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REVIEW ARTICLE

Platelet functions beyond hemostasis

S. S. SMYTH,* R. P. MCEVER,† A. S. WEYRICH,‡ C. N. MORRELL,§ M. R. HOFFMAN,¶ G. M. AREPALLY,** P. A. FRENCH,†† H. L. DAUERMAN,‡‡ and R. C. BECKER§§ FOR THE 2009 PLATELET COLLOQUIUM PARTICIPANTS[1]

**Lexington VA Medical Center and Division of Cardiovascular Medicine, Gill Heart Institute, University of Kentucky, Lexington, KY; †Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK; ‡Program in Human Molecular Biology & Genetics, University of Utah, Salt Lake City, UT; §Department of Molecular and Comparative Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD; ¶Pathology & Laboratory Medicine Service, Durham Veterans Affairs Medical Center, Durham, NC; **Department of Medicine, Division of Hematology, Duke University Medical Center, Durham, NC; ††Left Lane Communications, Chapel Hill, NC; ‡‡Cardiovascular Catheterization Laboratories, University of Vermont College of Medicine, Burlington, VT; and §§Cardiovascular Thrombosis Center, Duke Clinical Research Institute, Duke University Medical Center, Durham, NC, USA*

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Summary. Although their central role is in the prevention of bleeding, platelets probably contribute to diverse processes that extend beyond hemostasis and thrombosis. For example, platelets can recruit leukocytes and progenitor cells to sites of vascular injury and inflammation; they release proinflammatory and anti-inflammatory and angiogenic factors and microparticles into the circulation; and they spur thrombin generation. Data from animal models suggest that these functions may contribute to atherosclerosis, sepsis, hepatitis, vascular restenosis, acute lung injury, and transplant rejection. This article represents an integrated summary of presentations given at the Fourth Annual Platelet Colloquium in January 2009. The process of and factors mediating platelet–platelet and platelet–leukocyte interactions in inflammatory and immune responses are discussed, with the roles of P-selectin, chemokines and Src family kinases being highlighted. Also discussed are specific disorders characterized by local or systemic platelet activation, including coronary artery restenosis after percutaneous intervention, alloantibody-mediated transplant rejection, wound healing, and heparin-induced thrombocytopenia.

Keywords: adhesion, immune response, inflammation, platelets, secretion, transplantation.

Correspondence: Susan S. Smyth, Division of Cardiovascular Medicine, Gill Heart Institute, 900 S. Limestone Avenue, 326 Charles T. Wethington Building, Lexington, KY 40536, USA.
Tel.: +1 859 233 4511 ext. 4275; fax: +1 859 257 4845.
E-mail: susansmyth@uky.edu

[1]Participants in the 2009 Platelet Colloquium are listed in the Appendix.

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Platelets play important roles in several diverse processes beyond hemostasis and thrombosis, including promoting inflammatory and immune responses, maintaining vascular integrity, and contributing to wound healing. Platelets can recruit leukocytes and progenitor cells to sites of vascular injury and thrombosis; they store, produce and release proinflammatory and anti-inflammatory and angiogenic factors and microparticles into the circulation; and they spur thrombin generation. In experimental models, these functions have been shown to contribute to atherosclerosis, sepsis, hepatitis, vascular restenosis, acute lung injury, and transplant rejection.

This article represents an integrated summary of presentations given at the Fourth Annual Platelet Colloquium, held in Washington, DC on 22–24 January 2009, which focused, in part, on current knowledge regarding the role of platelets in vascular integrity, tissue repair, and immune responses.

