**PROTHROMBOTIC PLATELET SIGNALING
BY THE SCAVENGER RECEPTOR CD36**

by

KAN CHEN

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Thesis Advisor: Prof. Roy L. Silverstein

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CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

Kan Chen

candidate for the **Doctor of Philosophy** degree *.

(signed) **Thomas M. McIntyre**

(chair of the committee)

Roy L. Silverstein

Edward F. Plow

Gary E. Landreth

(date) **Sep 19th 2008**

*We also certify that written approval has been obtained for any proprietary material contained therein.

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Prothrombotic Platelet Signaling By the Scavenger Receptor CD36

Abstract

By

KAN CHEN

Platelet hyperactivity associated with hyperlipidemia may contribute to development of a pro-thrombotic state. We previously showed that oxidized LDL (oxLDL) formed in the setting of hyperlipidemia and atherosclerosis activated platelets in a CD36 dependent manner. We now show that MAP kinase JNK2 and its upstream activator MKK4 were phosphorylated in platelets exposed to oxLDL. Using *apoe*^{-/-} mice as a model of hyperlipidemia we showed that JNK was constitutively phosphorylated in platelets in a CD36-dependent manner. Inhibition of src kinase activity reduced JNK phosphorylation by oxLDL. Immunoprecipitations revealed that active phosphorylated forms of src kinases Fyn and Lyn were recruited to CD36 in platelets exposed to oxLDL. We also showed that Vav, a guanine nucleotide exchange factor for Rho/Rac small GTPases was phosphorylated upon oxLDL treatment. Co-immunoprecipitation revealed Vav-associated Fyn was phosphorylated upon oxLDL treatment. Pharmacological inhibition of the MAP kinase JNK or src family kinases abolished platelet activation by oxLDL *in vitro*. Using a murine carotid artery thrombosis model we demonstrated

CD36-dependent phosphorylation of platelet JNK within thrombi. Pharmacological inhibition of JNK prolonged thrombosis times in *wild type* but not *cd36* null mice *in vivo*. In addition, *in vitro* functional studies demonstrated that western diet feeding increased platelet aggregation from WT mice and this phenotype was absent in *vav*^{-/-} mice. These findings suggest that a specific CD36-dependent signaling pathway is required for platelet activation by oxLDL and may provide insights related to development of novel anti-platelet therapies more relevant to atherothrombosis than to normal hemostasis.

Chapter1 Background

1.1 CD36: structure and expression

CD36 is an 88 kd heavily N-glycosylated membrane protein. It is also referred to glycoprotein (GP) IV and is one of the major glycoproteins expressed on the platelet surface. It belongs to the class B scavenger receptor family, which also includes scavenger receptor class B type I (SR-BI), and lysosomal integral membrane protein II (LIMP-II). The cDNA for CD36 was first cloned using an expression cloning strategy based on its reactivity with monoclonal anti-CD36 antibodies¹. The human CD36 gene is located on chromosome 7q and consists of 15 exons. The 5'-untranslated region of CD36 is encoded by exon 1, 2 and part of exon 3. Part of exon 3, exon 4-13 and part of exon 14 encodes CD36 protein. The 3'-untranslated region is encoded by part of exon 14 or part of exon 14 and exon 15 depending on expression patterns^{2, 3}. The gene encodes a protein of 471 amino acids with a predicted molecular weight of 52,922 Da⁴. The actual molecular weight varies from ~80–90 kDa due to the presence of 10 N-linked glycosylation sites¹. According to the predicted structure, CD36 is a single-chain membrane protein with 2 short cytoplasmic domains and a large extracellular domain (Figure 1-1). Both tails are characterized by a pair of cysteine residues that are palmitoylated, which is important in localizing CD36 in lipid rafts^{5, 6}. The primary structure of CD36 is highly conserved among mammalian species cloned to date.

Figure 1-1 Schematic Representation of CD36 protein and its ligand binding sites. CD36 is characterized by having an intracellular N-terminus, two transmembrane domains linked by an extracellular loop and an intracellular C-terminus. The region between amino acids 93-120 is a binding site for Thrombospondin (TSP) -1 and -2. The region between amino acids 155-183 is a binding site for oxidized low density lipoprotein (oxLDL), advanced glycation endproduct (AGE) and apoptotic cells.

CD36 is widely expressed on a wide range of cells and tissues including microvascular endothelium⁷, adipocytes⁸, skeletal muscle⁹, dendritic cells^{10, 11}, epithelia of the retina¹², breast¹³, and intestine¹⁴, smooth muscle cells, erythroid precursors, platelets¹⁵, monocytes/macrophages^{16, 17}, and megakaryocytes. In Asian populations, ~3-8% individuals are CD36 deficient, which was initially referred to as the NaK^a negative immunophenotype. Among Nak^a negative individuals, about 90% have type II deficiency, expressing CD36 in monocytes but not in platelets. The remaining 10% are type I deficiency in which CD36 is absent in platelets, monocytes, endothelium and muscle¹⁸. Five mutations associated with type I deficiency have been identified. Among them, the predominant mutation is C478T, which results in the replacement of proline with serine at position 90 and the production of a misfolded protein¹⁹. The misfolded protein is then targeted for intracellular degradation. The genetic mechanism of type II deficiency is still not clear. Type I deficiency is also prevalent among African population, however, the most common mutation is T1264G in exon 10. The mutation produces a premature stop codon and the protein is not surface expressed²⁰.

1.2 CD36: a multi-functional receptor

Initially identified as a receptor for malaria-parasite-infected erythrocytes²¹, CD36 is now known as a multi-functional receptor. It recognizes a variety of

ligands and affects diverse biological processes including lipid metabolism, atherosclerosis, inflammation and angiogenesis²². On microvascular endothelial cells CD36 is a receptor for TSP-1 and -2 and mediates their anti-angiogenic signal (Figure 1-1). On adipocytes, heart and skeletal muscle, CD36 facilitates fatty acid transport and is thus also termed fatty acid translocase (FAT)²³⁻²⁵. On professional phagocytes CD36 serves as a scavenger receptor. It recognizes oxidatively modified low density lipoprotein (oxLDL), which is generated during athero-inflammatory conditions (Figure 1-1)¹⁶. Macrophage CD36 binds to and takes up oxLDL, resulting in foam cell formation. Foam cell formation is the early step during atherogenesis. CD36 also serves as a receptor for apoptotic cells, which are generated during inflammation, cancer and other circumstances as well (Figure 1-1)²⁶. Similarly, CD36 expressed on retinal pigment epithelium functions in the phagocytosis of rod outer segments²⁷. CD36 also binds to advanced glycation end products (AGE), which are generated during hyperglycemia (Figure 1-1)²⁸. Another ligand for CD36 is growth hormone-releasing peptide (GHRP), hexarelin and EP80317, a synthetic hexapeptide GHRP analog devoid of growth hormone releasing activity^{29, 30}. Most recently, our group showed that microparticles derived from endothelial cells, monocytes and platelets interact with platelet CD36. The interaction led to enhanced platelet activation³¹.

CD36 is evolutionarily conserved with orthologs found in invertebrate

model organisms including *Drosophila melanogaster* and *Caenorhabditis elegans*. Its ortholog *croquemort* in *Drosophila* is required for phagocytosis of apoptotic cells^{32, 33}. Another CD36 family member in *Drosophila* is involved in mycobacterial infection³⁴. A CD36-related receptor sensory neuron membrane protein (SNMP) is essential for pheromone detection in *Drosophila* in concert with odorant receptors³⁵. All these studies suggest that the pattern recognition property of CD36 is highly conserved through evolution, implying its role as a critical participant in the initial response of organisms to environmental stimuli as well as normal homeostasis.

In mice, an *N*-ethyl-*N*-nitrosurea (ENU) mutagenesis screen by Beutler's group has identified CD36 as a co-receptor for toll-like receptor (TLR) 2³⁶. Interestingly, although TLR2 can heterodimerize with TLRs 1 and 6 and sense triacylated lipopeptide and diacylated lipopeptide respectively, the co-receptor function is discharged by CD36 only during the sensing of diacylated lipopeptide by TLR2/6 heterodimer. Homozygous nonsense mutation of CD36 renders mice hypersusceptible to *Staphylococcus aureus* infection³⁶. Consistent with these studies, Stuart *et al* demonstrated that CD36 cooperates with TLR2/6 heterodimers to mediate lipoteichoic acid (LTA) signaling but not TLR9³⁷. It was also shown that the COOH-terminal cytoplasmic portion of CD36, especially Y463 and C464, is required for TLR2/6 signaling in response to LTA. Further, fluorescence resonance energy transfer (FRET) revealed that

TLR2/6 and TLR2/1 heterodimers pre-exist, however, heterotypic association of TLR2/6 and CD36 is ligand-induced³⁸. These studies suggest that CD36 is an important participant in innate immunity. In addition, CD36 is also involved in innate host response to fibrilla beta-amyloid, whose accumulation is associated with pathologic changes in the brain in Alzheimer's disease³⁹. Bamberger *et al* showed that a cell surface complex including CD36, CD47 and integrin $\alpha 6 \beta 1$ mediates the binding of microglial to beta-amyloid and the subsequent pro-inflammatory response⁴⁰. Later, it was shown that apolipoprotein C-II, a component of very low and high density lipoprotein forms amyloid fibrils that initiated a CD36-dependent pro-inflammatory response in macrophages⁴¹. These studies suggest CD36 may be part of a receptor complex that recognizes fibrillar proteins in general.

1.3 CD36: a signaling molecule

Despite having very short intra-cytoplasmic domains, CD36 has a signaling function, which was first suggested by studies showing that platelet CD36 co-precipitated with the src family kinases Fyn, Lyn and Yes⁴². This feature of CD36 was not shared by other major platelet membrane glycoproteins. On microvascular endothelial cells TSP-1 induces its anti-angiogenic, pro-apoptotic signal via CD36 and subsequent activation of Fyn, caspase-3, p38 MAP kinase and JNK1 MAP kinase (Figure 1-2)^{43, 44}. In

microglia (brain macrophage), the interaction between fibrillar beta-amyloid and CD36 assembles a multi-protein signaling complex that includes F-actin, Lyn, Fyn, Pax, Pyk-2 and Cas, leading to the phosphorylation of p44/42 MAP kinases and the Vav guanine nucleotide exchange factor (Figure 1-2)^{45, 46}. On macrophages, fibrillar amyloid apolipoprotein C-II activates a signaling cascade downstream of CD36 that includes Lyn and p44/42 MAPKs (Figure 1-2)⁴¹. Moreover, our group provided compelling evidence indicating that oxLDL activates JNK2 MAP kinase in a CD36-dependent manner in macrophages (Figure 1-2). Inhibition of JNK2 resulted in a significant reduction in uptake of oxLDL and prevention of foam cell formation⁴⁷. In macrophage/dendritic cells, CD36 also associates with TLR-2/6 upon the binding of some TLR-2 ligands and triggers a MyD88-mediated signaling cascade (Figure 1-2)^{36, 37}. The signaling results in the secretion of TNF alpha, IL-6 and IL-12. Altogether these studies suggest that CD36 signal transduction is complex and that even in the same cell different ligands may activate different pathways.

1.4 Platelets

Platelets are disk-shaped anucleate cells, which play an important role in hemostasis and thrombosis. After vascular injury, circulating platelets arrest on exposed collagen and von willerbrand factor (VWF). The engagement of

CD36 Signaling Cascades

Adapted from Moore KJ et al (2006)

Figure 1-2 Binding of ligands to CD36 trigger context-dependent signaling cascades. CD36 signaling initiated by pathological ligands including oxLDL, beta-amyloid and TSP1 has been partially defined. On macrophages, oxLDL triggers a CD36-initiated signaling pathway involving the src family kinases Lyn, and MAP kinases MEKK2 and JNK. On microglial/macrophages, binding of beta-amyloid or fibrillar apoCII to a complex including CD36, α 6 β 1 and CD47 leads to the activation of multiple signaling pathways including src family kinases Fyn, and Lyn and MAP kinase p44/42. On endothelial cells, TSP-1 induces activation of Fyn and MAP kinases p38 and JNK. CD36 also serves as a co-receptor for toll like receptor.

collagen with its surface receptors (GPVI and $\alpha_2\beta_1$) triggers intracellular signaling events which lead to platelet activation marked by activation of platelet integrin $\alpha_{IIb}\beta_3$ and secretion of platelet granular contents. The activation and adhesion of platelets allow the formation of platelet monolayer on extracellular matrix. Activated integrin $\alpha_{IIb}\beta_3$ can bind to fibrinogen. Because of its dimeric nature, fibrinogen can then cross link adjacent activated platelets leading to stable platelet aggregates. The further recruitment of circulating platelets to form aggregates depends on molecules released from platelets, most of which activate platelets through G-protein coupled receptors (GPCR), including adenosine diphosphate (ADP) and thromboxane A_2 (TxA₂). The aggregates formed by platelets will be further stabilized by signaling pathways including outside-in signals triggered by activation and clustering of integrin $\alpha_{IIb}\beta_3$ by its ligands including fibrinogen and VWF (Figure 1-3). It is estimated that the width of the gap between adjacent platelets range from 0 to 50 nm⁴⁸. The distance is short enough to allow the direct or indirect interaction between molecules expressed on one platelet and other molecules expressed on adjacent platelets. Indeed, additional interactions besides those mediated by $\alpha_{IIb}\beta_3$ have been identified, including Gas6 and its receptors, Eph kinases-ephrins, semaphorins, and CD40L⁴⁹⁻⁵³. These interactions serve to prevent the destabilization of the thrombus.

