Nucleotide-Binding Oligomerization Domain Protein 2 Deficiency Enhances Neointimal Formation in Response to Vascular Injury


Objective—Nucleotide-binding oligomerization domain protein 2 (NOD2) stimulates diverse inflammatory responses resulting in differential cellular phenotypes. To identify the role of NOD2 in vascular arterial obstructive diseases, we investigated the expression and pathophysiological role of NOD2 in a vascular injury model of neointimal hyperplasia.

Methods and Results—We first analyzed for neointimal hyperplasia following femoral artery injury in NOD2+/+ and NOD2−/− mice. NOD2−/− mice showed a 2.86-fold increase in neointimal formation that was mainly composed of smooth muscle (SM) α-actin positive cells. NOD2 was expressed in vascular smooth muscle cells (VSMCs) and NOD2+/− VSMCs showed increased cell proliferation in response to mitogenic stimuli, platelet-derived growth factor-BB (PDGF-BB), or fetal bovine serum, compared with NOD2+/+ VSMCs. Furthermore, NOD2 deficiency markedly promoted VSMCs migration in response to PDGF-BB, and this increased cell migration was attenuated by a phosphatidylinositol 3-kinase inhibitor. However, protein kinase C and c-Jun N-terminal kinase inhibitors exerted negligible effects. Moreover, muramyl dipeptide–stimulated NOD2 prevented PDGF-BB–induced VSMCs migration.

Conclusion—Functional NOD2 was found to be expressed in VSMCs, and NOD2 deficiency promoted VSMCs proliferation, migration, and neointimal formation after vascular injury. These results provide evidence for the involvement of NOD2 in vascular homeostasis and tissue injury, serving as a potential molecular target in the modulation of arteriosclerotic vascular disease. (Arterioscler Thromb Vasc Biol. 2011;31:2441-2447.)

Key Words: restenosis • vascular biology • nucleotide-binding oligomerization domain protein 2 • smooth muscle cells • vascular injury

Nucleotide-binding oligomerization domain protein 2 (NOD2), initially described as a susceptibility gene for Crohn disease and inflammatory bowel diseases,1,2 is an intracellular protein containing leucine-rich repeats similar to those found in Toll-like receptors (TLRs). NOD2 is the intracellular pathogen recognition receptor (PRR) responsible for recognition of bacterial peptidoglycans from Gram-positive and Gram-negative bacteria through its interaction with muramyl dipeptide (MDP).3 The expression of NOD2 has been reported in myeloid cells, particularly macrophages, neutrophils, and dendritic cells, as well as in Paneth cells in the small intestine.4,5 Moreover, NOD2 expression can be induced by the proinflammatory cytokines tumor necrosis factor-α and interferon (IFN)-γ in cultured intestinal epithelial cells.6 However, NOD2 expression and novel functions have been suggested in other cell types, including adipocytes, gingival and pulp fibroblasts, and vascular endothelial cells.6–10

The proliferation and migration of vascular smooth muscle cells (VSMCs) from the media into the intima contribute to pathological conditions, including restenosis after angioplasty, posttransplantation coronary artery disease, and hypertensive vasculopathy.11 These events can be induced by cytokines and growth factors, such as platelet-derived growth factor (PDGF) after vascular injury.12 PDGF stimulates intracellular signal molecules with Src homology 2 domains, including Src, phosphatidylinositol 3-kinase (PI3K), and ras/raf-1.13 Src and PI3K perform crucial functions related to actin reorganization, growth, and migration in response to PDGF in the VSMCs, and these activities are mediated by the activation of a family of serine/threonine-specific protein kinases and the mitogen-activated protein kinases.14 Interestingly, agonists of TLRs, including TLR3 and TLR9, have been suggested to regulate the production of PDGF.15 In addition, enhanced expression of TLRs (TLR1, TLR2, and TLR4) has been shown in human atherosclerotic plaques.16

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Animal experiments have also demonstrated that an absence of TLR2 or TLR4 in atherosclerotic-susceptible mice (low-density lipoprotein receptor-deficient or apolipoprotein E-deficient mice, respectively) results in a reduction in atherosclerotic lesion formation and that femoral artery injury in the presence of a TLR4 agonist (lipopolysaccharide) augments neointimal formation. Taken together, these studies suggest a role for PRRs in the pathobiology of vascular disease.

In this study, we found that NOD2 was expressed in VSMCs and that NOD2 regulated the proliferation and migration of cells exposed to factors such as PDGF-BB. We also showed that NOD2 contributed to vascular homeostasis and that the absence of NOD2 enhanced neointimal formation after vascular injury.

**Materials and Methods**

For any of the Materials and Methods not described in detail, please see the supplemental materials, available online at [http://atvb.ahajournals.org](http://atvb.ahajournals.org).

**Animals**

NOD2−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME) on a C57BL/6 (N7) genetic background, and the mice were further bred with C57BL/6 mice and then maintained by crossing either heterozygous or homozygous mutant mice within our animal facility at Harvard Medical School. The Standing Committee on Animal Care at Harvard Medical School approved all animal experimentation protocols of this study under the guidelines of our approved institutional animal care and use committee protocol.

**Femoral Artery Injury**

Endoluminal injury to the mouse left common femoral artery was performed as described. The contralateral right femoral arteries of the same mice undergoing endoluminal injury were used as noninjured control vessels. Noninjured and injured femoral arteries were harvested 28 days after femoral artery injury and fixed with 10% buffered formalin. Paraffin-embedded sections were sectioned as described.

**Histological Analysis and Immunohistochemistry**

Histological analyses were performed for histological and morphometric analyses. Vessel sections were stained for elastin (Sigma-Aldrich) and CD45 (BD Biosciences, Franklin Lakes, NJ), respectively.

**Reagents and Antibodies**

Protein kinase inhibitors (Enzo Life Science, Plymouth Meeting, PA), mouse and human PDGF-BB (PeproTech Inc., Rocky Hill, NJ), and MDP (Sigma-Aldrich) were used in the studies. All signaling antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA). All small interfering RNA (siRNA) reagents were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

**Culture of VSMCs**

Primary NOD2+/+ and NOD2−/− VSMCs from mice (mVSMCs) were obtained with the use of collagenase and elastase digestion of aortas as described previously. Human primary aortic smooth muscle cells (hVSMCs) were purchased from Clonetics Corp (San Diego, CA).

**Western Immunoblotting**

Western immunoblotting was performed as previously described.

**Transient Transfection**

The wild-type mouse and human VSMCs were transfected with mouse and human NOD2 siRNA and control siRNA as described by the manufacturer (Santa Cruz Biotechnology). The cells were used for migration and proliferation assays 48 hours after transfection.

