Role of platelets as mediators that link inflammation and thrombosis in atherosclerosis

Platelets and the Immune Response

Eduardo Fuentes Q.[1,4], Francisco Fuentes Q.[2], Vicente Andrés [3], Oscar M. Pello [3], Jaime Font de Mora [3], Iván Palomo G.[1, 4]

1. Department of Clinical Biochemistry and Immunohematology, Faculty of Health Sciences, Universidad de Talca, Chile.
2. School of Medicine, Faculty of Medicine, Universidad Católica del Maule, Talca, Chile.
3. Department of Epidemiology, Atherothrombosis and Imaging, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.
4. Centro de Estudios en Alimentos Procesados (CEAP), Conicyt-Regional, Gore Maule, R09I2001, Talca, Chile.

Address correspondence to:

Iván Palomo G., PhD

Departamento de Bioquímica Clínica e Inmunohematología

Facultad Ciencias de la Salud, Universidad de Talca, Talca, Chile

Casilla: 747, Talca, Chile

E-mail: ipalomo@utalca.cl

Telephone: 56-71-200493

Fax: 56-71-20048

Key words: Platelets, Inflammation, Atherosclerosis, Cardiovascular disease.

ABSTRACT

Platelets, crucial mediators of the acute complications of atherosclerosis that cause life-threatening ischemic events at late stages of the disease, are also key effectors of inflammation throughout plaque development through their interaction with endothelial and immune cells in the injured vessel wall. During the first steps of atherosclerosis, blood inflammatory leukocytes interact with the damaged endothelium in areas rich in platelet aggregates. In late stages of the disease, platelets secrete several inflammatory molecules, even without forming aggregates. These molecules exacerbate the inflammation and induce the transition from chronic to acute disease, featuring increased instability of the atherosclerotic lesion that results in plaque rupture and thrombosis. Moreover, platelets play an important role in vascular wall remodeling induced by chronic inflammation by controlling vascular cell differentiation and proliferation. In this review we discuss the role of platelets as cell mediators that link inflammation and thrombosis in atherosclerotic disease and their potential in the development of new therapeutic tools to fight cardiovascular disease.

INTRODUCTION

The incidence and prevalence of cardiovascular disease has increased significantly in recent years, due at least in part to the progressive aging of the population. According to the World Health Organization (WHO), by 2020 cardiovascular disease will become the leading cause of disability and death in the world [1, 2]. In most cases, myocardial infarction and stroke are the consequence of atherosclerotic plaque rupture and thrombus formation. Atherothrombosis is regulated by both genetic and environmental factors (e.g., dyslipidemia, hypertension, smoking, diabetes and obesity) [3, 4]. The development of atherosclerotic lesions is the result both of lipid accumulation in the sub-endothelial space in arteries and of a complex process of chronic inflammation characterized by endothelial dysfunction, leukocyte infiltration and activation of Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells vascular smooth muscle cells. Importantly, activated smooth muscle cells within the injured vessel wall undergo a de-differentiation process, acquiring a highly proliferative and migratory phenotype [5-7].

Circulating platelets in peripheral blood play a major role in maintaining blood homeostasis. Reports in the last decade have described the secretion by platelets of pro-inflammatory molecules that exacerbate the inflammatory response in the atherosclerotic plaque [8-10]. This event occurs during the initial injury to the endothelium as well as during later stages when the atherosclerotic plaque is destabilized [11, 12]. Platelets also interact directly with other cells of the immune system in physiological and pathological conditions [10]. In the context of atherosclerosis, platelets can adhere to endothelial cells and contribute to the recruitment of leukocytes involved in the local vascular inflammation [13, 14]. In this review we discuss the involvement of platelets in the initiation and development of the inflammatory process of atherosclerosis. We also describe the most relevant platelet molecules implicated in the interaction with immune cells and their activation toward the inflammatory phenotype.

