

ORIGINAL ARTICLE

Toll-like Receptor 4 and Vascular Cell Adhesion Molecule 1 in Monocyte-Endothelium Adhesion Induced by Lipopolysaccharide

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Background: Atherosclerosis potentially represents a chronic inflammatory disease in which both endothelial cells and monocytes are involved. Monocyte-endothelium adhesion, which is heavily dependent on the expression of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), is one of the key stages of atherogenesis. Toll-like receptor 4 (TLR4) has been found in atheroma and adventitia of human atherosclerotic coronary arteries. In TLR4-knockout mice studies, TLR4 contributes to atherogenesis via the nuclear factor-kappa B signal pathway, a process within which adhesion molecules are involved. Bacterial infection, such as Chlamydia pneumonia and lipopolysaccharide (LPS), is well known to act as the ligand of TLR4, triggering the overexpression of adhesion molecules and other inflammatory mediators, which can ultimately lead to atherogenesis.

Purpose: In this study, we explore the role of TLR4 in the monocyte-endothelium adhesion.

Method: The monocyte-endothelium adhesion triggered by LPS on human coronary artery endothelial cells was tested by anti-TLR4 antibody and anti-VCAM-1 antibody.

Results: The inhibition of TLR4 could suppress the overexpression of VCAM-1 in messenger RNA and protein levels. Both anti-TLR4 antibody and anti-VCAM-1 antibody interfere with monocyte-endothelium adhesion.

Conclusion: We conclude that LPS upregulates VCAM-1 by interacting with TLR4 and then enhances monocyte-endothelium adhesion. These findings may imply that the inhibition of TLR4 could be a potential target for atherosclerosis therapy.

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1. Introduction

Atherosclerosis and its complications, which include stroke, myocardial infarction, and peripheral arterial occlusion disease, remain the major causes of morbidity and mortality throughout the world.¹ Compelling evidence has already been provided to clearly demonstrate that atherosclerosis is a chronic inflammatory disease. The pathophysiology comprises consequential events, such as endothelial cell injury, monocyte adhesion, and migration and proliferation of smooth muscle cells in the subendothelial space.^{2,3}

Monocyte-endothelium adhesion, which is dependent on the interaction between adhesion molecules on the monocytes and endothelial cells, represents a key stage in atherogenesis.⁴

Inflammation is regulated by the immune system, which comprises both innate and adaptive immunity. Toll-like receptors (TLRs), including at least TLR2, TLR4, and TLR9, among others, are pattern-recognition receptors which, following their interaction with pattern-specific ligands, initiate innate immune responses.⁵

TLR4 has been found in atheroma and adventitia of human atherosclerotic coronary arteries by immunohistochemistry detection.⁶ It has also been documented in TLR4-knockout mice studies that TLR4 contributes to atherogenesis via the nuclear factor-kappa B signal pathway, a process within which adhesion molecules may be involved.⁷ TLR4 genetic polymorphisms are associated with atherogenesis complications, including myocardial infarction and stroke.^{8,9} All these findings point to the important role played by TLR4 in atherogenesis.

Bacterial infection, such as Chlamydia pneumonia and lipopolysaccharide (LPS), may act as the ligand of TLR4, triggering the overexpression of adhesion molecules, proinflammatory cytokines, and chemokines, all of which can ultimately lead to atherogenesis.^{10–13} In this study, we explore the role of TLR4 in the

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monocyte-endothelial adhesion induced by bacterial LPS and find that the vascular cell adhesion molecule 1 (VCAM-1) may be important in this process.

2. Methods

2.1. Cell culture

Human coronary artery endothelial cells (HCAECs) (Cell Applications, Inc., San Diego, CA, USA) were cultured in an endothelial cell basal medium (No. 210-500, Cell Applications, Inc., San Diego, CA, USA), which included a 10% Human Carotid Artery Endothelial Cells Growth Supplement (No. 212-GS, Cell Applications, Inc., San Diego, CA, USA); third- to seventh-generation cells were used in this study.

Following culturing in six-well plates for 24 hours, the HCAECs were treated with TLR4 or isotype antibodies at a concentration of 5 μ g/mL for 40 minutes to block TLR4. LPS of amount 100 ng/mL was then added into the cell culture plates for 6 hours. These cells were then harvested for reverse transcription polymerase chain reaction (RT-PCR).

Following culturing in 10-cm dishes for 48 hours, TLR4 or isotype control antibodies were added to the HCAECs at a concentration of 10 μ g/mL for 40 minutes to block the receptors. LPS (50 ng/mL) was then added into the cell culture plates for 12 hours. These cells were then harvested for Western blotting.