**Cell Migration and Proliferation Assays**

Cell migration was assessed using gelatin-coated 24-well chambers and polycarbonate membranes, 8-μm pores (Corning, Lowell, MA). Cell proliferation was determined using the Ez-Cytox Cell Viability Assay Kit (Daeillab Service Co Ltd, Seoul, Korea).

**Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from mouse and human VSMCs and tissues (spleen and aorta) using Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed using the TaqMan kit in a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA). Primers and probes were purchased from Applied Biosystems, and assay IDs were as follows: mouse NOD2, Mm00467543_m1; human NOD2, Hs00223394_m1; mouse NOD1, Mm00805062_m1; mouse TLR2, Mm00442336_m1; and mouse TLR4, Mm00445274_m1. Expression of mouse and human target genes was normalized to mouse GAPDH expression levels, respectively.

**Statistical Analysis**

Data are represented as mean±SD. For comparisons between 2 groups, we used the Student 2-tailed unpaired t test. For comparisons of timed series experiments, we performed Student paired t tests. For comparisons between more than 2 sets of experimental conditions, we used a 1-way analysis of variance (ANOVA). If significant, the ANOVA was followed by a protected Fisher least significant difference test and a Scheffe F-test. Statistically significant differences were accepted at P<0.05.

**Results**

To investigate the role of NOD2 in the vasculature, we examined the formation of neointimal lesions after femoral artery injury in NOD2+/+ and NOD2−/− mice. There were no differences in the structure of noninjured femoral arteries between NOD2+/+ and NOD2−/− mice (Figure 1A). The vessel size (inside area of external elastic lamina) of injured femoral arteries was not different between NOD2+/+ (43.21±0.82 mm², n=10) and NOD2−/− mice (41.86±0.56 mm², n=16) 28 days after injury. The medial areas were also similar between NOD2+/+ (12.33±0.23 mm², n=10) and NOD2−/− mice (11.57±0.19 mm², n=16). In the injured vessels of NOD2+/+ mice, neointimal thickening (area inside of internal elastic lamina excluding luminal area) was evident, although small (5.01±0.86 mm², n=10). In contrast, we observed a robust increase in intimal thickening in NOD2−/− mice after injury (16.85±0.75 mm², n=16) (Figure 1A and 1B). An absence of NOD2 increased the intimal/media ratio 2.86-fold, 1.54±0.22 in NOD2−/− compared with 0.37±0.21 in NOD2+/+ mice (Figure 1C). NOD2−/− neointima were composed mainly of smooth muscle α-actin positive cells. Both groups of mice showed sparse CD45-positive inflammatory cells after vascular injury in the neointima (Figure 1D). The arrows (red) highlight CD45-positive cells. These results suggest that NOD2 has a broad...
influence on the vascular response to injury, and an important physiological role in VSMCs.

In VSMCs, expression of NOD2 was not previously reported. Hence, we investigated whether NOD2 is expressed in VSMC, and whether NOD2 is induced by stimuli known to increase its expression in other cell types. Total RNA was isolated from the aortas of NOD2−/− and NOD2−/− mice. As shown in Figure 2A, mRNA expression of NOD2 was detected in NOD2+/+ but not in NOD2−/− aortas. However, mRNA expression of other PRRs (NOD1, TLR2, and TLR4) was not different in the aortas of NOD2+/+ and NOD2−/− mice (Figure 2A). Spleen was used as a positive control for mRNA expression of NOD2 and other PRRs. To investigate NOD2 expression in VSMCs, we harvested the cells from aortas of littermate mice. NOD2 mRNA was detected in NOD2+/+ VSMCs but not in NOD2−/− VSMCs (Figure 2B). In NOD2−/− VSMCs, expression levels of NOD1 and TLR2 mRNA were enhanced compared with NOD2+/+ VSMCs. However, TLR4 expression was slightly decreased in NOD2−/− VSMCs (Figure 2B). Some of these changes in PRR mRNA levels were modest; therefore, we will need to confirm whether these changes translate into an alteration in protein levels. Selective VSMC genes (calponins, caldesmon, smooth muscle Myh11) were expressed similarly in both NOD2+/+ and NOD2−/− VSMCs (Supplemental Figure I).

The tumor necrosis factor-α and IFN-γ are known to regulate NOD2 expression in other cell types, including intestinal epithelial cells. To investigate induction of NOD2 mRNA, we treated mVSMCs and hVSMCs with IFN-γ and MDP. Interestingly, expression of NOD2 mRNA was increased by IFN-γ in VSMCs but not by the NOD2 ligand MDP (Figure 2C). These data suggest that NOD2 mRNA is expressed in VSMCs and may have a role in VSMCs physiological and pathophysiological conditions.

To investigate potential mechanisms by which an absence of NOD2 leads to increased neointimal formation after vascular injury, we assessed VSMC proliferation, migration, and cell death in vitro. NOD2−/− VSMCs exhibited increased cell proliferation compared with NOD2+/+ VSMCs in response to mitogenic fetal bovine serum stimulation (Supplemental Figure II). We further analyzed whether NOD2 deficiency could increase cell proliferation in response to PDGF-BB, which is a well-known potent growth factor and chemoattractant for VSMCs, and released at sites of vessel injury. The proliferation of VSMCs was enhanced in
NOD2−/− (27.00±4.64%) compared with NOD2+/+ cells 4 days after PDGF-BB treatment (Figure 3A). In addition, treatment with siRNA-mediated NOD2 knockdown increased VSMC proliferation (Figure 3B). This increased proliferation of NOD2 deficient cells was not due to a difference in cell adhesion after plating, as adhesion was 77% in NOD2−/− cells and 75% in NOD2+/+ cells (P>0.05, not significant). Real-time RT-PCR was performed to verify downregulation of NOD2 expression by mouse NOD2 siRNA (Figure 3C).

