The effects of oxidized LDL in the inflammation of early stage atherosclerosis

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The effects of oxidized LDL in the inflammation of early stage atherosclerosis

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모든 이들께 감사 드리며 서진원 드림

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ABBREVIATION

AD	**atherogenic diet**
ApoB100	**apolipoprotein B100**
ApoE	**apolipoprotein E**
CCK-8	**cell counting kit-8**
CD	**cluster of differentiation**
DNA	**deoxyribonucleic acid**
dsDNA	**double-stranded DNA**
ECM	**extracellular matrix**
EDTA	**ethylenediaminetetraacetic acid**
ELISA	**enzyme-linked immunosorbent assay**
ER	**endoplasmic reticulum**
FBS	**fetal bovine serum**
FITC	**fluorescein isothiocyanate**
HDL	**high-density lipoprotein**
HMGB1	**high mobility group box 1**
HUVEC	**human umbilical vein endothelial cell**
IFN	**interferon**
Ig	**immunoglobulin**
IL	**interleukin**

IVIS	***in vivo* imaging system**
LDL	**low-density lipoprotein**
LDLR	**LDL receptor**
LOX-1	**lectin-like oxLDL receptor-1**
LPS	**lipopolysaccharide**
LXR	**liver X receptor**
M-CSF	**macrophage colony-stimulating factor**
MCP-1	**monocyte chemoattractant protein-1**
MeOH	**methyl alcohol**
mmLDL	**minimally modified LDL**
MMP	**matrix metalloproteinase**
MPO	**myeloperoxidase**
MRI	**magnetic resonance imaging**
NE	**neutrophil elastase**
NET	**neutrophil extracellular trap**
NO	**nitric oxide**
Nrf2	**nuclear erythroid-2 related factor**
oxLDL	**oxidized LDL**
PAGE	**polyacrylamide gel electrophoresis**
PBMC	**peripheral blood mononuclear cell**
PBS	**phosphate buffered saline**

PE	**phycoerythrin**
PMA	**phorbol 12-myristate 13-acetate**
PMN	**polymorphonuclear cell**
RNS	**reactive nitrogen species**
ROS	**reactive oxygen species**
SDS	**sodium dodecyl sulfate**
SR-A1	**scavenger receptor A1**
STAT	**signal transducers and activators of transcription**
T-CHO	**total cholesterol**
TBARS	**thiobarbituric acid reactive substances**
TG	**triglyceride**
TLR	**toll-like receptor**
TNF	**tumor necrosis factor**
WT	**wild type**

ABSTRACT

The effects of oxidized LDL in the inflammation of early stage atherosclerosis

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Atherosclerosis is a chronic inflammatory disease of the arterial wall. Many studies have shown that retention of low-density lipoprotein (LDL) and recruitment of monocytes into subendothelial space are critical early events in atherosclerotic development. Accumulated LDL in the arterial wall is undergone oxidative modification according to a variety of oxidizing agents and the exposure duration. For these reasons, LDL with different degrees of oxidation exist in the atherosclerotic plaque. However, there is no report regarding the degree of LDL oxidation and macrophage differentiation.

Therefore, the specific goal of the Part I study is to know the effects of degree of LDL oxidation on macrophage differentiation and polarization. Based on the level of lipid peroxidation, LDL is classified into high-oxidized LDL (high-oxLDL) and low-oxidized LDL (low-oxLDL). The differentiation profiles of macrophages were determined by surface receptor expression and cytokine secretion profiles in THP-1

cells and primary human monocytes. As a result, low-oxLDL induces macrophage polarization towards M1-like phenotype whereas high-oxLDL induces M2-like phenotype. Therefore, these results suggest that the degree of LDL oxidation influences the differentiation of monocytes into M1 or M2 macrophages and determines the inflammatory fate in early stages of atherosclerosis.

The Part II of my study focuses on neutrophils. Neutrophils are the first line of the defense against invading pathogens. Neutrophil extracellular traps (NETs) are most recently defined host defense mechanism to eliminate extracellular pathogens. Also, NETs have been implicated in chronic inflammatory diseases such as atherosclerosis. Recent studies have shown that NET components can be found in lesions associated with atherosclerosis. However, the role of NETs in early atherosclerosis is not identified clearly. Therefore, the effects of NETs during early atherosclerosis were tested in the Part II study using *ApoE*$^{-/-}$ mice and human neutrophils.

NETs were detected in *ApoE*$^{-/-}$ mice in early stage of atherosclerosis and circulating oxLDL induces NET formation. Especially, NETs induced by oxLDL promote endothelial dysfunction *via* MMP-9. Collectively, our results suggest that oxLDL-induced NET formation affects endothelial cells in early atherosclerosis and promotes LDL deposition into the intima.

Taken together with the results from the Part I and Part II, I suggest that the circulating oxLDL as well as intimal oxLDL play crucial roles for development of early atherosclerosis.

Key Words: atherosclerosis, LDL, oxidation, macrophage, differentiation, oxLDL, neutrophils, NETs, MMP-9, endothelial dysfunction

CHAPTER I. Literature review

1. Atherosclerosis

1.1. Introduction

Atherosclerosis is a complex, progressive vascular disease of the large- and medium-sized arteries, and is characterized by two main features: lipid retention and inflammation. It is a main cause of cardiovascular diseases such as myocardial infarction, sudden cardiac death, unstable angina, and stroke, and is responsible for nearly 50% of deaths in Western countries. This is likely to soon be the worldwide status.[1-3] For many years, atherosclerosis was believed to be merely a lipid storage disease in the vessel wall. However, atherosclerosis is much more complex than this, and it is now recognized as a chronic inflammatory disease of vessel walls, characterized by infiltration of a variety of immune cells such as monocytes, neutrophils, T cells, dendritic cells, and mast cells.[4]

1.2. Developmental process of atherosclerosis

Atherosclerotic plaque formation is initiated by accumulation of apolipoprotein B-containing lipoproteins, such as very low-density (VLDL) and low-density (LDL) lipoproteins, in the subendothelial space. This initiating process leads to endothelial activation and recruitment of monocytes. Consequently, early lesions appear. These ‘fatty streaks’ are characterized by lipid-laden foam cell formation by macrophages and lipid retention. Other immune cells, including dendritic and T cells, are also recruited, and lesions are established through the formation of a collagen-rich fibrous cap by vascular smooth muscle cells (VSMCs). Over time, accumulation of excess lipids and proinflammatory cytokines induces endoplasmic reticulum (ER) stress, resulting in

apoptotic cell death of macrophages.[5,6] These apoptotic cells are not sufficiently cleared by phagocytosis, a process also called 'efferocytosis,' in advanced lesions. As a result, a necrotic core, which is composed of apoptotic as well as necrotic cells, cell debris, inflammatory cytokines, proteases, and thrombotic factors, is established. The necrotic core contributes to thinning of the fibrous cap by smooth muscle cell death, together with degradation of the extracellular matrix by matrix metalloproteinases (MMPs) and proteases such as cathepsins. Eventually, a thinned fibrous cap causes plaque instability and rupture, which mediates thrombus formation through the release of pro-coagulant and thrombotic factors.[4,7,8]

1.3. Initiation of atherosclerosis

The key initiating step of atherosclerosis is the accumulation of LDL cholesterol in the subendothelial space, also known as the intima, and the oxidative modification of LDL. LDL particles are composed of esterified cholesterol and triglycerides (TG), surrounded by a shell of phospholipids (PL), free cholesterol, and apolipoprotein B100 (apoB100).[4] Circulating LDL particles penetrate the endothelial layer, and are deposited in the intima. Specifically, binding between ApoB100 and proteoglycans in the intimal extracellular matrix through ionic interaction amplifies the accumulation of LDL particles in the intima.[7,9] Subsequently, LDL particles undergo oxidative modification by various oxidizing agents such as reactive oxygen species (ROS), myeloperoxidase (MPO), and lipoxygenase (LO). These oxidized LDL (oxLDL) particles stimulate vascular endothelial cells, and the activated endothelial cells increase the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), on their surfaces, thus increasing attachment of the blood monocytes. Once monocytes adhere to the endothelium, they migrate into the intima in response to chemokines such as

monocyte chemoattractant protein-1 (MCP-1). Eventually, circulating monocytes are recruited to the inflamed sites, which is a crucial process in the development of early atherosclerosis.[8,10,11]

1.4. Monocyte differentiation into macrophages

Infiltrated monocytes differentiate into macrophages in response to macrophage colony-stimulating factor (M-CSF), which is secreted by endothelial cells and vascular smooth muscle cells in the arterial wall. Differentiated macrophages upregulate a variety of scavenger receptors, including SR-A1, CD36, and LOX-1 (lectin-like oxLDL receptor-1), and innate immune receptors such as Toll-like receptors (TLRs). These macrophages then take up oxLDL *via* scavenger receptors and develop into lipid-laden foam cells—a major hallmark of early-stage atherosclerotic lesions. Lipid-laden foam cells increase the expression of migration-inhibitory molecules such as netrin-1 and semaphorine 3E, decreasing macrophage migration from atherosclerotic lesions and amplifying foam cell accumulation in these lesions.[12] In addition, differentiated macrophages are activated by recruited T lymphocytes and macrophages to produce proinflammatory mediators such as ROS, reactive nitrogen species (RNS), inflammatory cytokines, and proteases.[7,13]

2. Macrophages in atherosclerosis

2.1. Macrophage phenotypes in atherosclerosis

Macrophages are principal contributors to the development and progression of atherosclerosis, and a variety of macrophage phenotypes are involved in this disease. In atherosclerotic lesions, macrophages face numerous micro-environmental signals, including cytokines, lipids, iron, and calcium, which can polarize and activate

macrophages. Macrophage phenotypes include classically activated M1 macrophages and alternatively activated M2 macrophages. Recently, additional stimulus-specific macrophage phenotypes were identified and termed Mox, Mhem, M(Hb), and M4. In addition, in response to the micro-environment milieu, polarized macrophages are skewed toward different types of macrophages, which is termed 'macrophage plasticity'.[14] Polarized macrophages in atherosclerotic lesions can influence progression and regression of these lesions; however, the relative ratio of macrophage subpopulations, rather than the absolute numbers in these populations, is the preferred parameter to measure lesion progression.[15,16]

2.2. Classically activated M1 macrophages

Classically activated M1 macrophages are induced by type 1 T helper cell (T_H1)-derived cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), as well as bacterial products such as lipopolysaccharide (LPS). Polarized M1 macrophages secrete proinflammatory cytokines and chemokines including interleukin-1β (IL-1β), TNF-α, IL-6, IL-12, IL-23, CXCL9, CXCL10, and CXCL11. In addition, M1 macrophages produce nitric oxide (NO) and ROS to eliminate invading pathogens. M1 macrophages increase the expression of class II major histocompatibility complex (MHC) molecules and costimulatory molecules such as CD80 and CD86. These proinflammatory properties are beneficial in acute infections; however, sustained induction of M1 macrophages can induce tissue damage and impair wound healing.[15,17]

2.3. Alternatively activated M2 macrophages

In contrast to M1 macrophages, alternatively activated M2 macrophages show anti-

inflammatory properties. M2 macrophages are polarized by type 2 T helper cell (T_H2)-mediated cytokines such as IL-4 and IL-13, and produce anti-inflammatory cytokines, including IL-10 and transforming growth factor-β (TGF-β).[18] Moreover, M2 macrophages typically express mannose receptor (MR, CD206), dectin-1, and CD163 on their cell surfaces. These M2 macrophages are associated with tissue repair, angiogenesis, and tumor progression.[16,19]

M2 macrophages are further classified into four distinct subgroups: M2a, M2b, M2c, and M2d. M2a macrophages are induced by IL-4 and IL-13, and express high levels of the mannose receptor (MR, CD206) and IL-1 receptor agonist. M2a macrophages contribute to tissue repair, and are referred to as 'wound-healing macrophages.' M2b macrophages are induced by immune complexes, Toll-like receptor (TLR) agonists, and IL-1 receptor ligands. These macrophages represent typical M2 characteristics; however, they also produce proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. M2c macrophages are induced by IL-10 and glucocorticoids, and they express high levels of Mer receptor tyrosine kinase (MerTK), which is involved in efferocytosis. M2b and M2c macrophages are referred to as 'regulatory macrophages.' Finally, M2d macrophages are induced by the combination of a TLR agonist and adenosine A_{2A} ligands, and they produce IL-10 and vascular endothelial growth factor (VEGF), but do not express the mannose receptor. These M2d macrophages have pro-angiogenic and pro-tumoral capacities.[15,20-22]

2.4. Stimulus-specific macrophages

In addition to M1 and M2 macrophages, macrophages with different phenotypes co-exist in atherosclerotic lesions. Mox macrophages are polarized by oxidized PL through transcription factor nuclear erythroid-2 related factor (Nrf2). These macrophages

display lower chemotactic and phagocytic capacities. Nrf2-dependent genes encoding proteins such as heme oxygenase-1 (HMOX-1), sulforedoxin-1 (Srx1), and thioredoxin reductase 1 (Txnrd1) are reported to be specific markers for Mox macrophages. Notably, Mox macrophages are a prominent subpopulation representing nearly 30% of the total number of macrophages in mouse lesions; however, they have not yet been reported in human lesions. M(Hb) macrophages are induced by hemoglobin/haptoglobin ingestion and characterized by high levels of MR and CD163, which are associated with hemoglobin clearance after plaque hemorrhage. In addition, M(Hb) macrophages show enhanced cholesterol efflux capacity related to liver X receptor (LXR)α and induce the expression of ferroportin, an iron exporter. As a result, M(Hb) macrophages contribute to the prevention of foam cell formation and decrease ROS production generated by intracellular iron. Mhem macrophages are polarized in response to heme stimulation, and activate transcription factors such as activating transcription factor (ATF)-1 and liver X receptor (LXR)β. Similar to M(Hb), Mhem show an enhanced cholesterol efflux capacity that inhibits foam cell formation. Finally, M4 macrophages are induced by the platelet-derived chemokine CXCL4, also known as platelet factor 4 (PF4). Typically, M4 macrophages express matrix metalloproteinase 7 (MMP7) and S100A8, a calcium binding protein, whereas they do not express CD163 completely.[15,16,22]

2.5. Localization of macrophage sub-populations in atherosclerotic lesions

Atherosclerotic lesions are an extremely complex microenvironment, with properties dependent on the specific spatial distribution of different macrophage subpopulations. M1 macrophages are detected predominantly in the lesion shoulder, which is the most rupture-prone area, and the necrotic core. On the other hand, M2 macrophages are mostly located in the adventitia and iron-rich neovascularization areas. In the fibrous

cap, similar amounts of both M1 and M2 macrophages exist. Moreover, M1 macrophages are exclusively found in unstable lesions of symptomatic patients following an acute ischemic attack, while M2 macrophages are predominant in lesions of asymptomatic patients. These observations indicate that M1 macrophages are the predominant macrophage subset in rupture-prone areas and in unstable lesions, and suggest that plaque instability might be the result of an imbalance between macrophages with M1 and M2 phenotypes.[15,22]

3. Oxidized low-density lipoprotein (OxLDL)

3.1. Oxidative modification of LDL

Oxidative modification of LDL in the intima is an important step in early atherosclerosis. LDL is a spherical particle with a diameter of approximately 22 nm to 27.5 nm.[23] One LDL particle is composed of approximately 600 molecules of free cholesterol (FC), 1600 molecules of cholesteryl esters (CE), 700 molecules of PL, 185 molecules of TG, and one molecule of apoB-100. The molecular composition of LDL particles is susceptible to change with oxidative modification. Notably, many exposed tyrosine and lysine residues in apoB100 can be directly oxidized by lipid oxidation products and, consequently, reactive aldehyde products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are attached covalently to apoB100.[24] Since oxLDL induces a variety of pro-atherogenic and proinflammatory effects such as endothelial activation and vascular smooth muscle cell proliferation, as well as promotes macrophage foam cell formation in early atherogenesis, the importance of oxidative modifications of LDL particles is highlighted.[25,26]

