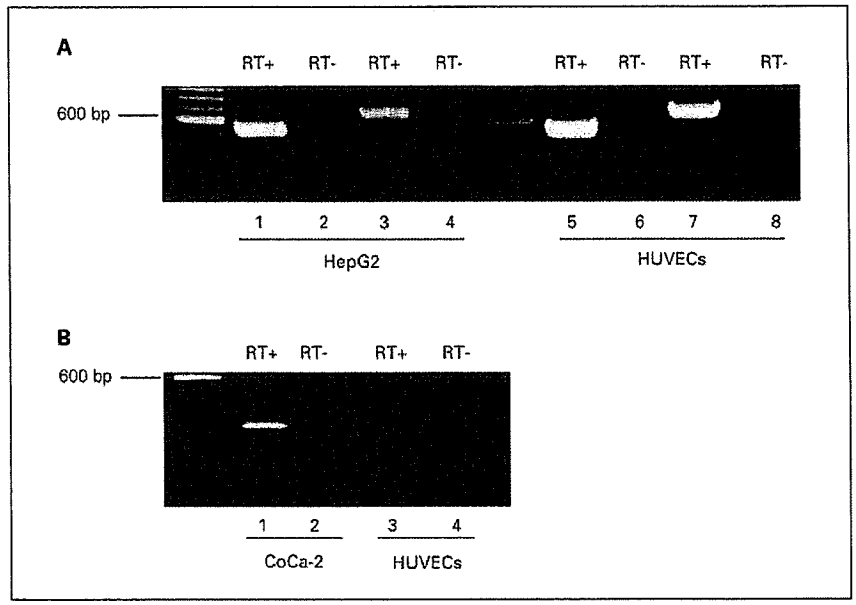


**Fig. 2.** RT-PCR demonstrating expression of SVCT-2 and SVCT-1 mRNA in HUVECs. The RT reaction was carried out with total RNA isolated from HepG2 cells, HUVECs and CoCa-2 cells. The RT product was used as a template for PCR amplification. The amplified DNA was separated on 1% agarose gel with ethidium bromide and visualized under UV. **A** By using two different primers for SVCT-2, the RT-PCR products corresponding to 517 bp (lanes 1 and 5) and 667 bp (lanes 3 and 7) were clearly detected. No RT-PCR product was obtained in the negative control where RT was not performed (lanes 2, 4, 6 and 8). **B** By using primers for SVCT-1, the RT-PCR product corresponding to 307 bp was detected in CoCa-2 cells, but not HUVECs. The results were representative of three different cell cultures in which the same results were obtained.

dent manner (fig. 1C). Kinetic analysis revealed that $K_m$ and $V_{max}$ values were 13.2 $\mu M$ and 0.13 nmol/min/mg, respectively. Next, we examined whether nonlabeled AA could competitively inhibit the uptake of $^{14}$C-AA. As shown in figure 1D, the Eadie-Hofstee plot demonstrated that the uptake of $^{14}$C-AA into HUVECs was inhibited by coincubation with nonlabeled AA in a competitive manner, indicating that AA was transported into HUVECs by some specific transport mechanism, not by simple diffusion.

*Uptake of AA by HUVECs Is Not Dependent on Extracellular Glucose*

Previous investigations have indicated that GLUT mediates the transport of DHAA, but not of AA. Thus, $^{14}$C-AA might be auto-oxidized to radiolabeled DHAA in the course of experiments, then transported into cells by GLUT. In our study, the concentrations of glucose in the transport buffer had no effect on the uptake of $^{14}$C-AA (fig. 1E).

*HUVECs Expressed mRNA of SVCT-2*

To examine whether SVCT-2 is expressed in HUVECs, RT-PCR was performed. Figure 2A is a representative result of RT-PCR of three independent cell cultures. By using two different primers for SVCT-2, RT-PCR products corresponding to 517 and 667 bp were clearly detected. No RT-PCR product was present in the negative control, where RT was not carried out. On the other hand, SVCT-1 mRNA was amplified in CoCa-2 cells, but not HUVECs (fig. 2B). These results suggested that human SVCT-2, but not SVCT-1, was expressed in HUVECs.

*Transfection of Antisense Oligonucleotide for SVCT-2 Suppressed the Uptake of AA*

To examine whether interference with the expression of SVCT-2 modulates the transport of AA, HUVECs were transfected with antisense oligonucleotides against SVCT-2. The uptake of AA was significantly suppressed by antisense oligonucleotide for SVCT-2 by 28% as compared with controls (fig. 3A). In contrast, the invert of the antisense oligonucleotide had no inhibitory effect on the uptake of AA, indicating that the uptake of AA into HUVECs is mediated by SVCT-2.

*Uptake of AA into HUVECs Is Significantly Inhibited by TPA and Phloretin*

Phloretin is reported to be a potent inhibitor of rat SVCT-1 and SVCT-2 [8], and there are several phosphorylation sites of protein kinase C (PKC) in human SVCT-2 [8]. Our examination of the effects of phloretin and TPA, a potent activator of PKC, on the uptake of AA by HUVECs showed that the uptake was strongly inhibited by approximately 71% at a concentration of 100 $\mu M$ phloretin (fig. 3B) and by 57% at a concentration of 100 n$M$ TPA (fig. 3C).

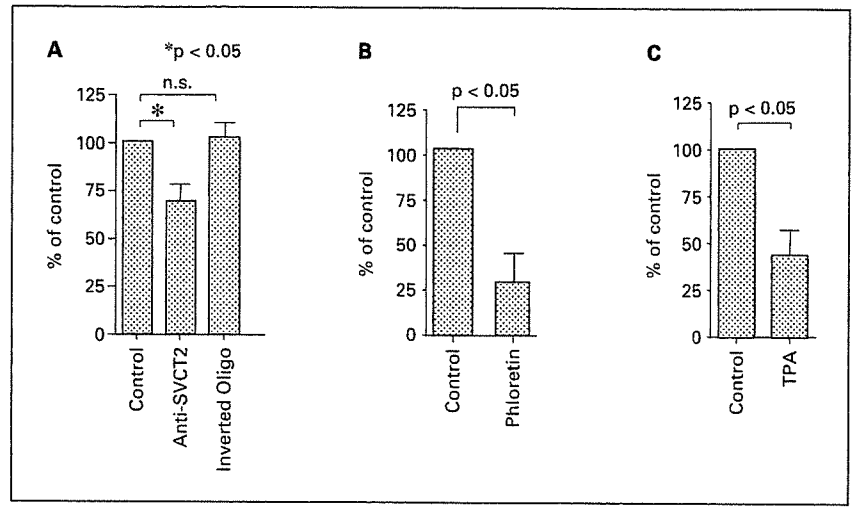


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**Fig. 3. A** Suppression of AA transport by an antisense oligonucleotide for SVCT-2. Antisense oligonucleotide was generated against nucleotides 387–411 of human SVCT-2 cDNA. The invert of the antisense oligonucleotide was used as negative control. After transfection, HUVECs were incubated for 48 h, and then the uptake of AA was determined. The uptake of AA was significantly suppressed by antisense oligonucleotide for SVCT-2 compared with controls. In contrast, the inhibitory effect of the invert of the antisense oligonucleotide was not significant. The results were obtained from nine wells of three different cell cultures. n.s. = Not significant. **B**, **C** Inhibitory effects of TPA (**B**) and phloretin (**C**) on sodium-dependent AA uptake. To examine the effects of TPA (100 n*M*) and phloretin (100 μ*M*), HUVECs were preincubated with these drugs for 30 min before the uptake study. After incubation, the activity of AA transport was assessed. The results were obtained from nine wells of three different cell cultures.

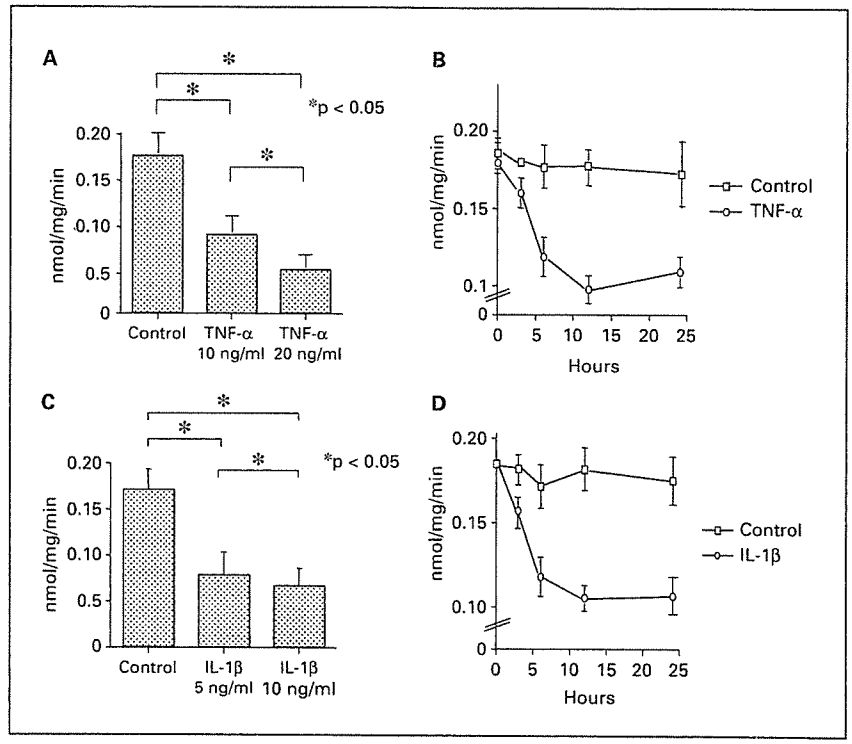


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**Fig. 4.** Inhibitory effects of TNF-α (**A**, **B**) and IL-1β (**C**, **D**) on AA transport into HUVECs. HUVECs were preincubated with TNF-α (**A**) or IL-1β (**C**) at the indicated concentrations for 24 h, and then the uptake studies were carried out. In time course experiments, the cells were preincubated with 20 ng/ml TNF-α (**B**) or 10 ng/ml IL-1β (**D**) for the indicated periods from 0 to 24 h, and then the uptake studies were carried out. TNF-α and IL-1β significantly suppressed the uptake of AA in a dose- and time-dependent manner. The results were obtained from nine wells of three different cell cultures.

