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Oxidized LDL binding to LOX-1 enhances MCP-1 expression in cultured human articular chondrocytes

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Summary

Objective: It has been suggested that oxidized low-density lipoprotein (ox-LDL) has some roles in progression of osteoarthritis. The purpose of this study is to investigate whether ox-LDL binding to lectin-like ox-LDL receptor 1 (LOX-1) enhances monocyte chemoattractant protein 1 (MCP-1) expression in cultured human articular chondrocytes (HACs).

Method: The time course and dose response of MCP-1 mRNA expression and MCP-1 protein release into medium following ox-LDL stimulation were investigated using quantitative Real time PCR (delta–delta Ct method) and enzyme-linked immunosorbent assay (ELISA), respectively. To examine the receptor specificity of ox-LDL action, HACs were preincubated with anti-human LOX-1 monoclonal antibody (TS92).

Results: A time-course study revealed that MCP-1 mRNA expression increased 5.09 ± 0.86 fold 12 h after ox-LDL stimulation compared to time-0. ox-LDL stimulation increased MCP-1 protein level in conditioned medium in a time-dependent manner. Increased MCP-1 level was evident 6 h after stimulation, reaching 830 ± 91 pg/ml at 24 h (33 ± 8 pg/ml at time-0). Dose responses of MCP-1 expression were also evident in mRNA and protein levels. Pretreatment with TS92 markedly suppressed these stimulating effects of ox-LDL, although that with non-specific IgG did not. Native LDL did not affect MCP-1 expression.

Conclusion: Our results suggest that ox-LDL enhances MCP-1 expression in HACs and supports the hypothesis that ox-LDL is involved in cartilage degeneration.

Key words: Oxidized LDL, MCP-1, Chondrocytes, LOX-1, Cartilage degeneration.

Introduction

Chemokines were originally identified by their chemotactic activity for inflammatory cells[1,2]. Besides the implicated involvement of the chemokine/chemokine receptor system in the pathogenesis of inflammatory joint diseases[3–5], accumulating evidence has indicated involvement of the system in cartilage degeneration in osteoarthritis[5–9], that is chondrocytes produce the chemokines[7] and express their receptors[8], whose interaction induces matrix metalloproteinase (MMP)-1, 3, 13 and *N*-acetyl-β-D-glucosaminidase in chondrocytes, inhibits proteoglycan synthesis by chondrocytes and enhances proteoglycan release from cartilage[8,9]. These findings strongly suggest that this system plays a key role in the cartilage degradation, possibly acting in an autocrine/paracrine manner.

Recently, it has been shown that oxidized low-density lipoprotein (ox-LDL) uptake through lectin-like ox-LDL receptor 1 (LOX-1) expressed on vascular endothelial cells is involved in endothelial activation and dysfunction in atherogenesis[10–12]. Interestingly, expression of LOX-1 and association with ox-LDL in chondrocytes were noted in zymosan-induced arthritis rats[13]. We recently demonstrated *in vitro* that ox-LDL binding to LOX-1 increases production of intracellular reactive oxygen species (ROS), resulting in activation of nuclear factor-kappaB (NF-κB)[14]. However, functional consequences caused by the ox-LDL-induced NF-κB activation have not been investigated.

It is known that ox-LDL increases monocyte chemoattractant protein 1 (MCP-1) expression in macrophages[15] and endothelial cells[16], and that ox-LDL-induced MCP-1 expression in endothelial cells plays an important role in monocyte transmigration into the subendothelial space[16,17]. The purpose of this study was to investigate whether ox-LDL binding to LOX-1 increases MCP-1 expression in cultured human articular chondrocytes (HACs).

Materials and methods

HAC CULTURES

HAC culture was performed using commercially available cryopreserved human normal chondrocytes according to the manufacturer's instruction (NHAC-kn, Cambrex Corp., East Rutherford, NJ, USA). After the cells had been thawed in a 37°C water bath, resuspended chondrocytes were seeded at a density of $1 \times 10^4/cm^2$ in growth culture medium (CGM BulletKit,

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Cambrex Corp., East Rutherford, NJ, USA) and incubated at 37°C in a humidified 5% CO_2 incubator. Chondrocytes cultured in the media were expanded in two passages. We first confirmed that the cells used maintained properties of chondrocytes after the cell expansion. Reverse transcription-polymerase chain reaction (RT-PCR) and Real time PCR showed that the cultured HACs without any stimulation constitutively expressed mRNA of both type II collagen and aggrecan gene (data not shown). After reaching 70% confluence in monolayers, cells were cultured in the serum-free culture medium for 12–24 h and then stimulated with various agents in the serum-free medium.

PREPARATION OF NATIVE LDL (n-LDL) and ox-LDL

Human LDL (density 1.019–1.063) was isolated from fresh plasma by ultracentrifugation as described previously[10]. LDL was oxidized at a protein concentration of 3 mg/ml by exposure to 7.5 μM $CuSO_4$ for 20 h at 37°C. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (10.7 nmol/mg protein) produced, and the increased mobility on agarose gel electrophoresis, due to increased negative charge, was compared with that of n-LDL (relative electrophoretic mobility was 3.25)[10].

PREPARATION OF ANTI-HUMAN LOX-1 MONOCLONAL ANTIBODY

Briefly, lysate of cells expressing human LOX-1 was immunized to the zenomouse[18], and hybridoma producing anti-human LOX-1 monoclonal antibody (TS92, which was called JTX92 in the past) was obtained according to the conventional method to prepare monoclonal antibody. TS92 was purified using protein A from serum-free medium of the hybridoma. The purity of the antibody was verified by SDS-PAGE. Specificity and blocking ability of the antibody against human LOX-1 were confirmed by western blot and suppression of DiI-labeled ox-LDL uptake by LOX-1, respectively (data not shown). This monoclonal antibody has been used in some experimental studies to block ox-LDL binding to human LOX-1 and to show presence of human LOX-1 in immunohistochemistry[11,12,19–21].

RT-PCR FOR LOX-1 mRNA

Total RNA (1 μg) extracted from cultured HACs using Isogen (Nippon Gene, Tokyo, Japan) was reverse transcribed using the OneStep RT-PCR kit (Qiagen Japan, Tokyo, Japan). Reverse transcribed material (1.5 μL) was amplified with Taq DNA polymerase (Bex, Tokyo, Japan) using primer pairs specific to human LOX-1 (sense primer, 5′-GGGGTACCCACCTACAT TATGCAGC-3′; antisense primer, 5′-CCGCTCGAGCGGCCTGGTTG CAAGCCTATAATC-3′). The LOX-1 PCR products were 834 bp long, respectively. For PCR amplification of LOX-1, 30 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s were used. In the same experiments, bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with equal efficiency as a relative internal reference. A primer pair for human GAPDH was used (sense primer, 5′-CTGCCGTCTAGAAAAACC-3′; antisense primer, 5′-CCAAATTCGTTGTCATACC-3′). The PCR product was 200 bp long. For GAPDH PCR amplification, 30 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s were used. The amplified samples were visualized on 1.5% agarose gels using ethidium bromide.

RNA EXTRACTION AND REAL TIME PCR ANALYSIS

Cells were seeded at 1×10^5 cells/well in a 24-well plate and were allowed to grow to 70% confluence. All experiments were set up in triplicate per same lot, and two different lots were used in this analysis.

Cell pellets were resuspended in 350 μl of RNeasy lysis buffer from the RNeasy kit (Qiagen Inc., Valencia, CA, USA) and homogenized through Qiashredder columns (Qiagen). RNA extraction was performed through RNeasy columns according to manufacturer's instructions. Extracted RNA was eluted with 30 μl of diethylpyrocarbonate (DEPC) water. Single strand cDNA was prepared from total RNA using random primer under standard conditions with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA from each sample was diluted and used for quantification of *MCP-1* and *β-actin* expression. Quantitative Real time PCR with total cDNA was performed using Perfect Real time Premix Ex-Taq™ (TAKARA BIO, Inc., Shiga, Japan) and following primer/probe sets (listed 5′ to 3′ in the order of forward primer, probe, reverse primer): *β-actin*, GGTCATCACCATTGGCAATG, CGGTTCCGCTGCCCTGAGGC, CCA CAGGACTCCATGCCC; *MCP-1*, AGTGTCCCAAAGAAGCTGTGA, TCAA GACCATTGTGGCCAAGGAGAT, CCTGAACCCACTTCTGCTTG. The primer sets were designed to span exons to distinguish cDNA from genomic DNA products. Probes were dual labeled with FAM and TAMRA (all primers and probes were purchased from Sigma Genosys, Tokyo, Japan). The PCR amplifications were performed with the 7100 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) at 95°C for 5 min followed by 35 cycles of 95°C, 30 s; 60°C, 30 s; and 72°C, 30 s. Quantification of gene expression was based on the cycle threshold (Ct) value for each sample. Delta Ct was calculated as (gene of interest Ct) − (*β-actin* Ct) using Sequence detector (Applied Biosystems) and Microsoft Excel (Microsoft corp., Redmond, WA, USA). The relative quantity of MCP-1 mRNA expressions was calculated by delta–delta Ct calculation as $2^{-((\text{treated sample delta Ct})-(\text{control sample delta Ct}))}$. The amplification efficiencies of the target and the endogenous reference were confirmed to be approximately equal by observing that the plot of cDNA dilution vs delta Ct is close to zero (<0.05). All experiments included negative controls consisting of no cDNA for each primer pair.

MCP-1 PROTEIN QUANTIFICATION

Chondrocytes were seeded at a density of 2×10^5/ml in 12-well culture plates and cultured for 5 days, reaching 70% confluence in monolayers. All experiments were set up in duplicate per same lot, and five different lots were used in this analysis.

After incubation of the chondrocytes under the indicated conditions, the conditioned medium was collected and centrifuged at $14{,}000 \times g$ for 5 min. The MCP-1 protein level (pg/ml) was measured in the supernatant using a human MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (sandwich method, Human MCP-1 Biotrak ELISA system, GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The absorbance at 450 nm was measured within 10 min of addition of a reaction-stopping reagent using a microplate reader (TEACAN SPECTRA Micro-Plate Reader 539-67021, MTX Lab System Inc., Vienna, VA, USA).

STATISTICAL ANALYSIS

Results are presented as means ± standard deviation (SD). Analyses of variance, Scheffe's tests, and un-paired Student's *t* tests were used for statistical assessments. A level of $P < 0.05$ was considered statistically significant.

Results

LOX-1 mRNA EXPRESSION IN CULTURED HACs

Previous studies had shown that LOX-1, one of the receptors for oxidized LDL, is expressed in cultured rat[22] and bovine[14] articular chondrocytes. To confirm LOX-1 expression in cultured HACs, changes in LOX-1 mRNA expression by ox-LDL and interleukin 1β (IL-1β) stimulation were investigated by RT-PCR. A time-course study revealed that basal level of LOX-1 mRNA expressed constitutively and that by addition of 0.1 ng/ml IL-1β and 50 μg/ml ox-LDL it reached a peak after 6 and 12 h, respectively (data not shown).

INCREASE IN MCP-1 mRNA EXPRESSION IN CULTURED HACs BY ox-LDL

After the HACs were washed with serum-free medium three times, they were stimulated with 50 pg/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL, and the time course of MCP-1 mRNA expression was investigated by Real time PCR. To examine the receptor specificity of ox-LDL action, HACs were pretreated with 40 μg/ml anti-human LOX-1 monoclonal antibody (TS92) for 30 min and then stimulated with 50 μg/ml ox-LDL. We tried some concentrations of TS92 in preliminary experiments to block LOX-1 and noted that 40 μg/ml TS92 provided consistent results in complete suppression of ox-LDL-induced MCP-1 expression. ox-LDL increased MCP-1 mRNA expression in HACs. Twelve hours after stimulation MCP-1 mRNA expression reached a peak and the mean increase in MCP-1 mRNA expression was estimated to be 5.09 ± 0.86 fold ($n = 3$) greater than time-0. Pretreatment of HACs with 40 μg/ml TS92 for 30 min significantly suppressed the increase in MCP-1 mRNA expression induced by ox-LDL, although that with non-specific IgG (human IgG, Equitech-Bio, Inc., Kerrville, TX, USA) did not [Fig. 1(A)].