INFLAMMATION, PLATELETS AND ATHEROSCLEROSIS

It is today well-established that numerous cellular and molecular inflammatory components participate during all stages of atherosclerosis, from early lesion development to vulnerable plaque formation [15, 16]. Atherosclerotic lesions typically have a prominent infiltrate of immune cells, composed mainly of monocytes/macrophages, T cells, dendritic cells and neutrophils. These cells are recruited from the bloodstream at sites of endothelial injury and locally produce a plethora of regulatory molecules that contribute to the onset and progression of atherosclerosis [17-19]. At late stages of the disease, monocytes/macrophages accumulate in the atheromatous lesion and secrete large quantities of extracellular proteases, including serine proteases, cathepsin and metalloproteinases, that increase plaque instability [20]. Moreover, apoptosis of neointimal macrophages provokes the release of collagen degrading proteases and tissue factor (TF), further promoting plaque vulnerability [21-23]. Immune cells also promote plaque instability by producing cathepsins and metalloproteinases that degrade interstitial collagen [24, 25]. Stable and vulnerable plaques differ in the composition of their inflammatory infiltrates [26], and current studies therefore focus on characterizing the immune cells localized at the plaque in order to identify markers of vulnerable plaques [27]. These markers would allow the identification of high risk patients before they start to develop an acute ischemic event [28, 29].

As indicated before, platelets play a major role in vascular inflammation through the production and release of pro-inflammatory mediators and by interacting with endothelial cells, leukocytes and vascular smooth muscle cells. Using the atherosclerosis-prone apolipoprotein E-deficient mouse model (ApoE-/-), it has been shown that activated platelets and platelet-leukocyte/monocyte aggregates promote formation of atherosclerotic lesions, suggesting their atherogenic potential [30]. This may be due in part to increased cell proliferation, a key process in the early stages of atherosclerosis both in animal models and in humans [31]. Indeed, platelet-derived growth factor (PDGF) down regulates the expression of the growth suppressor p27,

whose genetic ablation in ApoE-/- mice promotes excessive proliferation of vascular smooth muscle cells and neointimal macrophages and exacerbates atherosclerosis [32-35]. Another pro-atherogenic factor released by platelets is the chemokine stromal-cell derived factor-1 (SDF-1), which recruits hematopoietic precursors to incipient lesions where they proliferate and differentiate into new inflammatory cells [36]. In addition, after platelets get activated and form aggregates, they increase the secretion of other potentially pro-atherogenic molecules, such as growth-regulated oncogene-a (GRO-a), platelet factor-4 (PF-4), epithelial-neutrophil activating peptide (ENA-78), interleukin-8 (IL-8), monocyte chemo attractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α) and RANTES [37]. It is interesting to note that thrombin-mediated platelet activation is not required for the early development of atherosclerosis in ApoE-/- mice, suggesting that, if platelet activation is required for plaque formation in this animal model, platelet activators other than thrombin suffice [38]. For instance, P-selectin expression is critical for monocyte recruitment to sites of neointima formation after arterial injury in ApoE-/- mice and its complete absence attenuates plaque area in this experimental model (ref 39). Moreover, platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid artery in humans (ref 40).

Platelets also intensify the inflammatory process at all stages of atherosclerosis by expressing membrane molecules such as intercellular adhesion molecule-2 (ICAM-2), P-selectin, CD95L and CD40L. These molecules regulate several biological functions in the vessel wall, including cellular adhesion and aggregation, chemotaxis, survival and differentiation, and angiogenesis [41, 42].

It has also been shown that immunoglobulin complexes increase platelet expression of inflammatory molecules, such as soluble CD40L (sCD40L) and RANTES, in the absence of aggregation [43]. Moreover, platelet activation also promotes a conformational change in C-reactive protein (CRP), shifting it from the pentameric to the monomeric form, which is highly

abundant in inflammatory settings and which may play a role in the pathogenesis of atherosclerosis [44]. Recently, Kirbis *et al* have shown that increased levels of monomeric CRP can predict the risk of acute coronary syndrome independently of conventional cardiovascular risk factors [45].

