Platelets in atherosclerosis

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Summary

Beyond obvious functions in haemostasis and thrombosis, platelets are considered to be essential in proinflammatory surroundings such as atherosclerosis, allergy, rheumatoid arthritis and even cancer. In atherosclerosis, platelets facilitate the recruitment of inflammatory cells towards the lesion sites and release a plethora of inflammatory mediators, thereby enforcing and boosting the inflammatory milieu. Platelets do so by interacting with endothelial cells, circulating leukocytes (monocytes, neutrophils, dendritic cells, T-cells) and progenitor cells. This cross-talk enforces leukocyte activation, adhesion and transmigration. Furthermore, platelets are known to function in innate host defense through the release of antimicrobial peptides and the expression of pattern recognition receptors. In severe sepsis, platelets are able to trigger the formation of neutrophil extracellular traps (NETs), which bind and clear pathogens. The present antiplatelet therapies that target key pathways of platelet activation and aggregation therefore hold the potential to modulate platelet-derived immune functions by reducing cellular interactions of platelets with other immune components and by reducing the secretion of inflammatory proteins into the milieu. The objective of this review is to update and discuss the current perceptions of the platelet immune constituents and their prospect as therapeutic targets in an atherosclerotic setting.

Keywords

Inflammation, atherosclerosis, platelet immunology

Introduction

Platelets, anucleated cells of 1–2 μm in length and pinched-off multinuclear megakaryocytes in the bone marrow, have been acknowledged since 1865 for their primary physiological role in sensing damaged vessel endothelium and accumulating at sites of injury, where they initiate blood clotting (1). However, there is a constantly growing recognition that platelets modulate immune responses (2, 3). Platelets are well equipped to facilitate leukocyte recruitment to sites of vascular injury and inflammation (Fig. 1). Furthermore, platelets have been described to interact with bacterial pathogens and even express complement receptors (4). Platelets store and release antibacterial proteins (referred to as thrombocidins) which strengthens the concept that platelets are vital protagonists in immunity (3, 5). Thus, activated platelets have distinct modi operandi to initiate immune reactions (6).

Upon activation, platelets produce considerable quantities of cytokines and chemokines (7) which are released from the α-granules (8). Proteomic studies indicate that thrombin-stimulated platelets release more than 300 distinct proteins (9). Several of these secreted proteins have been identified in atherosclerotic lesions (10, 11). The release of inflammatory mediators boosts the inflammatory milieu at sites of platelet activation (e.g. vascular injury) leading to cell activation and cell migration/recruitment. Moreover, activated platelets express a multitude of immune receptors fortifying their immunological competence. These receptors enable platelets to interact with different leukocyte subsets and endothelial cells. Activated platelets interact with endothelial cells and induce the expression of cell adhesion molecules and chemokines, which in turn mediate leukocyte recruitment. Platelet activation also results in an increase in circulating platelet-leukocyte aggregates (PLA), which are protagonists of inflammatory reactions of the vessel wall (12, 13). In particular, platelet-monocyte complexes (PMC) have been observed in clinical conditions, where increased adhesion of platelets to monocytes is associated with ischaemic events (13, 14). Furthermore, platelets communicate with neutrophils (15, 16), dendritic cells (17, 18) and T cells (19). It is important to recognise that the communication or crosstalk between platelets and leukocytes or endothelial cells is often bidirectional (13). Platelets stimulate leukocyte differentiation into a pro-adhesive and pro-migratory phenotype, and the leukocytes secrete mediators that further activate the platelet.

Besides cellular interactions of platelets with blood cells and vascular cells, interactions with lipoproteins appear to alter platelet function (20). Platelets of hypercholesterolaemic patients show hyperaggregability in vitro and enhanced activity in vivo (21, 22). It is an emerging concept that platelets have the ability to bind, take up and transport modified lipoproteins (21). The purpose of this...
review is to explore and define the competence of platelets as inflammatory mediators in atherosclerosis and to introduce the concept that platelets are likely to be one of the first contestants of inflammation. In addition, we depict the potential of current and future antiplatelet drugs in trimming platelet-induced inflammation.

**From thrombus to inflammation**

In order to appreciate the inflammatory propensity of platelets, it is important to comprehend their elementary biology. Under physiological conditions, platelets circulate in a quiescent state. Resting platelets do not express P-selectin or CD40L and do not form PLAs. However, patients with advanced cardiovascular disease display a higher platelet activation status. The role of enhanced platelet activation in the progression from initial lipid retention in the arterial wall to clinical events of atherosclerotic plaque formation is still not completely understood (23). It is not known whether the platelet exerts its inflammatory functions by chronically interacting with endothelium and leukocytes, or if platelets, in time, cause multiple asymptomatic thrombi wherein they secrete their inflammatory mediators and promote leukocyte recruitment.