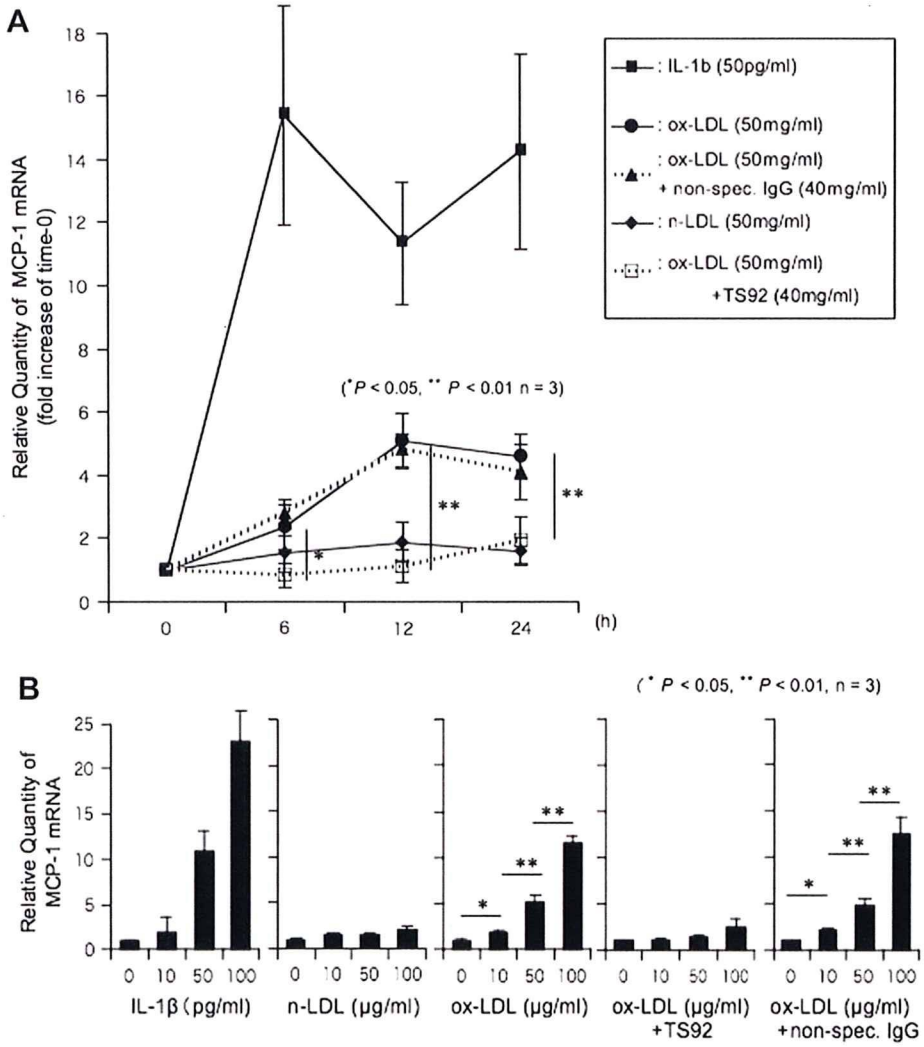

Fig. 1. Time and dose effects of ox-LDL on MCP-1 mRNA expression. HACs were incubated with 50 g/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL, and MCP-1 mRNA expression was investigated by Real time PCR at the indicated times (A). HACs were incubated with the indicated concentration of IL-1β, n-LDL or ox-LDL for 12 h, and MCP-1 mRNA expression was investigated by Real time PCR (B). Cells preincubated with 40 μg/ml anti-human LOX-1 mAb (TS92) or non-specific IgG for 30 min were also stimulated with ox-LDL. Relative quantity of MCP-1 mRNA was calculated by the delta–delta Ct method. Error bars indicate SDs ($n = 3$).

The dose dependency of ox-LDL stimulation for MCP-1 mRNA expression was then investigated. HACs were incubated with IL-1β (0, 10, 50, and 100 pg/ml), n-LDL (0, 10, 50, and 100 μg/ml) or ox-LDL (0, 10, 50, and 100 μg/ml) for 12 h, and MCP-1 mRNA expression was investigated by Real time PCR. HACs were pretreated with 40 μg/ml TS92 for 30 min and then stimulated with ox-LDL (0, 10, 50, and 100 μg/ml). IL-1β and ox-LDL increased MCP-1 mRNA expression in a dose-dependent manner. n-LDL did not significantly affect MCP-1 mRNA expression. The mean increase in MCP-1 mRNA expression was estimated to be 1.91 ± 0.32, 5.19 ± 0.67 and 11.6 ± 0.72 fold greater than control for cells, when stimulated with 10, 50 and 100 μg/ml ox-LDL, respectively. Pretreatment of HACs with TS92 significantly suppressed the increase [Fig. 1(B)].

INCREASES IN MCP-1 PROTEIN LEVEL INDUCED BY ox-LDL IN CONDITIONED MEDIUM

To examine whether ox-LDL stimulates MCP-1 production by HACs, MCP-1 levels in conditioned medium were determined by ELISA. First, the time-dependent effects of ox-LDL on MCP-1 production were observed in HACs incubated with 50 pg/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL. IL-1β stimulation increased MCP-1 production by chondrocytes, reaching a mean concentration 1803 ± 62 pg/ml 24 h after stimulation. ox-LDL stimulation also increased MCP-1 level in conditioned medium in a time-dependent manner. Increased MCP-1 level was evident 6 h after stimulation with ox-LDL, reaching 830 ± 71 pg/ml at 24 h. Pretreatment of HACs with TS92 significantly suppressed the increase in MCP-1 protein level by ox-LDL stimulation although that with non-specific IgG did not [Fig. 2(A)].

Next, the dose dependence of MCP-1 production was investigated after 24 h of stimulation with IL-1β (10, 50, and 100 pg/ml), n-LDL (10, 50, and 100 μg/ml) or ox-LDL (10, 50, and 100 μg/ml). IL-1β and ox-LDL dose-dependently increased MCP-1 protein level. The mean increase in MCP-1 level was estimated to be 7.5 ± 0.92, 11.5 ± 2.22 and 10.4 ± 1.75 fold greater than control with stimulation with 10, 50 and 100 μg/ml ox-LDL, respectively. Pretreatment

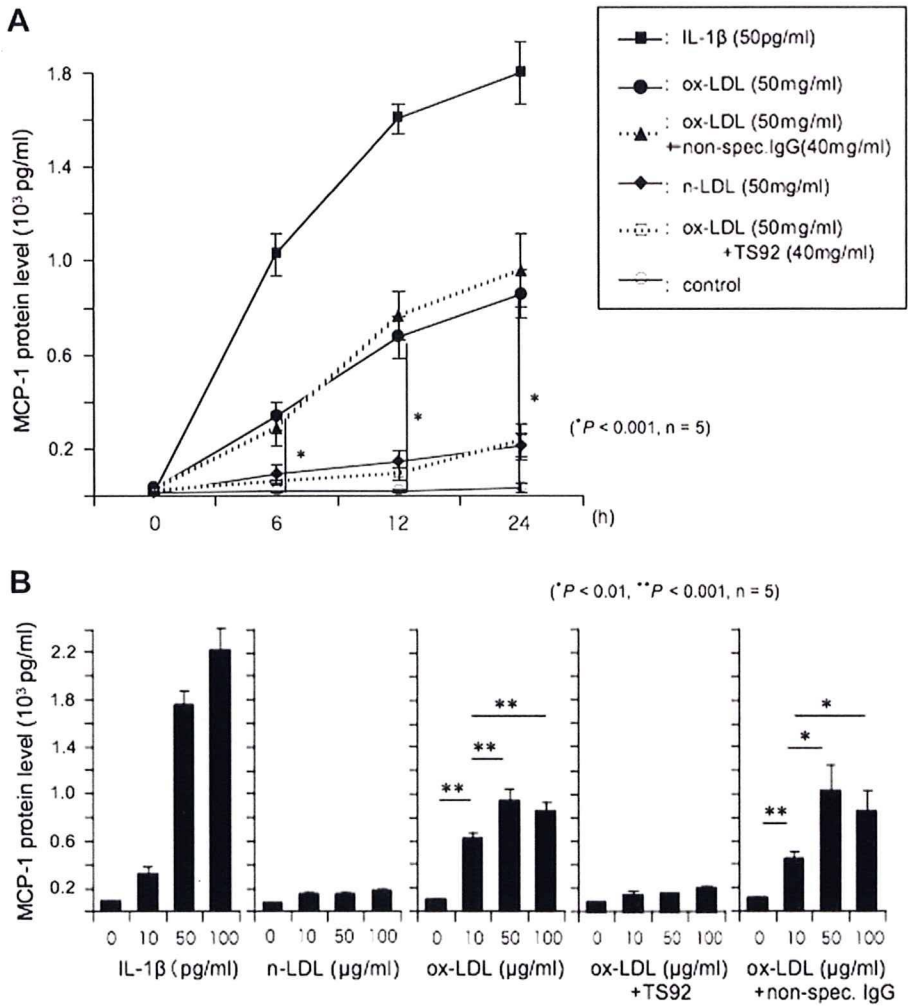

Fig. 2. Time and dose effects of ox-LDL on MCP-1 protein level. MCP-1 protein level was determined by ELISA of conditioned medium. Time dependent increases in MCP-1 level were observed when cells were incubated with 50 pg/ml IL-1β or 50 μg/ml ox-LDL (A). HACs were incubated with the indicated concentration of IL-1β, n-LDL or ox-LDL for 24 h (B). Cells preincubated with anti-human LOX-1 mAb (TS92) or non-specific IgG for 30 min were also stimulated with ox-LDL. Error bars indicate SDs ($n = 5$).

of HACs with TS92 significantly suppressed the increase in MCP-1 level by ox-LDL stimulation [Fig. 2(B)].

Discussion

Our data indicated that ox-LDL binding to LOX-1 enhanced MCP-1 expression in HACs. Increased MCP-1 mRNA expression and protein level in conditioned medium were evident 6 h after stimulation with ox-LDL and continued after 24 h, suggesting that MCP-1 upregulation by ox-LDL may have both a primary effect due to ox-LDL binding to LOX-1 and a secondary effect through enhancement of LOX-1 expression due to its ligand ox-LDL[11,12,14,22].

In the dose–response experiments, MCP-1 level reached a peak with ox-LDL stimulation at 50 μg/ml, while at 100 μg/ml of ox-LDL, the stimulation of MCP-1 level showed a decreasing trend, although expression of MCP-1 mRNA showed an significant increasing trend at both ox-LDL doses. This could be explained by a cytotoxic effect of high-dose ox-LDL. Nakagawa *et al.* reported that ox-LDL reduced chondrocyte viability through suppression of the PI3 kinase/Akt pathway[22]. We previously showed that ox-LDL reduces glycosaminoglycan synthesis with the decrease in cell viability[23]. Reduction in MCP-1 level with high-dose ox-LDL may therefore be attributable to the reduced chondrocyte viability.

In a previous *in vitro* study, we demonstrated ox-LDL-induced ROS results in the NF-κB activation in chondrocytes[14]. It is likely that one of functional consequences of the ox-LDL-induced NF-κB activation in chondrocytes is enhancement of MCP-1 expression, because the NF-κB is known as a nuclear transcription factor for chemokine expression[24–26]. Activation of mitogen-activated protein kinase (MAPK) may be another candidate of the signaling pathway involved in the ox-LDL-mediated MCP-1 expression. Actually, it has been demonstrated that activation of MAPK may play a critical role in signal transduction in ox-LDL-mediated MCP-1 expression in endothelial cells[16]. Interestingly, Pulai *et al.* have recently shown that fibronectin-fragment stimulation results in activation of MAPK in chondrocytes, which further trigger NF-κB activation, regulating MCP-1 expression[27].

In conclusion, we indicated in this study that ox-LDL enhances MCP-1 expression in cultured HACs. ox-LDL may play a significant role in progression of cartilage degeneration in osteoarthritis.

Conflict of interest

The authors declare they have no conflict of interest in connection with this paper.

