Platelets, thrombosis and inflammation: Recent data from pharmacological studies

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Abstract

Platelet adhesion as well as platelet recruitment and aggregation are implicated in thrombus formation. In platelets, thromboxane A2 is the main product of arachidonic acid via the enzyme cyclooxygenase-1 (COX-1), whose inhibition has been a target for the pharmacological inactivation of platelets. However, variability in platelet inhibition by aspirin, a COX-1 inhibitor, suggests that alternative mechanisms occur associated with platelet activation. The search for new
drugs to obtain an effective platelet inhibition has provided new insights into the role of platelets in cardiovascular disease. In addition, it has favoured the development of alternative agents targeting platelets, such as ADP receptor inhibitors (e.g., clopidogrel) and blockers of the GPIIb/IIIa receptors. Recent studies have associated thrombosis and inflammation. It has been suggested that activated platelets may act as enhancers of the inflammatory reactions and, therefore, the anti-inflammatory effects associated with the anti-platelet therapy may contribute in part to the clinical beneficial effects of various drugs in clinical trials. A better knowledge of the proteins and molecular pathways involved in the regulation of platelet activation will surely help identify new possible targets to develop more efficient anti-platelet drugs in the future.

Introduction

In the 19th century, there were two major hypotheses to explain the pathogenesis of atherosclerosis: the incrustation hypothesis, proposed by von Rokitansky in 1852 and the lipid hypothesis proposed by Virchow in 1856 [Reviewed in 1,2]. These two hypotheses focused on fibrin deposition, lipid accumulation and extracellular matrix formation, but the latter investigator suggested an inflammatory component of the disease [3,4]. However, lipoprotein retention and chronic inflammation are intimately related to and plays a role in plaque rupture and thrombosis. Therefore, these hypotheses were unified under the term “atherothrombosis”.

Platelets and thrombus formation

Platelet adhesion to the subendothelium as well as platelet aggregation and recruitment are implicated in thrombus formation. Thrombosis is one of the most important events in the pathogenesis of coronary occlusion and stroke. Platelet activation is a multicellular process with participation of platelets and other blood and vascular cell types [5,6].

When superficial endothelial cell disruption occurs, or when endothelium is dysfunctional and loses his antithrombotic properties, platelets adhere to the vascular wall [5]. In this adhesion process, different participating factors initially reduce the movement of platelets on the vascular surface. Different adhesion proteins expressed on the platelet surface are involved in this initial event. An important group of adhesion proteins are selectins, a family of proteins with three different members (E, L and P). P-selectin is present in alpha granules of platelets (see later) and in Weibel-Palade body of endothelial cells. P-selectin expression on platelet surface occurs during the degranulation process induced by multiple factors, such as collagen, epinephrine and ADP. P-selectin expressed on the platelet surface participates not only in platelet
rupture but also in the interaction of platelets with leukocytes, thus enhancing their proinflammatory activity. In the end, adhesion of platelets to the vascular wall depends on the interaction between diverse platelet membrane glycoproteins and subendothelial matrix compounds [7]. Among these, glycoproteins (GP) Ia and Ib participate in the adhesion mechanisms, while GP IIb/IIIa participates in both the adhesion of platelets to the vascular wall and platelet-platelet interaction (aggregation). The GP IIb/IIIa complex binds platelets with fibrinogen, fibronectin and von Willebrand factor, and thus adheres platelets to the vascular wall through the latter two factors [7].

**Secretion of active factors from platelets**

Platelets are anuclear cells derived from bone marrow megakaryocytes. Platelets circulate for approximately 10 days at a concentration range of 150,000 to 440,000/µl. Two different types of storage granules are present in platelets: dense granules, containing both nucleotides and serotonin, and alpha granules containing different types of proteins including hormones, adhesive proteins and pro-inflammatory proteins. Most of the latter granules’ constituents were synthesized in megakaryocytes, although proteins such as fibrinogen, albumin and IgG are endocytosed from plasma by these cells.