3.2. Oxidizing agents

Different types of oxidizing agents, which are divided into two main groups, are well defined. First, non-enzymatic oxidation of LDL is mediated by transition metal ions such as Cu^{2+}, Fe^{2+}, and heme. In particular, prolonged incubation of LDL with copper sulfate is a widely used *in vitro* model of extensively oxLDL. Overnight incubation of LDL with copper sulfate induces degradation of lipid parts such as phosphatidylcholine, as well as drastic alterations in apoB protein. This type of oxLDL is recognized by scavenger receptors, including CD36, SR-A1, and LOX-1, instead of native LDL receptor (LDLR). However, the contribution of copper sulfate-mediated oxLDL to the physiological oxidation of LDL remains controversial.[27] Compared to extensively oxLDL, incubation of LDL with copper sulfate for a short time (approximately 2–4 hours) or oxidation of LDL with enzymes such as lipoxygenase (LO), myeloperoxidase (MPO), NADPH oxidase, or nitric oxide synthase produces different types of oxLDL, which are termed 'minimally modified/oxLDL (mmLDL)'. These enzymes are generated by a variety of cells, including endothelial cells, vascular smooth muscle cells, macrophages, and neutrophils. Intriguingly, mmLDL is still recognized by native LDL receptor or Toll-like receptors rather than scavenger receptors, because these oxidizing agents do not induce substantial modifications in LDL.[24,28-30]

3.3. OxLDL in atherosclerosis

Oxidation of LDL is a stepwise process. With ongoing oxidation, the physicochemical properties such as charge, particle size, and lipid content are gradually changed. These alterations are dependent on the type of oxidizing agent, and they result in a variety of modifications with diverse physicochemical functions. For this reason, oxLDL in the intima is a heterogeneous mixture of bioactive compounds with variable

degrees of oxidation.[4,29] Depending on the degree or position of LDL oxidation, oxLDL can induce distinct biological effects in various cell types through different intracellular signaling pathways.[31-33] Variable effects have been reported in the literature, resulting from the use of different oxidizing agents to prepare oxLDL.[34]

OxLDL is known as a useful marker for atherosclerosis. It has been detected *in vivo* both in atherosclerotic lesions as well as in plasma.[35] Since elevated levels of circulating oxLDL were shown to correlate significantly with the severity of cardiovascular disease,[36] oxLDL levels are a possible prognostic marker for future cardiac events.[37-39] As mentioned, variable degrees of oxLDL exist in atherosclerotic lesions, and oxLDL can induce various biological effects, depending on the level of LDL oxidation. However, there are few reports on the degree of LDL oxidation and risk of atherosclerosis.[40]

4. Neutrophils in atherosclerosis

4.1. Neutrophil extracellular traps

Neutrophils are a component of the first line of innate immune defense against a broad spectrum of invading pathogens, including bacteria, fungi, and parasites. Neutrophils have three mechanisms to eliminate extracellular pathogens: phagocytosis (ingestion), degranulation (release of anti-microbial contents), and formation of neutrophil extracellular traps (NETs).[41] Neutrophils phagocytose and sequester pathogens into phagolysosomal compartments, where they kill many pathogens by generating ROS and releasing granule contents. They also secrete a variety of granular proteins such as myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, NADPH oxidase, and cathelicidin into the surrounding extracellular space, through a

process called 'degranulation', to eradicate nearby pathogens.[42,43] Although neutrophils mainly contribute to host defense against invading pathogens, they are also involved in the pathogenesis of various sterile inflammatory and autoimmune diseases such as atherosclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, and gout.[44,45]

4.2. NETs and their structure

Neutrophil extracellular traps (NETs) are the most recently identified mechanism of extracellular pathogen killing by neutrophils. NETs are formed from chromatin filaments that are approximately 15–17 nm in diameter and have backbones composed of DNA, histones, and nuclear protein HMGB1 (high-mobility group box 1). Histones occupy about 70% of the total proteins in these traps. The NET backbone is decorated with dotted, globular structures roughly 50 nm in diameter. The globular structures are composed of various primary (azurophilic) and secondary (specific) granular proteins including myeloperoxidase (MPO), NE, cathepsin G, lysozyme, cathelicidin, lactoferrin, and NADPH oxidase, as well as tertiary granular proteins such as matrix metalloproteinase 9. Interestingly, NETs appear, morphologically, not only as thin, elongated filaments, but also as cloud-like structures that occupy a 10–15-fold larger surface area than intact cells do.[46-49]

4.3. NET formation at the molecular level

NET formation is a process with several key steps: ROS generation, transport of NE from the granule to the nucleus, histone modification, disruption of the plasma membrane, and excretion of the NET structure. A variety of NET-formation inducers have been reported to date, including phorbol 12-myristate 13-acetate (PMA),

proinflammatory cytokines, chemokines, nitric oxide, HMGB1, calcium ionophore, and immune complexes. In addition, gram-positive staphylococci and gram-negative bacteria induce NET formation.[42,50] Upon stimulation with PMA and following NADPH oxidase-mediated ROS generation, NE in the primary granules translocates to the nucleus. In the nucleus, NE cleaves histone H1 and modifies the core histones, promoting chromatin decondensation. Later, MPO migrates into the nucleus, and is associated with decondensed chromatin. In addition to modifications by NE and MPO, peptidylarginine deiminase 4 (PAD4) induces citrullination in the core histones, which leads to weaker binding between histones and DNA.[46] NE and MPO cooperatively promote chromatin decondensation and eventually induce plasma membrane rupture and NET release. NET formation leads to cell death, also called NETosis, which is a unique type of neutrophilic cell death distinct from apoptosis and necrosis.[42,49,51]

4.4. The role of NETs in atherosclerosis

NET formation is critical to host defense; however, it is also implicated in atherosclerosis, systemic lupus erythematosus (SLE), thrombosis, sepsis, malaria, endothelial cell death, and ischemic reperfusion injury.[52-54] In atherosclerosis, NET formation activates leukocytes and platelets in the lumen and in the intima. NET formation in the intima also promotes the production of inflammatory cytokines and various proteases, which leads to plaque instability and thrombus formation.

NETs have been identified in murine and human atherosclerotic lesions, as well as in myocardial thrombi.[55] In addition, luminal NETs have been detected by intravital two-photon microscopy and immunofluorescence.[56-58] Specifically, NET components such as double-stranded DNA (dsDNA), nucleosomes, and MPO-DNA complexes were detected in patients with severe coronary atherosclerosis or calcified coronary arteries.

Moreover, plasma levels of nucleosomes and granular proteins such as MPO, MMP-2, and MMP-9 are elevated in patients with acute coronary syndrome. However, because the significance of NETs and their components in atherosclerosis is unclear, further studies are needed to clarify their roles in the disease.[59,60]

5. References

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CHAPTER II. Macrophage differentiation from monocytes is influenced by the lipid oxidation degree of low-density lipoprotein

1. Abstract

LDL plays an important role in atherosclerotic plaque formation and macrophage differentiation. However, there is no report regarding the degree of LDL oxidation and macrophage differentiation. Our study has shown that the differentiation into M1 or M2 macrophages is related to the lipid oxidation level of LDL. Based on the level of lipid peroxidation, LDL is classified into high-oxidized LDL (high-oxLDL) and low-oxidized LDL (low-oxLDL). The differentiation profiles of macrophages were determined by surface receptor expression and cytokine secretion profiles. Low-oxLDL induced CD86 expression and production of TNF-α and IL-12p40 in THP-1 cells, indicating an M1 macrophage phenotype. High-oxLDL induced mannose receptor expression and production of IL-6 and monocyte chemoattractant protein-1, which mostly match the phenotype of M2 macrophages. Further supporting evidence for an M2 polarization by high-oxLDL was the induction of LOX-1 in THP-1 cells treated with high-oxLDL but not with low-oxLDL. Similar results were obtained in primary human monocytes. Therefore, our results strongly suggest that the degree of LDL oxidation influences the differentiation of monocytes into M1 or M2 macrophages and determines the inflammatory fate in early stages of atherosclerosis.

Keywords: LDL, Oxidation, Macrophages, Differentiation

2. Introduction

Oxidized low-density lipoprotein (oxLDL) plays a critical role in atherosclerotic plaque formation, which is triggered by the persistence of lipid-laden macrophages[1] as well as interfering endothelial cell motility[2] in arterial wall. The differentiation of monocytes into macrophages, which accumulate oxLDL to form foam cells, is induced by oxLDL.[1,3] The process starts with endothelial activation in the sub-endothelial areas by oxLDL deposition.[4,5] Monocytes migrate into the intima guided by chemokines[6] and differentiate into macrophages. These macrophages then take up modified lipoproteins and, as they accumulate excess lipids, they form foam cells.[3] The foam cells die and release intracellular contents,[7] which induce an inflammatory reaction. This in turn attracts more macrophages into the lesion. During this process, LDL may be oxidized by enzymes, for example myeloperoxidase,[8,9] or by reactive oxygen species (ROS)[3] in the inflammatory microenvironment of the plaque. Although oxLDL is important for atherosclerotic inflammation, is no report about the degree of LDL oxidation and macrophage differentiation.

Macrophages are fundamental contributors in the development and progression of atherosclerosis. Monocytes that are recruited into the intima are exposed to a variety of stimuli, such as cytokines, lipids, iron, and calcium, which exist in complex microenvironments. These factors can influence the phenotypic polarization of macrophages and their subsequent activation.[10] Classically activated (M1) macrophages and alternatively activated (M2) macrophages are well-defined phenotypes. In addition, recent studies suggested that several subtypes of M2 macrophages (M2a, b, c, and d) and stimuli-induced subpopulations such as Mox, Mhem, M (Hb), and M4 macrophages exist in atherosclerotic lesions.[11]

Therefore, we hypothesized that LDL with different oxidation levels exists in the early atherosclerotic milieu and that the degree of LDL oxidation influences macrophage differentiation into classically activated (M1) or alternatively activated (M2) macrophages, which determines the inflammatory fate for atherosclerotic development. In this study, monocytes were treated with high-oxidized LDL (high-oxLDL) or low-oxidized LDL (low-oxLDL) and their surface receptor expression and cytokine secretion profiles were analyzed to determine if they differentiated into M1 or M2 macrophages.

3. Materials and methods

3.1. Reagents

Human native LDL and oxidized LDL were purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). During electrophoresis, low-oxLDL migrates further than the native LDL and is also defined by a low TBAR value (8.7–11.8 nmol/mg protein). High-oxLDL migrates 2.7 fold further than the native LDL and is defined by a high TBAR value (55.7–75.6 nmol/mg protein). Anti-human CD86-PE and anti-human CD206 (mannose receptor)-FITC antibodies were purchased from Beckman Coulter Inc. (Brea, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. Anti-LOX-1 and anti-tubulin antibodies were purchased from Abcam (Cambridge, MA, USA) and BioLegend (San Diego, CA, USA), respectively. Recombinant human M-CSF was purchased from BioLegend (San Diego, CA, USA). LPS (*E. coli* O26:B6), phorbol-12-myristate-13-acetate (PMA) and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Culture of THP-1 Cells

The human monocytic THP-1 cell line was purchased from ATCC and cultured in RPMI1640 medium containing 10% FBS and penicillin-streptomycin (100 units/ml and 100 μg/ml, respectively) at 37°C in a humidified 5% CO_2 incubator.

3.3. Oil Red O staining

THP-1 cells were cultured at 5×10^5 cells per well in 6-well plates and differentiated into macrophages with 100 ng/ml of PMA for 48 hr. Cell were then treated with native LDL, low-oxLDL or high-oxLDL (50 μg/ml each). After 24 hr, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then rinsed in 60%

isopropanol for 30 sec and stained with filtered Oil Red O working solution at 37°C for 30 min in darkness. Then cells were destained with 60% isopropanol for 30 sec and lipid accumulation was observed using bright-field microscope (IX71, Olympus, Tokyo, Japan).

3.4. Analysis of cell proliferation

The cell proliferation rate was measured using the colorimetric cell counting kit-8 (CCK-8) (Dojindo laboratories, Kyoto, Japan). THP-1 cells were cultured at 1×10^5 cells per well in 24-well plates and treated with native LDL, low-oxLDL or high-oxLDL (50 μg/ml each). After 24, 48 or 96 hr, the CCK-8 reagent was added to each well and cells were incubated for 30 min at 37°C. The supernatant was harvested and transferred to 96-well plates. The O.D. at 450 nm was determined with a spectrophotometer.

3.5. Flow cytometry

THP-1 cells were cultured at 2×10^5 cells per well in 12-well plates and treated with native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each). After 24 or 96 hr, cells were harvested and analyzed using flow cytometry to measure granularity and auto-fluorescence. To detect surface markers, cells were treated with native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each) for 96 hr and stained with PE-conjugated anti-human CD86 antibodies. Flow cytometric analysis was performed by a BD LSR II flow cytometer (BD Bioscience).

3.6. Confocal microscopy

THP-1 cells were cultured in 2-well chambered cover glasses at 2×10^5 cells per well and treated with native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each) for 96 hr.

Attached cells were stained with FITC-conjugated anti-human mannose receptor (CD206) antibodies and analyzed using FV1000 microscope (Olympus, Tokyo, Japan).

3.7. Isolation of human monocytes and observation of morphological changes

Blood was obtained from healthy donors after acquiring internal review board approval and informed consent (No: 4-2012-0088). Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation (density = 1.077) at 1,600 rpm for 30 min and human monocytes were purified from PBMCs using Monocyte Isolation Kit II (Miltenyi Biotec, Bergsch Gladbach, Germany). Staining for CD14 was performed to confirmed monocyte population and usually >95% of cells were positive. Human monocytes were cultured in RPMI1640 medium containing 10% FBS and penicillin-streptomycin (100 units/ml and 100 μg/ml, respectively) at 37°C in a humidified 5% CO_2 incubator. Human monocytes were cultured in 12-well plates at 2×10^5 cells per well and incubated with both M-CSF (50 ng/ml) and high-oxLDL or low-oxLDL (50 μg/ml each) for 7 days. Morphological changes were observed using bright-field microscope (IX71, Olympus, Tokyo, Japan).

3.8. Enzyme-linked immunosorbent assay (ELISA)

THP-1 cells were culture at 2×10^5 cells per well in 12-well plates and pre-treated with native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each) for 24, 48 or 96 hr. The medium was replaced with fresh medium and then cells were stimulated with LPS (100 ng/ml). Primary monocytes were cultured at 2×10^5 cells per well in 12-well plates and pre-treated with both M-CSF (50 ng/ml) and native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each) for 24, 48 or 72 hr. Primary monocytes were stimulated with LPS (20 ng/ml). Eighteen hours after LPS stimulation, culture supernatants were harvested and

stored at -80°C. ELISA was performed with a human cytokine TNF-α, IL-12p40, IL-6, and monocyte chemoattractant protein-1 (MCP-1) assay kit (BD Bioscience). The O.D at 450 nm was determined.

3.9. Western blot

THP-1 cells were seeded at 2×10^5 cells per well and treated with native LDL, low-oxLDL, or high-oxLDL (50 μg/ml each). After 24 and 48 hr, cells were harvested and lysed with lysis buffer. After centrifugation, total protein was stored at -80°C. Each 50 μg of protein samples was loaded in 12% SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare, NJ, USA). Then membranes were reacted with anti-LOX-1 or anti-tubulin antibodies. Protein bands were detected using a West-save up western blot detection kit (Ab frontier, Seoul, Korea).

3.10. Statistical analysis

One-way ANOVA analysis was conducted. $P<0.05$ was considered significant.