Acknowledgments

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Plasma Tetrahydrobiopterin/Dihydrobiopterin Ratio

— A Possible Marker of Endothelial Dysfunction —

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Background: Although endothelium-dependent vasodilatation has been used as a marker of endothelial dysfunction (ED), there have been no reliable plasma markers for ED. Oxidative stress, which is a major determinant of ED, oxidizes tetrahydrobiopterin (BH4), an essential cofactor of endothelial type nitric oxide synthase (eNOS), and resulted in the relative deficiency of BH4.

Methods and Results: In 163 patients with cardiovascular disorders, the plasma levels of BH4 and 7,8–dihydrobiopterin (BH2) by high performance liquid chromatography were measured and compared with the flow-mediated (FMD) vasodilatory response of the brachial artery, which was measured by ultrasonography. The effects of atorvastatin on plasma pteridine levels and FMD were examined in patients with multiple coronary risk factors. There was a positive relationship between FMD and plasma BH4 levels and a negative relationship between FMD and plasma BH2 levels. Subsequently, a strong positive relationship between FMD and the BH4/BH2 ratio ($r=0.585$, $P<0.0001$) was found. Although we did not find any significant relationship between pteridine levels and individual traditional risk factors, the BH4/BH2 ratio in patients with more than 2 risk factors showed significant reductions compared with that in those without risk factors. Statin treatment improved FMD in association with an increase in the plasma BH4/BH2 ratio.

Conclusions: Plasma pteridine levels were associated with endothelial dysfunction in cardiovascular disorders. (*Circ J* 2009; **73:** 955–962)

Key Words: Endothelial dysfunction; FMD; Plasma pteridine level; Statin

Endothelial dysfunction (ED) plays a critical role in the initiation and progression of atherosclerosis[1,2] and is associated with risk factors for coronary artery diseases, including smoking, hypertension, hyperlipidemia, diabetes mellitus and obesity.[3–7] Furthermore, endothelial function was demonstrated to serve as a predictor of cardiovascular events.[8,9] Therefore, the evaluation of endothelial function is important to determine the therapeutic strategy for atherosclerotic diseases.

Clinically, endothelial function has mostly been evaluated by the extent of endothelium-dependent relaxation (EDR), which is almost exclusively mediated by nitric oxide (NO). Particularly, flow-mediated vasodilatation (FMD) induced by reactive hyperemia following the release of a forearm-occluding cuff is an established method for assessing endothelial function.[10] By using this technique, the relationships between coronary risk factors and the ED have been assessed in many clinical studies, and the close linkage has been reported between endothelial function of the brachial artery and that of the coronary arteries.[11] Now it is well recognized that the extent of ED depends on the burden of coronary risk factors.[12]

There have been, however, no reliable plasma markers found for ED in humans. When EDR is used as a standard representing endothelial function, only limited numbers of clinical studies have shown a correlation between a given plasma marker and EDR.[13,14] Oxidative stress has been shown as an important factor leading to ED. Oxidative stress oxidizes tetrahydrobiopterin (BH4), an essential cofactor for endothelial type NO synthase (eNOS), to its oxidative form 7, 8–dihydrobiopterin (BH2) in vascular tissue, particularly in the endothelium.[15,16] The resultant relative deficiency of BH4 causes the uncoupling of the L-arginine-NO pathway (uncoupling of eNOS), which is at least partly involved in the ED in various vascular disorders.[16,17]

In certain pathological conditions such as renal failure, changes in plasma BH4 and BH2 have been reported.[18] As oxidative stress is the major factor damaging endothelial

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function and BH4 is a molecular target of reactive oxygen species (ROS)[15] we hypothesized that plasma levels of pteridine such as BH4 and BH2 might reflect endothelial function. In the present study, we measured plasma pteridine levels in patients with cardiovascular disorders, and examined the relationship between FMD in the brachial artery and plasma pteridine levels. We then examined the effects of HMG-CoA reductase inhibitors (statin) on plasma pteridine levels, which were demonstrated to improve the impaired EDR.

Methods

Subjects

From patients admitted to Kobe University Hospital from March 2006 to April 2008, 163 patients with cardiovascular disorders from which informed consent was obtained were used in the present study. Patients with acute coronary syndrome, those with heart failure, and those with an infectious disease were excluded from the study. In addition, patients with a percentage left ventricular ejection fraction of <40% were not included in the study. A clinical history and physical examination (blood pressure (BP), height, body weight, body mass index (BMI), waist circumference) were undertaken prior to the study. The left ventricular function was assessed by either transthoracic echocardiography or left ventricular angiography. Cardiovascular risk factors and medications were fully documented. An additional 46 patients with more than 2 coronary risk factors were used to examine the effects of treatment with atorvastatin (10 mg/day) for 3 months on plasma pteridine levels as well as the brachial endothelial function. Those 46 patients were randomly divided into the statin treatment group and the control group. Among the 23 patients enrolled in the control group (who did not receive statin treatment), 2 were dropped from the study protocol and 21 were re-examined 3 months later.

Coronary risk factors examined in the present study patients included hypertension, hypercholesterolemia, diabetes mellitus and cigarette smoking. Hypertension was defined as a systolic and/or diastolic pressure of ≥140 and/or 90 mmHg, respectively, or if the patient was being treated with anti-hypertensive drugs. Hypercholesterolemia was defined as serum cholesterol levels of ≥220 mg/dl or if the patient was under treatment. Diabetes mellitus was diagnosed by the presence of fasting plasma glucose (FPG) (≥126 mg/dl) or if the patient was being treated for diabetes mellitus. The Brinkman Index was obtained by taking the number of cigarettes smoked/day times the number of years smoking occurred.

All patients provided written informed consent, and the study was approved by the Institutional Review Board of the Kobe University School of Medicine.

Study Design

All medications including vasoactive drugs and statins, and smoking were stopped at least 12 h prior to the measurement of vasodilatory response. Blood samples were taken in the morning before the measurement of the vasodilatory response. In an additional group, re-examinations of both the brachial vasodilatory response and the blood sample measurements were conducted 3 months after the first examinations. To assess endothelial function in the brachial artery, FMD was measured by high-resolution ultrasonography in the early morning after overnight fasting[19] The diameter of the brachial artery was measured from B-mode ultrasound images using a 7.5-MHz linear array transducer (SSH-140A; Toshiba Medical Co, Tokyo, Japan). The brachial artery was scanned over a longitudinal section 3–5 cm above the antecubital fossa. When a satisfactory transducer position was found, the surface of the skin was marked, and the arm remained in the same position throughout the study. After baseline measurement of the diameter and the flow velocity in the brachial artery was conducted, a BP cuff placed around the forearm was inflated with a pressure of 220–250 mmHg, and released 5 min later. Fifteen minutes later, the second resting scan was recorded. A sublingual nitroglycerin spray (0.3 mg) was then administered, and the brachial artery was imaged 4 min later. Measurements were taken from the anterior to the posterior interface between the media and adventitia ("m" line) at the end diastole. The diameters at 4 cardiac cycles were analyzed for each scan, and measurements for the reactive hyperemia were taken 45–60 s after the cuff deflation to measure the peak diameter. Responses of the vessel diameter to the reactive hyperemia and nitroglycerin were expressed as the percentage increase to the baseline value of the diameter, and defined as FMD (%) and nitroglycerin-mediated vasodilation (NMD) (%), respectively. All scans were analyzed by the same experienced observers, who were blinded to the identity and the clinical and biochemical data of the subjects. As to the reproducibility of this ultrasound examination, the coefficient of variation for repeated measurements of FMD was 5.60±1.39% and the interobserver correlation coefficient of FMD was 0.985.

Biochemical Analysis

Blood for biochemical analysis was obtained from fasting venous samples. For measurements of plasma levels of pteridines (BH4 and BH2), we minimized the oxidation during the assay by adding 1,4-Dithioerythritol in blood sampling tubes, which has been revealed to inhibit oxidation of BH4 very effectively[20] Plasma levels of BH4 and BH2 were measured directly by high performance liquid chromatography (HPLC) with the electrochemical detection method, as previously described[21,22]

Total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), FPG and insulin, hemoglobin (Hb) A_{1c}, and high-sensitive C-reactive protein (CRP) were measured by using a standard assay. Insulin resistance was evaluated by a homeostasis model assessment of insulin resistance (HOMA-IR). The HOMA-IR value was calculated by using a formula: IR=fasting serum insulin (μU/ml)×FPG (mgl/dl)/405.

Statistical Analysis

Statistical analysis was conducted with a commercially available software package (Stat view ver. 5.0; SAS Institute Inc, Cary, NC, USA). The Spearman correlation coefficient analysis was used to assess associations between FMD and measured parameters. The significance of the difference between group means was analyzed by one-way ANOVA followed by a post-hoc test (PRISM 4.0, GraphPad). After 3 months, the differences between the statin group and the control group were tested by non-parametric analysis (Mann–Whitney U-test). We used a Student's paired t- or Wilcoxon-signed rank test to compare the value before and after each treatment, and the effects of statins were analyzed by using one-way ANOVA with a post-hoc test. The relationships between FMD and the risk factors, and each medi-

Table 1. Baseline Characteristics of Patients

No. of patients	163
Characteristics	
Age (years)	66.2±11.2
Gender (M/F)	91/72
Brinkman index	395.6±550.8
BMI (kg/m^2)	23.7±3.6
Waist circumference (cm)	85.1±10.8
SBP (mmHg)	125.2±20.3
DBP (mmHg)	66.8±11.3
Blood chemistry	
TC (mg/dl)	200.0±43.6
TG (mg/dl)	138.3±68.4
HDL-C (mg/dl)	53.7±18.1
LDL-C (mg/dl)	122.7±34.1
HbA$_{1c}$ (%)	5.9±1.1
FPG (mg/dl)	106.1±27.5
Underlining diseases (%)	
Coronary artery disease	65.6
Arrhythmia	16.6
Valvular disease	6.1
Hypertension	11.7
Medications (%)	
β-blockers	37.4
Ca-blockers	35.0
ACEI	16.6
ARB	43.6
Nitrates	27.0
Oral antidiabetics and insulin	30.7
Statins	33.7
Aspirins	51.5
Diuretics	27.0
Risk factors (%)	
Diabetes mellitus	38.7
Hypertension	68.7
Hyperlipidemia	69.9
Smoking	46.6

Data are mean±SEM.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HbA$_{1c}$, hemoglobin A$_{1c}$; FPG, fasting plasma glucose; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin type II receptor blocker.

cation, were assessed using multiple stepwise regression analysis. The effects of statin treatment on the ΔFMD and ΔBH4/BH2 ratio were also assessed by multiple stepwise regression analysis. Results were expressed as the mean± SEM and a P value of <0.05 was considered significant.

Results

The clinical characteristics of the study population are summarized in **Table 1**. Approximately 66% of patients were those with coronary artery disease. Nineteen patients were those with hypertension, 27 with arrhythmia, and 10 with valvular disease having preserved ventricular function. When patients were sub-grouped according to the number of coronary risk factors, most of the patients with coronary artery disease had more than 2 risk factors, whereas only a limited numbers of patients without coronary artery disease had more than 2 risk factors.