Platelet secretion

The ability of platelets to store and release bioactive mediators allows them to play an important role in modulating the function of other cells. Platelets contain three types of storage compartments – α-granules, dense granules, and lysosomes – whose contents are released into the circulation or translocated to the platelet surface upon platelet activation [1]. When stimulated by thrombin, the platelet releasate contains > 300 proteins [2]. A partial listing of platelet granule contents can be found in Table 1 [3]. Fibrinogen, von Willebrand factor (VWF), platelet factor 4 (PF4), transforming growth factor-β and platelet-derived growth factor are among the contents of α-granules, whereas dense granules are rich in ADP and serotonin. Although the release of cargo during exocytosis delivers many proteins into the circulation, the process also alters the composition of the platelet membrane, resulting in surface expression of

Table 1 Platelet granular and secreted molecules

α-Granules	Dense bodies
Platelet-specific proteins	ADP
Platelet factor 4	ATP
β-Thromboglobulin family*	Calcium
Multimerin	Serotonin
Adhesive glycoproteins	Pyrophosphate
Fibrinogen	GDP
von Willebrand factor	Magnesium
von Willebrand factor propeptide	Other secreted or released proteins
Fibronectin	Protease nexin I
Thrombospondin-1	Gas6
Vitronectin	Amyloid β-protein precursor (protease nexin II)
Coagulation factors	Tissue factor pathway inhibitor
Factor V	Factor XIII
Protein S	α_1-Protease inhibitor
Factor XI	Complement 1 inhibitor
Mitogenic factors	High molecular weight kininogen
Platelet-derived growth factor	α_2-Macroglobulin
Transforming growth factor-β	Vascular permeability factor
Endothelial cell growth factor	Interleukin-1β
Epidermal growth factor	Histidine-rich glycoprotein
Insulin-like growth factor I	Chemokines
Angiogenic factors	MIP-Iα (CCL3)
Vascular endothelial growth factor	RANTES (CCL5)
Platelet factor 4 (inhibitor)	MCP-3 (CCL7)
Fibrinolytic inhibitors	Gro-α (CXCL1)
α_2-Plasmin inhibitor	Platelet factor 4 (CXCL4)
Plasminogen activator inhibitor-1	ENA-78 (CXCL5)
Albumin	NAP-2 (CXCL7)
Immunoglobulins	Interleukin-8 (CXCL8)
Granule membrane-specific proteins	TARC (CCL17)
P-selectin (CD62P)	
CD63 (LAMP-3)	
GMP 33	

Adapted from Parise *et al*. [3], with permission. *Platelet basic protein, low-affinity platelet factor 4, β-thromboglobulin, and β-thromboglobulin-F. CCL, C–C motif ligand; CXCL, C–X–C motif ligand; ENA, epithelial cell-derived neutrophil-activating (peptide); GMP, granule membrane protein; Gro, growth-related oncogene; LAMP, lysosome-associated membrane protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NAP, neutrophil-activating peptide; RANTES, regulated on activation, normal T-cell expressed and secreted; TARC, thymus and activation-regulated chemokine.

P-selectin and an increase in the number of integrin $\alpha_{IIb}\beta_3$ [glycoprotein (GP) IIb–IIIa] molecules. The exposure of P-selectin is especially important for platelet–leukocyte interactions, given that this receptor mediates the initial interactions of leukocytes with activated platelets. P-selectin also serves as a C3b-binding protein to initiate complement activation on the platelet surface [4].

Platelet production of bioactive mediators

Platelets are not only storage houses for bioactive molecules, but also generate lipid-derived mediators such as thromboxane A_2 and participate in transcellular metabolism, which results in the production of both proinflammatory and anti-inflammatory molecules. In addition, platelets have several unique, extranuclear mechanisms for translating mRNA into protein in a 'signal-dependent manner', and can produce, among other proteins, interleukin-1β and tissue factor, which may link hemostasis and inflammation [5].

Platelet–leukocyte interactions

Analogously to the interactions of leukocytes with inflamed endothelium, leukocytes can roll on a template of adherent platelets, firmly adhere, and then transmigrate through the adherent platelets [6,7]. Although some of the receptor–ligand pairs and signaling molecules that mediate platelet–leukocyte interactions may differ from those involved in endothelial cell–leukocyte interactions (Fig. 1) [7], many of the fundamental aspects are similar. Rolling and adhesion of leukocytes on platelets or endothelial cells are regulated by adhesive receptors, cellular geometry, and, perhaps of greatest overall relevance, shear forces generated within flowing blood [8].