4. Results

4.1. Differential effects of the LDL oxidation degree on lipid uptake in THP-1 macrophages

The recognition of low-oxLDL in monocytes is mediated by LDL receptors and/or pattern recognition receptors, such as toll-like receptors (TLRs). TLR2 and TLR4 are the major TLR members activated by minimally modified LDL, such as low-oxLDL, and induce inflammatory responses in human macrophages and monocytes. However, these receptors do not mediate massive uptake of minimally modified LDL in the formation of foam cells. Indeed, we found that the accumulation of lipid was not significantly increased in cells treated with low-oxLDL compared to that in cells treated with native LDL, but lipid accumulation was significantly increased when cells were treated with high-oxLDL (Figure II-1)

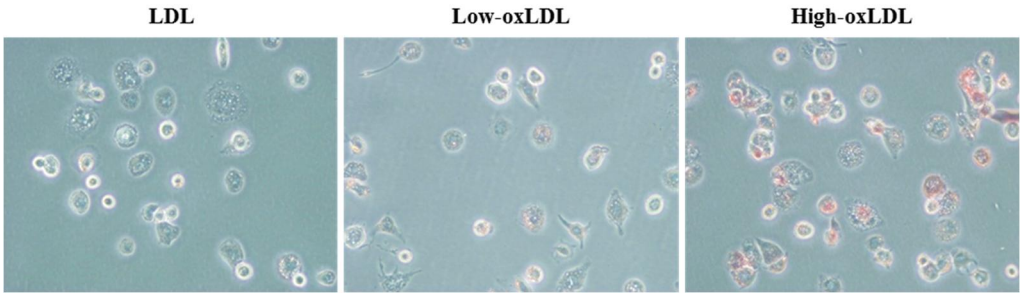


Figure II-1. Uptake of oxidized low-density lipoprotein (oxLDL) in PMA-differentiated THP-1 cells. THP-1 cells were differentiated into macrophages with PMA (100 ng/ml) for 48 hr. PMA-differentiated THP-1 cells were treated with differentially oxidized LDL (50 μg/ml) for 24 hr. Then, accumulated intracellular lipid droplets were stained by Oil-Red O and were observed using a bright-field microscope (× 200).

4.2. Differential effects of the LDL oxidation degree on granularity induction and proliferation of monocytes

To investigate the effects of the degree of LDL oxidation, we assessed differentiation and proliferation of THP-1 cells after treatment with native LDL, low-oxLDL, or high-oxLDL. Cell granularity (Figure II-2A) and auto-fluorescence (Figure II-2B) were significantly increased in cells treated with high-oxLDL compared to cells treated with native LDL or low-oxLDL. These effects by high-oxLDL increased over time. After treatment with high-oxLDL, the cell proliferation rate did not change compared to those of cells treated with native LDL. However, cell proliferation was significantly decreased in cells treated with low-oxLDL compared to that in cells treated with high-oxLDL (Figure II-2C). These results indicate that high-oxLDL increases granularity and auto-fluorescence whereas low-oxLDL decreases cell proliferation, suggesting that monocytes respond differently to degree of LDL oxidation.

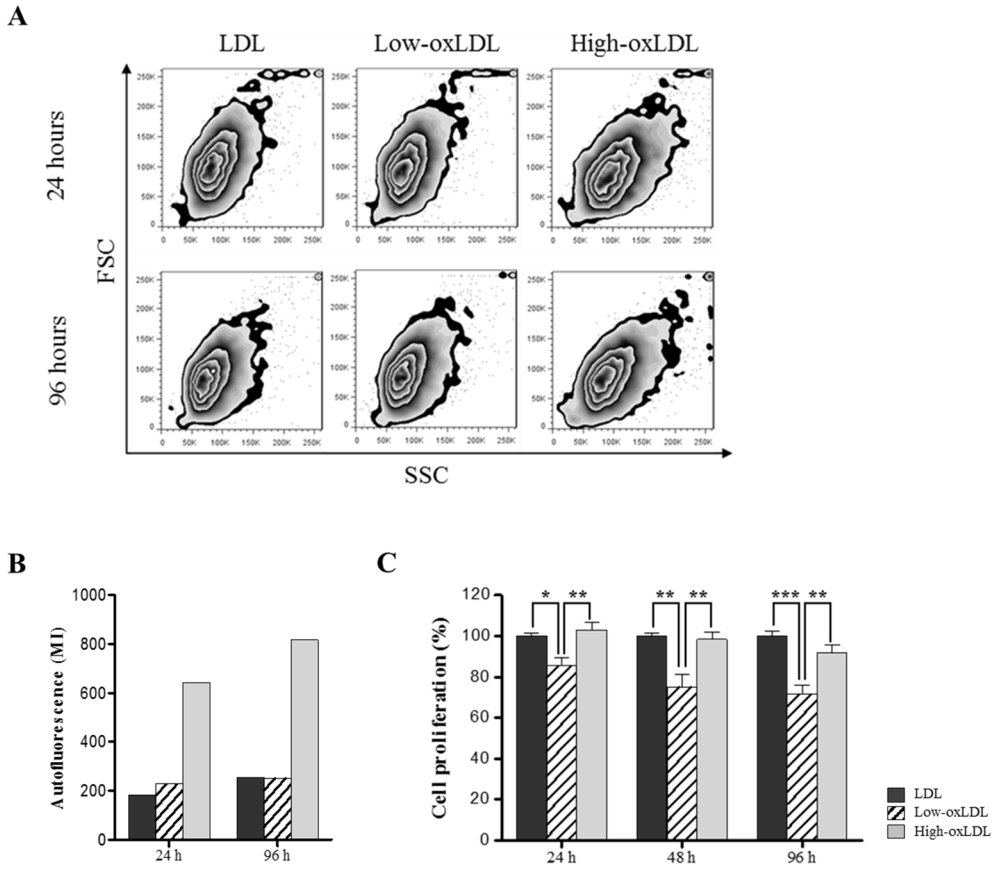
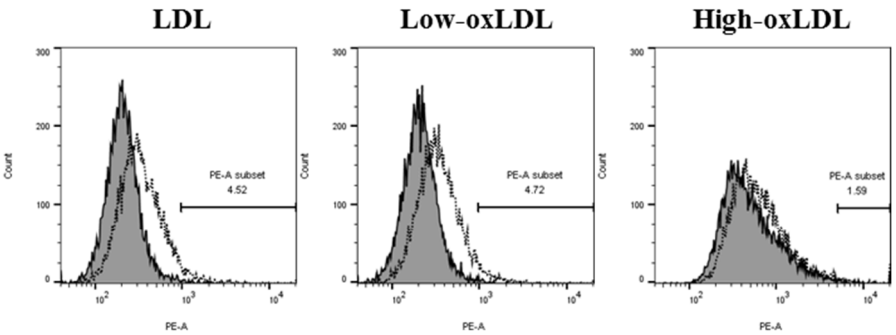


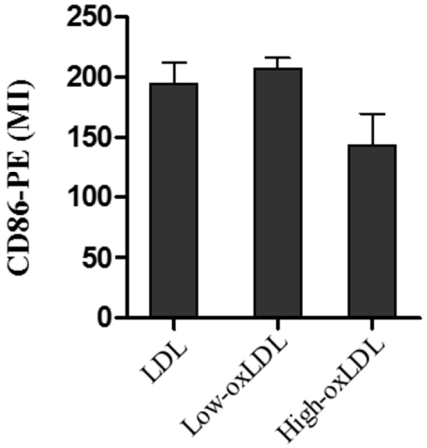
Figure II-2. Effects of oxLDL on granularity, auto-fluorescence, and proliferation. THP-1 cells were treated with differentially oxidized LDL (50 μg/ml) for 24, 48 or 96 hr. (A) Granularity and (B) auto-fluorescence were measured by flow cytometer. (C) Cell proliferation was assessed by CCK-8 assay. Data represent means ±S.E. of three independent experiments. One-way ANOVA analysis was used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

4.3. Effect of oxLDL on the expression of surface markers of macrophages

Next, we tested whether the degree of LDL oxidation influences the differentiation of monocytes into macrophages. When cells were treated with low-oxLDL, the expression level of the marker for M1 macrophages, CD86, was increased compared to that in cells treated with high-oxLDL (Figure II-3A). In contrast, the expression level of the marker for M2 macrophages, mannose receptor (CD206), was significantly increased in cells treated with high-oxLDL compared to that in cells treated with native LDL or low-oxLDL (Figure II-3B). These results indicate that the degree of LDL oxidation affects the differentiation of monocytes into different subtypes of macrophages.

A





B

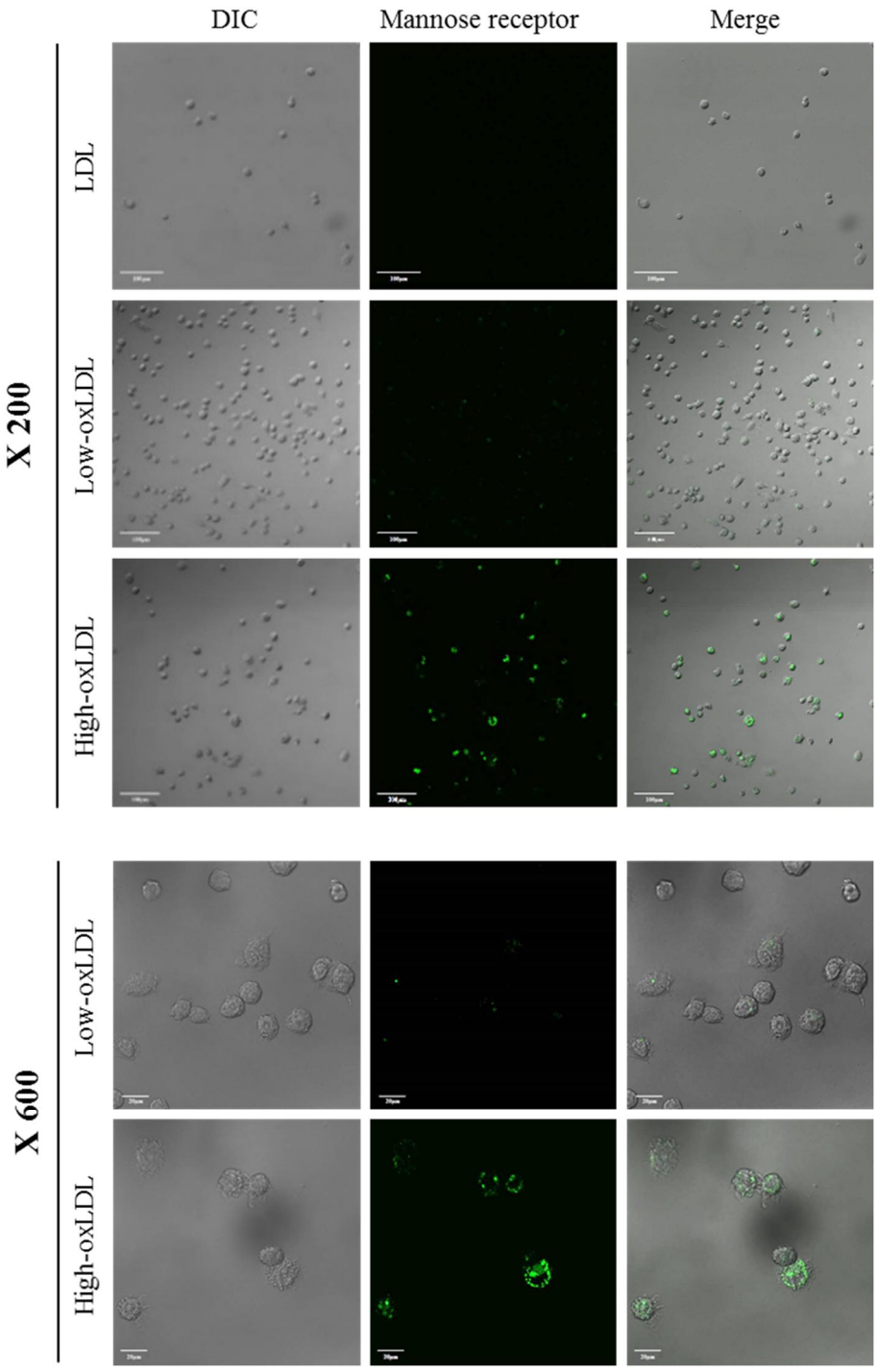


Figure II-3. Expression of CD86 and CD206 in response to oxLDL. THP-1 cells were treated with oxLDL (50 μg/ml) for 96 hr. (A) THP-1 cells were stained with anti-CD86-PE antibodies and analyzed by flow cytometry. (B) THP-1 cells were stained with anti-CD206-FITC antibodies and observed by confocal microscope (x 200 and x 600).

4.4. Effect of oxLDL on cytokine production

To assess cytokine production, THP-1 cells were pre-exposed to differentially oxidized LDL for 24, 48 or 96 hr and then stimulated with LPS. The production of TNF-α and IL-12p40 was decreased in cells pre-treated with high-oxLDL compared to those in cells pre-treated with low-oxLDL (Figure II-4A). However, the production of IL-6 and MCP-1 was significantly increased in cells pre-treated with high-oxLDL compared to cells pre-treated with low-oxLDL (Figure II-4B). These results suggest that macrophages secrete different sets of cytokines depending on the oxidation level of LDL. More interestingly, low-oxLDL and high-oxLDL induced opposite cytokine profiles.

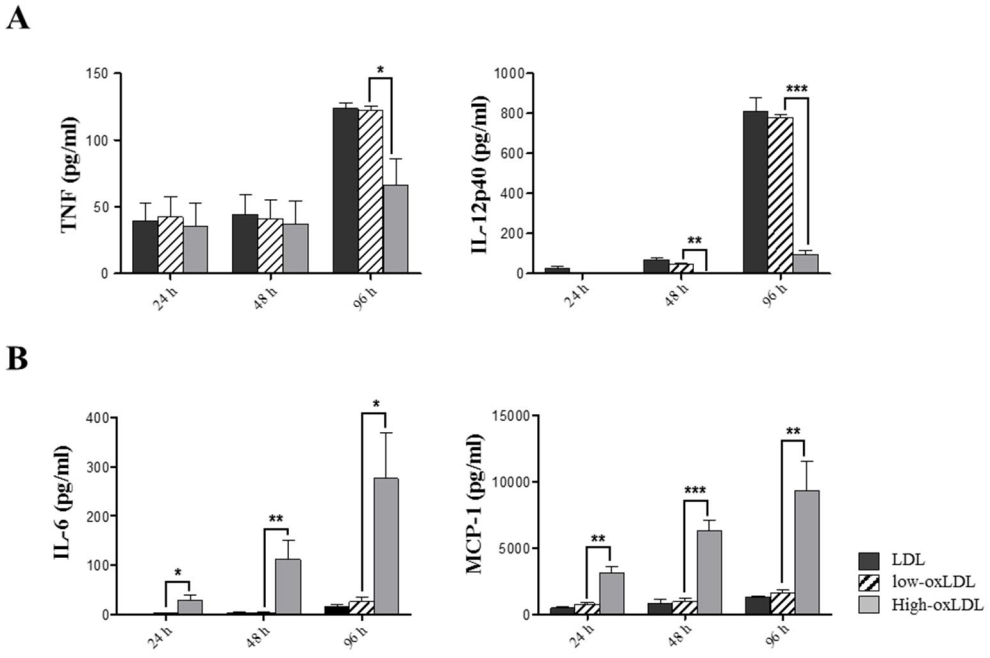


Figure II-4. Production of cytokines and chemokines in response to oxLDL. THP-1 cells were pre-treated with differentially oxidized LDL (50 μg/ml) for 24, 48 or 96 hr and treated with LPS (100 ng/ml) for 18 hr. Culture supernatants were harvested and the levels of TNF-α, IL-12p40, IL-6 and MCP-1 were measured by ELISA. Data represent means ±S.E. of three independent experiments. One-way ANOVA analysis was used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

4.5. Effects of oxLDL on LOX-1 expression

OxLDL binds to scavenger receptors, such as LOX-1, and also induces the expression of LOX-1 in macrophages. We tested the effect of oxLDL on the expression of LOX-1 in THP-1 cells. LOX-1 expression increased in cells treated with high-oxLDL compared to cells treated with native LDL or low-oxLDL after 24 or 48 hr. The expression of LOX-1 was slightly but not significantly decreased in cells treated with low-oxLDL compared to that in cells treated with native LDL (Figure II-5).