*Inflammatory Cytokines TNF-α and IL-1β Inhibit the Uptake of AA*

Since the inflammatory process plays an important role in atherosclerosis, the effects of inflammatory cytokines were examined. TNF-α and IL-1β significantly suppressed the uptake of AA in a dose- and time-dependent manner (fig. 4).

## Discussion

In the present study, we demonstrated that HUVECs can take up AA in a dose- and time-dependent manner, and that this uptake was dependent on the concentration of extracellular $Na^+$. RT-PCR demonstrated that mRNA of SVCT-2, but not of SVCT-1, was expressed in HU-

VECs. Furthermore, the transfection of antisense oligonucleotide against SVCT-2 into HUVECs significantly suppressed the uptake of AA. These findings suggested that human SVCT-2 was functionally expressed in HUVECs. Since TPA strongly suppressed the uptake of AA, the transport activity of AA by SVCT-2 was negatively regulated by PKC. These results are largely consistent with a previous report with regard to AA uptake in human lens epithelial cells, in which SVCT-2, but not SVCT-1, is expressed [13]. Furthermore, inflammatory cytokines inhibited the uptake of AA into endothelial cells.

Although the precise mechanism and regulation of transport of AA into the cytosol are not fully determined, there are two potential pathways, i.e. GLUT and SVCT. Because of the structural similarity of glucose and DHAA, the oxidative form of AA, GLUT has been proposed as a potential transporter in various cell types. In the present study, however, the transport of AA by HUVEC was largely dependent upon extracellular $Na^+$ (fig. 1A) and was not suppressed by glucose (fig. 1E). These findings indicate that SVCT, and not GLUT, is the main transporter of AA into HUVECs. Transfection experiments with antisense oligonucleotides confirmed the involvement of SVCT-2 in the uptake of AA. The suppression of SVCT-2 by antisense oligonucleotide was significant, but the inhibitory effect was partial. It is, therefore, speculated that the protein turnover of SVCT-2 is so slow that the inhibitory effects of antisense oligonucleotide are not fully elicited. It has been reported that phloretin suppressed the uptake of AA via SVCT-expressing *Xenopus* oocytes [8]. In the present investigation, this reagent significantly inhibited AA uptake, indicating the involvement of SVCT in endothelial cells. Ek et al. [12] demonstrated that phloretin did not affect the accumulation of AA for 2 h in HUVEC, but they did not evaluate the initial rate of AA uptake.

It has been reported that corneal endothelial cells take up DHAA seven times as rapidly as AA [17]. In the case of endothelial cells of the blood-brain barrier, DHAA is transported by GLUT-1 and then immediately reduced to AA in the cytosol [7]. Taken together with our findings, there is a possibility that the transport system and metabolism of AA is tissue specific.

Oxidative stress induced by ROS promotes atherosclerosis through several different mechanisms, including oxidative modification of low-density lipoprotein (LDL), endothelial injury induced by oxidized LDL and the induction of redox-sensitive genes such as ICAM-1 and VCAM-1. AA exerts profound effects on these processes and is reported to suppress cell-cell adhesion in various pathophysiological situations [18, 19]. It effectively scavenges superoxide and other ROS, and also prevents the oxidative modification of LDL by scavenging ROS in an aqueous environment. While scavenging ROS, any ascorbyl radicals formed are reduced back to AA by chemical reduction or enzymatic reduction mediated by thioredoxin reductase or glutaredoxin reductase. We have demonstrated that these important reducing molecules are expressed in human coronary arteries, and their expression changes dynamically with the progression of atherosclerosis [20]. Whether or not SVCT is expressed in coronary arteries is still unknown; however, it is interesting to speculate that the SVCT-AA system might play a protective role against coronary atherosclerosis cooperatively with thioredoxin and glutaredoxin.

AA elicits profound effects on the redox state in endothelial cells. Smith et al. [21] reported that supplementation with AA resulted in a significant increase in the GSH to GSSG as well as the NAD(P)H to $NAD(P)^+$ ratio. AA also increases the intracellular concentration of (6R)-5,6,7,8-tetrahydro-*L*-biopterin (BH4), a cofactor of endothelial NOS [22]. Therefore, the improvement of endothelial dysfunction by AA is, in part, mediated by the restoration of the BH4/BH2 balance. Given the importance of the intracellular redox state, it is speculated that cytoplasmic AA taken up through SVCT plays a critical role in the control of cellular functions in vascular endothelial cells.

Atherosclerosis is a chronic inflammatory disease. In atherosclerotic vessels, activated macrophages and smooth muscle cells produce inflammatory cytokines that alter the growth and survival of vascular cells and modify the extracellular matrix. Among the various cytokines, TNF-α and IL-1β play a pivotal role in such processes. TNF-α has pleiotropic cellular effects and may induce cachexia, microvascular coagulation and hemodynamic collapse. De Keulenaer et al. [23] reported that TNF-α induced the activation of NADH/NADPH oxidase, an important source of ROS in the vasculature, indicating that this proinflammatory cytokine could enhance the oxidative stress in the vasculature. In the present study, we demonstrated that the inflammatory cytokines TNF-α and IL-1β suppressed the activity of SVCT. Since previous studies indicated the involvement of PKC in intracellular signal transduction by TNF-α and IL-1, the activation of PKC may mediate the inhibitory effects of these cytokines. Recently, Liang et al. [24] reported that the activation of PKC reduced the catalytic transport efficiency of SVCT-2, whereas it resulted in the intracellular redistribution of SVCT-1 from the cell surface to the intracellular membrane. In light of these findings, it is

interesting to speculate that the activity of SVCT-2 is attenuated by inflammatory cytokines via the activation of PKC in atherosclerotic vessels, and that the disturbed uptake of AA into cells might lead to the progression of atherosclerosis.

Our investigation demonstrated that SVCT-2 was functionally expressed in human endothelial cells, and that inflammatory cytokines negatively regulated its activities. Our findings might provide a new insight into understanding the therapeutic treatment effect of AA in cardiovascular diseases.

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Regular Article

# Expression of Toll-like receptors on human platelets

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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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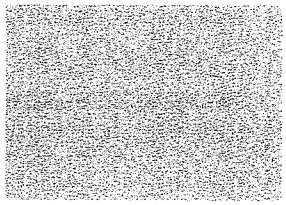

demonstrated that human platelets and Meg-01 cells expressed a family of TLRs for the first time, and our findings indicated that platelets might recognize antigens directly via TLRs. Our findings suggest a possibility that platelets have the ability to recognize the antigens via TLRs and that there 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

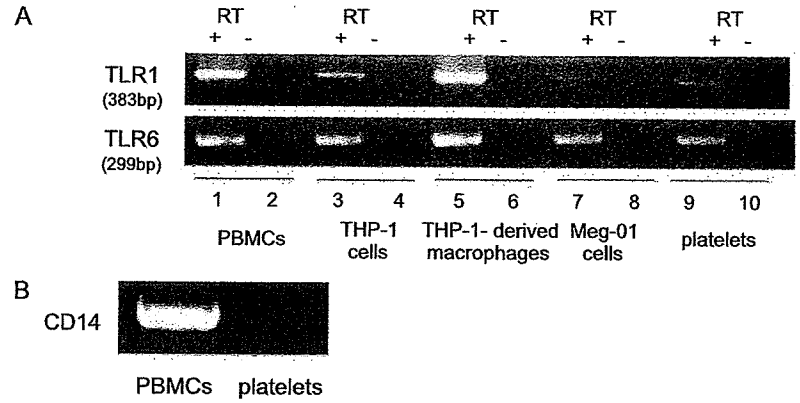


**Fig. 1** (A) Expression of TLR1 and TLR6 mRNA in Meg-01 cells and human platelets evaluated by RT-PCR. The RT reaction was carried out with total RNA isolated from PBMCs, THP-1 cells, THP-1-derived macrophages, Meg-01 cells and human platelets (RT+). Both TLR1 (383 bp) and TLR6 (299 bp) mRNA were expressed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