Next, the involvement of NOD2 deficiency in cell migration was measured using gelatin-coated 24-Transwell chambers. NOD2−/− VSMCs showed a 4.05±0.53-fold increased migration in response to PDGF-BB compared with NOD2+/+ VSMCs, 24 hours after PDGF-BB treatment, and the number of migrated cells was counted and represented as a graph (Figure 4A and 4B). Similarly, we observed increased hVSMCs migration in human NOD2 siRNA-treated cells in response to PDGF-BB, compared with control siRNA-treated hVSMCs (Figure 4C and 4D). Real-time RT-PCR was performed to verify downregulation of human NOD2 expression by human NOD2 siRNA (Figure 4E). These results indicate that in the absence of NOD2, VSMCs proliferate and migrate more rapidly in response to PDGF-BB. However, PDGF-BB did not alter mRNA expression of NOD2 (Supplemental Figure III). Interestingly, cells treated with lysophosphatidylcholine and H2O2, which are well known to induce cell death in VSMCs, showed only modest differences in viability between NOD2−/− and NOD2+/+ VSMCs (Supplemental Figure IV). These effects on viability were not nearly as dramatic as the enhanced proliferation and migration in NOD2−/− compared with NOD2+/+ VSMCs. Cell viability of NOD2−/− VSMCs was slightly increased by low concentrations of lysophosphatidylcholine but decreased by H2O2 compared with NOD2+/+ cells (Supplemental Figure IV). These results suggest that NOD2 plays an important role in the prevention of VSMC proliferation and, more dramatically, migration, without markedly affecting cell death.

PDGF-BB stimulates many intracellular signaling molecules including PI3K.13–14 To identify which signaling pathways mediate VSMC migration in the absence of NOD2, chemical inhibitors of protein kinase C, PI3K, and c-Jun N-terminal kinase were administrated to NOD2−/− VSMCs before PDGF-BB treatment, and migration was measured (Figure 5A). Remarkably, enhanced migration of NOD2−/− VSMCs was abolished when exposed to a PI3K inhibitor, LY294002, compared with NOD2+/+ VSMCs, in response to PDGF-BB stimulation (Figure 5A). The protein kinase C inhibitor GF10203X did not alter the enhanced migration of NOD2−/− VSMCs. A JNK inhibitor, SP600125, blunted the migration of VSMCs to PDGF-BB in both NOD2−/− and NOD2+/+ cells; however, NOD2−/− cells maintained their increased migration response in comparison with NOD2+/+ cells. Other signaling inhibitors, such as p38 and ERK inhibitors, did not alter the migration of NOD2−/− VSMCs (Supplemental Figure V). Thus, we focused on the PI3K pathway and its downstream target Akt. Levels of p-Akt (Ser473 and Thr308) returned to baseline by 15 minutes after PDGF-BB stimula-
Moreover, basal levels of p-Akt were not different between elevated throughout the time course (Figure 5B and 5C). In contrast, p-Akt levels (Ser473 and Thr308), total Akt, and GAPDH were determined at the indicated time points after PDGF-BB treatment. This panel denotes representative blots of 3 independent experiments, which are quantitated in C.

Figure 5. Inhibition of the phosphatidylinositol 3-kinase (PI3K) signaling pathway prevents increased migration in nucleotide-binding oligomerization domain protein 2 (NOD2)−/− vascular smooth muscle cells (VSMCs). A, VSMC migration assay was performed to measured migration of NOD2+/+ and NOD2−/− VSMCs in response to platelet-derived growth factor-BB (PDGF-BB) or PDGF-BB plus protein kinase C (PKC) inhibitor (GF10203X, 1 μmol/L), PI3K inhibitor (LY294002, 10 μmol/L), or c-Jun N-terminal kinase inhibitor (SP600125, 10 μmol/L). *P<0.05, increased migration of NOD2−/− compared with NOD2+/+. The number of migrated cells were measured and is represented as a graph. Values are mean±SD, n=4. **P<0.05 PDGF-BB vs PDGF-BB plus MDP in mouse or human VSMCs.

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PRRs are a class of innate immune response–expressed proteins that respond to pathogen-associated molecular patterns, yet they also respond to endogenous stress signals termed damage-associated molecular patterns.24–26 These stress signals may involve noninfectious danger signals,27,28 TLRs have been proposed to be a link between inflammation and atherosclerosis, and although infectious agents may accelerate atherosclerosis, they are not required for lesion formation.29 Taking into account these findings, along with the previously mentioned studies demonstrating that an absence of TLR2 or TLR4 in atherosclerotic mice reduce lesion formation17,18 and that exposure to a TLR4 agonist during femoral artery injury increases lesion formation,19 we hypothesized that NOD2 may have an important role in vascular biology, particularly after injury. In contrast to a deficiency of TLR2 or TLR4, NOD2−/− mice developed much larger neointimal lesions after vascular injury compared with NOD2+/+ mice (Figure 1A–1C), and these lesions were predominantly composed of VSMCs (Figure 1D). In this study, we demonstrate for the first time that NOD2 mRNA is expressed in aortas and primary VSMCs of mice and humans (Figure 2B and 2C), suggesting that NOD2 is important in vascular homeostasis and prevents neointimal formation. Interestingly, NOD2 expression has been previously shown in endothelial cells, another important cell type in vascular pathogenesis.9–10

Next, we wanted to determine whether the expression of NOD2 in VSMCs is functionally important, thus contributing to increased neointimal formation in NOD2−/− mice. NOD2 deficiency promoted increased proliferation and migration of VSMCs (Figures 3 and 4), supporting the concept that NOD2 may have an important protective role in vascular physiology. The effects of NOD2 deficiency on proliferation and migration of VSMCs were much more impressive than the effects of apoptotic agonists (Supplemental Figure IV). We also confirmed that cell proliferation and migration were enhanced by an acute downregulation of NOD2, as siRNA for NOD2 had similar effects as genetically deficient NOD2 VSMCs (Figures 3 and 4).

In NOD2−/− VSMCs, there is evidence of increased expression of NOD1 and TLR2, and a slight reduction in TLR4 (Figure 2B). We questioned whether the increased expression of NOD1 and TLR2 may be a compensatory response for the absence of NOD2. Cartwright et al reported
that selective NOD1 agonists cause a systemic inflammatory response, organ injury, and dysfunction of VSMCs, and Yang et al showed that TLR2 expression in VSMCs promoted inflammation within the arterial wall. However, in the present study, inflammatory cells were sparse in NOD2 deficient mice after vascular injury (Figure 1D), suggesting a different pathophysiology from that found in prior studies assessing injury due to NOD1 agonists and TLR2. However, we cannot totally exclude the fact that a compensatory increase in NOD1 and TLR2 may contribute to vascular lesion formation.

Interestingly, Cole et al established that the other PRR with a protective role in the arterial wall is TLR3. They demonstrated that TLR3 activated with poly(I:C), a TLR3 ligand, decreased neointimal formation in response to carotid injury, using a perivascular collar model. At the same time, mice deficient in both TLR3 and apolipoprotein E (apolipoprotein E−/−/TLR3−/−) developed accelerated early atherosclerotic lesion formation in the aortic root, compared with control mice (apolipoprotein E−/−/TLR3+/+). TLR3 is known to recognize double-stranded RNA, carried by some viruses, and recently single-stranded RNA has been shown to be an agonist of NOD2, along with NOD2 being protective during viral infections. These data, in conjunction with the vascular injury models, suggest that PRRs, such as NOD2 and TLR3, are more complex than just innate immune receptors. Moreover, although PRRs are important for the innate immune response, in the presence of noninfectious danger signals they can be quite different in their functions. For instance, PRRs can be either protective (NOD2 and TLR3) or injurious (NOD1, TLR2, and TLR4) in the vascular wall.