The mechanism of thrombus formation can be divided into four steps: platelet tethering, activation and firm adhesion, aggregation and platelet recruitment, and finally thrombus stabilisation (24). Platelet tethering (1) is mediated by glycoprotein (GP) Ibα, which is a component of the GPIb-V-IX complex. GPIbα is constitutively expressed on platelets and initiates platelet adhesion by binding to collagen bound von Willebrand factor (VWF) (25). Subsequent binding of GPVI to collagen leads to platelet activation (26, 27). GPVI is the major agonist for initial platelet activation and granule release (28). In addition, Massberg et al. showed that GPVI-collagen interactions are central in all major phases of thrombus formation, i.e. platelet tethering, firm adhesion, and aggregation (29). Thrombin is rapidly generated at the site of vascular injury and is considered the most potent platelet activator, leading to shape change, integrin activation, and granule secretion. Once firmly adherent, platelets start to spread and release the content of their granules (3). Adenosine diphosphate (ADP) and thromboxane A2 (TXA2) are released, further promoting activation leading to the conformational change of the integrin αIIbβ3 that increases its affinity for fibrinogen, VWF and fibronectin (30). In its active form, αIIbβ3 is the key molecule for platelet aggregation and stabilisation (24). During platelet activation, both growth-arrest-specific gene 6 (GAS6) and CD40 ligand (CD40L) are present in the platelet-platelet synapse and enhance stabilisation (31, 32). Ho-Tin-Noe et al. (33) recently demonstrated, in a mouse model of spontaneous tumour haemorrhage, that bleeding during thrombocytopenia involves leukocyte recruitment, which can be provoked by tumour necrosis factor (TNF)-α. Furthermore, depletion of neutrophils abolished the ability of TNF-α to cause bleeding in thrombocytopenic mice (33). Thus, inflammation significantly alters the procoagulant and anticoagulant setting, leading to enhanced crosstalk between platelets, leukocytes and endothelial cells. We therefore need to invest in studying the effects of platelets in atherosclerosis formation, which is considered to be predominantly a leukocyte driven disease and in the role of leukocytes in thrombus formation, a platelet driven process.

![Surface molecules, expressed by activated platelets, facilitating the cross-talk with leukocytes and endothelial cells.](image-url)
Surface molecules

Glycoproteins

Platelet glycoproteins GPIbα and GPIIb/IIIa (αIIbβ3) have important roles in platelet-endothelial interactions. Deletion of GPIbα results in severe bleeding, abnormal giant platelets, and severe thrombocytopenia (24). Massberg et al. showed in an elegant study that inhibition of platelet GPIbα significantly reduced both transient and firm adhesion to the vascular surface of the common carotid artery. In contrast, inhibition of αIIbβ3 (Fig. 1) had only partial effects on transient platelet adhesion but almost completely prevented firm attachment to endothelial cells in vivo (34). The main ligand of GPIbα is VWF; however, platelet GPIbα is also the best-characterised counter receptor for Mac-1, which indicates a strong link towards platelet-leukocyte interactions. In a mouse model of neointima formation, inhibition of Mac-1-GPIIa interactions after wire injury of the femoral artery reduced leukocyte accumulation and neointima thickening (35). αIIbβ3 is the key molecule for platelet aggregation and stabilisation (24). By forming a bridge via macromolecular proteins, such as VWF, fibrinogen, or fibronectin, αIIbβ3 mediates arrest of activated platelets to adhesion molecules intercellular adhesion molecule (ICAM)-1 and αvβ3 on endothelium. Signalling through the GPIb-IX-V receptor leads to the activation of αIIbβ3 (36). The activated conformation is maintained by continuous ADP stimulation otherwise it decreases and the thrombus becomes unstable (37). While the ADP signalling via P2Y12 is slow and persistent is the signalling via DAG-GEFI rapid and crucial for thrombus formation (38). In the process of thrombus formation can platelet aggregation already be initiated by changes in flow pattern (Fig. 3), and thrombus growth and stabilisation happens through platelet-platelet interactions mediated by αIIbβ3 (39). The knowledge about the role of αIIbβ3 in atheroclerosis in humans is scarce. We know that patients with Glanzman thrombasthenia are not protected from atherosclerosis despite functional absence of αIIbβ3 (40). A common single nucleotide polymorphism (SNP) known as the Leu33Pro polymorphism leads to increased platelet activation as determined by higher percentage of P-selectin-positive platelets. Carriers of this variant have been found to have the same plaque volume in the carotid artery as analysed by magnetic resonance imaging but interestingly a more vulnerable plaque phenotype (41). In mice, the genetic deletion of the α-subunit of αIIbβ3 in ApoE knockout mice resulted in less atherosclerosis by preventing platelet adhesion to both dysfunctional endothelial cells and extracellular matrix after wire injury induced endothelial denudation (42).

Another platelet-specific glycoprotein that mediates platelet-endothelium adhesion is glycoprotein VI (GPVI). GPVI is upregulated on circulating platelets in acute coronary syndrome (43), and GPVI (Fig. 1) binds to atherosclerotic endothelium and contributes to platelet-endothelium interactions. As a consequence, inhibition of GPVI decreases atherosclerosis progression (44). GPVI, which is actually known for its interaction with collagen, seems to contribute to weaker, but long-term platelet adhesion to the activated endothelium. Inhibition of this GPVI activity has beneficial effects on the morphology and function of the vessel wall in atherosclerosis. Besides its role in platelet-endothelium interactions, GPVI has recently been shown to mediate platelet-leukocyte interactions as well. Functional blockade of GPVI on platelets reduced monocyte firm adhesion to platelets (45), which only occurred at high shear rates. Platelet GPVI triggers interaction with monocytes by binding to the extracellular matrix metalloproteinase inducer (EMMPRIN, CD147), an immunoglobulin-like receptor expressed on monocytes (46). GPVI blockade did not reduce monocyte adhesion to platelets when venous shear conditions were applied; implying that platelet GPVI has diverse functions according to physiological conditions.