We then assessed the relationship between FMD and plasma pteridine levels. There was a positive relationship between FMD and plasma BH4 levels (**Figure 1A**, P< 0.0001, r=0.319) and a negative relationship between FMD and plasma BH2 levels (**Figure 1B**, P<0.0001, r=–0.439). Subsequently, we demonstrated a strong positive relationship between FMD and the BH4/BH2 ratio (**Figure 1C**, P< 0.0001, r=0.585). There were no correlations between NMD and plasma pteridine levels (data not shown). **Table 2** shows the basal clinical characteristics divided by the numbers of risk factors. When we examined the relationship between the plasma BH4/BH2 ratio and the total numbers of coronary risk factors, the BH4/BH2 ratio in patients with more

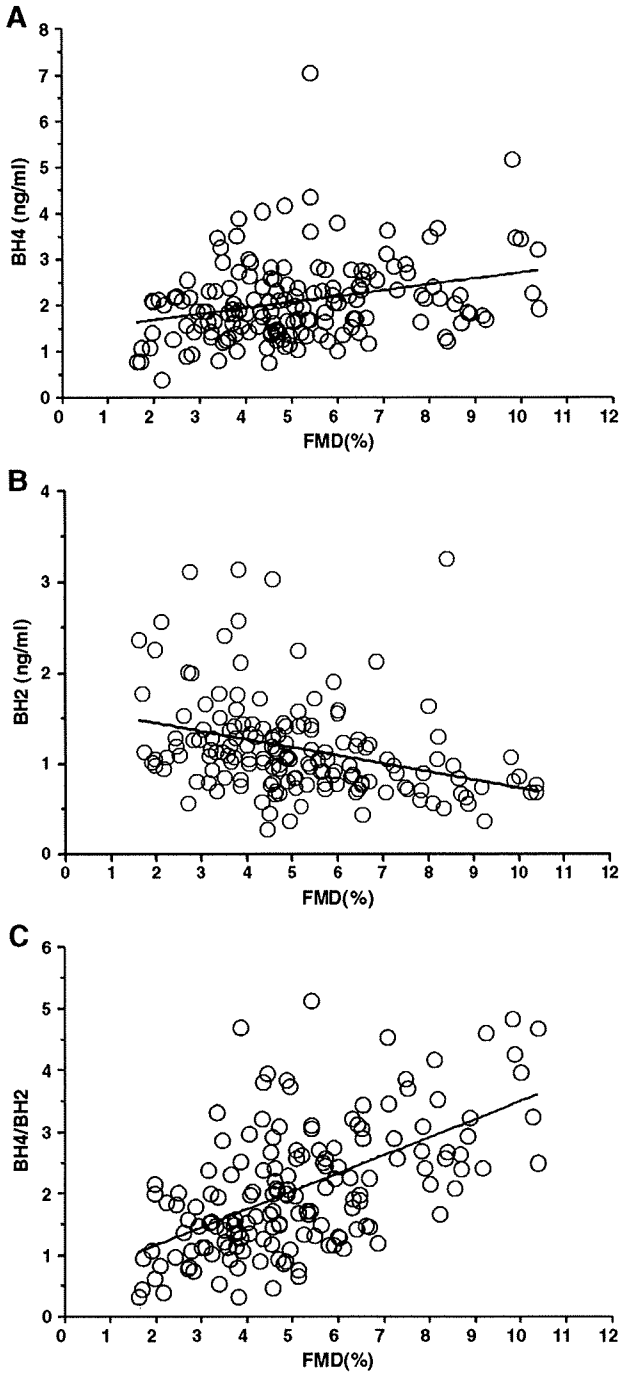

Figure 1. Relationship between flow-mediated vasodilatation (FMD) and plasma tetrahydrobiopterin (BH4) levels (**A**), plasma 7,8-dihydrobiopterin (BH2) levels (**B**), and the plasma BH4/BH2 ratio (**C**), respectively. There was a positive relationship between FMD and plasma BH4 levels (P<0.0001, r=0.319) and a negative relationship between FMD and plasma BH2 levels (P<0.0001, r=–0.439). Subsequently, a strong positive relationship between FMD and the plasma BH4/BH2 ratio was found (P<0.0001, r=0.585).

Table 2. Baseline Characteristics of Patients When Sub-Grouped by Numbers of Coronary Risk Factors

Coronary risk factors	Risk 0	Risk 1	Risk 2	Risk 3	Risk 4
No. of patients	11	25	59	50	18
Characteristics					
Age (years)	52.4±16.5	68.8±11.6	65.9±11.3	68.6±8.4	65.6±8.0
Gender (M/F)	4/7	10/15	33/26	27/23	17/1
Brinkman index	0	91.0±216.9	251.7± 369.8	538.8 ± 629.4	1,126±521.7
BMI (kg/m^2)	22.8±1.8	22.6±2.4	23.8±3.8	24.0±3.7	24.8±4.2
Waist circumference (cm)	77.8±8.4	81.6±9.1	84.3±10.8	87.3±10.6	89.9±11.2
SBP (mmHg)	114.9±9.0	125.4±15.3	122.2±23.5	127.1±21.1	135.8±13.4
DBP (mmHg)	65.5±6.5	68.3±9.8	67.0±15.1	65.8±9.4	67.4±6.3
Blood chemistry					
TC (mg/dl)	178.3± 20.0	198.8±46.2	206.2±42.7	195.2±44.4	205.3±46.9
TG (mg/dl)	80.8±19.3	110.3±42.6	150.9±81.9	134.4±60.3	173.7±57.2
HDL-C (mg/dl)	67.3±18.1	58.0±14.2	54.8±21.7	51.7±15.6	44.2±11.3
LDL-C (mg/dl)	97.6±17.5	120.1±36.6	128.0±32.5	118.8±32.7	132.5±40.0
HbA1c (%)	5.1±0.2	5.3±0.3	5.5±1.0	6.3±1.1	6.9±0.8
FPG (mg/dl)	86.9±9.3	93.0±7.6	101.4±20.2	114.7±32.2	120.7±37.6
IRI (μU/ml)	4.5±1.2	4.4±1.6	8.4±8.2	10.7±11.7	8.2±5.1
HOMA-IR	0.98±0.34	0.98±0.38	2.06±2.11	2.94±3.17	2.37±1.59
hs-CRP (mg/dl)	0.66±0.73	0.46±1.09	0.55±0.95	0.64±1.39	0.38±0.78
Left ventricular function					
LVEF (%)	62.2±8.1	60.0±9.3	52.4±12.2	56.1±9.3	51.5±6.4
Medication (%)					
β-blockers	9.1	32.0	37.3	38.0	61.1
Ca-blockers	9.1	28.0	39.0	46.0	16.7
ACEI	0	12.0	18.6	14.0	33.3
ARB	0	32.0	44.1	56.0	50
Nitrates	9.1	12.0	25.4	36.0	38.9
Statins	0	8.0	32.2	48.0	55.6
Oral antidiabetics and insulin	0	0	11.9	54.0	88.9
Aspirins	18.2	48.0	47.5	52.0	88.9
Diuretics	18.2	8.0	30.5	34.0	27.8
Underlining diseases (%)					
Coronary artery disease	27.3	40.0	61.0	84.0	94.4
Arrhythmia	63.6	32.0	11.9	8.0	5.6
Valvular disease	9.1	4.0	13.6	0	0
Hypertension	0	24.0	13.6	8.0	0
Risk factors (%)					
Hypertension	0	52.0	64.4	86.0	100
Diabetes mellitus	0	0	18.6	68.0	100
Hyperlipidemia	0	28.0	80.0	84.0	100
Smoking	0	20.0	37.3	68.0	100

Data are mean±SEM.
IRI, immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high-sensitive C-reactive protein; LVEF, left ventricular ejection fraction. Other abbreviations see in Table 1.

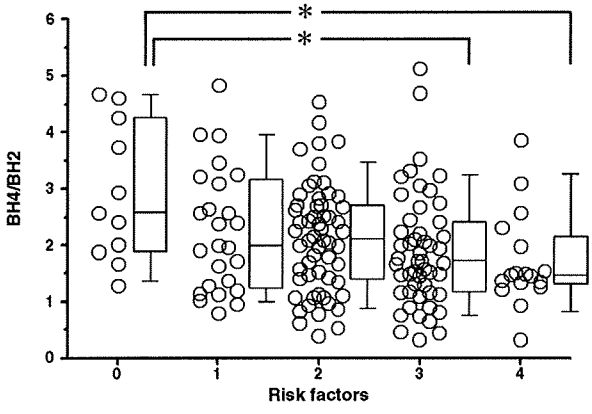

Figure 2. Relationship between the plasma tetrahydrobiopterin/7,8-dihydrobiopterin (BH4/BH2) ratio and the total number of coronary risk factors. A box-and-whisker plot shows the relationship between the plasma BH4/BH2 ratio and the total number of coronary risk factors. For each value of the coronary risk factors, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. *P<0.05.

than 2 risk factors showed significant reductions compared with that in those without risk factors (P<0.05) (**Figure 2**). As to the presence of each single risk factor examined, age, sex, and the Brinkman Index, which represents the severity of a smoking habit, showed a weak but significant negative correlation with the plasma BH4/BH2 ratio (β=–0.259, P=0.0009, β=–0.207 P=0.0084, and β=–0.251, P=0.0014, respectively). In the stepwise regression model, age and sex showed a significant negative correlation with the plasma BH4/BH2 ratio. There was no correlation between the Brinkman Index and the BH4/BH2 ratio. In addition, there were no correlations between the Log CRP levels and the BH4/BH2 ratio in the simple and multiple stepwise regression analyses. We then examined whether the BH4/BH2 ratio was associated with FMD, independently of risk factors. Multivariate analyses using stepwise regression models were carried out to analyze the relationships between FMD and risk factors including age, sex, abdominal circumference, BMI, Brinkman index, BP, HbA1c, FPG, HOMA-IR, TC, LDL-C, HDL-C, Log CRP and/or the BH4/BH2 ratio and medications such as calcium channel blockers, angiotensin converting-enzyme inhibitor (ACEI), angiotensin II receptor blocker (ARB) and statins. As demonstrated in

Table 3. Multiple Stepwise Regression Analysis of Variables Significantly Associated With FMD

	β	SE	F	P value
Model 1				
Age	−0.306	0.020	10.539	<0.001
Sex	−0.303	0.351	10.670	<0.001
Log CRP	−0.235	0.259	6.538	<0.001
HbA1c	−0.189	0.161	4.092	<0.001
		Multiple R^2=0.254		
Model 2				
BH4/BH2 ratio	0.438	0.157	24.892	<0.001
Age	−0.270	0.018	9.597	<0.001
Sex	−0.220	0.330	6.126	<0.001
		Multiple R^2=0.357		

Model-1 includes age, sex, abdominal circumference, BMI, Brinkman index, BP, HbA1c, FPG, HOMA-IR, TC, LDL-C, HDL-C, LogCRP and each medication such as Ca-blocker, ACEI, ARB and statin as independent variables.
Model 2 includes age, sex, abdominal circumference, BMI, Brinkman index, BP, HbA1c, FPG, HOMA-IR, TC, LDL-C, HDL-C, LogCRP, each medication such as Ca-blocker, ACEI, ARB, statin and BH4/BH2 as independent variables.
FMD, flow-mediated vasodilatory; β, standardized regression coefficient; SE, standard error; R, multiple correlation coefficient; BH4, tetrahydrobiopterin; BH2, 7,8–dihydrobiopterin; BP, blood pressure. Other abbreviations see in Tables 1,2.

Table 4. Multiple Regression Analysis of the BH4/BH2 Ratio and Numbers of Risk Factors Significantly Associated With FMD

	β	SE	P value
BH4/BH2	0.558	0.131	<0.001
Risk factor numbers	−0.147	0.128	0.0259

Abbreviations see in Table 3.

Table 5. Comparison of Parameters at the Basal Levels Before Treatment in Stain-Treated and Non-Stain Treated Groups

	Statin-treated	Non-statin treated	P value
No. of patients	23	21	
Characteristics			
Age (years)	67.1±6.9	64.3±11.3	NS
Gender (M/F)	15/8	12/9	
SBP (mmHg)	127.4±16.3	120.6±18.0	NS
DBP (mmHg)	67.7±9.8	69.1±9.5	NS
Blood chemistry			
TC (mg/dl)	229.4±30.5	226.6±42.5	NS
TG (mg/dl)	146.9±67.9	158.4±101.5	NS
HDL-C (mg/dl)	53.2±20.4	56.0±14.6	NS
LDL-C(mg/dl)	153.4±26.2	142.2±28.8	NS
HbA1c (%)	5.9±1.3	5.9±1.3	NS
FPG (mg/dl)	107.8±31.2	109.5±34.0	NS
BH4 (ng/ml)	2.03±0.93	1.75±0.53	NS
BH2 (ng/ml)	1.15±0.39	1.05±0.39	NS
BH4/BH2	1.96±1.06	1.96±0.94	NS
Ultrasonography			
FMD (%)	4.71±1.80	4.82±2.18	NS
NMD (%)	16.3±4.2	16.4±2.6	NS
Underlining diseases (%)			
Coronary artery disease	87.0	52.4	
Arrhythmia	8.7	14.3	
Valvular disease	0	9.5	
Hypertension	4.3	23.8	
Risk factors (%)			
Hypertension	65.2	47.6	
Diabetes mellitus	30.4	38.1	
Hyperlipidemia	87.0	71.4	
Smoking	39.1	47.6	

Data are mean±SEM.
NMD, nitroglycerin-mediated vasodilation. Other abbreviations see in Tables 1,3.