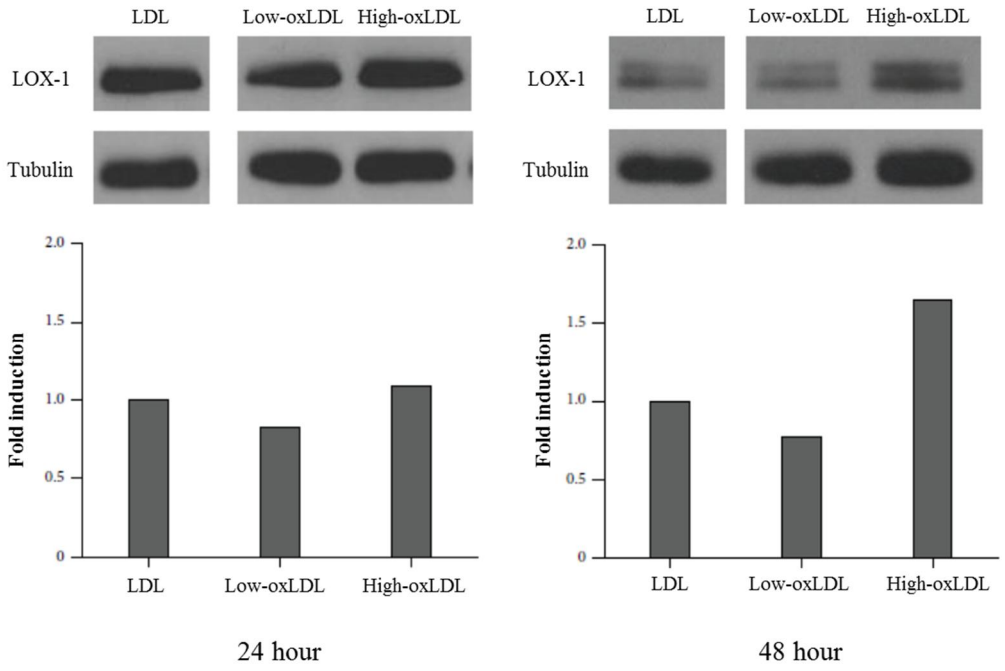


Figure II-5. Expression of LOX-1 in response to oxLDL. THP-1 cells were treated with differentially oxidized LDL (50 μg/ml) for 24 or 48 hr. Each 50 μg of protein samples was loaded and analyzed with anti-LOX-1 antibodies and anti-tubulin antibodies (Internal control).

4.6. Effects of oxLDL on morphological changes and cytokine production in primary monocytes

Human monocytes were isolated from purified peripheral blood mononuclear cells using Monocyte Isolation Kit II. To confirm the purity of isolated human monocytes, cells were stained with FITC anti-human CD14 antibody, which is the marker for human monocytes. The results show that >95% of the cells were positive (Figure II-6).

When human monocytes were treated with low-oxLDL, cells have changed into spindle-like shape, which is a unique feature of M1 macrophages whereas the treatment of high-oxLDL led to round cells, which represents M2 macrophages (Figure II-7A). The production of TNF-α and IL-12p40 was decreased in monocytes pre-treated with high-oxLDL compared to cells pre-treated with low-oxLDL (Figure II-7B). Moreover, the production of IL-6 and MCP-1 was significantly increased in monocytes pre-treated with high-oxLDL (Figure II-7C). These results support that the degree of LDL oxidation affects primary monocytes in a similar way to the monocytic cell line, THP-1 cells.

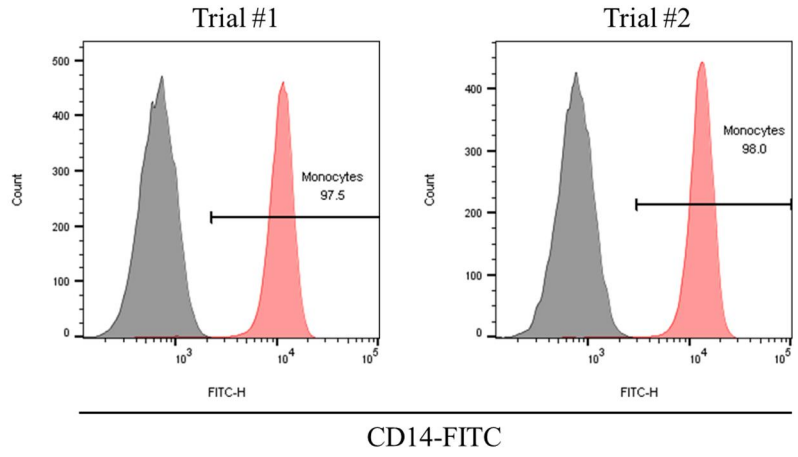


Figure II-6. Purity of isolated human primary monocytes. Human monocytes were purified from PBMCs using Monocyte Isolation Kit II and stained with human CD14 antibody. Flow cytometric analysis was performed using a BD LSR II flow cytometer.

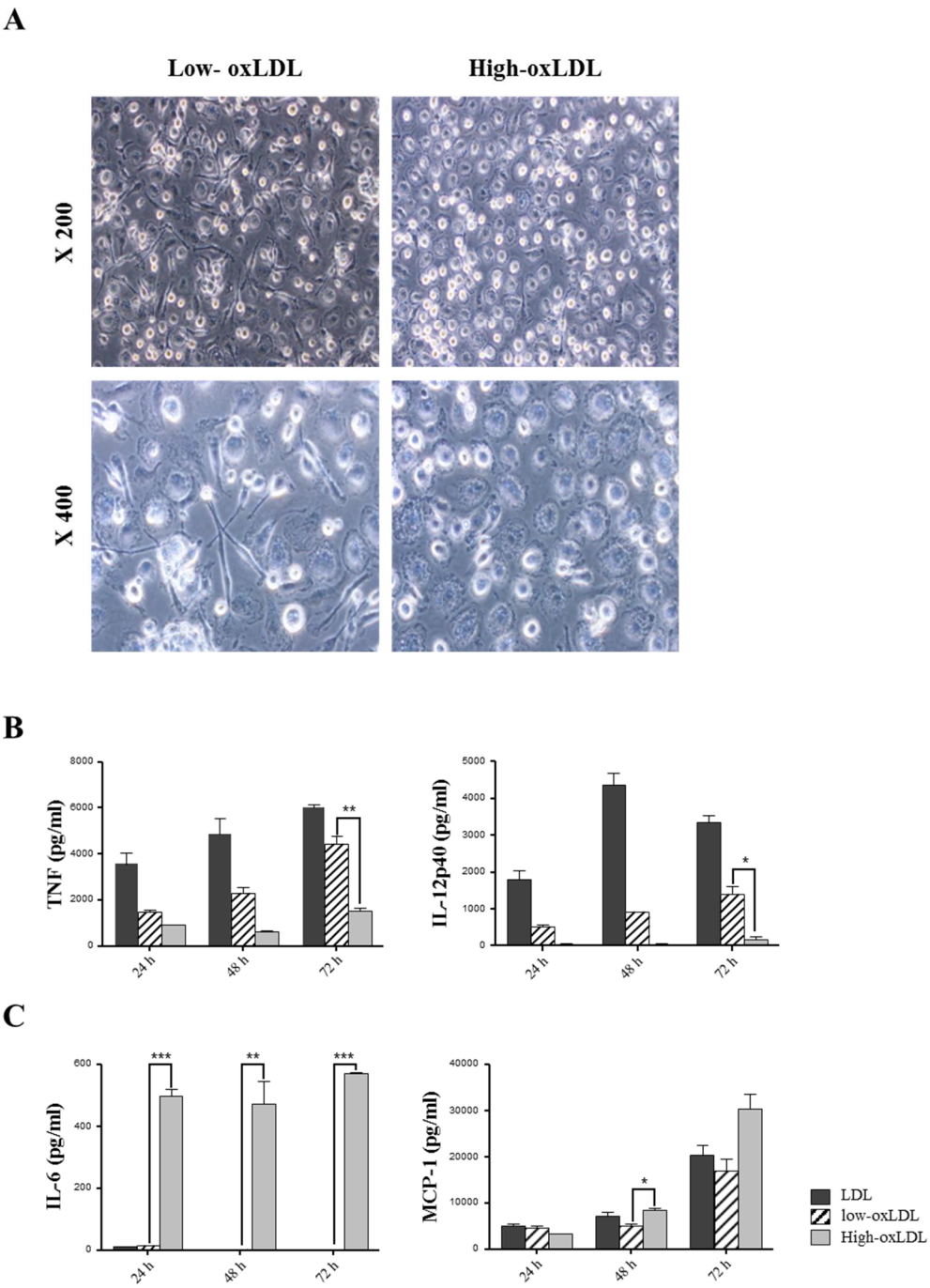


Figure II-7. Morphological changes and cytokine production in primary monocytes. (A) Human blood monocytes were isolated from PBMCs and cultured with both M-CSF (50 ng/ml) and differentially oxidized LDL (50 μg/ml each) for 7 days. Morphological changes were observed using bright-field microscope (x 200, 400). (B) Primary monocytes were pre-treated with both M-CSF (50 ng/ml) and differentially oxidized LDL (50 μg/ml) for 24, 48 or 72 hr and treated with LPS (20 ng/ml) for 18 hr. Culture supernatants were harvested and the levels of TNF-α, IL-12p40, IL-6 and MCP-1 were measured by ELISA. Data represent means ±S.E. and One-way ANOVA analysis was used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

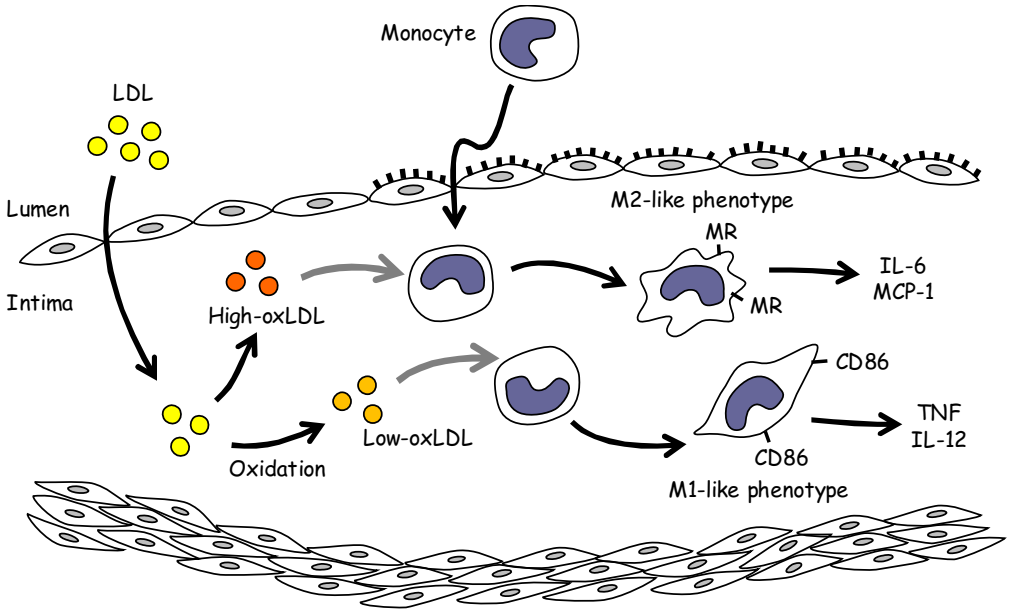


Figure II-8. Graphical summary of monocyte differentiation into M1 or M2 macrophages by differentially oxidized LDL. In early stage of atherosclerosis, LDL is accumulated in the intima and oxidized into a variety of degree of oxLDL. Low-oxLDL induces macrophage polarization towards M1 phenotype whereas high-oxLDL induces M2 phenotype. These results suggest that the degree of oxidation of LDL is the important factor for differentiation of monocytes into macrophages and may determine the inflammation status or even the development of atherosclerosis at early stages of the disease.

5. Discussion

During atherosclerotic plaque development, LDL can be oxidized to various degrees by ROS or enzymes released by macrophages in the sub-endothelial area.[3] This modified LDL, along with growth factors such as M-CSF, affects differentiation of monocytes into macrophages. Depending on the extent and duration of oxidation, the physicochemical properties of LDL, such as the charge, particle size and lipid content, are changed.[12] In addition, depending on whether the lipid or protein component is oxidized, oxidized LDL can induce different signaling pathways.[13] Our study investigated the effects of differentially oxidized LDL on the differentiation of macrophages. We used two types of oxLDL, which were classified as low-oxLDL or high-oxLDL based on the TBAR value for lipid peroxidation and relative electrophoretic mobility of LDL particles on agarose gels.[14,15]

OxLDL is known to stimulate cell proliferation and induces differentiation of monocytes and macrophages.[16,17] Treatment of THP-1 cells with high-oxLDL led to an increase in cell granularity and auto-fluorescence, which is a feature of differentiated macrophages.[18] Moreover, high-oxLDL did not change the cell proliferation rate compared to native LDL. In contrast, in cells treated with low-oxLDL the granularity and auto-fluorescence were not increased and cell proliferation was significantly decreased (Figure II-2). Next, as shown in Figure II-3, CD86 (M1 macrophages) was induced by low-oxLDL whereas mannose receptor (M2 macrophages) was induced by high-oxLDL. The results of the cytokine profiling showed an increased production of TNF-α as well as IL-12p40 (Figure II-4A), which reflects the typical phenotype of M1 macrophages,[19] whereas increased production of IL-6 and MCP-1 (Figure II-4B) reflects the M2 macrophage phenotype. M2 macrophages can be further classified into

four subtypes, namely M2a, M2b, M2c, and M2d. Among them, M2b macrophages secrete high levels of IL-6 and are referred to as 'regulatory macrophages'.[10] IL-6 is a pleiotropic cytokine that induces either pro-inflammatory or anti-inflammatory responses and is involved in a variety of conditions, such as obesity, arthritis, colitis, and sepsis. Generally, IL-6 is considered a pro-inflammatory cytokine, but its anti-inflammatory roles have been suggested in specific conditions.[20] Recent studies have shown that IL-6 promotes M2 activation while attenuating M1 activation. When myeloid cells were primed with IL-6, the STAT3-IL-4-STAT6 axis, which is a potent inducer of M2 polarization, was activated, whereas when monocytes were pre-treated with IL-6, lipopolysaccharide-induced production of pro-inflammatory cytokines was attenuated.[21] Moreover, IL-6 induces the polarization of tumor-associated macrophages, which are similar to obesity-associated M2 macrophages.[22] These observations indicate that IL-6 plays a 'counter-inflammatory' role in controlling metabolic homeostasis in low-grade and chronic conditions, such as obesity.

MCP-1 plays an important role in the recruitment of circulating monocytes to inflamed sites. MCP-1-mediated recruitment of monocytes contributes to pathogenesis, but several studies have shown that MCP-1 has a beneficial role in various inflammatory conditions. In the gut, lamina propria macrophages, which revealed an M2-like phenotype, produce large amounts of MCP-1. Additionally, a subset of lamina propria macrophages are recruited in response to MCP-1 and produce a large amount of IL-10 to maintain gut homeostasis in the steady state as well as to terminate excess inflammation in the intestine.[23] Furthermore, MCP-1 exerts beneficial effects in other inflammatory diseases such as ischemic heart diseases by inhibiting the generation of ROS and by healing necrotic areas in the myocardium.[24,25]

The exact roles of IL-6 and MCP-1 are still controversial. However, previous studies on the anti-inflammatory effects of IL-6 and MCP-1 support our results that high-oxLDL-induced differentiation of macrophages skews towards the M2-like phenotype rather than the M1 phenotype (Figure II-3B & II-4B).

Our results show that high-oxLDL induces IL-6 and MCP-1 production in macrophages, indicating that the expression of these cytokines may be a feature of M2 macrophages. While the interpretation of cytokine profiles might be difficult, most of our results for cytokine production and surface marker expression suggest that low-oxLDL induces M1 polarization, whereas high-oxLDL leads to M2 polarization.

Next, we assessed the expression of LOX-1. LOX-1 is a receptor for oxLDL and is expressed in a variety of cells, such as endothelial cells, muscle cells, monocytes, and macrophages.[2,26,27] After binding to oxLDL, LOX-1 is internalized; this induces endoplasmic reticulum (ER) stress that causes up-regulation of LOX-1, which occurs *via* a positive feedback loop between ER stress and LOX-1 expression, and macrophage differentiation into M2 macrophages together with foam cell formation.[27-29] Figure II-5 shows that treatment with high-oxLDL increased the LOX-1 expression of macrophages. These high LOX-1 levels amplify the signals from high-oxLDL and thus induce increased differentiation into M2 macrophages. A report using mesenchymal stem cells has demonstrated that treatments with oxLDL markedly increase expressions of LOX-1 and MCP-1,[30] but in which the degree of LDL oxidation was not clearly defined.

