Mini Review

Recognition of bacterial compounds by aortic endothelial cells activates Weibel-Palade body exocytosis

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The endothelial cell-specific granule Weibel-Palade body releases vasoactive substances capable of modulating vascular inflammation, thrombosis and atherogenesis. Pathogen recognition by endothelial Toll-like receptors (TLRs) is thought to play a crucial role in promotion of vascular inflammatory responses. However, the molecular basis for the early-phase responses of endothelial cells to pathogens has not been fully elucidated. We found that human aortic endothelial cells respond to several TLR ligands, including bacterial lipoteichoic acids and lipopeptides, but not lipopolysaccharides, to induce Weibel-Palade body exocytosis, accompanied by release of the storage component von Willebrand factor. In this mini-review, we briefly describe a novel function of endothelial TLRs and discuss its implications in aortic inflammation or atherogenesis.

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Introduction

Excess inflammatory responses of vessel walls or endothelium to invading pathogens have recently been suggested to be linked to atherogenesis[1,2]. Several common bacterial infectious agents or pathogens, such as *Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis* and other commensal bacteria, have been detected in vessel walls or atheroscrelotic lesions in humans[3-6]. However, the linkage between endothelial innate recognition of such pathogens and endothelial inflammatory responses has not been fully elucidated. For the detection of circulating bacteria in blood, several Toll-like receptors (TLRs) on endothelial cell surfaces are thought to identify molecular motifs that usually compose bacterial bodies or cell walls. TLR2 can detect a wide range of common bacterial constituents, such as lipoteichoic acids (LTA), peptidoglycans (PGN), bacterial di- or triacylated lipoproteins/lipopeptides, lipoarabinomannans, porins and fimbriae[7]. TLR1 and TLR6 further participate in the accurate discrimination of these molecules by TLR2[8]. TLR4 and TLR5 contribute to the recognition of lipopolysaccharides (LPS) and flagellin, respectively[8]. After recognition of cognate ago-

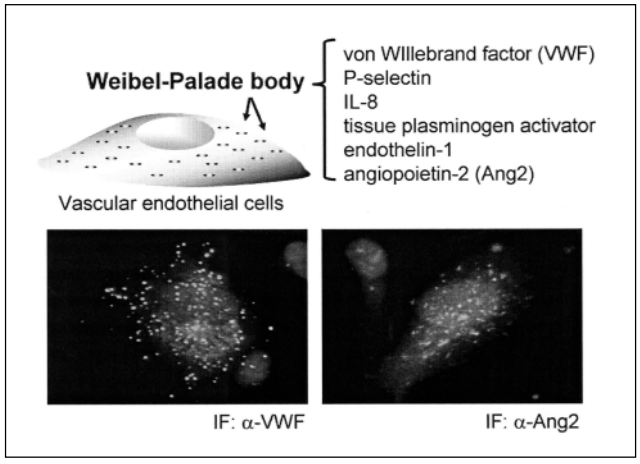

Fig. 1 The endothelial cell-specific granule Weibel-Palade body

Weibel-Palade bodies contain several vasoactive substances as shown in the figure. The presence of the granules in HAEC could be detected by immunofluorescent staining (IF) using antibodies to VWF (left picture) and Ang-2 (right picture).

nists, endothelial TLRs activate a signaling pathway utilizing MyD88, IL-1R-associated kinase (IRAK) 1 and TNFR-associated factor (TRAF) 6, which ultimately activate IκB and the release and translocation of active NF- κ B to the nucleus[8]. The endothelial NF-κB signaling pathway downstream of TLRs is thought to participate in the development of vascular inflammatory diseases or atherogenesis through the promotion of expression of large number of proinflammatory mediators and adhesion molecules[9-11]. However, it is still not known whether endothelial TLRs are primary initiators or modulators of vascular inflammatory diseases.

Induction of Weibel-Palade body exocytosis by bacterial constituents

We aimed to determine the early-phase inflammatory responses of human aortic endothelial cells (HAEC) to bacterial pathogens, especially their cell wall constituents. It is known that early endothelial activation involves dual phases: rapid translocation of P-selectin to the endothelial surface and slower synthesis and expression of adhesion molecules such as ICAM-1. The former process is accompanied by rapid exocytosis of Weibel-Palade bodies, endothelial cell-specific storage granules that contain vasoactive substances, including von Willebrand factor (VWF), P-selectin, IL-8, eotaxin-3, endothelin-1, CD63/lamp3, tissue plasminogen activator, angiopoietin-2 and osteoprotegerin[12] (Fig.1). These proteins are transported to the outside of the cell upon stimulation or vascular damage through Weibel-Palade body exocytosis and may control local or systemic biological effects, including vascular inflammation, thrombosis and atherogenesis[12].

