Original Article
Toll-like receptor 9 inactivation ameliorates ox-LDL injured HUVEC activity in vitro

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Received March 7, 2018; Accepted July 14, 2018; Epub September 15, 2018; Published September 30, 2018

Abstract: Background: Inflammatory responses that are induced by oxidized low density lipoprotein (ox-LDL) play substantial functions in atherogenesis, and could be elevated in diabetic patients. Toll-like receptor 9 (TLR9) is an innate inflammatory receptor, and is enhanced in human umbilical vein endothelial cells (HUVECs) under high glucose conditions. Ox-LDL-TLR9 pathway activation and further inflammation mediated by monocytes are involved in the atherosclerosis formation. Objective: The objective was to determine whether TLR9 plays a role on ox-LDL-induced inflammation in HUVECs. The function and impact of the ox-LDL-TLR9 pathway on the inflammatory responses in HUVECs was examined. Methods: HUVECs were treated with ox-LDL and then subsequently treated with IRS869, a special antagonist of TLR9, and ODN1826, a type B CpG oligodeoxynucleotide (ODN) which can inhibit the expression of TLR9 and activate TLR9, respectively. HUVEC viability was analyzed by CCK8, changes of mitochondrial membrane potential (MTP), and LOX-1, IκBα, pIκBα, NF-κBp65, pNF-κBp65 expression. Results: Compared with the control group, ox-LDL significantly reduced the viability of HUVECs. TLR9 inactivation improved the viability of HUVECs and reduced the decrease of MTP injured by ox-LDL. TLR9 inactivation could inhibit LOX-1’s expression and results in inactivation of NF-κB signaling pathway. Conclusion: TLR9 is involved in the inflammatory responses induced by ox-LDL in HUVECs. Inhibition of TLR9 could improve HUVECs survival activity and improve the status of growth damaged by ox-LDL, according to a preliminary protection.

Keywords: TLR9, IRS869, ODN1826, HUVECs, ox-LDL

Introduction
Atherosclerosis, a chronic inflammatory disease, is characterized by monocyte adherence to intrinsic endothelial cells and ox-LDL accumulation in the arterial wall [1]. Between the blood vessel wall and blood, blood endothelial cells maintain the integrity and function of the vascular structure. Elevated degrees of serum low-density lipoproteins (LDL) and endothelial disorder are regarded as a predilection for atherosclerosis [2]. Induction of endothelial cell activation, dysfunction, and subsequent monocyte adhesion and endothelial leakage is a crucial step for atherosclerotic plaque constitution that is induced via ox-LDL [3]. Endothelial cells (ECs) are in fact activated by ox-LDL via inducing the expression of oxidized low density lipoprotein receptor 1 (lectin-like oxidized low density lipoprotein receptor 1, LOX-1) and release inordinate reactive oxygen species [4]. In addition, ox-LDL significantly triggers activation of the nuclear factor kappa B predominate (nuclear factor kappa B, NF-κB) signaling pathway, and promotes its nuclear transformation, leading to oxidation of vascular endothelial cells and inflammatory lesions [5, 6]. Increased damage of vascular endothelial cells and secretion of a large number of inflammatory factors, promote monocyte accumulation and foam cell formation, formation of fatty streaks, and development of atheromatous plaques.

TLRs, pathogen pattern recognition receptors, are featured by the release and expression of cytokines and chemokines implicated in the progression and development of atherosclerosis [7]. TLR9 is an endosomal protein recognizing both autologous DNA and bacterial DNA. ODN1826 is a type B CpG ODN which can activate mouse TLR9 [8], while IRS869, a special antagonist of TLR9, can inhibit the expression of TLR9 and related cytokine. TLR9 detects unmethylated CpG DNAs or CpG ODN. Upon
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ligation with its cognate ligands, the MyD88 adapter protein is recruited by TLR9. Nuclear factor κB (NF-κB) dependent pro-inflammatory cytokines pathway are then initiated [9]. To date, TLR9 continues to be the only known receptor for immunostimulatory DNA [8, 10]. TLR9 was foremost identified in antigen presenting cells [10] consistent with their role in immune surveillance. TLR9 is likewise expressed in non-immune cells, including HUVECs [11], dermal microvascular [12], lymphatic endothelial cells [13], and endothelial cells of human atherosclerotic plaques [14]. The balance of TFPI and TF is altered by CpG DNA signaling via TLR9 in HCAECs, as reported earlier. Most studies hitherto have focused on implications of TLR4 or TLR2 on atherosclerosis, while data on the role of TLR9 in atherosclerosis is scarce. TLRs have been viewed as major culprits in the development of atherosclerosis, contributing to both clinical complications and its progression.