Lastly, we investigated the effects of differentially oxidized LDL in primary monocytes. Our study showed primary monocytes have changed into spindle-shape after treatment with low-oxLDL and into round cells after treatment with high-oxLDL.

It is well known that the shape of M1 macrophages adopt more spindle-like shape, whereas M2 macrophages are more rounded.[31,32] In addition to the morphological changes (Figure II-7A), the cytokines profiles, the decreased production of TNF-α and IL-12p40 as well as increased production of IL-6 and MCP-1 after treatment with high-oxLDL (Figure II-7B & II-7C), strongly support the role of differentially oxidized LDL in monocyte differentiation to M1 or M2 macrophages.

Taken together, our results for granularity, auto-fluorescence, cytokine profiles, and surface makers suggest that low-oxLDL induces macrophage polarization towards M1 phenotype whereas high-oxLDL induces M2 phenotype. As summarized in Figure II-8, our results suggest that the degree of oxidation of LDL is the important factor for differentiation of monocytes and macrophages and may determine the inflammation status or even the development of atherosclerosis at early stages of the disease.

6. References

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CHAPTER III. Neutrophil extracellular trap formation mediated by oxidized low-density lipoprotein affects endothelial cells in early atherosclerosis

1. Abstract

Neutrophil extracellular traps (NETs) are an important host defense mechanism to eliminate extracellular pathogens. They have also been implicated in chronic inflammatory diseases such as atherosclerosis. Recent studies have shown that NET components can be found in luminal and intimal lesions associated with atherosclerosis. However, the role of luminal NETs in early atherosclerosis is not well examined. In this study, we investigated the formation of NETs in early atherosclerosis, and searched for activating factors that promote NET formation. Wild type (WT) and *ApoE*$^{-/-}$ mice were fed an atherogenic diet for 4 wk, and luminal NET formation was evaluated by T2*-weighted MR imaging. NETs were detected in *ApoE*$^{-/-}$ mice after 2 wk and were more clearly detected after 3 wk. However, NETs were not detected in WT mice even after 4 wk. Next, neutrophils from WT mice or *ApoE*$^{-/-}$ mice were isolated and IL-8-induced NET formation was observed by confocal microscopy. In response to IL-8 neutrophils from *ApoE*$^{-/-}$ mice induced the NET formation, but neutrophils from WT mice did not. Along with NET formation, lipid accumulation in the arterial wall was increased in *ApoE*$^{-/-}$ mice compared to WT mice. Also, the plasma levels of oxLDL was significantly higher in *ApoE*$^{-/-}$ mice compared to WT mice.

The *ex vivo* study of oxLDL on NET formation was performed using human neutrophils. Although high-oxLDL as well as low-oxLDL induced the similar levels of NET formation in confocal analysis, high-oxLDL was found to induce more NETs in

Picogreen assay to detect dsDNA. Finally, the effect NETs on endothelial dysfunction was studied. The culture supernatants of neutrophils treated with oxLDL, which contain NET components, promote cell death as well as nitric oxide production in human endothelial cells. However, these effects were decreased with depletion of MMP-9, which is a component of NETs and detected highly in plasma and plaque lesions from atherosclerosis. Collectively, our results suggest that oxLDL-induced NET formation induces endothelial dysfunction *via* MMP-9 in early atherosclerosis and promotes LDL deposition into the intima. Thus, circulating oxLDL is a crucial component in the promotion of early atherogenesis.

Keywords: oxLDL, neutrophils, NETs, MMP-9

2. Introduction

Neutrophils are the components of the first line innate immune defense against invading pathogens such as bacteria, fungi, and parasites. They eliminate extracellular pathogens by phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs).[1,2] NETs are the most recently described extracellular killing mechanism. NETs are formed with a chromatin filament backbone and are decorated with dotted globular structures. Chromatin filaments are mainly composed of DNA and histones, whereas the globular structures consist of granular proteins, including neutrophil elastase (NE), myeloperoxidase (MPO), and matrix metalloproteinase-9 (MMP-9).[3,4] Although neutrophils mainly contribute to host defense against invading pathogens, they are also involved in the pathogenesis of various sterile inflammatory and autoimmune diseases such as atherosclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, and gout.[5]

A variety of inducers of NET formation have been reported to date, including phorbol 12-myristate 13-acetate (PMA), proinflammatory cytokines, chemokines, HMGB1, nitric oxide (NO), calcium ionophore, cholesterol crystals, and immune complexes.[6,7] In addition, gram-positive staphylococci and gram-negative bacteria induce NET formation through interactions between platelets and neutrophils.[8,9] Recently, oxidized low-density lipoprotein (oxLDL) was identified as a NET inducer, and was found to directly induce NET formation *via* TLR2 and TLR6 *in vitro*.[10]

OxLDL is a useful marker for atherosclerosis. It has been detected *in vivo* both in atherosclerotic lesions as well as in plasma.[11] Since elevated levels of circulating oxLDL were shown to correlate significantly with the severity of cardiovascular disease (CVD),[12] oxLDL levels are a possible prognostic marker for future cardiac events.[13-15]

Recently, several studies have shown that variable degrees of oxLDL exist in atherosclerotic lesions, and oxLDL can induce various biological effects, depending on the level of LDL oxidation.[16-19]

NETs have been identified in murine and human atherosclerotic lesions, as well as in myocardial thrombi.[20,21] A recent study implicated NETs in the development of atherosclerotic plaques.[22] Specifically, NET components such as double-stranded DNA (dsDNA), nucleosomes, and MPO-DNA complexes were detected in patients with severe coronary atherosclerosis or calcified coronary arteries. Moreover, plasma levels of nucleosomes, MMP-2, and MMP-9 were correlated with an increased risk of coronary stenosis.[23] More recently, the presence of luminal NETs was detected by intravital two-photon microscopy and immunofluorescence in early atherosclerotic lesions.[7,24] However, the exact role of luminal NETs in early atherosclerosis is not well defined.

We hypothesized that circulating oxLDL exist in the early stage of atherosclerosis, and stimulate neutrophils to promote NET formation on arterial walls. Eventually, NETs cause endothelial dysfunction and subsequent lipid accumulation in arterial walls. In this study, *ApoE*$^{-/-}$ mice were fed an atherogenic diet (AD), and then NET formation and neutrophil activities were measured, together with circulating oxLDL levels. In addition, we investigated whether oxLDL-primed human primary neutrophils induced endothelial dysfunction or cell death *ex vivo*.

3. Materials and methods

3.1. Reagents

Human LDL was purchased from Kalen Biomedical, LLC (Montgomery Village, MD, USA). Dil-LDL was purchased from Biomedical Technologies, Inc. (Stoughton, MA, USA). Poly-l-lysine solution, cyanogen bromide solution, barbital buffer, phorbol 12-myristate-13-acetate (PMA), and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). SYTOX Green and SYTOX Orange were purchased from Life Technologies (Eugene, OR, USA). Quanti-iT^{TM} PicoGreen dsDNA kit was purchased from Turner Biosystems, Inc. (Sunnyvale, CA, USA). A 24-well Transwell insert with a 5.0-μm pore polyester membrane was purchased from Costar (Corning, NY, USA). RQ1 RNase-free DNase was purchased from Promega (Madison, WI, USA). fluidMAG-D (50 nm) was purchased from Chemicell GmbH (Berlin, Germany). Cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was purchased from Duchefa Biochemie (Haarlem, Netherlands)

3.2. Antibodies

Anti-human neutrophil elastase (NE)-FITC antibody was obtained from Biorbyt (Cambridge, UK). Alexa Fluor 488 anti-human CD66a/c/e and Alexa Fluor 488 mouse IgG2b, κ isotype control antibodies were purchased from BioLegend (San Diego, CA, USA) and PE mouse anti-human CD66b and PE mouse IgM, κ isotype control antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-human MMP-9 antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA), and rabbit anti-histone H2A.x antibody was purchased from Bioss, Inc. (Woburn, MA, USA).

3.3. Experimental animals

C57BL/6J control and apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice (C57BL/6J background, male) were purchased from the Jackson Laboratory (CA, USA). At fifth week of age, the *ApoE*$^{-/-}$ and control mice were fed an AD containing 1.25% (w/w) cholesterol (D12336, Research Diet, NJ, USA) for 2–4 wk. All animal studies were performed in compliance with the Yonsei University Institutional Animal Care guidelines, and protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the Laboratory Animal Research Center at Yonsei University Health System (Permit Number: 2013-0331-5). All mice were maintained in a Specific Pathogen Free (SPF) facility of the Yonsei Laboratory Animal Research Center.

3.4. Measurement of plasma cholesterol levels

C57BL/6J control and *ApoE*$^{-/-}$ mice were fed an AD for 2 – 4 wk. At each time point, blood samples were collected in heparinized tubes and centrifuged at 1000 × g for 15 min. Supernatants were harvested and stored at -80°C until analysis. The levels of total cholesterol (T-CHO), triglycerides (TG), and high-density lipoprotein cholesterol (HDL) were measured using an HDL and LDL/VLDL Quantification Colorimetric Kit and Triglyceride Quantification Colorimetric Kit (BioVision, Mountain View, CA, USA).

3.5. Lipid accumulation in the aorta

C57BL/6J control and *ApoE*$^{-/-}$ mice were fed an AD for 2 – 4 wk. At each time point, a subset of mice were euthanized and vasculature was perfused at a rate of 1 mL/min with heparinized phosphate-buffered saline (heparin; 2%), followed by 4% paraformaldehyde, using a 25-gauge needle inserted into the left ventricle. Aortas were

carefully dissected from the mice, and adventitial lipids were removed. For Oil Red O staining, the isolated aortas were rinsed in 60% isopropanol for 30 sec and stained with filtered Oil Red O working solution at 37°C for 30 min in darkness. Then, aortas were destained with 60% isopropanol for 30 sec, and lipid accumulation was observed under a bright-field microscope. The percentage of Oil Red O-stained areas in the entire aorta area was determined using the Image J software (https://imagej.nih.gov/ij/).

3.6. *In vivo* imaging system (IVIS)

C57BL/6J control and *ApoE*$^{-/-}$ mice were fed an AD for 4 wk and Dil-tagged native LDL (20 μg) was injected intravenously. After 18 hr, the mice were euthanized and vasculature was perfused at a rate of 1 mL/min with heparinized PBS (heparin; 2%), using 25-gauge needle inserted into the left ventricle. Aortas were dissected from the mice, and accumulation of Dil-LDL in these aortas was measured using a Xenogen IVIS-200 (Alameda, CA, USA).

3.7. Magnetic resonance imaging (MRI)

C57BL/6J control and *ApoE*$^{-/-}$ mice were fed an AD for 2 – 4 wk. At each time point, 0.2 mg of histone H2A.x antibody-conjugated T2*-contrast were injected intravenously. After 30 min post injection, attached histone H2A.x antibodies in the lumen of carotid arteries were observed using MR imaging system

3.8. Isolation of polymorphonuclear neutrophils from human peripheral blood

Blood samples obtained from healthy donors were EDTA anti-coagulated, after acquiring internal review board approval (No: 4-2012-0088) and informed consent from each participant. PMNs were separated by PolymorphPrep^{TM} (Axis-Shield, Oslo, Norway) (density = 1.113 ± 0.001 g/mL; osmolality = 445 ± 15 mOsm) at 520 × *g* for

30 min. Contaminating erythrocytes were lysed with 3 mL of red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 7 min. Staining for CD66a/c/e and CD66b was performed to confirm the neutrophil population, and in general, >95% of cells were positive. Human neutrophils were cultured in RPMI 1640 medium containing 1% FBS and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) at 37°C in a humidified 5% CO_2 incubator.

3.9. Cytospin and Giemsa staining

Purified neutrophils were resuspended and diluted in cold PBS containing 2% FBS, to a concentration of 1×10^5 cells/mL. One hundred microliters of cell suspension was added to a Single Cytology Funnel (Fisher HealthCare, Norwich, UK) and spun at 800 rpm for 3 min, using a cytocentrifuge (Cytospin 3, Shandon). Sample slides were carefully removed from the cytocentrifuge and allowed to air-dry. Cells were fixed with cold pure MeOH for 5 min and allowed to air-dry. A 100-μL drop of Giemsa's azur eosin methylene blue working solution (Merck, Darmstadt, Germany) was placed on the slide glass, and slides were incubated for 30 min. Cells were then washed twice with distilled water. Stained cells were observed under a bright-field microscope (IX71, Olympus, Tokyo, Japan).

3.10. NET formation

Isolated neutrophils (1×10^5) were seeded on chamber slides (NUNC, NY, USA). Chamber slides were precoated with 0.001% poly-L-lysine before seeding cells for attachment. Cells were treated with native LDL, low-oxLDL, or high-oxLDL (100 μg/mL each) or PMA (10 ng/mL) for 3 hr at 37°C. After fixation, samples were stained with SYTOX Green and analyzed by confocal microscopy (FV1000, Olympus, Tokyo,

Japan).

3.11. Isolation of NETs

Isolated human neutrophils were seeded at 5×10^6 cells per well in six-well plates, and treated with native LDL, low-oxLDL, or high-oxLDL (100 μg/mL each) or PMA (10 ng/mL) for 4 hr at 37°C to induce NET formation. After stimulation, culture supernatants were removed very carefully and washed with 1 mL of PBS. Then, 1 mL of fresh RPMI 1640 medium was added and vigorously agitated by pipetting. Supernatants were harvested and centrifuged at 20 × *g* for 5 min. Supernatants were collected and used as stimulus.

3.12. Quantification of NETs

Isolated human neutrophils were cultured at 1×10^5 cells per well in 96-well plates and treated with native LDL, low-oxLDL, or high-oxLDL (100 μg/mL each) or PMA (10 ng/mL) for 4 hr at 37°C. Stimulated neutrophils were incubated with DNase I (5 U/mL) for 20 min at 37°C, and the reaction was stopped with 5 mM EDTA. Plates were centrifuged at 200 × *g* for 8 min. Supernatants were transferred to separate 96-well plates, and NET DNA in cell-free supernatants was measured using a Quanti-iT™ PicoGreen dsDNA kit according to the manufacturer's instructions. Extracellular DNA in supernatants was measured in a Victor X4 multilabel plate reader (Perkin-Elmer, CT, USA).

3.13. Immunodepletion of MMP-9 from NETs

Mouse anti-human MMP-9 antibodies were crosslinked with 50 μL of Dynabeads Protein G (Invitrogen Dynal, Oslo, Norway) for 20 min at room temperature. Crosslinked Dynabeads-MMP-9 antibody complex was incubated with isolated NET

supernatants for overnight at 4°C. MMP-9-depleted NET supernatants were isolated using magnetic field.

3.14. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and cultured in EGM™-2 BulletKit™ medium in 0.2% gelatin-coated plates at 37°C in a humidified 5% CO_2 incubator.

3.15. Cell viability

Cell viability was measured using a colorimetric Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kyoto, Japan). HUVECs were cultured at 5×10^4 cells per well in 96-well plates treated with conditioned medium containing NETs isolated from neutrophils. After 24 hr, CCK-8 reagent was added to each well and cells were incubated for 30 min at 37°C. The supernatant was harvested and transferred to 96-well plates. Optimal density (OD) values at 450 nm were determined using a spectrophotometer.

3.16. Enzyme-linked immunosorbent assay (ELISA)

Isolated human neutrophils were cultured at 2×10^5 cells per well in 48-well plates and treated with native LDL, low-oxLDL, or high-oxLDL (100 μg/mL each) or PMA (10 ng/mL) for 4 hr at 37°C. NETs were digested with DNase I (5 U/mL) for 20 min at 37°C, and the digestion was stopped with 5 mM EDTA. Plates were centrifuged at 1000 × *g* for 20 min, and supernatants were harvested and stored at −80°C. ELISA was performed with a human MMP-9 assay kit (R&D systems, Minneapolis, MN, USA). OD values at 450 nm were determined.