Table 3, the BH4/BH2 ratio was associated with FMD, independently of these risk factors. Furthermore, after adjusting the number of risk factors, the BH4/BH2 ratio was also associated with FMD independently (**Table 4**).

Table 5 shows the basal clinical characteristics of patients assigned to the atorvastatin protocol. Before treatment, there were no differences in blood chemistry including plasma pteridine levels and FMD value between the statin-treated and control groups. Stain treatment for 3 months did not change these parameters, the only exception being the

Table 6. Multiple Stepwise Regression Analysis Between the Change of FMD (ΔFMD) and the Changes of Parameters

	β	SE	F	P value
ΔBH4/BH2	0.455	0.363 Multiple R^2=0.207	5.468	<0.001

Table 6 includes ΔBP, ΔHbA1c, ΔFPG, ΔTC, ΔTG, ΔLDL-C, ΔHDL-C and ΔBH4/BH2 as independent variables. Abbreviations see in Tables 1–3.

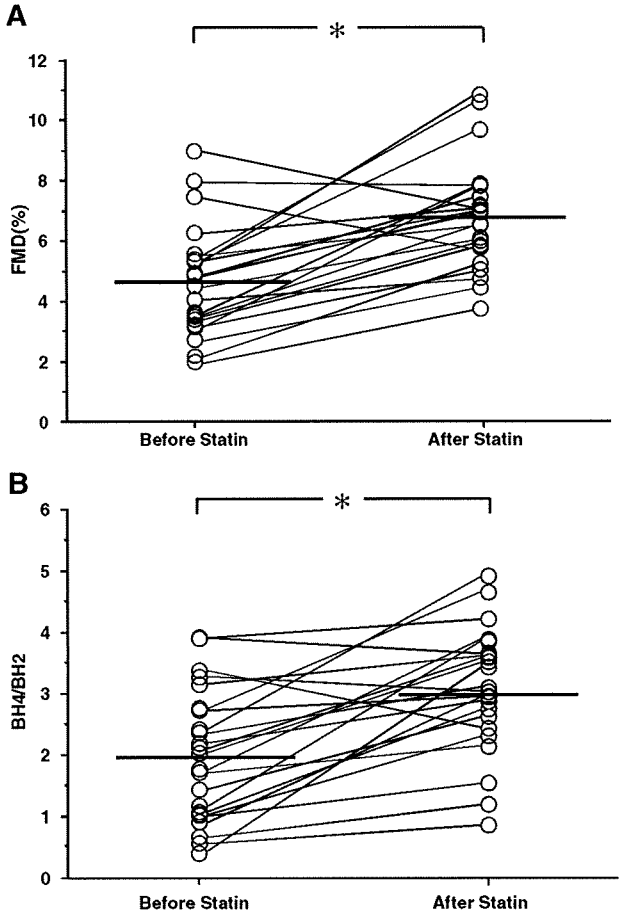

Figure 3. Effects of statin treatment on flow-mediated vasodilatation (FMD, **A**) and the plasma tetrahydrobiopterin/7,8–dihydrobiopterin (BH4/BH2) ratio (**B**). Statin treatment improved FMD. Along with these changes, the plasma BH4/BH2 ratio increased in most patients, with the exception of a few patients in whom improvement of FMD was minimal. The bars show mean values. *P<0.05.

plasma lipid profiles. TC decreased from 229.4±30.5 to 187.3±24.3 (mg/dl), and LDL-C from 153.4±26.2 to 109.4± 26.5 (mg/dl) by atorvastatin treatment. In the control group, there were no significant changes in parameters (as shown in **Table 5**) during the 3-month observation period. And as for FMD, statin treatment improved FMD (6.81±1.80%, P<0.05) (**Figure 3A**). Along with these changes, the plasma BH4/BH2 ratio increased in most patients, with the exception of a few patients in whom improvement of FMD was minimal (2.99±1.01, P<0.05) (**Figure 3B**). In contrast, in the control group who did not receive statin treatment, both FMD and the plasma BH4/BH2 ratio remained unchanged during the 3 months (4.71±1.59% and 1.87±0.78, respectively). We then assessed the relationship between ΔFMD and the ΔBH4/BH2 ratio by using multiple stepwise regression analysis including ΔBP, ΔHbA1c, ΔFPG, ΔTC, ΔTG, ΔLDL-C, ΔHDL-C and ΔBH4/BH2 as independent variables. As shown in **Table 6**, we found a significant correlation between the ΔFMD and the ΔBH4/BH2.

Discussion

In the present study, we examined plasma pteridine levels and endothelial function via the brachial artery FMD in patients with various cardiovascular disorders including coronary artery disease. We demonstrated that the extent of FMD was correlated with plasma pteridine levels. In association with a reduction in FMD, plasma BH4 levels decreased, BH2 levels increased and subsequently the BH4/BH2 ratio decreased. Particularly, the plasma BH4/BH2 ratio showed a strong positive correlation with FMD. The plasma BH4/BH2 ratio was negatively correlated with the total numbers of coronary risk factors. In addition, we showed that, in patients with multiple coronary risk factors, treatment with statins improved FMD in association with an increase in plasma BH4/BH2 ratio.

Among various factors produced by endothelial cells, NO produced by eNOS is most important in the control of endothelial function, and EDR is caused by eNOS-derived NO. NO is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of a NADPH-dependent NO synthase, which requires Ca^{2+}/calmodulin, FAD, FMN, and BH4 as cofactors. Although recently a catheter-type NO sensor has been extensively experimented with to measure NO synthase directly in animal models, it is difficult to apply in human studies[23,24] EDR has been used to assess the endothelial function, and clinically is most commonly evaluated by FMD in the brachial artery. Many experimental and human studies have demonstrated that coronary risk factors are associated with impaired endothelial function, which represents the reduced eNOS-derived NO bioactivity in vessels.

Among various mechanisms responsible for the impaired EDR, the increased breakdown of NO by superoxide is important. In vessels, a variety of enzymes, including NADPH oxidase and xanthine oxidase, produce superoxide. Recently, it has been revealed that eNOS itself produces superoxide when there is a relative deficiency of its essential cofactor, BH4?[21,25] In the presence of sub-optimal levels of BH4, electrons flowing from the reductase domain to the hem is diverted to molecular oxygen rather than to L-arginine, and thereby production of superoxide occurs?[26] BH4 is a molecular target for oxidative stress, and ROS such as peroxynitrite oxidizes BH4 to its oxidative form, BH2![15,27,28] Under conditions with elevated oxidative stress, it is assumed that BH4 levels decrease and its oxidative form, BH2, increases in the endothelium?[28] It is well known that the presence of atherosclerosis as well as coronary risk factors is associated with elevated oxidative stress. In the present study, therefore, oxidative stress is closely linked to impaired endothelial function, although the precise role of eNOS-derived superoxide was not elucidated.

Before this present study, a few clinical studies examined plasma levels of pteridine in patients with cardiovascular disorders![18,29,30] In patients with chronic renal failure, it was shown that the plasma BH4/BH2 ratio was reduced in association with the severity of renal failure and that there was a positive correlation between the BH4/BH2 ratio and creatinine clearance![18] Shinozaki et al showed that the plasma BH4/BH2 ratio was reduced in patients with insulin resistance?[29] They also demonstrated that the maximum coronary dilation induced by acetylcholine was positively correlated with insulin sensitivity and the plasma BH4/BH2 ratio. In the present study, we demonstrated that the plasma BH4/BH2 ratio was positively correlated with FMD in the brachial artery in patients with various cardiovascular disorders. Our finding is partly in accordance with the study of Shinozaki et al[29] and extended the importance of plasma pteridine measurements on the evaluation of ED. The present finding most likely indicates that plasma pteridine levels, particularly the BH4/BH2 ratio, can be used as a biomarker to evaluate ED.

Plasma BH2 levels increased, whereas BH4 levels did not change, in association with the increment of total numbers of coronary risk factors. Subsequently, the BH4/BH2 ratio significantly decreased as the numbers of coronary risk factors increased. It is very likely that the increased oxidative stress in the presence of coronary risk factors resulted in the oxidation of BH4 and an increase in BH2. Reduction of the plasma BH4/BH2 ratio might, therefore, represent the increased levels of oxidative stress. Although it is widely accepted that oxidative stress serves to impair endothelial function, only a limited numbers of studies have demonstrated the correlation between the plasma markers of oxidative stress, such as TBARS and oxLDL, and the extent of FMD in clinical studies?[31,32] In the preliminary study, we did not find correlations between plasma TBARS or 8-iso PGF2α levels and the plasma BH4/BH2 ratio or FMD (%) (data not shown). Although the source of plasma pteridine has not been clarified yet, it is assumed that plasma levels of pteridine reflect endothelial pteridine metabolism because the endothelium is directly in contact with blood and is the major site for formation of peroxynitrite that oxidizes BH4. Therefore, it is possible that the plasma BH4/BH2 ratio can serve as a more sensitive and specific marker of oxidative stress on the endothelium than commonly used plasma markers such as TBARS and 8-iso PGF2 , which are markers of total oxidative stress in a whole body. To prove this hypothesis, further studies will be required to investigate this.

Our finding is in contrast to that by Antoniades et al, who found no association between plasma pteridine levels and endothelial function in isolated vessels (saphenous veins or internal mammalian arteries) obtained from patients at the time of bypass surgery?[30] They also described that plasma pteridine levels are linked to systemic inflammation, because they found the association between plasma total pteridine levels and plasma CRP levels. The divergent results might be explained by the difference in patients' profiles. In our study, which is different from their study, most patients were not the subjects for coronary intervention, and CRP levels were relatively low. In their study, there were wide variations in plasma total pteridine, BH4 and BH2 levels, which are likely influenced by systemic inflammation rather than local vascular redox status. In our study, however, we did not find such a variation in plasma BH4 or BH2 levels, although our HPLC methods does not permit us to measure total pteridine. Therefore, it is likely that the plasma BH4/BH2 ratio can be used as a marker of endothelial function in the absence of overt systemic inflammation.

As a limitation of the present study, most patients recruited were taking various medications that could modify endothelial function and oxidative stress. Although all medications were stopped at least 12h prior to the study, their influence on the endothelial function and plasma pteridine metabolism probably did not disappear completely. In addition, we did not find a correlation between the Brinkman index and FMD. This might be related to the examinations performed during hospital admission when patients were not smoking cigarettes. However, our present study findings represented the overall relationship between FMD and plasma pteridine levels, and demonstrated the usefulness of plasma pteridine measurement to assess ED in the clinical situation, where many subjects were most likely taking medication.

In conclusion, the present study suggested that plasma pteridine levels, particularly the BH4/BH2 ratio, were associated with ED in the absence of recognizable systemic inflammation. The present study findings were obtained using a relatively small number of patients with heterogeneity, and, therefore, further studies conducted with a larger population will be needed to ascertain whether BH4/BH2 can be used as a marker of ED, and to address the issue of whether the measurement of the BH4/BH2 ratio serves as a useful tool in determining a therapeutic strategy for vascular disorders based on the improvement of endothelial function.

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Disclosure

We have no conflicts of interests.

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Antiplatelet Therapy and Stent Thrombosis After Sirolimus-Eluting Stent Implantation

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Background—The influences of antiplatelet therapy discontinuation on the risk of stent thrombosis and long-term clinical outcomes after drug-eluting stent implantation have not yet been addressed adequately.

