Endogenous Inflammatory Molecules Engage Toll-Like Receptors in Cardiovascular Disease

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Key Words

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Abstract

Innate immunity is important in the pathogenesis and progression of cardiovascular disease. Innate immune cells express various pattern-recognition receptors, among which also Toll-like receptors (TLRs). TLRs occur in atherosclerotic lesions where they are triggered by both exogenous (bacterial and viral pathogens) and endogenous (tissue damage-associated) molecules. Several endogenous TLR activators are described in relation to atherosclerotic disease or ischemia-induced cardiac injury. Experimental animal models have proved the role of TLR endogenous activators in disease initiation and further development. Nowadays, researchers aim to unravel the exact mechanisms involved in the endogenous ligand-dependent TLR signaling and to discover new potential activators, released during pathological conditions such as atherosclerosis and related ischemic manifestations. This review provides an overview of the currently known endogenous molecules which trigger innate immunity via TLRs in cardiovascular disease.

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Introduction

For decades, morbidity and mortality of the aging population due to cardiovascular disease (CVD) has been a burden for the health system and regrettably, we witness no revolution at the beginning of the 21st century. Along with a boost of hypertension, type II diabetes and obesity in the young generation, the incidence of CVD is rapidly increasing, with heart disease being the major cause of death in the modern world. The pathogenesis of CVDs is heterogeneous; infection, inflammation [1, 2] and even autoimmunity [3] are implicated. For most clinical presentations of CVD (such as stroke and acute coronary syndromes), atherosclerosis is the underlining pathological cause. Long considered a passive lipid deposition on the vessel wall, atherosclerosis is nowadays reckoned as a complex inflammatory process that involves both arms of immunity: innate and adaptive [4]. Immunity mediates the initiation, progression and eventual thrombotic complications of atherosclerosis [4]. The innate immune response rises quickly after a danger signal and combats foreign pathogens but also self-structures released under cell stress. It demands no prior ‘education’ of the immune cells and can recognize hundreds of different structures, therefore being labeled as ‘fast and blunt’. Fundamental

cells of the innate immune system, namely monocytes and macrophages, are present within atherosclerotic lesions and express various pattern-recognition receptors, among which, Toll-like receptors (TLRs). TLRs occur in atherosclerotic lesions [5] where they are triggered by both exogenous (bacterial and viral pathogens) and endogenous (tissue damage-associated) molecules [6]. Several endogenous ligands for TLRs are expressed during inflammation in atherosclerotic plaques or ischemic heart where they might play a role in disease pathogenesis and progression. With the use of experimental animal models, researchers have gained insight into the mechanisms behind endogenous ligand-induced TLR activation.

This review provides an overview of the endogenous molecules, released under cell stress during inflammation, which can trigger innate immunity via TLRs in CVDs.

The Role of TLRs in CVD

TLRs, especially TLR-2 and TLR-4, are extensively studied in CVD and implicated in atherosclerosis. TLRs are important components of innate immunity that bridge inflammation, infection and atherosclerosis [7]. Atherosclerosis is a chronic inflammatory process affecting the vasculature at predetermined sites. Lipid accumulation within the vessel intima leads, in time, to an accelerated inflammatory response that encompasses both innate and adaptive immune systems. Previous epidemiological studies have proved that exogenous bacterial ligands can induce an inflammatory response in the arterial wall and initiate atherosclerosis [8], and therefore, a link between atherosclerosis and TLRs has been established. Experimental data have shown that lipopolysaccharide (LPS) and peptidoglycan (PGN), both potent TLR activators, can accelerate plaque formation in atherosclerotic mice [6]. These results point to a potential role of TLR-2 and TLR-4 in the pathogenesis of atherosclerotic disease. Indeed, TLR-2 knockout mice crossed with low-density lipoprotein (LDL) receptor show a reduction in the degree of atherosclerosis [9]. TLR-4 knockout mice crossed with the apolipoprotein E (ApoE) knockout mice have reduced atherosclerosis compared to apoE knockout controls [10]. TLR-4-deficient mice also reveal reduced outward arterial remodeling [11]. apoE-deficient mice lacking TLR-4 and myeloid differentiation factor 8 (MyD88), a signal protein downstream TLR pathway, have reduced aortic atherosclerosis that is associated with reductions in cir-

culating levels of pro-inflammatory cytokine interleukin (IL)-12 or monocyte chemoattractant protein 1, plaque lipid content, numbers of macrophages and cyclooxygenase-2 immunoreactivity in their plaques [12]. Another group has reported reduced atherosclerosis through a decrease in chemokine levels and in macrophages infiltrating into plaques, in apoE/MyD88 null mice [13].