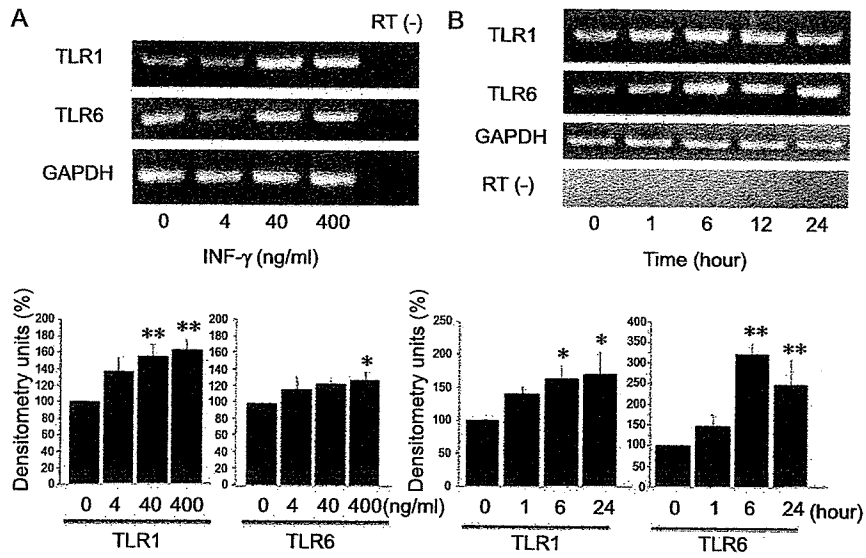


**Fig. 2** Effect of IFN-γ on the expression of TLR1 and TLR6 mRNA in Meg-01 cells. The cells were treated with IFN-γ at various concentrations (0–400 ng/ml, A) and for various times (0–24 h, B). The RT-PCR analysis of GAPDH expression was used as control. The graph depicts the mean ± S.E.M. of three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

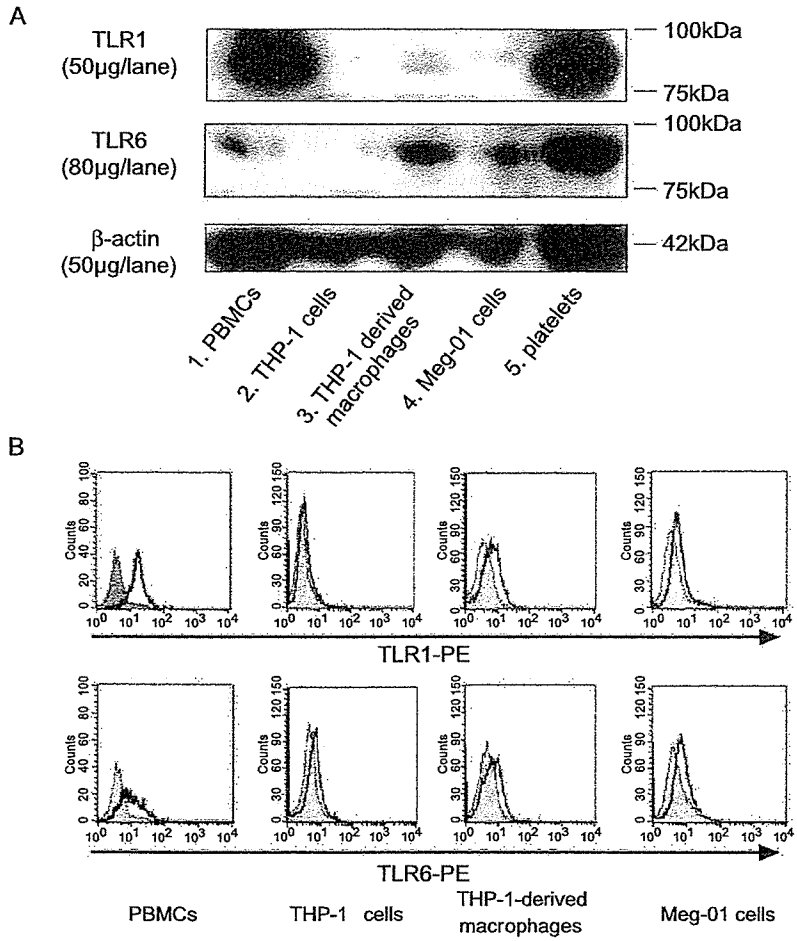


Fig. 3 (A) The protein expression of TLR1 and TLR6 detected by Western blotting. PBMCs are served as a positive control. The cell homogenates were separated on 10% polyacrylamide gels and immunoblotted with anti-TLR1 or anti-TLR6 antibodies. In human platelets, TLR1 and TLR6 proteins are expressed at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

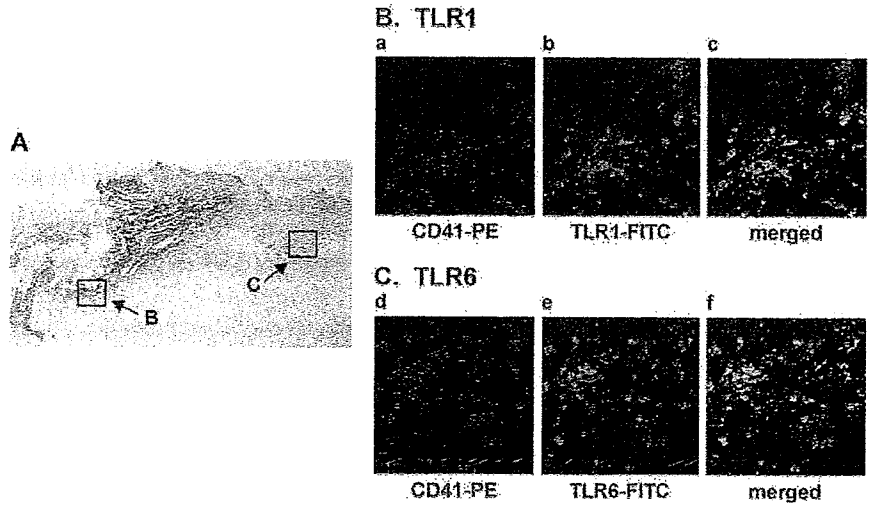


Fig. 4 Immunohistochemical analysis of TLRs in human coronary thrombi obtained from the patients of acute coronary syndrome. Double immunofluorescence was performed with CD41, a marker of platelets and anti-TLR1 (B: upper three panels) or anti-TLR6 (C: lower three panels) antibodies. Red fluorescence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

## A POTENTIAL ROLE IN INFLAMMATION*

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**Endothelial lipase (EL), a new member of the lipoprotein lipase gene family, plays a central role in high density lipoprotein metabolism. Previous studies indicated that EL is expressed in endothelial cells, macrophages, and smooth muscle cells in atherosclerotic lesions in human coronary arteries. However, the functional role of EL in the local vessel wall remains obscure. In this study, we evaluated the ability of EL to modulate monocyte adhesion to the endothelial cell surface. EL mRNA and protein levels were markedly increased in tissues of the mouse model of inflammation induced by lipopolysaccharide injection. Adhesion assays *in vitro* revealed that overexpression of EL in COS7 or Pro5 cells enhanced monocyte bindings to the EL-expression cells. Heparin or heparinase treatment inhibited EL-mediated increases of monocyte adhesion in a dose-dependent manner. Moreover, *ex vivo* adhesion assays revealed that the number of adherent monocytes on aortic strips was significantly increased in EL transgenic mice and decreased in EL knock-out mice as compared with wild-type mice. These results suggest that EL on the endothelial cell surface can promote monocyte adhesion to the vascular endothelium through the interaction with heparan sulfate proteoglycans. Thus, the up-regulation of EL by inflammatory stimuli may be involved in the progression of inflammation.**

The lipoprotein lipase gene family plays a crucial role in the lipid metabolism for the regulation of circulating lipoprotein levels and affects the process of atherosclerotic vascular diseases (1–4). The lipoprotein lipase gene family includes lipoprotein lipase (LPL)[1] and hepatic lipase (HL), both of which are synthesized and secreted by non-endothelial cells. We and other groups have reported a new member of the lipase gene family, endothelial lipase (EL). EL is mainly synthesized and secreted by vascular endothelial cells (5, 6). Previous studies have demonstrated that EL showed preferential substrate specificity for high density lipoprotein (HDL) (7). EL knock-out mice (*LIPG*−/−) showed an elevated plasma HDL-cholesterol (HDL-C) level. In contrast, human EL transgenic mice (h*LIPG*Tg) or overexpression of human EL in mice by adenovirus vectors showed decreased plasma HDL-C and apoA-I levels (6, 8). These data suggest that EL is a determinant of HDL levels in mice.

In addition to their functional role in lipoprotein metabolism at the endothelial cell surface, lipases possess non-enzymatic functions in the vascular wall. LPL within the vessel wall increases lipoprotein retention within the subendothelial cell matrix (9, 10) and in the aortic segment (11, 12). LPL can also enhance monocyte adhesion to endothelial cells (13, 14). The local expression of LPL in the vascular wall has been implicated in the accumulation of lipoproteins and the acceleration of atherosclerosis progression through this bridging function (15). Macrophage-derived LPL in the vessel wall is known to act as a proatherogenic molecule, determining susceptibility to atherosclerotic lesion formation (16, 17).