The selectin family of adhesive receptors mediates the initial stage of cellular rolling (Fig. 1) [9]. P-selectin plays an essential role in platelet–leukocyte contacts, whereas both P-selectin and E-selectin are present on endothelial cells and contribute to endothelial cell–leukocyte interactions. The third selectin, L-selectin, is present on leukocytes. The best-characterized

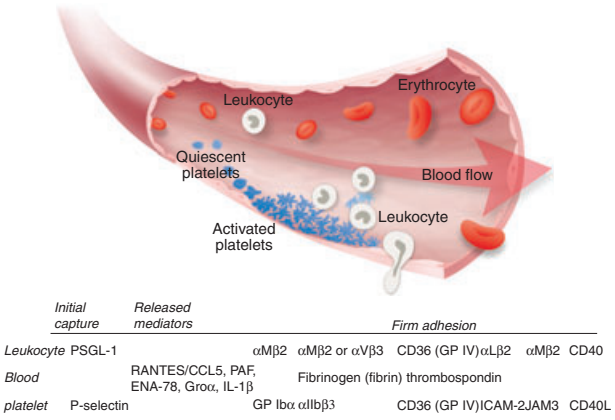

Fig. 1. Rolling, tethering, firm adherence and transmigration of leukocytes promoted by platelet deposition at sites of vascular injury. Platelets bind to exposed subendothelium at sites of vascular injury and can adhere to inflamed, activated endothelium. Activated, adherent platelets can recruit leukocytes to sites of injury or inflammation. Some of the key receptor pairs involved in initial and sustained platelet–leukocyte interactions are noted. ENA, epithelial neutrophil-activating peptide; GP, glycoprotein; Gro, growth-related oncogene; IL, interleukin; ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; PAF, platelet-activating factor; PSGL, P-selectin glycoprotein ligand; RANTES, regulated on activation, normal T-cell expressed and secreted. Illustration by Matt Hazzard, University of Kentucky; adapted from Wagner [7], with permission.

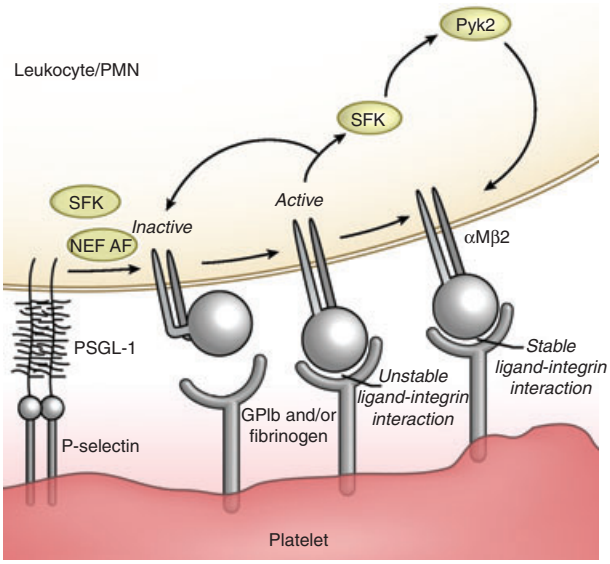

Fig. 2. Hypothetical scheme of a three-step model of $\alpha_M\beta_2$ activity during polymorphonuclear cell (PMN)–platelet adhesion. Step 1: platelet–PMN interactions mediated by P-selectin, P-selectin glycoprotein ligand (PSGL)-1, and G-protein-coupled receptors (GPCRs) induce initial activation and ligand binding to $\alpha_M\beta_2$. Step 2: generation of an outside-in, Src-family kinase (SFK)-Pyk2–mediated signal(s). Step 3: stabilization of integrin–ligand binding. AF, activating factor; GP, glycoprotein; NEF, negative factor. Adapted from Evangelista *et al*. [21], with permission.

leukocyte ligand for P-selectin is P-selectin glycoprotein ligand (PSGL)-1, which can interact with all selectin subtypes under inflammatory conditions [10]. Ligation of PSGL-1 transmits signals within the leukocyte that are necessary for adhesion mediated by leukocyte integrins [11]. Although unnecessary for leukocyte rolling on P-selectin, the cytoplasmic domain of PSGL-1 is essential for activation of leukocyte β_2 integrins [12].

