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Platelet α–granules: Basic biology and clinical correlates

Price Blair, Ph.D. and **Robert Flaumenhaft, M.D., Ph.D.***
Division of Hemostasis and Thrombosis, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215

Summary

α–Granules are essential to normal platelet activity. These unusual secretory granules derive their cargo from both regulated secretory and endocytotic pathways in megakaryocytes. Rare, inheritable defects of α–granule formation in mice and man have enabled identification of proteins that mediate cargo trafficking and α–granule formation. In platelets, α–granules fuse with the plasma membrane upon activation, releasing their cargo and increasing platelet surface area. The mechanisms that control α–granule membrane fusion have begun to be elucidated at the molecular level. SNAREs and SNARE accessory proteins that control α–granule secretion have been identified. Proteomic studies demonstrate that hundreds of bioactive proteins are released from α–granules. This breadth of proteins implies a versatile functionality. While initially known primarily for their participation in thrombosis and hemostasis, the role of α–granules in inflammation, atherosclerosis, antimicrobial host defense, wound healing, angiogenesis, and malignancy has become increasingly appreciated as the function of platelets in the pathophysiology of these processes has been defined. This review will consider the formation, release, and physiologic roles of α–granules with special emphasis on work performed over the last decade.

Keywords

α-granule; vesicle trafficking; endocytosis; secretion; hemostasis

Overview of platelet α–granules

Platelets are anucleate, discoid shaped blood cells that serve a critical function in hemostasis and other aspects of host defense. These cells are replete with secretory granules, which are critical to normal platelet function. Among the three types of platelet secretory granules – α–granules, dense granules, and lysosomes - the α–granule is the most abundant. There are approximately 50–80 α–granules per platelet, ranging in size from 200–500 nm.[1] They comprise roughly 10% of the platelet volume, 10-fold more than dense granules. The total α–granule membrane surface area per platelet is 14 μm^2, ~8-fold more than dense granules and approximately equal to that of the open canalicular system (OCS),[1] an elaborate system of tunneling invaginations of the cell membrane unique to the platelet.[2] The extra membrane

*Address correspondence to: Robert Flaumenhaft, M.D., Ph.D., Center for Life Science, Rm 939, Beth Israel Deaconess Medical Center, 3 Blackfan Circle, Boston, MA, 02215. Tel: 617-735-4005, Fax: 617-735-4000, Email: E-mail: rflaumen@bidmc.harvard.edu.

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provided by the OCS and α–granules enables the platelet to increase its surface area by 2–4-fold upon platelet stimulation and/or spreading.

Morphologic features observed by electron microscopy have historically defined α–granules. They include 1) the peripheral membrane of the granule, 2) an electron dense nucleoid that contains chemokines and proteoglycan, 3) a less electron dense area adjacent to the nucleoid that contains fibrinogen, and 4) a peripheral electronluscent zone that contains von Willebrand factor (vWf).[3] Not all zones, however, need to be observed in order to positively identify an α–granule. α–Granules have also been identified based on immunofluorescence studies. Staining of granule constituents such as P-selectin, vWf, and/or fibrinogen or other established markers identifies α–granules by this technique. However, α–granules appear to be heterogeneous with regard to cargo.[4,5] Absence of a particular α–granule marker does not preclude classification of a vesicular structure as an α–granule. Thus, the definition of α–granules may yet undergo further refinement as we learn more about their formation, structure, and content.

Formation of α–granules

Vesicle trafficking

The development of α–granules begins in the megakaryocyte, but continues in the circulating platelet. In the megakaryocyte, α–granules are derived in part from budding of small vesicles containing α–granule cargo from the trans-Golgi network (Fig. 1).[6,7] In other cell models, an orchestrated assemblage of coat proteins (e.g., clathrin, COPII), adaptor proteins (e.g., AP-1, AP-2, AP-3), fusion machinery (e.g., soluble NSF attachment protein receptors [SNAREs]), and monomeric GTPases (e.g., Rabs) mediate vesicle trafficking and maturation. Clathrin coat assembly likely functions, too, in trafficking of vesicles from the trans-Golgi network to α–granules in megakaryocytes. The clathrin-associated adaptor proteins AP-1, AP-2, and AP-3 are found in platelets[8,9] and are proposed to function in clathrin-mediated vesicle formation in platelets.[10] Mutations in the gene encoding AP-3, for example, results in impaired dense granule formation.[9] Clathrin-mediated endocytosis also functions in the delivery of plasma membrane into α–granules (Fig. 1). Vesicles budding off from either the trans-Golgi network or the plasma membrane can subsequently be directed to multivesicular bodies (MVBs).

MVBs found in most cells are endosomal structures containing vesicles that form from the limiting membrane of the endosome.[11,12] They are typically transient structures involved in sorting vesicles containing endocytosed and newly synthesized proteins. In megakaryocytes, MVBs serve in an intermediate stage of granule production.[13] Both dense granules and α–granules are sorted by MVBs.[13,14] Vesicles budding from the trans-Golgi network may be delivered directly to MVBs (Fig. 1).[13] Kinetic studies in megakaryocytes have demonstrated that transport of endocytosed proteins proceeds from endosomes to immature MVBs (MVB I, with internal vesicles alone) to mature MVBs (MVB II, with internal vesicles and an electron dense matrix) to α–granules. α–Granules within MVBs contain 30–70 nm vesicles, termed exosomes.[13] Some exosomes persist in mature α–granules and are secreted following platelet activation.[15] Although it is unknown whether all or most vesicle trafficking to α–granules proceeds through MVB, these observations indicate that MVB represent a developmental stage in α–granule maturation.

Maturation of α–granules continues in circulating platelets by endocytosis of platelet plasma membranes.[16–18] A clathrin-dependent pathway leading to the delivery of plasma membrane to α–granules has been described, as has a clathrin-independent pathway that traffics vesicles to lysosomes.[18] Unlike other cells, coated vesicles in platelets retain their clathrin coat throughout trafficking and for a period following fusion with α–granules.[17] Platelet endocytosis appears to be a constitutive activity of resting platelets. The molecular control of

endocytosis in platelets is not known, but may involve the Src family receptors Fyn, Fgr, Lck, and/or Lyn based on colocalization studies, their tyrosine-phosphorylation status,[19,20] and evidence of a role for Src family receptors in lymphocyte endocytosis.[21] Studies performed in dogs evaluating the accumulation of fibrinogen and immoglobulin, which are endocytosed by circulating platelets, show that levels of endocytosed, but not endogenous, α–granule proteins increase as platelets age.[22] This observation confirms that constitutive trafficking to α–granules continues throughout the lifespan of the platelet.

Protein Sorting

Many α–granule proteins are produced by megakaryocytes and sorted to α–granules via a regulated secretory pathway. These proteins are synthesized in the endoplasmic reticulum, exported to the Golgi for maturation, and subsequently sorted at the trans-Golgi network.[23] Trafficking of some well-known α–granule proteins synthesized in megakaryocytes, such as P-selectin, has been evaluated. Initial studies in heterologous cells indicated that the sorting sequence for P-selectin is contained within its cytoplasmic tail.[24–27] Subsequent studies, however, indicated that the cytoplasmic tail of P-selectin targets this adhesion molecule to storage granules in endothelial cells, but not in platelets.[25,28] This observation demonstrates that while some principles of protein sorting can be generalized among cell types, the mechanism of sorting of a particular protein can vary between cell types.