P-selectin

P-selectin (Fig. 1) is an integral membrane glycoprotein expressed by both platelets and endothelial cells. It is stored in α-granules of platelets and in Weibel-Palade bodies of endothelial cells and expressed upon activation. P-selectin plays an essential role in the initial recruitment of leukocytes to the sites of injury during inflammation (47). The importance of P-selectin in atherosclerosis has been shown in P-selectin-deficient animals that were protected from atherosclerotic disease (48). The role of platelet P-selectin in atherosclerosis progression was further elaborated by a study of Apoe-/- mice accelerated the formation of atherosclerotic lesions whereas mice injected with platelets that lacked P-selectin formed smaller lesions (49). In addition to platelet-endothelium interactions, P-selectin is also important in platelet-leukocyte interactions (Fig. 3). Through P-selectin, platelets bind to the P-selectin glycoprotein ligand 1 (PSGL-1) on monocytes. Upon activation, P-selectin triggers monocyte adhesion to platelets. This interaction is mediated by the interaction of P-selectin with monocyte PSGL-1, thus promoting the recruitment of monocytes to sites of inflammation.

Figure 2: Platelets transport a vast amount of inflammatory mediators. Upon activation, the release of chemokines will attract other cell types towards the site of inflammation and the release of cytokines will enhance the activation of both leukocytes and endothelial cells. In addition, thrombocidsins are released from platelet α-granules upon thrombin activation and have bactericidal effects.
(PSGL-1) on leukocytes, and thereby form multicellular aggregates, which promote the release of the chemokines CCL2, CCL5 and cytokines like interleukin (IL)-1β to further activate leukocytes and promote atherosclerosis (47, 49). PLAs tether and roll on endothelial cells with a higher avidity than unconjugated leukocytes enhancing endothelial activation and leukocyte transmigration (12). In addition, platelet specific P-selectin is also able to activate the endothelium by triggering Weibel-Palade-body release that leads to P-selectin-mediated rolling of leukocytes (47). This signifies that activated platelets in the circulation are competent to initiate an acute inflammatory response by provoking P-selectin expression on the endothelium and thus stimulate leukocyte rolling on the vessel wall. Platelet P-selectin is also required for the initial "rolling" of activated platelets on atherosclerotic arteries (49), whereas platelet GPIb and αIIbβ3 are crucial for platelet translocation and firm adhesion, respectively (34). In addition, P-selectin can be shed in a soluble form, (s)P-selectin, which is used as a marker for platelet and/or endothelial cell activation (50). In addition, increased levels of sP-selectin in a transgenic mouse model caused vascular complications, sP-selectin accelerated atherosclerosis formation and therefore, elevated sP-selectin has been suggested to be considered not only as a biomarker, but also as an active player in inflammation which probably enhances procoagulant leukocyte microparticle generation and integrin activation (51). A conceivable mechanism might be that sP-selectin binds and induces signalling via PSGL-1 on the leucocyte surface. The subsequent leukocyte activation leads to conformational changes in integrins such as Mac-1 and the release of microparticles.

**Co-stimulatory molecules**

CD40 ligand (CD40L), a member of the TNF-family, is one of the best characterised co-stimulatory molecules that, after binding to its receptor CD40, enhances immune responses and inflammation by inducing cell activation and differentiation (52, 53). Inhibition
of CD40L or CD40 results in a reduction of atherosclerotic plaque size and the induction of a stable plaque phenotype, which is particularly low in inflammation and high in fibrosis (54, 55). CD40 and CD40L are expressed on the majority of cell types present in atherosclerotic lesions, and in the circulation CD40L is mainly expressed on activated platelets (56). Platelet CD40L is expressed upon activation, and is able to bind CD40 on endothelium and different leukocyte subsets (56, 57). Platelet CD40L interaction with endothelial cells induces the release or upregulation of chemokines (e.g. CCL2 and CCL5), adhesion molecules (e.g. ICAM-1, VCAM-1), metalloproteases (e.g. MMP-1, -2, -3 and -9) and tissue factor (TF) (8). In addition, interaction of CD40L with endothelial cells can result in coronary endothelial dysfunction including reduced synthesis of nitric oxide (NO) and enhanced oxidative stress (58). Thus, CD40L-dependent platelet-endothelium interactions will provoke leukocyte recruitment and complement the progression of atherosclerosis.

We were recently able to demonstrate that platelet CD40L is not only important for platelet-endothelium crosstalk but also for the formation of platelet-leukocyte aggregates and for the recruitment of leukocytes to sites of injury (59). Also in our hands, injection of activated platelets increased atherosclerotic lesion formation; a process that we did not observe when activated CD40L-deficient platelets were administered. Moreover, we revealed that injection of activated platelets influenced the immune system by affecting T-cell subset distribution (59). Platelet activation increased the number of effector T cells, a T cell subset generally considered to be proatherogenic, and decreased the number of regulatory T cells (Tregs), a T cell subset known to be atheroprotective (60, 61). This platelet-induced decrease in Tregs was dependent on the expression of CD40L on platelets. Interestingly, these changes in T-cell homeostasis measured 12 hours (h) after platelet injection were only transient, as no differences could be observed 48 h after platelet infusion.

