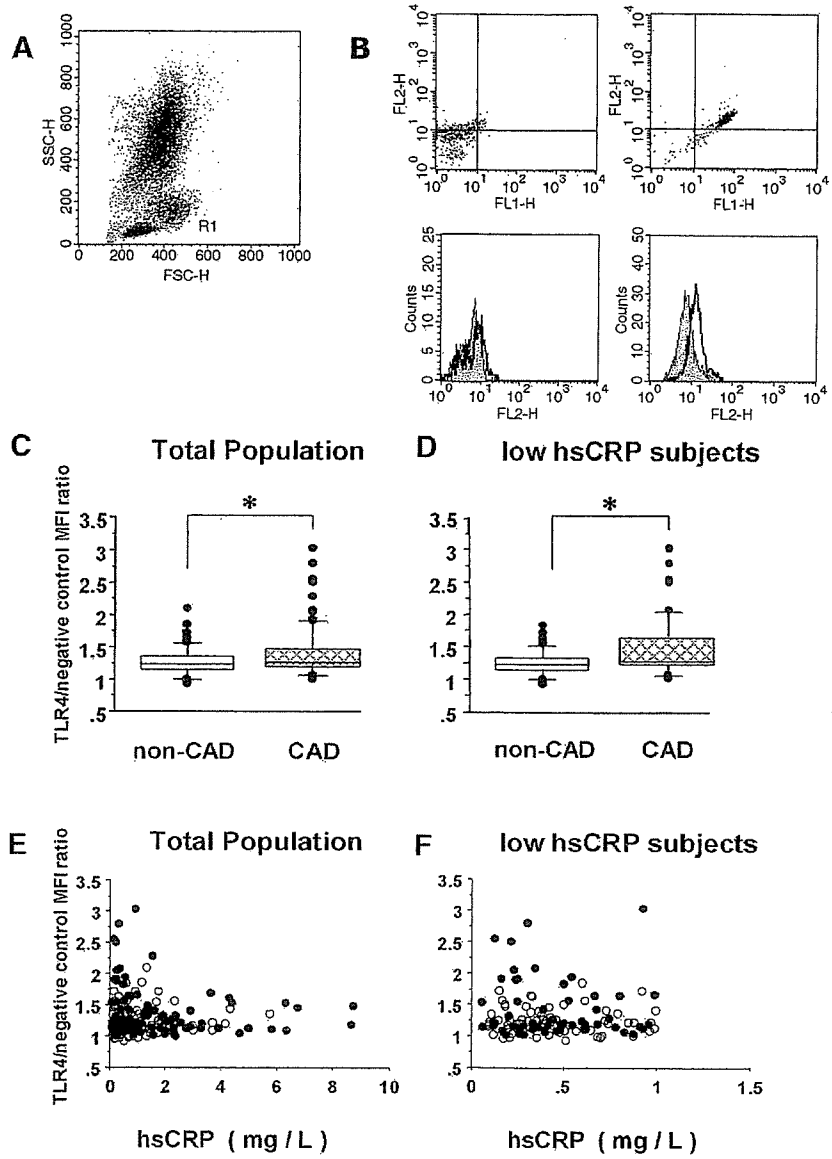

Fig. 2. The TLR4 expression levels on peripheral blood monocytes. (A) The FSC/SSC dot blot of peripheral blood cells from a CAD patient. We analyzed the gated area (R1). (B) The dot blots and histograms for CD14/TLR4 of the gated area R1. Upper figures: FL-1; CD14, FL-2, TLR4, lower figures: shaded area; isotype control, open area; TLR4. Upper left; isotype control, upper right; CD14/TLR4 double staining, lower left; non-CAD subject, lower right; CAD patient. The TLR4 expression levels on peripheral blood monocytes in the total population (C) and in the low-hsCRP subjects (D). Values are expressed as mean±25th percentile. *$P<0.05$, Mann–Whitney U test was used for statistical analysis. Relation between serum hsCRP levels and the TLR4 expression levels in the total population (E) and in the low-hsCRP subjects (F). Open circles; non-CAD group, closed circles; CAD group. Spearman's rank correlation coefficiency was used for statistical analysis.