Figure 1-3 A schematic representation of platelet activation. In resting platelets, P-selectin is in α granules in the intracellular space. The Integrin $\alpha_{IIb}\beta_3$ is in its inhibited state. Upon vascular injury, circulating platelets are arrested by von willebrand factor (VWF) and collagen. The binding of VWF and collagen to their receptors initiates several signaling pathways activating platelets. Upon platelet activation, α granules are released and P-selectin is expressed on platelet surface. The Integrin $\alpha_{IIb}\beta_3$ also change its conformation to its activated state and allow fibrinogen binding. Activated platelets release adenosine diphosphate (ADP) and Thromboxane A₂ (TxA₂), further contributing to platelet activation.

1.5 Src family kinases

Src family kinases are a family of prototypical modular signaling molecules. The conserved structure organization consists of 3 domains, an N-terminal src homology 3 (SH3) domain, a central src homology 2 (SH2) domain and a tyrosine kinase domain. SH2 domains are conserved protein sequences of about 100 amino acids. They bind to a phosphorylated tyrosine in the context of a peptide motif and represent the largest class of phosphotyrosine-recognition domains⁵⁴. SH3 domains are shorter protein sequences consisting of about 60 amino acids. They interact with proline-rich peptides in the context of a target protein and mediate the assembly of specific protein complexes. Src family kinases are normally kept in the autoinhibited state by an interaction between the SH2 domain and a C-terminal phosphotyrosine residue (Tyr 527). The interaction facilitates the binding of SH3 to a proline-rich motif within the linker between SH2 domain and tyrosine kinase domain, resulting in the inactivation of the kinase. Multiple mechanisms of activating src kinases are available including dephosphorylation of the C-terminal phosphotyrosine residue, binding of the SH2 domain to a competitive phosphotyrosine peptide and binding of the SH3 domain to a proline-rich motif peptide⁵⁵. These events convert src into active state and allow the auto-phosphorylation of Tyrosine 416 (Tyr 416) within the tyrosine kinase domain. There are 9 Src kinases in the human genome: Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and Yrk, of which at least four (Src, Fyn, Lyn and Yes)

are expressed in platelets.

1.6 Mitogen activated protein (MAP) kinases

Mitogen Activated Protein (MAP) Kinases are a family of protein kinases that phosphorylate specific serine and threonine residues of target substrates and regulate a wide variety of cellular processes including gene expression, mitosis, differentiation, and cell survival/apoptosis. They are part of a phospho-relay system composed of three sequentially activated kinases: MAP kinases (MAPKs), MAP kinase kinases (MKKs) and MAP kinase kinase kinase (MKKKs) (Figure 1-4)⁵⁶. A MKKK that is activated by extracellular stimuli phosphorylates a MKK on its serine and threonine residues, and then this MKK activates a MAP kinase through phosphorylation on its serine and tyrosine residues. This MAP kinase signaling cascade has been evolutionarily well-conserved from yeast to mammals. To date, six distinct groups of MAPKs have been identified in mammals including extracellular signal regulated kinases (ERK-1 and -2), c-Jun N-terminal kinases (JNK-1, -2 and -3), p38, and ERK5 (Figure 1-4).

1.7 C-Jun N-terminal kinase (JNK)

C-Jun N-terminal kinases (JNK) are also termed Stress-activated protein

Mitogen-Activated Protein Kinase Signaling Cascades

Figure 1-4 The signaling of Mitogen activated protein kinase (MAPK). MAP kinases signaling is a phosphorelay system composed of three sequentially activated protein kinase including MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). MAPK includes Erk1/2 (p44/42), p38 MAPK, JNK and Erk5/BMK1.

kinases (SAPK)⁵⁷. They were initially identified as kinases that bind and phosphophorylate c-Jun on Ser63 and Ser73 within its transcriptional activation domain. They are a family of MAP kinases that are responsive to environmental stress. There are ten isoforms of JNK derived from the three genes *jnk1*, *jnk2* and *jnk3*. *Jnk1* and *jnk2* are ubiquitously expressed while *jnk3* is mainly expressed in brain and testes.

JNKs are part of three sequentially activated kinases. The TXY motif in the activation loop of each JNK is dually phosphorylated by MKKs. MKK4 and MKK7 phosphorylate the threonine and tyrosine within the activation loop TXY motif (T¹⁸³ and Y¹⁸⁵ in JNK1) resulting in JNK activation. MKKs are in turn phosphorylated at specific serine or threonine residues within their activation loop.

JNKs are activated by a variety of stress stimulus including UV and gamma radiation, ceramide, inflammatory cytokines, growth factors and GPCR agonists. They are involved in various cellular processes including proliferation, apoptosis, motility, metabolism and DNA repair. Importantly, knowledge from biochemical studies and mouse models suggest an important role of JNK in the pathogenesis of several diseases including liver ischemia/reperfusion, neurodegenerative diseases, cancer, chronic inflammatory diseases (rheumatoid arthritis and atherosclerosis) and type 2 diabetes.

JNK signaling plays an important role in atherosclerosis⁵⁸. Using the *apoe*^{-/-} mouse model for atherosclerosis, it was shown that animals with genetic deletion of JNK2 (*apoe*^{-/-}/*Jnk2*^{-/-}) developed less atherosclerosis than control *apoe*^{-/-} animals. *Jnk1* deficiency did not reduce the level of atherosclerosis. Pharmacological JNK inhibitors were also able to reduce the level of atherosclerosis. In terms of the mechanism by which JNK is involved in atherosclerosis, our group demonstrated that in macrophages, which are key participants in the progression of atherosclerotic lesions, oxLDL initiates a CD36-dependent signaling pathway involving MEKK2 and JNK2. This results in the internalization of pro-atherogenic oxLDL⁴⁷. JNK2 is also responsible for the phosphorylation of scavenger receptors A (SR-A) and promoting additional lipid uptake through this receptor⁵⁸. These data suggest that JNK may be a potential therapeutic target in atherosclerosis.

1.8 Vav

The vav family is a group of signaling molecules known to play an important role in development and cell signaling. In mammalian cells the vav family has three identified members including Vav, Vav2 and Vav3. Vav proteins are composed of a calponin-homology (CH) domain, an acidic (Ac) region, the Dbl- (DH) and plekstrin-homology (PH) domains, a zinc finger (ZF)

domain, and two SH3 domains flanking a single SH2 region (Figure 1-5)⁵⁹. The Vav family has been studied extensively for their enzyme activity as guanosine nucleotide exchange factor (GEF) for Rho/Rac proteins. However, some recently identified roles of the vav family can not be attributed to this activity⁶⁰. For example, in T lymphocytes, vav expression activates the transcription factor nuclear factor of activated T cells (NF-AT) and subsequently induces transcription of the interleukin 2 (IL2) gene. Interestingly, catalytically inactive Vav was still able to induce the activation of NF-AT. Also, constitutively active Rac1, RhoA and Cdc42 are not capable of activating NF-AT. These data suggest that a GEF-independent property of Vav may play a role in this process. Indeed, before the discovery of Vav GEF activity, vav had been speculated to be an adaptor protein because of the presence of SH3-SH2-SH3 domains. Interestingly, a Vav protein lacking SH3-SH2-SH3 domain failed to activate NF-AT. These data imply that Vav protein may serve as an adaptor protein independent of its GEF activity.

The GEF activity of Vav proteins is tightly regulated by tyrosine phosphorylation. It has been demonstrated that the functional role of the Vav family co-evolved with tyrosine kinase pathways. In the non-phosphorylated state, Vav proteins are in auto-inhibited state and can not interact with their small G protein substrates. In contrast, upon phosphorylation, Vav proteins adapt an “open” configuration capable of interacting with their substrates (Figure 1-5).

Pawson T et al (2007)

Figure 1-5 The domain structure and the intramolecular interactions that regulates the Guanine nucleotide exchange factor (GEF) activity of Vav protein. Vav proteins is characterized having a calponin-homology (CH) domain, an acidic (Ac) region, the Dbl- (DH) and plekstrin-homology (PH) domains, a zinc finger (ZnF) domain, and two SH3 domains flanking a single SH2 region. The tyrosine phosphorylation in Ac region releases Vav from its autoinhibited state, becoming active Ganosine.

1.8 Platelet signaling

As described in chapter 1.4, although additional interactions between adjacent activated platelets are present, Activated $\alpha_{IIb}\beta_3$ allows the cross linking of platelets by fibrinogen and VWF, leading to platelet aggregate formation. The activation of $\alpha_{IIb}\beta_3$ is initially triggered by adhesion signaling triggered by collagen and VWF. There are at least two molecules responsible for collagen-induced platelet signaling: $\alpha_2\beta_1$ and GPVI. Among them GPVI is required for platelet responses to collagen. In current model, collagen binding results in clustering of GPVI and its associated γ -chain. The γ -chain phosphorylated by non receptor tyrosine kinases creates a docking site for Syk. Association of Syk activates Syk and leads to activation of the $\gamma 2$ isoform of phospholipase C (PLC $\gamma 2$). PLC $\gamma 2$ hydrolyzes PI-4,5-P₂ to form 1,4,5-IP₃ and diacylglycerol (DAG). IP₃ opens Ca⁺⁺ channel, raising the cytosolic Ca⁺⁺ concentration and triggering Ca⁺⁺ influx. DAG activates protein kinase C (PKC), allowing further signaling events to occur. In contrast, GPIb-IX-V serves as a major binding molecule for VWF. The engagement of GPIb-IX-V with VWF leads to cytoskeletal rearrangement and activation of $\alpha_{IIb}\beta_3$. Although the signaling pathway has not been completely understood, the current knowledge suggests that PLC $\gamma 2$ and subsequent Ca⁺⁺ release are essential and src family kinases are required for PLC $\gamma 2$ activation.

Activation of $\alpha_{IIb}\beta_3$ can be triggered by adhesion signaling as well as soluble agonists including ADP, thrombin, epinephrine, and thromboxane A₂. These soluble agonists are secreted upon activation of platelets by adhesion signaling. They are required for thrombus growth. Most of these agonists activate platelets via GPCR. In general, GPCR signaling triggered by secreted agonists result in three major events: Ca^{2+} mobilization and PKC activation, cytoskeleton reorganization and suppression of cyclic adenosine monophosphate (cAMP) synthesis. In particular, there are two ADP receptors in platelets: P2Y₁ and P2Y₁₂. P2Y₁ receptor is coupled to G_q and responsible for the activation of phospholipase C β (PLC β). P2Y₁₂ receptor is coupled to G_i, whose function is to suppress the synthesis of cAMP.

The role of MAP kinases and Vav in platelet biology has not been fully understood. Multiple lines of evidence suggest that p38 and/or ERK2 are activated in platelets in response to a diverse array of agonists including oxLDL⁶¹⁻⁷². Importantly, *p38* heterozygous deficient mice have prolonged thrombotic occlusion time in a FeCl₃-induced thrombosis model⁷³. The involvement of JNK in platelet biology has been studied to a limited extent with pharmacological inhibitors suggesting that JNK is involved in arterial thrombosis in mice. The role of Vav in platelet activation by collagen has been studied. Evidence suggests that Vav family proteins are required for platelet aggregation induced by collagen. However, the role of Vav in other aspects of

platelet activation has not been fully explored, although it was previously showed that thrombin induced the tyrosine phosphorylation of Vav in platelets⁷⁴.

1.9 Hyperlipidemia and atherosclerosis

Lipids are an essential part of our diet and have various biological functions. The majority of lipids in biological system act either as components of cell membrane or as energy storage resources. Some other lipids are active participants in signaling pathways by functioning as either intracellular messengers or extracellular messengers. Because of the water-insoluble nature of lipids, circulatory lipid transport is mediated by lipoproteins. A lipoprotein is a biochemical complex that contains both proteins and lipids. Low density lipoprotein (LDL) is a class of lipoprotein that transports triglycerides, phospholipids and cholesterol to target tissues and cells. It is formed from very low density lipoprotein (VLDL) by the action of lipoprotein lipase (LPL). High levels of LDL (hyperlipidemia) are very common in western populations and are associated with increasing risk for atherosclerosis. In the process of atherogenesis, LDL particles penetrate the wall of an artery and become oxidatively modified. OxLDL can then be internalized by monocyte-derived macrophages and trigger an array of pro-atherogenic events (Figure 1-6). Lowering LDL cholesterol levels can significantly reduce the risk of

cardiovascular disease.

Atherosclerosis is characterized by the formation of plaque within arteries. Upon plaque rupture, pro-thrombotic material exposed and released can activate platelets. Activated platelets can form occlusive thrombi, obstructing blood flow in the arteries. With the obstruction of blood flow, downstream tissues will be deprived of oxygen and nutrients. The event causes myocardial infarction and stroke, which are major complications of atherosclerosis.

1.10 Oxidized LDL and atherosclerosis

As described in chapter 1.9, the oxidation of LDL and the uptake of oxLDL by macrophages are early steps during atherogenesis. Abundant evidence supports the role of LDL oxidation including studies showing antibodies against oxidized but not native LDL recognize epitopes in atherosclerotic lesions from human subjects and animals⁷⁵⁻⁷⁹. In addition, LDL oxidized in cell-free systems affects normal functions of vascular cells, causing changes in cell motility, cell adhesion, cell viability, growth factor and cytokine secretion. Many of these changes are pro-atherogenic.

Endogenous LDL oxidation pathways are not completely understood. Initial studies often used LDL oxidized with chemicals in a cell-free system, including acetylated LDL, malondialdehyde (MDA)-modified LDL or methylated

Lindstrom A et al (1997)

Figure 1-6 A schematic representation of atherogenesis. During the initiation of atherosclerosis, circulating low density lipoprotein (LDL) particles and monocytes transmigrate into subendothelial space. LDL particles undergo oxidation becoming oxidized LDL and Monocytes derive macrophages. The scavenger receptors expressed on macrophage surface bind to and uptake oxidized LDL, resulting in foam cell formation.