Additionally, platelets constitutively express the receptor CD40. Danese et al. showed that CD40L-positive T cells induce platelet activation through a contact-mediated, CD40-dependent pathway (62). This resulted in RANTES release, which mediated T cell recruitment. Furthermore, ligation of platelet CD40 with a recombinant soluble CD40L augments P-selectin expression, α-granule and dense granule release and the typical shape change that is associated with platelet activation (63). These studies propose an important role for platelet CD40 in inflammation and atherogenesis. Further studies are required to clarify the precise character and significance of platelet CD40 in the setting of atherosclerosis.

Toll-like receptors (TLR): defense against pathogens

TLRs represent an increasingly appreciated class of immune receptors expressed in platelets, contributing to their immune cell function. The initial clearance of pathogens such as bacteria, viruses, fungi and parasites is initiated by TLRs. Through TLR expression, platelets bind infectious agents and deliver different signals for the secretion of cytokines and chemokines (64). TLRs recognise common pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, bacterial RNA and mucins. TLRs are expressed on antigen presenting macrophages, dendritic cells, and epithelial cells and have recently been discovered on platelets. TLR 1, 2, 4, 6, 8 and 9 are expressed on platelets (Fig. 1) (3). The best-known ligand for TLRs is LPS, which binds and activates TLR4. LPS has been shown to induce thrombocytopenia through TLR4-dependent recruitment of platelets to the lung. Several lines of evidence suggest that TLRs are involved in atherogenesis (65). TLR1, TLR2, and TLR4 are upregulated in human atheroma with active nuclear factor kappa B (NF-κB) co-localising with TLR2 and TLR4 in the plaques (66). Furthermore we know that genetic polymorphisms leading to a reduced functionality of TLR4 are associated with decreased atherogenesis and diminished reactions on gram-negative infections (67). ApoE-deficient mice lacking TLR4 displayed reduced aortic atherosclerosis, lower levels of circulating proinflammatory cytokines, and decreased lipid content in the plaques (68). Similarly, progression of atherosclerosis in Ldlr−/− mice lacking TLR2 is reduced with concomitant decreases in macrophage recruitment and proinflammatory cytokine levels (69). Additional support for the functional activity of platelet TLR4 comes from studies showing an in vivo role for LPS-stimulated platelets in triggering TNF-α secretion. Furthermore, in disorders like sepsis, platelet TLR4 contributes to thrombocytopenia through neutrophil-dependent pulmonary sequestration (64, 70). This suggests that platelets could be in part responsible for immune reactions against bacterial products and are a first-line defense against pathogens. Platelets trap bacteria through TLR expression and present LPS to adherent neutrophils (3, 71). In addition, platelets may directly kill bacteria by the secretion of antimicrobial agents (referred to as thrombocidins) (Fig. 2) (72). Taken together, we could anticipate a function for platelet TLRs in the progression of atherosclerosis by for example mediating platelet neutrophil interactions.

Chemokine receptors

The main function of chemokines and their receptors is to guide inflammatory cells in their migration to sites of inflammation. According to their ligands that they bind, chemokine receptors are classified into four groups, CXCR, CCR, CXR and CX3CR. To date, seven CXCR, 10 CCR, one CXR and one CX3CR receptor family members have been identified (73). The first evidence for the functional presence and membrane display of chemokine receptors on platelets was provided for CXCR4 (Fig. 1). In vitro studies revealed its presence on the megakaryocytic lineage from progenitors to platelets and demonstrated that its ligand stromal cell-derived factor-1 (SDF-1, CXCL12) is able to trigger adhesion of megakaryocytes to the endothelium and to induce migration of megakaryocyte precursors (74). Initially, platelets were reported to be unresponsive to CXCL12, but it was subsequently shown that although CXCL12 is a weak platelet agonist by itself, it has robust ac-
tivity in conjunction with low doses of ADP or thrombin. Furthermore, the functional expression of the chemokine receptors CCR1, CCR3, CXCR1, and CCR4 on platelets has been validated (75). CCR4 responds to the ligands CCL17 and CCL22 (monocyte-derived chemokine, MDC) (76). Also CX3CR1, the receptor for fractalkine (CX3CL1) is functionally expressed on platelets and promotes the upregulation of P-selectin and platelet adhesion to collagen and fibrinogen (77). Disruption of CX3CR1 in atherosclerosis prone mice results in a reduced plaque formation and a more stable plaque phenotype (78, 79). The rolling and adhesion mechanism requires the presence of auxiliary adhesive substrates like VWF interacting with platelet GPIb (80). A recent study observed that aspirin inhibits fractalkine expression in atherosclerotic plaques reducing atherosclerosis in ApoE-/- mice (81). Blocking platelet CX3CR1 appears advantageous since it might selectively inhibit platelet activation through atherosclerotic and inflamed endothelium (which is rich in CX3CL1), but would not affect injury-induced bleedings of unaltered endothelium. The specific functionality and inflammatory potential of singular platelet chemokine receptors needs to be further clarified by conditional knock-out mouse models using the cre-lox system.

