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Role of Nucleotide-binding and Oligomerization Domain 2 Protein (NOD2) in the Development of Atherosclerosis

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NOD2 (nucleotide-binding and oligomerization domain 2) was initially reported as a susceptibility gene for Crohn's disease, with several studies focused on elucidating its molecular mechanism in the progression of Crohn's disease. We now know that NOD2 is an intracellular bacterial sensing receptor, and that MDP-mediated NOD2 activation drives inflammatory signaling. Various mutations in NOD2 have been reported, with NOD2 loss of function being associated with the development of Crohn's disease and other autoimmune diseases. These results suggest that NOD2 not only has an immune stimulatory function, but also an immune regulatory function. Atherosclerosis is a chronic inflammatory disease of the arterial wall; its pathologic progression is highly dependent on the immune balance. This immune balance is regulated by infiltrating monocytes and macrophages, both of which express NOD2. These findings indicate a potential role of NOD2 in atherosclerosis. The purpose of this review is to outline the known roles of NOD2 signaling in the pathogenesis of atherosclerosis.

Key Words: Atherosclerosis, Chronic inflammatory disease, Immune balance, NOD2

NUCLEOTIDE BINDING AND OLIGOMERIZATION DOMAIN 2 PROTEIN (NOD2)

The nucleotide binding and oligomerization domain 2 protein (NOD2) is a member of the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family. It is also known as caspase recruitment domain-containing protein 15 (CARD15) and nucleotide-binding oligomerization domain (NOD)-like receptor with a CARD (NLRC2). The *NOD2* gene is located on chromosome 16q12-21, and encoded by the *CARD5* gene. However how to regulate the expression of NOD2 is not studied [1].

The NOD2 protein consists of two *N*-terminal caspase recruitment domains (CARD), a central nucleotide-binding domain (NBD; also known as a NOD domain), and a series of *C*-terminal leucine-rich repeats (LRPs). The LRP is similar to the repeats found in Toll-like receptors (TLRs), and senses muramyl dipeptide (MDP), a component of Gram-positive bacterial peptidoglycan [2]. Recognition of MDP by NOD2 triggers a conformational change in NOD2. NBD-mediated oligomerization of NOD2 is induced by ATP binding. The NBD domain contains a NACHT domain, winged helix and superhelical subdomains, which mediate NOD2 oligomerization [3-5]. NOD2 oligomerization promotes the recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIP2). Homotypic CARD-CARD interactions provide a large signaling platform to interact with RIP2 [6].

NOD2 is an intracellular bacterial-derived MDP sensing receptor, and is expressed in various cell subsets, particularly myeloid cells. NOD2-MDP interaction triggers innate immune responses. Alteration of these innate immune responses can result in several chronic inflammatory diseases, including Crohn's disease [2,7,8]. 118 NOD2 gene variants or polymorphisms have been reported [4]. The first report on NOD2 addressed the association of NOD2 variants with Crohn's disease [9], therefore, extensive studies have focused on the role of NOD2 with inflammatory bowel

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ABBREVIATIONS: ApoE, Apolipoprotein E; CARD15, caspase recruitment domain-containing protein; hnRNP A1, heterogeneous nucleoribonuclear protein A1; LRRs, leucine-rich repeats; MDP, muramyl dipeptide; MyD88, myeloid differentiation primary response gene 88; NACHT, NAIP (neuronal apoptosis inhibitos protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserine) and TP1 (telomerase-associated protein); NLRs family, NOD-like receptors family; NLRC2, NOD-like receptor with a CARD; NOD, nucleotide-binding and oligomerization domain; PAMP, pathogen-associated molecular patterns; PGE_2, Prostaglandin E_2; PGN, peptidoglycan; RIP2, receptor-interacting serine/threonine-protein kinase 2; PRPs, Pattern Recognition Proteins; TLRs, Toll-like receptors.

disease. The association of NOD2 with other diseases has been extensively studied and reviewed [3,5,9-13].