Trafficking of soluble proteins has also been evaluated. Study of the targeting of CXCL4 (also known as platelet factor 4) to α–granules has led to the identification of a signal sequence responsible for sorting chemokines into α–granules.[29,30] These experiments demonstrate that a four amino acid sequence within the exposed hydrophilic loop is required for sorting of CXCL4 into α–granules.[30] An analogous sequence was identified in the platelet chemokines RANTES and NAP-2.[30]

Soluble proteins must be incorporated into vesicles formed at the trans-Golgi network to become cargo within mature α–granules. A mechanism involving binding to glycosaminoglycans has been proposed for sorting small soluble chemokines. Mice that lack the dominant platelet glycosaminoglycan, serglycin, fail to store soluble proteins containing basically charged regions, such as CXCL4, PDGF, or NAP-2, in their α–granules.[31] This observation suggests that glycosaminoglycans may serve as a retention mechanism for chemokines possessing an exposed cationic region. A mechanism to incorporate larger soluble proteins into α–granules is by aggregation of protein monomers.[32] Although not formally proven to sort by aggregation, large, self-assembling proteins such as multimerin have been proposed to sort into immature vesicles by homoaggregation.[33] vWf self-assembles into large multivalent structures and is packaged into a discrete tubular structure within α–granules.[34,35] Heterologous expression of vWf can drive the formation of granules in cell lines possessing a regulated secretory pathway (e.g., AtT-20, HEK293, or RIN 5F cells), but not in cells lines that lack such a pathway (CHO, COS, or 3T3 cells).[36,37] While aggregation and glycosaminoglycan binding represent plausible mechanisms for sorting soluble proteins, alternative sorting receptors must exist for other endogenous α–granule proteins.

Plasma proteins are incorporated into α–granules via several distinct mechanisms of endocytosis. During receptor mediated endocytosis, a plasma protein is bound to a platelet surface receptor and subsequently internalized via a clathrin-dependent process. The most well-studied example is the incorporation of fibrinogen via integrin $\alpha_{IIb}\beta_3$.[38–42] Plasma proteins such as immunoglobulins and albumin incorporate into α–granules via pinocytosis.[43] The endocytosis of factor V by megakaryocytes involves two receptors. Following initial binding to a specific factor V receptor, subsequent binding to low-density lipoprotein receptor-related protein-1 (LRP-1) occurs and clathrin-dependent mediated endocytosis ensues.[44,45]

Endocytosis of α–granule proteins may occur at the level of the megakaryocyte, the platelet, or both.

Transport of α–granules into platelets

α–Granules formed in megakaryocytes must be distributed to platelets during megakaryopoiesis. Two models to account for organelle delivery during megakaryopoiesis include the fragmentation model and the proplatelet model. The fragmentation model predicts that the megakaryocyte demarcation membrane system divides the cell into regions, each containing their allotment of organelles.[46] The proplatelet model predicts that, in a profound terminal reorganization of the megakaryocyte, platelets form along extended projections termed proplatelets.[47–49] Recent studies have indicated that platelets are produced *in vivo* via the formation of proplatelets[50] and that α–granules are transported from megakaryocytes to α–granules on microtubule bundles.[51] These studies demonstrate that organelles within the megakaryocyte move from the cell body to the nascent platelets on microtubule tracks, powered by the microtubule motor proteins. Organelles move at a rate of 0.1–2 μm/min in what appears to be a random direction. They are captured in developing platelets by virtue of microtubule coils, which persist in platelets.[51]

Individual granules that move along proplatelet microtubule tracks appear to be heterogeneous with regard to cargo (Fig. 2).[4] Some α–granules stained with antibodies directed against vWf, but not fibrinogen. Others stained with antibodies against fibrinogen, but not vWf.[4] Additional antigen pairs such as vascular endothelial cell growth factor (VEGF) and endostatin, as well as basic fibroblast growth factor (bFGF) and thrombospondin-1, were also found to reside in different α–granule subpopulations.[4] Differential staining of α–granule subpopulations is observed in mature platelets as well.[4,5] Whether different α–granule subpopulations represent α–granules derived from different sources (e.g., endocytosis versus regulated secretory pathway), differentially sorted in MVBs, or separated by yet unknown mechanisms remains to be determined.

Defects in α–granule formation

Defects of α–granule formation have been described in both patients and mice. Gray platelet syndrome is the best known of the inherited disorders of α–granule formation (for review see [52]). This syndrome is heterogenous and its genetic underpinnings have yet to be elucidated. α–Granules are also severely reduced in Medich giant platelet disorder and the White platelet syndrome.[53,54] The molecular defects resulting in these syndromes, however, have not been identified.

α–Granule deficiency can result from mutations or deletions of specific transcription factors. For example, mice that lack Hzf, a zinc finger protein that acts as a a transcription factor, produce megakaryocytes and platelets with markedly reduced α–granules, mimicking Gray platelet syndrome.[55] Fibrinogen, PDGF, and vWf are nearly absent from Hzf-deficient platelets. However, no mutations in the orthologous *Hzf* gene were identified in a series of patients with Gray platelet syndrome.[56] Mutations in GATA1 have been described in patients with thrombocytopenia and markedly reduced or absent α–granules.[57,58] The downstream regulators involved in granule formation, however, have not been characterized for these transcription factor mutants.

Some mutations resulting in markedly decreased or absent α–granules occur in genes encoding proteins involved in vesicular trafficking. ARC syndrome results from mutations in the *VPS33B* gene.[59,60] VPS33B is a membrane-associated protein that binds tightly to and regulates the function of SNAREs.[60] VPS33B associates with α–granules in platelets.[59] Patients with this mutation possess α–granule-deficient platelets (Fig. 3), and their platelets

possess no detectable PF4, vWf, fibrinogen, nor P-selectin.[59] This observation indicates that loss of VPS33B effects incorporation of both endogenous and endocytosed proteins, as well as both soluble and membrane-bound proteins, into α–granules.[59] The number of dense granules in VPS33B-deficient platelets is somewhat increased, indicating that VPS33B function is not critical for dense granule formation. Isolated deficiencies of dense granule formation with normal α-granule formation, such as in the Hermansky-Pudlak syndrome, are well-described. These observations further support the premise that dense granule and α–granule formation require distinct membrane trafficking machineries.

That said, some mutations in vesicle trafficking proteins result in defects in both dense granule and α–granule formation, indicating aspects of commonality between the two pathways. Gunmetal mice with a mutant Rab geranylgeranyl transferase demonstrate a macrothrombocytopenia with a significant defect in both α–granule and dense granule production.[61,62] While the substrates of Rab geranylgeranyl transferase involved in granule formation have not all been elucidated, Rab27 is hypoprenylated in gunmetal mice and associates with both α–granules and dense granules.[61,63,64] Rab27 is a small GTP binding protein that regulates membrane trafficking.[65] Mice deficient in Rab27b demonstrate reduced numbers of α–granules and dense granules in their megakaryocytes and impaired proplatelet formation.[63] These observations suggest that Rab27b may coordinate proplatelet formation with granule transport. Other Rab proteins, including Rabs 1a, 1b, 3b, 5a, 5c 6a, 7, 8, 10, 11a 14, 18, 21, 27a, 27b, 32, 37 are present in platelets, associated with membranes, and may function in membrane trafficking and granule formation.[8,66]

Defects in membrane composition can also result in aberrant α–granule formation. Mice lacking the ATP-binding cassette half-transporter, ABCG5, suffer sitosterolemia, an accumulation of circulating plant sterols, and a macrothrombocytopenia characterized by large platelets with decreased granules.[67] Sitosterolemia secondary to ABCG5 deficiency also occurs in humans and results in macrothrombocytopenia.[68] The reason why the megakaryocyte membrane system is more sensitive than other cells to plant sterols is unknown. However, the observation that α–granule formation is impaired in this condition may inform strategies for studying how α–granule membranes form.