The relation between TLR-4 and atherosclerosis is strengthened by genetic analyses of polymorphisms in genes encoding TLR-4. However, large clinical studies have brought contradictory findings: TLR-4 polymorphism is related to a reduction [14] or no effect [15] on atherosclerosis and in intima-media thickness, to an increased risk of ischemic stroke [16] and MI [17], or to a decreased risk of acute coronary syndromes [18]. Others have reported no association between TLR-4 polymorphisms and the risk of acute ischemic events [19, 20].

Frantz et al. [21] were among the first to study the role of TLRs in the inflammatory response due to cardiac ischemia and they have triggered extensive research in the field of TLRs and cardiac ischemia-related pathological conditions. Their observation that TLR-4 is upregulated in heart failure [21] has been confirmed in TLR-4 knockout mice which are relatively protected against adverse ventricular remodeling after infarction [22]. The association of TLR-4 signaling with myocardial ischemia/reperfusion injury is documented in various studies [23–26], and, moreover, the pharmacological inhibition of TLR-4 signaling reduces infarct size [27]. In humans, circulating TLR-4-positive cells and TLR-4 signaling are increased in patients with unstable angina and myocardial infarction [28].

Similar experimental proofs are present for TLR-2, as a critical modulator of post-infarct injury and repair. TLR-2 knockout mice have higher survival rates and less expansive remodeling after infarction [29]. In a murine myocardial ischemia/reperfusion injury model, we and others have shown that lethal reperfusion injury is mediated by circulating TLR-2. Administration of a monoclonal anti-TLR-2 antibody at a clinically applicable time point reduces infarct size with approximately 50% and improves cardiac function [30, 31]. These data, together with the fact that the postinfarct period and TLR-2 and -4 signaling share similar immunological characteristics [32], suggest that TLR-2 and -4 are promising therapeutic targets in cardiac ischemia. It is expected that in the near future these promising compounds will enter clinical trials for their efficacy and feasibility.

Fig. 1. Schematic representation of potential toll-like receptor ligands (exogenous and endogenous).

Potential Endogenous Activators of TLRs: Expression and Role in CVD

TLRs were initially described as recognizing proteins for bacterial and viral antigens. Bacterial LPS [33], PGN [34], different bacterial lipoproteins (triacyl [35], diacyl [36]) and single [37] or double-stranded viral RNA [38] signal via TLRs. *Chlamydia pneumoniae*, an infectious agent which happens to be a potent TLR activator, is found in different atherosclerotic lesions [39]. In animal models *C. pneumoniae* can stimulate atherogenesis [40] and therefore is considered a therapeutic target for atherosclerosis [41]. PGN, a TLR-2 ligand, is present in the bacterial flora at all mucosal sites from where it can travel along the bloodstream via monocytes. PGN is an exogenous microbial-derived TLR ligand and activator that is widely present in the gut of each individual. PG-loaded monocytes adhere to the activated endothelium at sites of inflammation and are found in atherosclerotic plaques with features of unstable/rupture-prone lesions [42].

Along with the classical bacterial and viral antigen recognition, TLRs are able to respond to endogenous molecules released during tissue injury [43, 44]. Below we summarize (see also fig. 1) and discuss the role of potential TLR endogenous activators in the progression of CVDs.

Heat-shock proteins (HSPs) are present in most cells, serving as molecular chaperones, and play a role in cell

protection from damage in response to stress stimuli [45]. TLR-2 and TLR-4 are able to recognize and respond to the soluble forms of HSP60 [8], HSP70 [46, 47] and the endoplasmic reticulum HSP90 (GP96) [48]. Several groups have reported that in human mononuclear cells, human soluble HSP60 binds to TLR-4/CD14, leading to p38 mitogen-activated protein kinase activation [49], whereas in smooth muscle and epithelial cells chlamydial and human soluble HSP60 stimulates extracellular signal-regulated kinase 42/44 activation [50]. In macrophages and endothelial cells, the binding of soluble HSP60 to the TLR-4/CD14 complex leads to the activation of MyD88-NF- κ B pathways; the same TLR-4-mediated signal pathway is described for soluble HSP70 [51].