However, direct evidence for an effect of blocking TLR9 on accelerated atherosclerosis has been lacking. In this research, we investigated the role of TLR9 in the inflammatory response and induction via ox-LDL in HUVECs.

Materials and methods

Cell culture, treatment, and transfection

HUVECs were cultivated in the endothelial growth medium (EGM) (Promocell, USA) comprising 2% fetal calf serum (FCS), growth factors (epidermal growth factor, vascular endothelial growth factor, basic fibroblast growth factor, and insulin-like growth factor). Cells were maintained in endothelial cell basal medium (EBM, Promocell) supplemented with 1% FCS without growth factors prior to and throughout experiments.

For the experiments, HUVECs were stimulated with or without ox-LDL (0.1 mg/mL). In additional experiments, IRS869 or ODN1826 was added to HUVECs 6 h prior to adding ox-LDL. The treatment time was 24 hours.

Real-time polymerase chain reaction (PCR)

Total RNA was separated from HUVECs adopting the Qiazol reagent (Qiagen, Germany). Intracellular RNA was reverse-transcribed with SuperScript reverse transcriptase kit (Applied Biosystems, Germany). Real-time PCR was carried out utilizing Power SYBR Green PCR master mixture on a StepOne Plus real-time PCR system (Applied Biosystems, Germany). Target genes' expression were normalized to GAPDH. Target genes' relative expression were determined utilizing the 2^ΔΔCT approach. Primer sequences are as follows: TLR9 forward (5'-TGGACGGGAAGTACT-3') and reverse (5'-GCCACATTCTACAGGGATT-3'); LOX-1 forward (5'-GAGCTGCAAACTTTTCAGG-3') and reverse (5'-GCTCTTCATGCAAGAAGAG-3'); GAPDH forward (5'-TGACGGGAAGTACT-3') and reverse (5'-GCCACATTCTACAGGGATT-3').

Western blotting analysis

Total protein was separated from HUVECs utilizing RIPA buffer that was supplied with the protease inhibitor cocktail (Roche, Germany). Total protein concentration was gauged utilizing the BCA kit (Thermo Scientific, USA) in accordance with guidance of manufacturer. An equivalent quantity of total protein (20 μg) was subjected to 10% denaturing SDS gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, UK). After blocking with 5% non-fat dry milk, membranes were sequentially probed with primary antibody against proteins of interest that was followed by horseradish peroxidase (HRP)-linked secondary antibody. Blots were formulated utilizing chemiluminescence substrate (BioRad Laboratories, USA). Chemiluminescence signal intensity was determined utilizing Quantity One software (BioRad).

CCK8 analysis

Transduced HUVECs were plated at a density of 3,000 cells/well in 96-well plates. After placing for 16 hours, cells were serum starved or treated with ox-LDL (0.1 mg/mL) for an extra 24 hours. Throughout the last 4 hours of growth, WST-1 (Roche) was added to the media to gauge cell activity following the manufacturer's protocol. No less than four points were averaged for each condition, and the experiment was repeated three times with a representative experiment chosen.

Mitochondrial membrane potential (MTP) changing analysis

HUVECs were plated at a density of 3,000 cells/well in 96-well plates, and at the same time...
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Time, cells in each group were treated with the mitochondrial membrane potential detection reagent JC-1 (100 μl medium were added 10 μl JC-1 reagent), incubated at 37°C for 15 minutes in the CO₂ incubator. In accordance with recommendations from the manufacturer’s protocol, the wavelength of the FITC channel was utilized for dynamic observation. JC-1 dye was collected within the mitochondria followed by dependent potential. In normal mitochondria, JC-1 in the form of polymer cumulated in the mitochondrial matrix, demonstrated a strong red fluorescence (Ex = 585 nm, Em = 590 nm). As reported by color changes directly that reflect the change of mitochondrial membrane potential, mitochondrial depolarization was measurable via red fluorescence intensity.

Nitrite assay

As previously reported [15], Nitrite accumulation was measured in the culture medium of confluent HUVECs. Nitrite was colorimetrically quantified after adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μL culture medium. Absorbance at 550 nm was determined with a microplate reader (Molecular Devices). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

Statistical analysis

The data are expressed as mean with standard error (X ± SD). Data were compared utilizing Student’s t-test or one-way ANOVA followed by Tukey multiple comparison tests while more than two groups were compared (GraphPad Prism, version 5.01, GraphPad Software, Inc., USA). P value of below 0.05 was considered to be statistically significant.