Immobilized and released chemokines are also required for firm leukocyte adhesion and arrest. In reconstituted systems, immobilized chemokines trigger the arrest of leukocytes within 1 s [13,14], largely due to activation of G-protein-coupled receptors (GPCRs). Prominent among the chemokines released by platelets that influence leukocyte function and platelet–leukocyte interactions are PF4/CXCL4, RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5), and growth-related oncogene-α [15,16].

When leukocytes receive signals from both activated PSGL-1 and GPCRs, the expression of transcription factors, cytokines and chemokines is increased [17–20]. Leukocyte activation enhances the strength of the integrin bonds, leading to firm adhesion mediated by integrin $\alpha_M\beta_2$ (Mac-1) binding to GPIb and/or other ligands, such as fibrinogen bound to integrin $\alpha_{IIb}\beta_3$, on the platelet surface [5]. Signaling through Src-family kinases (SFKs) is required to sustain β_2 integrin activation [21]. An important downstream mediator of SFK-dependent signaling may be Pyk2, which is phosphorylated in leukocytes upon adhesion to platelets and is required to sustain platelet–neutrophil adhesion in murine and human cells (Fig. 2) [21]. As leukocytes undergo the transition from rolling to more firm adhesion, they become polarized through clustering of L-selectin and PSGL-1, and this promotes further leukocyte recruitment through leukocyte–leukocyte interactions [22,23].

Disorders involving local and systemic platelet activation

Given the number and variety of bioactive substances secreted by platelets (Table 1), it is unsurprising that they have been implicated in the development or severity of an array of disorders (Table 2) [11,24–36]. Some of these disorders may appear obvious (thrombosis and restenosis) but others may not (psoriasis and migraine).

Restenosis

The contribution of platelets to arterial injury and restenosis has been extensively studied in experimental models, and the information gained from these investigations provides a framework with which to understand the possible role of platelets in other inflammatory conditions. In a murine model of angioplasty wire-induced femoral artery injury, endothelial denudation is followed by a stereotypical response that includes platelet deposition, leukocyte recruitment, and altered arterial composition (Fig. 1). This response ultimately leads to development of intimal hyperplasia, the clinical correlate of which is restenosis [25]. A single layer of platelets is sufficient to promote recruitment of leukocytes, which is mediated by P-selectin [37]. P-selectin is required for the development of intimal hyperplasia, and chimeric mice lacking bone marrow-derived P-selectin are

protected from developing neointima. Integrin $\alpha_M\beta_2$ is also required for leukocyte recruitment to adherent platelets and for the development of intimal hyperplasia.

Work from the Simon group has established that, in the context of arterial injury, GPIb is the predominant platelet protein mediating binding of $\alpha_M\beta_2$. They developed both peptide and antibody reagents that specifically target the binding of $\alpha_M\beta_2$ to GPIb and prevent firm adhesion of human and murine leukocytes to adherent platelets under laminar flow conditions. Administration of these inhibitors in mice reduces leukocyte accumulation after wire-induced injury of the murine femoral artery, and inhibits cellular proliferation and neointimal thickening [25,38]. Likewise, disruption of SFK signaling in leukocytes, which is required to sustain activation of $\alpha_M\beta_2$, prevents leukocyte recruitment to adherent platelets and the development of intimal hyperplasia after endothelial denudation injury [21]. These studies establish several potential therapeutic strategies for targeting key pathways that promote platelet–leukocyte interactions and the inflammatory response leading to restenosis.