Neointimal formation is due, in part, to VSMC proliferation and migration mediated by cytokines and growth factors, such as PDGF, in the development of atherosclerosis and restenosis. In addition to increasing proliferation, migration and migration mediated by cytokines and growth factors, NOD2, TLR2, and TLR4) in the vascular wall.

involving excessive VSMCs proliferation and migration, such as occurs with restenosis after vascular injury.

Courivaud et al analyzed the role of NOD2 in the pathogenesis of atherosclerosis in humans and reported no corre-

lation between NOD2 gene polymorphisms and atherosclerosis after renal transplantation. They assessed the frequency of NOD2 gene polymorphisms from leukocytes of renal transplant recipients. Thus, it would be interesting to further assess the level of expression and the function of NOD2 polymorphisms in VSMCs from patients that develop other forms of vascular diseases, as the role of NOD2 may vary depending on disease models or patient populations.

In summary, NOD2 is expressed in mouse and human VSMCs. NOD2−/− mice develop more pronounced neointimal formation in response to vascular injury, and the proliferation and migration of NOD2−/− VSMCs to PDGF-BB stimulation is greater than in NOD2+/+ VSMCs. Moreover, activation of NOD2 in mouse and human VSMCs decreases their migratory response to PDGF-BB, suggesting that NOD2 plays a critical role in the function of VSMCs. These findings advance our understanding of PRRs in vascular injury and provide evidence that NOD2 may be an important therapeutic target in vascular diseases involving excessive VSMCs proliferation and migration.

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Disclosures
None.

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Online Figure I
Online Figure II

Fold increase O.D. (450 nm)

Day 0 1 2 3 4

NOD2+/+ 10% FBS
NOD2−/− 10% FBS

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Online Figure III

PDGF-BB (10 ng/ml)

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Online Figure IV
Online Figure V
NOD2의 결핍은 혈관손상 후 신생내막의 형성을 촉진한다.

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Summary

배경
동맥경화성 혈관협착에서 NOD2 (nucleotide-binding oligomerization domain protein 2)의 역할을 알아보기 위하여 혈관손상 실험동물모델에서 NOD2의 발현과 병태생리학적 역할에 관하여 연구하였다.

방법 및 결과
정상생쥐(NOD2+/+)와 NOD2 결핍생쥐(NOD2−/−)의 대퇴동맥 손상모델에서 NOD2 결핍생쥐는 대조군에 비하여 2.86배의 혈관평활근(vascular smooth muscle cells, VSMCs) 액틴 양성세포들로 구성된 신생내막의 증가를 보였다. NOD2는 VSMCs에서 발현되었으며, NOD2가 결핍된 VSMCs들은 NOD2가 발현된 VSMCs에 비하여 PDGF-BB (platelet-derived growth factor-BB) 등에 의한 분열 촉진 자극에 대하여 세포증식이 더 촉진되었다. 또한 NOD2 결핍은 PDGF-BB에 의한 VSMCs 이동을 촉진하였다. 이러한 VSMCs 이동은 phosphatidylinositol 3-kinase 억제제에 의하여 약화되었으며, protein kinase C와 c-Jun N-terminal kinase 억제제의 효과는 거의 관찰되지 않았다. 무라밀 디펩티드(muramyl dipeptide)로 자극된 NOD2는 PDGF-BB로 인한 VSMCs 이동을 억제하였다.

결론
NOD2는 VSMCs에서 발현되며, NOD2 결핍은 VSMCs 증식, 이동 및 신생내막 형성을 촉진한다. 이러한 결과는 NOD2가 동맥경화성 혈관협착 및 혈관손상에 따른 신생내막의 형성에 중요한 역할을 담당한다는 증거를 제시한다.
세균 등 외부물질이 체내에 들어오게 되면 체내의 면역계가 작동하여 세균에 있는 어떤 항원(pathogen)을 인식해 면역반응이 일어나는데, 이러한 외부물질의 항원을 인식하는 체내의 세포 수용체를 항원 인식 수용체(pathogen recognition receptor, PRR)라 한다. PRR은 면역체계의 활성제어에 가장 1차적이고 핵심적인 역할을 수행하며, 대표적인 PRR에는 TLR (toll-like receptor) family, NOD (nucleotide-binding oligomerization domain), RIG-I (retinoid inducible gene-I) family 등이 있다. 정상 체내에서는 이러한 PRR의 활성화에 의한 면역 및 염증반응의 시작과 해소가 조화롭게 진행이 되나 이러한 조화가 깨어질 경우 동맥경화, 염증성장질환, 당뇨병, 관절염, 암 등의 질환들이 유발된다. 따라서 PRR의 활성을 조절하는 것이 동맥경화 치료의 새로운 타겟이 될 수 있다.

이 연구에서는 이러한 PRR 중에서 특히 NOD2가 혈관손상 후 나타나는 신생내막 형성에 중요한 역할을 할 수 있다는 가설을 세웠다. TLRs (Toll-like receptors, TLR1, TLR2, TLR4)는 염증발생 후 죽상동맥경화증이 발생하는 기전에서 그 연결고리로 생각되어 왔으나, 신생내막 형성은 증가시키지는 않는다. TLR2 또는 TLR4가 결핍된 생쥐모델에서 심장내막 형성은 감소하고 대퇴동맥순상 실험 동안 TLR4 agonist에 노출시키면 신생내막 형성이 증가된다.

이와는 대조적으로, NOD2가 결핍된 생쥐들은 NOD2가 있는 쥐들과 비교하여 혈관손상 후에 신생내막 형성이 증가되었다. 또한 NOD2 결핍은 VSMCs의 증식과 이동을 촉진하였고 이는 NOD2가 혈관 생리학에서 중요한 보호역할을 한다는 개념을 제공한다. 또한 이 연구에서는 VSMCs 증식과 이동은 NOD2를 위한 siRNA에서 NOD2 VSMCs의 유전적 결핍과 비슷한 효과를 나타내는 NOD2의 급성하향조절에 의해서 증가한다는 것을 확인했다. 혈관손상 모델에서의 이러한 데이터는 NOD2와 TLR3와 같은 PRRs가 선천성 면역 수용체보다 더 복잡하다는 것을 시사한다. 이러한 발견은 혈관손상에서 항원 인식 수용체에 대한 우리의 이해를 진전시키고 과도한 VSMCs의 증식과 이동에 수반되는 신생내막의 형성기전에서 NOD2가 새로운 치료 목표로 중요할 수 있다는 증거를 제공한다.