Molecular mechanisms of α–granule release

α–Granule contents must be released from their intracellular repository in order to achieve their physiologic function. α–Granule contents are release when the α–granule membrane fuses with surface-connected membranes of the OCS or the plasma membrane.[69] SNAREs represent the core of the fusion machinery. They are membrane-associated proteins that are oriented to the cytosol (Fig. 4). SNAREs associated with granules are termed vesicular SNAREs (vSNAREs), while those associated with target membranes (e.g., OCS and plasma membrane) are termed tSNAREs. The association of vSNAREs with tSNAREs generates the energy required for membrane fusion.[70]

Known platelet vSNAREs include VAMP-2, -3, -7, and -8; while known platelet tSNAREs include syntaxins 2, 4, 7, and 11 and SNAP-23, -25, and -29.[71–77] Studies performed in mice deficient in specific VAMP isoforms indicate that VAMP-8 is the dominant vSNARE involved in α–granule release, while VAMP-3 and perhaps VAMP-2 play subordinate roles.[76,78] Syntaxins 2 and 4 both appear to function in α–granule release, which is unusual in that two syntaxin isoforms do not typically mediate release of the same granule.[72,79] There is no indication that SNAP-25 or SNAP-29 function in α–granule release, while the function of SNAP-23 is well-established.[72,79,80] The distribution of SNAREs in platelets provides a basis for several characteristics of α–granule secretion, including homotypic α–granule fusion and the fusion of α–granules with the open canalicular system and plasma membrane.[81] In addition

to their roles in α–granule release, SNAREs likely participate in platelet granule formation. However, this SNARE function yet to be evaluated in detail.

The function of SNAREs in platelet granule secretion must be tightly regulated so as to prevent the indiscriminant release of α–granule cargo. The (Sec1/Munc) SM proteins function as clamps to regulate the function of SNAREs. SM protein isoforms found in platelets include Munc13-4 and Munc18a, b, and c. Of these, Munc18c has been found to function in α–granule release.[82] Munc-18c is complexed primarily to syntaxin-4 in platelets.[82] An antibody that prevents association of Munc-18c with syntaxin-4 augments α–granule release, raising the possibility that Munc18c serves as a negative regulatory of SNARE function.[82] Munc13-4 functions in dense granule release as a downstream effector of Rab27;[83] however, its role in α–granule release is not known. CDCrel-1 is another syntaxin binding protein implicated in the regulation of α–granule release. Platelets from mice lacking CDCrel-1 demonstrate enhanced secretion in response to collagen.[84]

Many other chaperone proteins that bind to and direct the function of SNARE proteins have been described. A subset of these proteins has been found in platelets, and some of these function in α–granule secretion. NSF is a hexameric ATPase that is essential for most forms of membrane-trafficking, including regulated granule secretion.[85] The primary role of NSF is to disassemble SNARE complexes present on the same membrane (cis conformation) so that they are available to interact with cognate SNAREs on opposing membranes (trans conformation). Both inhibitory peptides and antibodies to NSF have been demonstrated to interfere with α–granule release from platelets.[79,86] Further evidence suggests that nitric oxide inhibits NSF regulation of α–granule release.[87] The soluble NSF-attachement protein (SNAP) α–SNAP binds and activates NSF.[88] In platelets, wild-type α–SNAP augments granule secretion, whereas a dominant-negative α–SNAP mutant (α–SNAPL294A) and antibodies directed at α–SNAP inhibit granule secretion.

Rab proteins and their effectors are capable of docking opposing membranes and seem to modify SNARE protein function. Rab proteins are the largest branch of the ras superfamily of GTPases. Rabs 3b, 6c, and 8 are phosphorylated upon platelet activation.[66,89] Rab GDP dissociation inhibitor (RabGDI), a general inhibitor of RabGTPases, inhibits α–granule but not dense granule release.[90] In addition, a dominant-negative mutant of His-tagged Rab4S22N (but not mutant His-Rab3BT36N) inhibits α–granule secretion but fails to affect dense granule release.[90] These data raise the possibility that Rab 4 is required for α–granule but not dense granule secretion. In nucleated cells, Rab proteins have been shown to function by binding to large effector proteins that have been proposed to interact with SNARE proteins directly or with proteins, such as NSF and Munc-18c, which mediate SNARE protein function[91] The Rab effector proteins in platelets that mediate α–granule release have not yet been identified.

Platelet α–granules content

α–Granule function derives from their contents. The content of α–granules includes both membrane bound proteins that become expressed on the platelet surface and soluble proteins that are released into the extracellular space. Most membrane bound proteins are also present on the resting plasma membrane[92] These proteins include integrins (e.g., α_{IIb}, α_6, β_3), immunoglobulin family receptors (e.g. GPVI, Fc receptors, PECAM), leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), tetraspanins (e.g., CD9) and other receptors (CD36, Glut-3)[93–97] The abundance of plasma membrane receptors residing in α–granule membranes suggests that endocytosis of plasma membrane contributes to the presence of adhesion molecules in α–granules[92] Not all membrane-associated α–granule proteins, however, are present on the plasma membrane (e.g., the integral membrane proteins fibrocystin L, CD109, P-selectin).[93]

Proteomic studies suggest that hundreds of soluble proteins are released by α–granules. It bears considering that proteins found in platelet releasate can originate from other platelet granules, cleavage of surface proteins, or exosomes. Nonetheless, the combination of proteomic studies evaluating releasate, isolated platelet α–granules, and isolated platelet dense granules provides creditable information regarding the identity of proteins released by α–granules.[93,98–101] Many of the proteins found in α–granules are present in plasma. This observation raises the question of whether the α–granule counterparts of plasma proteins differ in structure or function. Also, while many important bioactive proteins are present, concentrated, and even modified in platelet α–granules, establishing the physiologic importance of a particular α–granular protein is challenging. Nonetheless, as discussed below, there is evidence that secreted α–granule proteins function in coagulation, inflammation, atherosclerosis, antimicrobial host defense, angiogenesis, wound repair, and malignancy.

Functional roles of platelet α-granules

Coagulation

Platelets secrete many mediators of blood coagulation. Whereas platelet dense granules contain high concentrations of low molecular weight compounds that potentiate platelet activation (e.g., ADP, serotonin, and calcium), α-granules concentrate large polypeptides that contribute to both primary and secondary hemostasis. α–Granules secrete fibrinogen and von Willebrand factor (vWf), adhesive proteins which mediate platelet-platelet and platelet-endothelial interactions. α-Granular vWf constitutes 20% of the total vWf protein and is enriched in high molecular weight forms.[102,103] Studies in which normal bone marrow was transplanted into pigs with severe von Willebrand disease demonstrated that platelet vWf could partially compensate for lack of plasma vWf.[104] In a separate study, gene transfer resulting in ectopic expression of vWf in liver of mice with severe vWD led to normal thrombus formation following vascular injury.[105] These results suggest that α-granule vWf can contribute to, but is not necessary for, normal hemostasis and thrombus formation.