Methods and Results—In an observational study in Japan, 2-year outcomes were assessed in 10 778 patients undergoing sirolimus-eluting stent implantation. Data on status of antiplatelet therapy during follow-up were collected prospectively. Incidences of definite stent thrombosis were 0.34% at 30 days, 0.54% at 1 year, and 0.77% at 2 years. Thienopyridine use was maintained in 97%, 62%, and 50% of patients at 30 days, 1 year, and 2 years, respectively. Patients who discontinued both thienopyridine and aspirin had a significantly higher rate of stent thrombosis than those who continued both in the intervals of 31 to 180 days, 181 to 365 days, and 366 to 548 days after stent implantation (1.76% versus 0.1%, $P<0.001$; 0.72% versus 0.07%, $P=0.02$; and 2.1% versus 0.14%, $P=0.004$, respectively). When discontinuation of aspirin was taken into account, patients who discontinued thienopyridine only did not have an excess of stent thrombosis in any of the time intervals studied. Adjusted rates of death or myocardial infarction at 24 months were 4.1% for patients taking thienopyridine and 4.1% for patients not taking thienopyridine ($P=0.99$) in the 6-month landmark analysis.

Conclusions—Discontinuation of both thienopyridine and aspirin, but not discontinuation of thienopyridine therapy only, was associated with an increased risk of stent thrombosis. Landmark analysis did not suggest an apparent clinical benefit of thienopyridine use beyond 6 months after sirolimus-eluting stent implantation. (***Circulation.*** **2009;119:987-995.)**

Key Words: aspirin ■ follow-up studies ■ stents ■ coronary disease ■ thrombosis

Concerns have been raised about the safety of drug-eluting stents (DES), and certain issues remain unresolved.[1,2] First, although premature discontinuation of antiplatelet therapy is reported to be the most powerful predictor of stent thrombosis (ST) and adverse cardiovascular outcomes,[3–5] the relative contribution of discontinuation of either aspirin or thienopyridine on ST rates has not been addressed adequately. Furthermore, the optimal duration of dual-antiplatelet therapy has not been well established, although dual-antiplatelet therapy beyond 1 year has become commonplace in clinical practice. To address these issues, a large-scale, multicenter registry of patients undergoing sirolimus-eluting stent (SES) implantation was designed with prospective data collection on the status of antiplatelet therapy during follow-up.

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Methods

Study Population

The j-Cypher registry is a physician-initiated prospective, multicenter observational study in Japan enrolling consecutive patients undergoing SES implantation. The relevant review boards in all 37

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Table 1. Baseline Characteristics

Patient Characteristics	
No. of patients	10 778
Age, y	68.3±10.2
Age ≥80 y, n (%)	1362 (13)
Male, n (%)	8123 (75)
Body mass index	23.9±3.4
Hypertension, n (%)	8069 (75)
Diabetes mellitus, n (%)	4400 (41)
Taking insulin	996 (9.2)
Current smoking, n (%)	2119 (20)
eGFR <30 mL · min^{-1} · 1.73 m^{-2}, n (%)	
Without hemodialysis	522 (5.1)
With hemodialysis	594 (5.5)
ACS, n (%)	2308 (21)
STEMI	733 (6.8)
Non-STEMI	220 (2.0)
Unstable angina	1355 (13)
Prior MI, n (%)	3024 (28)
Prior stroke, n (%)	1007 (9.3)
Peripheral vascular disease, n (%)	1276 (12)
Prior heart failure, n (%)	1460 (14)
Prior PCI, n (%)	5179 (48)
Prior CABG, n (%)	787 (7.3)
Multivessel disease, n (%)	5392 (50)
Target of unprotected LMCA, n (%)	419 (3.9)
Ejection fraction, %	58.1±13.4
No. of vessels treated	1.22±0.47
Multivessel stenting, n (%)	2089 (19)
No. of lesions treated	1.37±0.66
Total No. of stents	1.75±1.04
Total length of stents, mm	38.9±25.6
Lesion and procedural characteristics	
No. of lesions	14 811
Lesion location, n (%)	
LAD	6138 (42)
LCx	3130 (21)
RCA	4913 (33)
LMCA	499 (3.4)
Saphenous vein graft	109 (0.7)
In-stent restenosis, n (%)	1895 (13)
Chronic total occlusion, n (%)	1348 (9.1)
Severe calcification, n (%)	1311 (8.9)
Bifurcation lesion, n (%)	2857 (19)
Side-branch stenting, n (%)	479 (3.2)
Lesion length ≥30 mm, n (%)	2146 (15)
Preprocedural reference diameter <2.5 mm, n (%)	4196 (29)
Use of intravascular ultrasound, n (%)	6681 (45)
Direct stenting, n (%)	3416 (23)
After dilation, n (%)	6491 (44)
Maximum inflation pressure, atm	17.9±3.3
No. of stents used	1.29±0.57
Length of stents used, mm	28.6±15.3
Minimal stent size, mm	2.89±0.37

eGFR indicates estimated glomerular filtration rate; STEMI, ST-elevation MI; non-STEMI, non–ST-elevation MI; LMCA, left main coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; and RCA, right coronary artery.

Continuous variables are expressed as mean±SD.

Table 2. Clinical Event Rates Through 2 Years

	Cumulative Event Rate, %		
	30 d	1 y	2 y
Death	0.7	3.7	7.2
Cardiac death	0.6	2.2	3.7
Sudden death	0.02	0.7	1.4
MI	0.3	0.9	1.5
Related to ST	0.2	0.4	0.7
Stroke	0.4	1.8	3.1
ST			
Definite	0.34	0.54	0.77
Definite/probable	0.46	0.68	0.91
Definite/probable/possible	0.46	1.38	2.48
Target-lesion revascularization	0.5	6.9	10.2
CABG surgery	0.1	0.9	1.5
Any coronary revascularization	2.5	19.2	25.9

participating centers (online-only Data Supplement, Appendix I) approved the study protocol. Written informed consent was obtained from all patients.

In an attempt to evaluate penetration of SES and to secure enrollment of truly consecutive patients, all patients undergoing percutaneous coronary intervention (PCI) in each center during the study interval were recorded on the PCI screening list by the technical staff in the catheterization laboratories. When SES implantation was undertaken, each patient was invited to participate in the j-Cypher registry. Although data entry was basically left to the individual sites, the clinical research coordinators (online-only Data Supplement, Appendix II) in the data management center (Kyoto University Hospital, Department of Cardiology) supported data entry when necessary. Obvious inconsistencies were resolved by inquiries to the site investigators and/or by audits against the original data sources. Follow-up data were obtained from hospital charts or by contacting patients and/or referring physicians at 30 days, 6 months, and 1 year and yearly thereafter. When death, myocardial infarction (MI), and ST were reported, the events were adjudicated with use of the original source documents by a clinical events committee (online-only Data Supplement, Appendix II).

Between August 2004 and November 2006, 15 155 patients were enrolled in the registry from among 29 555 consecutive patients recorded on the PCI screening list. SES penetration varied markedly across centers, with a median penetration rate of 53% (range 16% to 92%). After the exclusion of 2331 patients who were registered repeatedly because of PCI for restenosis or new lesions, 12 824 patients were enrolled in the registry for the first time. Of 19 675 target lesions, 17 050 were treated exclusively with SES. Treatment for the remaining 2625 lesions included bare-metal stent (BMS; 1259 lesions), combination of SES and other stent types (495 lesions), other DES (60 lesions), nonstent PCI (672 lesions), and failed procedure (139 lesions). Ultimately, 10 778 patients (84%) treated with SES exclusively constituted the study population for the present analysis.

Complete 1-year follow-up (median of 491 days; interquartile range 387 to 730 days) was achieved in 96% of patients. At 1-year follow-up, information was collected from the hospital charts (75% of cases) or by contacting patients (25% of cases). Additional information was obtained from the referring physicians in 6% of cases.

Definitions

Coronary angiographic parameters were assessed in each participating center either by visual assessment or by quantitative angio-

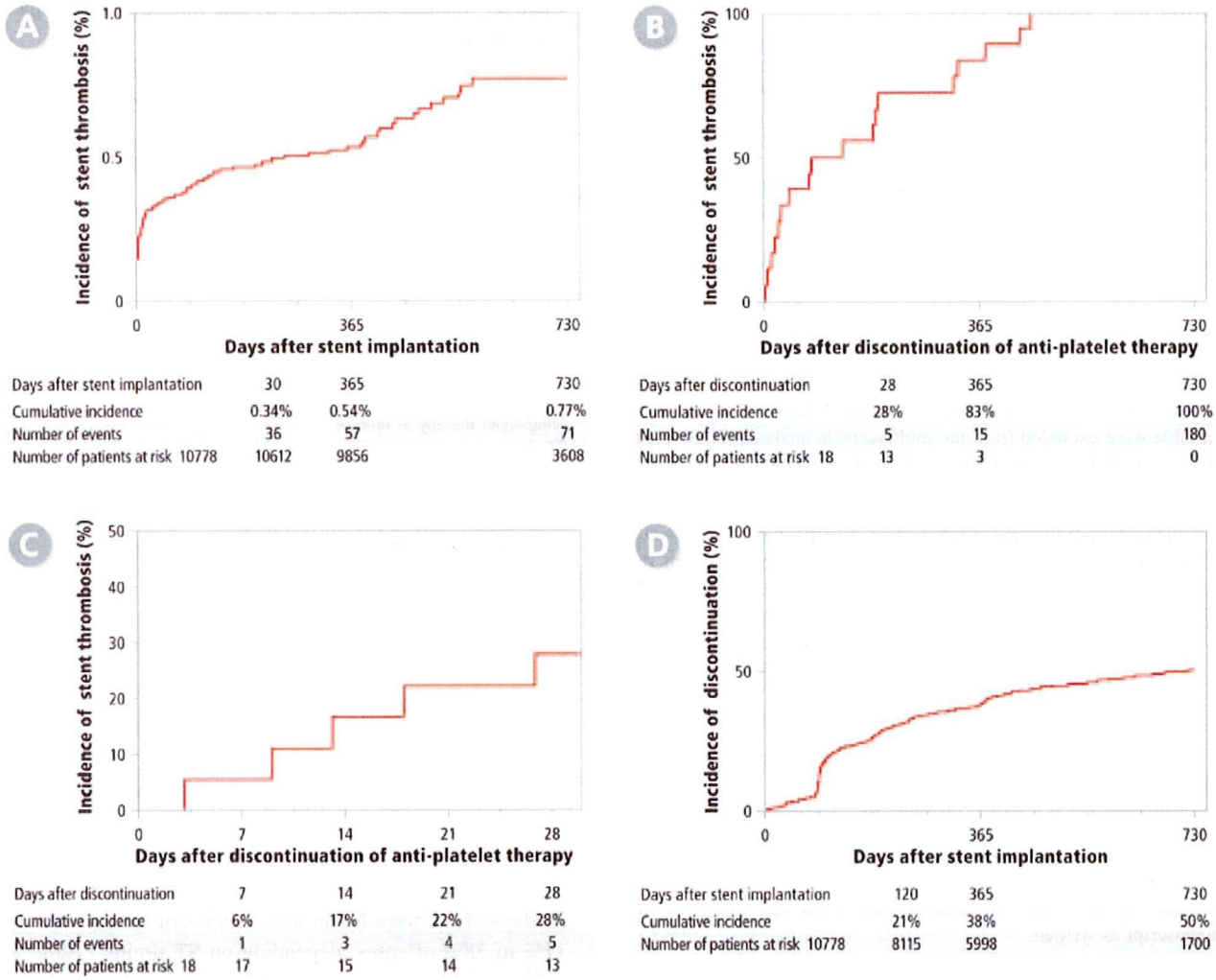


Figure 1. A, Kaplan–Meier curve showing the patient-based cumulative incidence of definite ST. B, Timing of ST after antiplatelet therapy discontinuation. C, Timing of ST within 28 days after antiplatelet therapy discontinuation. D, Kaplan–Meier curve showing the cumulative incidence of persistent thienopyridine discontinuation over time.

graphic measurement. Bifurcation lesion was defined as that involving a side branch of ≥2.2 mm in diameter.

Death was regarded as cardiac in origin unless obvious noncardiac causes could be identified. Any death during the index hospitalization was regarded as cardiac death. Sudden death was defined as unexplained death in previously stable patients. MI was adjudicated according to the definition in the Arterial Revascularization Therapy Study.[6] Within 1 week of the index procedure, only Q-wave MI was adjudicated as MI.