**Scavenger receptors**

Platelet CD36 (Fig. 1) is another recently discovered modulator of platelet reactivity. CD36, a member of the class B scavenger receptor family, binds several proteins such as collagen, thrombospondin and oxidised low-density lipoprotein (oxLDL) (82). CD36, in atherosclerosis research, has primarily been studied in macrophages where CD36 expression is known to accelerate atherosclerosis development by converting macrophages into foam cells (83). However, CD36 is highly expressed on platelets as well. Since oxLDL is a potent platelet activator, platelet-specific oxLDL-CD36 interactions induce platelet activation, upregulation of P-selectin and CD40L and intraplatelet reactive oxygen species (ROS) formation (84). Oxidation of LDL further contributes to atherosclerosis by generating lysophosphatidic acid (LPA), which promotes platelet shape change and aggregation (85). Besides oxLDL, platelets also respond to native LDL (nLDL). LDL fails to induce platelet aggregation and secretion but enhances responses initiated by thrombin, collagen and ADP (86). ApoER2 has been identified as the platelet receptor (87).

While high LDL cholesterol is a strong risk factor for atherosclerosis and influences cardiovascular mortality, elevated levels of high-density lipoprotein (HDL) are associated with cardiovascular health and have been considered to be atheroprotective (88). Although several drugs have been successfully developed to increase serum concentration of HDL, it has not led so far to significant effects on cardiovascular outcome (89).

The genetic deletion of CD36 in mice results in a phenotype, which develops less atherosclerotic lesions. In contrast, the deletion of scavenger receptor B1 (SR-B1) which is also expressed by platelets leads to severe atherosclerosis in mice (90). Unlike the LDL-receptor CD36, SR-B1 is responsible for taking up esterified cholesterol from HDL particles, which are heterogeneous and composed. This must be the explanation why usually atheroprotective high HDL levels (88) are increased in SR-B1 knockout mice but cause the opposite effect. These mice display enlarged and preactivated platelets, which have accumulated cholesterol. Since transplantation of SR-B1-deficient bone marrow in wild-type mice does not change HDL cholesterol levels, platelet activation or phenotype, high serum cholesterol is a prerequisite for enhanced platelet activation (90, 91). Recently, a SR-B1 mutation with impaired function was discovered in humans. Carriers of this SR-B1 variant exhibit a phenotype comparable to that of SR-B1 knockout mice including preactivated platelets and high HDL levels but so far without a detectable propensity to develop atherosclerosis (92).

This emerging concept that platelets bind and conceivably take up and transport lipoproteins uncovers tempting new mechanisms by which platelets initiate and contribute to atherosclerosis (20).

**Protease-activated receptors**

A role of thrombin for the initiation of atherosclerosis is testified by the presence of thrombin-generating activity in early atherosclerotic lesions (93). Thrombin receptor antagonists represent a novel class of anti-platelet agents that inhibit platelet activation. Thrombin catalyses the conversion of fibrinogen into fibrin, and is one of the most potent platelet activators (94). Thrombin-mediated platelet activation occurs via binding to protease-activated receptors (PARs). PARs have been described on platelets (Fig. 1), endothelial cells, smooth muscle cells, mononuclear cells and fibroblasts (95). To date, four PARs have been identified. PAR-4 (in mice) acts as the principal thrombin receptor. Thrombin binding to PAR-1 cleaves the aminoterminial end of the receptor, which leads to self-activation causing platelets to release ADP, serotonin, and thromboxane A2. In turn, these agonists activate other platelets, thus amplifying the signals facilitating thrombus formation (28). Consequently, PAR-1 signalling is important for platelet activation and clot formation but not for the formation of the initial platelet monolayer at sites of injury (94). PAR-1 antagonists may therefore tackle platelet-mediated thrombosis rather than platelet haemostasis. There are currently two selective reversible PAR-1 antagonists, vorapaxar (NCT00527943) and atorvapaxar (NCT00312052), in advanced clinical development.

**Functional adhesion molecules (JAM)**

The leukocyte integrin alpha M (Mac-1) harbours distinct binding sites for GPIbα, fibrinogen and JAM-C (JAM-3). The JAM family, a subclass within the Ig superfamily, plays a dual role by mediating...
leukocyte-endothelial cell contacts and regulates cell polarity (96, 97). JAM-A and JAM-C have been identified on platelets (98). JAM-C so far appears to be expressed solely on vascular cells and the megakaryocytic lineage. Under conditions of lower shear flow, the selective binding to Mac-1 was found to be responsible for the formation of neutrophil-platelet complexes and adhesion to surface-adherent platelets (99). JAM-A plays an important role for vascular repair mechanisms after endothelial injury (100). The role for its presence on activated platelets has to be further elucidated.

Released inflammatory mediators

Chemokines

Platelet activation results in the release of granule contents, which contain several chemokines stored in the α-granules (▶ Fig. 3). These chemokines are stored in the α-granules. Among them, CXCL4 (PF4), CCL5 (RANTES), CXCL7 (CTAP-III), and CXCL12 (SDF-1) mediate the adhesion of monocytes, PMN, and even progenitor cells (▶ Fig. 2). Among these chemokines are CXCL4 and CXCL7 specific for the megakaryocytic lineage whereas CCL5 and CXCL12 are expressed in a broad variety of cell types including activated T cells.