EL expression is regulated under various conditions. Inflammatory cytokines increased EL expression and enzymatic activity in cultured endothelial cells (18, 19). The EL expression is also detected in macrophages and smooth muscle cells within the atheromatous plaque of human coronary arteries (20). Interaction between the vessel wall and circulating monocytes is central for the initiation of atherosclerosis. Enhanced adhesion of monocytes to the vascular endothelium is believed to represent one of the earliest events in atherogenesis. Because of its expression in vessel wall and up-regulation by inflammatory cytokines, EL is likely to modulate the development of the atherosclerotic process. The aim of this study was to examine whether EL expression is regulated under inflammatory conditions *in vivo* and to clarify whether EL fulfills a "bridging function" between endothelial cells and monocyte/macrophages. In this study, we demonstrated that EL enhances human monocyte adhesion to the vascular endothelium and that EL expression is strongly up-regulated in the mouse systemic inflammation model. Our findings would further expand the functional role of EL in the vascular wall and provide new insights into endothelium-lipoprotein/blood cell interaction.

### MATERIALS AND METHODS

*Cell Culture*—THP-1, U-937, and COS7 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the manufacture's recommendations. COS7 cells that stably overexpress c-Myc-tagged human EL were generated as described

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[1] The abbreviations used are: LPL, lipoprotein lipase; EL, endothelial lipase; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; HL, hepatic lipase; hEL, human EL; h*LIPG*Tg, hEL transgenic mice; hLPL, human LPL; HSPG, heparan sulfate proteoglycan; *LIPG*−/−, EL knock-out mice; LPS, lipopolysaccharide; WT, wild-type.

previously (5). To generate COS7 cells that stably overexpress human LPL with an epitope tag (c-Myc) at the carboxyl terminus, reverse transcription PCR was performed with the RETROscript first strand synthesis kit (Ambion, Austin, TX), using the primers 5′-GGGAATTC-CCCACCATGGAGAGCAAAGCCCTGCTCC-3′ (forward) and 5′-GGTC-TAGACTCGAGTCACAGATCTTCTTCGGAGATAAGCTTCTGTTCGC-CAGACTTCTTCAGAGACTTGTC-3′ (reverse). The c-Myc-tagged LPL cDNA was cloned into the EcoRI-XhoI site of the pcDNA3.1 vector (Invitrogen) and sequenced by the dideoxy method. The c-*myc*-LPL expression construct was transfected into COS7 cells with Lipofectamine (Invitrogen). The cells were selected in the presence of 500 μg/ml G418. Levels of EL and LPL protein were determined by Western blotting using anti-c-Myc monoclonal antibodies (MBL, Nagoya, Japan). A mouse yolk sac endothelial cell line, Pro5 (21), was transfected with human EL cDNA using the Lipofectamine reagent and then selected in the presence of 500 μg/ml G418. The EL expression in the transfectants was determined by Western blotting using anti-human EL monoclonal antibodies (22). Mock (pcDNA3.1 vector)-transfected cells were used as a control.

*Mouse Model of Endotoxemia*—Male C57BL/6 mice (~20–25 g) were obtained from the Japan Charles River (Osaka, Japan). h*LIPG*Tg and *LIPG*−/− mice were described previously (8). A mouse model of lipopolysaccharide (LPS)-induced endotoxin shock was generated as described previously (23, 24). Briefly, mice were injected with LPS (50 mg/kg, intraperitoneal) from *Escherichia coli*, serotype 055:B5 (Sigma). Control animals were injected with normal saline (vehicle). Twelve or twenty-four hours later, mice were sacrificed with an overdose of pentobarbital, and tissues were excised for mRNA or protein extraction. In a set of experiments for lipid analysis, mice were injected with heparin (100 units/kg; Sigma). Thirty minutes later, whole plasma was obtained by cardiac puncture. Post-heparin plasma was obtained after centrifugation at 3000 × *g* for 10 min at 4 °C. Concentrations of total cholesterol, triglyceride, HDL-C, and phospholipid in the mouse plasma were determined using commercial kits (WAKO, Tokyo, Japan) (8). All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine.

*Northern Blot Analysis, RNase Protection Assay, and Immunoblot Analysis*—Total RNA was extracted from mouse tissues and cultured cells using the Isogen reagent (Nippon Gene, Tokyo). Northern blot was performed using a $^{32}$P-labeled mouse EL cDNA probe as described previously (5). For the RNase protection assay, cDNA fragments of human and mouse EL were obtained by reverse transcription PCR using the primers 5′-AGCTCTTGCTGCCTCTCTTG-3′ and 5′-TGA-CAGCCTTCTACACAGGG-3′ for human EL and 5′-TGATGGTTGCTA-CAGTGCTC-3′ and 5′-ACTACTAAAGGGTGTCTCGG-3′ for mouse EL. The cDNA fragments were cloned into the pCR II vector (Invitrogen) and linearized with BamHI. [$^{32}$P]UTP-labeled antisense riboprobe was synthesized with T7 RNA polymerase, and RNase protection assays were performed as described (25). Protection of human EL transcripts resulted in a labeled fragment of 203 nucleotides, and protection of mouse EL transcripts resulted in a labeled fragment of 144 nucleotides. Expression of cyclophilin was used for the housekeeping gene. Protection of cyclophilin transcripts resulted in a labeled fragment of 103 nucleotides. For immunoblot analysis, mouse tissues were homogenized in lysis buffer (10 mmol/liter Tris/HCl (pH 7.4), 150 mmol/liter NaCl, 2 mmol/liter $CaCl_2$, 1% Nonidet P-40, 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride, 40 units/ml aprotinin, and 15 g/ml leupeptin). Western blotting was performed using anti-EL polyclonal antibodies (26).

*In Vitro Monocyte Adhesion Assay*—COS7 or Pro5 cells were propagated on 6-well culture plates to form confluent monolayers. For monocyte labeling, THP-1 or U-937 cells were suspended in phosphate-buffered saline (1 × 10$^6$/ml) containing 1 μM calcein-AM (Molecular Probes, Eugene, OR) and incubated for 15 min at 37 °C. Labeled THP-1 cells were washed two times with phosphate-buffered saline and suspended in Hanks' buffered salt solution. THP-1 or U-937 cells (5 × 10$^5$/ml) were then added to monolayers of COS7 or Pro5 and incubated for 60 min with gentle rotation. Unbound cells were removed by gently washing with Hanks' buffered salt solution, and the number of binding monocytes was counted under fluorescent microscopy. In some experiments COS7 cells were incubated with monocytes in the presence of heparin (1–5 units/ml) to remove EL bound to their surfaces. To disintegrate cell surface heparan sulfate proteoglycans (HSPGs), COS7 and/or monocytes were treated with 1 unit/ml heparinase I for 30 min at 37 °C prior to the adhesion assay.

*Ex Vivo Monocyte Adhesion Assay*—Thoracic aorta was isolated from h*LIPG*Tg, *LIPG*−/−, and age-matched C57BL/6 (WT) mice. The surrounding tissue was gently cleaned. The aortas were opened longitudinally and fixed with fine needles on a slide glass. The *ex vivo* adhesion assay was performed as described previously (27). Cell suspension of fluorescein-labeled THP-1 cells (5 × 10$^5$/segment) was added on the aortic strip and incubated for 20 min at 37 °C. After non-adherent cells were gently washed with phosphate-buffered saline, the number of adherent cells was counted under fluorescence microscopy.

*Statistical Analysis*—Results are expressed as mean ± S.E. for the indicated number of experiments. The significance of variability among the experimental group means was determined by one-way analysis of variance followed by Bonferroni's test for samples. The level of statistical significance was set at $p < 0.05$.

## RESULTS

*EL Expression Is Up-regulated during Endotoxemia*—To examine whether EL expression is regulated *in vivo* by inflammation, a model of LPS-induced endotoxin shock was generated in C57BL/6 mice. EL mRNA expressions in mouse tissues were analyzed by Northern blotting. As shown in Fig 1*A*, EL mRNA levels were markedly up-regulated in tissues from LPS-injected mice compared with those from vehicle-treated mice. At 12 h after LPS injection, EL mRNA expression was significantly increased by 2.9-fold in the lung, 3.3-fold in the heart, 2.8-fold in the kidney, 2.1-fold in the liver, 2.5-fold in the spleen, and 3.3-fold in the aorta. We next investigated whether EL protein levels in these tissues are changed by the LPS treatment. At 24 h after LPS injection, the EL protein level in the aorta from LPS-treated mice was increased by 5-fold compared with that of vehicle-treated mice (Fig 1*B*). The increase of EL was detected after 12 h of LPS injection and peaked at 5-fold 24 h after injection (data not shown). Also, the LPS treatment significantly increased EL expression in the lung, heart, kidney, liver, and spleen by 2.1-, 2.6-, 4.2-, 1.5-, and 1.4-fold, respectively (Fig 1*B*). The secretion of EL into blood was analyzed by Western blotting using post-heparin plasma. Fig 1*C* showed that LPS administration increased the EL protein level in post-heparin plasma. Plasma EL level increased in a time-dependent manner, and after 24 h plasma levels of EL had increased 2-fold compared with those of the control groups (Fig 1*C*).