The three NOD2 gene mutations, the arginine to tryptophan conversion at amino acid residue 702 (R702W), the glycine to arginine change at 908 (G908R), and the frame shift-stop codon mutation at 1007 (1007fs), are commonly reported. Because G908R and 1007fs mutants are located in the LRP domain, these mutants cannot effectively recognize of microbial components, thus preventing NOD2 dimerization. Because the R702W mutant is located in the NBD domain, this mutant results in defective oligomerization. Downstream signaling of NOD2 is impaired by these mutations. These three mutants confer susceptibility to Crohn's disease rather than ulcerative colitis [4,11,14,15]. The association of various NOD2 gene mutations with several other rheumatic diseases has been studied in depth. However, the association of these three common variants and rheumatoid disease has not been demonstrated [4]. Only one study reported an association between R702W and psoriatic arthritis [16]. The association of NOD2 variants and atherosclerosis has not been shown. Only two reports regarding NOD2 and atherosclerosis were published in 2013 [17,18]. This review focuses on NOD2 immune regulation and the role of NOD2 in the pathogenesis of atherosclerosis.

ATHEROSCLEROSIS

Atherosclerosis is the most common pathological process of coronary artery and cerebrovascular disease, which, collectively, are the primary cause of sudden death worldwide [19]. Atherosclerosis is strongly associated with hypercholesterolaemia, hypertension, diabetes, smoking and genetic factors. Atherosclerosis begins with lipid deposition on the vessel wall and the alteration of endothelium hemostatic functions.

Such changes in endothelium hemostatic function promote inflammatory responses, including increased expression of adhesion molecules on endothelial cell, which leads to leukocytes penetration. Penetration of monocytes/macrophages into the intima of arteries is an important process in atherogenesis [20]. Reportedly, others inflammatory cells, including lymphocytes and mast cells, also contribute to plaque progression. The architectural changes in the intima characterize plaque formation. Atherosclerotic plaque is comprised of the necrotic core, calcified regions, accumulated modified lipids, and fibrous cap [21].

Atheroma progression is mediated by the production of inflammatory cells, such as vasoactive mediators, cytokines, and proteinases. Two major complications of atherosclerosis, plaque rupture and thrombosis, are regulated by inflammatory cells. Plaque rupture is caused by the disruption of fibrous cap integrity, which is mediated by matrix degrading enzymes from inflammatory cells; thrombosis is generally mediated by procoagulant molecules, such as tissue factor, but is also mediated by inflammatory cells. These points demonstrate that atherosclerosis is a chronic and multifactorial inflammatory disease and that disease progression is modulated by monocytes and macrophages, which are major players in innate immunity [21,22].

NOD2-MEDIATED INNATE IMMUNITY IN ATHEROSCLEROSIS

Innate immunity is the first line of host defense against pathogens. PRPs (Pattern Recognition Proteins) recognize common pathogen components known as pathogen-associated molecular patterns (PAMPs or microorganism associated molecular patterns, MAMPs). At least 50 PRPs have been reported in the mammalian system, and are calssified in to three groups: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NOD2 belongs to the NLR family.

Microorganisms are known to aggravate atherosclerosis. Various microbial agents have been reported in atheroma plaque, including *C. pneumonia*, *H. pylori*, Herpes simplex virus, Epstein-Barr virus, *Porphyromonas gingivalis*, etc [23-28]. However, antibiotics do not attenuate disease severity [29], and atherosclerosis is not altered in the experimental germ-free environment [30]. These experimental evidences suggest that microorganisms do not contribute to the development of atherosclerosis. However, it has been reported that TLR signaling mediates atherogenesis. TLRs initiate innate immune responses by sensing microbes on the myeloid cell surface. PAMPs recognition by TLR induces the myeloid differentiation primary response gene 88 (MyD88) activation, which then promotes NF-κB nuclear translocation via signaling cascades. In Apolipoprotein E (ApoE) and MyD88 double deficient mice or ApoE and TLR2/TLR4 double-deficient mice, atherosclerotic plaque formation and macrophage recruitment is reduced [31]. The effect of TLR1, TLR3, and TLR6 in atherogenesis is not clear, and that of TLR5, TLR8, and TLR9 is unknown. Taken together, these points suggest that atherosclerosis development is characterized by heterogeneous and complex immune balance.