PLATELET COMPONENTS THAT REGULATE THE INFLAMMATORY PROCESS

Platelets have been identified as regulators of the inflammatory processes that control both the initiation of the atherosclerotic lesion and plaque instability at late stages of disease progression (Figure 1) [46, 47]. This regulation is mediated by factors that are produced by activated platelets, such as CD40L, P-selectin, RANTES and toll-like receptors (TLRs).

CD40L. Immune and endothelial cells express CD40, a member of the superfamily of tumor necrosis factor (TNF) receptors. The ligand CD40L is similarly expressed in the plasma membranes of endothelial cells, T-lymphocytes and platelets [48-50]. However, platelets are the main source of the soluble form, sCD40L, contributing 95% of its circulating levels [51]. High levels of sCD40L have been associated with platelet activation together with increased numbers of neutrophils *in vivo*, suggesting a prognostic value in patients with advanced atherosclerosis and metabolic syndrome, and potential use as a biomarker of thrombosis [52, 53].

The major contributors to the release of circulating sCD40L from CD40L on the platelet surface are metalloprotease-2 [54] and glycoprotein (GP) Ib/IX/V complex (GPIb/IX/V), which induces sCD40L through the production of thromboxane A2 (TXA2) in patients with atherosclerosis [55]. sCD40L significantly increases platelet activation and aggregation through CD40-dependent TRAF-2/Rac1/p38 MAPK signaling [56], while blockade of this pathway with anti-CD40L antibodies can prevent or delay atherosclerosis progression [57, 58].

CD40/CD40L has been shown to induce macrophage-mediated liberation of TF, favoring a pro-thrombotic stage [59]. sCD40L is also able to bind macrophage 1 antigen (Mac-1), an integrin

expressed on monocytes/macrophages [60], resulting in the secretion of TF, pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and TNF-α) and myeloperoxidases [61]. CD40L can also induce maturation of dendritic cells, with a positive regulation of co-stimulatory molecules and the production of IL-12p40 [62]. CD40L also increases T CD8+ cell responses *in vitro* and *in vivo* [63], as well as the release of IgG from B lymphocytes [64]. CD40-CD40L interaction in neutrophils is PI3K-dependent and induces the production of reactive oxygen species, establishing a positive feedback loop for platelet activation by the redox environment [65]. In endothelial cells, sCD40L induces the expression of cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and the secretion of the pro-angiogenic chemokines MCP-1 and IL-8 [66, 67], and reduces the expression of thrombomodulin, thus promoting a pro-thrombotic state [68]. sCD40L also activates vascular smooth cells and fibroblasts [69, 70], two cell types that play an important role in the pathogenesis of atherosclerosis [71, 72].

P-selectin. P-selectin is a cell surface adhesion molecule stored in the Weibel-Palade bodies of endothelial cells and in α-granules in platelets [73]. P-selectin localizes to the cell surface of endothelial cells upon granule exocytosis and plays an essential role in the initial recruitment of leukocytes to sites of injury during inflammation by interacting with P-selectin glycoprotein ligand (PSGL1) [74, 75]. P-selectin expressed by neointimal macrophages may also contribute to the inflammatory response during atherosclerosis development [76-78]. P-selectin expression in platelets is mainly regulated by Gas6 [79] and low levels of fibrinogen [80]. Platelet-derived P-selectin seems to contribute to atherosclerotic lesion development [81] and arterial thrombogenesis by forming large stable platelet-leukocyte aggregates [82]. Notably, platelet-derived P-selectin, but not endothelial P-selectin, also plays a crucial role in the development of neointima formation after angioplasty/air desiccation injury of the mouse carotid artery [83].

sP-selectin is a soluble form of P-selectin produced *in vivo* by platelets either from alternative mRNA splicing that generates an isoform that lacks the transmembrane domain, or from

proteolytic cleavage of the membrane-bound form [84]. Evidence exists that sP-selectin is a biomarker of vascular disease, since its levels are increased in patients with acute coronary syndrome [85] or hypertension [86]. Moreover, measurement of circulating sP-selectin has been suggested for remote testing of platelet function in patients treated with clopidogrel and aspirin [87].

