In conclusion, older age, insulin treatment, low well-being, cognitive impairment and visual impairment were independently associated with functional disabilities of elderly patients with diabetes mellitus. At present, it is unknown whether psychological intervention and rehabilitation of physical, cognitive and visual impairment prevent functional decline or not. However, further growing understanding of underlying processes of functional disabilities could provide a basis to design effective strategies to delay functional decline in elderly patients with diabetes mellitus.

## Acknowledgments

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# Obesity and type 2 diabetes in Japanese patients

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# Obesity and type 2 diabetes in Japanese patients

Sir—The incidence and morbidity of type 2 diabetes mellitus is known to be higher in obese individuals, and is especially high in ethnic groups with high body-mass indices (BMI), such as Pacific Islanders and Pima Indians. However, few studies have compared the BMIs of type 2 diabetic patients from different ethnic groups, and this scarcity perhaps reflects the difficulties in obtaining large matched-sample cohorts from different ethnic groups.

Comparison of data from two prospective studies (table) reveals a striking difference in the average BMI of type 2 diabetic patients from two different ethnic populations—white individuals from the UK Prospective Diabetes Study (UKPDS)[1] and Japanese patients from the Japan Diabetes Complication Study (JDCS).[2] Both groups were similar in terms of numbers of patients, age, glycohaemoglobin $A_{1c}$ concentration, and disease duration. However, the BMI of white diabetic patients was much higher than that of the Japanese patients. Moreover, whereas the BMI of white diabetic patients was higher than that reported for non-diabetics of the same ethnic origin,[3] the BMI of Japanese diabetic patients was normal compared with that of the Japanese non-diabetic population.[4] The average BMI of the white UKPDS patients continued to increase during the 10 years after diagnosis, and although we do not have retrospective BMI data from the time of diagnosis in the JDCS cohort, there has been no significant increase in the average BMI over the 6 years of the study.

The origin of this large difference in BMI is unknown, but it might reflect differences in insulin secretion and sensitivity between the two ethnic groups. Unfortunately, this hypothesis is difficult to prove because plasma insulin concentrations were measured only at baseline in the UKPDS, and are not comparable with the subsequent insulin measurements taken in the JDCS. In general, diabetic patients who are obese have greater insulin secretion and lower insulin sensitivity (ie, insulin resistance) than non-obese diabetics, and the white population is regarded as more obese and insulin resistant than the east Asian population.[5]

Notwithstanding the lack of comparative insulin data, the higher systolic blood pressure and triglyceride concentrations in the UKPDS patients compared with those in the JDCS patients (table) could imply that they have insulin resistance syndrome, which is characterised by the accumulation of multiple cardiovascular risk factors. The lower fasting plasma glucose concentrations in the face of higher glycohaemoglobin concentrations in the UKPDS patients could suggest a more severe postprandial hyperglycaemia than that in the JDCS patients; postprandial hyperglycaemia is also known to be an independent risk factor for cardiovascular disease. This association might partly explain the higher incidence of cardiovascular complications in white diabetics than in east Asian diabetics.

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| | UKPDS (n=2015) | JDCS (n=2205) |
|---|---|---|
| Age (years) | 62 | 59 |
| Diabetes duration (years) | 9 | 11 |
| Blood pressure (mm Hg) | 140/80 | 132/77 |
| Fasting plasma glucose (mmol/L) | 8·14 | 8·75 |
| Glycohaemoglobin $A_{1c}$ (%) | 7·9 | 7·7 |
| Total cholesterol (mmol/L) | 5·3 | 5·2 |
| Triglycerides (mmol/L) | 1·53 | 1·40 |
| BMI (kg/m²) | 29·4 | 23·1 |
| Mean BMI of whole population | 24·1 | 22·7 |

**Comparison of mean baseline characteristics from the Japan Diabetes Complication Study (JDCS) and averaged year-9 data for white individuals from the UK Prospective Diabetes Study (UKPDS)**

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# Energy intake and obesity in Japanese patients with type 2 diabetes

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# Energy intake and obesity in Japanese patients with type 2 diabetes

Sir—Obesity is known to be one of the most important risk factors for the development and deterioration of type 2 diabetes. Nevertheless, we have previously revealed a discrepancy in body-mass indices (BMI) between white and Japanese patients with type 2 diabetes (about 29 kg/m$^2$ in white patients from the UK Prospective Diabetes Study [UKPDS] *vs* 23 kg/m$^2$ in Japanese patients from the Japan Diabetes Complications Study [JDCS]) whose other characteristics were very similar.[1] Moreover, by contrast with white patients with type 2 diabetes who have a higher BMI than does the white population as a whole (about 24 kg/m$^2$), the BMI of Japanese patients is similar to that of the general Japanese population, indicating that Japanese patients with type 2 diabetes are not obese, at least on average.[1]

We recently completed the baseline nutrition analysis of our JDCS patients. Comparing our results with those of the UKPDS,[2] we were surprised to find that the mean daily energy intake of both cohorts was almost the same despite the large differences in BMI and bodyweight (table). In other words, the UKPDS patients developed obesity with a relatively lower energy intake than the JDCS patients, considering the mean height difference between the groups. Accordingly, the daily energy intake per unit of bodyweight was 22% lower in the UKPDS patients than in the JDCS patients. Moreover, 19% of male (27% of female) patients in our cohort overate, taking more than 35 kcal per ideal bodyweight daily. Of those patients, 20% of men (29% of women) had a BMI greater than 25 kg/m$^2$, which is regarded as being overweight. Accordingly, only 3·8% of male and 7·8% of female patients in the JDCS study population had obesity associated with actual overeating.

This finding runs contrary to the conventional wisdom that the major pathophysiological background to type 2 diabetes is insulin resistance and obesity associated, at least to some extent, with excessive energy intake.

Obesity is known to have preceded and triggered the explosive increase in diabetes among Pacific Islanders and Pima Indians.[3] In Japan, however, despite the lack of a major increase in mean BMI and a decrease in mean total dietary intake since the 1970s, the prevalence of type 2 diabetes is now very high: a sixth of the adult population in Japan had known or strongly suspected diabetes in the most recent national survey. This proportion is much higher than in the European population,[3] and the prevalence is still increasing. The results of recent large-scale epidemiological surveys also suggested that a disturbance of insulin secretion rather than insulin resistance was strongly associated with the development of the disease in Japanese and Chinese patients, unlike in the European population.[4]

Risk factors other than obesity and insulin resistance seem to affect the development of type 2 diabetes in Japanese people. Additionally, even relatively mild obesity could have a major effect on the pathogenesis of diabetes in the Japanese population.[5]

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| | JDCS | UKPDS |
|---|---|---|
| Patients (men/women) | 1076 (576/500) | 108 (61/47) |
| Age (years) | 59·4 (7·4) | 55·1 (7·7) |
| Bodyweight (kg) | 58·6 (10·1) | 78·2 (12·2) |
| Body-mass index (kg/m$^2$) | 23·1 (3·0) | 27·9 (4·3) |
| Glycohaemoglobin A1C (%) | 7·7 (1·4) | 7·1 (1·5) |
| Total energy intake (kcal/day) | 1580 (398) | 1650 (424) |
| Men | 1778 (428) | 1797 (63)* |
| Women | 1598 (390) | 1439 (44)* |
| Total energy intake per kg weight (kcal/kg daily, mean) | 27·0 | 21·1 |

Values are mean (SD) unless otherwise indicated. *SE.