Table 1 Recognition of bacterial constituents by TLRs

Component	TLR recognition
E. coli O26:B6 LPS	TLR4
Salmonella minnesota R595 LPS	TLR4
Salmonella typhimurium flagellin	TLR5
S. aureus LTA	TLR2/TLR6
S. aureus PGN	TLR2
Pam_3CSK_4 (triacylated lipopeptide)	TLR2/TLR1
FSL-1 (diacylated lipopeptide)	TLR2/TLR6
MALP-2 (diacylated lipopeptide)	TLR2/TLR6

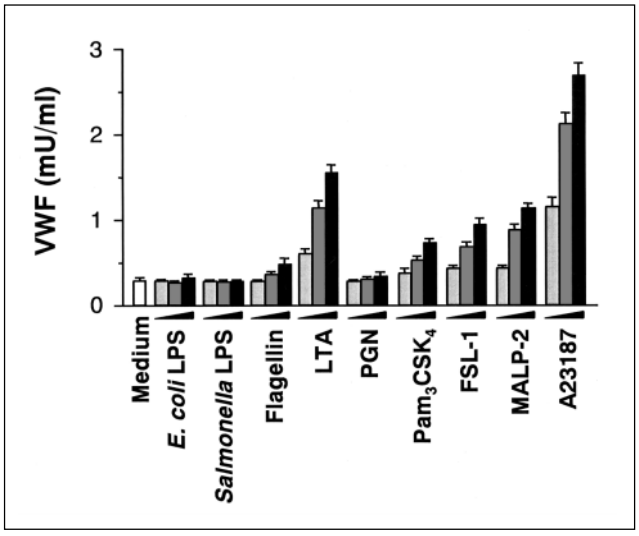

Fig. 2 VWF release by bacterial constituents

HAEC were stimulated with increasing concentrations of bacterial constituents shown in Table 1 for 60 min. The amount of VWF released into the culture medium was measured by ELISA.

As bacterial constituents, we prepared LPS from *E. coli* and *Salmonella minnesota*, flagellin from *S. typhimurium*, PGN and LTA from *Staphylococcus aureus* and the synthetic analogs of bacterial lipoproteins Pam_3CSK_4 (*N*-palmitoyl-S-dipalmitoylglyceryl CSKKKK), FSL-1 (*S*-dipalmitoylglyceryl CGDPKHPKSF) and MALP-2 (*S*-dipalmitoylglyceryl

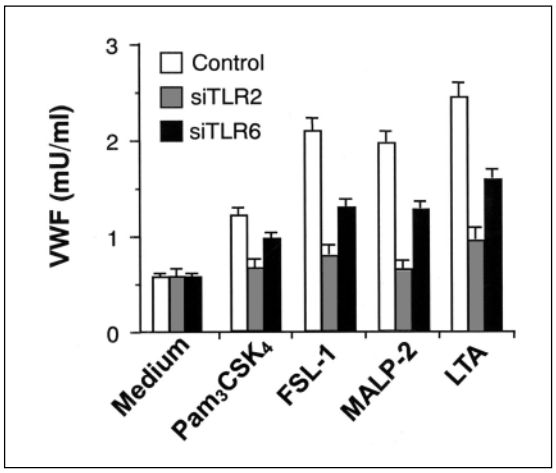

Fig. 3 Involvement of TLR2 and TLR6 in Weibel-Palade body exocytosis

HAEC transfected with specific siRNA oligonucleotides for mRNA of TLR2 or TLR6 were stimulated with the increasing concentrations of bacterial constituents shown in Table 1 for 60 min. The amount of VWF released into the culture medium was measured by ELISA.

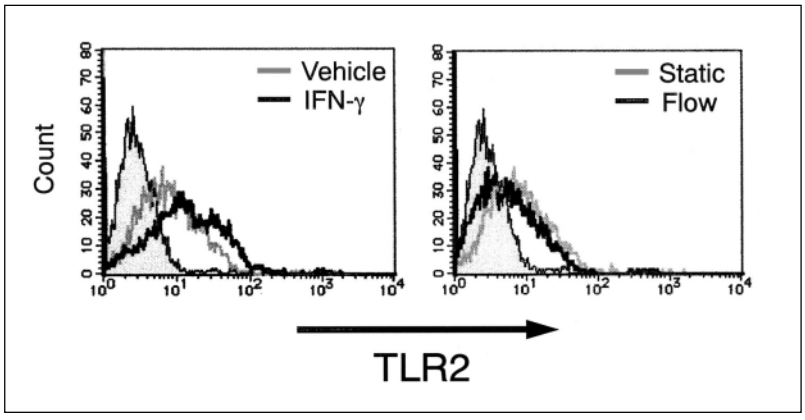

Fig. 4 Regulation of TLR2 expression in HAEC

HAEC were pretreated with IFN-γ or precultured under laminar flow for 12 h. The cell surface TLR2 expression was measured by flow cytometry.