Mice engineered to produce abnormally high plasma levels of sP-selectin exhibit enlarged infarcts in an ischemic stroke model, and increased susceptibility to atherosclerosis development in the ApoE−/− genetic background [88]. In addition to its role in cell adhesion, the interaction of P-selectin with PSGL1 is essential for the secretion of von Willebrand Factor by endothelial cells [89]. P-selectin also activates leukocyte Mac-1 and later antigen-4 (VLA-4), which intensify inflammation and facilitate adhesion of circulating platelets to the vascular endothelium [90]. Moreover, the P-selectin-PSGL1 interaction induces the "inside-out" activation of integrins $\alpha_L\beta2$ and $\alpha_M\beta2$ in leukocytes via signaling through Src kinases and Naf1 [91]. This process promotes cell-cell and cell-extracellular matrix interactions, since $\alpha_M\beta2$ binds several ligands (including iC3b, fibrinogen and heparin) and $\alpha_L\beta2$ binds ICAM-1 [92]. P-selectin-induced recruitment of neutrophils to the injured endothelium is primarily mediated by the activation of integrins ß2 and ß3 (CD11b/CD18 and CD41/CD61), further promoting the inflammatory response [93, 94]. Moreover, PSGL1 activation in monocytes and neutrophils induces superoxide anion production [95].

RANTES. Activated platelets secrete the chemokine RANTES, which binds to the CCR1 chemokine receptor in leukocytes and promotes their interaction with the endothelium in the inflamed vasculature [96]. In addition, RANTES facilitates the recruitment of low density lipoproteins (LDLs) to the sub-endothelial space. LDLs undergo oxidation that contributes to the inflammatory response and to the progressive increase of plaque vulnerability [97, 98]. Nanomolar concentrations of RANTES promote chemotaxis through the interaction with G

protein-coupled receptors (GPCRs) [99]. In contrast, at micromolar concentrations RANTES promotes the formation of aggregates that contribute to cell activation (proliferation, apoptosis and cytokine secretion) through mechanisms independent of GPCRs [99]. Cytoplasmic levels of RANTES correlate with CRP and fibrinogen levels in middle aged patients with cardiovascular risk factors, suggesting the potential of RANTES as a biomarker of atherosclerosis and associated cardiovascular disease [100, 101].

The function of RANTES in atherosclerosis is related to other platelet-derived molecules. For example, interactions between RANTES and PF-4 amplify the effect of PF-4 on monocytes by heterophilic interactions [102, 103]. Also, deposition of RANTES on the endothelium is promoted by platelet P-selectin [104], promoting the expression of the pro-atherogenic molecule MCP-1 by inflammatory monocytes [105].

Toll-like receptors (TLRs). TLRs are the main regulators of the adaptive and innate immune responses [106], and play important roles in atherosclerosis and pathological myocardial remodeling [107]. TLR4 and TLR9 are prominently expressed in the cytoplasm of human platelets [108], and their expression levels are doubled in activated platelets [109]. TLRs are activated by heat-shock proteins, components of the extracellular matrix, fibrinogen and myeloid related protein 8/14, resulting in the secretion of cytokines, chemokines and molecules related to the transition to an unstable plaque [110, 111]. It has been shown that lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway [112]. The binding of lipopolysaccharides to TLR4 also activates platelets, leading to the secretion of IL-1β and activation of their membrane-bound integrins GPIIb and IIIa [113]. In turn, active GPIIb and IIIa in platelets stimulate the secretion of MCP-1 and the expression of ICAM-1 and αvβ3 by endothelial cells, thus promoting monocyte and neutrophil recruitment [114, 115]. In addition, TLR4 activation increases platelet release of sCD40L and platelet-activating factor 4 (PAF4) [116]. However, another study suggests that stimulation of non-

platelet TLR2 and TLR4 during the advanced stages of atherosclerotic disease reduces the secretion of MIP-1 and RANTES in ApoE-/- mice [117]. Stimulation of TLR2 with Pam3CSK4, a synthetic agonist of TLR2/TLR1, activates GPIIb/IIIa and augments the expression of P-selectin on the platelet surface [118]. In addition, TLR2 activation promotes Ca^{2+}mobilization, TXA2 production and platelet aggregation mediated by the activation of P2X1 and ADP receptors [119]. The TLR2 signaling pathway further modulates the adhesion between platelets and immune cells and remodeling of their cytoskeleton [120].