NOD2 mediates bacterial clearance in myeloid cell cytoplasm and regulates innate immune responses [5,7,8,13]. NOD2 deficient mice are susceptible to bacterial infection, including *S. aureus* [32], *C. pneumonia* [33], *H. pylori* [34], and *B. anthracis* [35]. This susceptibility is due to the decreased proinflammatory cytokine production and leukocyte recruitment [35]. MDP, the ligand of NOD2 as described above, present in atherosclerotic plaque [36]. Taken together, we hypothesize that NOD2 may play some role as an immune regulator in atherosclerotic plaque.

It is reported that ApoE and NOD2 double-deficient mice are more susceptible to atherosclerosis [18]. Total serum cholesterol levels, serum inflammatory cytokine levels, leukocyte infiltration, and atherosclerotic plaque are elevated in ApoE and NOD2 double-deficient mice than ApoE deficient mice. Atherosclerosis development is enhanced by *P. gingivalis* infection, and this enhancement is more pronounced in ApoE and NOD2 double-deficient mice. When MDP is used to activate NOD2, serum inflammatory cytokine levels are decreased in ApoE deficient mice [18]. These results suggest that NOD2 has anti-inflammatory and anti-atherogenic effects. This is in contrast to TLRs, even though both NOD2 and TLRs are bacterial sensing receptors that initiate innate immune responses. However, the molecular mechanism of NOD2-mediated inflammatory regulation is not known. In this review, we will discuss a potential common signaling pathway that is both triggered by NOD2 and involved in atherosclerosis.

NF-κB SIGNALING, NOD2 AND ATHEROSCLEROSIS

MDP-NOD2 interaction triggers homotypic dimerization via CARD-CARD binding; this dimerization follows RIP2 recruitment and activation. In a step-wise fashion, RIP2 activates NF-κB, and AP-1 by TAK1 (TGF-β activated kinase 1) complex, and NEMO, p38, JNK and ERK MAPK pathway [3-6]. This results in the expression of several inflammatory cytokines, including TNF-α, IL-6, IL-8, CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 2 (CXCL 2). These cytokines induce the recruitment and priming of innate immune cells, including neutrophils and monocytes [37]. It has also been reported that NOD2 signaling inhibits activation of TLR2-induced c-Rel, an NF-κB subunit; TLR2-induced c-Rel activation is enhanced in NOD2 deficient mice [38]. The crosstalk between TLR4 and NOD2 is also reported. MDP-NOD2 signal inhibits TLR4-LPS induced IL-12 synthesis [39]. These observations suggest that NOD2 works as both a positive and negative regulator of NF-κB activation. The molecular mechanism of this ambivalent property of NOD2 is not clear, and may depend on microenvironmental conditions or activation context.

In experiments with ApoE and MyD88 double-deficient mice, the inhibition of TLR2- or TLR4- induced p65 NF-κB activation decreases atherosclerosis. However, inhibition of NF-κB activation by deletion of I-κB kinase 2 (IKK2), which is essential for NF-κB activation, in macrophages, increases atherosclerosis in LDL receptor deficient mice [40]. It is contradictory that both TNF-α, a pro-inflammatory cytokine, and IL-10, an anti-inflammatory cytokine, production are decreased in IKK2-deleted macrophages. These results suggest that pro- and anti-inflammatory balance is important to control the development of atherosclerosis. Taken together, we hypothesize that NOD2 works as a key player to regulate immune balance in atherosclerosis according to microenvironmental conditions. Understanding of NOD2 regulatory signaling in atherosclerosis may lead us towards the development of atherosclerosis treatment.

IL-10 AND NOD2 IN ATHEROSCLEROSIS

IL-10 is produced by macrophages, T cells, and B cells, and is associated with the differentiation of Th2 and Treg cells, therefore, it works as a broad-spectrum inflammation inhibitor [41]. Administration of IL-10 delays atherosclerosis development, and IL-10 and ApoE double deficient mice are more susceptible to atherosclerosis. It is clear that IL-10 has anti-atherogenic properties [22].