Results

TLR9 inactivation increased the viability of ox-LDL-mediated HUVECs

To investigate whether TLR9 activation had a direct effect on the viability of ox-LDL-mediated HUVECs, cell proliferation assays (Cell Counting Kit 8, CCK8) were performed in ox-LDL-mediated HUVECs, which were treated with TLR9 ligand C-phosphate-G (CpG) oligodeoxynucleotide ODN1826 (TLR9 agonist) and/or IRS869 (a special antagonist of TLR9). CCK-8 proliferation assay illustrated that cell proliferation was greatly reduced in HUVECs that were treated with ox-LDL in comparison with negative control (non ox-LDL treated HUVECs) (Figure 1A and 1B). Furthermore, compared with ox-LDL-mediated HUVEC, cell proliferation abilities were significantly promoted following the treatment of IRS869 in ox-LDL-mediated HUVECs (Figure 1A). In contrast, treatment of ODN1826 in ox-LDL-mediated HUVECs inhibited the cell growth rate (Figure 1B). Overall, these results illustrate that ox-LDL exerts anti-proliferative effects on HUVECs and TLR9 inactivation may ameliorate the viability of HUVECs injured by ox-LDL.

TLR9 inactivation inhibited the decrease of mitochondrial transmembrane potential induced by ox-LDL

Mitochondrial membrane potential plays an important role in maintain normal physiological functions of cells. Decreased cell viability may be caused by corresponding reduction in mitochondrial membrane potential. In order to confirm our speculation, the change of mitochondrial membrane potential (MTP) of HUVECs were observed by dynamic imaging system. Higher positions of mitochondrial membrane potential were marked by red fluorescence. The
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red fluorescence was not changed under the treatment of IRS869 or ODN1826 in ox-LDL-mediated HUVECs at first 10 minutes (Figure 2A and 2B). While red fluorescence signal persisted in HUVECs and later weakened rapidly in ox-LDL-mediated HUVECs. Furthermore, a lesser red fluorescence signal occurred following the treatment of ODN1826 in ox-LDL-mediated HUVECs and more red fluorescence signal was detected under the treatment of IRS869 in ox-LDL-mediated HUVECs (Figure 2A and 2B). These results show that IRS869 could reduce and delay the decrease of MTP induced by ox-LDL but have the opposite effect in ODN1826.

**TLR9 inactivation attenuates iNOS expression and decreased NO production in ox-LDL-mediated HUVECs**

To assess whether TLR9 induces iNOS expression in ox-LDL-mediated HUVECs, the cells were incubated IRS869 (a special antagonist of TLR9) with and/or ODN1826 (TLR9 agonist) for

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**Figure 2.** Mitochondrial transmembrane potential changes of IRS869 and ODN1826 on ox-LDL-mediated HUVECs. A. Detection of mitochondrial transmembrane potential changes of IRS869 (2 μM) and ODN1826 (2 μM) on ox-LDL (0.1 mg/mL)-mediated HUVECs at 10 and 50 minutes by fluorescent staining. B. Living cell imaging system showed mitochondrial transmembrane potential levels of IRS869 (2 μM) and ODN1826 (2 μM) on ox-LDL (0.1 mg/mL)-mediated HUVECs from 0 to 70 minutes.
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24 hours and then iNOS expression was measured. mRNA and protein expression of iNOS was hardly detectable in unstimulated HUVECs, but expression of iNOS was markedly upregulated in cells stimulated with ox-LDL (Figure 3A and 3B). While iNOS expression was attenuated in ox-LDL and IRS869-mediated HUVECs, in contrast, it was increased in ox-LDL and ODN1826-mediated HUVECs (Figure 3A and 3B). Consistent with iNOS, NO generation was time-dependently increased after ox-LDL and ODN1826 stimulating HUVECs and decreased in ox-LDL and IRS869-mediated HUVECs (Figure 3C).

**TLR9 inactivation inhibited expression of LOX-1 and inactivated NF-κB signaling pathway**

The molecules of LOX-1, IkBα, and NF-κBp65 are critical downstream signaling components in TLR9 pathway. To determine whether those molecules are involved in TLR9 inactivation, the mRNA and protein levels of TLR9 and the downstream molecules (LOX-1, IkBα, pIkBα, NF-κBp65 and pNF-κBp65) were determined by qRT-PCR and Western blotting. qRT-PCR results showed that the expression of TLR9, LOX-1, IkBα, and NF-κBp65 were significantly upregulated in HUVECs that were treated with ox-LDL compared to negative control (Figure 4A-D). Furthermore, under treatment of IRS869 or ODN1826 in ox-LDL-mediated HUVECs, expression of TLR9, LOX-1, IkBα, and NF-κBp65 was significantly downregulated or upregulated compared to those in ox-LDL-mediated HUVECs, respectively (Figure 4A-D). Consistent with the qRT-PCR results, the protein levels of TLR9, LOX-1, IkBα, NF-κBp65, pNF-κBp65 were upregulated in ox-LDL-mediated HUVECs except for the downregulation of pIkBα in ox-LDL-mediated HUVECs (Figure 4E and 4F). Meanwhile, compared with negative control, the markedly lower protein levels of TLR9, LOX-1, IkBα, NF-κBp65, pNF-κBp65 and higher protein level of pIkBα were detected following IRS869 treatment in ox-LDL-mediated HUVECs than those in non-treatment of IRS869 (Figure 4E and 4F). Altogether, these results demonstrate that TLR9 inactivation could inhibit the expression of LOX-1 and inactivate NF-κB signaling pathway induced by ox-LDL in HUVECs.