To examine whether the human EL promoter is regulated by LPS, h*LIPG*Tg mice in which human EL expression is driven by the human endogenous EL promoter were injected with LPS, and then mRNA levels of human EL, mouse EL, and cyclophilin were simultaneously determined by the RNase protection assay. As shown in Fig 1*D*, mouse EL levels in the tissues increased more in the LPS-injected mice than in the vehicle-treated mice. Also, the assay revealed a concomitant increase in human EL mRNA levels by LPS injection, suggesting that the human EL promoter is under the control of LPS *in vivo*.

To examine changes of lipid profiles by LPS treatment in our model, plasma levels of cholesterol, triglyceride, and phospholipid were measured (Table I). At 24 h after LPS treatment, the plasma total cholesterol level was increased by 31% ($p < 0.05$, $n = 12$) in WT mice. The triglyceride and phospholipid levels were also significantly increased by the LPS treatment by 321 and 25%, respectively. In contrast, the HDL-C level of WT mice was modestly but significantly decreased by 11% in response to LPS ($p < 0.05$, $n = 12$). In *LIPG*−/− mice the HDL-C level was not changed by the LPS treatment ($n = 12$). LPS administration increased plasma total cholesterol, triglyceride, and phospholipid levels in *LIPG*−/− mice.

*EL Acts as a Bridging Molecule*—To explore whether EL modulates interaction between the monocyte and the vessel wall, we examined the effects of EL overexpression on monocyte adhesion. Fibroblast cell lines, including the COS7 cell, did not express endogenous EL (data not shown). Therefore, we used COS7 cells constitutively overexpressing human EL (hEL-COS7) for cell adhesion assays utilizing two types of



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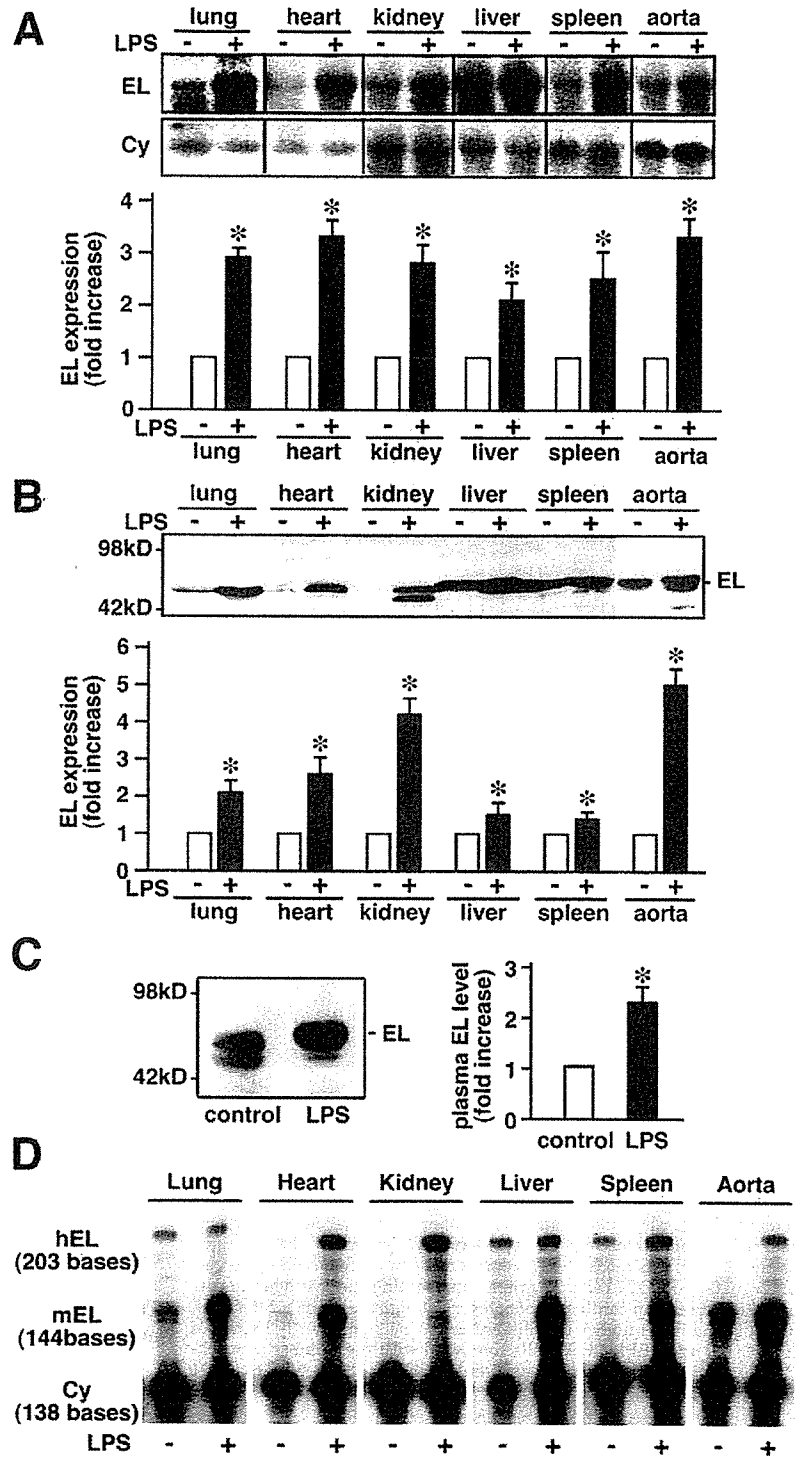


FIG. 1. **LPS up-regulates EL expression *in vivo*.** *A–C*, male C57BL/6 mice were treated with LPS (50 mg/kg, intraperitoneal). Mouse tissues were harvested after the indicated hours for EL mRNA analysis by Northern blotting (*A*). EL protein levels in mouse tissues (*B*) and post-heparin plasma (*C*) were analyzed by Western blotting using anti-EL polyclonal antibodies. LPS administration increases EL levels in various tissues and post-heparin plasma. *D*, RNase protection assay revealed that hEL expression and mouse EL (*mEL*) expression were increased by the treatment of LPS in EL transgenic mice. Values represent mean ± S.E. $*p < 0.05$ *versus* control. *Cy*, cyclophilin.

human monocyte/macrophage cell lines, THP-1 and U-937. The vector-transfected COS7 cell (mock-COS7) was used as a control. Confluent monolayers of the hEL-COS7 or mock-COS7 cells were incubated with THP-1 or U-937 cells, and the number of adherent monocytes was counted. As shown in Fig. 2, monocytes bound to the hEL-COS7 monolayer were significantly increased compared with those in mock-COS7; the number of THP-1 cells that bound to the hEL-COS7 and mock-COS7 monolayers was 78.9 ± 5.8 and 51.0 ± 2.6 cells/field, respectively ($p < 0.01$). Similar results were obtained with U-937 cells; the number of U-937 cells that bound to the hEL-COS7 and mock-COS7 monolayers was 47.4 ± 3.9 and 27.2 ± 3.4 cells/field, respectively ($p < 0.01$). These results indicate that EL overexpression can promote monocyte binding to the cell surface. It has been reported that LPL also promotes monocyte adhesion to the endothelial cell surface. To compare the abilities of these lipases in regard to monocyte adhesion, we generated COS7 cells stably overexpressing human LPL (hLPL-COS7) and employed the cell adhesion assay. The expression of EL and LPL in the transfectant was determined by

TABLE I
*Lipid profile of LPS-treated mice*

WT C57BL/6 and *LIPG−/−* EL knockout mice were treated with LPS or normal saline (vehicle). Concentrations of total cholesterol (Chol), triglyceride (TG), HDL-C, and phospholipid (PL) were determined by biochemical assays. Values represent mean ± S.E. ($n$ = 12 in each group).

| Mouse | Treatment | Chol | TG | HDL-C | PL |
|---|---|---|---|---|---|
| | | *mg/dl* | *mg/dl* | *mg/dl* | *mg/dl* |
| WT | Vehicle | 85.5 ± 5.5 | 38.2 ± 5.5 | 54.5 ± 2.1 | 156.3 ± 14.3 |
| WT | LPS | 112.1 ± 4.6[a] | 163.6 ± 16.2[a] | 48.2 ± 2.0[a] | 210.0 ± 8.3[a] |
| *LIPG−/−* | Vehicle | 130.9 ± 6.8[b] | 58.0 ± 3.9[b] | 74.4 ± 3.7[b] | 235.7 ± 23.6[b] |
| *LIPG−/−* | LPS | 191.8 ± 6.4[a] | 480.4 ± 97.3[a] | 71.9 ± 2.3 | 407.3 ± 14.2[a] |

[a] $p < 0.05$ *versus* vehicle-treated mice.
[b] $p < 0.05$ *versus* WT.