**Total energy intake and other characteristics of patients with type 2 diabetes from Japan Diabetes Complications Study (JDCS) and UK Prospective Diabetes Study (UKPDS)**

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Regular Article

# Expression of Toll-like receptors on human platelets

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**KEYWORDS**
Coronary artery disease;
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Inflammation;
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**ABSTRACT**

*Introduction*: Platelets play a crucial role in arterial thrombosis, which is the main cause of acute coronary syndrome. Some mycobacteriums, such as *Chlamydia pneumoniae*, were associated with progression of atherosclerosis and they are interacted with Toll-like receptors (TLRs), which have been defined as pathogen-associated molecular pattern recognition molecules in innate immunity. In the present study, we examined whether human platelets express TLRs. *Materials and methods*: Human platelets were obtained from healthy volunteers and the mRNA and protein level of TLRs on platelets and Meg-01 cells, megakaryoblastic cell line, were investigated. *Results*: Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that TLR1 and TLR6 mRNA were expressed in platelets and Meg-01 cells. Furthermore, interferon-γ up-regulated their mRNA levels in dose and time dependent manners after stimuli. Both TLR1 and TLR6 proteins in platelets were detected by Western blotting, and their expression of platelets was more than that of Meg-01 cells. Flow cytometry analysis revealed the expression of TLR1 and TLR6 on the cell surface of Meg-01 cells. Furthermore, immunohistochemical analysis using human coronary thrombi obtained from patients with acute coronary syndrome confirmed the expression of TLR1 and TLR6 on platelets. *Conclusion*: In summary, we

*Abbreviations:* TLR, Toll-like receptor; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger ribonucleic acid; HSP, heat shock protein; LPS, lipopolysaccharide; IFN-γ, interferon-gamma; CD, cluster differentiation; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PBMC, peripheral blood mononuclear cell; ACD buffer, acid-citrate dextrose buffer; PRP, platelet-rich plasma; RNase, ribonuclease; DNase, deoxyribonuclease; cDNA, deoxyribonucleic acid; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; PE, phycoerythrin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; *C. pneumoniae, Chlamydia pnumoniae*; HE, hematoxylin-eosin.

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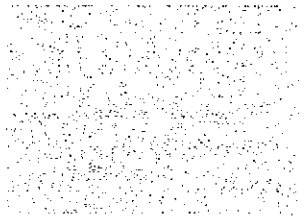

demonstrated that human platelets and Meg-01 cells expressed a family of TLRs for the first time, and our findings indicated that platelets might recognize antigens directly via TLRs. Our findings suggest a possibility that platelets have the ability to recognize the antigens via TLRs and that there are mechanistic relations between infectious inflammation and atherosclerotic vascular diseases.


## Introduction

Atherosclerosis is a chronic inflammatory disease. Inflammation occurs in response to vascular injury induced by oxidative stress and infection. Accumulating evidence indicates a close association of atherosclerotic diseases with chronic infection with ubiquitous pathogens such as *Chlamydia pneumoniae*. Recent studies have demonstrated a seroepidemiologic association with risk of cardiovascular events. Furthermore, immunohistochemistry has revealed the presence of microorganisms in atherosclerotic lesions. Very recently, Sasu et al. [1] demonstrated that Chlamydial heat shock protein (HSP) 60 stimulated proliferation of vascular smooth muscle cells via Toll-like receptor (TLR).

Inflammation induced by pathogens is a complex process of interaction between various soluble factors and inflammatory cells. It has become evident that a family of TLRs plays a crucial role in innate immunity as the first defense system against microbial infection [2]. TLR has been defined as a pathogen-associated molecular pattern recognition molecule. Microbial antigens, lipopolysaccharide (LPS) and bacterial HSPs interact with the extracellular domain of TLRs and subsequently activate multiple intracellular signaling pathways. TLR1, TLR2 and TLR4 are markedly expressed in human atherosclerotic vessels [3], and TLR4 in macrophages is up-regulated by oxidized low-density lipoprotein [4], strongly suggesting the association between TLRs and atherosclerotic vascular diseases.

Interferon-gamma (IFN-γ) is a proinflammatory cytokine, which contributes to atherogenesis via its various functions such as activation of human peripheral blood monocytes and enhancement of smooth muscle cells proliferation. $CD4^+$ T lymphocytes are reported to accumulate in atherosclerotic vessel walls through almost all stages and produce IFN-γ [5,6]. In the unstable plaques, IFN-γ secreted by T lymphocytes inhibits the collagen synthesis by vascular smooth muscle cells and activates macrophages, which secrete several proteases. These macrophages-derived proteases can break down the extracellular matrix and weaken the fibrous cap, rendering it susceptible to rupture and precipitation of acute coronary syndrome [7].

Platelets play a central role in arterial thrombosis superimposed on disrupted unstable plaques, which is the main cause of acute coronary syndrome. Spontaneous and agonist-induced hyperaggregation and hypersensitivity of platelets have also been implicated in pathogenesis of various cardiovascular disorders. These non-nucleated cells also have several immunomodulatory properties: activated platelets have been shown to induce inflammatory reaction on endothelial cells through the CD40 ligand originally identified on activated $CD4^+$ T cells, and to secrete various proinflammatory and anti-inflammatory mediators. Given the close association between platelets and inflammation, this cell type might express a family of TLR. In the present investigation, we examined through various approaches, whether platelets express any of the members of the TLR family. Furthermore, the effect of IFN-γ on their expression was investigated.

## Materials and methods

### Cell culture

THP-1 cells, human monocytic leukemia cell line, and Meg-01 cells, megakaryoblastic cell line, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The THP-1 cells were differentiated into macrophages by treatment with 100 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 h. Meg-01 cells were stimulated with IFN-γ (Diaclone, France) at various concentrations (0, 4, 40 and 400 ng/ml) for 6 h. For time course experiments, cells were stimulated with IFN-γ (40 ng/ml) for indicated periods (0, 1, 6, 12 and 24 h).

### Platelets and peripheral blood mononuclear cells (PBMCs) preparation

Under informed consent, blood was obtained from healthy volunteers who had taken neither aspirin nor other agents known to alter platelet function for at least 10 days before the study. Whole blood was drawn from the antecubital vein and mixed with acid-citrate dextrose buffer (ACD buffer: 85

mM trisodium citrate, 71 mM citric acid and 111 mM dextrose) at 9:1 v/v. Platelet-rich plasma (PRP) was prepared by centrifugation of mixed blood at 150 × *g* for 20 min at room temperature. To avoid the contamination of PBMCs, platelets were isolated by centrifugation of upper half of PRP at 1200 × *g* for 5 min and washed twice with platelet wash buffer (100 mM NaCl, 8.5 mM Tris, 8.5 mM dextrose and 1.0 mM EDTA). The contamination of PBMCs was less than $1/10^8$ platelet. A similar degree of contamination, using the same preparation method, was reported by others [8]. PBMCs were isolated from whole blood by density centrifugation with the use of Lymphoprep (AXIS-SHIELD PoC AS, Norway) according to the manufacture's instructions.

### Coronary thrombi of acute coronary syndrome obtained by intracoronary aspiration thrombectomy

Five patients with acute coronary syndrome who were treated with percutaneous coronary intervention with a RESCUE therombectomy catheter at Kobe Steel Hospital (Kakogawa, Japan) were investigated. Written informed consents were obtained from all patients. The occlusive thrombi were manually aspirated into a syringe through an outer catheter, and then the aspirated materials were immediately fixed in formalin for immunohistochemical analysis.

### Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated using the total RNA isolation kit (Isogen; NIPPON GENE, Japan) according to the manufacture's instructions. After RNA isolation, 1 μg of total RNA was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed to cDNA using RT-PCR kit (RETROscript™; Ambion, USA). PCR reactions were performed with *Taq* polymerase using following specific primers. The primer sequences were as follows: for TLR1, 5′-AAACGGTCTCATCCACGTTC-3′ (sense) and 5′-GAGCAATTGGCAGCACACTA-3′ (antisense); for TLR6, 5′-GGCCCAAGGAGAAAAGCAAAC-3′ (sense) and 5′-AGAGACTGGGCTGTCTCTAAC-3′ (antisense); for CD14, 5′-CGTGGGCGACAGGGCGTTCT-3′ (sense) and 5′-TAAAGGTGGGGCAAAGGGTT-3′ (antisense). PCR products were separated using 1.5% agarose gel and identified by ethidium bromide staining.

### Western blot analysis

The cell homogenates were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5.0% skim milk for 1 h at room temperature, incubated with goat anti-human TLR1 or TLR6 antibodies (Santacruz Biotechnology, USA) overnight at 4 °C, and then incubated with anti-goat horseradish-conjugated antibody for 1 h at room temperature. Detection of antigen was performed using the enhanced chemiluminescent detection method (ECL-plus; Amersham Bioscience, USA).

### Flow cytometry

The cells were frozen and thawed after fixation in 1% paraformaldehyde, and then incubated with goat anti-human TLR6 antibody, sequentially with phycoerythrin (PE)-conjugated anti-goat secondary antibody (Biomeda, USA). For TLR1, they were incubated with PE-conjugated anti-human TLR1 antibody (eBioscience, USA). After washing with PBS, cells were analyzed using the FACScan flow cytometer and CELLQuest software (Becton Dickinson, USA).

### Immunohistochemistry

Sliced formalin-fixed coronary thrombi were incubated with diluted blocking serum (5% FCS) for 1 h at room temperature, and then incubated with primary antibodies 2 overnight at 4 °C. The primary antibodies were mouse anti-human CD41 antibody (DAKO, Denmark) and goat anti-human TLR1 or TLR6 antibodies. The sections were washed with PBS, incubated with PE-conjugated anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (DAKO), and then analyzed with a confocal microscope (Carl ZEISS, Germany).

### Statistical analysis

The data are presented as mean ± S.E.M. of the indicated number of experiments. Differences were analyzed by one-way analysis of variance (ANOVA) and a post-hoc multiple comparisons test (Fisher's) and considered significant at $P<0.05$.

## Results

### Expression of TLR1 and TLR6 in human platelets

First, we examined the expression of various types of TLR mRNA in human platelets and Meg-01 cells by RT-PCR. Two members of TLRs, TLR1 and TLR6

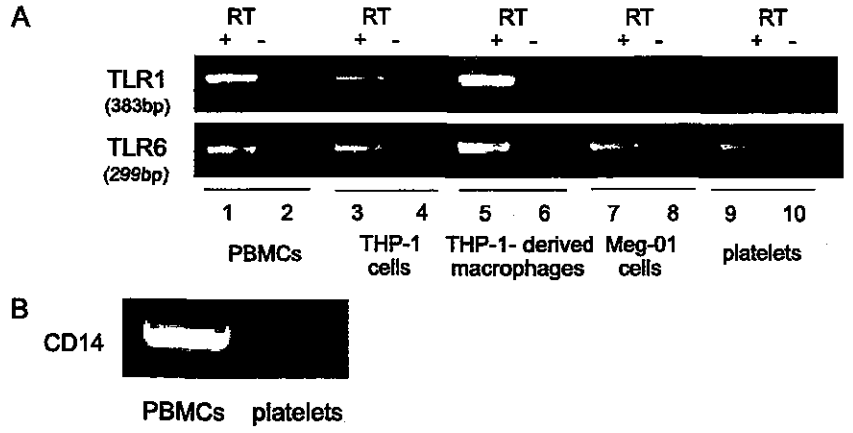


**Fig. 1** (A) Expression of TLR1 and TLR6 mRNA in Meg-01 cells and human platelets evaluated by RT-PCR. The RT reaction was carried out with total RNA isolated from PBMCs, THP-1 cells, THP-1-derived macrophages, Meg-01 cells and human platelets (RT+). Both TLR1 (383 bp) and TLR6 (299 bp) mRNA were expressed in not only monocyte cell line but also Meg-01 cells and human platelets. No RT-PCR product was obtained in the negative control where RT was not performed (RT − ). Lanes 1, 2: PBMCs; lanes 3, 4: THP-1 cells; lanes 5, 6: THP-1-derived macrophages; lanes 7, 8: Meg-01 cells; lanes 9, 10: human platelets. (B) Examination of CD14 mRNA in isolated platelets and PBMCs. CD14 mRNA was expressed only in PBMCs.

were detected. Other TLRs were detected in neither human platelets nor Meg-01 cells. The expression of TLR1 and TLR6 in human platelets as well as in Meg-01 cells is shown in Fig. 1A. No RT-PCR products were present in the negative control where RT was not carried out. To check the contamination of PBMCs, we examined the presence of CD14 mRNA, a specific marker of PBMCs, in the samples of platelets by RT-PCR. As shown in Fig. 1B, CD14 mRNA was not detected. Thus, the contamination of leukocytes in platelets was negligible. Sequencing the complementary DNA of TLR1 and TLR6, obtained from human Meg-01 cells by RT-PCR, revealed the identity of their sequences from the gene bank. These results confirmed that platelet TLRs were identical to those reported previously. Then, we investigated whether the mRNA expression level was regulated by IFN-γ in Meg-01 cells. Meg-01 cells were treated with various concentrations and for various times of IFN-γ (see

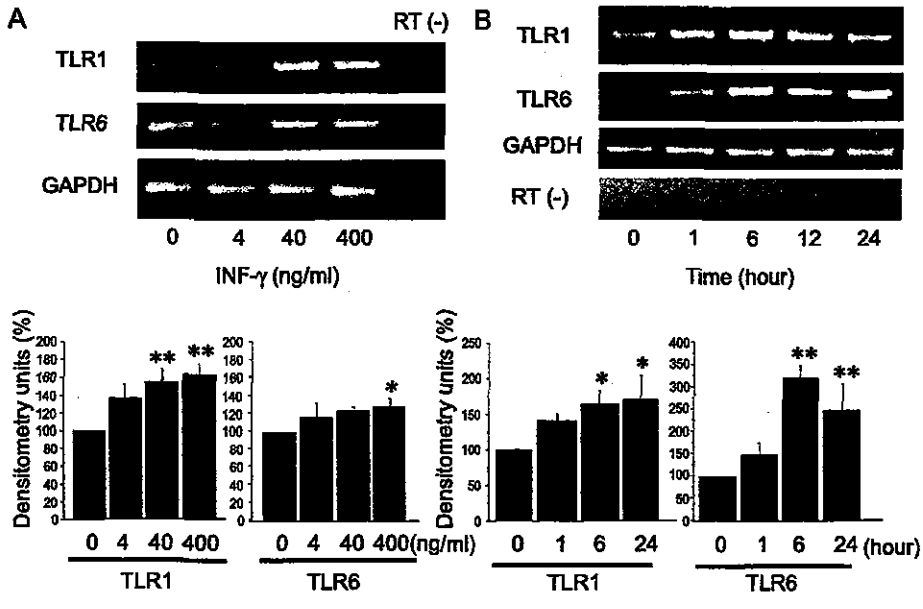


**Fig. 2** Effect of IFN-γ on the expression of *TLR1* and *TLR6* mRNA in Meg-01 cells. The cells were treated with IFN-γ at various concentrations (0–400 ng/ml, A) and for various times (0–24 h, B). The RT-PCR analysis of GAPDH expression was used as control. The graph depicts the mean ± S.E.M. of three independent studies. $^{*}P<0.05$, $^{**}P<0.01$ vs. treated with 0 ng/ml (A) or for 0 h (B).