Platelets have the ability to be captured by exposed collagen fibrils within an injured vessel wall, triggering a subsequent transformation in which the now-active platelets adhere tightly to the vascular wall and to each other [8]. Activated platelets undergo rapid cytoskeletal rearrangements, which allow them to maximize their contact area with and spread on the damaged vessel wall. The spread platelets act as a base upon which additional platelets can accumulate, sticking to those that arrive first. Therefore, platelets are circulating monitors of the integrity of the vascular wall. Once injury occurs and platelets begin to accumulate, the growing mass of platelets not only serve as an obstacle to further bleeding but also promotes thrombin generation by supplying a surface upon which more thrombin generates and stabilizes the thrombus [9]. There are several agonists which recognize specific receptors on the platelet surface. In response to these agonists, intracellular free calcium is increased and platelets shape change from disc to thorny sphere, and then several agents contained in platelet granules (a process known as platelet degranulation) are released. Two main metabolic pathways are implicated in this activation. Agonist interaction with its specific platelet receptor induces the stimulation of phospholipase C that activates the phosphoinositide pathway [5]. The generated products are inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 activates the release of intracellular calcium from platelet organelles and favours the increase of free calcium in the platelet cytosol. Diacylglycerol activates protein kinase C which phosphorylates proteins in
serine and threonin residues. This process is accompanied by the secretion of platelet alpha granules and the expression of the fibrinogen receptor GPIIb/IIIa.

Adenine-containing nucleotides are localized in dense granules, mainly as ADP and ATP -forming a complex with calcium and pyrophosphate ions-which do not participate in the pool of these nucleotides implicated in cell metabolism. Moreover, dense granules accumulate a significant amount of serotonin. All of these agents, ADP, ATP and serotonin, act in a synergistic manner with other agents to activate platelets, and are also modulators of both vascular tone and vascular integrity.

The main growth factors contained in alpha granules are: platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermic growth factor (EGF), and transforming growth factor-beta (TGF-β) [10]. Moreover, these granules contain pro-inflammatory proteins, namely interleukin-1β and the chemokines RANTES [11], CXC-chemokine platelet factor 4 [12] as well as CD40 ligand (CD40L) [13]. All these factors participate in the recruitment of leukocytes to the thrombus site. In this regard, it has been shown that platelets store CD40L in high amounts and release it within seconds after in vitro activation [13]. Ligation of CD40 by CD40L expressed on the endothelium surface of activated platelets increased the release of IL-8 and monocyte chemoattractant protein-1 (MCP-1), the main chemoattractants for neutrophils and monocytes [13]. Moreover, CD40L induces endothelial tissue factor expression [14].

**Cyclooxygenase activity and platelet activation**

Platelet stimulation with different agonists induces the release of arachidonic acid, that is metabolized enzymatically into three series of biologically active compounds collectively termed eicosanoids [15]: 1) prostanoids such as prostaglandins and thromboxane A2 (TxA2); 2) leukotriens, i.e., LTB4 and the hydroperoxyeicosatetraenoic acids (HPETEs); and 3) epoxyeicosatrienenoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs).

In platelets, thromboxane A2 is the main product of arachidonic acid via the enzyme cyclooxygenase-1 (COX-1). COX-1 catalyses the conversion of arachidonic acid to prostaglandin GG2 by its cyclooxygenase activity, and its subsequent reduction to PGH2 by its peroxidase activity. PGH2 is isomerized to TxA2 by the activity of TxA2 synthase [15]. Inhibition of cyclooxygenase activity has been a target for the pharmacological inactivation of platelets.

Acetylation of COX-1 by aspirin results in the blockade of arachidonic acid oxidation to PGG2 [16,17]. Aspirin has a short half-life (15-20 min) in the human circulation and is approximately 30-60-fold more potent in inhibiting
platelet COX-1 than monocyte COX-2, inducing a permanent inhibition of TxA2-dependent platelet function [18]. It is likely that COX-1 is inactivated by aspirin in relatively mature megakaryocytes and, since only 10% of the platelet pool is replenished each day, once-a-day dosing (75-325 mg) of aspirin can give rise to virtually complete inhibition of platelet TxA2 production.