Following culturing in 12-well plates for 12 hours, TLR4 or isotype control antibodies were added to the HCAECs at a concentration of 10 μ g/mL for 40 minutes to block the receptors. LPS (100 ng/mL) was then added into the cell culture plates for 12 hours. These cells were then prepared for adhesion assay.

Finally, human acute monocytic leukemia cell line (THP-1) cells (ATCC, Manassas, VA, USA) were cultured in an RPMI1640 medium (No. 464879, Gibco, San Diego, CA, USA) with a 10% fetal bovine serum (No. 26140-079, Gibco, San Diego, CA, USA).

2.1.1. RNA isolation, cDNA synthesis, and RT-PCR for VCAM-1 expression

Total RNA was extracted from the HCAECs using the trizol reagent (No. 15596018, Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was then synthesized by the use of M-MLV reverse transcription reagents (No. 19601, Mbiotech, Seoul, South Korea) with PCR being performed using Platinum Taq DNA Polymerase Kit (No. 10966030, Invitrogen, Carlsbad, CA, USA). The PCR conditions comprise 2- μ L cDNA for the template and 30 seconds of denaturation at 94°C, 30 seconds of individual annealing (at 56°C for VCAM-1 and 55°C for GAPDH), and 30 seconds of extension at 72°C. The VCAM-1 and GAPDH were individually amplified by 35 and 30 cycles.

The primer sequences for VCAM-1 were 5'-CTGTTGAGATCT-CCCCTGGA-3' (sense) and 5'-CGCTCAGAGGGCTGTCTATC-3' (antisense) for the template, with a predicted size of 128 bp. The primer sequences for GAPDH were 5'-GGTCTCCTCT GACTTCAACA-3' (sense) and 5'-GTGAGGGTCTCTCTTCTCT-3' (antisense) for the template, with a predicted size of 220 bp. Each PCR product (10 μ L) was then loaded onto 2% agarose gel stained with ethidium bromide, visualized under UV light, and then quantified using the NIH Image J1.37 software.

2.1.2. Western blot analysis of VCAM-1

The total membrane protein of the HCAECs was extracted by Mem-PER Eukaryotic Membrane Extraction Reagent Kit (No. 89826, PIERCE, IL, USA), with the samples subsequently being loaded into 10% SDS-PAGE gel and fractionated for 2.5 hours at 100 V. The proteins were then electroblotted onto a BioTrace PVDF membrane

(P/N 66543, Pall corporation, Port Washington, NY, USA) followed by incubation in a blocking buffer.

The membrane was incubated using the antihuman VCAM-1 IgG antibody (1:500; BBA19, R&D, Minneapolis, MN, USA), with the horseradish peroxidase-conjugated donkey antigoat serving as the secondary antibody (1:5000; sc-2020, Santa Cruz, CA, USA). An ECL chemiluminescence substrate (NEL104&105, Perkin Elmer, MA, USA) was used for the visualization of the VCAM-1-specific bands. We selected the guanine nucleotide-binding protein G(s) α subunit ($G_{\alpha s}$) protein as the internal control with an anti- $G_{\alpha s}$ rabbit IgG antibody (1:1000; sc-823, Santa Cruz, CA, USA) and a horseradish peroxidase-conjugated goat antirabbit secondary antibody (1:2500; ab6721, Abcam, Cambridge, UK). The membranes were then scanned by BioSpectrum AC Imaging System (UVP, USA) and quantified by UVP Vision Works Software.

2.1.3. Adhesion assay

The adhesion assay was performed using the CytoSelect Leukocyte-Endothelium Adhesion Assay Kit (CBA-210, Cell Biolabs, San Diego, CA, USA). THP-1 cells were labeled by Leuko Tracker for 30 minutes at 37°C and then centrifuged at 1000 rpm for 2 minutes. The culture medium was discarded, and the pellet was washed twice with serum-free medium, with the pellet ultimately being resuspended in the serum-free medium.

During incubation, the HCAECs were accompanied by 5×10^5 cells/well of Leuko Tracker-labeled THP-1 for 1 hour at 37°C. The nonadhered THP-1 cells were then removed, and the medium was discarded. Thereafter, every well was thoroughly washed three times using a wash buffer; the cells were treated with lysis buffer and then incubated and shaken for 5 minutes at room temperature.

The mixture was subsequently transferred to a 96-well plate suitable for fluorescence measurement. Fluorescence was measured by Varioskan Flash Microplate Spectrophotometer (Thermo Scientific, Rockford, IL, USA), with the analysis of the data being undertaken using the SkanIt software. The excitation wavelength was set at 480 nm, with a detection emission level of 520 nm.