$P<0.05$, after being adjusted for coronary risk factors, Fig. 2C, Table 2). Even in the low-hsCRP subjects, the TLR4 expression levels were also significantly higher in the CAD group than in the non-CAD group (1.22 [1.12–1.58] vs 1.20 [1.10–1.33]; ratio of TLR4 MFI to negative control MFI, $P<0.05$ after being adjusted for coronary risk factors, Fig. 2D, Table 2), though there were no differences in the serum hsCRP levels. TLR4 expression levels were affected by HDL-cholesterol levels, systolic blood pressure, and taking drugs of aspirin and nitrates. These parameters were selected by multiple regression analysis (Table 2), and TLR4 expression levels were significantly higher in CAD group even when these parameters were adjusted in analysis. Thus, both disease sensitivity and specificity of TLR4 expression were higher than those of hsCRP in low-hsCRP subjects. There was no correlation between the serum hsCRP levels and the TLR4 expression levels on peripheral blood monocytes ($P=0.17$, $\sigma=0.11$, Spearman rank correlation coefficient; Fig. 2E and F).

Disease activity and TLR4 expression on peripheral blood monocytes

We then investigated the relation between the serum hsCRP levels or the TLR4 expression levels on peripheral blood

Table 2
Logistic multiple regression analysis of various parameters TLR4 expression on peripheral blood monocytes

	Standardized coefficient of correlation	P value
Total population		
Systolic blood pressure	0.269	0.013
HDL cholesterol	−0.3	0.020
Taking aspirin	0.2	0.066
Taking nitrates	0.202	0.082
Low CRP subjects		
Systolic blood pressure	0.426	0.007
HDL cholesterol	−0.47	0.004

The parameters that P values were lower than 0.1 were shown.

monocytes and the clinical profile of CAD. The representative histograms of TLR4 expression are shown in Fig. 3A. In the total population, hsCRP levels in SAP or ACS patients were significantly higher than those in the non-CAD group ($P<0.01$, $P<0.01$, respectively), however, there were no differences in serum hsCRP levels between SAP and ACS patients (Fig. 3B). In the low-hsCRP subjects, as expected, there were no differences in serum hsCRP levels among the three groups (Fig. 3C).

On the other hand, the TLR4 expression levels on peripheral blood monocytes in both SAP and ACS patients were significantly higher than in the non-CAD group ($P<0.05$, $P<0.01$, respectively). Importantly, the TLR4 expression levels on peripheral blood monocytes in ACS patients were significantly higher than those in SAP patients ($P<0.01$), and these results were observed even in the low-hsCRP subjects (Fig. 3D and E).

TLR4 expression on peripheral blood monocytes changed in association with disease activity

In some patients with ACS, we examined the association of TLR4 expression with disease activity. All patients examined underwent PCI after blood sampling for TLR expression measurement and thereafter treated by conventional medications including aspirin, statins and angiotensin converting enzyme (ACE) inhibitors or angiotensin type 1 receptor blockers (ARBs). At 6 months after admission for ACS, patients were free of cardiac symptoms. Expression levels of TLR4 on peripheral blood monocytes were markedly decreased, but serum hsCRP levels remained unchanged (Fig. 4).

Discussion

In the present study, we examined the association of the TLR4 expression levels on PBMCs with coronary artery disease. Subjects with a serum hsCRP levels under 1 mg/L are considered to be at low coronary risk; however, 49% of the CAD group had a serum hsCRP levels under 1 mg/L in the present investigation. In these low-hsCRP subjects, the TLR4 expression levels on peripheral blood monocytes in the CAD group were significantly higher than those in the non-CAD group. The TLR4 expression levels on peripheral blood monocytes were particularly elevated in patients with ACS irrespective of their CRP levels.

It is well recognized that CRP is elevated in the serum of patients with CAD, suggesting that inflammation is closely involved in the pathophysiology of CAD.

Recent studies suggest that CRP itself may act to promote local inflammation. Immunohistochemical analysis demonstrated that CRP is expressed in atherosclerotic coronary arteries, and CRP localized at the vascular wall may enhance the generation of superoxide (Kobayashi et al., 2003). CRP in the bloodstream is, however, generated by hepatocytes in response to systemic inflammation, and the serum CRP levels do not reflect localized inflammation in the vasculature. Indeed, 49% of patients had low serum hsCRP levels in the present investigation, confirming the limitation of hsCRP as a marker of localized vascular inflammation. This nature of CRP may limit the usefulness of hsCRP as a sensitive coronary risk factor.