However, despite the consolidated clinical beneficial effects of aspirin, variability in aspirin-mediated platelet inhibition has subsequently been documented among normal subjects, in patients with cerebrovascular disease, stable CAD and those presenting for coronary artery bypass surgery. Indeed, prospective studies have confirmed the relationship between aspirin resistance and cardiovascular risk, and demonstrated that the magnitude of this risk may not be trivial [19].

Several authors have postulated a genetic theory to explain the existence of aspirin resistance [20,21]. In fact, a polymorphism of the COX-1 gene resulting in a reduction of COX activity could cause a decreased production of TxA2 and possibly eliminate the need for aspirin [20]. However, if the COX gene is dysfunctional, TxA2 may be produced by mechanisms other than COX-1. In this regard, another COX isoform, COX-2, has been described. COX-2 protein is undetectable in most cells and is not found in platelets; however, it can be induced by pro-inflammatory stimuli such as cytokines and growth factors, as well as shear stress, in the vascular wall [22, 23]. Although aspirin can inhibit both COX-1 and COX-2, more than 90% inhibition of TxA2 is needed for aspirin to be effective; it could thus be speculated that a genetic COX-2 overexpression may contribute to aspirin resistance as a result of incomplete TxA2 suppression [20]. However, the mechanism for aspirin resistance is uncertain, and it probably involves a combination of clinical, biological and genetic features affecting platelet function.

**Non-COX-1-dependent platelet inhibition**

A search for new drugs to obtain an effective platelet inhibition favoured the development of ADP receptor inhibitors such as clopidogrel. This compound is a structural analogue of ticlopidine. Clopidogrel does not act on the COX pathway but avoid the action of ADP on platelets [24]. Platelet activation related to ADP is mediated by a family of receptors named as P2X and P2Y, that are linked to ion channels and G proteins, respectively. There are presently two different P2Y receptor subtypes, with low and high affinity for ADP. The high affinity P2Y receptors are linked to the platelet shape change that is not inhibited by clopidogrel. The low affinity P2Y receptors are coupled to inhibition of adenilate cyclase, an enzyme that produces cyclic AMP and reduces platelet activation. Clopidogrel binding to the low affinity ADP receptors ensures cyclic AMP formation as the main mechanism of its anti-platelet activation effect.
Another approach to reduce platelet activation is the blockade of the GPIIb/IIIa receptors. As mentioned above, GPIIb/IIIa receptors mediate the interaction platelet-platelet through fibrinogen binding. These receptors also recognize other proteins such as von Willebrand factor and fibronectin [25, 26]. The specificity of GPIIb/IIIa receptors to bind to these different proteins is regulated by two peptidic sequences: the RGD region (arginine, glycine, aspartic acid) and the KQAGDV region (lysine, glutamine, alanine, glycine, aspartic acid, valine) [27]. Different drugs to inhibit GPIIb/IIIa receptors have been developed; among them, quimeric antibodies (abciximab), cyclic heptapeptides (eptifibatide) and non-peptidic inhibitors (tirofiban) [28]. It is important to know that abciximab, eptifibatide and tirofiban all can produce thrombocytopenia immediately after their administration in a small proportion of patients. The mechanism of this effect, which usually occurs within hours but may occasionally be delayed, is likely to involve the immune system. Thus, patients who develop severe thrombocytopenia upon re-exposure to abciximab have IgG antibodies that recognize platelets sensitized with the intact monoclonal antibody 7E3 from which the murine sequences in abciximab are derived [29].

**Involvement of platelets in inflammation**

During the last years, several investigators have associated thrombosis and inflammation, particularly in the setting of acute coronary syndromes. In histological samples, a systematic infiltration of blood inflammatory cells, i.e., lymphocytes and monocytes, have been observed at the site of the plaque rupture where platelet thrombus is formed [30]. Later studies have demonstrated that activated platelets stimulated the inflammatory capability of leukocytes by enhancing the release of oxygen-derived free radicals, such as superoxide anion [31]. The P-selectin protein expressed on the surface of activated platelets interacts with leukocytes, increasing superoxide anion release.