Section 2). TLR1 and TLR6 mRNA level in Meg-01 cells was up-regulated in a dose-dependent manner (Fig. 2A)., and the peak level was observed 6 h after stimulated by IFN-γ (Fig. 2B).

Next, we assessed the protein level of TLRs by Western blotting. PBMCs were shown as a positive control. Both TLR1 and TLR6 protein were strongly expressed in human platelets. TLR6 protein was also expressed in THP-1-derived macrophages and Meg-01 cells, whereas the levels of TLR1 protein expression were different among these cell types (Fig. 3A). TLR1 protein was not detected in Meg-01 cells, and it was weakly expressed in THP-1-derived macrophages.

To further confirm the expression of TLRs on cell surface, flow cytometry was applied. Not only TLR 1 but also TLR6 were detected on Meg-01 cells (Fig. 3B). As same as the result of Western blotting, the expression level of TLRs on THP-1-derived macrophages was higher than that on THP-1 cells. These results suggest that cell differentiation might influence their expression.

## Expression of TLRs on coronary thrombus

Double immunofluorescence of TLRs and CD41, a specific marker of platelets, was carried out on tissue sections of coronary thrombi obtained from

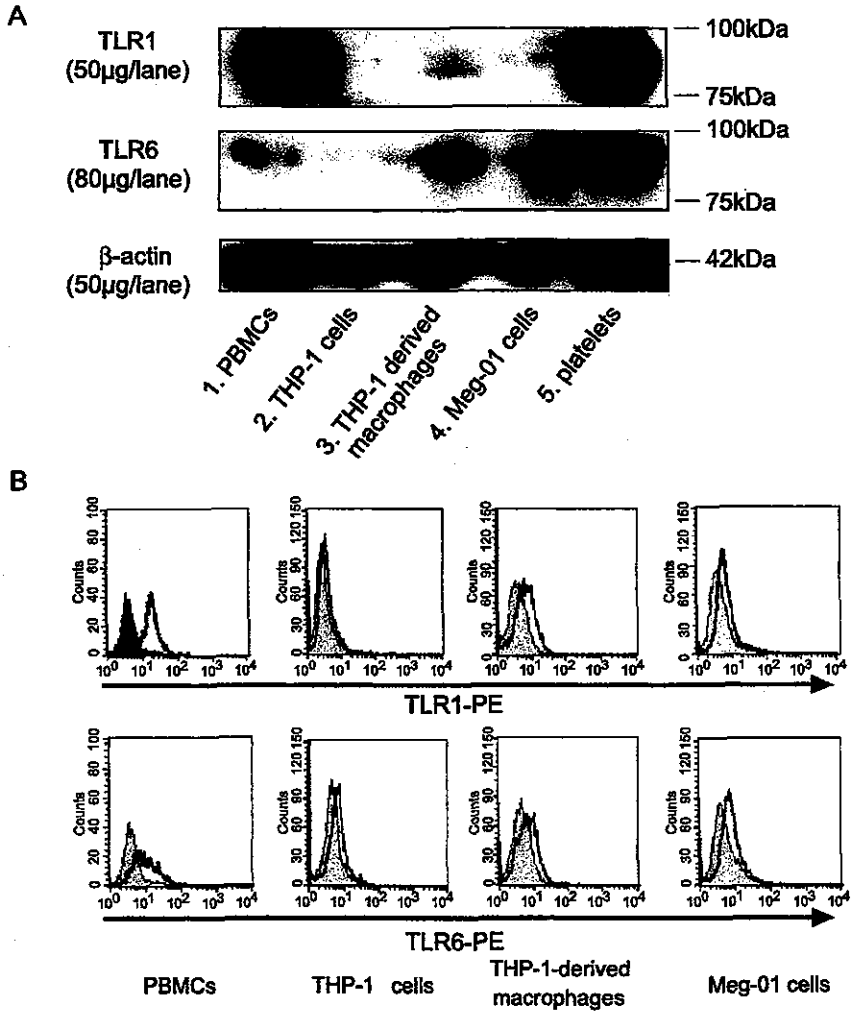


**Fig. 3** (A) The protein expression of TLR1 and TLR6 detected by Western blotting. PBMCs are served as a positive control. The cell homogenates were separated on 10% polyacrylamide gels and immunoblotted with anti-TLR1 or anti-TLR6 antibodies. In human platelets, TLR1 and TLR6 proteins are expressed at very high level. Lane 1: PBMCs, lane 2: THP-1 cells, lane 3: THP-1-derived macrophages, lane 4: Meg-01 cells, lane 5: human platelets. (B) TLR expression on Meg-01 cells demonstrated by flow cytometry. The shaded area indicates isotype control. The expressions of TLR1 (upper panels) and TLR6 (lower panels) on PBMCs were shown as a positive control. Both TLR1 and TLR6 were also expressed on Meg-01 cells. The expression levels of TLR1 and TLR6 on THP-1 cells were increased by differentiation to THP-1-derived macrophages.

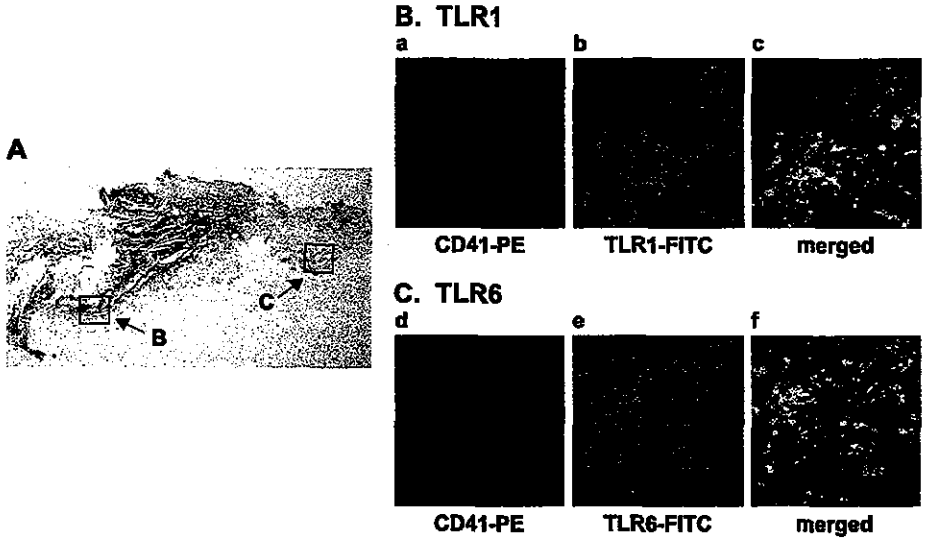


Fig. 4 Immunohistochemical analysis of TLRs in human coronary thrombi obtained from the patients of acute coronary syndrome. Double immunofluorescence was performed with CD41, a marker of platelets and anti-TLR1 (B: upper three panels) or anti-TLR6 (C: lower three panels) antibodies. Red fluorescence signals indicate CD41 (panels a and d). Green fluorescence signals indicate TLRs (panels b and e). TLRs are expressed in human platelets on thrombi obtained from coronary arteries (yellow signals, panels c and f). Panel A shows coronary thrombotic tissue stained with HE. Representative figure ($n=5$) is shown and similar results were observed in all examinations. Original magnification: panel A: ×40, panels B and C: ×400.

five patients with acute coronary syndrome. Representative images of anti-CD41 antibody for platelets (red signals), the immunoreactivity of TLR1 or TLR6 (green signals), and their colocalization (yellow signals) are shown in Fig. 4. A large number of platelets on human coronary thrombi expressed TLR1 and TLR6.