HSPs have been found to be highly expressed in cardiovascular tissues and to induce inflammatory responses. These proteins are expressed during the development of atherosclerosis and elicit an immune response as auto-antigens [52]. HSP60 is observed in the cell mitochondria (endothelial, smooth muscle cells and mononuclear cells of the carotid and aortic plaque specimens) [53] and is transported to the cell membrane in response to stress stimuli. Auto-antibodies against HSP60 (demonstrated in patients with heart attacks and strokes) bind to cell membrane-exposed HSP60 and therefore induce damage of the cell [54].

HSPs are secreted under stress stimuli and the soluble forms have pro-inflammatory activities [55]; Chen et al. [56] have demonstrated that autologous HSP60 serves as a danger signal to the innate immune system, which results in pro-inflammatory responses, including the production of tumor necrosis factor (TNF)- α , IL-12, and IL-15. The soluble HSP60 is found in high concentrations in the serum of subjects with prevalent/incident carotid atherosclerosis [55]. The intensity of HSP60 expression in human plaque specimens positively correlates with the atherosclerotic severity [53, 57].

Furthermore, the expressions of HSP60 and the stress-inducible form of HSP70 correlate with the development of atherosclerotic lesions in the aortic tree of apoE-deficient mice [58]. Both HSPs are present on the aortic root and endothelium at lesion-prone sites of apoE knockout mice, before infiltration of mononuclear cells. The same study has shown a temporary expression of HSP60 and HSP70 on all major cell types in lesion-predisposed areas during atherogenesis [53].

Taken together, these results demonstrate that HSP60 and HSP70 are involved in both initiation as well as progression of atherosclerosis.

High-mobility group box-1 (HMGB-1) is described as a nonhistone, chromatin-associated nuclear protein [59]. HMGB-1 appears to have two distinct functions in cellular systems. First, it is an intracellular regulator of transcription, and second, HMGB-1 can occupy an extracellular role in which it promotes tumor metastasis and inflammation [60, 61]. Extracellular HMGB-1 participates in inflammatory processes and stimulates the secretion of TNF- α and different pro-inflammatory cytokines [60, 62]. HMGB-1 binds to TLR-2 and TLR-4 [63] and induces activation of endothelial cells and macrophages/monocytes with subsequent release of pro-inflammatory cytokines, chemokines and adhesion molecules. In human atherosclerotic plaques, HMGB-1 is highest in fibrofatty lesions where it is expressed and secreted by macrophages [64]. Along with an increase in HMGB-1 levels, also a concomitant decrease in HMGB-1 inhibitory proteins such as the anticoagulant protein thrombomodulin, which binds and sequesters HMGB-1 [65], is documented. In addition, the receptors for HMGB-1, namely TLR-2, TLR-4 and receptor of advanced end glycation products (RAGE), are upregulated in atherosclerotic lesions [66, 67]. Thus, HMGB-1 might be an important molecule linking innate immunity and inflammation in the pathogenesis of atherosclerosis.

HMGB-1 plays a pathogenic role in ischemic injury, because its circulating levels are increased shortly after

ischemia/reperfusion, and its inhibition is protective against liver ischemia/reperfusion injury [68]. Moreover, the expression levels of TLR-2, TLR-4 and RAGE are increased after cardiac injury [69], which might facilitate HMGB-1 to mediate an inflammatory response. In a murine myocardial ischemia/reperfusion injury model, HMGB-1 administration increases infarct size, while its inhibition significantly reduces infarct size via RAGE [70]. Several other HMGB-1-inhibiting agents (such as ethyl pyruvate, green tea and adrenomedullin) preserve cardiac function after myocardial ischemia [71, 72], consistent with a pathogenic role of HMGB-1 in CVDs.