ST was defined according to the Academic Research Consortium definition.[7] Not only sudden death but also those deaths without enough information to exclude sudden death were regarded as possible ST. Unless otherwise noted, definite ST assessed on an individual patient basis was used as the end point for ST, because this was the end point used in a recent large-scale registry for DES.[8]

Antiplatelet Therapy

The recommended antiplatelet regimen was aspirin (≥81 mg daily) indefinitely and thienopyridine (200 mg of ticlopidine or 75 mg of clopidogrel daily) for at least 3 months. Duration of antiplatelet therapy was left to the discretion of each attending physician.

Dates of discontinuation of aspirin and thienopyridine were reported separately on the follow-up forms. When discontinuation was intended to be temporary, the dates the medications were restarted were also reported. When the attending physician intended to discontinue medications permanently, dates related to the restarting of medications after discontinuation were not systematically reported. Persistent discontinuation was defined as withdrawal that lasted at least 2 months.

ST incidences were evaluated according to the status of aspirin therapy and thienopyridine therapy. Analyses were made by time intervals after index PCI (ie, within 30 days, 31 to 180 days, 181 to 365 days, 366 to 548 days, and 549 to 730 days) in accordance with a previous report.[4] Those patients in whom occurrence of ST could be evaluated throughout the given intervals of interest were eligible for the analysis. Patients with known discontinuation of therapy for any duration until the end of the given intervals were assigned to the discontinuation group of patients without ST. In patients with ST, only discontinuation before the onset of ST was evaluated. Patients with acute ST and those with ST during the prior intervals were excluded from the analysis.

The influence of prolonged dual-antiplatelet therapy on clinical outcome was assessed with the so-called landmark analysis reported previously, which is a form of survival analysis that classifies patients on the basis of some nonoutcome event that occurs during follow-up (eg, discontinuation of thienopyridine at 6 months).[9] Eligible patients were those patients who continued taking aspirin and were free from death, MI, stroke, or ST at the 6-month landmark point.

Statistical Analysis

Categorical variables were compared with the χ^2 test. Continuous variables are expressed as mean±SD unless otherwise indicated. Continuous variables were compared with the Student t test or Wilcoxon rank sum test based on the distribution. Cumulative incidence was estimated by the Kaplan–Meier method, and differences were assessed with the log-rank test.

A Cox proportional hazard model was used to identify independent risk factors of ST. We used the variables listed in supplemental Table I as potential independent variables. The continuous variables were dichotomized by clinically meaningful reference values or median values. To determine the independent risk factors, we first selected variables with P values <0.05 in the univariable Cox models and for which proportional hazard assumptions were acceptable on the plots of log (time) versus log [−log (survival)] stratified by the variable. We then included them simultaneously in the multivariable models. Patients with missing values for any selected variable were excluded from the multivariable analysis. The robustness of independent risk factors for ST that were identified by the full model without selection of variables was confirmed by both forward and backward selection procedures.

Landmark analysis was conducted as described previously.[9] We computed the propensity score using logistic regression, with the dependent variable being continued thienopyridine use at 6 months and with the 23 independent variables listed in supplemental Table I. Next, we computed the adjusted survival curves of groups with and without thienopyridine use at the 6-month landmark using the Cox proportional hazard model in conjunction with methods described by Ghali et al,[10] adjusting for the propensity score and the above-mentioned 23 covariates.

All analyses were conducted by a physician (Takeshi Kimura) and an independent statistician (Takeshi Morimoto) with the use of SAS software version 9.1 (SAS Institute Inc, Cary, NC) and S-Plus version 7.0 (Insightful Corp, Seattle, Wash). All reported P values are 2-sided.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Baseline Characteristics

It was common for patients in the present study to have high-risk features, such as age ≥80 years, diabetes mellitus, renal failure, or unprotected left main disease (Table 1). Complex lesions such as chronic total occlusion, long lesions, and small vessels were also common. However, only 21% of patients presented with acute coronary syndrome (ACS). Procedures were characterized by use of high inflation pressure and a high rate of intravascular ultrasound guidance.

Clinical Outcome

Cumulative incidences of death, cardiac death, and sudden death at 2 years were 7.2%, 3.7%, and 1.4%, respectively (Table 2). Incidence of MI was 1.5% at 2 years. MI related to ST constituted 45% of all MIs, or 0.7% at 2 years. Incidence of target-lesion revascularization was 10.2% at 2 years.

Incidence, Clinical Sequelae, and Predictors of ST

Cumulative incidences of ST at 2 years were 0.77% for definite ST, 0.91% for definite or probable ST, and 2.48% for all ST (Table 2). Incidences of definite ST were 0.34% (95% confidence interval [CI] 0.23% to 0.45%) at 30 days, 0.54% (95% CI 0.4% to 0.68%) at 1 year, and 0.77% (95% CI 0.58% to 0.96%) at 2 years (Figure 1A). The slope of the linear portion of the cumulative incidence curve of ST between 30 days and 2 years was 0.2% per year. Clinical sequelae within 30 days of ST were MI in 85% to 95% of cases and death in 11% to 38% of cases, depending on ST timing (Table 3).

Table 3. Clinical Sequelae of ST and Antiplatelet Therapy at the Time of ST

	Early ST (≤30 d) (n=36)	Late ST (31–365 d) (n=21)	Very Late ST (366–730 d) (n=14)
Interim TVR, %	0	9.5	14
Clinical sequelae within 30 days of ST, %			
Death	11	38	18
MI	85	95	91
Q-wave infarction	56	70	83
Non–Q-wave infarction	29	25	8
Emergency CABG	11	0	0
Antiplatelet therapy at time of ST, %			
On dual antiplatelet therapy	86	57	36
On aspirin alone	8.3	14	43
On thienopyridine alone	0	4.8	0
Off both	2.8	24	21
Unknown	2.8	0	0
Discontinuation of thienopyridine, n	1	8	9
Median interval (IQR) between thienopyridine discontinuation and ST, d	9	29 (14–174)	196 (82–404)

TVR indicates target-vessel revascularization; IQR, interquartile range.

Univariable predictors for ST are shown in supplemental Table I. Multivariable analysis identified ACS (hazard ratio [HR] 2.53, 95% CI 1.3 to 4.92, P=0.006) and heart failure (HR 2.33, 95% CI 1.12 to 4.84, P=0.02) as independent predictors of early ST. Independent predictors of late or very late ST included hemodialysis (HR 6.86, 95% CI 3.05 to 15.45, P<0.001), end-stage renal disease (estimated glomerular filtration rate <30 mL · min^{-1} · 1.73 m^{-2}) without hemodialysis (HR 5.33, 95% CI 2.0 to 14.15, P<0.001), side-branch stenting (HR 3.5, 95% CI 1.36 to 9.03, P=0.01), and smoking (HR 2.36, 95% CI 1.17 to 4.76, P=0.02).

Discontinuation of Antiplatelet Therapy and ST

During the index hospitalization, aspirin and thienopyridine were administered in 98.9% and 99.5% (ticlopidine 96.9% and clopidogrel 2.6%) of patients, respectively. Cilostazol was administered in 3.2% of patients at the time of hospital discharge.

The status of antiplatelet therapy immediately before the onset of ST was known for the vast majority of patients with ST, except for 1 patient who presented with cardiogenic shock. The majority of patients (86%) with early ST were taking dual-antiplatelet therapy at the time of ST. The prevalence of dual therapy was 57% for late ST and 36% for very late ST, respectively (Table 3). Among 18 patients who

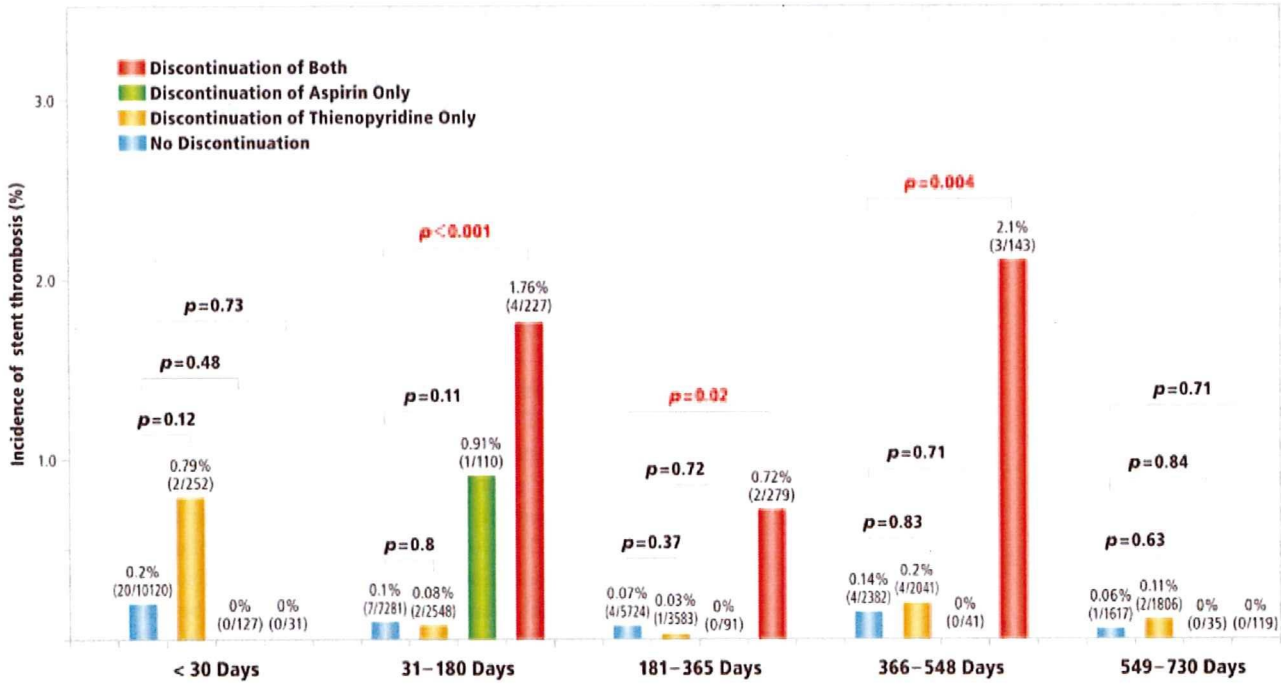


Figure 2. Relationship between thienopyridine and/or aspirin discontinuation and ST by time interval after stent implantation.

had ST after any antiplatelet therapy discontinuation, the majority of ST events occurred >1 week after discontinuation (Figure 1B and 1C).

Thienopyridine use was maintained in 97%, 62%, and 50% of patients at 30 days, 1 year, and 2 years, respectively. A steep rise was found at ≈3 months in the cumulative incidence curve of persistent discontinuation of thienopyridine (Figure 1D); however, a corresponding steep rise was not observed in the cumulative ST incidence curve (Figure 1A).

With regard to the relation between aspirin and/or thienopyridine discontinuation and ST, patients who discontinued both aspirin and thienopyridine had a significantly higher ST rate than those who continued both agents in the intervals of 31 to 180 days, 181 to 365 days, and 366 to 548 days after stent implantation (1.76% versus 0.1%, $P<0.001$; 0.72% versus 0.07%, $P=0.02$; and 2.1% versus 0.14%, $P=0.004$, respectively; Figure 2). When discontinuation of aspirin was considered, discontinuation of thienopyridine therapy only was not associated with increased ST risk in any of the time intervals.

Landmark Analysis Based on Thienopyridine Use

At 6 months, thienopyridine use was maintained in 7247 (73%) of 9875 patients eligible for the landmark analysis. Patients taking thienopyridine had significantly more complex characteristics, although some of these statistically significant differences might not be clinically relevant (Table 4). After adjustment for differences in baseline characteristics, the rates of death, MI, death or MI, and a combined end point of cardiac death, MI, or stroke at 24 months were not different between the 2 groups with or without thienopyridine therapy in the 6-month landmark analysis (Figure 3; Table 5).

The influence of ACS on the 6-month landmark analysis was evaluated. The cumulative rate of death or MI was significantly higher in patients with ACS than in those without ACS (Figure 4A); however, rates of death or MI beyond 6 months were similar in both groups (Figure 4B). Adjusted rates of death or MI at 24 months in the 2 groups either taking or not taking thienopyridine therapy at 6 months were similar in patients with or without ACS (Figure 4C and 4D).