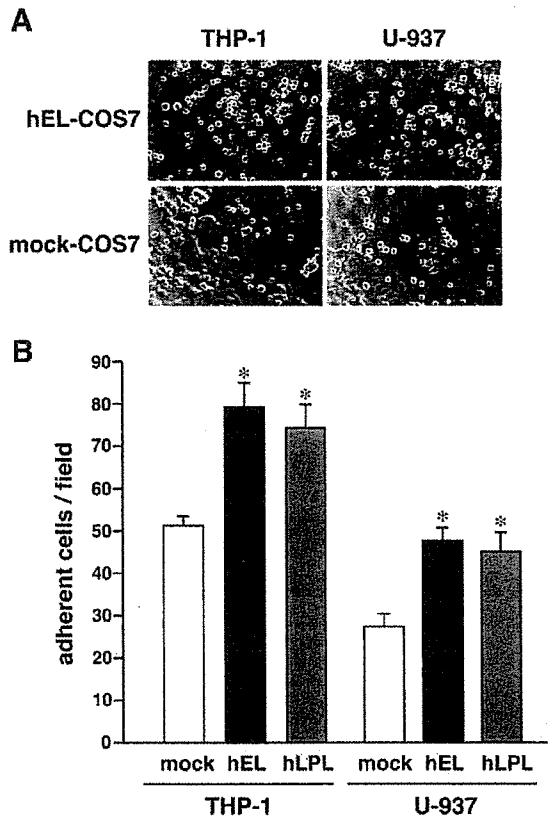


FIG. 2. **EL increases monocyte binding to COS7 cells.** Confluent monolayers of COS7 cells constitutively expressing hEL or hLPL were incubated with 5 × $10^5$/ml THP-1 or U-937 cells. Unbound cells were removed by gentle washes, and the number of binding monocytes was counted under microscopy. *A*, representative images of the adhesion assay. Control was mock-COS7, representing monocyte binding to vector-transfected COS7 cells. *B*, graph representing the average number of adherent cells on confluent monolayers at eight random ×200 fields, showing that the number of adherent cells was significantly higher in hEL-COS7 and hLPL-COS7 cells compared with the number in mock-COS7 cells (*mock*). Values represent mean ± S.E. *, $p < 0.01$ *versus* mock.

Western blot using an anti-c-Myc antibody. As depicted in Fig 2*B*, the number of monocytes bound to hLPL-COS7 was significantly higher than that bound to mock-COS7, *i.e.* by 1.5-fold ($p < 0.01$). When the protein level was standardized using the c-Myc antibody, there was no significant difference in the number of adherent THP-1 or U937 cells between hEL-COS7 and hLPL-COS7 monolayers (Fig 2*B*). Thus, it was suggested that these lipases have nearly equivalent abilities in regard to monocyte binding.

It has been demonstrated that other lipase members, including LPL and HL, require HSPGs to localize distribution to the luminal surface of the blood vessel wall. Therefore, we next examined the effect of heparin and heparinase on the monocyte adhesion to EL-transfected cells. The confluent monolayers of transfectants were incubated with THP-1 in the presence of heparin or vehicle. As shown Fig 3*A*, treatment with 5 units/ml heparin did not have any effect on monocyte binding to mock-COS7 cells. However, heparin treatment completely abolished the monocyte binding to hEL-COS7 cells. The inhibitory effect of heparin was dose-dependent, and significant inhibition was achieved at a concentration as low as 1 unit/ml (Fig 3*B*). Identical results were obtained with another monocyte/macrophage cell line, U-937 (Fig. 3*A*, *right*).

To confirm the involvement of HSPGs, we examined whether degradation of HSPGs by heparinase treatment affects monocyte binding to the EL-expressing cells. As depicted in Fig 4*A*, pretreatment of hEL-COS7 cells with 1 unit/ml heparinase I completely abolished monocyte binding. In contrast, heparinase did not affect the monocyte binding to mock-COS7 cells. These results indicate that increased cell binding between monocytes and EL-expressing cells is mediated by HSPGs. To clarify whether EL-mediated monocyte binding require HSPGs on the monocyte, THP-1 cells were pretreated with 1 unit/ml heparinase I for 30 min prior to the assay. Whereas heparinase I did not show significant effects on the THP-1 binding to mock-COS7 cells, treatment of THP-1 cells with heparinase I blocked the monocyte adhesion to hEL-COS7 (Fig 4*B*). Pretreatment of both COS7-monolayers and THP-1 cells with heparinase I did not show an additional inhibitory effect on the cell binding (Fig 4*B*). Thus, EL-mediated cell binding between monocytes and EL-expressing cells requires cell surface HSPGs on both the monolayer and the monocytes.

To verify the EL effect on cell adhesion in the other cell type, Pro5 cells were transfected with human EL cDNA. This cell line is considered to be an embryonic endothelial progenitor and expresses EL. The cells were grown to confluent monolayers, and the cell adhesion assays were performed using THP-1 cells. As is shown in Fig. 5, hEL overexpression resulted in a significant 52% increase in monocyte adhesion to the cell. These findings suggest that EL levels may modulate the binding activity of monocytes to the endothelial cell surface.

*Up-regulation of EL in the Vessel Wall Promotes Monocyte Adhesion*—To address whether EL regulates monocyte binding to the vessel wall, we evaluated monocyte binding to the blood vessel from h*LIPG*Tg mice and *LIPG−/−* mice by an *ex vivo* adhesion assay. h*LIPG*Tg mice were shown to overexpress human EL on the endothelial cell surface of thoracic aorta. The thoracic aortas were isolated from h*LIPG*Tg, *LIPG−/−*, or WT mice and incubated with fluorescein-labeled THP-1 cells. Unbound cells were gently washed, and the number of adherent cells was counted under fluorescent microscopy. The number of THP-1 cells that bound to the aortic strips from h*LIPG*Tg mice was increased 68% compared with that from WT mice. In contrast, the number of THP-1 cells that bound to the aortic strip from *LIPG−/−* mice was decreased compared with that in WT mice (Fig. 6).

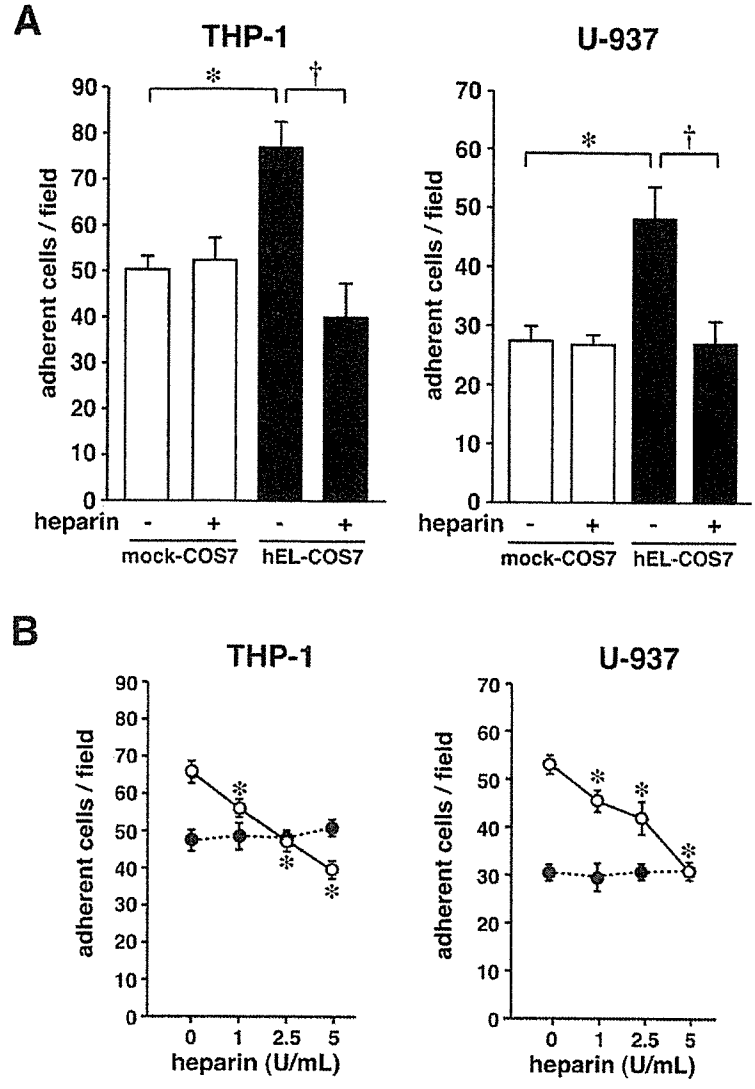


FIG. 3. **Heparin treatment inhibits monocyte binding to EL-expressing cells.** Confluent monolayers of COS7 cells constitutively expressing human EL (hEL-COS7) were treated with heparin (1–5 units/ml) or vehicle and incubated with THP-1 or U-937 cells. Control was mock-COS7, representing monocyte binding to vector-transfected COS7 cells. *A*, heparin treatment (5 units/ml) significantly diminished the augmented cell adhesion to hEL-COS7 cells. *, $p < 0.01$ *versus* mock without heparin treatment; †, $p < 0.01$ *versus* vehicle treatment. *B*, graphs showing the dose-dependent effect of heparin treatment on the cell adhesion to hEL-COS7 cells (*open circle*) or mock-COS7 cells (*closed circle*). Values represent mean ± S.E. *, $p < 0.01$ *versus* vehicle treatment.