PLATELET COMPONENTS THAT CONTROL CELL DIFFERENTIATION

Recent studies have shown that platelets are also involved in the differentiation of immune and endothelial precursor cells. For example, in co-cultures with platelets, CD34+ stem cells differentiate into CD68-immunoreactive macrophages capable of forming foam cells [121]. This interaction of bone marrow cells with platelets involves the intervention of P-selectin and the GPIIb integrin [122]. SDF-1 secretion by activated platelets contributes to vascular remodeling through the recruitment and differentiation of bone marrow precursors [123, 124]. This process is mediated by CXCR4, CD184 and vascular endothelial growth factor receptor-1 (VEGFR-1) [125]. This SDF-1-dependent mechanism favors neo-vascularization of ischemic organs in which platelets accumulate (e.g. heart, liver and brain), thus contributing to their functional and structural repair [126]. Nevertheless, SDF-1 production by activated platelets can also trigger noxious responses by inducing a pro-inflammatory phenotype in hematopoietic precursors recruited to the arterial wall [127].

CONCLUSIONS

In addition to their well-recognized role in promoting thrombus formation after atherosclerotic plaque rupture, platelets exert pro-atherogenic actions by exacerbating inflammation at all stages of atherosclerosis development. This is achieved in part by facilitating the interaction between immune and endothelial cells through molecules such as sCD40L, P-selectin, RANTES, TLRs,

and SDF-1, even in the absence of platelet aggregation. These new findings provide evidence that platelet activation is an attractive therapeutic target for prevention of the transition from chronic to acute inflammation and the subsequent formation of atherosclerotic plaque and thrombosis.

ACKNOWLEDGEMENTS

We are grateful to Simon Bartlett for English editing. Work in the author's laboratories is supported by Centro de Estudios en Alimentos Procesados (CEAP), Conicyt-Regional, Gore Maule, R09I2001, Talca, Chile (Proyecto Basal); The Spanish Ministry of Science and Innovation (MICINN) and the European Regional Development Fund (grant SAF2010-16044); Instituto de Salud Carlos III (ISCIII) (Red de Enfermedades Cardiovasculares [RECAVA], grant RD06/0014/0021). O.M.P. holds a Juan de la Cierva contract (MICINN). The CNIC is supported by the MICINN and the Pro-CNIC Foundation.

Declaration of interest

The authors report no declarations of interest.