CXCL5 is an extensively characterised platelet chemokine in atherosclerosis. It causes the selective migration of human blood monocytes and T cells (101, 102). In mice, platelets have been demonstrated to deliver CCL5 to the monocyte surface and the endothelium resulting in increased leukocyte adhesion to the vascular wall. The role of CCL5 and its receptors CCR1 and CCR5 in atherosclerosis have been addressed in a number of experimental studies. CCR1 and CCR5 are predominantly expressed on monocytes, macrophages and T cells. As already stated, injection of activated platelets in atherosclerosis-prone mice leads to the endothelial deposition of CCL5 and CXCL4 (49). This process is P-selectin dependent. Furthermore, inhibition of CCL5 receptors results in decreased lesion size, both in atherosclerosis and vascular injury (11, 49, 103, 104). Not all proatherosclerotic functions of CCL5 may be exclusively platelet-associated since CCL5 is expressed and secreted by numerous cell types. However, since the perfusion of purified activated platelets over endothelial cells in vitro and in the carotid artery ex vivo (11) or their injection in vivo (49) led to increased monocyte recruitment and atherosclerosis, there is enough evidence to regard platelets as the principal source of CCL5 in atherogenesis.

CXCL4 was the first chemokine discovered in releasates from platelets (105). CXCL4 differs from most other chemokines in that it exerts its biological activity at much higher concentrations (micromolar range). CXCL4 is a lysine-rich chemokine belonging to the CXC subfamily, is synthesised mainly by megakaryocytes, and exists as a tetramer in α-granules. Although CXCL4 has been found in other cell types, it appears due to its exuberant expression to be most relevant in platelets. In the presence of appropriate co-stimuli, such as TNF-α, CXCL4 induces exocytosis and firm neutrophil adhesion to endothelium (106, 107). CXCL4 is localised in fatty streaks and atherosclerotic lesion in humans, and CXCL4 expression in the atherosclerotic lesion correlates with histological and clinical severity of disease, indicating its role in human atherosclerosis (108). As a consequence, a significant decrease in atherosclerotic lesion formation in the absence of CXCL4 has been reported (10).

A point of great importance is that CCL5 and CXCL4 are found to engage in heterophilic interactions (109). This heteromeric complex between CCL5 and CXCL4 is a more potent inducer of monocyte arrest onto activated endothelial cells than either chemokine alone, indicating a synergistic enhancement of the monocyte-recruiting function of CCL5 (10). Immunoprecipitation studies have revealed that these chemokines exist in a preformed complex in platelets, suggesting that platelets carry this heterodimer to specifically promote monocyte arrest under inflammatory conditions (10). Therapeutic potential may lie in the interference with chemokine heteromerisation since fewer side effects can be anticipated than with complete chemokine inhibition (73, 110). Disrupting the CCL5/CXCL4 heterodimer attenuates monocyte accumulation during atherogenesis. A synthetic peptide, termed CKEY2, has been designed that actively competes with CXCL4 for binding to CCL5 (10).

CXCL12 (stromal cell-derived factor-1, SDF-1t) is expressed in atherosclerotic lesions and has proatherogenic properties by recruiting leukocytes into the subintimal space. CXCL12 plays an essential role in neointima formation after arterial injury as it has been demonstrated to attract bone marrow-derived smooth muscle cell progenitors in Apoe−/− mice after wire injury (111). Recent genome-wide-association studies have further found a relationship with myocardial infarctions by identifying a variation on chromosome 10q11 near the CXCL12 gene as a powerful predictor for the susceptibility of coronary atherosclerosis which is causally linked to the increased expression of CXCL12 (112, 113). On the other hand, CXCL12 plasma levels have been found to be decreased in the genetic CXCL12 risk variant (114). Collectively, platelets contain a multitude of chemokines that link platelet activation to the recruitment of immune cells by displaying or depositing chemokines on cell surfaces and activating their cognate receptors, resulting in enhanced integrin activity. The interference with immobilisation sites, oligomerisation or synergistic heteromerisation of chemokines at sites of inflammation will be explored to provide novel avenues for therapeutic targeting. Notably, for pleiotropic chemokines such as CXCL12, strategies have to be developed enabling antagonists to take effect in a cell specific environment.

Cytokines IL-1β, TGF-β and sCD40L

IL-1β has been recognised as a main mediator of platelet-induced endothelial cell activation, and an increased expression of IL-1β in arteries was found in hyperlipidaemic animals (115, 116). The activity of IL-1β expressed by platelets appears to be associated with
the platelet surface, and co-incubation of endothelial cells with thrombin-activated platelets induces IL-1β-dependent secretion of IL-6 and IL-8 from endothelial cells (8, 117). Activated platelets enhance endothelial secretion of the chemokine CCL2 in an IL-1β-dependent manner (118). CCL2 is a chemokine important in monocyte recruitment throughout atherogenesis (119, 120) and is also induced by platelet TGF-β (59). Furthermore, platelet released IL-1β increases the endothelial expression of adhesion molecules. Expression of ICAM-1 and αvβ3 on endothelial cells is significantly enhanced by activated platelets via IL-1β (8). By enhancing chemokine release and upregulation of adhesion receptors, platelet-derived IL-1β will promote leukocyte recruitment into the lesion. In addition, adhesion of platelets to the endothelium initiates the activation of NF-κB in endothelial cells. This process was also shown to depend on platelet IL-1β (121).