Adhesive receptors found in α-granules also participate in platelet adhesion. Components of the vWf receptor complex, GPIbα-IX-V, the major receptor for fibrinogen, integrin $\alpha_{IIb}\beta_3$, and the collagen receptor, GPVI, are found in α–granules.[92,97] Although these receptors are constitutively expressed on the platelet plasma membrane, an estimated one-half to two-thirds of $\alpha_{IIb}\beta_3$ and one-third or more of GPVI reside in α-granule membranes in resting platelets and are expresssed following activation.[94–97]

Platelet α-granules contain a number of coagulation factors and co-factors which participate in secondary hemostasis. Factors V, XI, and XIII each localize in α-granules and are secreted upon platelet activation.[106] Factor V is endocytosed from the plasma and is stored complexed to the carrier protein multimerin, while factors XI and XIII are endogenously synthesized in megakaryocytes.[107–109] Some platelet-derived coagulation factors are structurally different from their plasma counterparts. In contrast to plasma pools, platelet-derived factor V is released in a partially activated form that exhibits substantial cofactor activity prior to thrombin activation.[110] Purified platelet-derived factor V/Va is more resistant to inactivation by activated protein-C or Ser(692) phosphorylation than its plasma counterpart.[111] Likewise, washed platelets from both normal and plasma factor XI-deficient donors correct the clotting defects observed in factor XI-deficient plasma.[112] Platelet α-granules also contain the inactive precursor of thrombin, prothrombin, and significant stores of high molecular weight kininogens, which augment the intrinsic clotting cascade.[93,106] In addition, platelets release inhibitory proteases, such plasminogen activator inhibitor-1 (PAI-1) and α_2-antiplasmin, which limit plasmin-mediated fibrinolysis.106 Degradation of α–granule proteins by plasmin secondary to upregulation of urokinase plasminogen activator in megakaryocytic α–granules, [113] as observed in the Quebec platelet disorder, results in a bleeding diathesis.[114] Patients with

Gray Platelet Syndrome lack α–granules and also present with a bleeding diathesis.[52] These observations demonstrate a role for α–granule proteins in hemostasis.

Platelets also contribute to hemostatic balance by secreting numerous proteins that limit the progression of coagulation. α-Granules store antithrombin, which cleaves activated clotting factors in both the intrinsic and extrinsic pathways, and C1-inhibitor, which degrades plasma kallikrein, factor XIa, and factor XIIa. Platelets secrete tissue factor pathway inhibitor (TFPI),[115] protein S,[116] and protease nexin-2 (amyloid β-A4 protein), which inhibits factors XIa and IXa.[117] The anticoagulant properties of platelet-derived protease nexin-2 have been demonstrated in transgenic mouse models, as specific and modest overexpression of protease nexin-2 in platelets both reduces *in vivo* cerebral thrombosis and increases intracerebral hemorrhage.[118] α-Granules store the proteinase plasmin and its inactive precursor plasminogen. The observation that α–granules are heterogenous and may be differentially released could explain how they contribute both anti- and pro-coagulants to regulate coagulation. However, the possibility that pro- and anticoagulants are separated into different populations and that these populations are differentially released has not been evaluated.

Inflammation

Accumulating evidence demonstrates that platelets contribute to the initiation and propagation of the inflammatory process.[119] Platelet α–granules function in inflammation both by expressing receptors that facilitate adhesion of platelets with other vascular cells and by releasing a wide range of chemokines.

Adhesive interactions generally result in mutual activation and in the propagation of the inflammatory phenotype of each cell. Fibrinogen, fibronectin, vitronectin, and vWf, contribute to firm platelet-endothelial adhesion by forming cross-bridges between platelet GPIIb-IIIa and endothelial $\alpha_V\beta_3$ integrin or ICAM-1.[120,121] However, although these proteins are found in α-granules, the specific contributions of platelet-, endothelial- and plasma-derived pools have not been established. P-selectin, which translocates from α-granules to the platelet surface membrane following platelet activation, participates in platelet interactions with endothelial cells, monocytes, neutrophils, and lymphocytes [122,123]. *In vitro* and *in vivo* studies demonstrate that platelets, regardless of their activation state, are able to transiently adhere to the intact, activated endothelium.[124,125] These interactions are mediated by endothelial P-selectin binding to constitutively-expressed platelet P-selectin glycoprotein-1 (PSGL-1) or platelet GPIbα.[124,126] Platelet-endothelial interactions are strengthened following platelet activation, as platelet P-selectin binds to endothelial PSGL-1.[127] A recent study using intravital fluorescence microscopy demonstrates the importance of P-selectin and PSGL-1 in these interactions, as platelet adherence to an inflamed endothelial wall was significantly reduced following immunoneutralization of either P-selectin or PSGL-1.[128]

P-selectin also mediates platelet interactions with PSGL-1-expressing immune cells. Activated platelets bind to circulating immune cells in the blood stream, and surface-adherent platelets facilitate the recruitment, rolling, and arrest of monocytes, neutrophils, and lymphocytes to the activated endothelium.[123,129,130] Platelets have been shown to play a critical role in the recruitment of neutrophils to damaged lung capillaries. Platelet depletion, P-selectin neutralization, or selective knockout of P-selectin in the hematopoietic compartment reduces neutrophil recruitment and inhibits the development of acute lung injury.[131,132] Adherence to platelets induces a host of proinflammatory responses in immune cells, including activation of adhesion receptor complexes, secretion of chemokines, cytokines, proteases, and pro-coagulants, and promotion of cellular differentiation.[119] In platelets, these interactions promote further activation and granular secretion.

Platelet α–granules also influence inflammation by secreting high concentrations of proinflammatory and immune-modulating factors. These mediators induce recruitment, activation, chemokine secretion, and differentiation of other vascular and hematologic cells.[133] In some cases, these chemokines feed back to stimulate chemokine receptors on the platelet surface, thereby causing platelet activation, secretion, and perpetuation of the inflammatory cycle. α-Granules contain a wide range of chemokines including CXCL1 (GRO-α), CXCL4, CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES).[133] Among these, CXCL4 and CXCL7 are the most abundant.[134] For example, platelets contain 20 μg of CXCL4/10^9 cells, and following thrombin stimulation the serum concentration of CXCL4 rises to 5–10 μg/ml, approximately 1000-fold greater than normal plasma.[135,136] Platelets are considered the major cellular source of CXCL4 and CXCL7, whereas platelet secretion of other chemokines is thought to merely augment their secretion by other cells.[121] CXCL4 has been shown to induce neutrophil adhesion and degranulation, monocyte activation, and monocyte differentiation to macrophages and foam cells.[137–139] In coordination with CCL5, CXCL4 is also able to induce adhesion of monocytes to endothelial cells.[140] The most abundant platelet-derived chemokine, CXCL7, can be sequentially and proteolytically cleaved to form four distinct chemokines – PBP, CTAP-III, β-TG, and NAP-2.[134] It is only NAP-2, however, that is thought to display significant chemotactic activity.[141] Numerous studies demonstrate that CXCL7 induces neutrophil chemotaxis and adhesion to endothelial cells.[134,142]

Atherosclerosis—Atherosclerosis represents an important example of the role of platelet α–granule function in inflammation. Atherosclerosis is a chronic inflammatory disease characterized by the infiltration of immune cells into the subendothelial layers of the arterial wall (Fig. 5).[127,143] Platelets are increasingly thought to play a central role in both the initiation and progression of the disease.[144] Platelets influence atherogenesis by adhering to activated endothelial cells and depositing chemotactic mediators on the endothelial surface. Platelets also interact with leukocytes, facilitating their migration into the arterial wall.[121] In multiple mouse models of atherosclerosis (LDL-receptor$^{-/-}$ and apolipoprotein E$^{-/-}$), P-selectin deficiency is associated with decreased formation of fatty streaks and reduction in plaque lesion size.[145,146] Specific knockout of platelet P-selectin reduces lesion development by 30% in apoE$^{-/-}$ mice. A subsequent study demonstrated that platelet, not endothelial, P-selectin is required for neointimal formation following vascular injury.[147,148] In apoE$^{-/-}$ mice, injection of activated wild-type, but not P-selectin deficient, platelets increased monocyte arrest on atherosclerotic lesions and, subsequently, the size of the lesion.[149] P-selectin has also been shown to be necessary for platelet deposition of CCL5 on inflamed and atherosclerotic endothelium.[150]