**Discussion**

Vascular endothelial cells are the main barrier of the blood vessel wall, while its dysfunction and injury are the first steps of atherosclerosis. It is already well known that ox-LDL plays a key role in the development of atherosclerosis, including endothelial dysfunction, macrophage invasion, and foam cell formation [16, 17]. In our study, the model of ox-LDL induces HUVECs injury had been successfully performed in the experiment. Our data demonstrate that ox-LDL exerts anti-proliferative effects on HUVECs and TLR9 inactivation may ameliorate the viability of HUVECs injured by ox-LDL.

Mitochondria as cellular “power plants”, is the basis of eukaryotic cell survival. Mitochondrial membrane potential changes could lead to electron transport and oxidative phosphorylation obstacles, resulting in decreasing ATP production [18]. Furthermore, the decrease of the ATP can decrease ATP dependent ion channels open, causing gain of ion balance disor-
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...ders, as well as in vivo and in vitro calcium overload in the mitochondria [19], increased mitochondrial damage, and even cause apoptosis or necrosis [20]. Our experiment results show that mitochondrial membrane potential was reduced in HUVECs treated with ox-LDL. While TLR9 inactivation could reduce and delay the decrease of mitochondrial membrane potential induced by ox-LDL.

The damage of endothelial cells induced was affected by binding of ox-LDL and its receptor LOX-1 on endothelial cells [21]. The expression of LOX-1 mRNA and protein were upregulated through ox-LDL in a dose-dependent manner. After the combination of both, the production of a large number of reactive oxygen species and reduction of NO was generated by cells through the mitochondrial electron transport chain. Previous studies showed that iNOS expression is upregulated with subsequent synthesis of large quantity of NO following injury to the vasculature [22]. NO is an important pro-inflammatory and pro-atherogenic mediator. Enhancement of NO level likely plays a significant role in the development of atherosclerosis.

Figure 4. Expression levels of TLR9, LOX-1, IkBα, pIkBα, NF-κBp65, and pNF-κBp65 protein of ODN1826 and IRS869 on ox-LDL-mediated HUVECs. HUVECs were dealt with ox-LDL (0.1 mg/mL, 24 hours) with or without pretreatment with IRS869 (2 μM, 12 hours) or ODN1826 (2 μM, 12 hours), and the expression levels of TLR9 (A), LOX-1 (B), IkBα (C), NF-κB (D) mRNA were determined by quantitative real-time PCR analysis. (E) The protein levels of TLR9, LOX-1, IkBα, pIkBα, NF-κBp65, pNF-κBp65 were analyzed by Western blotting. (F) TLR9, LOX-1, IkBα, pIkBα, NF-κBp65, pNF-κBp65 protein levels were analyzed by Western blotting gray value statistics. *, P<0.05; **, P<0.01.
It is well known that TLR9 inactivation inhibits iNOS expression in M1 macrophage infiltration [24]. The results in the study show that TLR9 inactivation remarkably attenuates iNOS in the mRNA and protein level, and decreases NO production in ox-LDL-mediated HUVECs.

Although pro-inflammatory effect of TLR9 has firmly been established, its pro-inflammatory signaling pathway, especially in ox-LDL-mediated HUVECs, remains to be completely defined. It is reported that CpG ODN activates the TLR9-MyD88-ERK1/2 pathway in formation of foam cells [25]. Additionally, ODN1826, the agonist ligand of TLR9, can significantly enhance perilipin 3 expression in RAW264.7 cells [26]. Alternatively, compelling evidence has suggested that activation of TLR9 facilitates formation of foam cells in an NF-κB- and IRF7-dependent manner [27, 28]. Consistent with those findings, our results indicate that inactivation of TLR9 downregulated LOX-1, IκBα, NF-κBp65 and alleviated atherosclerosis progression.

In conclusion, our data provide novel observations that TLR9 inactivation increases mitochondrial transmembrane potential, decreases NO production, and promotes cell viability. Our study may be valuable for deciphering cross talk between the autoimmune response and atherosclerosis and provide a promising therapeutic strategy for atherosclerosis, given that atherosclerosis is a multifactorial disease in which diverse mechanisms are involved.

Acknowledgements

This study was supported by research grants from the Shanghai Pudong New District Key Specialty Construction Foundation (grant. nos. PWZzk2017-17).

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