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Regular Article

Expression of Toll-like receptors on human platelets

Rio Shiraki[a], Nobutaka Inoue[a,*], Satoru Kawasaki[b], Asumi Takei[b], Makoto Kadotani[b], Yoshio Ohnishi[b], Junya Ejiri[a], Seiichi Kobayashi[c], Ken-ichi Hirata[a], Seinosuke Kawashima[a], Mitsuhiro Yokoyama[a]

[a] *Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho, Chuo, Kobe 650-0017, Japan*
[b] *Department of Cardiovascular Medicine, Kobe Steel Hospital, Kakogawa, Japan*
[c] *Division of Surgical Pathology, Department of Biological Informatics, Kobe University Graduate School of Medicine, Kobe, Japan*

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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.

* Corresponding author. Tel.: +81-78-382-5846; fax: +81-78-382-5859.
E-mail address: nobutaka@med.kobe-u.ac.jp (N. Inoue).

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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.
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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

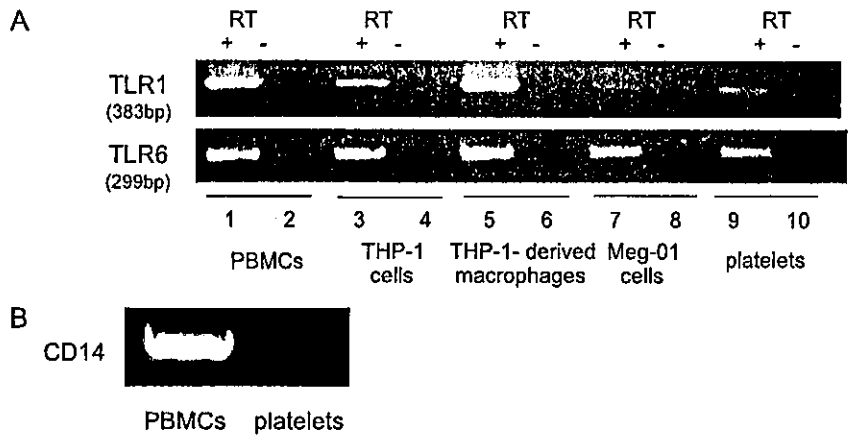

Fig. 1 (A) Expression of TLR1 and TLR6 mRNA in Meg-01 cells and human platelets evaluated by RT-PCR. The RT reaction was carried out with total RNA isolated from PBMCs, THP-1 cells, THP-1-derived macrophages, Meg-01 cells and human platelets (RT+). Both TLR1 (383 bp) and TLR6 (299 bp) mRNA were expressed 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

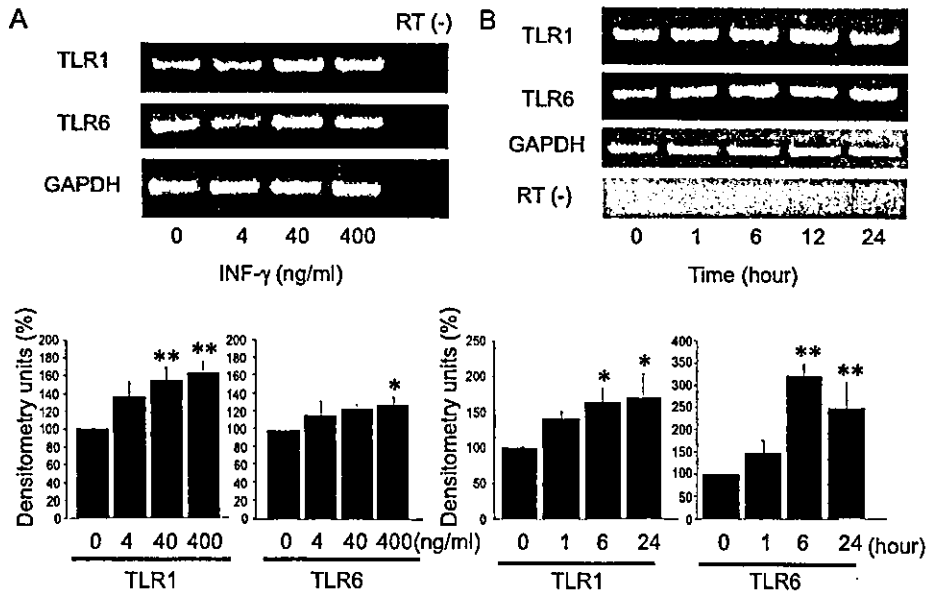

Fig. 2 Effect of IFN-γ on the expression of TLR1 and TLR6 mRNA in Meg-01 cells. The cells were treated with IFN-γ at various concentrations (0–400 ng/ml, A) and for various times (0–24 h, B). The RT-PCR analysis of GAPDH expression was used as control. The graph depicts the mean ± S.E.M. of three independent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