## Discussion

In the present study, we demonstrated for the first time that mRNA and protein of TLR1 and TLR6, members of the TLR family, were expressed in human platelets as well as Meg-01 cells, cell line of megakaryocytes and immunohistochemistry revealed their expression on the platelets of coronary thrombi in acute coronary syndrome. Furthermore, IFN-γ up-regulated the mRNA level of TLR1 and TLR6 in Meg-01 cells. Thus, the expressional regulation is likely under the control of inflammatory cytokines.

The TLR family has recently been identified as a major component of pathogen-associated molecular-pattern-recognition molecules [9]; it locates at the very front line of the innate immune system. Recent evidence suggests a possibility that TLRs also play an important role in pathogenesis of atherosclerosis. For example, Edfeldt et al reported the expression of TLR1, TLR2 and TLR4 was markedly enhanced in human atherosclerotic plaques, and their expression was associated with nuclear factor-κB [3]. Furthermore, it is reported that TLR4 expression on macrophages was up-regulated by oxidized LDL [4]. In the present investigation, TLRs were clearly detected in coronary thrombi from patients with acute coronary syndrome. These findings strongly suggest the implication of TLRs in the pathogenesis of atherosclerotic vascular diseases.

Ligands for TLR1 and TLR6 have not been fully determined; however, it is proposed that they mediate an immune response cooperatively with TLR2. Takeuchi et al. [10] reported that TLR1 interacted with TLR2 to recognize the lipid configuration of the mycobacterial lipoprotein. TLR6 was reported to associate with TLR2 and recognize the mycoplasmal lipopeptide [11]. Interestingly, it is reported that TLR6 enhanced the response of TLR2, whereas TLR1 inhibited it [12]. In the present study, transcripts of TLR1 and TLR6 were detected in platelets. The interaction among these TLRs deserves further investigation.

The ability of infectious agents to promote atherosclerosis has been demonstrated experimentally as well as clinically. High rates of *C. pneumoniae* were detected in coronary and aor-

tic atheroma from autopsy or atherectomy materials. The implication of infection is supported by the clinical findings that high titers of candidate pathogens are associated with increased cardiovascular events. Recently, Naghavi et al. [13] reported that infection with influenza virus promoted the intimal proliferation and the formation of platelet thrombosis in apoE knockout mice. Although the precise mechanisms linking infection and vascular diseases are still unclear, it is speculated that components of pathogens such as LPS, lipopeptide, or peptidoglycan activate various cell types, including platelets, through TLRs, and that inflammatory cytokines generated by these activated cells mediate the progression of atherosclerosis as well as the enhancement of coagulability and thrombogenicity.

There is a report indicating that the expression of TLRs is related to cellular differentiation. TLRs, TLR1 to TLR9, were expressed in osteoclast precursors, whereas only TLR2 and TLR4 were prominently expressed in mature osteoclasts [14]. In the present study, the expressional patterns of TLRs in platelets were different from those in Meg-01, leading to the speculation that the differentiation from magakaryocytes to platelets modulates the expression of TLRs. Further investigation is needed to clarify the regulatory mechanism of TLR expression.

Previous reports indicate that platelets have receptors for bacterial antigen and HSP, and their interaction modified dendritic cell maturation [15]. Our findings explain, in part, these observations; however, their physiological and pathophysiological roles of platelet TLRs need further investigation.

In summary, we demonstrated that human platelets expressed a family of TLRs, and our findings indicated that platelets might recognize antigens directly via TLR for the first time. Given the importance of inflammatory responses, it is speculated that platelet TLRs are key molecules linking infection and vascular disease.

### Acknowledgements

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# Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis

Seinosuke Kawashima, Mitsuhiro Yokoyama

*Abstract*—Atherosclerosis is associated with an impairment of endothelium-dependent relaxations, which represents the reduced bioavailability of nitric oxide (NO) produced from endothelial NO synthase (eNOS). Among various mechanisms implicated in the impaired EDR in atherosclerosis, superoxide generated from dysfunctional eNOS has attracted attention. Under conditions in which vascular tissue levels of tetrahydrobiopterin (BH4), a cofactor for NOS, are deficient or lacking, eNOS becomes dysfunctional and produces superoxide rather than NO. Experimental studies in vitro have revealed that NO from eNOS constitutes an anti-atherogenic molecule. A deficiency of eNOS was demonstrated to accelerate atherosclerotic lesion formation in eNOS knockout mice. In contrast, eNOS overexpression with hypercholesterolemia may promote atherogenesis via increased superoxide generation from dysfunctional eNOS. Thus, eNOS may have 2 faces in the pathophysiology of atherosclerosis depending on tissue BH4 metabolisms. An improved understanding of tissue BH4 metabolisms in atherosclerotic vessels is needed, which would help in developing new strategies for the inhibition and treatment of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2004;24:998-1005.)

**Key Words:** endothelial nitric oxide synthase ■ atherosclerosis ■ tetrahydrobiopterin ■ superoxide ■ nitric oxide

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS), which requires $Ca^{2+}$/calmodulin, FAD, FMN, and tetrahydrobiopterin (BH4) as the cofactors.[1–4] In the vessels, NO is produced from the endothelium by constitutive expression of the endothelial isoform of NOS (eNOS), which is activated by mechanical stress such as blood shear-stress and stimulation with agonists such as bradykinin and acetylcholine. NO has a variety of functions, but its action as the endothelium-derived relaxing factor (EDRF) is the most important for the maintenance of vascular homeostasis.[5] An impairment of endothelium-dependent relaxations (EDR) is present in atherosclerotic vessels even before vascular structural changes occur and represents the reduced eNOS-derived NO bioavailability. Endothelial dysfunction as characterized by an impairment of EDR, and thereby reduced eNOS-derived NO bioactivity, is the critical step for atherogenesis. Among various mechanisms responsible for the impaired EDR, the increased NO breakdown by superoxide is important, and there is augmented production of superoxide in atherosclerotic vessels. Recently, it was revealed that under certain circumstances, eNOS becomes dysfunctional and produces superoxide rather than NO. The pathophysiological role of dysfunctional eNOS has attracted attentions in vascular disorders, including atherosclerosis. This review focuses on the role of dysfunctional eNOS on atherosclerotic vessels and refers to the possible role of dysfunctional eNOS on atherogenesis.

## Impaired EDR in Atherosclerosis

All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension, and smoking are associated with impaired EDR.[6–8] Although the underlining mechanisms of the reduced EDR are multifactorial, its most important cause is a derangements of the eNOS/NO pathway, which include the reduced activity and expression of eNOS, decreased sensitivity to NO, and increased degradation of NO by reaction with superoxide.[8] Regarding the expression of eNOS at the vessel wall, it may be reduced in advanced atherosclerosis, possibly because of reduced transcription and/or increased instability of eNOS mRNA caused by cytokines.[9] However, most animal models with atherosclerosis demonstrate the unchanged or rather augmented expression of eNOS, at least in early atherosclerosis, despite the presence of impaired EDR.[10,11]

The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia. Pro-atherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) and lysophosphatidylcholine, inhibit signal transduction from receptor activation to eNOS activation.[12–14] Hypercholesterolemic serum and LDL upregulate caveolin abundance, augments caveolin–eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells.[15,16] Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) and N-monomethylarginine (NMA) are also revealed to be involved in the mechanisms of reduced EDR in atherosclerosis.[17,18]