최근 관상동맥질환, 말초혈관질환 및 대동맥질환 같은 혈관질환의 치료로서 혈관 내 시술이 점점 많은 비중을 차지하고 있다. 혈관 내 시술 시 혈관내막에 필연적으로 외상이 가하게 되고, 이로 인한 동맥경화의 유발은 치료 효과를 감소시키는 요인이 된다.

이 연구에서는 NOD2 결핍 동맥경화 생쥐모델에서 정상생쥐에 비하여 혈관손상 후 신생내막 형성이 현저하게 증가됨을 확인하였다. NOD2가 신생내막 형성의 억제작용이 있음을 밝히고, NOD2리간드의 개발로 동맥경화 치료의 새로운 타겟으로의 가능성을 제시한 것이다. 즉, NOD2 리간드를 이용한 동맥경화의 치료적 접근뿐만 아니라 NOD2 리간드가 도포된 스텐트 등을 개발하여 시술 시 혈관내막의 외상에 의한 동맥경화의 악화를 방지하여 치료 효과를 향상시킬 수 있을 것으로 기대된다.
REFERENCES
Nucleotide-Binding Oligomerization Domain Protein 2 Deficiency Enhances Neointimal Formation in Response to Vascular Injury


**Objective**—Nucleotide-binding oligomerization domain protein 2 (NOD2) stimulates diverse inflammatory responses resulting in differential cellular phenotypes. To identify the role of NOD2 in vascular arterial obstructive diseases, we investigated the expression and pathophysiological role of NOD2 in a vascular injury model of neointimal hyperplasia.

**Methods and Results**—We first analyzed for neointimal hyperplasia following femoral artery injury in NOD2<sup>+/+</sup> and NOD2<sup>−/−</sup> mice. NOD2<sup>−/−</sup> mice showed a 2.86-fold increase in neointimal formation that was mainly composed of smooth muscle (SM) α-actin positive cells. NOD2 was expressed in vascular smooth muscle cells (VSMCs) and NOD2<sup>−/−</sup> VSMCs showed increased cell proliferation in response to mitogenic stimuli, platelet-derived growth factor-BB (PDGF-BB), or fetal bovine serum, compared with NOD2<sup>+/+</sup> VSMCs. Furthermore, NOD2 deficiency markedly promoted VSMCs migration in response to PDGF-BB, and this increased cell migration was attenuated by a phosphatidylinositol 3-kinase inhibitor. However, protein kinase C and c-Jun N-terminal kinase inhibitors exerted negligible effects. Moreover, muramyl dipeptide–stimulated NOD2 prevented PDGF-BB-induced VSMCs migration.

**Conclusion**—Functional NOD2 was found to be expressed in VSMCs, and NOD2 deficiency promoted VSMCs proliferation, migration, and neointimal formation after vascular injury. These results provide evidence for the involvement of NOD2 in vascular homeostasis and tissue injury, serving as a potential molecular target in the modulation of arteriosclerotic vascular disease. (Arterioscler Thromb Vasc Biol. 2011;31:2441-2447.)

**Key Words:** restenosis ■ vascular biology ■ nucleotide-binding oligomerization domain protein 2 ■ smooth muscle cells ■ vascular injury

Nucleotide-binding oligomerization domain protein 2 (NOD2), initially described as a susceptibility gene for Crohn disease and inflammatory bowel diseases, is an intracellular protein containing leucine-rich repeats similar to those found in Toll-like receptors (TLRs). NOD2 is the intracellular pathogen recognition receptor (PRR) responsible for recognition of bacterial peptidoglycans from Gram-positive and Gram-negative bacteria through its interaction with muramyl dipeptide (MDP). The expression of NOD2 has been reported in myeloid cells, particularly macrophages, neutrophils, and dendritic cells, as well as in Paneth cells in the small intestine. Moreover, NOD2 expression can be induced by proinflammatory cytokines tumor necrosis factor-α and interferon (IFN)-γ in cultured intestinal epithelial cells. However, NOD2 expression and novel functions have been suggested in other cell types, including adipocytes, gingival and pulp fibroblasts, and vascular endothelial cells.

The proliferation and migration of vascular smooth muscle cells (VSMCs) from the media into the intima contribute to pathological conditions, including restenosis after angioplasty, posttransplantation coronary artery disease, and hypertensive vasculopathy. These events can be induced by cytokines and growth factors, such as platelet-derived growth factor (PDGF) after vascular injury. PDGF stimulates intracellular signal molecules with Src homology 2 domains, including Src, phosphatidylinositol 3-kinase (PI3K), and ras/raf-1. Src and PI3K perform crucial functions related to actin reorganization, growth, and migration in response to PDGF in the VSMCs, and these activities are mediated by the activation of a family of serine/threonine-specific protein kinases and the mitogen-activated protein kinases. Interestingly, agonists of TLRs, including TLR3 and TLR9, have been suggested to regulate the production of PDGF. In addition, enhanced expression of TLRs (TLR1, TLR2, and TLR4) has been shown in human atherosclerotic plaques.

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Animal experiments have also demonstrated that an absence of TLR2 or TLR4 in atherosclerotic-susceptible mice (low-density lipoprotein receptor-deficient or apolipoprotein E–deficient mice, respectively)\textsuperscript{7,18} results in a reduction in atherosclerotic lesion formation and that femoral artery injury in the presence of a TLR4 agonist (lipopolysaccharide) augments neointimal formation.\textsuperscript{19} Taken together, these studies suggest a role for PRRs in the pathobiology of vascular disease.

In this study, we found that NOD2 was expressed in VSMCs and that NOD2 regulated the proliferation and migration of cells exposed to factors such as PDGF-BB. We also showed that NOD2 contributed to vascular homeostasis and that the absence of NOD2 enhanced neointimal formation after vascular injury.

**Materials and Methods**

For any of the Materials and Methods not described in detail, please see the supplemental materials, available online at http://atvb.ahajournals.org.

**Animals**

NOD2\(^{-/-}\) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) on a C57BL/6 (N7) genetic background, and the mice were further bred with C57BL/6 mice and then maintained by crossing either heterozygous or homozygous mutant mice within our animal facility at Harvard Medical School. The Standing Committee on Animal Care at Harvard Medical School approved all animal experimentation protocols of this study under the guidelines of our approved institutional animal care and use committee protocol.