Platelets are an important storage vehicle of transforming growth factor β (TGF-β) (122). Platelets contain the largest amount of TGF-β1 in the body and release it after activation/degranulation (3). Nevertheless, its role in platelets remains unclear. Of interest, patients with immune thrombocytopenia (ITP) have low levels of TGF-β and shortages in CD4+CD25+FOXP3+ Tregs. When these patients are treated with thrombopoietin receptor (TPO-R) agonists, the levels of TGF-β1 and Treg activity positively correlated with the degree of improvement in platelet counts (123). In atherosclerosis, TGF-β has been extensively studied and has been recognised as an anti-inflammatory and pro-fibrotic cytokine (124, 125). In addition, since TGF-β1 is released from activated platelets during thrombotic events, or occurs at low levels upon interaction with endothelium, platelet-specific TGF-β release should be able to modulate immunity and inflammation in atherosclerosis and to induce matrix production (125).

Upon platelet activation, CD40L is cleaved from the platelet surface over a period of minutes to hours to generate an 18 kDa sCD40L molecule, which is equivalent to the T cell-released sCD40L (53). Enhanced levels of sCD40L are found in patients with cardiovascular disorders and could be used as a biomarker to predict the presence and extent of coronary artery disease and acute coronary syndrome (126). Furthermore, it has recently been shown that sCD40L is an important platelet primer, predisposing platelets to enhanced thrombus formation in response to vascular injury (127). sCD40L induces tissue factor expression on monocytes, endothelial cells, and vascular smooth muscle cells and is crucial in the stabilisation of thrombi, which is mediated via αIIbβ3 (32). It has been reported that αIIbβ3 antagonists inhibit the release of sCD40L from activated platelets. Apart from mediating platelet aggregation, αIIbβ3 also contributes to primary platelet adhesion to endothelial cells. αIIbβ3 mediates arrest of activated platelets to ICAM-1 and αvβ3 (42, 128). Although there is still some controversy on the function of sCD40L, we have to consider the potential of sCD40L to mediate inflammatory events within the vasculature.

Serotonin

Serotonin (5-hydroxytryptamin, 5-HT) is a neurotransmitter involved in sexual behaviour, appetite, sleep and mood (129). Dysfunction of the 5-HT system can lead to psychiatric diseases, such as depression, schizophrenia, addiction and autism. At the same time, 5-HT is a peripheral hormone that is transported in high quantities by platelets (Fig. 2), stored in dense granules, and is released upon activation of platelets (130). To promote haemostasis, activated platelets release 5-HT, which induces vasoconstriction and amplifies platelet activation at sites of vessel wall injury (131). Furthermore, release of 5-HT is a potent augmentative stimulus for platelet aggregation (130, 132). Platelets express the 5-HT transporter (SERT) and the 5-HT2 receptor (5-HT2R). Sarpgorale (Anplag®) blocks serotonin-induced platelet aggregation by selectively inhibiting the serotonin receptor 5-HT2 and may improve coronary microcirculation (133). A different modality for reducing peripheral 5-HT levels is the chronic use of the antidepressant 5-HT-selective reuptake inhibitor drugs (SSRIs) such as fluoxetine (better known as prozac) (134). Indeed, chronic treatment with SSRIs was shown to significantly reduce myocardial infarction risk (135). However, such treatment is not favourable, due to possible side effects (gastrointestinal side effects, reduced libido) and drug interactions. In addition, Dees et al. demonstrated recently that platelet-derived 5-HT provides an important link between vascular disease and tissue fibrosis (136). Systemic sclerosis (SSc) patients have ongoing endothelial cell damage and activation resulting in activation of platelets. The authors revealed that 5-HT induced extracellular matrix synthesis and that inactivation of 5-HT30 effectively prevented the onset of experimental fibrosis and ameliorated established fibrosis. Moreover, 5-HT has been described as an inflammatory mediator. Monocytes treated with 5-HT displayed improved capacity to stimulate T cells in vitro and reduced vulnerability to Fas-FasL-mediated apoptosis (137). Exogenous 5-HT stimulated the activation of ERK1/2 and NFkB in naïve T cells, promoting their activation and proliferation (138). This is consistent with findings that dendritic cells cultured from monocytes express 5-HT receptors and react after stimulation with 5-HT by secreting pro-inflammatory mediators and modulating the immune response towards TH2 (139). Taken together, we could speculate that 5-HT/5-HT receptor signalling could be a protagonist of atherogenesis as well.