There was no correlation between serum hsCRP levels and TLR4 expression levels on peripheral blood monocytes, particularly in the low-CRP range in our study (Fig. 2E and F). The results of these figures suggest that the elevated TLR4 expression levels in the absence of elevated hsCRP implied the presence of mechanisms that regulate TLR4 expression independently of the regulation of hsCRP expression. It seems that expression levels of TLR4 on peripheral blood monocytes reflect more sensitively the local atherosclerotic events than serum hsCRP levels. The elevation of TLR4 expression provides new insight into pathophysiology of coronary artery disease from the standpoint of inflammatory and immune responses in the pathogenesis of atherosclerotic disease. Recent investigations revealed that microbial antigens such as lipopolysaccharides and bacterial heat shock proteins interact with the extracellular domain of TLRs and subsequently activate multiple intracellular signaling pathways leading to induction of inflammatory responses (Shor et al., 1992; Akira and Hemmi, 2003). For example, Sasu et al. demonstrated that Chlamydial heat shock protein 60 stimulated the proliferation of vascular smooth muscle cells via TLRs (Sasu et al., 2001). Furthermore, TLRs recognize not only microbial antigens but also endogenous factors (Akira and Hemmi, 2003). Thus, pro-atherogenic mechanisms by various atherogenic factors seem to be mediated through TLRs.

Recent studies reveal that TLRs have an important role in the differentiation of macrophages (Karlsson et al., 2004), which is one of the essential processes in atherogenesis. Furthermore, stimulation through TLR4 is important for differentiation of phagosomes (Blander and Medzhitov, 2004), or osteoclasts (Kikuchi et al., 2001). TLRs are up-regulated by several inflammatory cytokines such as interferon-gamma and tumor necrosis factor alpha (Miettinen et al., 2001; Wolfs et al., 2002), and, therefore, these inflammatory cytokines might promote differentiation and activation of macrophages, leading to the progression of atherosclerosis, via