CCL5 and other chemokines found in platelet α-granules, including CCL2, CCL3, CXCL4, and CXCL12, have been detected in atherosclerotic plaques. In mouse models, pharmacological inhibition or genetic mutation of these chemokines and/or their receptors decrease the progression of atherosclerosis.[133,151–155] CXCL4-deficient, ApoE$^{-/-}$ mice show reduced lesion size.[154] Disruption of proinflammatory interactions between CXCL4 and CCL5 inhibit atherosclerosis in hyperlipidemic mice.[151] Unlike CXCL4, however, which derives from platelets, many of these chemokines are secreted by multiple cell types. Functional studies directly assessing the contribution of platelet-derived chemokines to atherogenesis have been limited. Bone marrow reconstitution in chimeric mice and platelet-specific conditional knockouts will help define the direct contribution of α–granular chemokines to atherogenesis.[121,156]

Antimicrobial Host Defense

Although it was once thought that platelets promoted infection by facilitating the adhesion of microbes to the vessel wall, it is now understood that platelets play a significant role in host defense against pathogenic microorganisms.[157,158] Numerous studies have demonstrated that platelets are among the first blood cells to recognize endothelium damaged by microbial colonization and to accumulate at sites of infected endovascular lesions.[157,159–161] Platelets rapidly associate with vegetations in infective endocarditis and accrue at sites of suppurative thrombophlebitis.[161] Platelets interact directly with viruses, bacteria, fungi, and protozoa.[162–166] Recent studies, for example, have demonstrated that platelets bind to erythrocytes infected with plasmodium and kill the parasite.[166]

Platelet α-granules contain proteins with direct microbicidal properties, a group collectively referred to as platelet microbicidal proteins.[121] Many of the chemokines secreted by activated platelets – including CXCL4, thymosin-β4, the derivatives of CXCL7 (PBP, CTAP-III, NAP-2), and CCL5 (RANTES) – are microbicidal.[167,168] Truncation of CTAP-III and NAP-2 at their C-terminus generates two additional peptides, thrombocidins-1 and -2 (TC-1, TC-2), which are bactericidal *in vitro* against some strains of *Bacillus subtilis*, *Staphylococcus aureus*, and fungicidal for *Cryptococcus neoformans*.[169] In the setting of infective endocarditis, *in vitro* susceptibility of staphylococcal extracts to thrombocidin-1 correlated well with clinical severity and prognosis.[170] Another microbicidal protein, thymosin-β4, localizes to platelet α-granules.[168] While it is debatable whether the local concentration of these secreted proteins are more important for antimicrobial activity or chemoattraction of leukocytes, multiple studies using combined *in vitro* and *in vivo* techniques have demonstrated that platelets are clinically relevant to host defense.[170–173]

α–Granules also contain complement and complement binding proteins, which facilitate the clearance of microorganisms from the circulation. Platelet α-granules secrete complement C3 and complement C4 precursor, which participate in the complement activation cascade.[93] P-selectin binds C3b, localizing the inflammatory response to sites of vascular injury.[174] Platelets α–granules also contain regulators of complement activation, such as C1 inhibitor.[175] Platelet factor H secreted from α–granules regulates C3 convertase in the alternative pathway.[176] Despite these suggestive observations, the impact of α–granule deficiency on complement activation and regulation is not known.

Mitogenesis

Angiogenesis—That platelets support angiogenesis is well-established.[177,178] However, the molecular mechanisms of this function are just beginning to be understood.[179] Platelet α-granules contain a variety of both pro- and anti-angiogenic proteins. Growth factors stored in α-granules include vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF).[106,180] These angiogenic activators collectively promote vessel wall permeability and recruitment, growth, and proliferation of endothelial cells and fibroblasts. Although these growth factors are secreted by a variety of inflammatory cells, the rapidity with which platelets accumulate at sites of vascular injury makes them a relevant source of mitogenic mediators. For example, VEGF concentrations are elevated 3-fold during the first minutes after plug formation following forearm incision.[181] VEGF also accumulates inside platelet thrombi formed *in vivo*.[182] Platelet-derived VEGF and FGF-2 exert trophic effects on cultured endothelial cells.[183] In an *ex vivo* rat aortic ring model, platelet-derived VEGF, bFGF, and PDGF promote sprouting of new blood vessels.[184] α-Granules contain other pro-angiogenic mediators, including angiopoietin, CXCL12 (SDF-1α), and matrix metalloproteinases (MMP-1, -2, and -9). Platelet-derived CXCL12 has

been reported to induce recruitment of CD34+ progenitor cells to arterial thrombi *in vivo* and promote differentiation of cultured CD34+ cells to endothelial progenitor cells.[155,185]

α–Granules also contain established inhibitors of angiogenesis. TSP-1, a major constituent of α-granules, acts as a potent inhibitor of endothelial cell proliferation and stimulates endothelial cell apoptosis.[186] *In vivo* studies demonstrate that TSP-1 inhibits revascularization in a model of hind limb ischemia, while TSP-deficient mice show accelerated revascularization. The proangiogenic activity in TSP-deficient mice is directly related to increased platelet secretion of CXCL12.[186] CXCL4 also displays antiangiogenic properties, likely by preventing VEGF binding to its cellular receptor and by interfering with the mitogenic effects of FGF.[187,188] In a rat aortic ring model in which platelet-derived VEGF and FGF induced endothelial sprouting, immunoneutralization of CXCL4 in the releasate further amplified neovascularization.[184] Platelet α-granules contain other anti-angiogenic proteins, including angiostatin, endostatin, and tissue inhibitors of metalloproteinases (TIMPs-1 and -4). Recent studies suggest anti-angiogenic proteins may be packaged in different α-granule subpopulations than pro-angiogenic proteins [4]. There is some evidence that secretion of pro- versus anti-angiogenic stores may be agonist-specific.[4,189]

Wound Healing—Platelets promote wound healing in several *in vitro* and *in vivo* models.[180] Although many of these studies and applications involve preparations that include platelets, isolated platelet supernatants enriched in α–granule proteins are sufficient to support wound healing. *In vitro* studies demonstrate that platelet releasate increases the proliferation and migration of osteogenic cells.[190] Platelet releasate also stimulates proliferation of human tendon cells in culture and promotes significant synthesis of VEGF and HGF.[191] Studies in dogs demonstrated that platelet releasate in a collagen sponge promotes periodontal tissue regeneration.[192] Healing of cutaneous wounds is also promoted by platelet releasate in diabetic rats.[193]

Perhaps the strongest evidence that platelet releasate promotes wound healing is the use of "platelet-derived wound healing factor" (PDWHF) in the treatment of chronic wounds. PDWHF is a FDA-regulated preparation of the supernatant of washed, thrombin-stimulated platelets. This supernatant is added to microcrystalline collagen to generate PDWHF.[194] In placebo controlled studies, PDWHF accelerated wound healing in the settings of chronic diabetic foot ulcers.[195,196] PDWHF has also been used to treat leg ulcers in the setting of β-thalasemia intermedia.[197] The mechanism by which PDWHF promotes healing remains largely unstudied; however, upregulation of $\alpha_V\beta_3$ in capilliaries surrounding treated, but not untreated wounds, has been observed.[198] Not all studies using PDWHF have demonstrated a positive effect on wound healing compared to placebo.[199] Differences in results may be secondary to differences in platelet concentrations used for preparations or different patient populations.

Malignancy—Platelets have been implicated in tumor stability, growth, and metastasis. Acute thrombocytopenia results in rapid tumor destabilization and intratumor hemorrhage.[200] This observation implies an ongoing requirement for platelets in maintaining tumor stability. Infusion of resting, but not degranulated, platelets prevents thrombocytopenia-induced tumor bleeding (Fig. 6), suggesting that platelet granules contribute to tumor stability.[200] Angiopoietin-1 may enhance intratumor stability in this model,[200] however, the specific α–granule constituents that stabilize tumors remain to be identified.