LDL. However, studies have suggested that these chemically modified LDL do not share many biological properties with oxidized LDL. Later, it was demonstrated that vascular cells including endothelial cells, smooth muscle cells, activated monocytes, neutrophils and macrophages were able to oxidize LDL, although the mechanism may vary depending on cell type⁸⁰⁻⁸⁴. Mechanisms involving metal ions, superoxide anion, hydrogen peroxide and peroxidases have been suggested and studied extensively^{85, 86}. Interestingly, myeloperoxidase (MPO), a secreted heme protein product of activated monocytes is present in human atherosclerotic lesions⁸⁷. With collaborators, our group has identified a novel, physiologically relevant mechanism of LDL oxidation by reactive nitrogen species (RNS) generated by MPO. LDL oxidized in this system, unlike LDL oxidized in most cell-free systems, is a specific ligand for CD36 and does not bind scavenger receptor A (SRA) I/II⁸⁸⁻⁹¹. Studies have shown the presence of MPO-dependent nitration and chlorination of proteins and lipids *in vivo*, suggesting that this pathway is pathophysiologically relevant. Also, recent clinical studies have linked the level of MPO to coronary disease risk in human subjects⁹².

1.11 OxPC_{CD36}

Studies with LDL oxidized by MPO-generated RNS termed NO₂LDL have shown that the CD36-binding property resides in the lipid portion of the particle

and that the interaction of oxidized phosphatidylcholine (oxPC) with CD36 accounts for more than 90% of the binding. In a systematic attempt to understand the exact nature of oxPC that serves as ligands for CD36, Podrez *et al* identified eight phospholipid species (Figure 1-7)^{93, 94}. Four are derived from oxidation of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (PAPC) and four from 1-palmitoyl-2-lineoyl-*sn*-glycero-3-PC (PLPC). They all share the unmodified 1-palmitoyl, 3-glycero phosphocholine backbone. The *sn*-2 acyl groups are truncated, containing terminal γ -hydroxy (or oxo) – α , β -unsaturated carbonyl. These phospholipid species are now referred to as oxPC_{CD36}. Synthetic oxPC_{CD36} incorporated into phospholipid liposomes are able to inhibit NO₂LDL binding to CD36 expressed on transfected cells. Of physiological relevance, high performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) studies further showed the presence of oxPC_{CD36} *in vivo* with enrichment in atherosclerotic lesions⁹³. In sum, these data suggest these phospholipid species are indeed endogenous phospholipid ligands responsible for CD36 binding *in vivo*.

1.12 CD36 and platelet function

As described in chapter 1.1, CD36 is also referred to as GPIV and is one of the major glycoproteins expressed on the platelet surface. Although CD36 was also first isolated and structurally characterized in platelets⁹⁵, a

A

B

PAPC series n=1	X	Y	PLPC series n=5	X	Y
HOdiA-PC	OH	OH	HDdiA-PC	OH	OH
KOdiA-PC	=O	OH	KDdiA-PC	=O	OH
HOOA-PC	OH	H	HODA-PC	OH	H
KOOA-PC	=O	H	KODA-PC	=O	H

Podrez, E. A. et al. J. Biol. Chem. 2002;277:38517-38523

Figure 1-7 A schematic overview of oxidized phosphatidylcholines that serve as CD36 ligands. A. the shared structural motifs conserved among oxidized PC species that support CD36 binding activity. Products generated from oxidized PAPC and PLPC are similar except for the chain length of the truncated oxidized fatty acid esterified to the *sn*-2 position of lysophosphatidylcholine. R_1 , fatty acid such as palmitate or oleate. n , chain length (number of methylene groups). B. the structures, chemical names, and abbreviations of identified phospholipid species with CD36 binding activity derived from oxidized PAPC and PLPC

biological role for platelet CD36 has only recently been detected. Initial studies suggested platelet CD36 was one of the receptors for collagen. However, platelets from CD36 null human subjects or CD36 null mice did not have any defect in binding or responding to collagen *in vitro*⁹⁶. Recently Podrez *et al* in collaboration with our group showed that platelets bind oxLDL via CD36⁹⁷, and that CD36-oxLDL interaction led to platelet activation. Similar to macrophage CD36, platelet CD36 bind oxLDL via the recognition of oxPC_{CD36}. Synthetic oxPC_{CD36} incorporated into liposomes activated platelets *in vitro*. With *in vivo* thrombosis models (discussed later in chapter 1.13), our group showed that *apoe*^{-/-} mice on high-fat western diet had shorter thrombosis time in comparison to wild type mice, however, this phenotype was rescued by genetic depletion of CD36. With LC/ESI/MS/MS, it was showed that the level of oxPC_{CD36} was increased in the plasma of hyperlipidemic mice. These data convincingly showed that platelet CD36 links hyperlipidemia and oxidative stress with a prothrombotic phenotype. Related to this finding, our group demonstrated that platelet CD36 may play a more general role in normal thrombus formation. In a FeCl₃-induced thrombosis model, our group showed that CD36 deficiency rendered mice less sensitive to vascular injury³¹. Further studies demonstrated that cell-derived microparticles (MPs) were generated during vascular injury and they bind platelet CD36, sensitizing platelets to low dose of thrombotic stimulus. These results suggest platelet CD36 may function as a general sensor of endogenous ligands generated during pathological

states. Thus, defining the mechanism by which CD36 signals in platelets, the subject of this dissertation, is important in helping to understand the nature of prothrombotic states.

1.13 Murine models of vascular thrombosis

Thrombotic complications associated with various pathological conditions are the leading cause of morbidity and mortality in western populations. However, current antiplatelet therapies have major drawbacks regarding efficiency and safety issues. *In vivo* murine models of thrombosis are thus necessary to dissect the complexity of thrombosis and test the effect of potential antiplatelet therapies. Often, fluorescently-loaded platelets are used to visualize thrombus formation in arterioles and venules. Falati et al have developed a confocal intravital microscopy system with fluorescent labeling⁹⁸. The system provided brightfield images and recorded the accumulation of platelets at the injury site. Thus, the dynamics of clot formation can be precisely assessed. Multiple methods have been developed to produce thrombosis by vascular injury. One common method is to use ferric chloride as a thrombogenic agent. It was first applied in the rabbit inferior vena cava. The method was then adapted to rat carotid arteries⁹⁹. The ferric chloride model was first adapted to mice in 1998¹⁰⁰. In that study, a single 1X2 millimeter strip of whatman filter paper was soaked in a solution of 10% ferric chloride. The

strip was then topically applied to the surface of carotid artery. The strip was removed after 3 minutes and the residue ferric chloride was washed away by saline. The thrombus formation was then monitored until complete cessation of blood flow. In general, the dose of ferric chloride and the time of application may vary depending on mouse strains and protocols. This method has also been adapted to study the inferior vena cava and the microvasculature.

Additional methods to induce thrombosis include intravenous injection of collagen and epinephrine, mechanical injury as well as photochemical injury. In a photochemical injury model, Rose Bengal is injected. The vessel is then exposed to 543 nm wavelength light. The light induces reactive oxygen species formation at the target site¹⁰¹. Reactive oxygen species are potent participants to endothelial injury resulting in thrombosis.

It should be noted that each model has its advantages and disadvantages. For example, it has been suggested that ferric chloride-induced thrombosis is dependent on collagen/GPVI signaling, while photochemical injury model is more close to thrombosis induced by thrombin. It is still controversial which model may be more physiological-relevant. Also, thrombosis after vascular injury is dependent on injury site. The mesenteric thrombosis model allows the examination of thrombosis in a moderate shear, small artery system. In contrast, carotid injury model may represent a high shear, atheroma-prone

large artery environment.

Chapter2 Materials and Methods

2.1 Materials

Chemicals

The DAB visualization system for immunohistochemistry (EnVision™ Doublestain Kit) was from DAKO (Carpinteria, CA). Thrombin receptor activated peptide (SFLLRN) was from Bachem (Torrance, CA). SP600125, LY294002, Go6983 and AG1879 were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Antibodies

Anti-CD36 antibody FA6-152 was obtained from Abcam (Cambridge, MA). The blotting antibody for Lyn, PE-conjugated anti-CD62P, FITC-conjugated PAC-1 and mouse anti-CD41 antibody were from BD Biosciences (San Jose, CA). Antibodies to Fyn, Lyn and Vav were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-phospho-tyrosine antibody (4G10) was from Upstate Biotechnology, Inc (Norwalk, CT). Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-rat IgG antibody were from Invitrogen (Carlsbad, CA). All other antibodies were obtained from Cell Signaling Technology, Inc (Danvers, MA).

Lipoprotein

LDL was isolated from fresh plasma by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/mL fraction. Final preparations were placed in 50 mM sodium phosphate (pH 7.0) and stored under N₂ until use⁴⁷. LDL protein concentration was determined by the Lowry assay. All LDL concentrations were given in terms of their protein content. Native LDL (nLDL) was oxidized with CuSO₄ for 24 hours at 37 °C and extent of oxidation was determined by measuring malondialdehyde (MDA) formation as thiobarbituric acid reacting substances (TBARS). MDA values were given as nmol/mg LDL protein. The amount of MDA in the starting nLDL material was between 0.6-0.8 nmol/mg LDL protein. OxLDL with final MDA values between 2-10 nmol/mg were used in these studies to avoid any potential disruption of the LDL particles.

2.2 Methods

Human Platelets and flow Cytometry

Whole blood was collected from healthy human volunteers in 0.109 M sodium citrate (1:9 dilution). All studies involving human blood are in accordance with the Cleveland Clinic Institutional Review Board (IRB).

These donors were not taking aspirin, non-steroidal anti-inflammatory drugs or any other medication. Platelet rich plasma (PRP) was obtained by centrifugation at 100 g for 12 minutes at room temperature. Washed platelets were prepared by centrifuging PRP at 600 g for 10 minutes in the presence of 100 nM prostaglandin E₁ (PGE₁). The platelet pellet was washed twice with Pipes/saline/glucose (5 mM Pipes, 145 mM NaCl, 4 mM KCl, 50 μ M Na₂HPO₄, 1 mM MgCl₂-6H₂O, and 5.5 mM glucose) in the presence of 100 nM PGE₁ and then resuspended in Modified Tyrode's Buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 5 mM HEPES, 0.1% glucose and 0.35% bovine serum albumin, pH 7.2). Platelet concentration was adjusted to 2×10^8 platelets/ml, unless otherwise indicated. CaCl₂ (final concentration of 2 mM) and MgCl₂ (final concentration of 1 mM) were added immediately before platelet stimulation.

As described in chapter 1.4, platelet activation is marked by activation of platelet integrin $\alpha_{IIb}\beta_3$ and secretion of platelet granular contents. P-selectin is within α granules. It is only expressed on the platelet surface when platelets are activated. Thus P-selectin surface expression and integrin $\alpha_{IIb}\beta_3$ conformation change are important events showing platelet activation status. PE-conjugated anti-CD62P antibody and FITC-conjugated PAC-1 antibody were used to detect these events. PAC-1 antibody only recognizes activated form of integrin $\alpha_{IIb}\beta_3$. For flow cytometry analysis, resting washed platelets or

washed platelets activated by various agonists under non-stirring conditions were incubated with antibodies or isotype controls for 30 minutes and analyzed using a Guava PCA-96 instrument (Guava Technologies, Inc) and FlowJo software (Treestar, Inc). Platelets from 3-4 different donors were used for all studies.

Immunoprecipitation and immunoblotting

For immunoblotting, platelets were lysed in 2 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , and 1 $\mu\text{g}/\text{ml}$ leupeptin, and protein concentrations were measured with a Bio-Rad Protein Assay Kit based on the method of Bradford. Immunoblotting was performed following standard protocols. After chemiluminescence detection of phosphorylated proteins, blots were stripped and re-probed with antibodies to total relevant proteins for normalization. For immunoprecipitation, pre-cleared lysates containing 500 μg proteins were incubated with 3 μg of antibodies to Lyn immobilized on protein-A agarose beads overnight at 4°C. Beads were washed, boiled in 2 x SDS-PAGE loading buffer, and the bound material was analyzed by immunoblotting. For co-immunoprecipitation studies, cells were treated with Dithiobis-succinimidyl-propionate (DSP) before lysis in buffer

containing 1% CHAPS. Band intensities were quantified with ImageJ software (Bethesda, MD).

Murine platelet studies

Vav null were obtained from Tybulewicz's group¹⁰². *Cd36* null mice were originally created from 129svj ES cells, and backcrossed 6x to C57Bl/6¹⁰³. After the last backcross, heterozygotes were mated to create background matched wild type and null mice for experiments in the present studies. The background-matched *cd36* null and wild type lines were maintained as homozygotes by sibling mating. *apoe;cd36* double null were created from a cross between 4x backcrossed *cd36* null and *apoe* null (>20x backcrossed to C57Bl/6; backcross #5) mice to yield double heterozygotes. These were mated back to the *apoe* null (backcross #6) and screened for *apoe* null- *cd36* heterozygotes of each gender. These were then mated to yield background matched strains of *apoe* null and *apoe;cd36* double null mice⁴⁷. These lines are maintained as homozygotes by sibling mating. To isolate platelets, mice were anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg) and 600 μ l blood obtained by cardiac puncture into syringes containing 100 μ l acid citrate dextrose (ACD; 75 mM trisodium citrate dehydrate, 38 mM citric acid monohydrate and 100 mM glucose) and 500 μ l Ca^{2+} - and Mg^{2+} -free PBS. Blood was centrifuged at 100 g for 10 minutes to collect PRP. PRP was then

subjected to centrifugation at 325 g for 5 minutes in the presence of PGE₁ to pellet platelets.