MDP-NOD2 activation alone cannot induce the IL-10 production from myeloid cells. However, co-stimulation with PGN (peptidoglycan, TLR2 ligand), but not LPS (TLR4 ligand), induces IL-10 secretion. In this pathway, IL-10 transcription is regulated by phosphorylation of heterogeneous nucleoribonuclear protein A1 (hnRNP A1), and p38 MAPK [42]. It is also reported that the three major NOD2 mutants, particularly 1007fs, inhibit IL-10 transcription following TLR2 activation. Crohn's disease patients carrying the 1007fs mutant have impaired hnRNP A1 phosphoylation, and IL-10 secretion. IL-10 impairment may cause uncontrolled chronic inflammatory disease. Normal MDP-NOD2 signal cannot inhibit IL-10 production followed by TLR2 stimulation, but 1007fs patients have IL-10 production impairment followed the strong TLR2 stimulation. It can say that 1007fs mutants gain of this IL-10 inhibitory functions [42].

ApoE deficient mice challenged with MDP produce more IL-10 and have less atherosclerotic lesion. ApoE and NOD2 double-deficient mice produce more IL-10, despite having large atherosclerotic lesions. Based on the impairment of IL-10 production in 1007fs mutant patients, it is possible that the 1007fs mutant may show a gain of function to inhibit IL-10 suggesting that patients with the 1007fs NOD2 mutant may be more susceptible to atherosclerosis. Although this has not been reported, it has been shown that inflammatory bowel disease patients have a greater risk of developing atherosclerosis [43,44].

PGE_2 AND NOD2 IN ATHEROSCLEROSIS

Prostaglandin E_2 (PGE_2) is an inflammatory lipid molecule in the eicosanoid family, the synthesis of which is initiated by the release of arachidonic acid (AA) from membrane glycerophospholipids through cytosolic phospholipase A2a. PGE_2 synthesis depends on the activity of cyclooxygenase-1, cyclooxygenase-2, and PGE_2 synthases (PGESs). It has been reported that TLR4 activation in macrophages drives PGE_2 production by the upregulation of cyclooxygenase-2 through the NF-κB pathway, and that PGE_2 has a proinflammtory role [45]. However, the effect of PGE_2 in atherosclerosis is still controversial. C-reactive protein (CRP) which inhibits PGE_2 expression increase in atherosclerotic plaque; PPAR-γ induces PGE_2 expression, also increases in atherosclerotic plaque. PGE_2 can modulate platelets adhesion on atherosclerotic plaque, however, PGE_2 receptor antagonists does not modulate atherosclerosis [46]. Considering these results, it is possible that PGE_2 can play different roles, either anti- or pro-atherogenic, depending on the disease state.

NOD2 is highly expressed in atherosclerotic lesions and PGE_2 is upregulated by MDP treatment in ex vivo cultured human carotid atherosclerotic plaque [17]. These results indicate that enhanced NOD2-mediated PGE_2 signaling is involved in atherogenesis. PGE_2 has both proinflammatory and anti-inflammatory effects depending on cell types and its receptor subtypes, suggesting that the function of NOD2 in atherosclerosis is ambivalent. From these reports, the question remains, as to how NOD2 regulates this immune balance, and what confers susceptibility against some diseases.

MDP induces inflammation and a vulnerable plaque phenotype [36], and PGE_2 production [17]. PGE_2 mediates platelet aggregation on ruptured plaque [46]. This suggests that the effect of MDP-NOD2 signal may be more important on vulnerable plaque formation not whole atherosclerosis pathogenesis. Because the most critical and fatal event in atherosclerosis is vulnerable plaque rupture, understanding this mechanism will provide new insights to predict and treat atherosclerosis and its complications.

