Cholesterol in platelet biogenesis and activation

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Abstract
Hypercholesterolemia is a well-established risk factor for atherothrombotic disease, largely attributed to its impact on lesional cells in atherosclerotic plaques such as macrophages and their critical roles in inflammation, atherogenesis, plaque rupture and athero-thrombosis. Platelets are involved in immune and inflammatory responses and directly impact atherogenesis, primarily by modulating immune and inflammatory effector cells. There is emerging evidence that hypercholesterolemia increases the risk of atherosclerosis and arterial thrombosis by modulating platelet biogenesis and activity. This review highlights recent findings on the impact of aberrant cholesterol metabolism on platelet biogenesis and activity and their relevance in atherosclerosis and thrombosis.

Platelets and atherosclerosis
Platelets are best known for their roles in hemostasis and thrombosis. However, there is an increasing appreciation of the critical roles of platelets in immunity and inflammation in both health and disease. Atherosclerosis is a lipid-driven chronic inflammatory disease which involves localized recruitment of myeloid and immune cells including neutrophils, monocytes and lymphocytes to large and medium sized arteries. Platelets appear to play a key role in recruitment of these inflammatory effector cells. Activated platelets interact with endothelial cells of inflamed or atherosclerotic arteries and deposit platelet-derived cytokines such as CCL5 (Rantes) or CXCL4 (PF4) onto the surface of endothelial cells, facilitating recruitment of leukocytes into the lesions. Genetic deficiency of CCL5 and CXCL4 or pharmacological disruption of the functional heteromerization between CCL5 and CXCL4 decreases atherogenesis in animal models. Activated platelets form aggregates with neutrophils and monocytes and the ensuing crosstalk between platelets and leukocytes also plays a key role in inflammatory cytokine production, leukotriene biosynthesis and reactive oxygen species production, with pro-inflammatory consequences in the vasculature. Platelet-leukocyte aggregates (PLA) are an independent risk factor for atherothrombotic disease and promote atherogenesis in mouse models (Fig. 1). An elegant recent study from Sreeramkumar et al demonstrated that neutrophils bound to endothelium scan the bloodstream for activated platelets, leading to neutrophil polarization and formation of PLA and facilitating neutrophil migration into inflamed blood vessels. This interaction of platelets with leukocytes is initiated by the binding of platelet P-selectin to P-selectin ligand which localizes to the uropod (tail) of neutrophils as they bind to endothelium. Disruption of platelet/leukocyte interactions via genetic deficiency of P-selectin or by anti-P-selectin blocking antibodies decreases leukocyte
recruitment and atherogenesis. Consistently, infusion of activated wild type but not P-selectin deficient platelets into Apoe<sup>−/−</sup> mice increases atherosclerosis. In addition to modulating migration and recruitment of leukocytes, platelet-leukocyte interactions could potentially impact atherosclerosis and athero-thrombosis by modulating other activities of leukocytes. Neutrophil extracellular traps (NETs) generated during NETosis promote venous and arterial thrombosis in animal models and recent evidence suggests a role of NETs in atherogenesis and atherothrombotic disorders as well. Interestingly, activated platelets interact with neutrophils and promote NETosis, which appears to require P-selectin/PSGL-1 interactions. Thus, activated platelets could potentially promote atherosclerosis and thrombogenesis by facilitating NET generation. Other roles of platelets in atherosclerosis have been reviewed and discussed elsewhere.