Alloantibody-mediated transplant rejection

Platelets have recently been implicated in the development of alloantibody-mediated transplant rejection. Here, antibodies target the endothelial cell major histocompatibility complex (MHC)-1 within the transplanted tissue. Antibodies to human leukocyte antigen activate human endothelial cell exocytosis and leukocyte trafficking [39]. Alloantibodies also appear to recruit and activate platelets via platelet Fc receptors. In addition, alloantibodies can activate complement, which then causes damage to the endothelium, with the corresponding recruitment of platelets [39].

Experiments in a murine model of MHC mismatch, in which skin from B10A mice is transplanted onto BalbC or B6 nude mice, have shed light on the dynamic interactions between platelets and white cells in the endothelium of transplanted tissue [40]. At 7 days after transplant, when blood flow had been established within grafted tissue, Morrell *et al.* injected fluorescently labeled platelets from the recipient strain of mice with either an IgG_{2a} or IgG_1 monoclonal antibody targeting the donor MHC, and examined the results in real time by intravital microscopy. Repeated injections of IgG_{2a} alloantibody resulted in sustained platelet–endothelial cell interactions and vascular pathology, including the release of VWF, the creation of small platelet thrombi, and complement deposition [40]. Continued platelet–endothelium interactions depended on activation of complement. Platelets recruited leukocytes to sites of alloantibody deposition, increased transplant P-selectin and complement expression, and increased transplant vascular cell adhesion molecule expression.

Wound healing

Components of the hemostatic system, including coagulation factors and platelets, are required for wound healing. After vascular injury, clots that are rich in platelets and fibrin form a scaffold for healing. Thrombin, in addition to its procoagulant role in transforming fibrinogen into fibrin, also acts a chemoattractant for macrophages, stromal cells, and endothelial cells [41–43], has growth factor mitogenic activities, and supports angiogenesis [44]. Platelets contribute to wound healing by promoting thrombin generation and by secreting a wide range of growth factors, cytokines and chemokines that directly influence the reparative process [45]. Observations from experimental models of hemophilia B provide evidence supporting the role of hemostasis in wound healing. Mice with hemophilia B (absence of factor IX) have delayed and histologically abnormal healing [46]. Correction of thrombin generation at the time of wounding, by administration of human FIX or FVIIa, did not restore normal healing in mice with hemophilia B [47], but instead increased late bleeding during healing, primarily at sites of angiogenesis. Thus, adequate functioning of the coagulation system appears to be necessary for normal wound healing, beyond the establishment of initial hemostasis.

Vascular integrity

Platelets are also critical to the overall maintenance of vascular integrity. In 1969, Gimbrone *et al.* [48] showed that thyroid glands being stored for transplantation had preserved vascular integrity (no disruption of the endothelium) if they were perfused with platelet-rich plasma rather than platelet-poor plasma. Kitchens and Weiss [49] showed that rabbits made thrombocytopenic by injecting antiplatelet serum displayed histologic evidence of endothelial thinning as compared with animals not injected. In fact, petechiae commonly form in the setting of thrombocytopenia, because of red cells extravasating through small channels in the endothelium [50]. According to one investigation, approximately 18% of platelet turnover can be attributed to support of vascular integrity [51]. The role of platelets in maintaining the vasculature during normal or healing angiogenesis may differ from their role under pathologic conditions. For example, platelet adhesion is required to stabilize angiogenic vessels at healing sites, but not tumor vessels [53] or in the setting of inflammation [54]. Platelets are required to maintain endothelial barrier function in inflamed and tumor vessels through processes that may involve produced and/or released platelet products, rather than adhesive events.

Heparin-induced thrombocytopenia

Platelet-derived antigens can trigger immune-mediated responses, the most common of which is heparin-induced thrombocytopenia (HIT). Thrombocytopenia affects an estimated 30–40% of inpatients receiving heparin for ≥ 4 days, with 10% of patients suffering from immune-mediated HIT [55]. Immune-mediated HIT results from antibodies being developed against PF4–heparin complexes.