**Femoral Artery Injury**

Endoluminal injury to the mouse left common femoral artery was performed as described.\textsuperscript{20} The contralateral right femoral arteries of the same mice undergoing endoluminal injury were used as noninjured control vessels. Noninjured and injured femoral arteries were harvested 28 days after femoral artery injury and fixed with 10% formalin. Paraffin-embedded tissues were sectioned as described.\textsuperscript{21}

**Histological Analysis and Immunohistochemistry**

Femoral arteries were harvested for histological and morphometric analyses. Vessel sections were stained for elastin (Sigma-Aldrich, St Louis, MO), and the intimal and medial areas were measured using National Institutes of Health Image software. Vessel sections were stained for VSMCs and leukocytes, using antibodies against smooth muscle \(\alpha\)-actin (Sigma-Aldrich) and CD45 (BD Biosciences, Franklin Lakes, NJ), respectively.

**Reagents and Antibodies**

Protein kinase inhibitors (Enzo Life Science, Plymouth Meeting, PA); mouse and human PDGF-BB (PeproTech Inc., Rocky Hill, NJ), and MDP (Sigma-Aldrich) were used in the studies. All signaling antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA). All small interfering RNA (siRNA) reagents were purchased from Santa Cruz Biotechnology. Inc (Santa Cruz, CA).

**Culture of VSMCs**

Primary NOD2\(^{+/+}\) and NOD2\(^{-/-}\) VSMCs from mice (mVSMCs) were obtained with the use of collagenase and elastase digestion of aortas as described previously.\textsuperscript{22} Human primary aortic smooth muscle cells (hVSMCs) were purchased from Clonetics Corp (San Diego, CA).

**Western Immunoblotting**

Western immunoblotting was performed as previously described.\textsuperscript{23}

**Transient Transfection**

The wild-type mouse and human VSMCs were transfected with mouse and human NOD2 siRNA and control siRNA as described by the manufacturer (Santa Cruz Biotechnology). The cells were used for migration and proliferation assays 48 hours after transfection.

**Cell Migration and Proliferation Assays**

Cell migration was assessed using gelatin-coated 24-well chambers and polycarbonate membranes, 8-μm pores (Corning, Lowell, MA). Cell proliferation was determined using the Ez-Cytox Cell Viability Assay Kit (Daelilab Service Co Ltd, Seoul, Korea).

**Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from mouse and human VSMCs and tissues (spleen and aorta) using Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed using the TaqMan kit in a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA). Premade primers and probes were purchased from Applied Biosystems, and assay IDs were as follows: mouse NOD2, Mm00467543_m1; human NOD2, Hs00223394_m1; mouse NOD1, Mm00805062_m1; mouse TLR2, Mm00442346_m1; and mouse TLR4, Mm00445274_m1. Expression of mouse and human target genes was normalized to mouse peptidyl-prolyl isomerase A or human GAPDH expression levels, respectively.

**Statistical Analysis**

Data are represented as mean±SD. For comparisons between 2 groups, we used the Student 2-tailed unpaired \(t\) test. For comparisons of timed series experiments, we performed Student paired \(t\) tests. For comparisons between more than 2 sets of experimental conditions, we used a 1-way analysis of variance (ANOVA). If significant, the ANOVA was followed by a protected Fisher least significant difference test and a Scheffe F-test. Statistically significant differences were accepted at \(P<0.05\).

**Results**

To investigate the role of NOD2 in the vasculature, we examined the formation of neointimal lesions after femoral artery injury in NOD2\(^{+/+}\) and NOD2\(^{-/-}\) mice. There were no differences in the structure of noninjured femoral arteries between NOD2\(^{+/+}\) and NOD2\(^{-/-}\) mice (Figure 1A). The vessel size (inside area of external elastic lamina) of injured femoral arteries was not different between NOD2\(^{+/+}\) (43.21±0.82 mm\(^2\), \(n=10\)) and NOD2\(^{-/-}\) mice (41.86±0.56 mm\(^2\), \(n=16\)) 28 days after injury. The medial areas were also similar between NOD2\(^{+/+}\) (12.33±0.23 mm\(^2\), \(n=10\)) and NOD2\(^{-/-}\) mice (11.57±0.19 mm\(^2\), \(n=16\)). In the injured vessels of NOD2\(^{+/+}\) mice, neointimal thickening (area inside of internal elastic lamina excluding luminal area) was evident, although small (5.01±0.86 mm\(^2\), \(n=10\)). In contrast, we observed a robust increase in intimal thickening in NOD2\(^{-/-}\) mice after injury (16.85±0.75 mm\(^2\), \(n=16\)) (Figure 1A and 1B). An absence of NOD2 increased the intimal/media ratio 2.86-fold, 1.54±0.22 in NOD2\(^{-/-}\) compared with 0.37±0.21 in NOD2\(^{+/+}\) mice (Figure 1C).

NOD2\(^{-/-}\) neointima were composed mainly of smooth muscle \(\alpha\)-actin positive cells. Both groups of mice showed sparse CD45-positive inflammatory cells after vascular injury in the neointima (Figure 1D). The arrows (red) highlight CD45-positive cells. These results suggest that NOD2 has a broad
influence on the vascular response to injury, and an important physiological role in VSMCs.

In VSMCs, expression of NOD2 was not previously reported. Hence, we investigated whether NOD2 is expressed in VSMC, and whether NOD2 is induced by stimuli known to increase its expression in other cell types. Total RNA was isolated from the aortas of NOD2\(^{+/+}\) and NOD2\(^{-/-}\) mice. As shown in Figure 2A, mRNA expression of NOD2 was detected in NOD2\(^{+/+}\) aortas but not in NOD2\(^{-/-}\) aortas. However, mRNA expression of other PRRs (NOD1, TLR2, and TLR4) was not different in the aortas of NOD2\(^{+/+}\) and NOD2\(^{-/-}\) mice (Figure 2A). Spleen was used as a positive control for mRNA expression of NOD2 and other PRRs. To investigate induction of NOD2 expression in VSMCs, we harvested the cells from aortas of littermate mice. NOD2 mRNA was detected in NOD2\(^{+/+}\) VSMCs but not in NOD2\(^{-/-}\) VSMCs (Figure 2B). In NOD2\(^{-/-}\) VSMCs, expression levels of NOD1 and TLR2 mRNA were enhanced compared with NOD2\(^{+/+}\) VSMCs. However, TLR4 expression was slightly decreased in NOD2\(^{-/-}\) VSMCs (Figure 2B). Some of these changes in PRR mRNA levels were modest; therefore, we will need to confirm whether these changes translate into an alteration in protein levels. Selective VSMC genes (calponins, caldesmon, smooth muscle Myh11) were expressed similarly in both NOD2\(^{+/+}\) and NOD2\(^{-/-}\) VSMCs (Supplemental Figure I).