Platelet aggregation assay

Platelet aggregation in vitro was assessed at 37 °C in an aggregometer type 500 VS (Chrono-log, Haventown, PA) with stirring at 1200 rpm. Agonists were ADP (2 to 5 μ M).

Immunofluorescence

PRP from mice blood was spread on silanized slides (DAKO, Carpinteria, CA) and platelets were allowed to adhere for 30 minutes at room temperature. Cells were then fixed in acetone at -20°C for 5 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking non-specific binding with 5% bovine serum albumin, slides were incubated with a rabbit antibody to phosphorylated JNK (final 1:25 dilution) and a rat antibody to CD41 (final 1:100 dilution) in PBS overnight at 4°C. CD41, also known as integrin IIa, is a surface marker specific for platelets. Cells were then incubated with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-rat IgG antibodies for 1 hour at room temperature. Coverslips were mounted with VectaShield (Vector Laboratories) and fluorescence images were taken with a Leica TCS-SP2 AOBS upright confocal microscope. Fluorescence

intensities of 30 randomly selected platelets from 3 different mice were analyzed by Image J software (Bethesda, MD). The platelet area was identified by CD41 staining, and the mean fluorescence intensity per platelet was calculated.

Murine carotid artery thrombosis

Twelve week old wild type (WT) mice and *cd36* null (*cd36*^{-/-}) mice matched for body weight were anesthetized with ketamine (90 mg/kg)/xylazine (15 mg/kg) and the right jugular veins and the left carotid artery exposed via a middle cervical incision. For some studies that do not need platelet transfusion, 100 µl of rhodamine 6G (0.5 mg/ml) was injected directly into the right jugular vein to label platelets. The left carotid artery was stripped of adventitia and a piece of black plastic was placed under the vessel to reduce the background fluorescence. A 1x2 mm piece of filter paper saturated with 12.5% FeCl₃ solution was applied to the carotid artery for 1 minute. The filter paper was removed, and the vessel was rinsed with saline. Thrombus formation was observed in real-time under a Leica DMLFS fluorescent microscope with an attached Gibraltar Platform (EXFO, Quebec, Canada) and a water immersion objective at 10x magnification. Time to thrombosis was determined through visual inspection using real time video image capture with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2

software (Norpix, Montreal, Canada). The end points were set as either cessation of blood flow for >30 seconds or no occlusion after 30 minutes, in which case the time was recorded as 30 minutes.

At the end of the experiment the carotid arteries were harvested, fixed in 4% formaldehyde and embedded in paraffin. Cross sections (4-6 μ m) were cut through the thrombi and analyzed by immunohistochemical staining with an antibody to phospho-JNK. Sections were counter stained with hematoxylin and images taken with a Leica DMR upright microscope equipped with a Retiga Exi Cooled CCD camera with a Liquid Crystal Tunable RGB filter (Qimaging, Surry, BC Canada). Experimental groups consisted of three WT mice and three *cd36*^{-/-} mice. For quantification, images were scored on the basis of staining intensity by a blind observer. Scores 0-3 were allocated as follows: 0, negative; 1, weak staining; 2, moderate staining and 3, intense staining.

Platelet transfusion studies

Experimental groups consisted of 10 WT and 10 *cd36*^{-/-} mice. Recipient WT and *cd36*^{-/-} mice were exposed to 11Gy of external beam irradiation from a Cesium 137 source to induce thrombocytopenia with platelet counts <5% of normal after 5 days⁹⁷. Donor platelets were then isolated from wild type and

cd36^{-/-} donor mice as mentioned above, re-suspended in 500 μ l Ca^{2+} and Mg^{2+} free PBS, and incubated with Calcein AM (final concentration of 0.5 mg/ml) in the presence or absence of the JNK inhibitor SP600125 (final concentration of 400 nM) for 30 minutes at room temperature. Calcein AM is a non-fluorescent, cell permeable compound that is hydrolyzed by intracellular esterases into the fluorescence anion Calcein. Two $\times 10^9$ donor Calcein-labeled platelets were then injected through the jugular vein of thrombocytopenic mice 10 minutes prior to carotid injury with 12.5% FeCl_3 to allow the transfused platelets to reach equilibrium in the circulation.

Mass spectrometric analysis of phospholipids

LC-ESI/MS/MS were used to quantify plasma oxPC species⁹⁷. Briefly, 4 ng of internal standard, ditetradecyl phosphatidylcholine (DTPC) was added to 200 μ l plasma and extracted phospholipids three times following the method of Bligh and Dyer⁹⁷. Combined extracts were dried under nitrogen flow, resuspended in 120 μ l 85% (vol/vol) methanol and stored under argon at -80 °C. Mass spectrometric analysis was performed within 24 hours of plasma collection. 40 μ l of the extract were introduced onto a 2690 HPLC system (Waters) and separated phospholipids through a C18 column (2 $\times$ 150 mm, 5 μ m octadecyl silane, Phenomenex) under gradient conditions at flow rate of 0.2 ml/min as described. The HPLC column effluent were then introduced onto a

Micromass Quattro Ultima triple quadrupole mass spectrometer (Micromass) and analyzed by positive electrospray ionization in the multiple reaction monitoring (MRM) mode. Following collision-induced fragmentation with argon gas, phosphatidylcholine molecules generated a diagnostic product ion with mass-to-charge ratio (m/z) 184, corresponding to the protonated phosphocholine headgroup. The ion pairs for the MRM transitions monitored were each molecular ion $[MH]^+$ of the phosphatidylcholines and the diagnostic product ion (m/z 184). Calibration curves were constructed with a fixed amount of DTPC and varying amounts of each synthetic phosphatidylcholine.

Statistical analysis

Each experiment was repeated at least 3 times with different platelet donor or different mice. Values are expressed as Mean $\pm$ SE. The statistical significance was evaluated using an unpaired t test or ANOVA or Mann whitney if appropriate and considered statistically significant when $p < 0.05$.

Chapter3 OxLDL-induced platelet activation is CD36-dependent

3.1 Introduction and rationale

CD36 is expressed on platelets. Although CD36 was first isolated and structurally characterized from platelets, the functional role of CD36 on platelets remained not clear until recently⁹⁵. As described in chapter 1.12, our lab has developed strong evidence suggesting that CD36 might transduce cellular signals and render platelets hyperactive. In addition, Plow et al showed that the monoclonal antibody OKM5, which recognizes CD36, induced platelet aggregation, secretion, and conformation change of fibrinogen receptors¹⁰⁴. Although F(ab')₂ fragments of the antibody did not have stimulatory effects on platelet function, they did block the activation of platelets by the intact antibody, which suggested that the CD36 epitope was indispensable in this process. Actually, almost all CD36 antibodies tested had stimulatory effect on platelets. Additionally, some studies have suggested a role for anti-CD36 auto-antibodies in the pathogenesis of several platelet disorders including thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). In both cases, plasmas from patients show reactivity against CD36,

raising the possibility that anti-CD36 auto-antibodies are responsible for thrombocytopenic events occurring in both TTP and HUS conditions^{105, 106}.

As a scavenger receptor, CD36 recognizes ligands generated in pathological states, including oxLDL, which occurs in settings of hyperlipidemia/atherosclerosis, advanced glycated end products (AGE), which occur in diabetes, and the surface of apoptotic cells, which can be generated in inflammation and cancer^{10, 16, 28}. Among them, we are particularly interested in oxLDL because of its pathogenic role in atherosclerosis. Atherosclerosis is the leading cause of death in populations with a western life style. Evidence suggest that oxidation of LDL plays a key role in the formation of lipid loaded foam cells and the pathogenesis of atherosclerotic plaque. Antioxidants have been shown to be protective against lesion development in some animal models and oxLDL-derived epitopes have been detected in atherosclerosis lesions. In addition, oxLDL likely participates in the acute clinical complications of coronary and cerebrovascular atherosclerosis; i.e. heart attack and stroke. These events are usually triggered by plaque rupture or erosion with subsequent exposure of thrombogenic plaque components to blood. Studies also showed that low concentrations of oxLDL circulate in human plasma of patients with coronary artery disease¹⁰⁷.

Here, we sought to test the hypothesis that oxLDL-CD36 interaction

modulates platelet function, rendering them hyperactive. To address this, we established flow cytometry-based assay to assess the extent of platelet activation.

3.2 OxLDL activates human platelets

As described in chapter 2.2, platelet activation is marked by activation of platelet integrin $\alpha_{IIb}\beta_3$ and secretion of platelet granular contents. To test the ability of oxLDL to activate platelets, we assessed platelet α -granule secretion by measuring surface expression of P selectin with anti-P selectin antibody in flow cytometry. We also assessed platelet activation by measuring surface PAC-1 antibody binding as an assay for integrin $\alpha_{IIb}\beta_3$ activation. PAC-1 antibody only recognizes the activated form of $\alpha_{IIb}\beta_3$. We used non-treated platelets (control) and platelets treated with native LDL as controls. We determined that treating platelets with 50 ug/ml Cu^{++} oxidized LDL activated platelets, leading to increasing surface exposure of P-selectin (Figure 3-1A) and conformation change of $\alpha_{IIb}\beta_3$ integrin (Figure3-1B). These results suggested that oxLDL activates platelets and is consistent with the result from Podrez *et al* that oxLDL induced platelet activation (also described in chapter 1.12).

Figure 3-1 OxLDL-induced platelet activation is CD36-dependent. Platelets were incubated with 50 $\mu\text{g/ml}$ oxLDL or native LDL and then analyzed by flow cytometry for binding of PE-labeled anti-CD62P antibody to detect α -granule secretion (A) or FITC-labeled PAC-1 antibody to detect $\alpha_{\text{IIb}}\beta_3$ activation (B). Platelets from a CD36 null donor were incubated with oxLDL or 10 μM ADP.

3.3 OxLDL activates platelets in a CD36-dependent manner

We screened platelet donors for CD36 expression by flow cytometry using the specific antibody to human CD36 and identified a CD36-null donor. Platelets from this donor were then used to determine if CD36 was necessary for oxLDL-induced platelet activation. Platelets were isolated from a normal control and the CD36 null donor as described above. They were incubated with 50 ug/ml oxLDL for 30 minutes. In contrast to the activating effect of oxLDL on normal control platelets, platelets from CD36 null donor were not activated by oxLDL, while they responded normally to ADP (Figure 3-1C). These results suggest that OxLDL activates platelets in a CD36-dependent manner, which is consistent with the previous result that platelets from CD36-null mice showed significantly reduced activation by OxLDL in comparison to platelets from wild type mice. These results demonstrated conclusively that CD36-oxLDL interactions lead to platelet activation.

Chapter4 OxLDL induces phosphorylation of JNK in platelets

4.1 Introduction and rationale

It is now well established that despite having very short intra-cytoplasmic domains, CD36 can serve as a signaling molecule. Studies in other cellular systems have linked the signaling function of CD36 to recruitment/activation of src-family kinases and activation of specific mitogen activated protein (MAP) kinases. For example, on microvascular endothelial cells TSP-1 induces a CD36-dependent anti-angiogenic, pro-apoptotic signal via activation of Fyn, caspase-3, and p38 MAP kinase⁴³. On macrophages, exposure to oxLDL leads to recruitment of Lyn and activation of JNK-2 in a CD36 dependent manner⁴⁷. Inhibition of JNK resulted in significant reduction in uptake of oxLDL and foam cell formation. These studies suggest a context-dependent mechanism for CD36 signaling involving specific src and MAP kinases. In platelet biology, MAP kinases have not been studied in detail, although it has been demonstrated that the P2Y₁ ADP receptor activates p38 MAP kinase, and p38 deficient mice have prolonged thrombotic occlusion time in a ferric chloride (FeCl₃)-induced thrombosis model^{73, 108}. We thus hypothesized that CD36-mediated platelet activation might involve specific members of the src and MAP kinase family.

4.2 OxLDL induces phosphorylation of JNK and MKK4 in platelets

As described in chapter 4.1, we recently showed that the specific MAP kinase JNK was a key component of a macrophage CD36-initiated signaling cascade induced by oxLDL, thus, we investigated whether JNK was involved in platelet CD36 signaling induced by oxLDL. We assessed the phosphorylation state of JNK by immunoblot with phospho-specific antibody to JNK. We also used total antibody to JNK to assess protein loading. Human platelets were isolated as described in chapter 2.2. Isolated platelets were incubated with 50 ug OxLDL for 30 minutes and resuspended in lysis buffer. The controls were non-treated platelets and platelets treated with native LDL. We found that human platelets exposed to oxLDL but not native LDL, had a 3-5 fold increase in phosphorylation of JNK and its upstream activator MKK4 (Figure 4-1A). The results suggest that OxLDL induced the phosphorylation of JNK and MKK4 in platelets.