2.1.4. Inhibition of THP-1 adhesion to HCAECs by the anti-VCAM-1 antibody

The HCAECs were cultured in the gelatin-coated 12-well plates for 24 hours, with LPS subsequently being added at a concentration of 100 ng/mL for 12 hours. Following incubation, anti-VCAM-1 antibody (BBA19, R&D, Minneapolis, MN, USA) was added into the cell-cultured plates at a concentration of 1.5 μ g/mL for 1 hour. After discarding the culture medium, the culture plates were then washed with serum-free medium and prepared for adhesion assay.

2.2. Statistic analysis

The results were tested on multiple comparisons by analysis of variance. Bonferroni test was applied for further analysis. In addition, the comparisons between HCAECs treated by LPS with and without anti-TLR4 antibody or anti-VCAM-1 antibody were tested by use of Student's *t* test. The analysis comprises three sets of independent experiments, and *p* < 0.05 indicates statistically significant values.

3. Results

3.1. The effects of LPS and anti-TLR4 antibody on VCAM-1 mRNA

The HCAECs treated by LPS exhibited a significant increase in VCAM-1 expression in mRNA levels; however, the anti-TLR4 antibody achieved significant suppression of the upregulation in VCAM-1 expression induced by LPS. No similar effects, in terms of

changing the mRNA levels in VCAM-1 expression, were discernible for the isotype control antibody, either with or without LPS treatment (Figure 1).

3.2. The effects of LPS and anti-TLR4 antibody on VCAM-1 protein

The HCAECs treated by LPS exhibited a significant increase in protein levels in VCAM-1 expression; however, the anti-TLR4 antibody achieved significant suppression of this upregulation in protein levels in LPS-induced VCAM-1 expression. No similar effects, in terms of changing the protein levels in VCAM-1 expression, were discernible for the isotype control antibody, either with or without LPS treatment (Figure 2).

3.3. The effects of LPS and anti-TLR4 antibody on THP-1 adhesion

The HCAECs treated by LPS exhibited a significant increase in THP-1 adhesion; however, the anti-TLR4 antibody achieved significant suppression of such LPS-induced adhesion. This adhesion remained unchanged by the isotype control antibody, either with or without LPS treatment (Figure 3).

3.4. The effects of LPS and anti-VCAM-1 antibody on THP-1 adhesion

The HCAECs treated by LPS showed a significant increase in THP-1 adhesion; however, the anti-VCAM-1 antibody also achieved significant suppression of such LPS-induced adhesion (Figure 4).

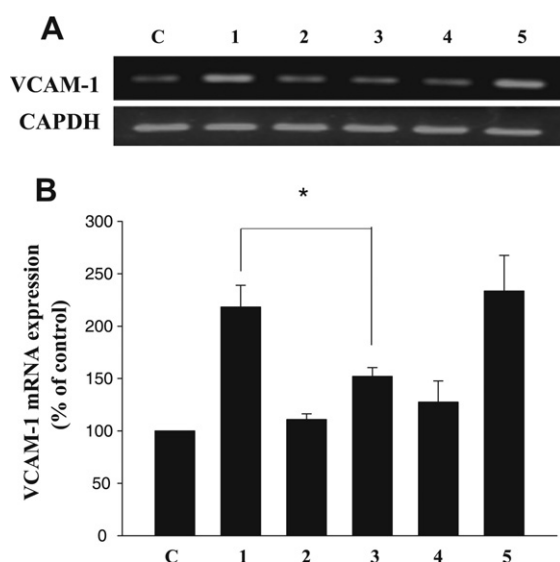


Figure 1 VCAM-1 expression in mRNA levels in HCAEC treated with LPS, anti-TLR4 antibody, and isotype control antibody. (A) is a representation of RT-PCR, and (B) is the VCAM-1 expression in mRNA (as a percentage of control). C refers to the control; 1 refers to HCAEC treated with only LPS (100 ng/mL) for 6 hours; 2 refers to treatment with only the anti-TLR4 antibody (5 μ g/mL); 3 refers to pretreatment with anti-TLR4 antibody for 40 minutes followed by LPS (100 ng/mL) for 6 hours; 4 refers to treatment with only isotype control antibody (5 μ g/mL); and 5 refers to pretreatment with isotype control antibody (5 μ g/mL) for 40 minutes followed by LPS (100 ng/mL) for 6 hours. The analysis comprises three sets of independent experiments with error bars also being shown. * indicates $p < 0.05$. VCAM-1 = vascular cell adhesion molecule 1; mRNA = messenger RNA; HCAEC = human coronary artery endothelial cell; LPS = lipopolysaccharide; TLR4 = toll-like receptor 4; RT-PCR = reverse transcription polymerase chain reaction.

