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Toll-like Receptor-4 and Lipoprotein Accumulation in Macrophages

Yury I. Miller^{1,*}, Soo-Ho Choi¹, Longhou Fang¹, and Richard Harkewicz²

¹Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093

²Department of Chemistry and Biochemistry, and Pharmacology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093

Abstract

Excessive lipid accumulation in macrophages, also known as foam cell formation, is a key process during the development of atherosclerosis, leading to vascular inflammation and plaque growth. Recent studies have identified a new mechanism of macrophage lipid accumulation in which minimally oxidized LDL (mmLDL) and its active components, polyoxygenated cholesteryl ester hydroperoxides, are involved in endogenous activation of toll-like receptor-4 (TLR4), which in turn leads to robust cytoskeletal rearrangements and macropinocytosis due to recruitment of spleen tyrosine kinase (Syk) and activation of its downstream cytoskeletal signaling targets. In hyperlipidemic environments, mmLDL-induced, TLR4- and Syk-dependent macropinocytosis leads to substantial lipid accumulation in macrophages and monocytes, which may constitute an important mechanism of foam cell formation in atherosclerosis. A novel hypercholesterolemic zebrafish model of early stages of atherosclerosis was used to demonstrate that the TLR4 deficiency significantly reduces the in vivo rate of macrophage lipid accumulation in vascular lesions.

Introduction

Atherosclerosis initially develops as a pathology of lipid accumulation in the vascular wall and then gradually progresses into a chronic vascular inflammatory disease. A major atherogenic process connecting lipid deposition in the vessel wall with inflammation is the formation of lipid-loaded macrophage “foam” cells. The process of foam cell formation involves retention of low-density lipoprotein (LDL) in the vascular wall and its modification, including oxidation and enzymatic degradation, followed by excessive uptake of modified LDL by macrophages, recruited to the sites of vascular lipid accumulation. Unregulated uptake of modified LDL by macrophages, combined with downregulated efflux of lipids, stimulates expression of pro-inflammatory cytokines by macrophages, antigen presentation, secretion of matrix-degrading enzymes, and often results in cell death, thereby promoting further lesion development and its eventual rupture.

The mechanisms of uptake of modified LDL by macrophages are diverse and in part reflect the multitude of LDL modifications. LDL is a macromolecule consisting of hundreds of

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*Corresponding author, yumiller@ucsd.edu, telephone 858-822-5771, fax 858-534-2005.

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molecules of phospholipid (PL), cholesteryl ester (CE), free cholesterol (FC) and triglyceride (TG), and one 512 kDa molecule of apoB100 protein. Polyunsaturated acyl chains in PL, CE and TG, as well as FC and many amino acids in apoB100, are highly susceptible to oxidation. Thus, LDL oxidation, a common *in vivo* modification of LDL, produces a myriad of lipid and protein oxidation products, many of them exhibiting distinct biological activities. Depending on the type of LDL modification, macrophages would use different binding receptors and different uptake mechanisms to remove these altered self-antigens from the tissue. For example, specific oxidized phosphocholine-containing PLs in oxidized LDL (OxLDL) bind to the scavenger receptor CD36 and mediate OxLDL uptake by macrophages (Webb and Moore 2007, Hartvigsen et al. 2009). In addition to the specific scavenger receptor-mediated uptake, macrophages can internalize LDL via fluid phase uptake, or macropinocytosis, which could be constitutive in M-CSF-differentiated macrophages or induced via activation of PKC (Kruth et al. 2005, Zhao et al. 2006). A comprehensive review of the lipoprotein uptake mechanisms and the discussion of their relevance to the formation of foam cells *in vivo* can be found in several recent articles (Witztum 2005, Webb and Moore 2007). In this paper, we will describe a novel uptake pathway, which combines the features of both specific receptor recognition via the toll-like receptor-4 (TLR4) and non-specific lipoprotein uptake via macropinocytosis, and discuss its relevance to foam cell formation *in vivo*.