As previously mentioned, platelets contain pro-inflammatory related proteins such as cytokines and quimokines. Indeed, although the platelet may be considered as a pro-inflammatory cell by itself, a presently unresolved issue is to define its true role in inflammation. The common, final pathway in platelet activation is the platelet surface expression and activation. There are several works that have attributed additional effect for blockade of GPIIb/IIIa receptors than merely the inhibition of platelet aggregation and adhesion. In this regard, administration of abciximab reduced pro-inflammatory properties of monocytes associated with integrin Mac-1 activation. Moreover, abciximab also reduced both the number of platelets adhered to leukocytes and the circulating levels of various inflammation biomarkers in patients after percutaneous revascularization [32]. Some of these properties of abciximab
have been accounted for by its properties as a chimeric antibody which may favour its interaction with other targets besides GPIIb/IIIa receptors, rather than affecting platelet proinflammatory pathways. However, a recent study by Molero et al [33] demonstrated that tirofiban, a more specific GPIIb/IIIa inhibitor, reduced the inflammatory response in guinea-pigs myocardium and circulating leukocytes induced by infusion of endothelin-1, a vasoconstrictor peptide with inflammatory activity. Moreover, reduction of circulating platelets was associated with a diminished inflammatory response in the myocardium after endothelin-1 infusion [33]. In the same line, Gawaz et al [34] demonstrated that activated platelets enhanced the expression of adhesion molecules in cultured endothelial cells. Taken together, these findings suggest that platelets have a direct participation in the inflammatory reaction at least as inflammatory enhancers. The fact that blockade of GPIIb/IIIa receptors reduced the inflammatory response in circulating leukocytes and in the myocardium suggests that other anti-platelet drugs such as aspirin and clopidogrel may also prevent inflammation.

In the Physician’s Health Study of primary prevention carried out among 22,071 male physicians, aspirin reduced the incidence of myocardial infarction compared with that observed with placebo [35]. Moreover, it has been reported that patients who developed a cardiac event while taking aspirin were more likely to experience unstable angina than acute myocardial infarction, the most dramatically situation within the acute coronary syndromes [36]. However, there is an apparent failure of aspirin to prevent the coronary syndrome although it may be only partial since the drug still has the potential to modify the natural history of the disease towards a less severe acute outcome. In contrast, no differences have been observed in the characteristics of acute coronary syndromes after prior treatment with β-blockers, oral nitrates or calcium antagonists [37]. Whether these different clinical observations may be related to a reduction in inflammation induced by aspirin is still unproven. However, it has also been observed that patients taking aspirin showed reduced levels of pro-inflammatory markers, such as IL-6 and intracellular adhesion protein-1 (ICAM-1), during the acute phase of the unstable angina when compared with patients with no previous aspirin use [38].

Accordingly, the aforementioned Physician’s Health Study showed that the effect of aspirin in preventing a first myocardial infarction was directly related to the level of C-reactive protein, supporting the notion that aspirin may exert beneficial actions at least in part by its anti-inflammatory properties [39,40]. Furthermore, aspirin, independently of its anticyclooxygenase activity, can inhibit the nuclear translocation of the transcription factor nuclear factor-κB (NF-κB), which promotes the expression of several inflammatory mediators [41].
All these observations raise two main questions: 1) What is the dose of aspirin inducing an anti-inflammatory effect?. The putative answer to this question is that even low doses of the drug (75-300 mg/day) can show this effect. In this regard, Cyrus et al [42] demonstrated that low-dose aspirin reduced vascular inflammation, associated with plaque stabilization, in a mouse atherosclerosis model. The second question referred to above is whether the antiinflammatory effect of aspirin depends on platelet inhibition or on its direct action on pro-inflammatory cells (i.e., leukocytes and other resident cells in the vascular wall), or both.

Recent results have shown that inhibition of platelet activation by clopidogrel reduced the expression of the pro-inflammatory proteins CD40L and tissue factor in ischemic coronary arteries of rabbits [43]. Moreover, the expression of endothelial nitric oxide synthase, an enzyme downregulated under inflammatory conditions, was maintained in the ischemic coronary vessel after clopidogrel treatment; supporting the anti-inflammatory effect of this drug [43]. Consistent with these observations, clopidogrel pre-treatment of patients undergoing percutaneous coronary intervention reduced both the expression of CD40L in platelets and circulating IL-6 levels [44]. Moreover, clopidogrel inhibits the platelet-leukocyte interaction after ADP and thrombin stimulation in patients with atherosclerotic vascular disease [45-47].