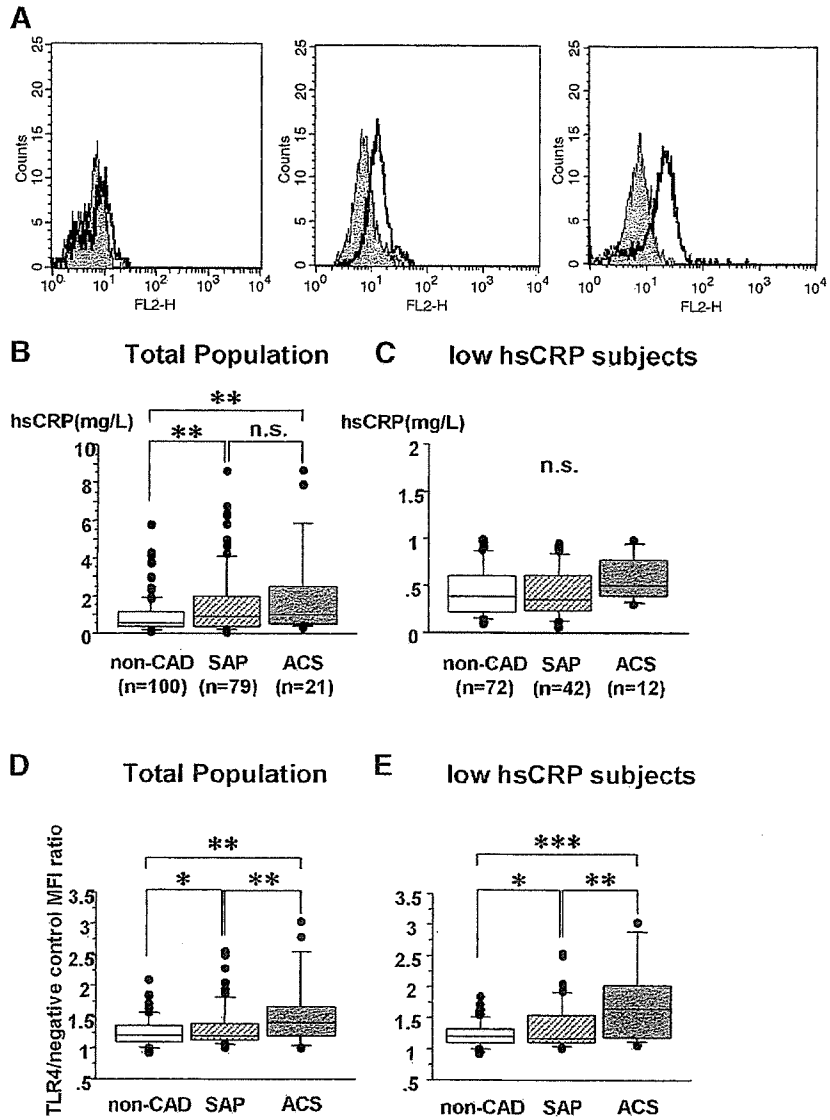

Fig. 3. Disease activity and TLR4 expression. (A) The representative TLR4-histograms of non-CAD subject (left), SAP patient (middle), and ACS patient (right). Relations between serum hsCRP levels (B and C) or TLR4 expression levels on peripheral blood monocytes (D and E) and the clinical profile. In the total population; B and D, in the low-hsCRP subjects; C and E. Values are expressed as mean±25th percentile $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Kruskal–Wallis rank test was used for statistical analysis.

TLRs. The finding that TLR4 expression on peripheral blood monocytes was elevated in patients with stable angina may reflect not only that stimuli for TLR4 expression were increased even in stable angina but also that the up-regulated TLR4-mediated signals lead to progression of atherosclerotic lesion formation.

In previous reports, individuals carrying Gly alleles at 299 of the TLR4 gene had less responsibility to lipopolysaccharides and lower serum CRP levels compared with wild type (Arbour et al., 2000). Since the report that the Asp299Gly polymorphism of the TLR4 gene decreased the risk of atherosclerotic disease (Kiechl et al., 2002), other reports have been published with conflicting results (Ameziane et al., 2003; Yang et al., 2003; Reismann et al., 2004). Thus, a controversy still exists with regard to the association between TLR4 Asp299Gly polymorphism and cardiovascular diseases. The present study showed the close association of TLR4 expression levels on peripheral blood monocytes with the occurrence of cardiovascular diseases, however none of the patients carried Gly alleles at 299 in the TLR4 gene (data not shown). It might be due to the ethnical background. Indeed, it was reported that there were few Asp299Gly polymorphisms of TLR4 gene in Japanese population (Okayama et al., 2002), however, the population of this study is too small to evaluate this important point unfortunately. Taken together, these findings suggest that the Asp299Gly polymorphism of TLR4 gene did not influence the results of the present study.

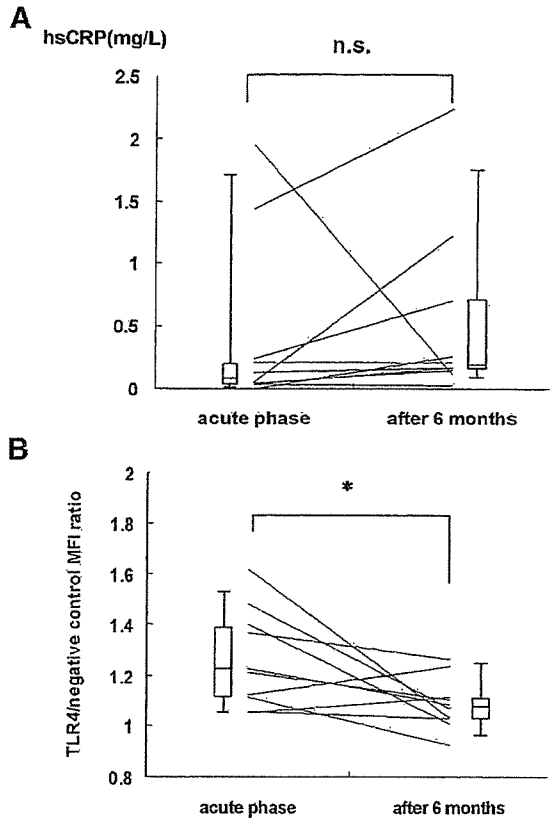

Fig. 4. The association of TLR4 expression levels on peripheral blood monocytes with the disease activity. The levels of hsCRP and TLR4 expression on peripheral monocytes were evaluated in eleven patients with ACS at acute phase and 6 months after the onset. HsCRP levels were not changed (A), however, TLR4 expressional levels on peripheral blood monocytes decreased at 6 months after the onset (B). $*P<0.05$, Wilcoxon's rank test was used for statistical analysis.