4.3 OxLDL induces the phosphorylation of JNK in platelets in a time-dependent and dose-dependent manner.

To investigate the time kinetics of JNK phosphorylation induced by OxLDL, we treated isolated platelets with 50 ug/ml OxLDL for 1, 5, 15 and 30 minutes. To assess dose-dependent effects of OxLDL on JNK phosphorylation in

Figure 4-1 OxLDL induces phosphorylation of JNK2 and MKK4 in platelets. Washed human platelets (2×10^8 /ml) containing 2 mM CaCl_2 and 1 mM MgCl_2 were incubated with native LDL or various concentrations of oxLDL over varying time points and then lysed. The lysates were analyzed by immunoblot with antibodies specific for phospho-JNK (p-JNK2, A, B, C), phospho-MKK4 (p-MKK4, A). The membranes were then stripped and re-probed with antibodies to the total relevant proteins to normalize the protein loaded. (D) Platelets were incubated with 50 µg/ml native LDL (Lane 2) or oxLDL with PBS (Lane 3), 0.5 U/ml apyrase (Lane 4) or 1 mM RGDS (Lane 5) for 5 minutes and then lysed. The lysates were analyzed by immunoblot as above for phospho-JNK (p-JNK) and total JNK. Results are representative of at least 3 independent experiments from different donors. The bar graph represents quantification of the phosphorylation of JNK2 (ratio of phosphorylated/total) expressed as relative values when compared with platelets without any treatment (A, B) or platelets treated with 0.2 µg/ml oxLDL (C). $n=5$ for A, $n=3$ for B and $n=4$ for C. c- control, nLDL- native LDL, oxLDL- oxidized LDL, * $p < 0.05$ when compared to control or nLDL treatment.

platelets, we treated isolated platelets with 0.2, 1, 5, 25 and 100 $\mu\text{g/ml}$ for 30 minutes. We found phosphorylation was time-dependent (Figure 4-1B) with detectable levels after one minute and reaching maximum by 15 minutes. The rapid kinetics suggests that the signaling is triggered by surface receptor-ligand interaction. We also found that JNK phosphorylation was also concentration-dependent (Figure 4-1C). Concentrations as low as 5 $\mu\text{g/ml}$ were effective at activating JNK with maximal effect seen at 25 $\mu\text{g/ml}$, suggesting the physiological relevance of the signaling events triggered by oxLDL.

4.4 The phosphorylation of JNK in platelets by OxLDL is independent of platelet secretion and outside-in signaling

Platelet activation is marked by activation of platelet integrin $\alpha_{\text{IIb}}\beta_3$ and secretion of platelet granular contents. Activated Integrin $\alpha_{\text{IIb}}\beta_3$ then mediates signaling events termed outside-in signaling that are induced by fibrinogen binding. Secreted platelet granular contents include ADP. Secreted ADP further activates platelets via binding to P2Y receptors. To exclude the possibility that platelet secretion and outside-in signaling contribute to the phosphorylation of JNK induced by OxLDL, we used Apyrase (0.5 U/ml) to hydrolyse secreted ADP. We also used an RGDS peptide (1mM) to block

fibrinogen binding and thereby outside-in signaling. We found that treatment of platelets with RGDS peptide or apyrase did not block JNK activation by oxLDL, indicating that phosphorylation was not dependent on outside-in integrin signals or secreted ADP (Figure 4-1D).

4.5 JNK phosphorylation by oxLDL is decreased in CD36 -/- platelets

We treated platelets from a CD36 null donor with 50 ug/ml oxLDL and assessed JNK phosphorylation in platelets. In comparison to platelets from a CD36 positive donor, JNK phosphorylation by oxLDL was decreased (Figure 4-2). Although this is the only available donor and basal JNK phosphorylation was relatively high in platelets from this donor, the data suggest that oxLDL-mediated JNK phosphorylation in platelets is in a CD36-dependent manner.

4.6 OxLDL induces phosphorylation of p38 and ERK in platelets

We also assessed the phosphorylation state of other MAP kinases p38 and ERK by immunoblot with phospho-specific antibodies. We used total relevant antibodies to assess protein loading. Isolated platelets were incubated with 50 ug OxLDL for 30 minutes and resuspended in lysis buffer. The controls

Figure 4-2 Phosphorylation of JNK2 induced by oxLDL is CD36-dependent. Platelets from a CD36 null donor or CD36 positive donor were incubated with 50 µg/ml oxLDL and then analyzed by immunoblot as in figure 4-1 for JNK phosphorylation.

A

B

Figure 4-3 OxLDL induces phosphorylation of p38 and ERK2 in platelets. Washed human platelets (2×10^8 /ml) containing 2 mM CaCl_2 and 1 mM MgCl_2 were incubated with 50 ug/ml native LDL or oxLDL and then lysed. The lysates were analyzed by immunoblot with antibodies specific for phospho-p38 (A) or phospho-ERK (B). The membranes were then stripped and re-probed with antibodies to the total relevant proteins to normalize the protein loaded.

were non-treated platelets and platelets treated with native LDL. We found that human platelets exposed to oxLDL but not native LDL, had increases in phosphorylation of both p38 and ERK (Figure 4-3). The data was also consistent with findings from another group showing that oxLDL induces phosphorylation of p38 in platelets.

Chapter5 Elevated levels of platelet JNK phosphorylation in vivo are associated with hyperlipidemia and are CD36-dependent

5.1 Introduction and rationale

As described in Chapter 4, we have demonstrated that OxLDL induced the phosphorylation of JNK in platelets. To further test if JNK phosphorylation in platelets is induced *in vivo*, we used *apoe*^{-/-} mice as a model of hyperlipidemia. ApoE is an apolipoprotein component of lipoprotein particles that are primarily synthesized in the liver. ApoE is essential to the metabolism of lipoprotein particles especially those that are triglyceride-rich. ApoE genetic variation in humans has a significant impact on plasma lipoprotein profiles and susceptibility to atherosclerosis, thus *apoe*^{-/-} mice were developed in 1992¹⁰⁹. These mice are severely hyperlipidemic, especially when they are challenged with a high fat western-type diet. The severe hyperlipidemia is mainly due to an increase in the levels of very low and intermediate density lipoproteins. *Apoe*^{-/-} mice develop visible atherosclerotic lesions starting around 10 weeks of age.

Our group, with collaborators, recently identified specific oxidized phospholipids in oxLDL that serve as high affinity ligands for CD36 (described

in chapter 1.11)^{93, 94}. With LC-ESI/MS/MS Podrez *et al* demonstrated that these oxidized lipids are present in the plasma of western diet fed *apoe*^{-/-} mice⁹⁷. With mesenteric and carotid thrombosis models, Podrez *et al* demonstrated that the time to thrombotic occlusion after induction of injury was significantly shorter in *apoe*^{-/-} mice than in WT mice. This hyperlipidemia induced pro-thrombotic phenotype was rescued by genetic deletion of CD36 in the *apoe* null background. All these results suggest oxidized lipid ligands transduce pro-thrombotic signals in a CD36-dependent manner. To characterize these signals, we assessed the phosphorylation state of JNK in platelets from *apoe*^{-/-} mice. To test CD36 dependency, we also used *apoe*^{-/-}; *cd36*^{-/-} mice.

5.2 Basal phosphorylation of JNK is increased in resting platelets from hyperlipidemic mice

To characterize pro-thrombotic signals, we isolated platelet-rich plasma (PRP) from WT and *apoe*^{-/-} mice fed normal chow or high fat “western” diets for 3 months beginning at 6 weeks of age. Platelets in platelet-rich plasma (PRP) were allowed to adhere to silanized slides. We performed immunofluorescence staining with phospho-specific antibody to JNK to assess the phosphorylation state of JNK. We also used CD41 antibodies as a marker for platelets. We examined the presence of phosphorylated JNK in these

platelets by confocal fluorescence microscopy. We found minimal phospho-JNK2 expression in resting platelets from WT mice fed a chow diet (Figure 5-1B). Platelets from *apoe*^{-/-} mice on chow diet showed a modest increase in JNK phosphorylation, whereas platelets from *apoe*^{-/-} mice on the western diet showed a marked increase in phospho-JNK staining ($p < 0.001$ compared to those from either of the chow fed strains, Figure 5-1A,B). As additional confirmation, we also examined the level of phospho-JNK by immunoblot (Figure 5-2) and found an increase in platelets from *apoe*^{-/-} mice on western diet compared to platelets from *apoe*^{-/-} mice on chow diet. These results show that the phosphorylation of JNK in platelets was induced by *in vivo* hyperlipidemia.

5.3 Diet-induced phosphorylation of JNK in platelets from hyperlipidemic mice is CD36-dependent

As described in chapter 5.2, we showed that the phosphorylation level of JNK was elevated in platelets from *apoe*^{-/-} mice. To test if this is CD36-dependent, we use *apoe*^{-/-}; *cd36*^{-/-} mice fed on high-fat western diet. By immunofluorescence microscopy we found that diet-induced JNK phosphorylation was completely eliminated in *apoe*^{-/-} mice that were also deficient in CD36 (Figure 5-1A, B). Flow cytometry analysis of platelets in

Figure 5-1 Basal phosphorylation of JNK is increased in resting platelets from hyperlipidemic mice in a CD36-dependent manner. (A) Platelets from WT and *apoe*^{-/-} mice maintained on normal chow diet and *apoe*^{-/-} mice and *apoe*^{-/-};*cd36*^{-/-} mice maintained on high fat (HF) diet for 3 months at the age of 6 weeks were stained for phosphorylated JNK (Red), CD41 (Green) was stained simultaneously for platelet identification. Original magnification was 63X4 for all panels. Scale bar=20 μ m. (B) Fluorescence intensity of 30 randomly selected platelets from 3 different mice (10 for each from 3 random fields) were determined with ImageJ software, and adjusted to the area of CD41 staining of the same platelet, used for p-JNK quantification. The bar graph represents Mean $\pm$ SE fluorescence values.

suspension confirmed that those from *apoe*^{-/-};*cd36*^{-/-} mice on western diet had significantly lower levels of phospho-JNK than those from *apoe*^{-/-} mice on western diet did (n=3, P< 0.05 Figure 5-3). By immunoblot we also demonstrated that diet-induced phosphorylation JNK phosphorylation in *apoe*^{-/-} platelets was not observed in platelets from *apoe*^{-/-};*cd36*^{-/-} mice on western diet (Figure 5-2). These data demonstrate that the interaction between endogenous oxidized lipid ligands and CD36 triggers a signaling cascade leading to JNK activation in platelets.

Figure 5-2 Basal phosphorylation of JNK is increased in resting platelets from hyperlipidemic mice in a CD36-dependent manner. The lysates of platelets from *apoe*^{-/-} mice maintained on normal chow diet and *apoe*^{-/-} mice and *apoe*^{-/-}*cd36*^{-/-} mice maintained on high fat (HF) diet for 3 months were analyzed by immunoblotting for the level of phospho-JNK as well as total JNK. The bar graph represents quantification of the phosphorylation of JNK (ratio of phosphorylated/total).

Figure 5-3 Platelets from *apoe*^{-/-} mice fed a high-fat diet have elevated level of phosphorylated JNK in comparison to *apoe*^{-/-}; *cd36*^{-/-} mice. Platelets from *apoe*^{-/-} mice and *apoe*^{-/-}; *cd36*^{-/-} mice maintained on high fat (HF) diet for 3 months at the age of 6 weeks were stained for phosphorylated JNK. The staining intensity was measured in flow cytometry. The bar graph represents mean fluorescence intensity (MFI) from three different mice.

Chapter6 Recruitment of Fyn and Lyn kinases by platelet CD36 is essential for oxLDL-induced signaling events

6.1 Introduction and rationale

Previous studies demonstrated that the src family kinases Fyn, Lyn, and Yes were co-precipitated from platelet membrane lysates with anti-CD36 antibodies⁴². In addition, Fyn and/or Lyn has /have been shown to be involved in CD36-initiated signaling leading to MAP kinase activation in macrophages, microglia and endothelial cells. In macrophages, with GST-CD36 cytoplasmic tail fusion protein, our group previously showed by pull-down assays that CD36 associated with a signaling complex including Lyn⁴⁷. We also demonstrated that the amount of Lyn associated with CD36 and the extent of its phosphorylation was increased in macrophages incubated with OxLDL. In microglia, it has been demonstrated beta-amyloid triggered a CD36-initiated signaling cascade involving Fyn, Lyn and MAP kinase p44/42 and beta-amyloid induced the association of Lyn with CD36⁴⁵. In microvascular endothelial cells, thrombospondin-1 results in the recruitment of Fyn to CD36 and increase in the phosphorylation of CD36-associated Fyn⁴³. All these results suggest that Src family kinases Fyn and/or Lyn play a role in CD36-initiated signaling cascades. To investigate the role of src family kinases

in OxLDL-mediated signaling in platelets, we used a specific src kinase inhibitor AG1879 and also performed co-immunoprecipitation with a CD36 monoclonal antibody.

6.2 OxLDL-induced JNK2 phosphorylation is mediated by src family kinases

To investigate the mechanism by which oxLDL induces JNK phosphorylation we used a panel of specific pharmacological inhibitors. We also used treatment with DMSO alone as a control. We found that pre-treatment of platelets with the broad spectrum src kinase inhibitor AG1879 blocked oxLDL induced JNK2 activation, while the PI3 Kinase inhibitor LY294002 and the broadly active PKC inhibitor GO6983 had minimal effect (Figure 6-1A). These data suggest that JNK activation is downstream of src family kinases and independent of PI3K or PKC signaling pathways.

6.3 OxLDL results in the recruitment of Fyn and Lyn to platelet CD36

To test the possibility that a physical association between CD36 and Fyn and/or Lyn may have a functional role in CD36-mediated signaling in platelets, we performed co-immunoprecipitation with a CD36 monoclonal antibody. We

Figure 6-1 Recruitment of src family kinases to platelet CD36 is essential for oxLDL-mediated signaling. (A) Human platelets were incubated with the JNK inhibitor SP600125, src inhibitor AG1879, PKC inhibitor GO6983 or PI3K inhibitor LY294002 for 30 minutes prior to incubation with 50 µg/ml oxLDL. The platelet lysates were then analyzed by immunoblot as in figure 1 for JNK phosphorylation. (B, C) Platelets were incubated with oxLDL or native LDL and then lysed. CD36 was precipitated by FA6 anti-CD36 IgG. Precipitates were analyzed by immunoblot with antibodies to CD36, phospho-src (Y416), Fyn and Lyn (B). The supernatant after immunoprecipitation was also analyzed by immunoblot with antibodies to phospho-src (416), Fyn and Lyn (C). (D) Platelets were incubated with 50 µg/ml oxLDL or native LDL and then lysed. Lyn was immunoprecipitated from lysates and the precipitates were analyzed by immunoblot with a phosphotyrosine antibody (4G10) and Lyn antibody.

then examined the precipitates for the presence of src kinases and their activation state by immunoblot. We found that the amounts of Fyn and Lyn in CD36 immunoprecipitates were markedly increased upon oxLDL treatment (Figure 6-1B). Src family kinases have two tyrosine phosphorylation sites; phosphorylation in the activation loop increases kinase activity while phosphorylation in the C terminus renders the kinases inactive. We used an antibody specific to the phosphotyrosine in the activation loop and found that the Fyn and to a lesser extent Lyn recruited to CD36 after oxLDL exposure were in the “active” state (Figure 6-1B). In contrast, oxLDL did not increase the amount of “active” tyrosine phosphorylated Fyn or Lyn in the non-CD36-associated fraction (Figure 6-1C). These data suggest that oxLDL induced the recruitment of actively phosphorylated Fyn and Lyn to platelet CD36 and this interaction may contribute to downstream platelet CD36 signaling events.