The tumor necrosis factor-\(\alpha\) and IFN-\(\gamma\) are known to regulate NOD2 expression in other cell types, including intestinal epithelial cells.\(^4\) To investigate induction of NOD2 mRNA, we treated mVSMCs and hVSMCs with IFN-\(\gamma\) and MDP. Interestingly, expression of NOD2 mRNA was increased by IFN-\(\gamma\) in VSMCs but not by the NOD2 ligand MDP (Figure 2C). These data suggest that NOD2 mRNA is expressed in VSMCs and may have a role in VSMCs physiological and pathophysiologic conditions.

To investigate potential mechanisms by which an absence of NOD2 leads to increased neointimal formation after vascular injury, we assessed VSMC proliferation, migration, and cell death in vitro. NOD2\(^{-/-}\) VSMCs exhibited increased cell proliferation compared with NOD2\(^{+/+}\) VSMCs in response to mitogenic fetal bovine serum stimulation (Supplemental Figure II). We further analyzed whether NOD2 deficiency could increase cell proliferation in response to PDGF-BB, which is a well-known potent growth factor and chemotactrant for VSMCs, and released at sites of vessel injury.\(^12\) The proliferation of VSMCs was enhanced in
NOD2<sup>−/−</sup> (27.00±4.64%) compared with NOD2<sup>+/+</sup> cells 4 days after PDGF-BB treatment (Figure 3A). In addition, treatment with siRNA-mediated NOD2 knockdown increased VSMC proliferation (Figure 3B). This increased proliferation of NOD2 deficient cells was not due to a difference in cell adhesion after plating, as adhesion was 77% in NOD2<sup>−/−</sup> cells and 75% in NOD2<sup>+/+</sup> cells (P>0.05, not significant). Real-time RT-PCR was performed to verify downregulation of NOD2 expression by mouse NOD2 siRNA (Figure 3C).

Next, the involvement of NOD2 deficiency in cell migration was measured using gelatin-coated 24-Transwell chambers. NOD2<sup>−/−</sup> VSMCs showed a 4.05±0.53-fold increased migration in response to PDGF-BB compared with NOD2<sup>+/+</sup> VSMCs, 24 hours after PDGF-BB treatment, and the number of migrated cells was counted and represented as a graph (Figure 4A and 4B). Similarly, we observed increased hVSMCs migration in human NOD2 siRNA-treated cells in response to PDGF-BB, compared with control siRNA-treated hVSMCs (Figure 4C and 4D). Real-time RT-PCR was performed to verify downregulation of human NOD2 expression by human NOD2 siRNA (Figure 4E).

These results suggest that NOD2 plays an important role in the prevention of VSMC proliferation and, more dramatically, migration, without markedly affecting cell death.

PDGF-BB stimulates many intracellular signaling molecules including PI3K. To identify which signaling pathways mediate VSMC migration in the absence of NOD2, chemical inhibitors of protein kinase C, PI3K, and c-Jun N-terminal kinase were administrated to NOD2<sup>+/+</sup> and NOD2<sup>−/−</sup> VSMCs before PDGF-BB treatment, and migration was measured (Figure 5A). Remarkably, enhanced migration of NOD2<sup>−/−</sup> VSMCs was abolished when exposed to a PI3K inhibitor, LY294002, compared with NOD2<sup>+/+</sup> VSMCs, in response to PDGF-BB stimulation (Figure 5A). The protein kinase C inhibitor GF10203X did not alter the enhanced migration of NOD2<sup>−/−</sup> VSMCs. A JNK inhibitor, SP600125, blunted the migration of VSMCs to PDGF-BB in both NOD2<sup>+/+</sup> and NOD2<sup>−/−</sup> cells; however, NOD2<sup>−/−</sup> cells maintained their increased migration response in comparison with NOD2<sup>+/+</sup> cells. Other signaling inhibitors, such as p38 and ERK inhibitors, did not alter the migration of NOD2<sup>−/−</sup> VSMCs (Supplemental Figure V). Thus, we focused on the PI3K pathway and its downstream target Akt. Levels of p-Akt (Ser473 and Thr308) were acutely induced by PDGF-BB in NOD2<sup>+/+</sup> VSMCs; however, the levels returned to baseline by 15 minutes after PDGF-BB stimula-
Figure 5. Inhibition of the phosphatidylinositol 3-kinase (PI3K) signaling pathway prevents increased migration in nucleotide-binding oligomerization domain protein 2 (NOD2)/vascular smooth muscle cells (VSMCs). A, VSMC migration assay was performed to measured migration of NOD2+/− and NOD2−/− VSMCs in response to platelet-derived growth factor-BB (PDGF-BB) or PDGF-BB plus protein kinase C (PKC) inhibitor (GF10203X, 1 μmol/L), PI3K inhibitor (LY294002, 10 μmol/L), or c-Jun N-terminal kinase inhibitor (SP600125, 10 μmol/L). *P<0.05, increased migration of NOD2+/− vs NOD2−/− VSMCs; †P<0.05, decreased migration of NOD2−/− vs NOD2+/− VSMCs. Values are mean±SD, n=6. B, Phosphorylated Akt (p-Akt Ser473 and p-Akt Thr308), total Akt, and GAPDH were determined at the indicated time points after PDGF-BB treatment by Western blotting. This panel denotes representative blots of 3 independent experiments, which are quantitated in C. C, Normalized phosphorylation of Akt levels by total Akt expression are represented as a graph. *P<0.05 vs PDGF-BB (0 minute) in NOD2+/− VSMCs, †P<0.05 vs PDGF-BB (0 minute) in NOD2−/− VSMCs, and ‡P<0.05, increased phosphorylation of Akt (Ser473 or Thr308) in NOD2+/− vs NOD2−/+/− VSMCs. NS indicates not significant. Values are mean±SD, n=3.

Figure 6. Activation of nucleotide-binding oligomerization domain protein 2 (NOD2) inhibits migration of vascular smooth muscle cells (VSMCs) in response to platelet-derived growth factor-BB (PDGF-BB). A, Mouse and human VSMCs were pre-treated with muramyl dipeptide (MDP) (2 μg/mL) or vehicle (−MDP) for 30 minutes, followed by administration of PDGF-BB (20 ng/mL) or vehicle (−PDGF-BB). After 12 hours, PDGF-BB, migrated cells were stained using the Crystal Violet staining kit. B, The number of migrated cells were measured and is represented as a graph. Values are mean±SD, n=4. *P<0.05 PDGF-BB vs PDGF-BB plus MDP in mouse or human VSMCs.