Very recently, Methe et al. demonstrated that TLR4 expression on circulating monocytes and serum heat shock protein 60 levels was elevated in patients with ACS (Methe et al., 2005). They also showed that serum from patients with ACS activated TLR4-transfected Chinese hamster ovary cells through TLR4 (Methe et al., 2005). Their work was the first report in humans on the association of TLR4 on circulating monocytes and coronary artery disease. In their study the relation between serum hsCRP levels and TLR4 expression levels was not assessed, and no difference was found in TLR4 expression levels between patients with stable angina and control subjects. We speculate that these different results may be due to difference of methodology in the TLR4 expression level measurement. Indeed, the examination of the level of mRNA would provide more information, however, we did not examine it due to several reasons. Our investigation is a population study, and it is impossible to isolate mRNA of peripheral blood monocytes from all subjects including patients with acute coronary syndrome in our institute. Nevertheless, we think that the protein levels of TLR reflect the function more precisely than the levels of mRNA.

Study limitations

This research is a case-control study; therefore, the usefulness of the obtained results in the primary and secondary prevention of coronary events is not known. To confirm the TLR4 expression level on peripheral blood monocytes as a coronary risk, it is necessary to perform a prospective investigation in a large population.

Conclusion

Even in the low-hsCRP subjects, the TLR4 expression levels on peripheral blood monocytes of the CAD group were significantly higher than those of the control group. Moreover, the TLR4 expression levels on peripheral blood monocytes are likely a better marker for the CAD activity than serum hsCRP levels. The combined measurement of serum hsCRP and TLR4 expression on peripheral blood monocytes, especially among low-hsCRP subjects, might serve as a new marker that reflects coronary risk and vulnerable patients more precisely than serum hsCRP levels alone.

Acknowledgments

This work was supported by Grant for Research on Cardiovascular Disease from Japan Heart Foundation/Pfizer Japan Inc., Grant for Clinical Vascular Function, and Grant in Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

We thank Dr. Toru Miyajima in Kobe Steel Hospital for his help in getting the blood samples from the Kobe Steel Hospital.

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Arteriosclerosis, Thrombosis, and Vascular Biology

Author Disclosures

Tomofumi Takaya: No disclosures

Ken-ichi Hirata: No disclosures

Tomoya Yamashita: No disclosures

Masakazu Shinohara: No disclosures

Naoto Sasaki: No disclosures

Nobutaka Inoue: No disclosures

Toyotaka Yada: No disclosures

Masami Goto: No disclosures

Akiko Fukatsu: No disclosures

Toshio Hayashi: No disclosures

Nicholas J Alp: No disclosures

Keith M Channon: No disclosures

Mitsuhiro Yokoyama: No disclosures

Seinosuke Kawashima: No disclosures

A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression

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Total words 4951 counts **Abstract** 199 words **Figure** 5 **Table** 1

Abstract

Objective: When the availability of tetrahydrobiopterin (BH4) is deficient, endothelial nitric oxide synthase (eNOS) produces superoxide rather than NO (uncoupled eNOS). We have shown that the atherosclerotic lesion size was augmented in apolipopritein E-deficient (ApoE-KO) mice overexpressing eNOS due to the enhanced superoxide production. In this study, we addressed the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus non-specific anti-oxidant strategies in restoring eNOS coupling.

Methods and Results: We crossed mice over-expressing eNOS in the endothelium (eNOS-Tg) with mice over-expressing GTP-cyclohydrolase I (GCH), the rate-limiting enzyme in BH4 synthesis, to generate ApoE-KO/eNOS-Tg/GCH-Tg mice. As a comparison, ApoE-KO/eNOS-Tg mice were treated with vitamin C. Atherosclerotic lesion formation was increased in ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice. GCH over-expression in ApoE-KO/eNOS-Tg/GCH-Tg mice increased vascular BH4 levels and reduced plaque area. This reduction was associated with decreased superoxide production from uncoupled eNOS. Vitamin C treatment failed to reduce atherosclerotic lesion size in ApoE-KO/eNOS-Tg mice, despite reducing overall vascular superoxide production.