We also performed immunoprecipitation with anti-Lyn antibody and probed the tyrosine phosphorylation state with a phosphotyrosine specific antibody 4G10 and found that there was no significant increase in total Lyn tyrosine phosphorylation after exposure to oxLDL (Figure 6-1D). This result further showed that the ligand-induced recruitment of Fyn and Lyn to platelet CD36 may represent the mechanism by which platelet CD36 transduce signals to MAP kinase JNK.

Chapter7 OxLDL induces the phosphorylation of Vav and Vav-associated Fyn in platelets

7.1 Introduction and rationale

As described in chapter 1.8, Vav is known as a GEF for Rac/Rho proteins⁵⁹. This activity is strictly controlled by tyrosine phosphorylation by src family kinases including Fyn and Lyn. Importantly, in microglial cells, Vav was shown to be involved in a CD36-initiated signaling cascade induced by beta-amyloid. Interestingly we have shown that ligand-induced recruitment of phosphorylated Fyn and Lyn by platelet CD36 is essential for OxLDL-induced signaling events. Thus, we hypothesize that Vav may play a functional role downstream of Fyn and Lyn, transducing a CD36-initiated signaling cascade in platelets.

7.2 OxLDL induces the phosphorylation of Vav in platelets in a time and dose-dependent manner

To test for a functional role of the inducible association between CD36,

Fyn and Lyn in Vav activation, we immunoprecipitated Vav from platelets and assessed its tyrosine phosphorylation state by immunoblot using 4G10 antibody. Human platelets were isolated as described in chapter 2.2. We found Vav phosphorylation in platelets was induced by 50 ug/ml oxLDL (~ 2 fold increases, Figure 7-1). 50 ug/ml native LDL also induced modest Vav phosphorylation (Figure 7-1). To investigate kinetics of Vav phosphorylation by oxLDL, we treated isolated platelets with 50 ug/ml OxLDL for 1, 5, 15 and 30 minutes. To assess dose-dependent effect of OxLDL on Vav phosphorylation in platelets, we treated isolated platelets with 0.2, 1, 5, 25 and 100 ug/ml oxLDL for 30 minutes. We found phosphorylation was detectable after one minute and sustained for at least 30 minutes (Figure 7-2A). We also found that Vav phosphorylation was concentration-dependent (Figure 7-2B). Concentrations as low as 1 µg/ml were effective at inducing Vav phosphorylation with maximal effect seen at 25 µg/ml. These data suggest that the phosphorylation of Vav in platelets was induced by OxLDL and Vav phosphorylation may be one of the early signaling events downstream of platelet CD36 upon OxLDL binding.

7.3 OxLDL induces the phosphorylation of Vav-associated Fyn

Vav has been shown to be a selective substrate for Fyn under conditions

Figure 7-1 OxLDL induces phosphorylation of Vav in platelets. Washed human platelets (2×10^8 /ml) containing 2 mM CaCl_2 and 1 mM MgCl_2 were incubated with 50 ug/ml native LDL or oxLDL for 30 minutes and then lysed. Vav was immunoprecipitated by anti-Vav antibody. The precipitates were analyzed by immunoblot with anti-phosphotyrosine antibody (EG10). The membranes were then stripped and re-probed with antibodies to the total relevant proteins to normalize the protein loaded.

A

B

Figure 7-2 OxLDL induces phosphorylation of Vav in platelets in a time-dependent and dose-dependent manner. Washed human platelets (2×10^8 /ml) containing 2 mM CaCl_2 and 1 mM MgCl_2 were incubated with various concentrations of oxLDL over varying time points and then lysed. Vav was immunoprecipitated by anti-Vav antibody. The precipitates were analyzed by immunoblot with anti-phosphotyrosine antibody (EG10). The membranes were then stripped and re-probed with antibodies to the total relevant proteins to normalize the protein loaded. Results are representative of at least 3 independent experiments from different donors.

of low-affinity T cell receptor mediated cell signaling¹¹⁰. This signaling pathway involving Fyn, Vav and Rac-1 is required for the cytoskeletal reorganization that leads to the formation of the immunologic synapse¹¹⁰. With the broad spectrum src family kinase inhibitor AG1879, we showed that pharmacological inhibition of src family kinases significantly reduced the phosphorylation of Vav induced by oxLDL in platelets (Figure 7-3A). To investigate the role of Fyn in the phosphorylation of Vav induced by OxLDL in platelets, we performed co-immunoprecipitation with anti-Vav antibody. We then performed immunoblot with EG10 antibody to assess the amount the phosphorylated Fyn associated with Vav. We found that OxLDL increased the amount of phosphorylated Fyn associated with Vav (Figure 7-3B). To further investigate if the increase in phosphorylated amount of Fyn associated with Vav is due to the phosphorylation of Fyn or the increase in the association between Fyn and Vav, we also performed immunoblot with Fyn antibody. We found that there was no increase in the association between Fyn and Vav (Figure 7-3B). To further confirm that, we performed co-immunoprecipitation with Fyn antibody and immunoblot with Vav antibody. We showed that there was also no increase in the association between Fyn and Vav (Figure 7-3C). These results suggest OxLDL induced the phosphorylation of Vav-associated Fyn in platelets and Fyn may be upstream kinase involved in the phosphorylation of Vav triggered by oxLDL.

Figure 7-3 OxLDL induces the phosphorylation of Vav-associated Fyn (A) Human platelets were incubated with src inhibitor AG1879 for 30 minutes prior to incubation with 50 μ g/ml oxLDL. The platelet lysates were then analyzed by immunoprecipitation and immunoblot as in figure 9-1 for Vav phosphorylation. (B) Platelets were incubated with oxLDL and then lysed. Fyn was co-immunoprecipitated by anti-Vav antibody. The precipitates were analyzed by immunoblot with anti-phosphotyrosine antibody (EG10). The membranes were then stripped and re-probed with antibodies to the total relevant proteins to normalize the protein loaded. (C) Platelets were incubated with 50 μ g/ml oxLDL and then lysed. Vav was co-immunoprecipitated by anti-Fyn antibody from lysates and the precipitates were analyzed by immunoblot with anti-Fyn and anti-Vav antibody.

Chapter8 OxLDL-induced activation of platelets in vitro is mediated by JNK and src family kinases

8.1 Introduction and rationale

As described in previous chapters, OxLDL induced a CD36-initiated signaling cascade involving src family kinases Fyn and Lyn and MAP kinases JNK and p38. We also showed that OxLDL activates human platelets in a CD36-dependent manner. To further investigate if src family kinases or MAP kinases play a functional role in platelet activation by OxLDL. We used JNK inhibitor SP600125, p38 inhibitor SB203580 as well as broad spectrum src kinase inhibitor AG1879 in a flow-cytometry based P-selectin expression assay. JNK inhibitor SP600125 is an anthrapyrazdone reversible ATP-competitive inhibitor with selectivity over 20 fold for JNK against a range of other kinases¹¹¹. In cells, it dose-dependently inhibits the phosphorylation of c-Jun. It is a common strategy using to identify a role for JNK in various pathological conditions.

8.2 Pharmacological inhibition of JNK, p38 and src blocks platelet activation by OxLDL

We studied the functional role of JNK in oxLDL-induced platelet activation

using a flow-cytometry based assay for surface exposure of P-selectin as a marker for platelet activation. We found that specific pharmacological inhibition of JNK by SP600125 reduced platelet activation in response to oxLDL by ~40% (Figure 8-1A). The inhibitor had minimal effect on platelet activation by other agonists as exemplified by ADP and TRAP (Figure 8-1B, C). These results suggest that JNK may be specifically involved in OxLDL-induced platelet activation, but other pathways are also involved. Consistent with our previous findings that oxLDL induced p38 phosphorylation, we found that pharmacological inhibitor of p38 by p38 inhibitor SB203580 also blocked platelet activation by oxLDL (Figure 8-2A). In contrast to the JNK inhibitor, the p38 inhibitor also reduced platelet activation by ADP (Figure 8-2B).

To investigate the functional role of src family kinases in OxLDL-mediated platelet activation, we used src kinase inhibitor AG1879 in a flow-cytometry based assay for surface exposure of P-selectin as a marker for platelet activation. We also found that inhibition of src family kinases by AG1879 blocked oxLDL-induced platelet activation by ~55% (Figure 8-3). This result demonstrated that src family kinases contribute to oxLDL-induced platelet activation.

Figure 8-1 OxLDL-induced platelet activation of platelets is mediated by JNK. Human platelets were incubated with the specific JNK inhibitor SP600125 (final concentration of 20 μ M) for 30 minutes prior to incubation with 50 μ g/ml oxLDL (A) or 10 μ M ADP (B) or 2 μ M TRAP (C) and analyzed by flow cytometry with PE-labeled anti-P-selectin antibody. Control platelets were treated with the vehicle, DMSO. Results are representative of at least 3 independent experiments from different donors. The bar graph represents mean fluorescence intensities measured with flow cytometry. The error bars are expressed as Mean $\pm$ SE. $n=4$ for A or $n=3$ for B, C.

Figure 8-2 OxLDL-induced platelet activation of platelets is mediated by p38. Human platelets were incubated with p38 inhibitor SB203580 (10 μ M) for 30 minutes prior to incubation with 50 μ g/ml oxLDL (A) or 10 μ M ADP (B) and then analyzed by flow cytometry with PE-labeled anti-P-selectin antibody. Control platelets were treated with the vehicle, DMSO.

Figure 8-3 OxLDL-induced platelet activation of platelets is mediated by src kinases. Human platelets were incubated with src family kinase inhibitor AG1879 (10 μ M) for 30 minutes prior to incubation with 50 μ g/ml oxLDL. Control platelets were treated with the vehicle, DMSO. Results are representative of at least 3 independent experiments from different donors. The bar graph represents mean fluorescence intensities measured with flow cytometry. The error bars are expressed as Mean $\pm$ SE. $n=3$.

Chapter9 Activation of CD36-JNK signaling axis contributes to thrombosis *in vivo*

9.1 Introduction and rationale

The role of MAP kinases in platelet activation has not been fully explored. Multiple lines of evidence suggest that p38 and/or ERK2 are activated in platelets in response to a diverse array of agonists including oxLDL⁶¹⁻⁷². Importantly, *p38* heterozygous deficient mice have prolonged thrombotic occlusion time in a FeCl₃-induced thrombosis model⁷³. The involvement of JNK in platelet biology has been studied to a limited extent with pharmacological inhibitors suggesting that JNK is involved in arterial thrombosis in mice. As described in previous chapters, we have demonstrated that MAP kinase JNK plays an essential role in a CD36-initiated signaling cascade leading to platelet activation. To further explore the functional role of this CD36-JNK signaling axis *in vivo*, we studied a carotid artery thrombosis in mice.

9.2 CD36-dependent activation of JNK promotes *in vivo* thrombus formation

We induced carotid artery thrombosis in 12 week old male WT and *cd36*

null mice by topical application of 12.5% ferric chloride as previously described. After the vessel was allowed to become completely occluded, the carotid arteries were removed and sectioned across the thrombi. To determine if JNK signaling occurs during thrombosis *in vivo* we performed immunohistochemical analysis of these thrombi using a phospho-specific antibody to JNK. For quantification of phosphorylated JNK staining, images were scored on the basis of staining intensity by a blinded observer (0 = negative; 1 = weak; 2 = moderate; 3 = intense). As shown in Figure 8-1, phospho-JNK was detected in thrombi from both WT and *cd36*^{-/-} mice. The staining intensity, however, was significantly lower ($p=0.003$) in thrombi from *cd36*^{-/-} mice, suggesting that CD36-mediated JNK signaling occurred during thrombus formation *in vivo* (Figure 9-1A, B). As an alternative approach to quantify phospho-JNK levels we dissected carotid artery thrombi from WT and *cd36*^{-/-} mice and examined pooled lysates from 9 thrombi in each group by immunoblot. Thrombi from *cd36*^{-/-} mice had ~16% less phospho-JNK than those from WT mice (Figure 9-2). These data suggest that CD36 contributed to JNK phosphorylation during thrombus formation *in vivo*.