Discussion

PRRs are a class of innate immune response–expressed proteins that respond to pathogen-associated molecular patterns, yet they also respond to endogenous stress signals. The expression of NOD1 and TLR2 may be a compensatory response to a deficiency of TLR2 or TLR4 in atherosclerotic mice reduce lesion formation and severity compared with NOD2−/− mice. NOD2 deficiency promoted increased proliferation and migration of VSMCs (Figures 3 and 4), supporting the concept that NOD2 may have an important role in vascular biology, particularly after injury. In contrast to a deficiency of TLR2 or TLR4, NOD2−/− mice developed much larger neointimal lesions after vascular injury compared with NOD2+/+ mice (Figure 1A–1C), and these lesions were predominantly composed of VSMCs (Figure 1D). In this study, we demonstrate for the first time that NOD2 mRNA is expressed in aortas and primary VSMCs of mice and humans (Figure 2B and 2C), suggesting that NOD2 is important in vascular homeostasis and prevents neointimal formation. Interestingly, NOD2 expression has been previously shown in endothelial cells, another important cell type in vascular pathogenesis.

Next, we wanted to determine whether the expression of NOD2 in VSMCs is functionally important, thus contributing to increased neointimal formation in NOD2−/− mice. NOD2 deficiency promoted increased proliferation and migration of VSMCs (Figures 3 and 4), supporting the concept that NOD2 may have an important protective role in vascular physiology. The effects of NOD2 deficiency on proliferation and migration of VSMCs were much more impressive than the effects of apoptotic agonists (Supplemental Figure IV). We also confirmed that cell proliferation and migration were enhanced by an acute downregulation of NOD2, as siRNA for NOD2 had similar effects as genetically deficient NOD2 VSMCs (Figures 3 and 4).

In NOD2−/− VSMCs, there is evidence of increased expression of NO1 and TLR2, and a slight reduction in TLR4 (Figure 2B). We questioned whether the increased expression of NO1 and TLR2 may be a compensatory response for the absence of NOD2. Cartwright et al reported...
that selective NOD1 agonists cause a systemic inflammatory response, organ injury, and dysfunction of VSMCs,\(^1\) and Yang et al showed that TLR2 expression in VSMCs promoted inflammation within the arterial wall.\(^2\) However, in the present study, inflammatory cells were sparse in NOD2 deficient mice after vascular injury (Figure 1D), suggesting a different pathophysiology from that found in prior studies assessing injury due to NOD1 agonists and TLR2. However, we cannot totally exclude the fact that a compensatory increase in NOD1 and TLR2 may contribute to vascular lesion formation.

Interestingly, Cole et al established that the other PRR with a protective role in the arterial wall is TLR3.\(^3\) They demonstrated that TLR3 activated with poly(I:C), a TLR3 ligand, decreased neointimal formation in response to carotid injury, using a perivascular collar model. At the same time, mice deficient in both TLR3 and apolipoprotein E (apolipoprotein E\(^{-/-}\)/TLR3\(^{-/-}\)) developed accelerated early atherosclerotic lesion formation in the aortic root, compared with control mice (apolipoprotein E\(^{-/-}\)/TLR3\(^{-/-}\)). TLR3 is known to recognize double-stranded RNA, carried by some viruses, and recently single-stranded RNA has been shown to be an agonist of NOD2, along with NOD2 being protective during viral infections.\(^4\) These data, in conjunction with the vascular injury models, suggest that PRRs, such as NOD2 and TLR3, are more complex than just innate immune receptors. Moreover, although PRRs are important for the innate immune response, in the presence of noninfectious danger signals they can be quite different in their functions. For instance, PRRs can be either protective (NOD2 and TLR3) or injurious (NOD1, TLR2, and TLR4) in the vascular wall.

Neointimal formation is due, in part, to VSMC proliferation and migration mediated by cytokines and growth factors, such as PDGF, in the development of atherosclerosis and restenosis.\(^5\) In addition to increasing proliferation, migration is markedly increased in NOD2\(^{-/-}\) VSMCs in response to PDGF-BB (Figure 4A and 4B). In particular, the PI3K signaling pathway performs crucial functions related to actin reorganization, growth, and migration in response to PDGF-BB in VSMCs, and these activities are mediated by the activation of a family of serine/threonine-specific protein kinases.\(^6\) In the experiment shown in Figure 5A, increasing migration in of NOD2\(^{-/-}\) VSMCs was blocked in the presence of a PI3K inhibitor. Interestingly, NOD2\(^{-/-}\) VSMCs showed sustained Akt phosphorylation, which is the downstream target molecule of PI3K in response to PDGF-BB (Figure 5B and 5C). These data suggest that NOD2 may negatively regulate the PDGF-BB signaling pathway, even though PDGF-BB did not increase mRNA expression of NOD2 (Supplemental Figure III). However, more detailed mechanism(s) need to be elucidated. Another very important finding was that MDP prevented PDGF-BB-induced migration of mouse and human VSMCs (Figure 6). These findings indicate that modulation of NOD2 signaling in VSMCs may be beneficial in treating vascular diseases involving excessive VSMCs proliferation and migration, such as occurs with restenosis after vascular injury.

Courivaud et al analyzed the role of NOD2 in the pathogenesis of atherosclerosis in humans and reported no correlation between NOD2 gene polymorphisms and atherosclerosis after renal transplantation.\(^7\) They assessed the frequency of NOD2 gene polymorphisms from leukocytes of renal transplant recipients. Thus, it would be interesting to further assess the level of expression and the function of NOD2 polymorphisms in VSMCs from patients that develop other forms of vascular diseases, as the role of NOD2 may vary depending on disease models or patient populations.

In summary, NOD2 is expressed in mouse and human VSMCs. NOD2\(^{-/-}\) mice develop more pronounced neointimal formation in response to vascular injury, and the proliferation and migration of NOD2\(^{-/-}\) VSMCs to PDGF-BB stimulation is greater than in NOD2\(^{+/+}\) VSMCs. Moreover, activation of NOD2 in mouse and human VSMCs decreases their migratory response to PDGF-BB, suggesting that NOD2 plays a critical role in the function of VSMCs. These findings advance our understanding of PRRs in vascular injury and provide evidence that NOD2 may be an important therapeutic target in vascular diseases involving excessive VSMCs proliferation and migration.

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**Disclosures**

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**References**