Established anti-platelet drugs and their anti-inflammatory potential

Aspirin (acetylsalicylic acid, ASA) is known as the ‘reference’ anti-platelet drug. It acts as an irreversible inhibitor of cyclooxygenase (COX-1), an enzyme involved in the synthesis of thromboxane A2, an important amplifier of platelet aggregation (140). An expanding body of evidence designates anti-inflammatory and anti-atherosclerotic properties to the current anti-platelet drugs (141). Inhibition of platelet activation reduces the release of inflamma-
tory mediators and decreases leukocyte recruitment to sites of injury. Therefore, aspirin administration has been reported to reduce the development and progression of atherosclerotic lesions in hyperlipidaemic mice (142, 143). Atherosclerotic lesions contained increased levels of smooth muscle cells, while the number of inflammatory cells was strongly reduced. These plaques are the equivalent of stable plaques in humans. Additionally, aspirin induces the formation of nitric oxide (NO)-radicals in the body, which decreases inflammation by decreasing leukocyte adhesion (144). There is also evidence to suggest that aspirin modulates signalling through NF-κB (145). NF-κB is a transcription factor required for the expression of a plethora of genes encoding molecules involved in the regulation of inflammation, apoptosis, and cell proliferation. Many of these NF-κB-regulated genes have been implicated directly or indirectly in atherosclerosis (146).

ADP plays a pivotal role in platelet activation and aggregation. Consequently, ADP receptors P2Y₁₂ and P2Y₁ (▶ Fig. 2) are targets for antithrombotic drugs. P2Y₁₂ is the target of the antithrombotic drugs from the thiopyridine group such as clopidogrel, while P2Y₁ is a potential target for new anti-platelet compounds. Clopidogrel is a selective ADP P2Y₁₂ receptor antagonist. It is a prodrug requiring extensive metabolism by cytochrome P450 (CYP) isoenzymes in the intestines and liver, before the active metabolite can bind to platelets. The active metabolite irreversible binds to the P2Y₁₂ receptor and blocks ADP interactions for the remainder of the platelet’s lifespan. Also the use of clopidogrel has been associated with anti-inflammatory effects. Clopidogrel significantly reduced plaque size and augmented its stability in the atherosclerosis prone ApoE⁻/⁻ mice (147). Clopidogrel is known to decrease the expression of CD40L and P-selectin on platelets and has been associated with reduced CRP levels (148, 149). Therefore, clopidogrel inhibits platelet-leukocyte interactions and platelet-endothelium adhesion. Moreover, clopidogrel prevents platelet-dependent ROS production in polymorphonuclear leukocytes (PMN) (150). It is important to note that not all studies reveal anti-inflammatory effects of anti-platelet therapy. Findings even suggest potential inflammatory effects of clopidogrel in patients with coronary artery disease (151). In addition, the ability of aspirin to directly lower levels of inflammatory markers is not supported by recent studies in healthy volunteers (141). Overall, the anti-inflammatory properties of anti-platelet agents are likely to be a result of decreased platelet activation and therefore, indirect.

The development of αIIbβ₃ (GPIIb/IIIa) antagonists (abciximab, epifibatide and tirofiban) has become an attractive strategy for anti-platelet therapy with a strong and specific effect. αIIbβ₃ on resting platelets exhibits very low affinity for its ligands fibrinogen and VWF. Platelet activation leads to conformational changes of αIIbβ₃ enhancing its affinity for fibrinogen and VWF (152, 153). Ligation of αIIbβ₃ through fibrinogen and VWF leads to clustering and outside-in signalling, which is important in cytoskeletal rearrangement in the process of aggregation (154). Consequently, platelets secrete their α-granules, activate other receptors, promote further aggregation and retract clots. The primary mechanism of action of the integrin αIIbβ₃ antagonists is therefore not inhibition of platelet activation but of the final common pathway of platelet-to-platelet aggregation (155). Clinically they are applied in percutaneous coronary interventions and stent implantation in the setting of an acute coronary syndrome with a high risk for an adverse outcome. Thus an assessment of anti-atherosclerotic effects of a permanent αIIbβ₃ inhibition remains speculative.

**Conclusion**

Activated platelets have emerged as culprits in inflammation (▶ Fig. 3). They provide the link between inflammation, thrombosis and atherosclerosis. The inflammatory repertoire of platelets ranges from enhancing leukocyte recruitment to host defense. Upon activation, platelets release a collection of cytokines, chemokines and even antibacterial proteins, and platelets express an excess of inflammatory receptors. Besides integrins, platelets express co-stimulatory molecules which activate other cell types and scavenger receptors, a group of receptors that recognise modified lipids. In addition, TLRs provide platelets with a link towards host defense. Platelets can be considered as sentinel cells that detect inflammation and distribute inflammatory mediators that will boost the inflammatory environment, recruit other inflammatory cell types and potentiate the recruited cells in their inflammatory engagement. Therefore we would like to encourage the concept that platelets are the first in line to initiate atherosclerotic immune responses. They are abundantly present in the circulation, have the ammunition to fight inflammation and are able to orchestrate immune responses by communicating with the white blood cell fraction. However, we have to admit that this concept is only emerging. We still don not know how platelets interact with the different leukocyte subsets in vivo, or what drives them. Is it lipids? We need to invest in our pursuit to uncover the initial “danger” signal that prompts platelets to participate in atherosclerosis. Finally, we should investigate in the potential of anti-platelet drugs in damping the inflammatory properties of platelets. Since antiplatelet drugs reduce platelet activation, they should also reduce the inflammatory capacity of platelets. Nevertheless conflicting results have been published, and we are not able to proclaim a prominent anti-inflammatory effect of the known therapies. Novel therapeutic targets could be more effective in damping both platelet-induced thrombosis and inflammation.

**Conflict of interest**

None declared.

**References**