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The accelerated degradation of NO by increased superoxide from vessel wall is demonstrated as another important mechanism of the reduced EDR in hyperlipidemia and atherosclerosis.[8] Superoxide production from atherosclerotic vessels is augmented in human and animal models with atherosclerosis.[19–22] The endothelium is important as a source of superoxide production, and its denudation decreases superoxide production from vessels with atherosclerosis but has no effects in normal vessels without atherosclerosis.[19] Animal models of hyperlipidemia and atherosclerosis demonstrate an excess vascular superoxide flux that is linked to reduced NO bioactivity. As an evidence for the involvement of superoxide in the impaired EDR in atherosclerotic vessels, the restoration of EDR by antioxidants and superoxide dismutase has been shown.[20,23,24] In rabbit aortas with high-cholesterol diet-induced atherosclerosis, the impaired vasodilatory responses to acetylcholine and A23187 were restored by chronic treatment with polyethylene-glycolated SOD.[20] Antioxidants improve EDR in human and animal models with atherosclerosis.[25–27] In particular, vitamin C is effective in the restoration of EDR associated with most risk factors for atherosclerosis, including hypercholesterolemia, hypertension, diabetes mellitus, and smoking.[28–30]

## Superoxide Production From Vessels

Superoxide is produced by a variety of enzymes, including xanthine oxidase, cyclooxygenase, and NADPH oxidase. Among them, NADPH oxidase plays a major role in vascular cells.[31,32] In normal vessels, NADPH oxidase is present in adventitial fibroblasts. In atherosclerotic vessels, increased expression of subcomponents of NADPH oxidase has been found.[33–36] In the early stage of atherosclerosis, superoxide seems to be produced from NADPH oxidase localized in the endothelium; in advanced atherosclerosis, vascular smooth muscle cells serve as the major source of NADPH oxidase-derived superoxide.[37]

However, in vitro biochemical studies demonstrated that NOS can independently produce superoxide under certain conditions.[38–41] The catalytic mechanisms of NOS involve flavin-mediated electron transport from C-terminal–bound NADPH to the N-terminal heme center, where oxygen is reduced and incorporated into the guanidine group of L-arginine, giving rise to NO and L-citrulline. The eNOS-mediated superoxide generation is primarily regulated by BH4 availability. In the presence of suboptimal concentrations of BH4, activation of NOS leads to "uncoupling of NOS" and subsequent production of superoxide.[42–45] In "uncoupled NOS," electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to L-arginine; thereby, production of superoxide occurs. The ability of NOS to produce superoxide was first demonstrated in neuronal NOS (nNOS) and then extended to eNOS.[46,47] In the recombinant bovine eNOS, the heme moiety was identified as the main source for superoxide production.[45] In endothelial cells, a close link between cellular BH4 levels and NO synthesis was demonstrated, suggesting that an optimal concentration of BH4 is essential for NO production. The precise role of BH4 in the formation of NO is not completely understood, but it is postulated that BH4 donates electrons from the reductase domain to the ferrous–dioxygen complex in the oxygenase domain.[48,49] It is also demonstrated that addition of exogenous BH4 increases NO production and decreases superoxide production from endothelial cells.[40] As mentioned later in this article, there is an interaction between NADPH oxidase and eNOS, and it is thought that superoxide produced by NADPH is involved in the uncoupling of eNOS.

## Exogenous BH4 and eNOS Function

It has been demonstrated in clinical and animal studies that acute administration of BH4 improves endothelial dysfunction associated with hypercholesterolemia, atherosclerosis, hypertension, and cigarette smoking.[50–53] These data have been presented as evidence for the presence of "uncoupled eNOS," which produces superoxide rather than NO, leading to impaired EDR. Laursen et al clearly demonstrated the production of superoxide from eNOS.[54] In apolipoprotein E-knockout (apoE-KO) mice, they showed the increased vascular superoxide production from the endothelium, which was associated with impaired EDR. Incubation of vessels with sepiapterin, a precursor to BH4, improved EDR and decreased superoxide production.

As in the study of Laursen et al, sepiapterin has been shown to restore endothelial function in acute studies, however, sepiapterin may not always be effective when vessels are exposed to it for a long time.[55–57] Sepiapterin is an oxidized BH4 analogue that generates BH4 by enzymatic reduction of sepiapterin reductase and dihydrofolate reductase. It is reported that relatively long-term (6 hours) incubation of hyperlipidemic rabbit vessels with sepiapterin resulted in a further derangement of vasodilatory response to endothelium-dependent agonists.[58] In addition, incubation of canine cerebral arteries with high levels of sepiapterin was shown to reduce EDR significantly, despite an increase in vascular BH4 levels. It is revealed that a high concentration of sepiapterin can serve as a pro-oxidant and thereby oxidizes BH4 to dihydrobiopterin (BH2).[49] Sepiapterin may increase BH2 rather than BH4 in the tissues, and the increased BH4 levels potentially compete with BH4 for eNOS binding and worsen eNOS uncoupling.

## Vascular Pteridine Metabolism in Atherosclerosis

The presence of eNOS dysfunction as a mechanism of impaired endothelial function seems to be well-recognized now. However, only limited information is available on pteridine metabolism in the vessel wall in diseased states. In normal vascular tissue, >60% of total BH4 is present in the endothelium.[38,56] Endothelial cells from diabetic BioBreeding (BB) rats have a marked reduction in BH4 contents.[59] In the insulin resistance rat model induced by high-fructose diet, a modest reduction of BH4 levels in the aortas was associated with impaired EDR.[60] Furthermore, as compared with control rats, the levels of 7,8-dihydrobiopterin and biopterin, the oxidized form of BH4, were increased in the aortas of diabetic BB rats. Plasma BH4 levels were decreased in SHR with established hypertension.[61] Recently, it was reported that BH4 content was reduced and the content of oxidized forms of BH4 was increased in vessels from mice with deoxycorticosterone (DOCA)-salt hypertension.[62]

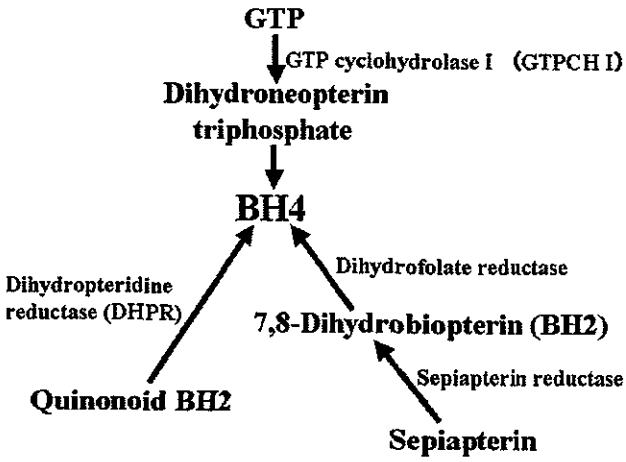


**Figure 1.** Scheme of BH4 biosynthesis.

Regarding hyperlipidemia and atherosclerosis, Vasquez-Vivar et al reported that BH4 levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits.[58] We have also demonstrated the BH4 levels in the aortas were decreased ≈50% in apoE-KO mice with marked hypercholesterolemia compared with normocholesterolemic wild-type mice.[63] In contrast, d'Uscio et al reported that in the aortas of apoE-KO mice with moderate hypercholesterolemia, BH4 levels were increased by ≈1.8-fold compared with those in control mice.[64]