C57BL/6J control and *ApoE*$^{-/-}$ mice were fed an AD for 2–4 wk. At each time point, blood samples are collected in heparinized tubes and centrifuged at 1000 × *g* for 15 min. Supernatants were harvested and stored at -80°C until analysis. The levels of oxLDL in plasma were measured using a mouse oxidized LDL ELISA kit (Cloud-Clone Corp., Houston, TX, USA).

3.17. Oxidative modification of LDL and characterization of oxLDL

Human LDL was purchased from Kalen Biomedical, LLC (Montgomery Village, MD, USA). LDL (1 mg/ml) was then dialyzed against 1X PBS at 4°C for 24 hr, twice. After dialysis, LDL was incubated with 5 μM $CuSO_4$ for 2–24 hr at 37°C. The oxidation was stopped by the addition of 1 mM EDTA. After oxidation, all lipoproteins were sterilized using 0.22 μm syringe filter. Protein concentration was determined using the BCA protein assay reagent (Thermo Scientific, Rockford, IL, USA). The degree of oxidation was estimated as thiobarbituric acid reactive substances (TBARS) value using OXItek TBARS Assay Kit (Enzo, Farmingdale, NY, USA) and electrophoretic mobility of native LDL and oxidized LDL was observed by agarose gel electrophoresis. Relative electrophoretic mobility (REM) was defined as ratio of the migration distances from the origin by oxLDL compared to native LDL.

3.18. Nitric oxide measurement

HUVECs were cultured at 5 × 10^4 cells per well in 96-well plates and treated with conditioned medium containing NETs isolated from neutrophils for 24 hr at 37°C. Supernatants were transferred to separate 96-well plates and stored at -80°C. The levels of NO in culture supernatants were measured using a Nitric Oxide Colorimetric Assay Kit (BioVision, Mountain View, CA, USA).

3.19. Statistical analysis

Data represent means ±S.D. One-way ANOVA and Student's independent t-tests were conducted. $P<0.05$ was considered significant.

4. Results

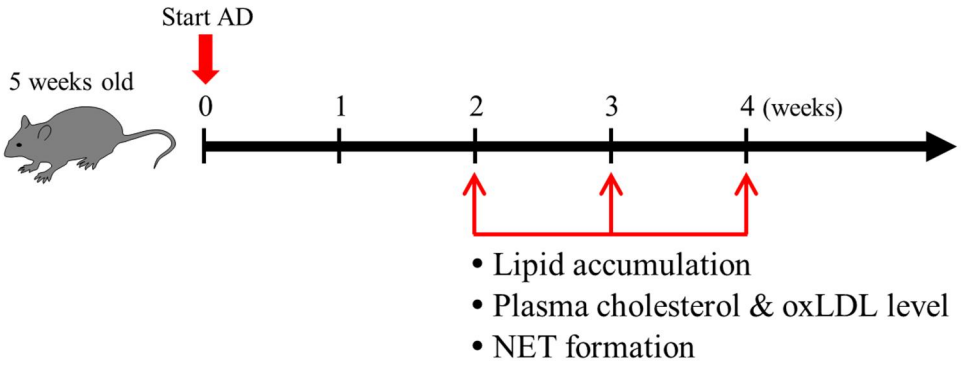


Figure III-1. Schematic representation of the animal model. C57BL/6 and *ApoE*$^{-/-}$ mice were fed an atherogenic diet (cholesterol 1.25%) for 2–4 wk. NET formation, lipid accumulation, neutrophil activity and plasma circulating oxLDL levels were detected using MR imaging, *In-vivo* imaging system (IVIS), trans-well system and oxLDL specific ELISA kit.

4.1. Effects of AD on plasma cholesterol levels, weight gain, and food consumption

AD has been shown to induce nutritional dyslipidemia in *ApoE*$^{-/-}$ mice. Therefore, we monitored plasma cholesterol profiles, including T-CHO, TGs, and HDL, as well as weight gain and food consumption. The levels of T-CHO and TGs were dramatically increased in AD-fed *ApoE*$^{-/-}$ mice compared to those in WT mice (Figure III-2A & III-2B). In contrast, HDL cholesterol was significantly decreased in AD-fed *ApoE*$^{-/-}$ mice after third week (Figure III-2C). These results correlated with the typical features of dyslipidemia and hyperlipidemia. Body weight and food intake showed no significant difference between the two groups (Figure III-2D & III-2E).

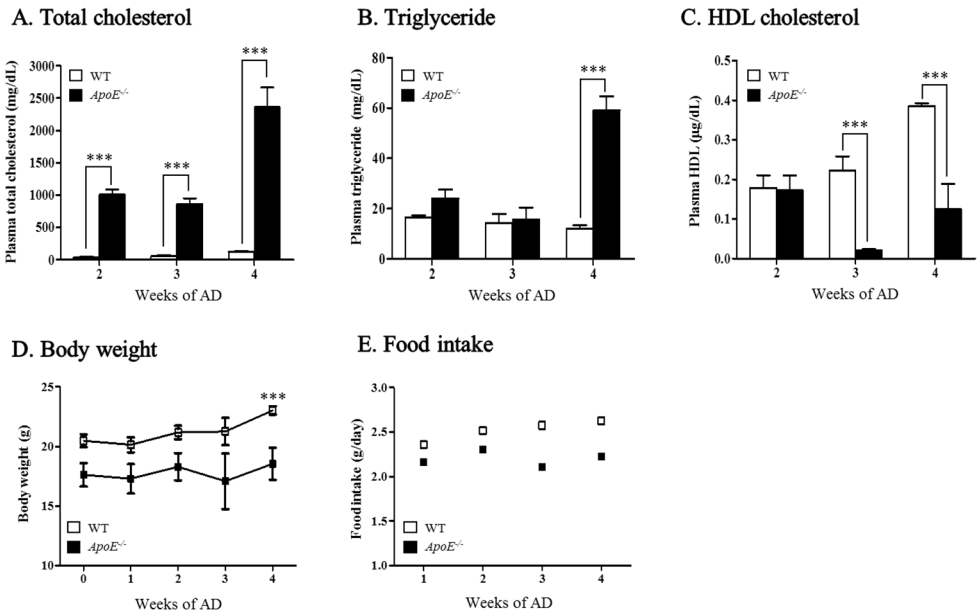


Figure III-2. Plasma cholesterol profiles, body weight and food intake in WT and *ApoE*$^{-/-}$ mice. WT and *ApoE*$^{-/-}$ mice were fed an atherogenic diet (AD) for 2 – 4 wk. (A-C) The levels of T-CHO, TGs, and HDL were measured using HDL, LDL/VLDL and Triglyceride Quantification Colorimetric Kit. n=3 mice per group. Student's independent t-tests were used to determine significance (D, E) During an AD, the body weight and food intake were measured. One-way ANOVA analysis was used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

4.2. Effects of AD on lipid accumulation in the aorta

The key initiating step of atherosclerosis is the accumulation of LDL cholesterol in the intima. Therefore, we assessed lipid accumulation in the aorta. First, we used Dil-tagged native LDL as an exogenous lipid. After 4 wk of AD, Dil-tagged LDL was injected into mice intravenously. The accumulation of Dil-LDL was increased in aortas isolated from AD-fed *ApoE*$^{-/-}$ mice compared with that from WT mice (Figure III-3A). Next, endogenous lipid accumulation was observed by Oil Red O staining. Correlating with the results presented above, lipid accumulation in the aorta was significantly increased in AD-fed *ApoE*$^{-/-}$ mice compared with that in WT mice (Figure III-3B).

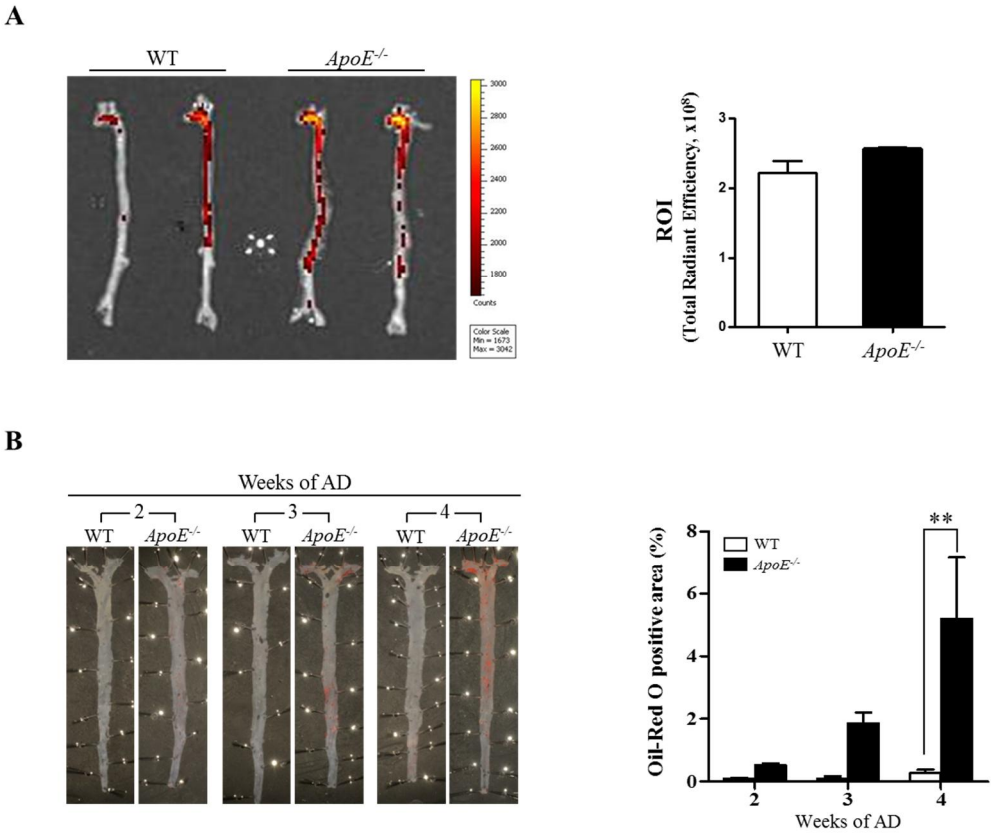


Figure III-3. Accumulation of lipid in the aorta. (A) WT and *ApoE*$^{-/-}$ mice were fed an AD for 4 wk and Dil-tagged native LDL (20 μg) was injected intravenously. After 18 hr, aortas were dissected from mice and accumulation of Dil-LDL in the aorta was measured using IVIS system. (B) WT and *ApoE*$^{-/-}$ mice were fed an AD for 2 – 4 wk. At each time point, aortas were dissected from mice and adventitial lipids were removed. Isolated aortas were stained with Oil Red O solution and observed using bright-field microscope. n=3 mice per group. Student's independent t-tests were used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

4.3. Effects of AD on the formation of luminal NETs in carotid arteries

To investigate whether luminal NETs were present in the early stages of atherosclerosis, control and *ApoE*$^{-/-}$ mice were fed an AD for 2–4 wk. We introduced histone H2A.x antibody-conjugated T2*-contrast agents to detect NET structures and observe NET formation in the carotid arteries, using magnetic resonance imaging (MRI). The formation of luminal NETs was detected in AD-fed *ApoE*$^{-/-}$ mice after 2 wk, and these luminal NET structures were more clearly detected after 4 wk. However, luminal NET were not observed in AD-fed WT mice (Figure III-4).

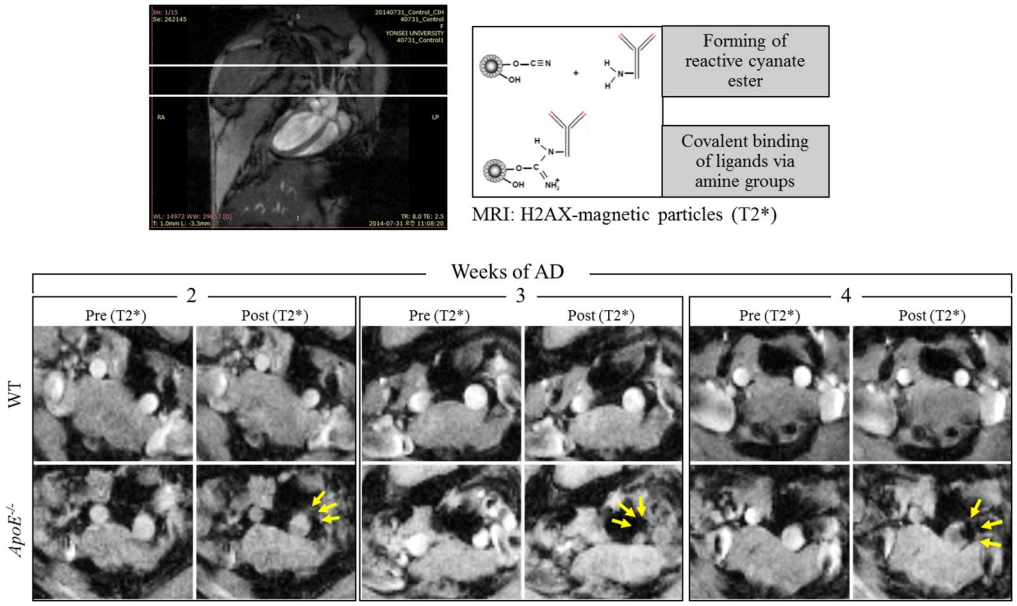


Figure III-4. The formation of luminal NET in mouse carotid arteries. WT and *ApoE*$^{-/-}$ mice were fed an AD for 2–4 wk. At each time point, histone H2A.x antibody-conjugated T2*-contrast agents were injected intravenously. After 30 min post injection, attached histone H2A.x antibodies in the lumen of carotid arteries were observed using MR imaging system.

4.4. Effects of AD on plasma levels of oxLDL and neutrophil activity

OxLDL is known as a useful marker for atherosclerosis, and the presence of oxLDL in plasma has been reported. Therefore, we assessed the oxLDL levels in plasma. The levels of plasma oxLDL were dramatically increased in AD-fed *ApoE*$^{-/-}$ mice after 2 wk, compared to that in WT mice, and these levels more increased up to 4 wk (Figure III-5A). Next, we tested the ability to induce NET formation in an *ex vivo* experiment. Neutrophils isolated from AD-fed *ApoE*$^{-/-}$ mice induced NET formation *via* IL-8 stimulation. In contrast, neutrophils isolated from WT mice did not induce NET formation after IL-8 stimulation (Figure III-5B).

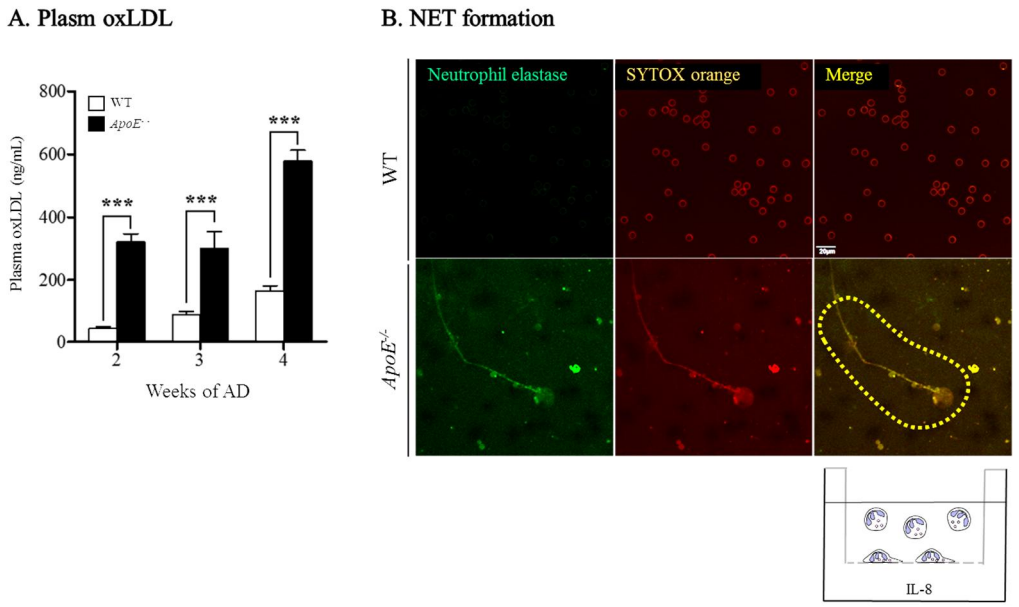


Figure III-5. The levels of plasma oxLDL and NET formation *ex vivo* experiment.