Conclusion: In contrast to vitamin C treatment, augmenting BH4 levels in the endothelium by GCH over-expression reduced the accelerated atherosclerotic lesion formation in ApoE-KO/eNOS-Tg mice, associated with a reduction of superoxide production from uncoupled eNOS.

Key words: eNOS uncoupling, Tetrahydrobiopterin, Vitamin C, Atherosclerosis, Apolipoprotein E-deficient mice

Condensed abstract

In apolipoprotein E-deficient mice over-expressing eNOS in the endothelium, augmenting BH4 levels in the endothelium by GTP-cyclohydrolase I over-expression was more efficient to reduce the accelerated atherosclerotic lesion formation and superoxide production from uncoupled eNOS compared with chronic vitamin C treatment.

Nitric oxide (NO) derived from endothelial NO synthase (eNOS) is a critical signaling molecule in the vasculature, and has a range of anti-atherogenic effects [1]. In eNOS/apolipoprotein E (ApoE) double-knockout mice, atherosclerosis is increased, suggesting a protective effect of eNOS-derived NO [2, 3]. However, certain vascular diseased states are associated with an increase rather than a decrease in the expression of eNOS [4, 5]. We have shown that endothelium-targeted over-expression of eNOS in ApoE-KO (ApoE-KO/eNOS-Tg) mice surprisingly resulted in decreased endothelial NO availability, increased vascular superoxide production, and accelerated atherosclerosis [6].

These observations could be explained by a relative deficiency of the co-factor tetrahydrobiopterin (BH4) causing eNOS 'uncoupling' [7], where the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. In ApoE-KO/eNOS-Tg mice, dietary BH4 supplementation reduced superoxide production and increased NO availability, although it was unclear whether this was a general anti-oxidant effect of BH4 or a specific effect on eNOS coupling [6]. Indeed, dietary supplementation with the anti-oxidant vitamin C can also reduce vascular oxidative stress, increase BH4 levels, and was sufficient to improve the depressed endothelium-dependent relaxation in ApoE-KO mice fed a high fat diet [8], although a specific effect on eNOS coupling was not investigated. These pre-clinical data suggest a potential role for vitamin C therapy in vascular disease, yet large scale clinical trials have failed to demonstrate an effect on major clinical endpoints in human atherosclerosis [9, 10]. It is possible that interventions targeted at specific redox mechanisms may be more effective than non-specific anti-oxidant strategies for treatment of vascular disease states.

Substantial evidence points to an important role for BH4 in regulating eNOS coupling in atherosclerosis. Indeed, even in the absence of vascular disease, eNOS over-expression in the endothelium without a concomitant increase in BH4 levels can result in eNOS uncoupling, but augmenting endothelial BH4 levels, by over-expression of the rate-limiting enzyme in BH4 biosynthesis, GTP-cyclohydrolase I (GCH), is able to restore eNOS/BH4 stoichiometry and eNOS coupling [11].

In this study, we sought to address the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus non-specific anti-oxidant strategies in restoring eNOS coupling. We used the ApoE-KO/eNOS-Tg mouse as a model of

eNOS uncoupling in atherosclerosis, and investigated the relationships between eNOS protein, BH4 availability and atherosclerotic plaque progression. Furthermore, we compared the strategy of directly augmenting endothelial BH4 levels by transgenic GCH over-expression, versus vitamin C supplementation.

Methods

Experimental design

The animals used in these experiments were offspring from breeding between ApoE-KO/eNOS-Tg mice (ApoE-KO mice over-expressing a bovine *eNOS* transgene in the endothelium) and ApoE-KO/GCH-Tg mice (ApoE-KO mice over-expressing a human *GCH* transgene in the endothelium), as previously reported in detail [6, 12]. The background strain was C57BL/6 mouse. ApoE-KO, ApoE-KO/eNOS-Tg and ApoE-KO/eNOS-Tg/GCH-Tg mice were generated and fed standard mouse chow from weaning. Experimental mice were euthanized at 16 weeks of age and organs used for histological and biochemical analysis. In parallel experiments, another group of ApoE-KO/eNOS-Tg mice were treated with 500 mg/kg body weight/day vitamin C dissolved in drinking water starting from 4 weeks of age until 16 weeks of age.