The tissue levels of BH4 are determined by a balance between its production and degradation. As shown in Figure 1, BH4 is synthesized from GTP via a *de novo* pathway by the rate-limiting enzyme guanosine 5′-triphosphate (GTP) cyclohydrolase I (GTPCH I). Alternatively, the synthesis of BH4 can occur via a so-called salvage pathway, which uses BH2 as a substrate. Therefore, the reduced activity or expression of GTPCH I results in the decreased BH4 levels in the tissue. In the insulin resistance rat model, Shinozaki et al reported that GTPCH I activity in the aorta was significantly lower than that of control rats.[65] We also found the reduced vascular GTPCH I activity in apoE-KO mice fed a "high-cholesterol diet" (S Kawashima et al, article under submission). Although the activity of GTPCH I is augmented by inflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$, which are activated in atherosclerotic vessels, GTPCH I gene expression is reduced by oxidized LDL.[66–68] The mechanisms of the reduced GTPCH I activity in the aortas of apoE-KO mice are currently under investigation. However, the tissue levels of BH4 are also determined by their gradation, namely by their oxidation to 7,8-dihydrobiopterin.[38] Studies in vitro showed that BH4 can be rapidly oxidized by reactive oxygen species such as peroxynitrite.[62,69] In DOCA-salt hypertensive mice, it was demonstrated that superoxide produced by NADPH oxidase led to the formation of peroxynitrite in reaction with NO, which induced uncoupling of eNOS. With elevated oxidative stress, the oxidation of BH4 is enhanced and vascular tissue levels of 7,8-dihydrobiopterin increase. Therefore, the discrepant results in vascular BH4 levels in hyperlipidemia and atherosclerosis can be at least partly explained as caused by the difference in the levels of oxidative stress. The studies of Vasquez-Vivar et al and ours were conducted in animals with severe hypercholesterolemia, which is likely associated with high oxidative stress, and d'Uscio et al used animals with mild hypercholesterolemia.[58,63,64]

It has been proposed that in addition to the absolute availability of BH4, the ratio of BH4/7,8-dihydrobiopterin, the ratio of reduced and oxidized biopterin, is important for determining the rates of NO production versus uncoupled superoxide formation from eNOS.[60,70] Only the completely reduced (tetrahydro) form of biopterin supports NOS coupling of NADPH oxidation to NO synthesis. Partially oxidized analogues of BH4 enhance rates of superoxide formation from purified eNOS in the presence of saturating L-arginine concentration.[58] Therefore, oxidative stress causes "uncoupling" of eNOS not only by decreasing $BH_4$ levels but also by increasing the ratio of BH4/7,8-dihydrobiopterin. Then, generation of superoxide and peroxinitrite from dysfunctional (uncoupled) eNOS induces a further reduction of BH4 availability.[54]

The mechanism of the improvement of endothelial dysfunction by vitamin C includes its effects on BH4.[71,72,73] Vitamin C not only scavenges superoxide but also enhances NO synthase activity. Vitamin C increases the Kmax of NOS enzyme without any effects on L-arginine. It is postulated that, by its reductase capacity, vitamin C chemically stabilizes BH4, but a recent study of Kuzkaya et al showed that vitamin C reduces the intermediate product of the reaction between peroxynitrite and BH4, BH3, back to BH4.[74] Saturated ascorbic acid levels in endothelial cells are necessary to protect BH4 from oxidation to provide optimal condition for cellular NO synthesis.

## eNOS and Atherogenesis

As described, it seems to be established now that in hyperlipidemia and atherosclerosis, eNOS is dysfunctional and produces superoxide, which is implicated in endothelial dysfunction and impaired EDR. However, only limited information is available on how eNOS dysfunction affects atherogenesis. A substantial body of evidence in vitro suggests that eNOS-derived NO acts as anti-atherogenic molecule.[75–78] NO from eNOS inhibits leukocyte–endothelial adhesion, vascular smooth muscle migration and proliferation, and platelet aggregation, all of which are important steps in atherogenesis. Although the exact mechanisms are still not well defined and although there is still some controversy, chronic treatment with L-arginine, a substrate for NOS, inhibits atherosclerotic lesion formation in animal models of atherosclerosis, such as diet-induced atherosclerosis models of rabbits and LDL-receptor knockout mice.[79,80] On the contrary, NOS inhibitors like L-NAME significantly accelerate atherosclerotic lesion development, suggesting that inhibition of endogenous NO synthesis facilitates the progression of atherosclerosis.[81,82] Although little information is available for NOS gene transfer in atherosclerotic lesion formation, local adenovirus-mediated nNOS gene transfer to atherosclerotic carotid arteries rapidly reduces adhesion molecule expression and inflammatory cell infiltration in cholesterol-fed rabbits, indicating an anti-atherogenic role of endogenous NO in vivo.[83]

## eNOS Gene Engineered Mice as a Tool to Study the Role of eNOS in Atherogenesis

Recently, eNOS gene-engineered mice have been used to clarify more directly the role of eNOS/NO system on atherogenesis. Knowles et al first demonstrated that a genetic lack of eNOS resulted in enhanced atherosclerosis in association with hypertension in apo E/eNOS double-knockout mice, which were produced by crossing apo E-KO mice with eNOS knockout (eNOS-KO) mice.[84] Based on the positive correlation between blood pressure and the size of atherosclerotic lesions in aortas, they suggested that an elevation of blood pressure was responsible for the increases in the lesion size in these mice. More recently, their group reported that the hypertensive and atherogenic effects of eNOS deficiency in apoE-KO mice depended on the presence of endogenous sex hormones.[85] By use of gonadectomized apo E/eNOS double-knockout mice, they suggested that in the absence of sex hormones, eNOS had little effect on blood pressure and atherogenesis, although which hormones were responsible for these effects were not identified. Kuhlencordt et al also reported that eNOS deficiency promoted atherosclerosis in apo E/eNOS double-knockout mice.[86] Fed with a "Western-type" diet, apo E/eNOS double-knockout mice showed significant increases in aortic lesion area, which were associated with peripheral coronary atherosclerosis and aortic aneurysm formation. Later, they showed that these changes were not inhibited by hydralazine treatment, which reduced blood pressure to the levels comparable to those of apoE-KO mice and concluded that hypertension did not account for the accelerated atherosclerosis and aortic aneurysm formation.[87] Therefore, although the participation of elevated blood pressure and sex hormones remains to be further clarified, these reports indicated that the absence of endogenous eNOS-derived NO caused by the lack of eNOS gene accelerates atherosclerosis.

In contrast, recently Shi et al reported the paradoxical reduction of atherosclerotic lesion size in high-cholesterol diet-induced atherosclerosis in eNOS-KO mice compared with wild-type mice.[88] They fed mice a "high-cholesterol diet" for 12 weeks and then examined the lesion size in the aortic sinus. They found that eNOS-KO mice had much smaller aortic sinus lesions than did wild-type mice. L-NAME, the NOS inhibitor, reduced LDL oxidation by endothelial cells from wild-type mice but not from eNOS-KO mice. Based on these findings, they speculated that eNOS may contribute to the oxidation of LDL under the circumstance of hypercholesterolemia, and that the absence of eNOS-mediated LDL oxidation may lead to the reduction of atherosclerotic lesion formation in eNOS-KO mice. They did not refer to the mechanisms of eNOS-mediated LDL oxidation, but it is very likely that superoxide from the dysfunctional eNOS was involved in the mechanisms. This study raised the possibility that eNOS may act to accelerate atherogenesis under certain conditions such as hypercholesterolemia.