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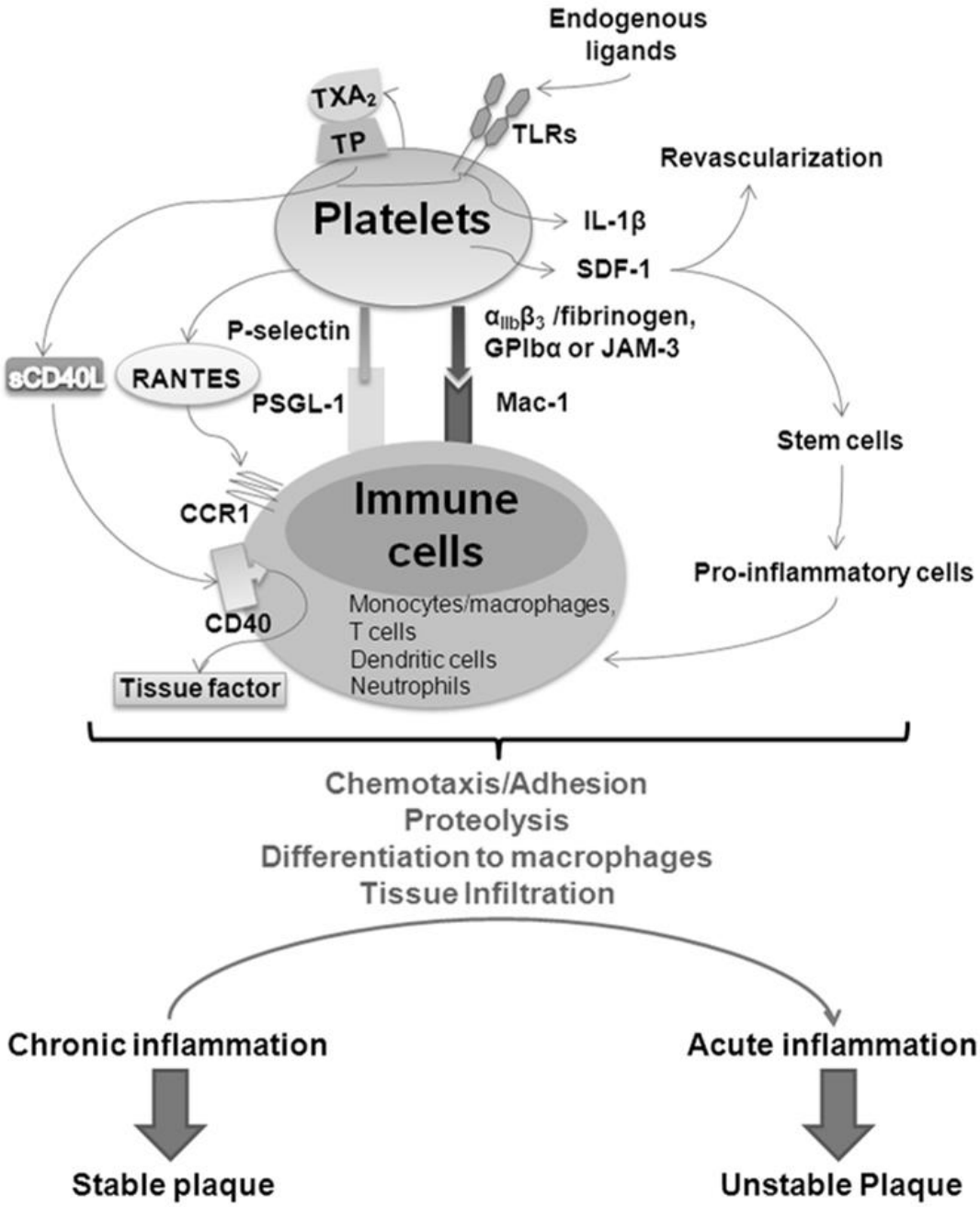

Figure 1. Role of platelets in the vascular inflammatory response associated with atherosclerosis. Platelets promote inflammation in the arterial wall in part through interaction with immune cells. Even without forming aggregates, platelets produce inflammatory molecules (sCD40L, P-selectin, RANTES and TLRs) that contribute to all stages of atherosclerosis, from chronic phases of atheroma initiation and growth to acute disease characterized by plaque rupture and ensuing thrombosis and ischemic events (myocardial infarction or stroke). Through the recruitment and differentiation of progenitor cells mobilized from the bone marrow, SDF-1 produced by platelets can have both beneficial effects (enhancement of angiogenesis) and harmful effects (exacerbation of inflammation) in the artery wall. CCR1, Chemokine (C-C motif) receptor 1; JAM-3,Junctional adhesion molecule 3; TXA_2, Thromboxane A2; TP, Thromboxane A2 receptor; TLRs, Toll-like receptors; IL-1β, Interleukin-1 beta; SDF-1, Stroma-cell derived factor-1; GPIbα, glycoprotein Ibα; PSGL-1, P-Selectin Glycoprotein Ligand 1; Mac-1, Macrophage 1 antigen.