Minimally oxidized LDL and polyoxygenated cholesteryl ester hydroperoxides

Our laboratory focuses on studying the composition and biological effects of minimally oxidized LDL (mmLDL), which represents an early form of oxidized LDL modified by 15-lipoxygenase (15LO). Human 15LO and mouse 12/15LO have been proposed to play a major role in *in vivo* LDL oxidation and the development of human and experimental murine atherosclerosis, as reviewed in (Zhao and Funk 2004). The family of 12/15LO enzymes is conserved between various animal and plant species and includes human 15LO, mouse 12/15LO (both expressed in macrophages), soybean lipoxygenase (SLO) and others. The classic reaction catalyzed by 12/15LO is the oxygenation of arachidonic acid at carbons 12 or 15 (hence the name of the LO enzyme) and the formation of 12(S)- or 15(S)-hydroperoxyeicosatetraenoic acid. 12/15LO (unlike 5LO) is capable of oxygenating not only free fatty acids (FFA) but also polyunsaturated acyl chains in PL and in CE (Yamamoto 1992), suggesting its role in oxidation of LDL and thereby in the development of atherosclerosis. Indeed, the importance of 12/15LO in the development of diet-induced atherosclerosis has been established in several murine models, including 12/15LO knockout and transgenic mice (Huo et al. 2004, Zhao and Funk 2004, Poeckel et al. 2009). The 12/15LO^{-/-}, apoE^{-/-} double knockout mice on a high-fat diet have less atherosclerosis, significantly lower titers of autoantibodies against OxLDL in plasma and lower isoprostane levels in urine as compared to apoE^{-/-} mice, indicating that 12/15LO is important in LDL oxidation *in vivo*. Overexpression of 15LO in mouse macrophages paradoxically reduced atherosclerosis, which may be due to increased synthesis of 15LO-dependent anti-inflammatory eicosanoids (Merched et al. 2008).

An incubation of LDL with isolated 12/15LO or with the cells expressing 12/15LO produces hydroperoxides of three classes: FFA-OOH, PL-OOH and, most profusely, CE-OOH (Yamamoto 1992, Ezaki et al. 1995, Harkewicz et al. 2008). Yoshimoto and co-workers suggested a highly plausible mechanism of how intracellular 12/15LO mediates oxidation of extracellular LDL and particularly of its CE residing in the hydrophobic core of the lipoprotein (Takahashi et al. 2005). According to their hypothesis, LDL binds to macrophage LDL receptor related protein-1 (LRP-1), which in turn induces 12/15LO translocation from the cytosol to the cell membrane. An exchange of CE between LDL and the cell in an LRP-1-dependent manner leads to 12/15LO-mediated oxygenation of the CE. Further, LRP-1 contributes to the efflux of

oxidized CE back to the LDL particle. This mechanism agrees well with the known preferential oxygenation of CE by 12/15LO expressing cells (Ezaki et al. 1995). Accumulation of CE hydroperoxides has been documented in human atherosclerotic lesions and in the lesions of apoE^{-/-} and LDLR^{-/-} mice fed a high-fat diet (Upston et al. 2002, Leitinger 2003, Harkewicz et al. 2008).

A liquid chromatography – mass spectrometry analysis of mmLDL demonstrated the presence of polyoxygenated CE hydroperoxides in which cholesterol is not oxidized but the arachidonate acyl chain carries a hydroperoxide at the 15th carbon and several other functional groups with 1 to 4 additional oxygen atoms (Figure 1). These CE hydroperoxides, which can be also generated by direct incubation of cholesteryl arachidonate with soybean 15LO (the product of this reaction is abbreviated as 15LO-CE), induced many (but not all) of the same biological responses in macrophages as did mmLDL, including membrane ruffling and actin polymerization, activation of ERK1/2 and its downstream targets (Harkewicz et al. 2008, Choi et al. 2009). The presence of a hydroperoxide group in 15LO-CE is critical because a hydroperoxide-reducing agent, ebselen, abrogated the biological activity of both mmLDL and 15LO-CE.

TLR4 mediates biological effects of mmLDL and 15LO-CE

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that sense the presence of numerous pathogen-associated molecular patterns (PAMPs). Activation of PRRs has been widely implicated in signaling mechanisms that contribute to chronic inflammatory diseases, including atherosclerosis. While PRRs were originally postulated to recognize only exogenous pathogens, they are now increasingly documented to respond to endogenous modified self, such as mmLDL and OxLDL. We have postulated that such modified LDL could become endogenous PAMP and initiate low-grade, but sustained PRR-mediated inflammation and other immune responses (Miller et al. 2003a).