PF4 is synthesized in megakaryocytes, packaged into platelet α-granules, and released upon platelet activation. It normally

Table 2 Disorders associated with platelet activation and platelet–leukocyte interactions

Acute lung injury [11]	Interactions of platelets, leukocytes and ECs are critical to pathogenesis; key molecules include P-selectin and the eicosanoid thromboxane A_2
Atherosclerosis, thrombosis, restenosis [24,25]	Interactions of platelets, leukocytes and ECs trigger autocrine and paracrine activation, leading to leukocyte recruitment to the vascular wall. Platelet-induced chronic inflammation of the vascular wall leads to atherosclerotic lesions and atherothrombosis; $\alpha_M\beta_2$ engagement of GPIbα is critical to the biological response to vessel injury
Inflammatory hepatitis [26]	Bilateral ductal ligation induced intravascular platelet aggregates in the liver in mice, increased platelet adhesion in postsinusoidal venules, and massive platelet accumulation in liver sinusoids. P-selectin mediated cholestasis-induced platelet accumulation
Inflammation in obesity [27]	In *ob*/*ob* mice and mice with obesity induced by a high-fat diet, visceral adipose tissue showed increased leukocyte–EC–platelet interactions in the microcirculation, increased P-selectin expression, formation of monocyte–platelet conjugates, and upregulated expression of adhesion molecules on macrophages and ECs
Inflammatory bowel disease [28]	Patients with Crohn's disease or ulcerative colitis showed increased platelet surface expression of P-selectin and GP53, increased circulating platelet aggregates, incteased platelet aggregability *in vitro* and increased serum β-thromboglobulin levels as compared with healthy controls
Migraine [29]	Platelet activation and leukocyte–platelet aggregation were significantly increased during migraine headaches in patients without aura vs. control volunteers in flow cytometry assays, but not in migraine patients with aura. All patients had an increased baseline level of platelet activation, and triptan drugs appeared to downregulate platelet aggregation. Possible role for release of serotonin from platelets
Psoriasis [30]	Spontaneous platelet hyperaggregability and plasma levels of β-thromboglobulin were significantly increased in male patients with psoriasis vs. control subjects. Platelet regeneration time was significantly shorter in patients with the disease
Reperfusion-induced inflammation after CPB [31]	In 12 patients undergoing CABG, IL-6 levels and platelet CD62 expression rose early during reperfusion after CPB. Leukocyte–platelet microaggregates also formed during surgery and persisted during reperfusion
Rheumatoid arthritis [32]	In 16 patients with active disease, platelet count and CRP and IL-6 levels were elevated and correlated with each other, whereas neutrophil count, platelet volume and myeloperoxidase level also mirrored disease activity but did not correlate with other markers
Sepsis [33,34]	Disseminated intravascular platelet activation can occur in cases of systemic inflammation, such as the response to sepsis, contributing to microvascular failure and thus organ dysfunction. Platelets can also be directly involved in the inflammatory response – platelet TLR-4 binding to ligands on adherent neutrophils leads to robust neutrophil activation and formation of neutrophil extracellular traps, which retain their integrity under flow conditions and ensnare bacteria within vessels
SLE [35]	Patients with SLE had higher levels of platelet microparticles, CD62P expression, annexin V, IL-1β, IL-4, IL-6, GM-CSF, and TNF-α, and soluble factors (serum IL-2R, thrombomodulin, HLA-1, β_2-microglobulin, VCAM-1, PECAM-1, P-selectin, E-selectin) vs. control patients. Levels of IL-4, IL-6, β_2-microglobulin, IL-2R, VCAM-1, P-selectin and E-selectin were particularly high in SLE patients with elevated thrombomodulin levels, as were levels of CD62P expression, annexin V, and microparticles
Transplant rejection [36]	Platelet interactions with dendritic cells, T cells and B cells can contribute to vasculopathy in transplants. Activated platelets secrete chemokines to recruit helper and cytotoxic T cells; activated T cells then stimulate platelets, through CD40–CD154 interactions, to secrete more chemokines, thereby recruiting more T cells. P-selectin/PSGL-1 stimulation enhances platelet–T-cell interactions. Antibody production stimulated through increased helper T-cell function can activate complement, creating another activation loop when platelets express receptors for antibodies and complement

CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass; CRP, C-reactive protein; EC, endothelial cell; GM-CSF, granulocyte–macrophage colony-stimulating factor; GP, glycoprotein; HLA, human leukocyte antigen; IL, interleukin; IL-2R, interkeukin-2 receptor; PECAM, platelet–endothelial cell adhesion molecule; PSGL, P-selectin glycoprotein ligand; SLE, systemic lupus erythematosus; TLR, toll-like receptor; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

binds to heparin-like molecules in the vascular endothelial wall, but it can also bind circulating heparin in patients treated with unfractionated or low molecular weight heparin. Macromolecular PF4–heparin complexes, which can approach 1 μm in diameter *in vitro* [56], stimulate production of 'HIT antibodies', a subset of which activates platelets [57]. The most serious manifestation of HIT is thrombosis, which can occur in up to 75% of patients [57].

Experimental models for the study of HIT have been developed in mice, by means of intravenous injection of murine PF4 and heparin. In this model, C57BL/6 mice show a greater immune response to the murine PF4–heparin antigen than do BALB/c mice [58]. More recent efforts have focused on stimulating endogenous PF4 release by means of systemic activation of platelets and concomitant injection of heparin at the time of platelet activation to trigger the formation of PF4–heparin complexes.

Conclusion

In conclusion, knowledge regarding the role of platelets in the development and severity of various disorders beyond thrombosis continues to emerge, particularly in the arenas of inflammation and the immune response. Although we have focused on the role of platelets in inflammatory, immune and wound-healing responses, other findings have implicated platelets as regulators of atherosclerosis, angiogenesis, tumor progression, metastasis, and other diverse processes (see Table 2 for more detail). These observations not only expand our view of the role of platelets outside of hemostasis, but also suggest that antiplatelet therapy may have applications beyond thrombosis.

Addendum

All authors provided critical writing or revision for intellectual content and granted final approval of the version to be published. S. S. Smyth and P.A. French wrote the first draft of the manuscript, with concept and design input and additional critical analysis and interpretation from H. L. Dauerman and R. C. Becker.

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Disclosure of Conflict of Interests

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Appendix: participants in the 2009 Platelet Colloquium

Gowthami M. Arepally, Duke University Medical Center, Durham, NC; Richard C. Becker, Duke University Medical Center and Duke Clinical Research Institute, Durham, NC; Deepak L. Bhatt, Brigham and Women's Hospital and the VA Boston Healthcare System, Boston, MA; Jaehyung Cho, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; Harold L. Dauerman, University of Vermont College of Medicine, Burlington; Daniel D. Gretler, Portola Pharmaceuticals, Inc., South San Francisco, CA; Maureane R. Hoffman, Durham VA Medical Center, Durham, NC; Jay Horrow, AstraZeneca, Wilmington, DE; Neal S. Kleiman, The Methodist DeBakey Heart and Vascular Center, The Methodist Hospital, Houston, TX; Richard Kocharian, The Medicines Company, Parsippany, NJ; A. Michael Lincoff, Lerner Research Institute, Cleveland Clinic Coordinating Center for Clinical Research, Cleveland, OH; Juan Maya, AstraZeneca, Wilmington, DE; Rodger P. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK; Craig N. Morrell, The Johns Hopkins University School of Medicine, Baltimore, MD; Jayne Prats, The Medicines Company, Waltham, MA; Christopher P. Rusconi, Regado Biosciences, Inc., Durham, NC; Susan S. Smyth, University of Kentucky, Lexington; John Strony, Schering-Plough Research Institute, Kenilworth, NJ; Henry Sun, The Johns Hopkins Hospital, Baltimore, MD; Enrico P. Veltri, Schering-Plough Research Institute, Kenilworth, NJ; Andrew S. Weyrich, University of Utah, Salt Lake City, UT; Stephen D. Wiviott, Brigham and Women's Hospital and Harvard Medical School, Boston, MA; Jeremy P. Wood, University of Vermont, Burlington, VT.

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