9.3 Platelet JNK inhibition prolongs time to thrombosis in a CD36-dependent manner.

To study the effect of JNK in thrombosis *in vivo*, WT or *cd36* null mice were exposed to 11Gy of external beam irradiation from a Cesium 137 source

Figure 9-1 JNK is phosphorylated during thrombus formation in a CD36-dependent manner. (A) Representative images of Immunohistochemical detection of phosphorylated JNK (p-JNK) in carotid thrombi from WT and *cd36*^{-/-} mice. No staining with non-immune IgG control showed specificity. Brown indicated positive staining. Red font number indicates the score of the appropriate image. Scale bar=100 μ m. (B) The bar graph represents Mean $\pm$ SE of staining score of 5 sections from 3 thrombi in each group. (C) Lysates of 9 pooled carotid artery thrombi dissected from WT or *cd36*^{-/-} mice were analyzed by immunoblot for phospho-JNK. The membrane was then stripped and re-probed with antibodies to the total JNK to normalize the protein loaded. The gel was scanned and band intensities quantified with NIH ImagePro software.

to induce thrombocytopenia with platelet counts <5% of normal after 5 days. Platelets obtained from syngeneic donor mice were labeled with Calcein AM (final concentration of 0.5 mg/ml) in the presence or absence of the JNK inhibitor SP6000125 (final concentration of 400 nM for 30 min, Figure 9-3). This dose is the minimum active dose and was chosen to avoid potential off-target effects. 2×10^9 donor platelets were injected through the jugular vein of thrombocytopenic mice 10 minutes prior to carotid injury with 12.5% FeCl₃ to allow the transfused platelets to reach equilibrium in the circulation. As shown in Figure 9-4A, inhibition of platelet JNK significantly prolonged the time to thrombosis in mice transfused with WT platelets (2-sample t test, $p=0.01$) but had no effect in mice transfused with *cd36*^{-/-} platelets (2-sample t test, $p=0.37$). Figure 9-4B shows representative fluorescence images from these studies. These data strongly suggest that CD36 mediated JNK signaling promotes platelet activation and thrombus formation *in vivo*.

Figure 9-2 Strategy for the platelet transfusion studies. WT or CD36 null mice were exposed to 11Gy of external beam irradiation from a Cesium 137 source to induce thrombocytopenia with platelet counts <5% of normal after 5 days. Platelets obtained from syngeneic donor mice were labeled with Calcein AM (final concentration of 0.5 mg/ml) in the presence or absence of the JNK inhibitor SP6000125 (final concentration of 400 nM for 30 min). 2×10^9 donor platelets were injected through the jugular vein of thrombocytopenic mice 10 minutes prior to carotid injury with 12.5% FeCl₃ to allow the transfused platelets to reach equilibrium in the circulation.

Figure 9-3 JNK inhibition prolongs occlusion times in a CD36 dependent manner *in vivo* after carotid artery injury. Carotid thrombosis times were assessed by intravital video microscopy in irradiated thrombocytopenic WT and *cd36*^{-/-} mice transfused with platelets from donor animals of identical genotype. Arteries were injured by topical application of FeCl₃ (12.5%). Platelets were incubated with the JNK inhibitor SP600125 (400 nM) or vehicle control for 30 minutes prior to transfusion. (A) Bar graph represents occlusion time. Data are expressed as Mean $\pm$ SE (n=5). (B) Representative fluorescence photograph of the time course of thrombus formation with WT and *cd36*^{-/-} platelets treated with SP600125 and vehicle control.

Chapter10 Diet-induced prothrombotic phenotype is mediated by Vav

10.1 Introduction and rationale

It has been previously demonstrated that platelets from hyperlipidemic animals and patients have an increase in *in vitro* aggregation response⁹⁷. In particular, platelet aggregation in response to ADP was elevated in *apoe*^{-/-} mice challenged with high-fat dietary compare with *apoe*^{-/-} mice on normal chow. To investigate whether this effect of high-fat feeding on platelet aggregation can be observed in WT mice, we tested *in vitro* aggregation of platelets from WT mice fed on either high-fat western diet or normal chow. We also used platelets from *vav*^{-/-} mice fed on either high-fat western diet or normal chow.

10.2 High-fat feeding increases aggregation of platelets in WT mice

To test the effect of high-fat feeding on *in vitro* aggregation of platelets in WT mice, we tested the aggregation response of platelets in WT mice fed on high-fat western diet for two weeks as well as WT mice on normal chow. We used ADP as an aggregation agonist and tested *in vitro* aggregation in

aggregometer. The dose of ADP we used initially was 5 μ M. We found that platelets from WT mice on high-fat diet aggregated more robustly in response to 5 μ M ADP than those from WT mice on normal chow (Figure 10-1B). To further confirm that, we further lower the dose of ADP to 2 μ M. We found that high-fat diet also increased the aggregation of WT platelets in response to 2 μ M ADP (Figure 10-1A, B). These results suggest that a prothrombotic phenotype is also present in WT mice fed on high-fat western diet.

10.3 Diet-induced aggregation of platelets is mediated by Vav

We have shown in chapter 9 that Vav phosphorylation in platelets can be induced by OxLDL, raising the possibility that Vav may transduce the prothrombotic signal triggered by OxLDL. To test this possibility, we tested the ADP-induced aggregation of platelets in *vav*^{-/-} mice either on western diet or normal chow. We found that the diet-induced aggregation phenotype we observed in WT mice was absent in *vav*^{-/-} mice (Figure 10-1A, B). These results suggest that Vav may mediate the diet-induced prothrombotic signal in platelets. To exclude the possibility *vav* deficiency does not affect diet-induced hyperlipidemia, we examined the weights of *vav*^{-/-} animal fed on either normal chow or western diet. We showed that western diet significantly increased the weights of *vav*^{-/-} mice (Figure 10-2). These results suggest that Vav is not

A

B

Figure 10-1 High-fat feeding enhances the aggregation response of WT platelets but not *vav*^{-/-} platelets in response to low doses of ADP. (A) Platelets from WT and *vav*^{-/-} mice on chow or western diet were stimulated with 2 uM ADP. Aggregation was assessed turbidimetrically with a dual-channel aggregometer. Shown are representative curves. (B) Aggregation amplitude of platelets from WT or *vav*^{-/-} mice on chow or western diet incubated with 2 or 5 uM ADP as indicated.

Figure 10-2 Vav deficiency has no effect on diet-induced hyperlipidemia. *Vav*^{-/-} mice were put on chow diet or western diet for two weeks. They were then weighed. The bar graph represents weights from 4 different mice.

Involved in causing hyperlipidemia and the defect we observed between *vav*^{-/-} and wild type animals are not due to different responses to diet.

10.4 OxPC_{CD36} is present in the plasma from *vav*^{-/-} mice on western diet

Previous studies have identified a novel family of oxidized choline glycerophospholipids (oxPC_{CD36}) that serve as high-affinity ligands for CD36^{93, 94}. They are derived from PAPC and PLPC. It has been demonstrated that these CD36 phospholipid ligands were present in the plasma from hyperlipidemic *apoe*^{-/-} mice. The interaction between platelet CD36 and these endogenous CD36 phospholipid ligands play an essential role in the association of the prothrombotic phenotype with hyperlipidemia and oxidant stress. Since we showed that Vav may mediate the prothrombotic signal induced by hyperlipidemia in chapter 10.3, we then tested if CD36 phospholipid ligands oxPC_{CD36} were also present in the plasma from *vav*^{-/-} mice on high-fat western diet. We identified the presence of oxPC_{CD36} by liquid chromatography–electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) analyses as control experiments. We showed that there was significant amount of oxPC_{CD36} present in the plasma from *vav*^{-/-} mice on high-fat western diet (Table 10-1). This result further suggests that endogenous CD36 phospholipid ligands are present in the plasma *vav*^{-/-} mice on western diet.

Table 10-1 The level of oxPC_{CD36} in the plasma of *vav*^{-/-} mice on western diet

Sample Text	Analyte concentration (nM)	Analyte/Precursor (mmol/mol)
HodiA-PC	14.49	2.31
KodiA-PC	3.26	4.00
HOOA-PC	71.5	4.00
KOOA-PC	5.65	1.01
HDdiA-PC	7.73	1.83
KDdiA-PC	9.70	2.32
HODA-PC	27.35	6.55
KODA-PC	20.93	4.96

Chapter11 Implication, Problems and Pitfalls

11.1 Discussion and future direction

Hyperlipidemia is defined as the presence of elevated levels of lipoprotein in the bloodstream. It is considered as a one of the significant risk factors for atherosclerosis. Atherosclerosis is a chronic inflammatory disease taking place in the arterial walls. It is characterized by the formation of multiple plaques within the vessel walls. Advanced atheromatous plaques are prone to rupture, resulting in thrombosis. In the coronary circulation this leads to acute coronary syndromes and in the carotid and cerebral circulation this leads to transient ischemia attack (TIA) and stroke. Acute coronary syndromes are the leading cause of morbidity and mortality in the western world.

Platelets play a key role in atherothrombosis. During plaque rupture, circulating platelets are exposed to thrombogenic materials released from plaque and form occlusive thrombi. Hyperlipidemic individuals have enhanced platelet reactivity, aggravating the thrombotic risks¹¹². Enhanced platelet reactivity is also seen in hyperlipidemic animal models, such as apolipoprotein E-deficient mice on high-fat diet¹¹³.

While the role of antiplatelet therapies in the prevention of acute coronary syndromes has been well recognized, the mechanism of enhanced platelet

activity associated with hyperlipidemia had not been not clear until very recently. Hyperlipidemic individuals normally have elevated plasma level of low density lipoprotein (LDL) as well as decreased plasma level of high density lipoprotein (HDL). Hyperlipidemia is also associated with oxidant stress, resulting in the generation of oxidized LDL (oxLDL). OxLDL binds to scavenger receptors including CD36 and Scavenger receptor A (SRA) expressed on macrophage surface. These scavenger receptors bind and take up oxLDL, resulting in the foam cell formation. Foam cell formation is the first step during atherogenesis.

In addition to the effect of oxLDL on macrophages, several studies have shown that LDL subjected to various methods of *in vitro* oxidation, including by exposure to metal ions or HOCL (Hydrochlorous acid), can influence platelet function^{62, 63, 66, 67, 114-121}. These studies suggest that the effect of oxLDL on platelets may account for enhanced platelet activity associated with hyperlipidemia. However, the molecular mechanism by which oxLDL modulates platelet function was unclear until recently. As described in chapter 1.12, Podrez *et al* recently showed that oxLDL bound to human and mouse platelets via platelet CD36⁹⁷. Podrez *et al* also showed that CD36-oxLDL interactions lead to platelet activation. In our studies described here, we confirmed that oxLDL activates platelets and showed that platelets from one CD36 null human subject were not activated, suggesting that oxLDL-mediated

platelet activation is CD36 dependent. In addition, these studies also suggest ligand bound platelet CD36 may modulate platelet function. As described in chapter 1.2 and 3.1, as a scavenger receptor, CD36 recognizes ligands generated in pathological states, including oxLDL, advanced glycated end products (AGE), which occur in diabetes, and the surface of apoptotic cells and cell-derived microparticles (MP), which can be generated in inflammation and cancer^{10, 16, 28, 31}. Our results, together with the data from Podrez et al, also imply a more general role of platelet CD36 as a sensor for pathological conditions and provide a potential explanation for prothrombotic conditions associated with other life-threatening diseases including inflammation, cancer and diabetes. Consistent with this concept, Ghosh et al in our laboratory showed that cell-derived microparticles also enhanced platelet activity in a CD36-dependent manner. With these encouraging data, it will be interesting to assess if platelet CD36 is a link between prothrombotic phenotype and other diseases associated with elevated level of circulating microparticles including metabolic syndrome, diabetes, cancer and inflammation. These further studies may also address the question if the expression level of platelet CD36 may be predictive for thrombotic risk under pathological condition.

The mechanism by which CD36-oxLDL interaction promotes platelet reactivity was unknown before the study described here. CD36 is a multi-functional receptor. Besides serving as a receptor for oxLDL, CD36

facilitates long chain fatty acid (LCFA) transport. Genetic linkage analysis has shown that CD36 deficiency underlies insulin resistance in a spontaneous hypertensive rat (SHR)¹²². Thus, global inhibition of CD36 function may be problematic. In addition, existing antiplatelet therapies have major drawbacks regarding safety and efficiency because most of them target normal platelet activation rather than pathological platelet activation¹²³. In these regards, the knowledge about downstream signaling pathway triggered by the interaction between oxLDL and platelet CD36 may help the development of antiplatelet therapies specifically targeting atherothrombosis without affect normal hemostasis.

As described in chapter 1.3 and 4.1, a context-dependent mechanism for CD36 signaling involves specific src and MAP kinases. Interestingly, oxLDL induced JNK phosphorylation in macrophage via CD36. Thus, we hypothesized that oxLDL-CD36 interaction may induce JNK phosphorylation in platelets. Indeed, we showed that the phosphorylation of JNK was induced in platelets by oxLDL. The phosphorylation of JNK in this process was time-dependent and dose-dependent. The results are consistent with recent studies from other laboratories showing that MAP kinases, including JNK have a significant role in platelet biology. Pharmacological inhibition of JNK in a model of arteriolar and venular thrombosis in mice suggested a role in arterial but not venular thrombosis¹²⁴. It was recently demonstrated that JNK was activated after thrombin exposure and during collagen-induced platelet

aggregation¹²⁴. In the latter process, ADP release was required for JNK activation, although ADP alone was not sufficient to induce JNK activation. In contrast, we demonstrated that oxLDL-induced JNK activation was not dependent on ADP release. Integrin outside-in signaling was also not required for JNK activation induced by oxLDL, suggesting oxLDL-platelet interactions directly trigger the signaling cascade. We also showed that JNK phosphorylation from one CD36-null donor was decreased. However, because of the availability of other CD36-null donors, we addressed CD36-dependency later with *cd36*^{-/-} mice we generated in the laboratory (described in chapter 5.3).

Our studies and those of others have also shown that another member of the MAP kinase family, p38, is phosphorylated in platelets after exposure to oxLDL⁶⁷. Whether p38 and JNK work synergistically or independently in the process of platelet activation by oxLDL remains to be determined.