The work from our laboratory has demonstrated that mmLDL binds to CD14, the receptor for bacterial lipopolysaccharide (LPS), and activates macrophages via TLR4/MD-2 (Miller et al. 2003c). Importantly, all mmLDL preparations have been tested for the presence of endotoxin and only endotoxin-free samples were used in experiments. Moreover, in contrast to the LPS stimulation, the most robust TLR4-mediated effects of mmLDL were in the regulation of cytoskeleton and to a lesser degree in the expression of proinflammatory cytokines (Miller et al. 2003c, 2005, Harkewicz et al. 2008, Choi et al. 2009, Bae et al. 2009). 15LO-CE also induced rapid and extensive macrophage spreading in a TLR4-dependent manner. Downstream from TLR4, as we discovered, spleen tyrosine kinase (Syk) was the major signaling molecule transducing the mmLDL signal, while MyD88 involvement was insignificant. mmLDL induced the recruitment of Syk to a TLR4 signaling complex, TLR4 and Syk phosphorylation, activation of a Vav1-Ras-Raf-MEK-ERK1/2 signaling cascade, phosphorylation of paxillin, and activation of Rac, Cdc42 and Rho GTPases, and the formation of a N-WASP/Arp2 complex – all are signaling components of cytoskeleton regulation, leading to dramatic, actindependent membrane ruffling (Figure 1). In addition, mmLDL induced TLR4-independent activation of PI3K (Miller et al. 2003b, 2005), which, in combination with Rho activity, facilitates ruffles closure into endosomes. These mmLDL-induced cellular events result in macropinocytosis, as is evident from phase contrast microscopy as well as from the increased uptake of small molecules and of high-molecular weight dextran (Choi et al. 2009).

TLR4-dependent lipid accumulation in macrophages and monocytes

Most importantly, mmLDL-induced and TLR4- and Syk-dependent macropinocytosis results in excessive intracellular lipid accumulation (Figure 1). Not only does mmLDL mediate its own uptake by macrophages, but because of the nature of fluid phase uptake,

macropinocytosing macrophages internalize all lipoproteins present in the media in the vicinity of the cell. This has been demonstrated with mmLDL-induced uptake of native LDL and, even more remarkably, with the uptake of OxLDL (Choi et al. 2009). Although OxLDL is internalized via highly specific and efficient CD36-, SRA- and other scavenger receptor-mediated binding, addition of mmLDL significantly increased the OxLDL uptake, suggesting that, under these conditions, a certain proportion of OxLDL was internalized independently of saturable scavenger receptor-mediated mechanisms. mmLDL itself did not bind to CD36. In contrast, knockdown of TLR4 or Syk abolished macrophage lipoprotein accumulation stimulated by either mmLDL or 15LO-CE (Choi et al. 2009).

It is generally believed that macrophage lipid accumulation occurs exclusively in the vessel wall. Indeed, as discussed above, differentiated tissue macrophages use multiple and diverse mechanisms to internalize OxLDL, mmLDL, enzymatically modified LDL and LDL retained by its binding to the extracellular matrix as reviewed in (Witztum 2005, Webb and Moore 2007, Tabas et al. 2007). Foam cells have previously been observed in the circulation of non-human primates after months of hypercholesterolemia and were thought to represent lipid-laden macrophages that entered the circulation from the intima (Faggiotto et al. 1984). However, under hyperlipidemic conditions, in addition to excessive accumulation of lipid by tissue macrophages, peripheral blood monocytes can also accumulate lipoproteins. Recent studies demonstrate that circulating monocytes of familial hypercholesterolemia (FH) patients accumulate lipid, with a clear trend of increased lipid accumulation from control subjects to heterozygous FH to homozygous FH patients (Mosig et al. 2008). Because FH patients lack a functional native LDL receptor (LDLR), monocyte lipid accumulation should occur in an LDLR-independent manner. In our experiments, an intravenous injection of fluorescently labeled mmLDL in wild type mice resulted in its rapid accumulation in circulating monocytes, which was significantly attenuated in TLR4-deficient mice (Choi et al. 2009). A number of lipid-loaded circulating monocytes correlated with the plasma concentration of cholesterol. As TLR4 is highly expressed on the surface of circulating monocytes in patients with cardiovascular disease (Methé et al. 2005, Geng et al. 2006), and cholesteryl ester hydroperoxides are present and stable in plasma, lipid uptake by monocytes in circulation via a TLR4-dependent mechanism may contribute to monocytes' pathological roles in atherogenesis. Peripheral blood monocytes are a source of tissue macrophages, which in large part determine the initiation, progression and outcomes of atherosclerosis. Monocytes already enriched in neutral lipids even before immigration into the artery intima may be more susceptible to further lipid accumulation, as well as having altered metabolic properties.