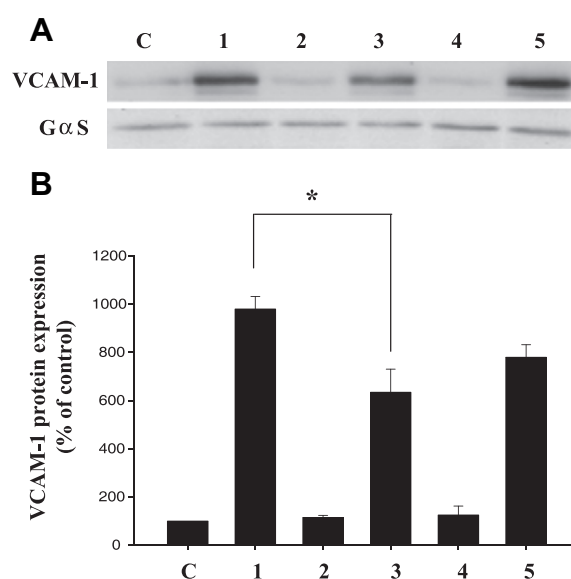


Figure 2 VCAM-1 expression in protein levels in HCAEC treated with LPS, anti-TLR4 antibody, and isotype control antibody. (A) is a representation of Western blot, and (B) is the VCAM-1 expression in protein levels (as a percentage of control). C refers to the control; 1 refers to HCAEC treated with only LPS (50 ng/mL) for 12 hours; 2 refers to treatment with only the anti-TLR4 antibody (10 μ g/mL); 3 refers to pretreatment with anti-TLR4 antibody (10 ng/mL) for 40 minutes followed by LPS (50 ng/mL) for 12 hours; 4 refers to treatment with only isotype control antibody (10 μ g/mL); and 5 refers to pretreatment with isotype control antibody (10 μ g/mL) for 40 minutes followed by LPS (50 ng/mL) for 12 hours. The analysis comprises three sets of independent experiments with error bars also being shown. * indicates $p < 0.05$. VCAM-1 = vascular cell adhesion molecule 1; HCAEC = human coronary artery endothelial cell; LPS = lipopolysaccharide; TLR4 = toll-like receptor 4.

4. Discussion

Atherosclerosis (arteriosclerosis) is known to be a major contributor to cardiovascular disease;¹⁴ indeed, there are many atherosclerosis risk factors that induce vascular endothelium injury, such as

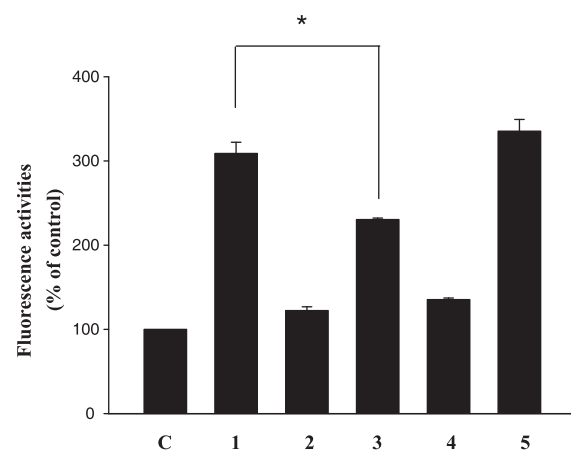


Figure 3 Adhesion assay of THP-1 to HCAEC, with anti-TLR4 antibody, by percentage of control in fluorescence activities. C refers to the control; 1 refers to HCAEC treated with only LPS (100 ng/mL) for 12 hours; 2 refers to treatment with only the anti-TLR4 antibody (10 μ g/mL); 3 refers to pretreatment with anti-TLR4 antibody (10 ng/mL) for 40 minutes followed by LPS (100 ng/mL) for 12 hours; 4 refers to treatment with only isotype control antibody (10 μ g/mL); and 5 refers to pretreatment with isotype control antibody (10 μ g/mL) for 40 minutes followed by LPS (100 ng/mL) for 12 hours. The analysis comprises three sets of independent experiments with error bars also being shown. * indicates $p < 0.05$. THP-1 = human acute monocytic leukemia cell line; HCAEC = human coronary artery endothelial cell; TLR4 = toll-like receptor 4; LPS = lipopolysaccharide.