Discussion

The main findings of the present study are that discontinuation of both aspirin and thienopyridine, but not discontinuation of thienopyridine therapy only, is associated with an increased ST risk and that no apparent clinical benefit is received from thienopyridine use beyond 6 months after SES implantation. More than 2 years ago, concerns were raised about DES safety.[1,2] Although more recent reports from registries and meta-analyses of randomized, controlled trials provided data supporting the relative safety of DES compared with BMS,[11,12] a cohort study conducted in Bern, Switzerland, and Rotterdam, Netherlands, demonstrated that definite ST continues to occur at the constant rate of 0.6% per year from 30 days to 3 years after DES implantation.[8] The present ongoing analysis using the same ST end point also showed that ST remained a continuous hazard up to 2 years after SES implantation, although the cumulative incidence of ST appeared to be considerably lower than that in the Bern and Rotterdam cohorts.[8]

We can suggest several potential reasons for the markedly lower rate of early ST in the present registry. First, there might be ethnic differences in the propensity for ST. We reported a 0.9% rate of early ST in 320 Japanese patients undergoing planned BMS implantation with an antithrom-

Table 4. Baseline and Procedural Characteristics in the 2 Groups Analyzed by Landmark Analysis at 6 Months

	Taking Thienopyridine (n=7247)	Not Taking Thienopyridine (n=2628)	*P*
Age, y	68.1±10.2	68.3±10.0	0.44
Male, %	76	75	0.12
Body mass index, kg/m^2	24.0±3.4	24.0±3.4	0.98
Hypertension, %	76	74	0.03
Diabetes mellitus, %	41	39	0.2
Current smoking, %	19	21	0.11
eGFR <30 mL · min^{-1} · 1.73 m^{-2}, %	9.7	8.0	0.009
ACS, %	20	22	0.03
Prior MI, %	28	28	0.62
Prior stroke, %	9.3	8.1	0.06
Peripheral vascular disease, %	11	12	0.54
Prior heart failure, %	13	10	<0.001
Prior PCI, %	50	44	<0.001
Prior CABG, %	7.3	7.0	0.58
Multivessel stenting, %	21	16	0.14
Target of unprotected LMCA, %	4.1	2.6	<0.001
Target of proximal LAD, %	49	49	0.51
Target of chronic total occlusion, %	12	9.7	0.005
Target of in-stent restenosis, %	17	15	0.046
Target lesion <2.5 mm in diameter, %	33	33	0.93
Intravascular ultrasound use, %	49	44	<0.001
Side-branch stenting, %	4.9	2.5	<0.001
Total length of stents, mm	39.8±26.5	36.0±22.5	<0.001

eGFR indicates estimated glomerular filtration rate; LMCA, left main coronary artery; and LAD, left anterior descending coronary artery.

Continuous variables were expressed as mean±SD.

botic regimen that included aspirin and warfarin.[13] This early ST rate appears to be markedly lower than the 2.7% in 550 US patients reported in the Stent Anticoagulation Restenosis Study using the same antithrombotic regimen and the same BMS.[14] Second, the incidence of ACS presentation, which is an established risk factor for early ST, was lower in the present study population than in the Bern and Rotterdam cohorts[9] (21% and 59%, respectively). Third, only 3% of patients in the present study discontinued thienopyridine within 30 days of SES implantation compared with the 14% discontinuation rate reported in the PREMIER registry (Prospective Registry Evaluating Myocardial Infarction: Events and Recovery).[5] We cannot provide a clear explanation for the lower rate of late and very late ST in the present study population compared with that in the Bern and Rotterdam cohorts,[8] because the mechanisms of late and very late ST have not yet been well clarified.

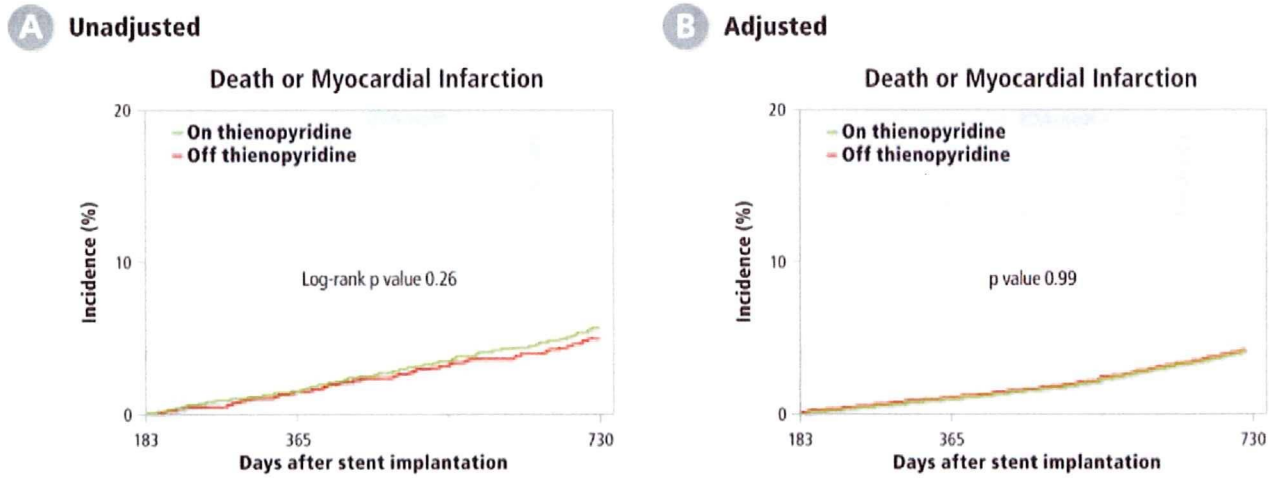
Although no randomized study has evaluated the role of dual-antiplatelet therapy in DES, the benefit of dual-antiplatelet therapy in preventing ST within 1 month after BMS implantation has been well established from randomized trials.[14,15] The TRITON-TIMI 38 trial (Trial to Assess Improvements in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis In Myocardial Infarction) also demonstrated that more intensive antiplatelet therapy with prasugrel was associated with a marked reduction in ST rate compared with standard antiplatelet therapy with clopidogrel.[16] Reduction of the ST rate in the prasugrel group was predominantly seen within 30 days after stent implantation, although the reduction in the rate of late ST was also of borderline significance. Therefore, the role of intensive antiplatelet therapy in reducing early ST appears to be firmly established.

However, the role of thienopyridine therapy in reducing late ST beyond 1 month after stent implantation has not been well addressed. Although premature discontinuation of antiplatelet therapy has been reported to be the most powerful predictor of ST and/or adverse outcome,[3–5] these previous reports did not discriminate the relative impact of discontinuation of either aspirin, thienopyridine, or both agents. The present analysis demonstrated that withdrawal of both thienopyridine and aspirin, but not of thienopyridine therapy alone, was associated with increased ST risk beyond 1 month after SES implantation. Aspirin withdrawal was reported to be responsible for admission with an ACS in 51 (4%) of 1236 patients, with a mean delay between aspirin cessation and hospitalization of 10±2 days.[17] It is noteworthy that late and very late ST at a mean of 16±7 months after BMS implantation was responsible for ACS in 10 (20%) of these 51 patients.

Furthermore, only one third of ST events after discontinuation of antiplatelet therapy (mostly thienopyridine) occurred within the first 28 days after discontinuation. This might lead to a discussion about whether or not a direct link in fact exists between discontinuation of antiplatelet therapy and ST, particularly very late ST.

Previous prospective studies demonstrated a clinical benefit of the prolonged use of thienopyridine for up to 1 year in patients undergoing PCI with BMS,[18,19] primarily in the setting of ACS. Extrapolation of these findings to DES might make it appear reasonable to advocate adherence to dual-antiplatelet therapy for 1 year after DES implantation; however, the present study results suggest that it is also reasonable to discontinue thienopyridine and adhere to aspirin monotherapy in situations in which continuation of dual-antiplatelet therapy appears to be otherwise clinically irrelevant. Furthermore, because the majority of ST events occurred >1 week after discontinuation of antiplatelet therapy, it appears important to make the duration of discontinuation as short as possible if discontinuation is unavoidable.

The optimal duration of dual-antiplatelet therapy has not been well established. A single-center observational study of 1216 DES patients and 2393 BMS patients reported that use of clopidogrel at 6 and 12 months was associated with a lower incidence of death or MI at 24 months in patients with DES but not in patients with BMS.[9] On the other hand, a similar



Days after stent implantation	183	365	730
Cumulative incidence, On thienopyridine		1.6%	5.8%
Number of events		115	268
Number of patients at risk	7247	6918	2405
Cumulative incidence, Off thienopyridine		1.5%	5.0%
Number of events		39	91
Number of patients at risk	2628	2533	1072

Days after stent implantation	183	365	730
Cumulative incidence, On thienopyridine		1.1%	4.1%
Cumulative incidence, Off thienopyridine		1.1%	4.1%

Figure 3. Unadjusted and adjusted cumulative incidences of death or MI using the 6-month landmark analysis.

analysis in 671 diabetic patients reported that use of clopidogrel at 6 months was associated with a significantly lower incidence of death or MI at 18 months in patients with BMS but not in those with DES.[20] Relatively small sample sizes in these subgroup analyses to evaluate hard clinical end points might be 1 of the reasons for the discrepancy. The present analysis of a larger number of patients by the same method demonstrated a similar long-term clinical outcome regardless of thienopyridine use at 6 months in SES patients. Given the increased risk of bleeding and the huge economic burden with prolonged dual-antiplatelet therapy,[18,19,21] the optimal duration of dual-antiplatelet therapy should be defined by prospective randomized trials evaluating the net clinical benefit after considering both ischemic events and bleeding complications.

The present study has several important limitations. First, baseline characteristics and procedural characteristics such as the high rate of intravascular ultrasound guidance in the present cohort might be markedly different from practices outside Japan. Also, ticlopidine was used as a thienopyridine antiplatelet agent in the vast majority of patients, in contrast to the use of clopidogrel in most other studies. These and other ethnic differences might make it difficult to apply the findings in the present study outside of Japan. Second, although data on antiplatelet therapy use were collected prospectively, no attempt was made to verify compliance with antiplatelet medications for patients in whom discontinuation was not reported; this might well have led to overestimation of compliance. In addition, we did not systematically evaluate the restarting of antiplatelet therapy after persistent discontinuation, which could have resulted in the potential underestimation of medication use. In fact, in the 6-month landmark analysis, 13% of patients who underwent repeated revascularization >6 months after the first procedure were likely to have restarted thienopyridine. The limitation of a landmark analysis is that it only examines specific points in time. A Cox proportional hazards model with a time-dependent covariate (thienopyridine discontinuation) might be able to examine the continuous risk of thienopyridine discontinuation. Furthermore, when follow-up information was obtained by contact with patients, dates of discontinuation of aspirin and thienopyridine were based on retrospective recall by the patients or relatives, which suggests a potential for recall bias. Third, unmeasured confounders related to thienopyridine discontinuation might be present because of the observational study design. Fourth, the number of patients at risk at 2-year follow-up was limited. Therefore, the results of the present study might be valid only during the first year after SES implantation. Finally, bleeding complications were not evaluated, which made it impossible to evaluate the net clinical efficacy of dual-antiplatelet therapy.

Table 5. Unadjusted and Adjusted 24-Month Outcomes Based on 6-Month Thienopyridine Use

	No. of Events					Unadjusted Event Rates, %				Adjusted Event Rates, %			
	No. at Risk at 6 Months	Death	MI	Death or MI	Cardiac Death, MI, or Stroke	No. at Risk at 24 Months	Death (P=0.22)	MI (P=0.81)	Death or MI (P=0.26)	Cardiac Death, MI, or Stroke (P=0.6)	Death (P=0.9)	MI (P=0.42)	Death or MI (P=0.99)	Cardiac Death, MI, or Stroke (P=0.79)
On thienopyridine	7247	238	41	268	228	2424	5.2	0.9	5.8	4.9	3.4	0.6	4.1	4.0
Off thienopyridine	2628	79	17	91	82	1078	4.4	0.8	5.0	4.3	3.4	0.8	4.1	4.1