**Cholesterol and platelet activation**

Hyperlipidemia as exemplified by familial hypercholesterolemia is associated with increased platelet activation and an underlying pro-coagulant state. Hyperlipidemia primes platelets and increases platelet activation in response to various agonists. Plasma cholesterol levels appear to have a critical role in modulating platelet activity as hypercholesterolemia increases platelet activation more potently than hypertriglyceridemia. Hyperlipidemia increases platelet activation likely via multiple mechanisms. Oxidized LDL or oxidized phospholipids, which are increased in hyperlipidemia, serve as ligands of platelet CD36 and activate platelets. Oxidized lipids also promote formation of pro-coagulant tissue factor microparticles derived from monocytes. In vitro cholesterol-loading also increases human platelet activation. HDL is a cholesterol acceptor and HDL promotes cholesterol efflux. HDL has been shown to mediate various antithrombotic effects albeit some of the effects are via the impact on the vasculature such as reducing endothelial cell surface expression of adhesion molecules. Infusions of a reconstituted HDL (rHDL) preparation reduced ex vivo platelet activation in diabetic subjects, likely by promoting cholesterol efflux from platelets. These findings in humans are consistent with observations in animal studies. A striking example is the markedly increased platelet activation and thrombosis in Scarb<sup>−/−</sup> mice. Scarb<sup>−/−</sup> mice have an unusually high plasma unesterified-to-total cholesterol ratio, reflecting impaired delivery of cholesterol by HDL from plasma and peripheral tissues back to the liver via hepatic scavenger receptor class B type I (SR-BI). While the major function of hepatic SR-BI is to mediate selective uptake of cholesterol and cholesteryl ester from HDL, SR-BI mediated cellular unesterified cholesterol efflux to HDL has been reported. The net unesterified cholesterol influx or efflux facilitated by SR-BI likely
depends on the type of cells and the relative cholesterol/phospholipid ratio in the cell membrane versus in HDL\textsuperscript{40, 41}. While SR-BI is expressed in platelets, platelet cholesterol overload due to markedly increased unesterified cholesterol content in plasma lipoproteins but not intrinsic SR-BI deficiency in platelets or other hematopoietic cells is responsible for the heightened platelet activation in \textit{Scarb1\textsuperscript{-/-}} mice\textsuperscript{29}. As a consequence, \textit{Scarb1\textsuperscript{-/-}} mice developed spontaneous occlusive arterial thrombosis and premature death when introduced into hypercholesterolemic \textit{apoE\textsuperscript{-/-}} or \textit{Ldlr\textsuperscript{-/-}} background\textsuperscript{42, 43}. Despite this accumulating evidence indicating a direct effect of cholesterol enrichment on platelet activation, the underlying mechanisms have not been elucidated. Cholesterol accumulation in plasma membranes disturbs membrane structures, particularly the cholesterol-rich specialized microdomains of lipid rafts\textsuperscript{44}. Enhanced signaling of cell surface receptors located in lipid rafts has been reported in various hematopoietic effector cells in response to membrane cholesterol accumulation\textsuperscript{45, 46}. Thus, plasma membrane cholesterol accumulation in platelets could potentially alter the membrane structure and affect signaling via surface receptors. One study suggests that rHDL infusions suppress platelet activation by reducing lipid raft assembly\textsuperscript{35}. SR-BI is a receptor for HDL and there is evidence that HDL suppresses thrombin-induced platelet aggregation by binding to SR-BI and generating inhibitory signals\textsuperscript{47}. In contrast, rHDL can suppress platelet activation via a mechanism independent of SR-BI\textsuperscript{35}, likely due to the fact that the cholesterol-free, phospholipid-rich rHDL can promote passive cholesterol efflux independent of transporters such as ABCA1, ABCG1 or SR-BI.

**Cholesterol and platelet biogenesis**

Platelets are derived from megakaryocytes and the latter from megakaryocyte progenitor cells (MkP) in the bone marrow and spleen\textsuperscript{48}. Thrombopoietin (TPO) and its cognate receptor C-MPL acts as a key growth factor signaling pathway in megakaryopoiesis and platelet biogenesis\textsuperscript{49}. Genetic deficiency of TPO or C-MPL causes marked thrombocytopenia\textsuperscript{49} while increased signaling in this pathway results in elevated platelet production and thrombocytosis\textsuperscript{50}. Plasma lipid levels have long been linked to platelet biogenesis and/or turnover. An analysis of two independent studies involving ~10,000 participants indicates a positive correlation of non-HDL cholesterol levels with platelet counts\textsuperscript{51}, consistent with some other studies\textsuperscript{52, 53}. Hyperlipidemia also is associated with shortened platelet survival and increased turnover, particularly in the setting of overt atherosclerosis\textsuperscript{54-56}. Together, these findings suggest promotion of platelet production by hypercholesterolemia (Fig. 1). TPO and C-MPL function to maintain platelet homeostasis, with TPO/C-MPL signaling in HSCs, MkPs and megakaryocytes regulating
megakaryocyte and platelet production and megakaryocyte and platelet c-MPL acting as a sink for plasma TPO and mediating its internalization and turnover. Recent studies indicate an additional mechanism regulating platelet production in which aged desialylated platelets bind the hepatic Ashwell-Morell receptor and thereby stimulate hepatocyte TPO production. Increased platelet turnover is often associated with increased platelet biogenesis via elevated TPO. The newly generated platelets are larger, more dense and RNA rich and these so-called reticulated platelets are generally more active than the more mature platelets.