## DISCUSSION

The adhesion of monocytes to the arterial endothelium is a common feature linking the inflammation and development of atherosclerosis (28–30). The present study demonstrated for the first time evidence that EL expression was strongly up-regulated by LPS stimulation *in vivo* and that EL may act as a bridging molecule between the vessel wall and circulating monocytes. Overexpression of EL in cultured monolayers enhanced the binding of monocytes to EL expression cells. The release of EL from the cell surface by heparin completely abolished the ability of EL to enhance monocyte adhesion. Degradation of HSPGs by treatment with heparinase also suppressed the EL-mediated monocyte adhesion. These data indicate that EL-mediated cell adhesion requires interaction with HSPGs. The bridging function of the lipase family has been extensively characterized in LPL. The present results indicate that the effect of EL on monocyte adhesion is nearly equivalent to that of LPL. Furthermore, the aortic endothelium from h*LIPG*Tg mice showed an increased binding to monocytes, and *LIPG*−/− mice showed decreased monocyte binding, indicating that the bridging function of EL is relevant *in vivo*.

Treatment of mice with LPS increased EL mRNA and protein levels in the aorta, lung, heart, kidney, liver, and spleen. EL up-regulation in mouse tissues was accompanied by an EL increase in post-heparin plasma. This is the first report demonstrating that EL expression was increased during acute inflammation *in vivo*. This up-regulation of EL by inflammation is one of the unique characteristics of EL, because the expression of LPL and HL is known to be down-regulated by inflammatory cytokines (31, 32). The physiological significance of increased EL expression in inflammation remains speculative. However, given that EL may act as a bridging molecule, we speculate that the increase of EL levels may play a role in the progression of inflammation by the recruitment of monocyte macrophages through its bridging function. In addition, it has been reported that inflammatory cytokines up-regulate EL enzymatic activities in vascular endothelial cells (19). During acute inflammation, affected organs require energy to repair damaged tissues. Damaged cells may require cholesterol for new membrane synthesis during cell repair and regeneration. Under inflammatory conditions, furthermore, angiogenesis occurs to supply blood flow to repair the tissue, and endothelial cell proliferation is essential for the process of angiogenesis. EL is known to be increased in tube-forming endothelial cells (5). Throughout these cellular responses to inflammation the increased expression of EL may supply fatty acids generated from triglyceride and the phospholipids of lipoproteins through its lipase activity as a fuel source to injury tissues.

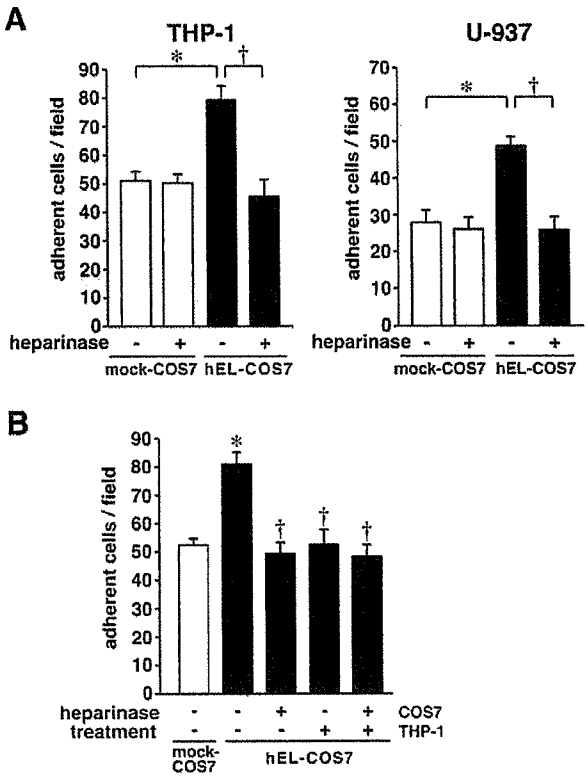


FIG. 4. **Heparinase treatment abolished the increased monocyte binding by EL.** *A*, confluent monolayers of COS7 cells constitutively expressing human EL (hEL-COS7) were treated with heparinase I (1 unit/ml) prior to the adhesion assay with THP-1 or U-937 cells. Control was mock-COS7, representing monocyte binding to vector-transfected COS7 cells. Values represent mean ± S.E. *, $p < 0.01$ *versus* mock without heparinase treatment; †, $p < 0.01$ *versus* vehicle treatment. *B*, confluent monolayers of hEL-COS7 cells and/or THP-1 cells were treated with heparinase I (1 unit/ml) prior to the adhesion assay. Values represent mean ± S.E. Treatment of COS7 or monocytes with heparinase I totally suppressed the ability of EL to enhance monocyte adhesion to hEL-COS7 cells, suggesting the involvement of EL though heparan sulfate proteoglycans. *, $p < 0.01$ *versus* mock; †, $p < 0.01$ *versus* vehicle treatment.

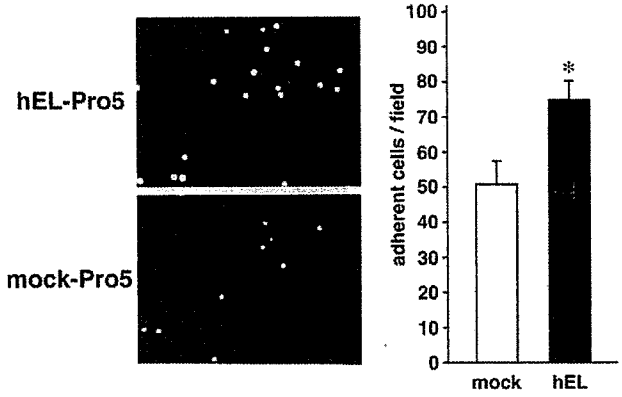


FIG. 5. **EL-overexpression in yolk sac cells enhanced monocyte adhesion.** Confluent monolayers of a yolk sac cell line (Pro5) constitutively expressing hEL were incubated with labeled 5 × $10^5$/ml THP-1 cells. Mock represents monocyte binding to the vector transfected COS7 cells. The number of binding monocytes was counted under microscopy. Values represent mean ± S.E. *, $p < 0.01$ *versus* mock

Although the acute increase of EL levels during the inflammation may be beneficial to the host defense, a prolonged induction of EL may result in undesirable consequences. Ath-

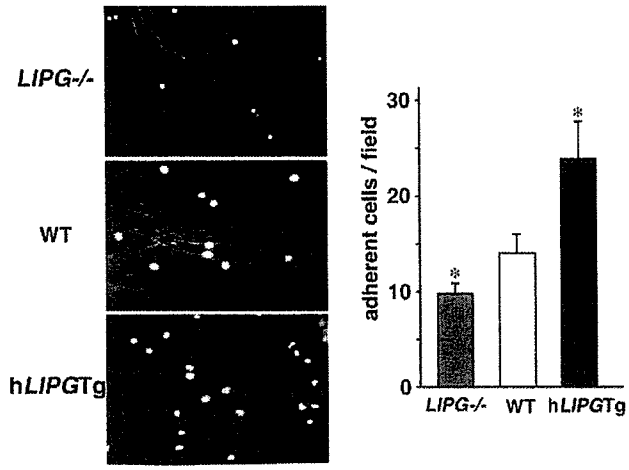


FIG. 6. **Gene dosage effect of EL expression in the vessel wall on monocyte binding.** Thoracic aortas were isolated from h*LIPG*Tg, *LIPG*−/−, and WT mice and incubated with fluorescein-labeled THP-1 cells (5 × $10^5$/segment). The number of adherent monocytes was counted under fluorescence microscopy. Values represent mean ± S.E. *, $p < 0.05$ *versus* WT.

erosclerosis is now considered to be a chronic inflammatory process (33). The earliest events in atherosclerosis include monocyte recruitment into subendothelial spaces. Previous studies demonstrated that EL is substantially expressed in infiltrating macrophages and smooth muscle cells as well as in endothelial cells within atherosclerotic plaques of human coronary arteries (20). Moreover, it has been reported that EL mediates the uptake and binding of HDL and regulates the selective uptake of HDL-associated cholesterol esters (34). Another study reported that EL mediates the binding and uptake of LDL as well as HDL (35). The increase of EL expression and its activity at the site of chronic inflammation may also increase the uptake of lipoprotein-associated cholesteryl ester into vascular cells. Taken together, EL induction in local and chronic inflammation may contribute to monocyte/macrophage recruitment and lipoprotein accumulation in the vascular wall. Thus, the present data imply that EL may modulate the progression of vascular diseases (36).

It has been shown that systemic inflammation can perturb lipoprotein metabolism and result in fundamental changes in the plasma concentrations of lipids and lipoproteins (37). In particular, severe infection or inflammation is associated with a decrease in the HDL-C level (38–40). Furthermore, chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus are also accompanied by reduced plasma HDL-C levels (41). In the present study, plasma HDL-C levels in WT mice are decreased by 11% in the LPS-injected mice compared with those in control mice. On the other hand, HDL-C levels in *LIPG*−/− mice were not changed with LPS treatment. Considering that the overexpression of EL decreases the plasma HDL-C level in mice (6, 8), the up-regulation of EL in the present study could, at least in part, account for the reduced HDL-C levels in inflammation. A previous study showed an increase in LPL and HL expression in *LIPG*−/− mice (42). It has been reported that a number of molecules, including secretary type phospholipase A2, LPL, and HL, are regulated in LPS-induced endotoxemia. We speculate that lipid profile in LPS-induced inflammation may be regulated by the net effect of these molecules. In this context, EL may have a local role in the pathophysiology of inflammation and atherosclerosis beyond its action on plasma lipid levels.