TLR4-dependent lipid accumulation *in vivo*: a zebrafish model

The macrophage is a professional scavenger and as such it employs various, often redundant, mechanisms of uptake, both specific and non-specific. We suggest that in the environment of lipid-rich fatty streaks, the specificity of mechanisms used by macrophages to take up lipoproteins increases with progressing degree of LDL oxidation. In early atherogenesis, TLR4 as a sensor for CE hydroperoxides in mmLDL may be a key regulator of macropinocytosis – a non-specific liquid phase uptake of lipoproteins. In later stages of atherosclerosis, the more advanced OxLDL would then lead to more specific mechanisms of OxLDL (OxPL) recognition and CD36-dependent endocytosis. The question is how to verify, in *in vivo* settings, the quantitative importance of the TLR4-dependent uptake mechanism.

To obtain quantitative data, we have suggested to determine kinetic rates of *in vivo* lipid uptake by wild-type and TLR4-deficient macrophages (Stoletov et al. 2009). For this purpose, we used optically transparent zebrafish larvae as an animal model for monitoring the kinetics of macrophage lipid accumulation in live animals. We found that feeding zebrafish a high-cholesterol diet (HCD) resulted in remarkable hypercholesterolemia and lipoprotein oxidation,

accompanied by the formation of fatty streaks and myeloid cell recruitment to major blood vessels. TLR4-deficient and wild-type mouse macrophages were injected into zebrafish larvae in which HCD has induced the formation of fatty streaks. Because zebrafish larvae are transparent and we used fluorescently labeled cells and fluorescent dietary lipids, we were able to monitor macrophage lipid uptake *in vivo*, directly in the environment of a fatty streak. Using this model, we found that the rate of *in vivo* lipid uptake by TLR4-deficient macrophages was significantly lower compared to the uptake by wild-type macrophages, supporting the results of our *in vitro* experiments (Stoletov et al. 2009, Choi et al. 2009). Similar measurements with macrophages lacking other receptors and signaling molecules could be used in the future to measure a set of rate constants to compare different mechanisms of foam cell formation. An advantage of the optically transparent zebrafish model is that unlike in cell culture experiments in which lipoproteins are modified *in vitro* and then added at arbitrary concentrations to the cells, in our settings, macrophages are exposed to a multitude of lipoprotein modifications occurring *in vivo*. There is no interference of the adaptive immune system in these experiments, as it is yet immature at this stage of larval development. In addition, larvae can be maintained for several days at 35–36°C to accommodate the optimal temperature range for transferred mammalian cells. Because the majority of the aspects of macrophage biology are conserved from worms to mammals, the zebrafish model will be useful in understanding the involvement of macrophages in the processes of atherogenesis in humans and enable informed design of therapies targeting foam cells.

Role of TLR4 in the development of atherosclerosis

Although kinetic studies of macrophage lipid uptake, as we have performed with mouse cells transferred into optically transparent zebrafish, are difficult to replicate in mammals, in general, mouse experimental studies support the role of TLR4 in the development of diet-induced atherosclerosis. The expression of TLR4, as well as of TLR2, has been found in endothelial cells and macrophages within human and mouse atherosclerotic lesions (Xu et al. 2001, Mullick et al. 2008). A deficiency in TLR4 or MyD88 (an adaptor molecule for many TLRs) attenuates the development of atherosclerosis in hyperlipidemic apoE^{-/-} mice, likely owing to the reduction in macrophage recruitment to atherosclerotic lesions (Michelsen et al. 2004, Bjorkbacka et al. 2004). Overexpression of both TLR2 and TLR4 in the intima of carotid arteries of hyperlipidemic rabbits significantly augmented atherosclerosis, although transfection of only TLR4 or only TLR2 resulted in little changes in atherosclerosis (Shinohara et al. 2007).