(A) WT and *ApoE*$^{-/-}$ mice were fed an AD for 2–4 wk. At each time point, plasma samples were harvested and stored at -80°C until analysis. The levels of oxLDL in plasma were measured using mouse oxidized low-density lipoprotein ELISA kit. n=3 mice per group. Student's independent t-tests were used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001). (B) Neutrophils were isolated from AD-fed WT and *ApoE*$^{-/-}$ mice. Purified neutrophils were seeded into transwell inert and stimulated by IL-8. Formation of NET was observed using confocal microscope.

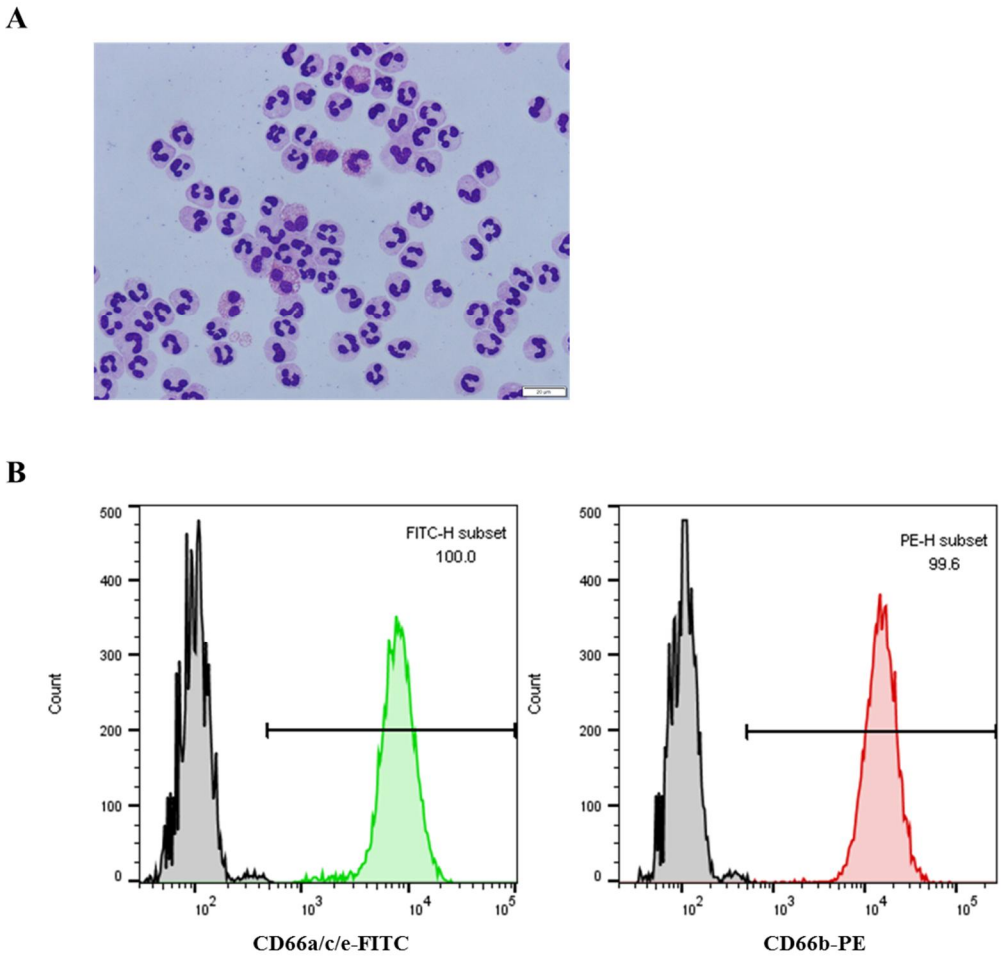


Figure III-6. Purity of isolated polymorphonuclear cells. PMNs were separated by PolymorphPrep^{TM} from human blood. (A) Cytospin and Giemsa staining and (B) staining for CD66a/c/e and CD66b was performed to confirm the neutrophil population, and in general, >95% of cells were positive.

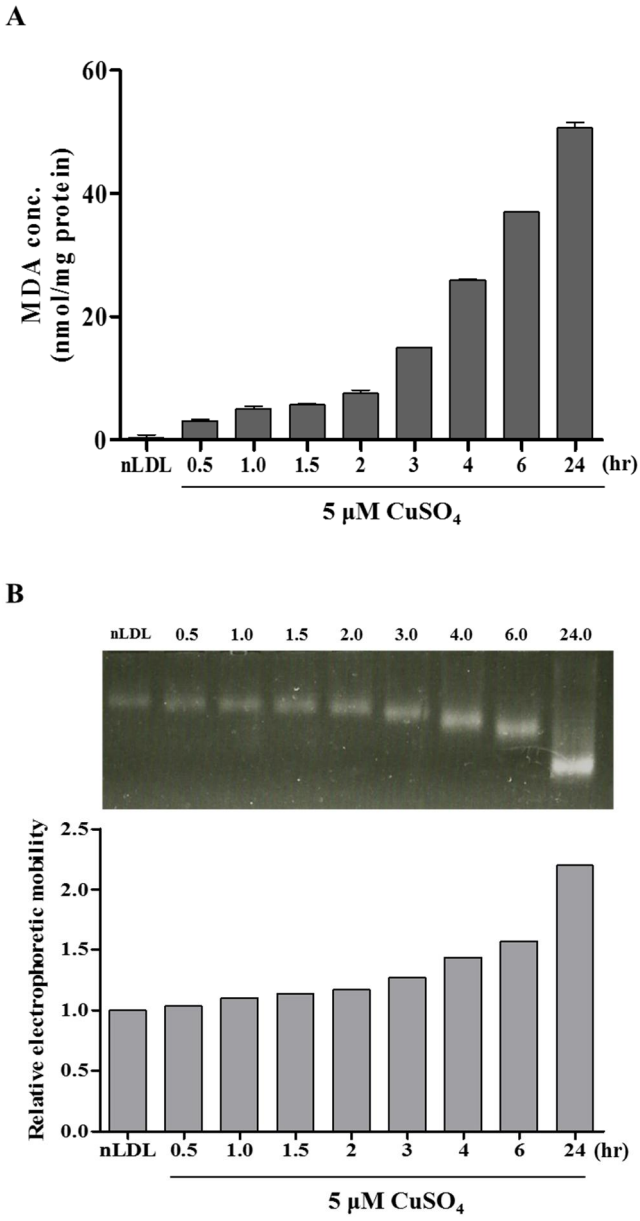
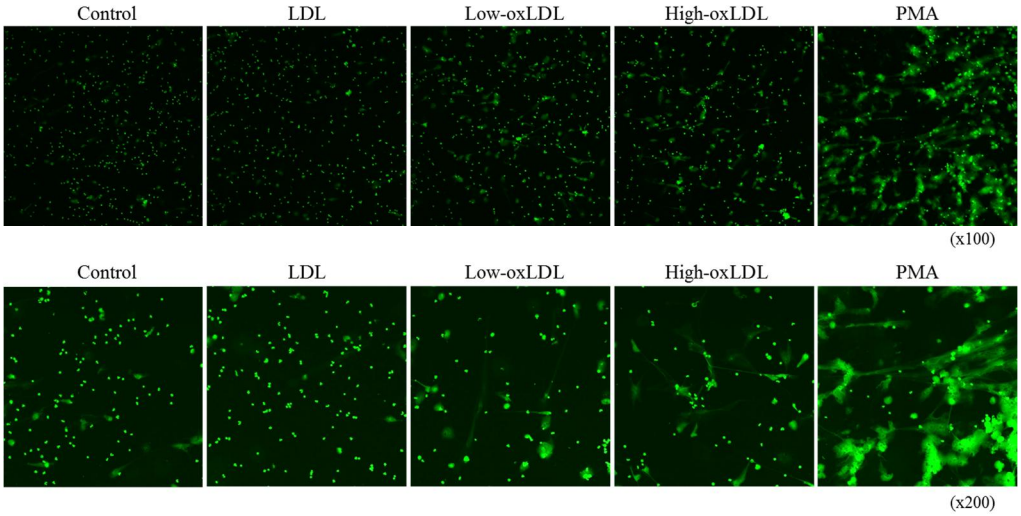


Figure III-7. Characterization of oxLDL. Native LDL (1 mg/ml) was incubated with 5 μM $CuSO_4$ for 0.5–24 hr at 37°C. The degree of oxidation was estimated as (A) thiobarbituric acid reactive substances (TBARS) value and (B) electrophoretic mobility of native LDL and oxidized LDL was observed by agarose gel electorphoresis.

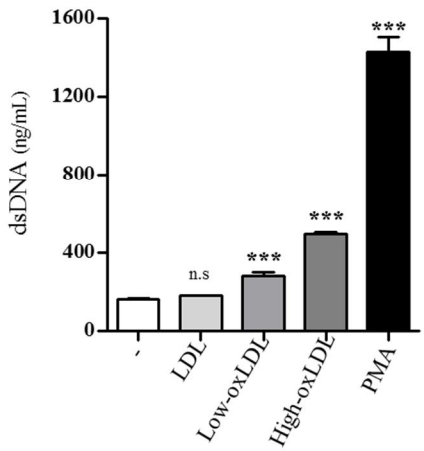
4.5. Effects of oxLDL in human neutrophils

We then tested whether oxLDL promotes NET formation in vitro. Neutrophils were isolated from human blood and stimulated with native LDL, low-oxLDL, or high-oxLDL. NET structures were stained with SYTOX green, and the released ds-DNA was measured using a PicoGreen kit. NET formation was observed in cells treated with either low- or high-oxLDL, but not with native LDL. Interestingly, high-oxLDL induced NET formation more effectively (Figure III-8A). Similarly, NET formation increased with increase in the degree of LDL oxidation (Figure III-8B).

A. Confocal imaging by SYTOX green



B. dsDNA in supernatants



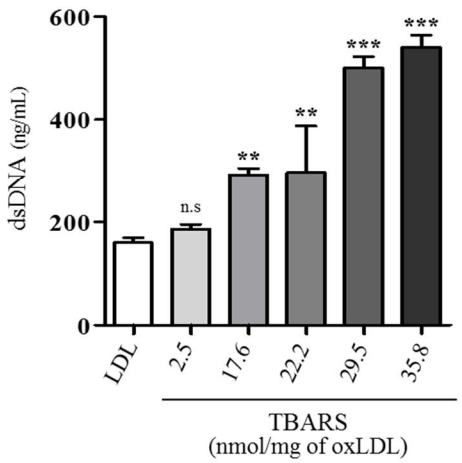
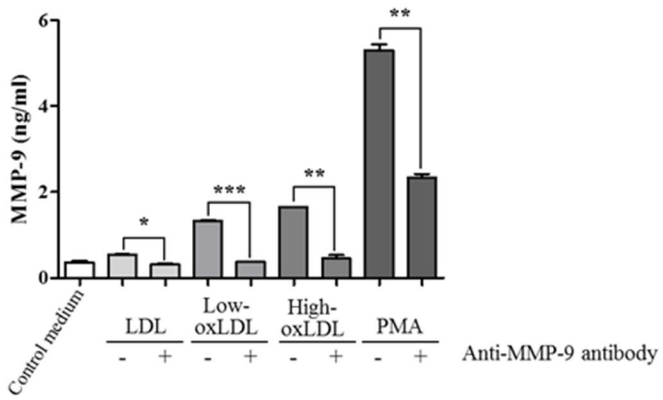


Figure III-8. NET formation in human neutrophils. (A) Isolated human neutrophils were stimulated with native LDL, low-oxLDL, or high-oxLDL (100 μg/ml each) or PMA (10 ng/ml) for 3 hr at 37°C. NET formation was stained with SYTOX Green and analyzed using confocal microscope. (B) Isolated human neutrophils were treated with native LDL or various degrees of oxLDL (100 μg/ml each) or PMA (10 ng/ml) for 4 hr at 37°C. NET-DNA in cell-free supernatants was measured with Quanti-iT^{TM} PicoGreen dsDNA Kit. Data represent means ±S.D. of three independent experiments. One-way ANOVA analysis was used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

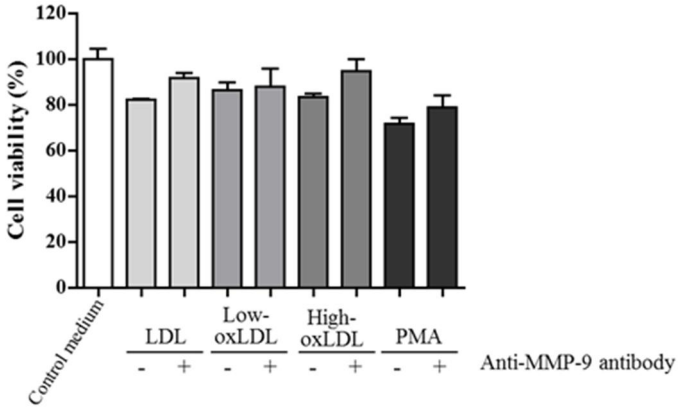
4.6. Effects of NETs on endothelial cells

Finally, we investigated the effects of NETs on endothelial cell dysfunction. First, we determined MMP-9 secretion levels induced by oxLDL. Based on its capacity to degrade the extracellular matrix (ECM), regulate tissue architecture, and induce proteolysis, MMP-9 has been implicated in atherogenesis and vascular damage. The levels of secreted MMP-9 increased in cells treated with either low- or high-oxLDL (Figure III-9A). Next, HUVECs were treated with conditioned medium containing NET-associated MMP-9. Cell viability decreased in cells treated with conditioned medium with either low- or high-oxLDL, but showed a slight recovery upon pretreatment with MMP-9 antibody (Figure III-9B). In addition, conditioned medium-treated HUVECs produced massive amounts of NO; however, the cells treated with MMP-9-depleted conditioned medium showed slightly reduced production of NO (Figure III-9C).

A. MMP-9



B. Endothelial cell death



C. Nitric Oxide production

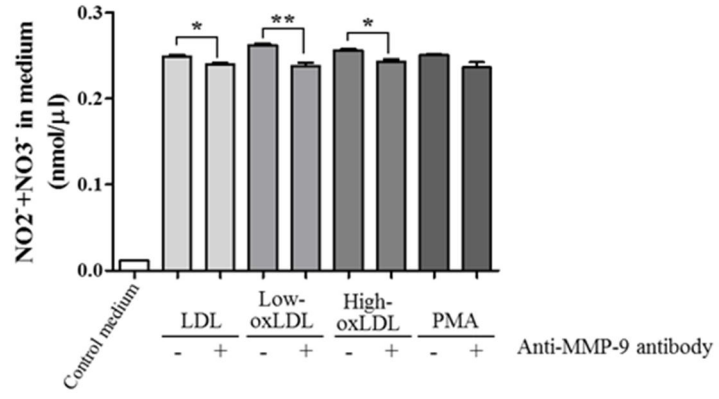


Figure III-9. Endothelial cell viability and NO production in response to NET. (A) Isolated human neutrophils were treated with native LDL, low-oxLDL, or high-oxLDL (100 μg/ml each) or PMA (10 ng/ml) for 4 hr at 37°C. NETs were digested with DNase I (5 U/ml) for 20 min at 37°C. Cell-free supernatants were harvested and stored at -80°C. ELISA was performed with a human MMP-9 assay kit. HUVECs were cultured with conditioned medium containing NETs isolated from neutrophils. After 24 hr, (B) cell viability and (C) NO production were measured by the CCK-8 and Nitric Oxide Colorimetric Assay Kit, respectively. Data represent means ±S.D. of three independent experiments. Student's independent t-tests were used to determine significance (*P<0.05, **P< 0.01, ***P< 0.001).