Collectively, the aforementioned data support the hypothesis that activated platelets may act as enhancers of the inflammatory reaction associated with cardiovascular diseases. Therefore, the anti-inflammatory effects associated with anti-platelet activation may contribute in part to the clinical beneficial effects of agents such clopidogrel as observed in clinical trials.

Considering that inflammation seems to be associated with the clinical outcome of cardiovascular patients, it could be anticipated that inhibition of platelet activation might modify the prognosis of these patients in the long run. However, there are not current data which allow to definitively establish this notion. In this regard, a recent study has demonstrated that at least 80% inhibition of platelet activation was related to reduce circulating levels of some pro-inflammatory biomarkers. However, if the degree of platelet inhibition was lower (20-50 %), the opposite effect was observed [48]. This opens the question of whether an individualized control of the degree of platelet inhibition should be performed in each patient. Interestingly in this regard, it is beginning to be recognized the existence of genetic polymorphisms affecting some proteins involved in platelet activation, such as GPIIb/IIIa receptors; suggesting that platelets from different individuals may have different activity degree [20]. Therefore, a better knowledge of the agents, proteins and molecular pathways involved in the regulation of platelet activation might help identify new possible targets to develop more efficient anti-platelet drugs. From this perspective, new technologies such as proteomics may prove to be a
powerful tool in understanding platelet function. In fact, although platelet proteomics is a young research field, remarkable advances have already been accomplished. Thus, more than 300 proteins released by human platelets after thrombin activation have currently been identified, and even some of them were previously unknown to be present in human platelets [49,50]. Thus, the use of proteomics or alternative technologies, may help characterize agents from the vascular wall or blood cells as novel agonists or antagonists of platelet activity in the near future. Recent evidence suggests that PTH and parathyroid hormone (PTH)-related protein (PTHrP) may be one of these factors (see later).

**PTH and PTHrP : Emerging roles in platelets and inflammation**

PTHrP was discovered in 1987 as the causative factor of humoral hypercalcemia of malignancy. However, PTHrP is now known to be widespread in normal foetal and adult tissues including epithelia, mesenchymal tissues, endocrine glands, the central nervous system and blood vessels [51]. PTH and PTHrP are the products of separate genes located on distinct chromosomes. Due to N-terminal sequence homology between PTHrP and PTH, both peptides interact with a common receptor, the G-protein-coupled PTH1 receptor (PTH1R) [52]. The activation of PTH1R stimulates cAMP formation, protein kinase (PK) C and mitogen-activated protein kinase (MAPK) activities in different cell types [53,54]. By posttransductional processing, the three different isoforms of PTHrP originated by alternative splicing of the PTHrP gene, different fragments are formed; among them, only the N-terminal fragment, PTHrP (1-36), binds to PTH1R in the same manner as PTH (1-34) in target cells [51,52].

In contrast to PTH, which acts as a classical endocrine hormone, PTHrP exerts its effects locally in an autocrine/paracrine and even intracrine fashion [51,52]. Therefore, PTHrP unlike PTH, does not circulate in appreciable amounts in normal subjects. Among the most potent inducers of PTHrP are vasoconstrictors including angiotensin II, serotonin, endothelin-1, noradrenaline, bradykinin and thrombin [52]. PTHrP is also induced in vascular smooth muscle cells in response to mechanical stimuli. PTHrP has been shown to induce vaso relaxant effect in many vascular beds including heart and mammary gland throughout the activation of PTH1R. Indeed, mice over-expressing either PTHrP or the PTH1R in vascular smooth muscle cells have reduced systemic blood pressure; consistent with the prediction that PTHrP acts as a local vasodilator [53]. PTHrP has been found in the myocardium, mainly in the atria and vessels but lower levels also in ventricle [52]. PTHrP is abundantly expressed in coronary endothelial cells [54]. Several
evidences have suggested that many cardiac effects of PTHrP can not be mimicked by PTH [52].