Primary tumors are highly dependent on the development of an adequate blood supply. As previously discussed, platelets contain both pro- and antiangiogenic factors and support angiogenesis. In animals bearing malignant tumors, platelets selectively accumulate angiogenesis regulators.[201] In a canine model of cutaneous mast cell tumor, VEGF concentrations in activated platelet-rich plasma correlate well with microvascular density, mast

cell density, and malignancy grading.[202] Cancer patients have increased levels of serum VEGF and angiopoietin-1, which decrease after tumor resection.[203–206] Platelet α-granules from cancer patients contain elevated levels of VEGF and angiopoietin-1[207,205,208] and platelets constitute a significant source of circulating VEGF in cancer patients.[209]

Early evidence that platelets function in cancer progression derive from studies showing decreases in lung metastases following induction of thrombocytopenia.[210–213] Platelet infusion reverses the effect on tumor metastasis. Adhesion of platelets to tumor cells is thought to facilitate tumor metastasis. Potential mechanisms by which platelet adhesion may facilitate tumor metastasis include cloaking tumor cells from immune surveillance and assisting their egress from the circulation.[214,215] Adhesive proteins found in α-granules mediate direct interactions between tumors and platelets. P-selectin can mediate initial interactions by binding to mucins on the tumor surface.[216] Global deficiency of P-selectin is associated with reduced tumor growth and metastasis, but specific knockout of platelet P-selectin has not been examined.[217] Vitronectin and fibronectin in platelet releasate enhance the adhesion of tumor cells to cultured endothelial cells under shear in a $\alpha_V\beta_3$-dependent manner.218 In addition, platelet releasate can induce expression of tumor-proteases that enhance invasiveness.[219] Future studies using platelet-specific knockouts of α-granule-derived mediators will enhance our understanding of the role of the platelet in angiogenesis and tumor metastasis.

Perspective

The diversity of physiologic functions influenced by platelets – coagulation, inflammation, microbial host defense, wound healing, and malignancy – raises the question how the platelet can modulate so many varied processes. Proteomic data cataloging individual α–granule proteins demonstrate that the α–granule possesses a wide array of bioactive proteins, implying participation of α–granule proteins in varied physiologic functions. The vast majority of these proteins, however, are also found in either plasma and/or other vascular cells, leaving the contribution of the α–granule source uncertain. Assessment of the α–granule pool of a specific protein in a given physiologic function will require bone marrow reconstitution in chimeric mice and/or platelet-specific conditional knockouts.

Evaluation of α–granule contents also demonstrates that α–granules contain many proteins with opposing activities: pro- and anticoagulants, proteases and their inhibitors, pro- and antiangiogenic proteins. To understand the role of α–granules in various physiologic functions, a more detailed understanding of how the activity of these contents is regulated is required. Do molar concentrations of various components dictate which activity is dominant? Is the activity of releasate regulated in a kinetic manner, with rapid onset of pro-coagulant or pro-angiogenic activities that are subsequently controlled by lagging inhibitory activities? Is the activity of these components regulated by differential release of heterogenous α–granule subpopulations?

An appreciation of the role of α–granule release in multiple physiologic functions raises the question of whether this activity can be controlled for therapeutic benefit. The observation that the biosynthetic pathway to dense granule and α–granule formation diverge at a relatively proximal step in granule formation suggests that α–granule formation could be specifically inhibited. Therapeutics that render platelets selectively deficient in either dense or α–granules could be useful in some clinical settings. Understanding the differential release of α–granules versus dense granules and, perhaps, differential release of distinct α–granule subpopulations may also inform strategies of controlling α–granule participation in disease processes. A more detailed understanding of the role of α–granules in disease states will be required for optimally targeting such strategies to specific disease entities.

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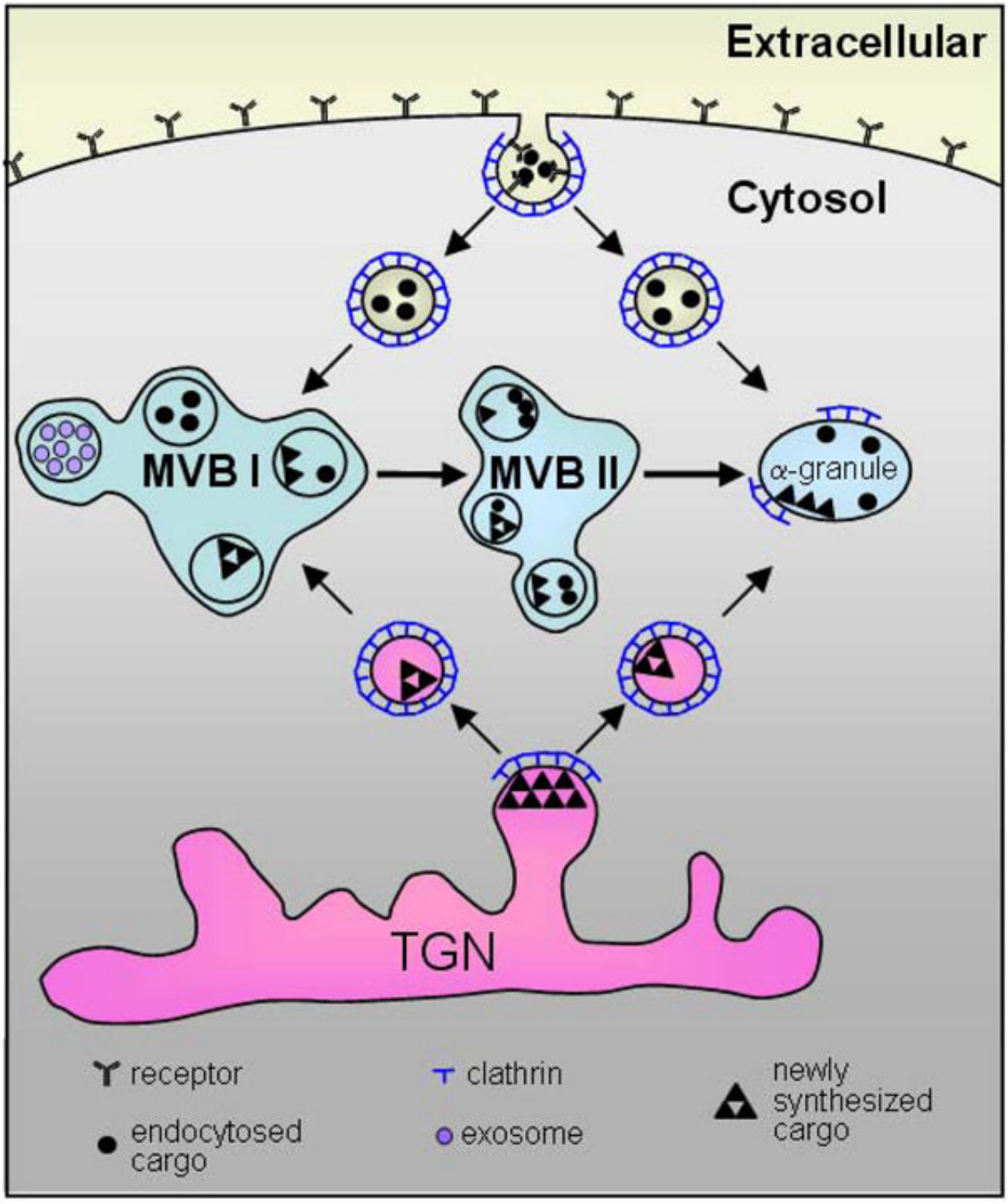

Figure 1. Working model of α–granule formation in megakaryocytes

α–Granule cargo derives from budding of the trans-Golgi network (TGN) and endocytosis of the plasma membrane. Both processes are clathrin-mediated. Receptor-mediated endocytosis is depicted in this figure; however, pinocytosis of α–granule cargo can also occur. Vesicles can subsequently be delivered to multivesicular bodies (MVBs), where sorting of vesicles occurs. It is possible that vesicles may also be delivered directly to α–granules. Some vesicles within MVBs contain exosomes. MVBs can mature to become α–granules.