Hypercholesterolemia is positively associated with the mean volume of platelets and ploidy of megakaryocytes in humans, suggesting the possibility that hypercholesterolemia primes platelets and increases platelet activity by promoting platelet production. This is expected to increase the risk of CHD. Consistently, there is strong evidence that mean platelet volume and counts of reticulated platelets are positively associated with acute coronary syndrome, and one study shows that baseline platelet counts are independent risk factors for acute coronary syndrome. These findings in humans have been recapitulated in animal studies. Unesterified cholesterol accumulation in platelets of Scarb−/− mice led to increased platelet turnover and clearance from circulation. This resulted in increased platelet biogenesis and produced juvenile platelets with greatly increased volume, likely as a consequence of the feedback regulation. Thus, the highly activated platelets in the circulation in Scarb−/− mice could be the result of the combined effect of increased platelet biogenesis as well as a direct impact of platelet cholesterol enrichment on platelet activation. Moreover, dietary hypercholesterolemia led to thrombocytosis and leukocytosis as a result of bone marrow hematopoietic progenitor cell mobilization and altered interactions of megakaryocytes with endothelial cells due to disturbed CXCL12 and CXC4 signaling. However, the diet used in this study contained a very high cholesterol content as well as cholate and was pro-inflammatory; thus, effects may not be directly attributable to a direct impact of cholesterol in bone marrow progenitor populations. A recent study indicates that damage associated molecular patterns promote bone marrow hematopoietic progenitor cell mobilization and extramedullary hematopoiesis. Nevertheless, another study assessed the impact of hypercholesterolemia induced by a high fat high cholesterol diet in Ldlr−/− mice relative to the chow-fed Ldlr−/− mice on hematopoietic cells and showed an expansion of the pool of bone marrow hematopoietic stem and progenitor cells (HSPC), in association with increased myelopoiesis. This study in younger mice examined platelet counts and found no change. However, we have detected increased platelet counts in ~12 month old chow-fed Ldlr−/− mice (~12% increase, unpublished observation). Despite these
observations, how hypercholesterolemia modulates platelet biogenesis or turnover at the molecular level remains enigmatic.