T CELL MEDIATE IMMUNITY AND NOD2 IN ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease, with both innate and adaptive immune responses contributing to disease initiation and progression. It has been demon-

strated that Foxp3$^+$ expressing Treg cells have a protective effect against atherosclerosis. Similarly, IL-10 or TGF-β, which is important for Treg differentiation, has an anti-atherogenic effect. ApoE deficient mice have less Treg cells than control mice [38,47]. Treg cells are known to inhibit T cell activation and maintain immune balance, thereby modulating disease progression. Th1 response is known as a pro-atherogenic process by producing proinflammatory cytokines, such as IFN-γ, and IL-6, and regulating antibody production from B cells. IFN-γ injection induces plaque growth, and IFN-γ or and IFN-γ receptor deficient mice have smaller atherosclerotic plaques [48]. The Th2 immune effect in atherosclerosis is still controversial. Severe hypercholesterolemia in ApoE deficient mice induces a switch in the Th2 immune response; however, IL-4 production does not prevent the development of atherosclerosis. IL-17 production is increased in Ldlr or ApoE deficient mice fed a Western diet to develop atherosclerosis. ApoE and IL-17 double-deficient mice are less susceptible to atherosclerosis. These results suggest that Th17 immunity is pro-atherogenic [48].

As an intracellular receptor against microbes, NOD2 mediates innate immune response. In contrast, NOD signaling does not strongly induce co-stimulatory molecule expression on DCs. Whereas TLRs primarily induce a Th1 immune response, NOD2 promotes a Th2 immune response. Indeed, NOD2 actually inhibits the TLR2 induced Th1 immune response. Secretion of IL-12, a major cytokine that induces Th1 differentiation from macrophages, by PGN treatment is reduced by MDP co stimulation [38]. These results suggest that NOD2 may shift the immune balance to Th2 from Th1, thus causing the anti-atherogenic Th2 immune response. NOD2 also regulates Th17 cell responses. NOD2 signaling affects the production of IL-23, an important cytokine for Th17 differentiation, from dendritic cells by miR-29 upregulation [5,49]. In regards to atherosclerosis, it is possible that NOD2 reduction of the Th17 immune response in atheroma plaque contributes to its anti-atherogenic effect.

DISCUSSION

Systemic immune network balance determines atherosclerosis progression, but the mechanism is not clear.

The Amar group reported that atherosclerosis development is increased when NOD2 signaling is impaired [18]. It is similar that the 1007fs mutant disrupts TLR2 signaling, even though downstream signaling through RIP2 is impaired. Further studies are required to address the association of the gain of function (inhibit TLR2 signal) of 1007fs, or other mutants, with atherosclerosis development. The Yan group reported that MDP mediated innate immune signaling induces PGE_2 production in atherosclerotic plaque [17]. Can NOD2 signaling mediate the vulnerable plaque formation? If NOD2 induces the vulnerable plaque formation, it will work as pro-atherogenic. However, it is not consistent with the study on NOD2 deficient mice as it shows the anti-atherogenic effect. These findings lead the question what is the role of NOD2 in atherosclerosis.

118 NOD2 variants have been associated with various diseases, including Crohn's disease and other rheumatoid diseases [4]. Many genes conferring susceptibility to atherosclerosis also have been reported [50]. However, the genetic association of NOD2 variants with atherosclerosis has not been reported until recently. The risk of atherosclerosis development is higher in inflammatory bowel disease patients [43,44]. Based on these data, it is possible that a relationship exists between NOD2 mutation and atherosclerosis development.

There are 22 known NLRs in humans [12], all sharing a domain structures. Particularly, NOD1 has a similar structure to NOD2; NOD1 has only one CARD domain, while NOD2 has two. NOD1 is widely expressed in whole tissue, NOD2 expression is limited to myeloid cells, keratinocytes, and epithelial cells of intestine, lung, and oral cavities [3,51-55]. Only one paper reported on NOD1 and atherosclerosis in 2015; administration of the NOD1 ligand, FK565, to ApoE deficient mice accelerated atherosclerosis development [56]. NOD2 has quite different effects, leading to the question of what causes such differences.

In this review, we discussed the possible role of NOD2 signaling in atherosclerosis pathogenesis; we also summarized two recent studies revealing the relationship between NOD2 and atherosclerosis. Based on these reported events, several questions are raised here. Undoubtedly, elucidating these questions will provide new insights into the mechanisms of host defense and the pathogenesis of inflammatory diseases.

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