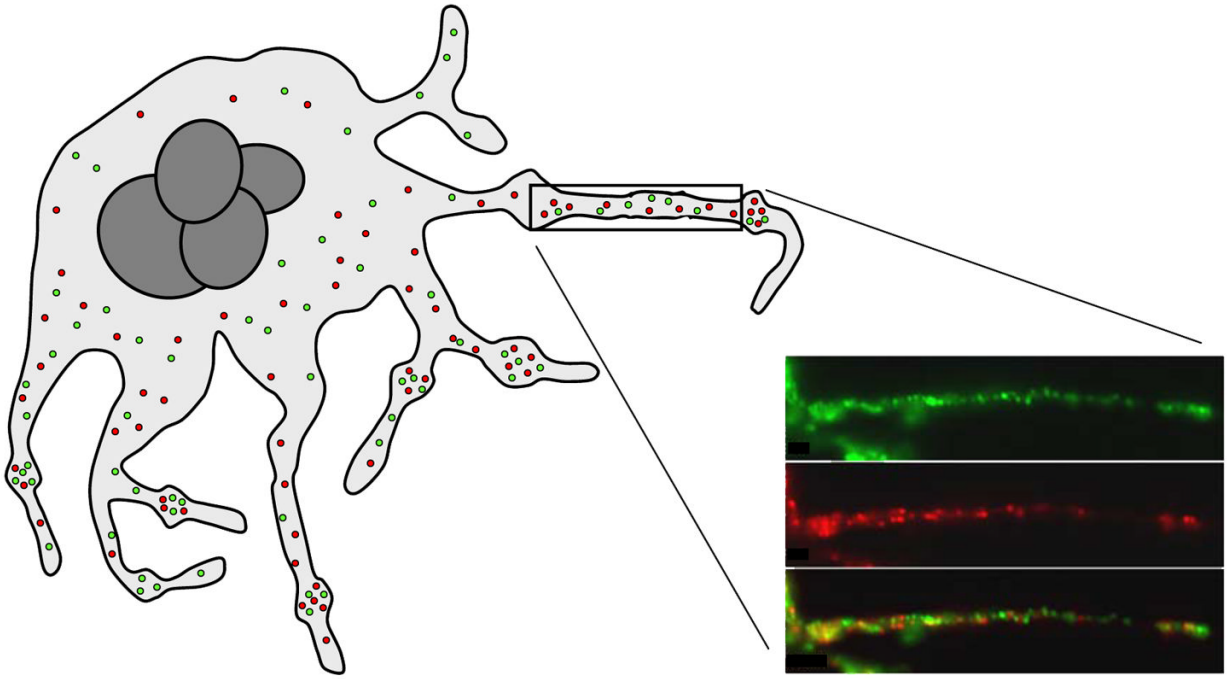

Figure 2. Model of transport of α–granules during platelet formation

Platelet α–granules are transported along microtubules from the megakaryocyte cell body through long pseudopodial extensions termed proplatelets. Platelets form as bulges along the length of these extensions. α–Granules are maintained in the nascent platelets by coiled microtubules. The insert demonstrates subpopulations of α–granules containing distinct cargos being transported along a proplatelet. α–Granules containing fibrinogen are shown in green, while those containing vWf are shown in red. (Insert from Italiano et al., *Blood*, 111:1227–1233.)

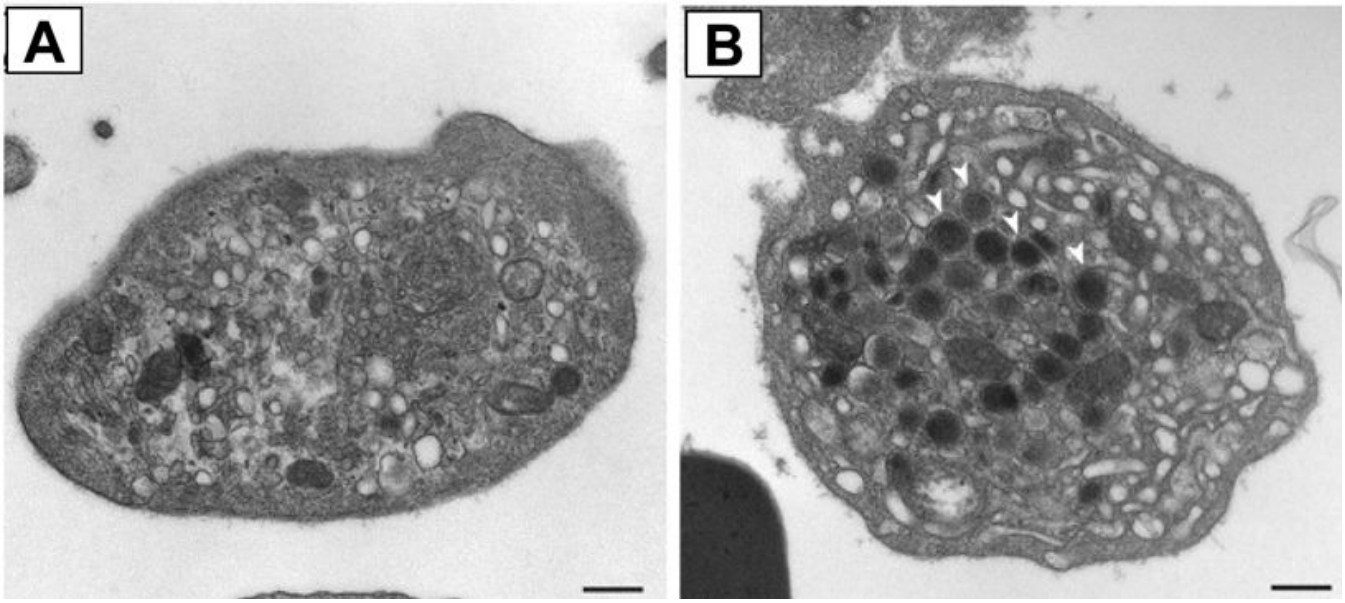

Figure 3. Absence of α–granules in platelets from patients harboring a mutation in VPS33B

Thin-section transmission electron micrographs of platelets (A) from a fetus with a mutation in VPS33B and (B) platelets from an unaffected fetus. Abundant α–granules indicated with white arrows in control platelets are lacking in platelets with mutant VSP33B. *Bar, 500 nm*. (Adapted from Lo et al., *Blood*, 106:4159–4166).