All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine. All commercial drugs besides those mentioned specifically were purchased from Sigma Chemical Co. (MO).

Plasma lipid analyses, vitamin C levels and hemodynamic analyses

After overnight fasting, blood was collected by the cardiac puncture under anesthetic using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation of the blood for 10 minutes at 5500 g at 4 °C and stored at -80 °C. Concentrations of plasma total cholesterol and triglyceride were determined by use of an automated chemistry analyzer. High density lipoprotein cholesterol levels were quantified by enzymatic reaction using a commercially-available kit (Wako, Osaka, Japan). Plasma concentrations of vitamin C were measured with reverse-phase high-performance liquid chromatography (HPLC).

Heart rate and systolic blood pressure were measured at 16 weeks of age using a tail cuff photoelectric device. All measurements were repeated six times for each mouse.

Measurement of vascular biopterin concentrations

Mice were anesthetized and the aorta sample was dissected from the ascending aorta to the iliac bifurcation. The dissected aorta was placed in a mixture of 0.5 M perchloric acid

containing 0.1 mM disodium EDTA, 0.1 mM $Na_2S_2O_3$ for protein separation and 0.1 mM ascorbic acid to prevent oxidation. Then the aorta was homogenized on ice in 200 μL homogenate buffer. After centrifugation (15000 g for 10 min) and filtration (0.45 μm pore size; Millex-HV Filter Unit, Millipore, Billerica, MA), we measured vascular BH4 and dihydrobiopterin (BH2) concentrations by HPLC developed by Tani et al [13]. Briefly, by post-column $NaNO_2$ oxidation with a reversed-phase ion-pair LC system, BH4 and BH2 were detected fluorometrically at wave lengths of 350 nm for excitation and 440 nm for emission (LC-10 series, Shimadzu, Kyoto, Japan). Protein concentrations of aortic homogenates were measured by the Bradford method and BH4 or BH2 concentration was corrected for protein concentration.

Analysis of eNOS protein

The levels of total eNOS protein in aorta homogenates were analyzed by western blotting, and in addition, to investigate the ratio of eNOS dimer to monomer, western blotting was performed by use of non-boiled lung homogenates and low-temperature SDS-page as previously described [14, 15].

Superoxide production in the aortic endothelium

First, we measured overall superoxide production from aortas. The aorta was cut into four pieces (5 mm length per each piece), which were incubated with the Cu-Zn superoxide dismutase inhibitor for 30 min at 37 °C. Then vascular superoxide production levels were measured by chemiluminescence with 10 μM lucigenin (bis-N-methylacridinium nitrate).

By this method, however, we cannot differentiate endothelium-derived superoxide from superoxide produced in other vascular cell components. We, therefore, measured in situ superoxide production from vessel wall by use of dihydroethidium (DHE; Molecular Probe, OR) [16]. Briefly, the unfixed tissues embedded in OCT were cut into 10 μm thick sections, and incubated with or without 5 mM Nω-nitro-L-arginine methyl ester (L-NAME) for 5 minutes at 37 °C, and then incubated with 2 μM DHE at 37°C for 10 minutes in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope (Carl ZEISS, Germany). DHE fluorescence from high-power (x 200) images was quantified by automated image analysis using the Image J software (National Institutes of Health, MD). For quantification of endothelial cell ethidium fluorescence, fluorescence (intensity x area) was measured only on the luminal side of

the internal elastic lamina. For each vessel, mean fluorescence was calculated from 3 separate high-power fields taken in each sections of the vessel to produce n=1.

Atherosclerotic lesion assessment at the aortic sinus

At 16 weeks of age, mice were anesthetized and the aorta was perfused as above. The aorta was dissected from the middle of the left ventricle to the aortic arch, and fixed with 4% paraformaldehyde for 16 hours. The samples were cut in the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compounds (Tissue-Tek, CA). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Sudan III. For quantitative analysis of atherosclerosis, the total lesion area of five separate sections from each mouse was obtained with the use of the Image J [6].

Statistical Analysis

Data were expressed as means±SEM. One-way ANOVA or two-way ANOVA were used to compare the differences among the four experimental groups and then appropriate post hoc analysis with Bonferroni correction for each result was performed. Values of $p<0.05$ were considered statistically significant.