Extradomain A of fibronectin (EDA), generated by regulated fibronectin RNA splicing [73], is absent from healthy plasma and extracellular matrix and yielded only during pathological tissue alterations. EDA is expressed after tissue injury due to inflammation and stimulates production of various cytokines [74] via TLR-4 through NF- κ B pathway [75]. In atherosclerotic plaques, fibronectin abundantly contains EDA, whereas in the adjacent unaffected vessel wall, fibronectin lacks EDA. In a model of atherogenetic-predisposed mouse (apoE knockout), systemic as well as local (plaque) EDA levels are elevated, while mice lacking both apoE and EDA have decreased atherosclerosis (as much as 67%) [76]. The observed effect is justified by EDA involvement in plasma lipoprotein metabolism and in macrophage foam cell formation. These observations imply a pro-atherogenic role for EDA in the pathogenesis of atherosclerosis. The findings in mice are strengthened by those in humans: our group has previously reported, in human carotid atherosclerotic plaques, high levels of fibronectin containing EDA in fibrous (more stable) and low levels in highly inflamed (unstable) lesions [77]. Additionally, patients with carotid atherosclerotic stenosis (>75%) and no clinical-associated complaints reveal high EDA plaque levels contrary to patients with clinical manifestations of carotid stenosis which have low EDA plaque levels. Although the exact role of EDA in the pathogenesis of atherosclerosis remains to be elucidated, there is no doubt that EDA is a molecule linking innate immunity and inflammation in atherosclerosis.

Myeloid-related protein (MRP)-8 and -14, also known as S100A8 and S100A9, are two calcium-binding proteins mainly expressed in cells of myeloid origin, particularly in monocytes and neutrophils [78]. Both proteins are secreted by activated monocytes and neutrophils and have pro-inflammatory effects [79, 80]. Upon cell activation, the two proteins form a complex, MRP-8/14, that translocates to the cytoskeleton and plasma membrane where

it is secreted [81]. Intracellularly, MRP-8 and MRP-14 essentially regulate phagocyte (monocytes and neutrophils) migration by integrating the calcium and mitogen-activated protein kinase transduction pathways, thereby controlling reorganization of the phagocyte microtubular system [82]. The secreted MRP-8/14 complex exerts antimicrobial activity [83], stimulates IL-8 production by airway epithelial cells [84] and transports arachidonic acid to endothelial cell targets affecting pathological responses in inflammation and atherosclerosis [85]. MRP-8, MRP-14 and MRP-8/14 are expressed in both mouse [86] and human atherosclerotic plaques [87]. MRP-14 and MRP-8 are expressed by subsets of macrophages during inflammation in different tissues [88] as well as in atherosclerotic lesions [87, 89].

Taking advantage of mice lacking functional MRP-8/14 complexes, Vogl et al. [90] have identified the endogenous MRP-8 and MRP-14 as activators of TLR-4. Their study shows that even though leukocytes of the MRP-14 knockout mice have a normal TLR-4 and CD14 (TLR-4 co-receptor for LPS) cell-surface expression, they have an impaired response toward LPS stimulation with less TNF- α production and secretion when compared to the wild-type leukocyte; addition of extracellular MRP-8/14 compensates for the reduced response in TNF- α of MRP-14 knockout leukocytes. Further analysis has shown that MRP-8 alone stimulates TNF- α RNA and protein secretion and has an additive effect on LPS-induced TNF- α secretion. Moreover, the same study has shown that leukocytes from mice with nonfunctional TLR-4 show no response to MRP-8. Direct binding of MRP-8 to TLR-4/MD2 complex is confirmed by surface plasmon resonance studies [90]. These findings prove that MRP-8/14 acts as an endogenous TLR-4 activator and is involved in amplification of LPS effects on leukocytes.

Libby et al. [86] have recently shown that MRP-8/14 is critical for the biological response to vascular injury. Using mice lacking functional MRP-8/14 complexes, the authors demonstrate reduced atherosclerotic lesion area and macrophage accumulation within the lesions in MRP-14 knockout mice, supporting a role for MRP in plaque formation and development.

In human carotid endarterectomy specimens, we have previously described an association between high MRP-8, MRP-14 and MRP-8/14 levels and the features of rupture-prone lesions [87], suggesting a role for MRP proteins in plaque destabilization and disruption. Additionally, we identified MRP proteins expression in a subset of nonfoam plaque macrophages, and in vitro, human monocyte-derived macrophages expressing MRP pro-

teins do not acquire a foamy phenotype when fed human oxidized LDL. This indicates MRP proteins as potential regulators of macrophage phagocyte capabilities, although additional proof is required.

Systemic MRP-8/14 complex relates to cardiac ischemic events: plasma MRP-8/14 levels are elevated in patients with acute coronary syndromes compared to patients with stable coronary artery disease or with normal coronary arteries [91]. Moreover, levels of systemic MRP-8/14 are elevated prior to markers of myocardial necrosis (myoglobin, creatine kinase – MB and troponin) and associated with increased risk of recurrent cardiovascular events compared to patients with lower MRP-8/14 levels [92]. Plasma MRP-8/14 concentrations are higher in patients with ST-segment elevation myocardial infarction compared to patients with stable coronary artery disease [93], and the risk of the first cardiovascular event increases with each quartile of plasma MRP-8/14.