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# Transplantation of Cardiotrophin-1–Expressing Myoblasts to the Left Ventricular Wall Alleviates the Transition From Compensatory Hypertrophy to Congestive Heart Failure in Dahl Salt-Sensitive Hypertensive Rats

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**OBJECTIVES** We investigated whether autologous transplantation of skeletal myoblasts (MB) transferred with cardiotrophin-1 (CT-1) gene could retard the transition to heart failure (HF) in Dahl salt-sensitive (DS) hypertensive rats.

**BACKGROUND** Although MB is a therapeutic candidate for chronic HF, little is known about the efficiency of this strategy when applied in nonischemic HF. Cardiotrophin-1 has potent hypertrophic and survival effects on cardiac myocytes. We hypothesized that transplantation of CT-1–expressing myoblasts could provide cardioprotective effects against ventricular remodeling in DS hypertensive rats.

**METHODS** The DS rats were fed a high salt diet for 6 weeks and developed left ventricular (LV) hypertrophy at 11 weeks. At this stage, animals underwent MB to the myocardium with skeletal myoblasts transferred with CT-1 gene using retrovirus (transplantation of CT-1–expressing myoblasts [MB + CT], n = 31) or myoblasts alone (MB, n = 31). The sham group rats were injected with phosphate-buffered saline (n = 24).

**RESULTS** At 17 weeks, MB and MB + CT groups showed a significant alleviation of LV dilation and contractile dysfunction compared with the sham group. The degree of alleviation was significantly greater in the MB + CT group than the MB group (LV end-diastolic dimension: sham 7.06 ± 0.14 mm, MB 6.51 ± 0.16 mm, MB + CT 6.24 ± 0.07 mm; fractional shortening: sham 32.1 ± 1.4%, MB 38.5 ± 1.5%, MB + CT 43.2 ± 0.8%). Histological examination revealed that the myocyte size was 20% larger in the MB + CT group at 17 weeks than in the age-matched sham group. Upregulation of renin-angiotensin and endothelin systems during the transition to HF was attenuated by myoblast transplantation, and this effect was enhanced in the MB + CT group.

**CONCLUSIONS** Transplantation of skeletal myoblasts combined with CT-1-gene transfer could be a useful therapeutic strategy for HF. (J Am Coll Cardiol 2004;43:2337–47) © 2004 by the American College of Cardiology Foundation

Despite medical and surgical advances, heart failure (HF) is still a major cause of death. Because mature cardiac myocytes cannot re-enter the cell cycle and the adult heart lacks functional repair mechanisms, myogenic cell transplantation into the damaged myocardium is a promising approach to the treatment for end-stage HF (1). Recent experimental studies have demonstrated that intramyocardial skeletal myoblast transplantation improves cardiac function after myocardial infarction (2–5). Clinical trials with skeletal myoblast transplantation are also on the way (6,7). However, little is known about the efficiency of this strategy when applied in global HF of nonischemic causes (8–10).

The Dahl salt-sensitive (DS) hypertensive rats undergo the transition from compensatory hypertrophy to congestive HF (11). When they are fed a high salt diet after the age of 6 weeks, they develop systemic hypertension and concentric left ventricular (LV) hypertrophy at 11 weeks, followed by marked LV dilation and contractile dysfunction at 15 to 20 weeks (11). Thus, they have been used as an animal model for nonischemic hypertension-based HF. We investigated whether autologous transplantation of skeletal myoblasts (MB) could retard the transition from compensatory hypertrophy to HF in DS hypertensive rats.

Skeletal myoblast transplantation is also useful as a tool for cell-mediated gene therapy, providing sustained local expression of recombinant proteins in the heart (12,13). Cardiotrophin-1 (CT-1), a member of the interleukin-6 superfamily, induces hypertrophy and prolongs survival of cardiac myocytes in vitro (14–16). It has been reported that CT-1 expression in the myocardium is upregulated in chronic HF (17,18). However, the pathophysiologic signif-

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**Abbreviations and Acronyms**

| | |
|---|---|
| Ang II | = angiotensin II |
| CT-1 | = cardiotrophin-1 |
| DS | = Dahl salt-sensitive |
| EDD | = end-diastolic dimension |
| ET-1 | = endothelin-1 |
| %FS | = percent fractional shortening |
| HF | = heart failure |
| LV | = left ventricle or left ventricular |
| MB | = transplantation of skeletal myoblasts alone |
| MB + CT | = transplantation of cardiotrophin-1–expressing myoblasts |
| PWT | = posterior wall thickness |

icance of CT-1 in the transition from compensated to decompensated HF is not fully elucidated (18). We accordingly analyzed the feasibility and efficiency of transplanting skeletal myoblasts that are transferred with CT-1 gene using retrovirus in preserving cardiac function of DS hypertensive rats. We hypothesized that this strategy could provide cardioprotective effects against ventricular remodeling in combination with functional benefits of skeletal myoblast-derived muscle.

## METHODS

**Animal model and isolation of primary skeletal myoblasts.** All animal experiments were performed in accordance with the guidelines for animal experimentation at Kobe University Graduate School of Medicine. Male DS rats were obtained from Japan SLC Co. Ltd (Hamamatsu, Japan). After weaning, the rats were fed a low salt diet (0.3% NaCl) until the age of six weeks; thereafter, a high salt diet (8% NaCl) was started. The myoblast culture process was performed according to the method reported previously (2). In brief, skeletal muscles were harvested from hind limb skeletal muscle of DS rats, and then they were minced and seeded on polystyrene plates. Tissue pieces were incubated at 37°C for 48 h in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, Missouri) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 500 μg/ml penicillin (all from Sigma) to allow primary satellite cell (myoblast) isolation. When cells began to migrate out of tissue, tissue pieces were removed. Myoblast growth densities were maintained at <70% to avoid the differentiation into the myotube (2).

**Retrovirus-mediated CT-1–gene transfer.** The full-length complementary deoxyribonucleic acid of the rat CT-1 was obtained from total ribonucleic acid in neonatal rat ventricular cells using reverse transcriptase-polymerase chain reaction. Sense and antisense primers for rat CT-1 were 5′-TCTATGAGCCAGAGGGAGGGAAGTCTG-3′ and 5′-TATTCAGGCAACGCCCCCTGGCAC-3′, respectively (14). The polymerase chain reaction fragment was confirmed by deoxyribonucleic acid sequencing and then inserted into pLN plasmid (generously provided by Dr. Noriyuki Kasahara, USC Institute for Genetic Medicine, Los Angeles, California) (19) to construct a retroviral transfer vector encoding the rat CT-1 complementary deoxyribonucleic acid sequence (pLN-CT-1). Retroviral vector encoding CT-1 was produced as described previously, with slight modifications (19,20). Briefly, a 100-mm dish of nonconfluent 293T cells were transfected with 15 μg of pLN-CT-1, 15 μg of pHIT 60 (i.e., cytomegalovirus [CMV] gag pol encoding plasmid), and 10 μg pHIT 123 (CMV ecotropic envelope) using the calcium phosphate coprecipitation method (21); 16 h after transfer, the media were adjusted to a final concentration of 10 mM in sodium butyrate. After 8-h incubation, cells were washed and incubated in fresh medium without sodium butyrate. Conditioned medium containing retrovirus was harvested 16 h later and filtered through 0.45-μm Millipore-HA (Millipore Co., Bedford, Massachusetts). For transfer of CT-1 gene into the myoblasts, cells were infected overnight with a dilution of virus stock in cultured medium supplemented with 8 μg of polybrane per ml. Infections were performed twice for efficient transfer into myoblasts.

**Cell transplantation.** At the age of 11 weeks, animals underwent thoracotomy under general anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg) and autologous transplantation to the myocardium with CT-1–transferred myoblasts (MB + CT group, n = 31) or myoblasts alone (MB group, n = 31). Cells ($1 \times 10^6$) were harvested and resuspended in 0.15 ml of phosphate-buffered saline, followed by intramyocardial injection 10 to 15 times into the anterior aspects of the LV free wall with a 26G needle. Accordingly, approximately $1 \times 10^5$ cells were injected in each site. In the sham group (n = 24), the same volume of phosphate-buffered saline was injected. The surgical wounds were repaired, and the rats were returned to the cages to recover.

**Echocardiographic and hemodynamic studies.** Systolic blood pressure and heart rate were measured by a tail-cuff method (Muromachi Kikai, Japan).

At the age of 11, 15, and 17 weeks, transthoracic two-dimensional echocardiography (SONOS 5500, Philips Medical Systems Corp., Andover, Massachusetts) was performed under light anesthesia with sodium pentobarbital. A 12-MHz Ultraband Sector Transducer (Philips Medical Systems Corp.) probe was used. Left ventricular end-diastolic dimension (EDD), end-systolic dimension (ESD), and LV posterior wall thickness (PWT) were determined from the M-mode tracing based on the short-axis view of the LV at the papillary muscle level. Left ventricular percent fractional shortening (%FS) was calculated as: [(EDD − ESD)/EDD] × 100.

At the age of 17 weeks, animals underwent direct cardiac catheterization via subdiaphragmatic approach to measure LV pressure under light anesthesia. The catheter was connected to a pressure transducer, and continuous measurements of LV pressure and heart rate were recorded using a Maclab system (Bioresearch Center, Nagoya, Japan). Animals were allowed to breathe spontaneously dur-