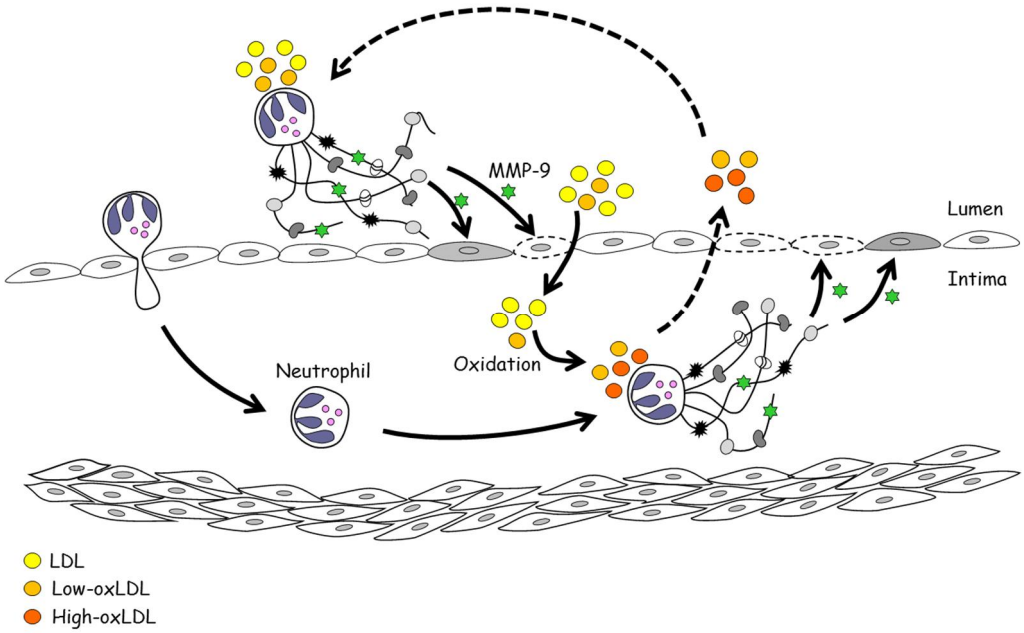


Figure III-10. Graphical summary of role of luminal NET on endothelial dysfunction. In early stage of atherosclerosis, circulating oxLDL promotes neutrophil extracellular trap formation in lumen of arterial walls. Luminal NET incites endothelial dysfunction and cell death. As a result, circulating lipoproteins are more easily penetrate into the intima through endothelial leaky junctions.

5. Discussion

The key initiating step of atherosclerosis is endothelial cell dysfunction that leads to the expression of adhesion molecules, secretion of chemokines,[25] and eventually the recruitment of circulating monocytes into the inflamed sites.[26-28] Accumulation of oxLDL in the subendothelial space is a process that prominently promotes endothelial dysfunction in early atherosclerosis.[29] Other cardiovascular risk factors such as dyslipidemia and shear stress are also related to endothelial cell dysfunction.[30-32]

Recently, NETs have emerged as an important contributor to atherosclerosis.[33] NET components such as dsDNA, nucleosomes, MPO-DNA complexes, and MMPs have been detected in the intima as well as in plasma.[20] Interestingly, luminal NETs were also detected in atherosclerotic lesions in a mouse model and in human samples.[24] However, the relationship between luminal NETs and endothelial dysfunction in early atherosclerosis is not well understood. Therefore, our study focused on detecting luminal NETs at an early stage of atherosclerosis and investigated the effects of luminal NETs on endothelial dysfunction.

ApoE$^{-/-}$ mice are a genetically well-established animal model of atherosclerosis. AD-fed *ApoE*$^{-/-}$ mice exhibit nutritional dyslipidemia as well as accelerated plaque formation.[34] Notably, AD-fed mice showed elevated plasma T-CHO, TGs, and LDL/VLDL cholesterol; however, levels of HDL cholesterol were reduced when compared with that in standard chow-fed animals.[35,36] Similarly, plasma levels of T-CHO and TGs were significantly increased in *ApoE*$^{-/-}$ mice compared with WT mice; however, HDL cholesterol levels were dramatically decreased in *ApoE*$^{-/-}$ mice after 3 wk of AD (Figure III-2). Since dyslipidemic conditions are associated with an increased risk of atherosclerosis, we assessed the accumulation of lipids in the aorta.

Subendothelial retention of apolipoprotein B-containing lipoproteins is a prerequisite for the initiation of atherosclerosis.[37] Entrapment of LDL in the intimal ECM by proteoglycans through ionic interactions amplifies the accumulation of LDL particles in this space.[38,39] This process is the basis of the "response-to-retention" hypothesis for atherosclerosis.[40] As shown in Figures III-3A & III-3B, lipid accumulation in the aorta increased in AD-fed *ApoE*$^{-/-}$ mice compared with WT mice. Further, lipid accumulation dramatically increased at 4 wk.

NET formation is a crucial component in host defense; however, it has also been implicated in atherosclerosis. A recent study showed that luminal NETs could be detected in early atherosclerotic lesions.[24] Therefore, we investigated the formation of NETs in vivo at a very early stage of atherosclerosis. As shown in Figure III-4, NET formation was detected in *ApoE*$^{-/-}$ mice after 2 wk of AD and was more clearly detected after 4 wk. Showing results similar to those for MRI, neutrophils isolated from *ApoE*$^{-/-}$ mice induced NET formation via IL-8 stimulation (Figure III-5B). OxLDL is a potential inducer of NETs under physiologic conditions, and elevated levels of circulating oxLDL are significantly correlated with the severity of CVD.[12] Indeed, oxLDL plasma levels dramatically increased in *ApoE*$^{-/-}$ mice compared with those in WT mice (Figure III-5A). Further, we tested the effects of oxLDL on NET formation in primary human neutrophils. As shown in Figures III-8A & III-8B, oxLDL induced NET formation, but high-oxLDL induced NET formation more effectively. Similarly, NET formation increased with increase in the degree of LDL oxidation (Figure III-8B). These variable effects of oxLDL on NET formation probably occurred because each oxLDL uses a distinct intracellular signaling pathway.[16,17]

LDL is a spherical particle with a diameter of approximately 22–27.5 nm.[41] In a

quiescent state, on a small percentage of LDL particles penetrates the endothelium by transcytosis.[32] The endothelium is the endothelial cell monolayer that lines the interior surface of blood vessels. It mechanically and metabolically separates the vascular wall from circulating blood. In pathologic conditions such as dyslipidemia and hypertension, the endothelium undergoes a constant process of injury and repair in response to mechanical and chemical insults.[42] As a result, the endothelium not only becomes dysfunctional, but endothelial cells can lose their integrity, progress to senescence, detach, and enter circulation. Eventually, leaky junctions are formed around apoptotic cells,[43] which are the main pathways for LDL transport. Therefore, our results suggest that oxLDL-induced NET formation affects lipid accumulation via endothelial dysfunction.

Finally, we investigated the molecular mechanism by which NET formation induces endothelial dysfunction. Matrix metalloproteinases (MMPs) are a family of enzymes that can cleave all components of the ECM. MMPs are linked to endothelial dysfunction because they are associated with the loss of endothelial cell adhesion to the ECM, and thus the loss of survival signals.[44,45] In addition, MMPs are involved in the disruption of endothelial cell-to-cell adhesive junctions that control the transendothelial flux of fluid and macromolecules.[46] MMPs are highly expressed in atherosclerotic lesions. Notably, MMP-9 is abundant in advanced atherosclerotic plaques, and elevated levels of circulating MMP-9 are associated with plaque instability.[47] As shown in Figure III-9A, the levels of NET-associated MMP-9 were increased upon oxLDL treatment. In addition, oxLDL-induced NET formation affected cell viability as well as NO production (Figures III-9B & III-9C). NO plays a key role in maintaining vascular functions, such as vascular tone, blood pressure, immune response, and oxidation-

sensitive mechanisms.[48] The effect of NO production on cellular processes is concentration dependent. As shown in Figure III-9C, NET treatment induced significant NO production accompanied by cell death. This probably occurred because higher (micromolar) concentrations of NO favor pathways that lead to cell cycle arrest, apoptosis, and senescence.[49]

Taken together, our results suggest that, in early atherosclerosis, neutrophils stimulated by circulating oxLDL induce the formation of luminal NETs in the aorta. Eventually, luminal NETs induce endothelial dysfunction, at least partially through NET-associated MMP-9 (Figure III-10).

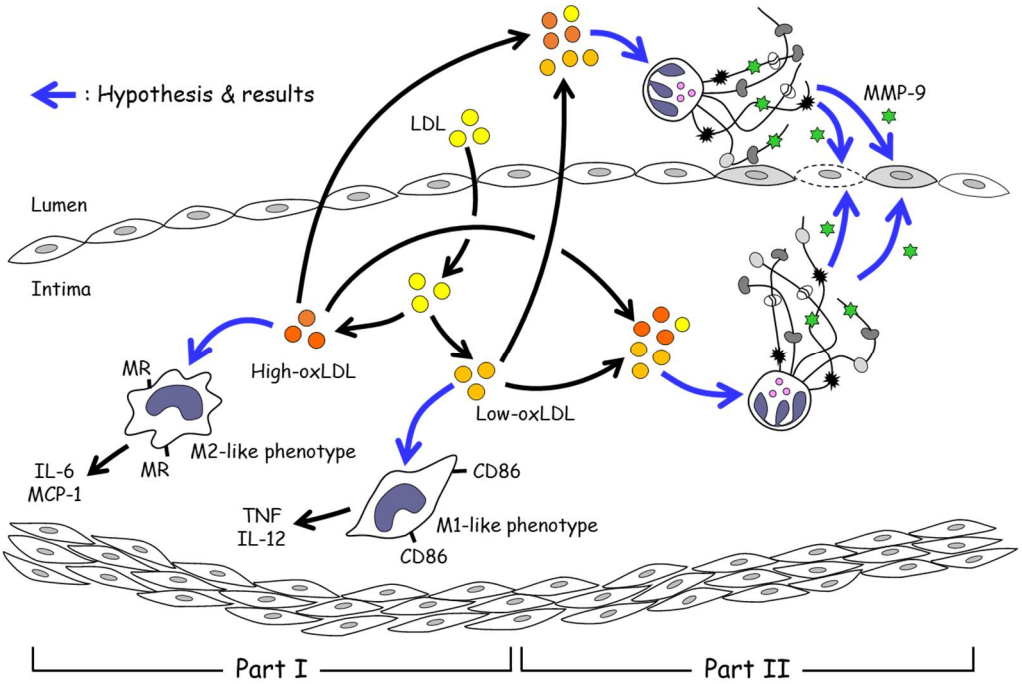


Figure III-11. Graphical summary of Part I and Part II. OxLDL plays a role in early atherogenesis by affecting macrophage differentiation as well as enhancing NET formation, which may be influenced by the oxidation degree of LDL.

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Abstract (in Korean)

동맥경화 초기 단계 염증에서

산화된 LDL 콜레스테롤의 역할

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서진원

동맥경화는 대표적인 만성염증질환으로 혈관 내 지질의 침착과 염증이라는 두 가지 큰 특징을 나타낸다. 동맥경화반은 동맥 혈관벽에 LDL 형태의 콜레스테롤이 축적되면서 형성되기 시작한다. 내막 지역 내 축적된 LDL 입자는 산화기전 및 산화반응 시간에 의해 각기 다른 정도로 산화되는데, 이러한 이유로 내막 내에 존재하는 산화된 LDL 입자는 한 가지의 분자 형태가 아닌, 다양한 형태의 산화된 LDL 입자의 집합을 의미한다.

본 논문의 첫 번째 연구에서는 서로 다른 정도의 산화된 LDL 입자가 대식세포의 분화 및 극성에 어떠한 영향이 미치는지에 대해 연구하였다. 지질의 산화 정도에 따라 산화 정도가 낮은 LDL과 산화 정도가 높은 LDL을 단핵구 세포주(THP-1 세포)에 처리하고, 표면 수용체 발현 및 싸이토카인

분비를 측정하여 대식세포의 분화 양상에 미치는 영향을 관찰하였다. 그 결과 산화 정도가 낮은 LDL을 처리했을 때, CD86의 발현과 TNF-α 및 IL-12p40의 분비가 유도되었으며, 반면 산화 정도가 높은 LDL을 처리한 경우 만노오스 수용체의 발현과 IL-6 및 MCP-1의 분비가 증가된 것을 관찰하였다. 더불어 산화 정도가 높은 LDL은 LOX-1의 발현을 유도하였다. 이러한 결과를 통해 산화 정도가 낮은 LDL은 전염증성(M1) 대식세포로의 분화를 유도하며, 반면 산화 정도가 높은 LDL은 항염증성 (M2) 대식세포로의 분화를 유도함을 확인하였다. 사람 말초혈액 단핵구를 사용한 실험에서도 유사한 결과를 확인하였다. 이러한 결과는 동맥경화 초기 단계에서 LDL 입자의 산화 정도가 병변 내 염증의 방향을 결정하는 대식세포의 분화에 영향을 미칠 수 있음을 제시한다.

두 번째 연구에서는 동맥경화 초기 단계에서 호중구의 역할을 관찰하였다. 호중구는 선천면역반응의 가장 최전선에 있는 면역세포로 박테리아, 곰팡이와 기생충 등 외부로부터 들어온 미생물을 제거한다. 호중구가 미생물을 제거하는 기전으로 식균작용, 탈과립과 더불어 최근 호중구 세포외 덫(neutrophil extracellular trap, NET)이 밝혀졌다. NET 형성은 숙주방어뿐 아니라 동맥경화 발달의 중요한 요인으로도 밝혀졌으며, 마우스와 사람의 동맥경화 병변의 내막과 혈관 내강에서 NET 구조가 확인되었다. 그러나 동맥경화에서 NET의 역할에 대해서는 아직 알려지지 않았다. 그러므로 두 번째 연구에서는 혈관 내강 내 형성된 NET이 내피세포의 기능에 어떠한 영향을 미치는지 관찰하였다.

야생형 및 *ApoE* 결핍 마우스에 4주간 동맥경화 유발 식이를 한 후 혈관 내강 내 NET의 형성 및 호중구의 활성도를 측정하였다. 그 결과 *ApoE* 결핍 마우스에서 2주 후부터 혈관 내강 내 NET 형성이 관찰되는 반면, 야생형 마우스에서는 4주 후에도 NET 형성이 관찰되지 않았다. 더불어 야생형 마우스와 비교하여 *ApoE* 결핍 마우스로부터 분리한 동맥에서 지질의 침착이 현저히 증가함을 관찰하였다. 그리고 시험관내 실험에서 4주간 동맥경화 유발 식이 후 *ApoE* 결핍 마우스로부터 분리된 호중구는 야생형 마우스로부터 분리된 호중구와 비교하여 IL-8 에 의한 NET 형성이 증가되었다. 다음으로 NET을 유도할 가능성이 있는 인자로 추정되는 산화된 LDL 입자의 혈중 내 농도를 관찰한 결과 *ApoE* 결핍 마우스에서 산화된 LDL입자의 농도가 야생형 마우스와 비교하여 의미 있게 증가하였다. 마지막으로 산화된 LDL 입자에 의해 유도된 NET의 구성 성분 중 하나인 기질금속단백질분해효소-9(matrix metalloproteinase, MMP-9)이 내피세포의 기능에 어떠한 영향을 미치는지 관찰하였다. 그 결과 산화된 LDL에 의해 유도된 NET은 내피세포의 세포죽음 및 산화질소의 생성을 증가시켰으나, MMP-9을 고갈시켰을 때 이러한 반응이 감소하였다. 이를 통해 동맥경화 초기 단계에서 혈중 내 산화된 LDL에 의해 유도된 NET에 포함된 MMP-9이 내피세포의 기능장애 및 세포죽음을 일으켜 내막으로 지질의 침착을 더 유도할 것으로 추정된다.

본 연구에서는 이상의 두 실험 결과를 통해 혈중 및 내막에 존재하는 산화된 LDL 입자가 대식세포 관련 염증 반응과 NET을 통한 내피세포 기능장애를 유발하여 동맥경화 초기 단계를 촉진하는데 핵심적인 요소임을 제

시한다.

핵심되는 말: atherosclerosis, LDL, oxidation, macrophage, differentiation, oxLDL, neutrophils, NET, MMP-9, endothelial dysfunction

Publication list

Seo JW, Yang EJ, Yoo KH, Choi IH. Macrophage Differentiation from Monocytes Is Influenced by the Lipid Oxidation Degree of Low Density Lipoprotein. Mediators Inflamm 2015;2015:235797.

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