CGNNDESNISFKEK). These compounds are known to be recognized by cognate TLRs[13] as shown in Table 1. We examined the release of VWF, an essential molecule for the formation of Weibel-Palade bodies[14], after stimulation of HAEC with these bacterial constituents for 60 min. We found that LTA, Pam_3CSK_4, FSL-1, MALP-2 and, to a lesser extent, flagellin induced release of VWF in a dose-dependent manner (Fig.2). The stimulatory activity of PGN was very weak. Interestingly, the TLR4 agonist LPS did not activate Weibel-Palade body exocytosis despite its potent activity to induce IL-8 production after stimulation of HAEC for 4 h, suggesting that TLR4 lacks the ability to induce exocytosis. This may be related to evidence that TLR4 intracellularly localizes in artery endothelial cells[15]. We must further investigate the differential potential of endothelial TLRs in the induction of Weibel-Palade body exocytosis.

Induction of TLR2-mediated Weibel-Palade body exocytosis

We focused on TLR2-mediated Weibel-Palade body exocytosis, because several TLR2 agonists could activate potent responses (Fig.2). HAEC express TLR2 at a weak but evident level on their cell surface (Fig.4), whereas expression of TLR2 and responsiveness to TLR2 agonists have been reported in human endothelial cells from other endothelial beds such as HUVEC[16]. We found that 'knockdown' of TLR2 expression by siRNA almost completely suppressed the magnitude of VWF release by all TLR2 agonists in HAEC (Fig.3). TLR6 interference also significantly decreased the activities of LTA, FSL-1 and MALP-2 and even Pam3CSK4 (Fig.3). Therefore, endothelial recognition of bacterial compounds by TLR2 and TLR6 directly contributes to Weibel-Palade body exocytosis.

Regulation of TLR2-mediated Weibel-Palade body exocytosis

Recently, complete deficiency of TLR2 in atherosclerosis-prone LDLR-null mice was shown to lead to an apparent reduction in the formation of lesions even in the absence of known exogenous TLR2 agonists[17]. Interestingly, the progression of lesions was indicated to be probably caused by aortic endothelial cells, not cells from bone marrow[17]. Indeed, several proper-

ties of endothelial TLR2 have been implicated in the disease development. Specifically, endothelial TLR2 expression is enhanced by inflammatory stimuli, such as TNF-α, IFN-γ and LPS[18]. Indeed, the expression level of TLR2 is known to be increased at atherosclerotic lesions in humans[19]. We examined whether regulation of endothelial TLR2 expression modulates exocytosis. We found that increased endothelial TLR2 expression after IFN-γ treatment (Fig.4) increased the magnitude of TLR2-mediated exocytosis of Weibel-Palade body, suggesting enhanced responsiveness of endothelial cells to pathogens in an inflamed or atherosclerotic lesion. Physiologic fluid shear stress has been suggested to have atheroprotective effects *in vivo*, since atherosclerosis preferentially occurs in an area of disturbed flow or a low level of shear stress, whereas regions with steady laminar flow and physiologic shear stress are protected. Disturbed flow or a low level of shear stress has been reported to regulate expression of various regulatory molecules of endothelial activation, by which atherosclerotic processes may be accelerated in the sites. Physiologic fluid shear stress is known to decrease endothelial TLR2 expression via impaired activity of the transcriptional factor SP1[20]. Indeed, TLR2 expression slightly decreased in HAEC incubated under laminar flow (Fig.4). We found that laminar flow decreased TLR2-mediated exocytosis. Thus, TLR2 agonist-induced Weibel-Palade body exocytosis may be influenced by surface expression level of physiologically or pathologically regulated endothelial TLR2.

Conclusions

Our findings show a linkage between endothelial innate recognition of circulating bacteria and early-phase endothelial inflammatory responses. These findings may provide a new insight into the roles of endothelial TLRs in the initiation and modulation of the development of vascular inflammation and atherosclerosis.

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