Acknowledging all these findings, we conclude that MRP proteins are endogenous activators of TLR-4 occurring during atherosclerosis or acute coronary syndromes, therefore exacerbating the inflammatory response associated to those diseases.

Minimally modified LDL (mmLDL) is a pro-inflammatory and pro-atherogenic lipoprotein that, in contrast to oxidized LDL, is not recognized by macrophage scavenger receptors [94]. mmLDL possesses pro-atherogenic properties; for example, it induces monocyte adhesion to endothelial cells, production of colony stimulating factors, monocyte chemotactic protein-1 and tissue factor [94–96]. mmLDL also has pro-inflammatory capabilities; it is present in atherosclerotic plaques where it binds CD14 and TLR-4 [97] on macrophages, and induces TLR-4-dependent cytokine expression [98]. In addition, mmLDL promotes TLR-4/MD-2-dependent actin polymerization and spreading of macrophages [97]. Recently, Choi et al. [99] have shown that mmLDL enhances macrophage macropinocytosis, which results in lipid accumulation in macrophages and foam cell formation, by binding to a functional TLR-4 on macrophages. Additionally, the authors injected mice with mmLDL, and have proved its uptake by circulating monocytes. Their new data indicate that circulating monocytes, under hyperlipidemic conditions, can accumulate lipoproteins which might lead to their activation and enhanced migration into the vessel intima.

The active component in mmLDL, responsible for most of the observed effects is lipoxygenase (15LO)-cholesteryl esters. Atherosclerosis-prone mice (ApoE or LDL receptor knockout) lacking 12/15 LO (the mouse homo-

logue of human 15LO) have reduced atherosclerosis [100]; furthermore, enhanced expression and activity of 12/15LO has been reported in macrophages and endothelial cells in atherosclerotic plaques in mice [101].

All these imply that mmLDL constitutes an endogenous activator of TLR-4 and could be involved in sustained inflammation in atherosclerosis.

Free fatty acids promote insulin resistance and atherosclerosis in mice [102, 103] and are proposed as endogenous activators of TLR-2 and TLR-4 in these diseases. A saturated fatty acid, namely lauric acid, can activate TLR-2 in 293T cells and TLR-4 in RAW264.7 cells [104]. Similarly, an oleate/palmitate mixture of fatty acids induces TLR-4 signaling in 293T cells and causes inflammation in wild-type but not in TLR-4-deficient adipocytes [105]. Palmitate causes TLR-4-dependent $\text{I}\kappa\text{B}\alpha$ degradation in elicited peritoneal macrophages [102]. However, recent publications suggest that fatty acids do not directly stimulate TLR-4 or TLR-2, and that the observed effects might be due to sample contamination with TLR-4-stimulating bacterial LPSs (endotoxin), TLR-2-stimulating lipopeptide products or with an unidentified TLR endogenous ligand [106, 107]. Therefore, the mechanisms linking high-fat diets with TLR-associated pathologies such as atherosclerosis and insulin resistance remain to be discovered.

Summary

CVD is an inflammation-driven malignancy which involves innate immune responses partially due to TLR signaling. TLRs are emerging as promising therapeutic targets for atherosclerosis and cardiac ischemia due to their involvement in disease pathogenesis. TLRs are triggered by classical bacterial and viral particles (such as *C. pneumoniae* and PGN), but recent data suggest that endogenous molecules (such as HSPs, HMGB-1, EDA and MRPs) released from cells undergoing damage can also bind and stimulate these receptors. Exogenous and endogenous TLR activators have proved to be important contributors to the progression of atherosclerosis and related thrombotic complications.

TLR-related research has gained much attention and continues to be a dynamic field since TLR discovery in humans. In the near future, studies will reveal new potential endogenous ligands and provide more mechanistic insight into their biological functions.

Especially endogenous activators are appealing for both researchers and clinicians, since they are yielded only during pathological conditions and therefore might represent useful diagnostic tools and therapeutic targets. In addition, pharmacological interventions antagonizing these endogenous molecules will likely cause fewer side effects.

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