We have examined the effects of eNOS overexpression on atherosclerotic lesion formation with the use of transgenic (eNOS-Tg) mice that overexpress eNOS mainly in the endothelium.[89,90] We crossed eNOS-Tg mice with apo E-KO mice and fed them a "high-cholesterol diet." Unexpectedly, the atherosclerotic lesion areas were significantly larger in eNOS-overexpressing apo E-KO (apo E-KO/eNOS-Tg) mice compared with control apo E-KO mice.[63] In apoE-KO/eNOS-Tg mice, we found the presence of eNOS dysfunction, demonstrated by lower NO production relative to eNOS protein levels and enhanced superoxide production in the endothelium. We also found decreased vascular BH4 levels and increased 7,8-dihydrobiopterin levels in apo E-KO/eNOS-Tg mice. Therefore, chronic overexpression of eNOS does not inhibit, but rather accelerates atherosclerosis under hypercholesterolemia. In contrast, van Haperen et al also crossbred apo E-KO mice with another line of eNOS transgenic mice that they created and reported that atherosclerotic lesion size was reduced by eNOS overexpression.[91] Regarding the mechanisms, they cited the reductions of blood pressure and plasma cholesterol levels. In their study, eNOS overexpression was associated with 20- to 25-mm Hg reduction in mean blood pressure and a ≈15% decrease in plasma cholesterol levels. Although the difference in promoter by which eNOS was targeted to the endothelium is possibly involved, the discrepancy between their study and ours can be explained at least partly by a difference in the balance between NO and superoxide production from the endothelium. The increase of plasma cholesterol levels achieved by the "Western-type" diet that they used was much modest compared with that we achieved by feeding a "high-cholesterol" diet. Therefore, it is speculated that oxidative stress in the hypercholesterolemic mice of van Haparen et al was not increased as much as that in our model, although they did not describe oxidative stress and eNOS function in their model.

As mentioned, increasing evidence demonstrates the presence of eNOS dysfunction in hyperlipidemia and atherosclerosis. It is conceivable that dysfunctional eNOS may promote atherogenesis under certain pathological conditions that alter the balance between eNOS protein levels and tissue pteridine metabolism. Under pathological conditions with severe hyperlipidemia, there exists an increase in oxidative stress, which determines the extent of eNOS uncoupling and the resultant generation of superoxide from eNOS. In contrast to NO, superoxide is a pro-atherogenic molecule, and antioxidants have been demonstrated to inhibit atherosclerotic lesion formation.[92] The marked increase in superoxide in association with decreased NO production would promote atherogenesis. However, it is totally unclear whether acceleration of atherogenesis by dysfunctional eNOS occurs only under a specific condition with severe hypercholesterolemia or whether it may take place under other pathological conditions with elevated oxidative stress. The role of eNOS dysfunction on atherogenesis needs further studies (Table).

## Therapeutic Implication

It is important to define a therapeutic intervention for atherosclerosis from the standpoint of dysfunctional eNOS. Although the role of BH4 in the regulation of eNOS function is still not well understood, supplementation with exogenous BH4 is effective for the treatment of endothelial dysfunction. We found that supplementation with BH4 inhibits atherosclerotic lesion formation in apo E-KO mice.[63] Although the detailed mechanisms are unclear, it is conceivable that in

**Atherosclerotic Lesion Formation in eNOS Gene-Engineered Mice**

| Model of Atherosclerosis | Lesion Size | Reference |
|---|---|---|
| eNOS-KO Mice cross-breeding with apo E-KO mice *(caused by hypertension or sex hormones?)* | Augmented | 84, 85 |
| eNOS-KO Mice cross-breeding with apo E-KO mice (unrelated to hypertension) | Augmented | 86, 87 |
| eNOS-KO Mice, diet-induced atherosclerosis | Reduced | 88 |
| eNOS-Tg Mice, cross-breeding with apo E-KO mice | Augmented | 63 |
| eNOS-Tg Mice, cross-breeding with apo E-KO mice | Reduced | 91 |

addition to the simple removal of superoxide by its antioxidant effect, exogenous BH4 improved pteridine metabolism at the vessel wall and led to restore normal eNOS function. However, the effect of sepiapterin on atherosclerosis lesion formation has not been reported yet and it may not be effective. It is necessary to further clarify pteridine metabolism in the tissues, particularly in the vascular wall. GTPCH could be a rational target to augment endothelial BH4 and normalize eNOS activity in endothelial dysfunction. As for the strategy for augmenting GTPCH activity, GTPCH 1 gene transfer in vitro to human endothelial cells augments intracellular BH4 levels in association with an increase in enzymatic activity of eNOS to produce NO.[93] Recently, Alp et al generated transgenic mice overexpressing GTPCH I solely in the endothelium.[94] They reported that in the rat model of streptozotocin-induced diabetes, overexpression of GTPCH I augmented endothelial BH4 levels, improved the impaired vascular function, and decreased superoxide production from vessels. They suggested that a small increase in BH4 levels in the tissue was sufficient to maintain normal eNOS function. The beneficial effects of GTPCH I gene transfer was also confirmed by a very recent study of Zheng et al, who reported that ex vivo gene transfer of human GTPCH I to the aortic segments from DOCA-salt hypertensive rats reversed BH4 deficiency in the vascular tissue and improved EDR.[95]

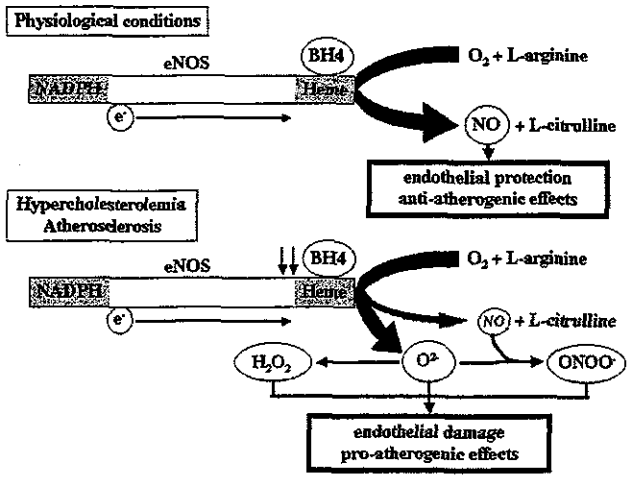


**Figure 2.** Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherogenesis. Under physiological conditions, tissue levels of BH4 are optimal for eNOS catalytic activity, and activation of eNOS generates NO and L-citrulline. NO generated by eNOS serves as an anti-atherogenic molecule. With hypercholesterolemia and atherosclerosis, when oxidative stress is increased, tissue levels of BH4 are reduced. In the presence of suboptimal levels of BH4, activation of eNOS leads to "uncoupling of NOS" with subsequent generation of superoxide rather than NO. Superoxide and, subsequently, peroxinitrite and hydrogen peroxide serve to damage endothelial cells and thus may promote atherosclerosis.

The anti-atherogenic property of drugs may also be evaluated from the standpoint of their effects on GTPCH. Statins are shown to increase eNOS protein levels in endothelial cells. Hattori et al demonstrated that statins increased GTPCH I mRNA in vascular endothelial cells and led to an elevation of intracellular BH4 levels.[96] These effects may be partly responsible for the anti-atherogenic action of statins.

However, simply augmenting NOS protein levels under pathological conditions such as hyperlipidemia may not increase NO but instead augment superoxide production, resulting in detrimental rather than beneficial effects. Therefore, a strategy directed at increasing NOS protein levels in association with maintaining its enzymatic activity is needed.[97,98] (Table 1, Figure 2)

## Summary

It is now being widely recognized that eNOS becomes dysfunctional and produces superoxide rather than NO in hyperlipidemia and atherosclerosis. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in atherosclerotic vessels. It seems to be widely accepted that eNOS with normal function inhibits atherogenesis by producing NO. However, although further studies are needed, recent reports on eNOS gene-engineered mice raised the possibility that dysfunctional eNOS may serve to promote atherosclerotic lesion formation under severe hypercholesterolemia (Figure 2). For the development of eNOS dysfunction, an abnormality in BH4 metabolism in vascular tissue seems to be fundamental. However, little is known about BH4 metabolism in vascular tissue, particularly in diseased states including atherosclerosis. We need an improved understanding of tissue BH4 metabolisms in atherosclerotic vessels in relation to conditions in which eNOS dysfunction develops. It would be intriguing to know whether dysfunctional eNOS participates in the pathogenesis of vascular disorders other than atherosclerosis.

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