Eitzman *et al* showed previously *apoe*^{-/-} mice on high-fat have shorter thrombosis time, suggesting the presence of a prothrombotic phenotype¹¹³. Podrez *et al* later showed that CD36 deficiency in the *apoe*^{-/-} background rescues the prothrombotic phenotype⁹⁷. LC-ESI/MS/MS revealed that the level of oxPC_{CD36} lipids is elevated more than 10-fold in western diet fed *apoe*^{-/-} mice. In studies described here, with *apoe* null mice fed a high fat “western”

diet as a model of hyperlipidemia and oxidant stress, we demonstrated that there was an increase in the level of phosphorylated JNK in platelets from *apoe*^{-/-} mice on western diet in compare to those from WT mice on chow diet. Together with previous results, we showed that JNK phosphorylation in platelets was induced by oxLDL *in vitro* as well as hyperlipidemia *in vivo*. The hyperlipidemia-induced JNK phosphorylation was probably mediated by CD36 endogenous ligands such as oxPC_{CD36}. Interestingly, we found that JNK phosphorylation in platelets was significantly elevated in *apoe*^{-/-} mice fed on high-fat western diet in comparison to those fed a normal chow diet. Since *apoe*^{-/-} mice have a defect in lipoprotein metabolism regardless of their diet, our results indicate that JNK phosphorylation in platelets was not only caused by the increase in lipoprotein concentration. Both elevated levels of lipoproteins and high-fat western diet are necessary to induce platelet signaling, rendering platelets hyperactive.

With *apoe*^{-/-}; *cd36*^{-/-} mice on western diet, we showed that the elevated levels of phosphorylated JNK in platelets was normalized in the absence of CD36 in *apoe* null background. This data suggest that hyperlipidemia-induced JNK phosphorylation was CD36-dependent. The results correlate the previous findings that CD36 deficiency rescued the prothrombotic state of *apoe*^{-/-} mice, suggesting that the increased basal JNK activity contributed to CD36-dependent platelet hyper-reactivity associated with hyperlipidemia. The

contribution of JNK to enhanced platelet activity associated with hyperlipidemic mice will need to be addressed in *apoe*^{-/-}; *jnk2*^{-/-} and *apoe*^{-/-}; *jnk*^{-/-} mice in the future.

The precise function of JNK in platelet biology also remains to be determined. It is known to be involved in a wide variety of diverse cellular processes through transcription-dependent and transcription independent mechanisms⁵⁷. Since platelets are anucleate cells and the time course of platelet activation is very rapid, it is unlikely that JNK action would be transcription-dependent. It will be important to define substrates of JNK in platelets to further define its functional role. Since MAP kinase is closely related to cytoskeletal function, we hypothesize that in platelets, JNK is involved in a signaling cascade resulting in cytoskeletal reorganization. One potential candidate as the target of JNK is 14-3-3 zeta. It has been shown that 14-3-3 is phosphorylated by JNK in nucleated cells and responsible for translocation of Bax to mitochondria, triggering cytochrome c release and apoptosis¹²⁵. Studies also demonstrated that in platelets, 14-3-3 proteins play an important role in cytoskeletal reorganization¹²⁶. These data suggest that 14-3-3 may be downstream molecules of JNK mediating its biological effect.

How platelet CD36 transduces signal in platelets was not clear, although it has been demonstrated previously that platelet CD36 physically associates

with src family kinases Fyn, Lyn and Yes. In studies presented here, we have shown that pharmacological inhibition of src family kinases blocked JNK phosphorylation. This result suggests that JNK phosphorylation induced by oxLDL is mediated by src family kinase members. Given the fact that platelet CD36 physically associates with Fyn, Lyn and Yes, we demonstrated by co-immunoprecipitation that the recruitment of src family kinases Fyn and Lyn by platelet CD36 indeed play an essential role in platelet CD36 signaling triggered by oxLDL. There was no change in the levels of “active” Fyn or Lyn in the fractions not associated with CD36. These studies thus suggest that CD36 functions to assemble a signaling complex in a ligand-dependent manner. It may represent a common mechanism for how CD36 transduces signals regardless of cell types and the nature of ligands. The mechanism by which the recruitment of src family kinases to CD36 transduces signals is not clear. It is possible that CD36 clusters active Fyn and Lyn in specific plasma membrane domains and that this allows sufficient kinase activity to induce downstream signaling events and eventually MAP kinase activation. A well described example of cluster signaling is the Raf-Ras-MEK-ERK cascade¹²⁷. Recent imaging techniques have showed that GTP-Ras formed a discrete signaling nanocluster, and ablation of critical scaffold proteins abolished nanocluster formation and ERK activation. In the case of CD36 signaling in platelets, it is possible that CD36 clusters active Fyn and Lyn upon ligand binding, triggering localized signaling events. This model provides an

explanation for the requirement of src kinase activity in CD36 signaling as shown by pharmacological inhibition, even in the absence of global changes in src activation.

Signaling events immediately downstream of src family kinases have not been defined. The studies outlined here have identified the Rho/Rac GEF Vav as a critical modulator transducing pro-thrombotic signals and that the tyrosine phosphorylation of Vav by oxLDL was mediated by src family kinases. The studies also provide insights into the mechanism how Fyn mediates the tyrosine phosphorylation of Vav in platelets in this process. Interestingly, both Fyn and Lyn have been implicated in the tyrosine phosphorylation of Vav in other cell types. Co-immunoprecipitation revealed that Vav-associated Fyn but not Lyn underwent an increase in tyrosine phosphorylation following oxLDL stimulation. These studies suggest that oxLDL induced the phosphorylation of Vav-associated Fyn as a mechanism to up-regulate the tyrosine phosphorylation of Vav in platelets. Further studies are in need to address if Vav is upstream of JNK in prothrombotic signaling pathway; To address this question *in vivo*, we are currently breeding *apoe*^{-/-}; *vav*^{-/-} mice. With these mice in hand, we will be able to examine if platelets from western diet fed *apoe*^{-/-}; *vav*^{-/-} mice have decreased level of phosphorylated JNK in compare to those from *apoe*^{-/-} mice.

In an attempt to determine if JNK phosphorylation may contribute to oxLDL-mediated platelet activation, we demonstrated that pharmacological inhibition of JNK reduced significantly platelet activation by oxLDL, mimicking the effect of CD36 deficiency. Interestingly, we found that pharmacological inhibition of JNK had no effect of platelet activation by other physiological agonists exemplified by ADP. This data implies that JNK may be specifically involved in platelet activation by ligands generated under pathological condition. These pathological ligands including oxLDL are sensed by platelet CD36, triggering CD36 signaling and leading to pathological platelet activation. We also showed that pharmacological inhibition of src family kinases reduced platelet activation by oxLDL. In sum, these data suggest that platelet CD36 signaling involving src family kinases and MAP kinase JNK plays a functional role in platelet activation by oxLDL. It should be noted pharmacological inhibition of JNK or src did not result in complete inhibition of platelet activation by oxLDL. These results raised possibility that alternative activation pathways were involved. Consistent with that, we also showed oxLDL induced phosphorylation of p38 in platelets. It will be interesting to see if pharmacological inhibition of both JNK and p38 may result in complete block the effect of oxLDL on platelets.

Ghosh *et al* in our laboratory showed that *cd36* null mice are less sensitive to carotid injury³¹. This suggests that CD36 may play a role in the

normal function of platelets in response to injury. One likely explanation for hypo-sensitivity of platelets from CD36 null mice is that there exist some putative CD36 ligand(s) other than oxLDL-like particles and CD36 deficiency blocks the signaling initiated by these ligands. We then hypothesized that these CD36 ligands will contribute to platelets activation through the same signaling cascade triggered by oxLDL-CD36 interaction, which involves JNK. According to this hypothesis, the inactivation of JNK should also mimic CD36 deficiency in the wild type background. With carotid artery thrombosis model in chow-fed mice, we first showed that phosphorylation of platelet JNK during thrombus formation *in vivo* was, in part, CD36-dependent. Further, with a specific JNK inhibitor, we demonstrated that indeed inactivation of platelet JNK produced a significant anti-thrombotic effect in a CD36-dependent manner. Altogether, these data suggest that platelet CD36-JNK signaling axis contributes to thrombosis *in vivo*. The nature of the CD36 ligands remains to be defined. With the data from Ghosh *et al*, it is intriguing to speculate the endothelial cell-derived microparticles could function in this capacity³¹. It will be interesting to address if cell-derived microparticles are able to induce JNK phosphorylation in platelets *in vitro*. It is possible that CD36-JNK signaling axis has more general role in transducing prothrombotic signals from pathological ligands other than oxLDL.

Podrez *et al* previously demonstrated that *apoe*^{-/-} mice fed on high-fat

western diet had significantly increased thrombotic response in comparison to WT as well *apoe*^{-/-} mice on normal chow⁹⁷. Given the fact that *apoe*^{-/-} mice have defects in lipoprotein metabolism regardless of the diet content, the studies suggest that both dysregulated lipoprotein levels and high-fat dietary contribute to the pro-thrombotic state associated with hyperlipidemia. The effect of high-fat diet on platelet reactivity has not been carefully examined. In this study, we challenged C57Bl/6 WT mice with high-fat feeding for 2 weeks and compared the ADP-induced aggregation of platelets from these high fat fed mice with those from mice on normal diet. In this study, aggregometry revealed that there was a diet-induced increase in ADP-induced aggregation response of platelets from C57Bl/6 mice on western diet. The data suggest that high fat diet alone may be enough to trigger a pro-thrombotic state. Interestingly, we also demonstrated that enhanced platelet reactivity was absent in *vav*^{-/-} mice in the C57Bl/6 background. We also confirmed that oxPC_{CD36} are present in the plasma from these *vav*^{-/-} mice on western diet. Altogether, these results suggest that Vav may be a critical modulator transducing diet-induced pro-thrombotic signals in platelets.

The role of Vav in platelet biology has not been extensively studied. Previous studies showed that the tyrosine phosphorylation of Vav was induced by thrombin and collagen^{74, 128, 129}. Vav also contributed to platelet activation by both thrombin and collagen. Our study extended our knowledge about the

functional role of Vav in platelets, showing that tyrosine phosphorylation was induced by oxLDL and that Vav plays an essential role in transducing a pro-thrombotic signal. The precise role of Vav remains to be determined. The absence of diet-induced aggregation response in *vav*^{-/-} mice could be explained by a role of Vav in inside-out signaling or outside-in signaling by the integrin $\alpha_{IIb}\beta_3$.¹³⁰ Although Vav is best known for its GEF activity toward Rac/Rho, Vav also functions as an adaptor protein in platelets (described in chapter 1.8). It also remains to be determined whether Vav functions as GEF or adaptor protein in transducing pro-thrombotic signals.

In sum, we have identified a platelet CD36-initiated signaling pathway triggered by oxLDL (Figure 11-1). In this pathway, oxLDL binds to CD36. Upon binding, CD36 recruits phosphorylated Fyn and Lyn. The signaling complex then induces subsequent phosphorylation of MKK4 and JNK. Phosphorylated Fyn also induces Vav phosphorylation. The signaling pathway may be involved in clinical association between dyslipidemia and prothrombotic phenotype. Targeting this pathway may provide therapeutic strategies for atherothrombosis rather than normal hemostasis. It is to be assessed if Vav is upstream of JNK in this pathway. It is also to be determined the function of Vav in platelets. Previous studies suggest in platelets Vav may modulate integrin signaling and thus platelet aggregation.

11.2 Problems and pitfalls

As described in chapter 1.4, platelets are anucleate cells. They can not be genetically manipulated. With *cd36*^{-/-} mice and *vav*^{-/-} mice, we were able to perform experiments showing CD36-dependency and Vav-dependency of signaling events. However, the remaining functional experiments were mostly addressed with pharmacological inhibitors due to the availability issues of corresponding genetic engineered mice. Although src family kinase inhibitor AG1879 and JNK inhibitor SP600125 were proven to be highly specific and in our experiments we tended to use minimal action dose of inhibitors, the potential off-target effect can not be excluded. It will be interesting to examine our CD36 signaling model further with *Jnk2*^{-/-} mice, *fyn*^{-/-} and *lyn*^{-/-} mice.

OxLDL is a complex particle and can contain a variety of biologically active lipids other than CD36 ligands, including lysophosphatidylcholine (LPC), platelet-activating factor (PAF), lysophosphatidic acid (LPA), and 9- and 13-HODE¹³¹. The present studies identified JNK and Vav as critical modulators for pro-thrombotic signals. Podrez *et al* also revealed the presence of oxPC_{CD36} in the plasma of mice. The data suggests that oxPC_{CD36} may be responsible for platelet CD36 signaling, however, it remains to be determined if oxPC_{CD36} induces JNK phosphorylation and Vav phosphorylation. The role of these other lipids in this process remains unknown. Others have shown that

Figure 11-1 A schematic overview of platelet CD36 signaling outlined in this study. Upon oxLDL binding, the C-terminal cytoplasmic tail of CD36 recruits src family kinases Fyn and Lyn in their active phosphorylated form. These events result in the downstream signaling events including the phosphorylation of JNK and its upstream activator MKK4. Active phosphorylated Fyn also induces Vav phosphorylation. Phosphorylated Vav serves as guanine nucleotide exchange factor (GEF) for small GTPases: Rho, Rac and Cdc42. It remains to be determined if Vav is upstream of JNK. Also, it is speculated that Vav may modulate integrin signaling and thus platelet aggregation, in which case, Vav play some functional role other than GEF.

LPA in oxLDL can induce platelet shape change via a specific G-protein coupled LPA receptor. The signaling pathways triggered by LPA involve tyrosine phosphorylation of specific proteins including Syk and an increase of cytosolic Ca^{2+} ¹¹⁶. It is also possible that LPA or PAF receptors may work in a synergic way with oxPC_{CD36} and contribute to pro-thrombotic signals.

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