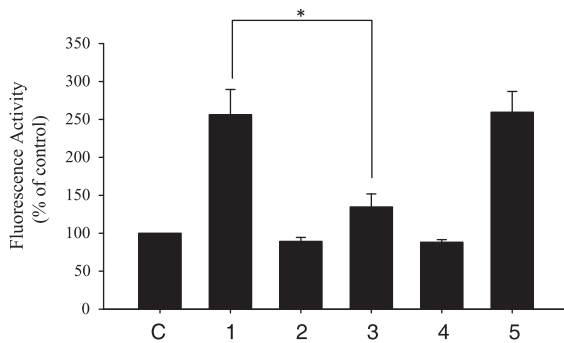


Figure 4 Adhesion assay of THP-1 to HCAEC, with anti-VCAM-1 antibody, by percentage of control in fluorescence activities. C refers to the control; 1 refers to HCAEC treated with only LPS (100 ng/mL) for 12 hours; 2 refers to pretreatment with LPS (100 ng/mL) for 12 hours followed by anti-VCAM-1 antibody (1.5 µg/mL) for 1 hour; 3 refers to treatment with only the anti-VCAM-1 antibody (1.5 µg/mL) for 1 hour; 4 refers to treatment with only isotype control antibody (1.5 µg/mL); and 5 refers to pretreatment with isotype control antibody (1.5 µg/mL) for 1 hour followed by LPS (100 ng/mL) for 12 hours. The analysis comprises three sets of independent experiments with error bars also being shown. * indicates $p < 0.05$. THP-1 = human acute monocytic leukemia cell line; HCAEC = human coronary artery endothelial cell; VCAM-1 = vascular cell adhesion molecule 1; LPS = lipopolysaccharide.

hypertension, dyslipidemia, and diabetes.¹⁵ Infection has also been shown to be an important factor responsible for triggering atherosclerosis and the associated cardiovascular diseases.^{16,17}

However, further analysis has shown atherosclerosis to be a chronic inflammatory disease of vessels;² thus, if we can succeed in eliminating the overall impacts of infection on vessels and then suppress the related inflammation, this could potentially serve as an effective treatment strategy for the future prevention of cardiovascular diseases. TLR4 is a transmembrane receptor that is involved in inflammatory responses to bacterial endotoxins, such as LPS, and other ligands.¹⁸ Endothelial cells may be activated by microbial ligands through TLRs with the resultant expression of adhesion molecules, such as VCAM-1, and other inflammatory mediators, all of which are known to be atherogenic.¹⁹ TLR4-mediated inflammatory activation of HCAECs by LPS has also been reported before (Figure 5).²⁰

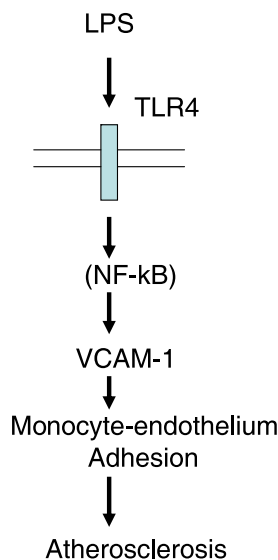


Figure 5 The scheme of this study. LPS upregulates VCAM-1 by interacting with TLR4, and VCAM-1 is involved in monocyte-endothelium adhesion, which is the key step of atherosclerosis. LPS = lipopolysaccharide; VCAM-1 = vascular cell adhesion molecule 1; TLR4 = toll-like receptor 4.

Evidence is emerging to indicate that human atherogenesis is mediated in part via the TLR4/nuclear factor-kappa B pathway.^{7,21} In a previous study among ethnic Chinese patients, we found that the TLR4 genetic polymorphism, C119A, was associated with ischemic stroke.⁹ In the present study, we go on to present *in vitro* evidence that TLR4 inhibition by means of its own antibody could suppress the overexpression of VCAM-1 but not E-selectin (data not shown) and monocyte-endothelium adhesion induced by LPS in HCAECs. However, high dose of anti-TLR4 antibody still could not completely block the overexpression of VCAM-1 and the increase of adhesion of monocytes on endothelium after treatment of LPS. We speculate that although TLR4 is the main receptor of LPS for further inflammatory responses, there should be other receptors or signaling pathways corresponsive to the VCAM-1 expression and subsequent adhesion enhancement. In addition to THP-1 cells, primary macrophages or monocytes, which are more physiologically relevant, could be applicable to do these experiments.

Furthermore, by achieving VCAM-1 inhibition in this study, we have essentially proven that VCAM-1 is involved in the monocyte-to-endothelial cell adhesion that is induced by LPS. Both the overexpression of VCAM-1 and monocyte-endothelium adhesion are found to play important roles in atherogenesis. Thus, our findings may imply that TLR4 inhibition could be a potential target for future atherosclerosis therapy as has been suggested by others.^{22,23}

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