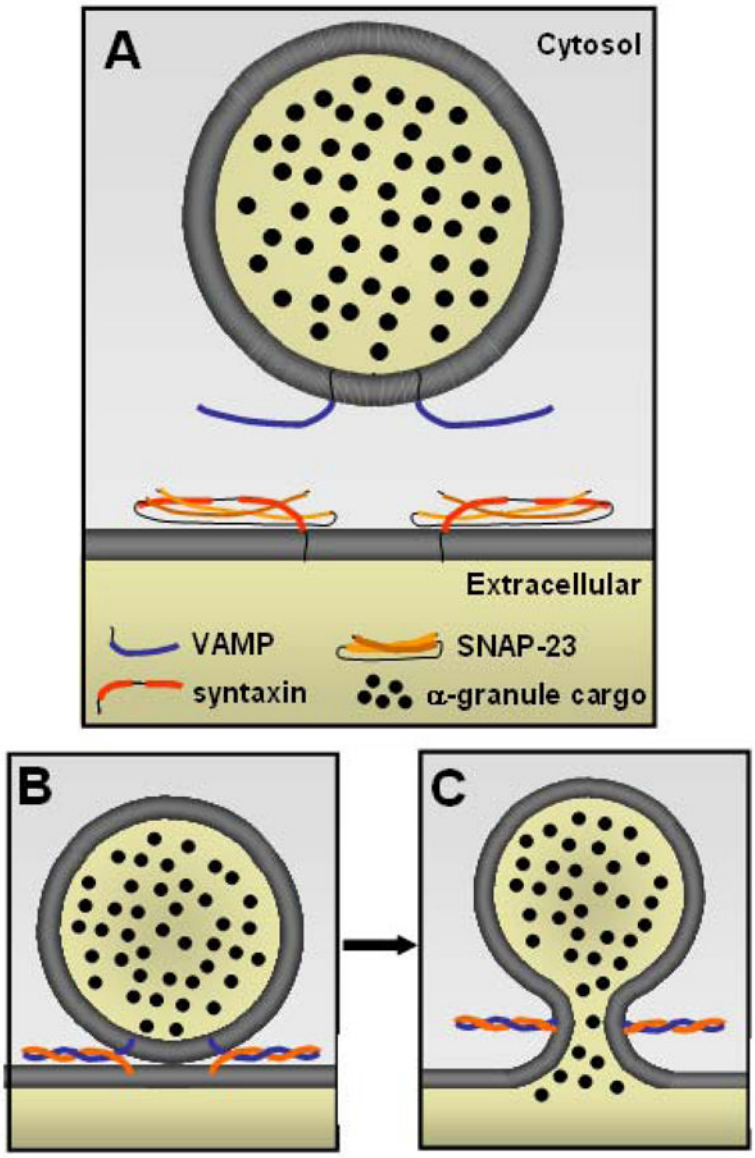

Figure 4. Role of SNAREs α–granule membrane fusion

A) The primary vSNARE mediating platelet α–granule secretion is VAMP-8, with VAMP-3 and perhaps VAMP-2 serving subordinate functions. Platelet tSNAREs include syntaxins and SNAP-23. Syntaxin-2 and 4 participate in α–granule release. Coiled-coil domains (*bolded*) within vSNAREs (*blue*) and tSNAREs (*orange*) interact, forming a twisted 4-helical bundle. B) Interaction of the coiled-coil domains brings the opposing membranes of the granule and target membrane into close apposition. C) Binding of vSNAREs and tSNAREs generates energy required for membrane fusion. Pore formation with release of granule contents subsequently ensues.

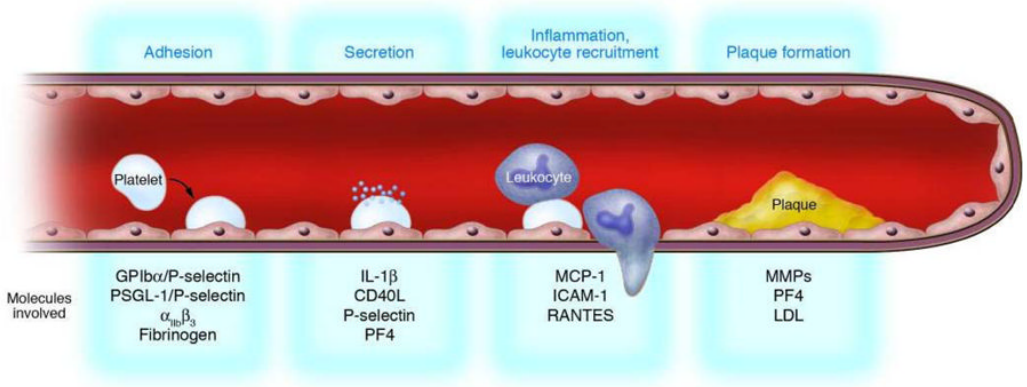

Figure 5. Hypothetical model of atherogenesis triggered by platelets

Activated platelets roll along the endothelial monolayer via GPIbα/P-selectin or PSGL-1/P-selectin. Thereafter, platelets firmly adhere to vascular endothelium via β3 integrins, release proinflammatory compounds (IL-1β, CD40L), and induce a proatherogenic phenotype of ECs (chemotaxis, MCP-1; adhesion, ICAM-1). Subsequently, adherent platelets recruit circulating leukocytes, bind them, and inflame them by receptor interactions and paracrine pathways, thereby initiating leukocyte transmigration and foam cell formation. Thus, platelets provide the inflammatory basis for plaque formation before physically occluding the vessel by thrombosis upon plaque rupture. (Adapted from Gawaz et al., *J. Clin. Invest*., 115:3378).

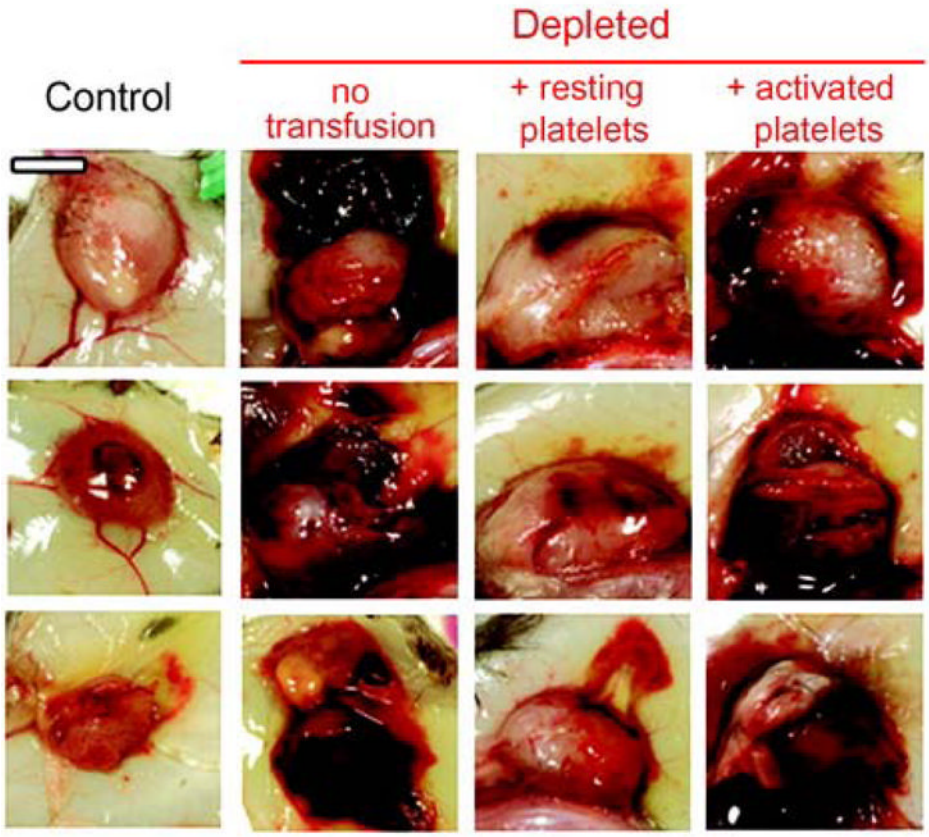

Figure 6. Degranulated platelets are unable to prevent thrombocytopenia-induced tumor bleeding

At day 8 after tumor cell implantation, mice were injected with either the control IgG (*Control*) or the platelet-depleting IgG (*Depleted*). A subset of mice was transfused 30 min before the induction of thrombocytopenia with tyrode buffer (*no transfusion*) or 7×10^8 of either resting or activated platelets and s.c. Lewis lung carcinoma cells were photographed 18 h later. *Bar*, 5 mm. (Adapted from Ho-Tin-Noe et al., *Cancer Res*, 68:6851–6858)