**Mechanisms of cholesterol homeostasis in MkPs**

Cholesterol homeostasis in hematopoietic cells is maintained in part by mechanisms involving ATP-binding cassette transporters and apolipoproteins such as apolipoprotein E (ApoE) which promote cellular cholesterol efflux. We showed increased myelopoiesis in ABCA1/ABCG1 or apoE deficient mice due to increased cellular cholesterol accumulation in HSPCs as a result of defective cholesterol efflux. At the molecular level, this was attributed to the increased cell surface levels and signaling of common β subunit of IL-3 and GM-CSF receptors. Increased myelopoiesis resulted in leukocytosis and accelerated atherosclerosis in these models. In subsequent studies, we found that deficiency of hematopoietic ABCG4, a transporter highly homologous to ABCG1 and actively promoting cholesterol efflux to HDL, increased atherosclerosis in association with increased platelet counts but without any change of plasma TPO levels in hypercholesterolemic Ldlr−/− mice. Selective thrombocytosis in bone marrow ABCG4 deficiency in combination with restricted Abcg4 expression in MkPs but no or low expression in platelets suggested selectively increased megakaryopoiesis and platelet production. Indeed, MkPs and megakaryocytes but not HSPCs or progenitors of other hematopoietic lineages were increased in Abcg4−/− mice. As a result, hematopoietic ABCG4 deficiency led to defective cholesterol efflux to HDL and increased free cholesterol accumulation in MkPs including plasma membranes, in association with increased C-MPL levels on the surface of Abcg4−/− MkPs, increased cell proliferation in response to TPO, increased megakaryopoiesis and more pronounced increase in platelet counts in response to TPO injection. The increased cell surface C-MPL levels in Abcg4−/− MkPs were because of blunting of the negative feedback regulation of C-MPL in response to TPO and involved a defective activation of Lyn kinase and c-CBL E3 ligase. Lyn kinase, a palmitoylated membrane protein, seems to act as a membrane cholesterol sensor. Increased membrane cholesterol in Abcg4−/− MkPs may increase Lyn association with the membrane and decrease its tyrosine kinase activity in response to TPO, causing defective phosphorylation of c-CBL. This disrupts the negative feedback regulation of C-MPL and leads to increased TPO/c-MPL signaling and platelet production (Fig. 2). In addition to increased atherosclerosis, Abcg4−/− mice also showed accelerated arterial thrombosis, in association with increased reticulated platelets, platelet/leukocyte complexes, and platelet-derived microparticles, all with proven proatherosclerotic and prothrombotic properties. These studies link increased platelet...
production, initiated from aberrant cholesterol metabolism in its lineage progenitor cells, to accelerated atherosclerosis and arterial thrombosis.

Infusion of rHDL reduced MkP proliferation and platelet counts in wild-type mice but not in Abcg4+/− mice. The therapeutic potential of rHDL infusions in the control of platelet overproduction was exemplified by the finding that in a mouse model of essential thrombocytopenia induced by BM cell expression of a mutant form of c-C-MPL found in human subjects with essential thrombocytopenia71, rHDL reduced the platelet count in mice receiving Abcg4+/+ but not Abcg4−/− BM cells.68

In addition to ABCG4, ABCB6 also is highly expressed in MkPs. and hematopoietic ABCB6 deficiency increases atherogenesis in hypercholesterolemic Ldlr−/− mice, in association with selectively increased MkPs, megakaryocytes, total and reticulated platelet counts and platelet activity72. Unlike ABCG4, ABCB6 is reported to have transporter activity for porphyrin but not cholesterol73. The detailed molecular mechanism linking ABCB6 deficiency to increased MkP proliferation and platelet production is unknown but increased oxidative stress in Abcb6−/− MkPs has been suggested to contribute to these phenotypes.

In summary, platelets have critical roles in atherosclerosis and athero-thrombosis. The accumulation of cholesterol in platelets or their progenitors, reflecting hypercholesterolemia or defective cholesterol efflux pathways, markedly increases platelet biogenesis, turnover and activity, potentially contributing to atherogenesis and athero-thrombosis. While the mechanistic understanding of these processes is at an early stage, gaining further insights into the regulation of platelet production and activation by cholesterol and other lipids could open a new window on treatments for athero-thrombosis.
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Conflict of interest disclosure

The authors declare no competing financial interests.


Fig. 1. Hypercholesterolemia is a risk factor for atherothrombotic disease by promoting platelet production and activation. Hypercholesterolemia promotes megakaryopoiesis, platelet (PLT) biogenesis and myelopoiesis, leading to leukocytosis. Hypercholesterolemia also increases platelet activation, likely by elevating platelet production and direct impact on platelets. Activated platelets form platelet-leukocyte aggregates (PLA), which are further increased in leukocytosis. PLA is an independent risk factor for atherothrombotic disease.
Fig. 2. Working model. HDL-mediated cholesterol efflux from MkPs via ABCG4 increases activity of the palmitoylated Lyn kinase, c-CBL phosphorylation and activation as well as c-MPL degradation, limiting MkP proliferation and platelet production.
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