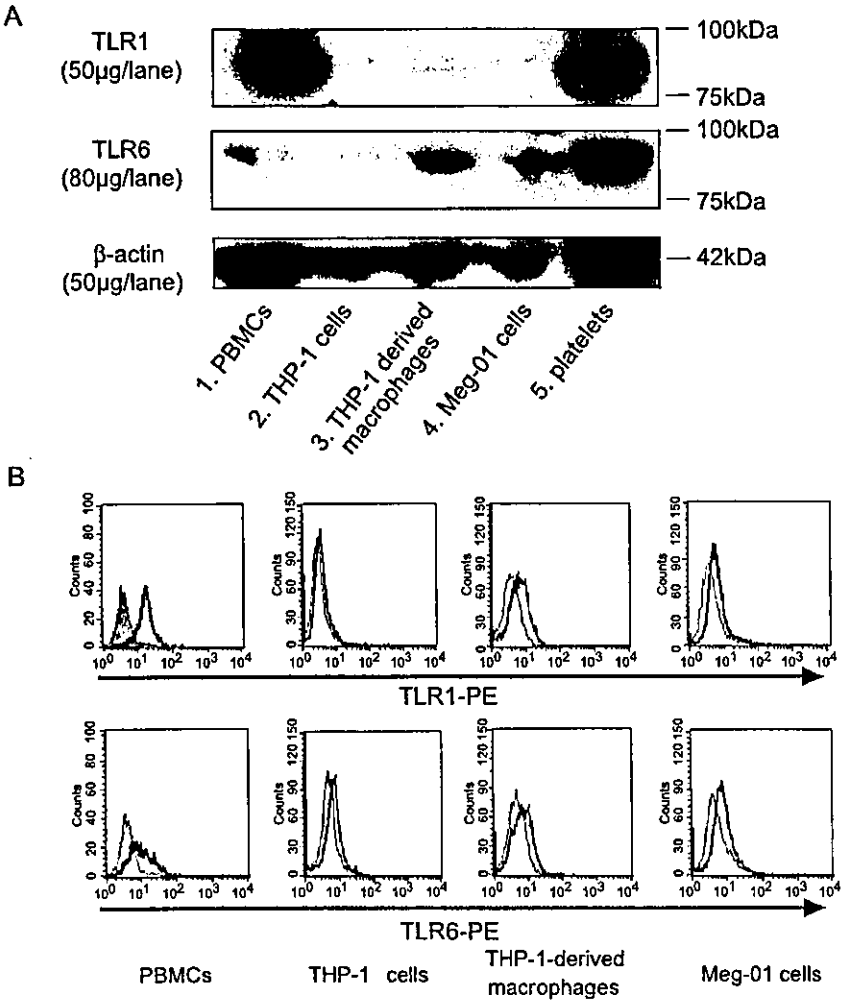

Fig. 3 (A) The protein expression of TLR1 and TLR6 detected by Western blotting. PBMCs are served as a positive control. The cell homogenates were separated on 10% polyacrylamide gels and immunoblotted with anti-TLR1 or anti-TLR6 antibodies. In human platelets, TLR1 and TLR6 proteins are expressed at 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.

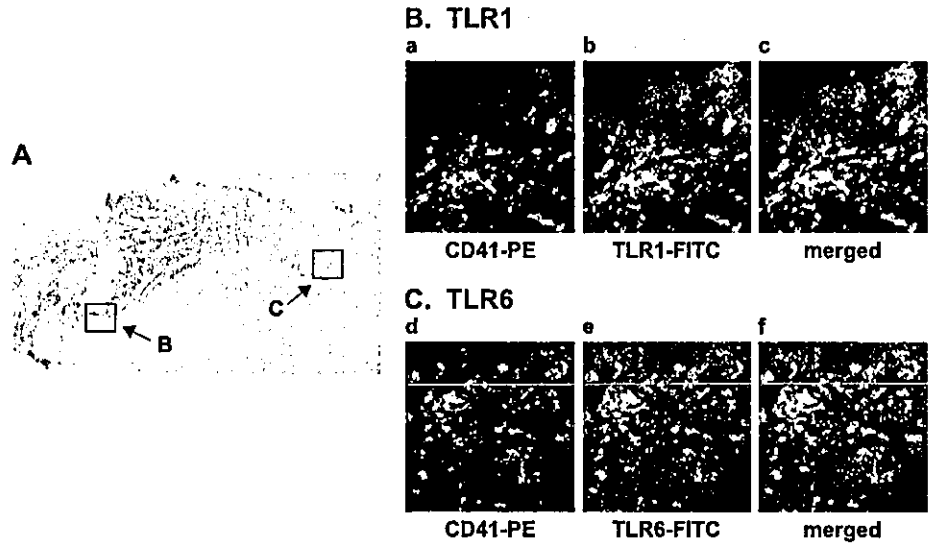

Fig. 4 Immunohistochemical analysis of TLRs in human coronary thrombi obtained from the patients of acute coronary syndrome. Double immunofluorescence was performed with CD41, a marker of platelets and anti-TLR1 (B: upper three panels) or anti-TLR6 (C: lower three panels) antibodies. Red fluorescence 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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