Evidence for the role of TLR4 in the development of human atherosclerosis is mixed. Kiechl et al. first reported that the common Asp299Gly TLR4 polymorphism (loss-of-function) was associated with a decreased risk of carotid artery and femoral artery atherosclerosis and cardiovascular cause of death (Kiechl et al. 2002). This TLR4 polymorphism was associated with lower plasma levels of pro-inflammatory cytokines, soluble adhesion molecules and acute phase proteins. In a different study, the Asp299Gly TLR4 polymorphism was also associated with a significantly reduced risk of acute coronary events, independent of other coronary risk factors (Boekholdt et al. 2003, Ameziane et al. 2003). In addition, carriers of the variant Asp299Gly allele better responded to statin therapy than other study subjects (Boekholdt et al. 2003, Holloway et al. 2005). However, the Asp299Gly polymorphism was not associated with coronary artery stenosis, cerebral ischemia, or progression of atherosclerosis in patients with familial hypercholesterolemia (Netea et al. 2004, Yang et al. 2003, Reismann et al. 2004). Because the above studies evaluated different clinical manifestations of atherosclerosis, the observed discrepancies are not necessarily contradictory. A large, prospective study is needed to establish a role of TLR4 polymorphism in cardiovascular disease.

Conclusions

The studies described in this brief review suggest a novel mechanism of lipid accumulation in macrophages via TLR4-dependent macropinocytosis. TLR4 is activated by endogenously derived polyoxygenated cholesteryl ester hydroperoxides present in minimally oxidized LDL and in atherosclerotic lesions. Further downstream, Syk recruitment to TLR4 mediates actin polymerization, membrane ruffling and macropinocytosis, a liquid phase uptake mechanism leading to significant lipid accumulation in macrophages. The *in vivo* relevance of the TLR4-dependent mechanism has been suggested in experiments with hypercholesterolemic zebrafish. The optical transparency of zebrafish permits temporal studies in a live animal, which may be useful in future experiments to compare the rates of macrophage lipid uptake via different mechanisms. Such comparative kinetic studies are important, since the formation of lipid-laden macrophage foam cells is a hallmark and a rate-limiting step in the initiation and progression of atherosclerosis. Because the processes regulating macrophage lipid accumulation are complex and diverse, it will be important to identify one or several key elements controlling foam cell formation as potential targets for atherosclerosis therapy.

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Figure 1.

Minimally oxidized LDL induces TLR4-dependent lipoprotein uptake by macrophages. Polyoxygenated CE hydroperoxides, with a generic structure shown in the top-center portion of the Figure ($n = 1 - 4$), are responsible for cytoskeletal rearrangements induced by mmLDL in macrophages. The TLR4/MD-2 receptor complex is required for the cytoskeletal effects of mmLDL and OxCE. The TLR4-dependent signaling pathway involves recruitment of Syk, likely via the Syk N-terminal SH2 domain binding to a phospho-tyrosine in the C-terminal domain of TLR4, phosphorylation of Syk, with subsequent activation of Vav1, Ras, Raf, MEK1 and ERK1/2. Downstream from ERK1/2, Cdc42, Rac and paxillin are activated, inducing actin polymerization and robust plasma membrane ruffling as shown in the bottom-left image (green,

F-actin; blue, nucleus). In addition, ERK1/2 mediates Rho activation, and mmLDL activates PI3K (in a TLR4-independent manner); both Rho and PI3K regulate ruffles closure into large endosomes. In the process of such a liquid phase uptake occurring in lipoprotein-rich environments, large quantities of lipoproteins become captured, including mmLDL and also native LDL and OxLDL. The result is the formation of lipid-laden macrophage foam cells, as the one shown in the bottom-right image (red, Oil Red O staining of intracellular neutral lipid). Note that this mechanism combines a specific receptor (TLR4) recognition of distinct oxidized lipid moieties in mmLDL and the non-specific macropinocytic uptake of a variety of lipoproteins present in the vicinity of a macrophage.