Funk et al [55] have suggested that PTHrP may be a cytokine-like peptide, and showed that it can act as a member of the cascade of proinflammatory cytokines, such as tumor necrosis factor and interleukin-1 [56]. Immunoreactivity of PTHrP has been demonstrated in the thickened intima induced either by balloon injury of rat aorta or by placing a non-obstructive cuff around rat femoral artery; as well as in the restenotic and primary atherosclerotic lesions of human coronary arteries [57,58]. It is noteworthy that in the latter, staining for PTHrP was observed within regions where macrophages were predominant [58]. Additional findings further support the pro-inflammatory properties of PTHrP. Thus, Jiang et al [59] have shown that PTHrP upregulates IL-1β-induced NO synthesis; and it is well known the relationship between the inducible form of this enzyme and inflammation.

Atherosclerosis, platelets and PTHrP

Atherosclerosis is characterized by arterial luminal narrowing associated with thickening of the arterial intima. A large number of biologically active substances such as growth factors, cytokines and vasoactive peptides have been considered to play an important role in the atherogenic and restenotic processes [60,61]. Disruption of the atherosclerotic plaque has been associated with a proinflammatory phenomenon but also with a thrombotic process. Indeed, thrombosis plays a key role in the pathogenesis of unstable angina, acute myocardial infarction and sudden death. Plaque rupture exposes the subendothelial content of the vessel wall to circulating blood elements, thus facilitating the access of PTHrP contained in the plaque to circulating blood cells.

As previously mentioned, PTHrP immunoreactivity has been detected in human atherosclerotic lesions [57], but the putative effect of PTHrP on platelet activation has been demonstrated yet. Some investigators have reported conflicting effect of PTH on platelet activation. Some investigators have shown that acute administration of physiological concentrations of PTH increases intracellular Ca^{2+} levels in platelets, suggesting platelet activation. On the other hand, other authors showed no reduction or even no effect of PTH on platelet activation [62-64]. We recently observed that exposure to either PTH or PTHrP failed to modify by itself platelet activation, but enhanced platelet aggregation induced by several proaggregating agents [65]. This finding might open new insights into the pathophysiological relevance of these proteins in the setting of acute coronary syndromes. In this regard, a previous study has reported an increase in PTH serum levels the first day after the onset of acute myocardial ischemia [66].
Platelets and diabetes

It is well established that diabetes mellitus increases the risk of cardiovascular diseases including coronary heart disease and stroke. Type II diabetes is associated with insulin resistance and hyperinsulinemia and is often part of a metabolic syndrome which comprises hypertension, dyslipemia, decreased fibrinolysis and increased procoagulating factors. Moreover, patients with diabetes have a poor prognosis compared to non-diabetic patients when they experience a major vascular event [67,68].

Thrombosis appears to contribute significantly to the increased risk of diabetic patients. In this regard, differences in platelet function have been described between diabetic and nondiabetic subjects. Platelets from type I and II diabetic patients exhibit enhanced platelet aggregation activity early in the course of the disease. Indeed, platelets from these patients produce less NO and prostacyclin, and contain reduced antioxidant levels, associated with increased aggregability. Moreover, serum fibrinogen levels are also elevated in patients with diabetes. Diabetic patients also have an increased platelet population expressing adhesion molecules associated with platelet activation, such as GP IIb/IIIa, lysosomal GP53, thrombospondin and P-selectin. An additional interesting observation is that diabetic patients with vascular disease may have a greater rate of platelet turnover, which may reduce the ability of the antiaggregating drugs to exert their action [68]. It is remarkable that plasma levels of PTHrP seems to be elevated in type-II diabetes mellitus patients compared with controls [69]. Interestingly in this regard, a recent study has shown an increased in the PTHrP/PTH1R system in the kidney of experimental diabetes, which adversely affects the course of diabetic nephropathy [see chapter Diabetic nephropathy as an inflammatory disease in this book].

Future considerations

It has now become clear that besides their role in thrombosis, platelets have a direct involvement in the inflammatory response. Therefore, inflammation and thrombosis are linked rather than separate entities. It is hoped that the use of new technologies, namely pharmacogenomics and proteomics, may supply new insights to help us understand the complex platelet pathology in cardiovascular diseases.

References