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Antiplatelet Agents

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Antiplatelet Agents

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Foreword

Platelets: Past, Present, and Future

This volume will be greatly welcomed by the biomedical community worldwide for bringing knowledge about platelets and their involvements up to date. Herewith are a few, I hope, relevant thoughts.

The Past: Platelets were discovered by Bizzozero in 1882. Knowledge about them increased slowly until the mid-twentieth century. I came to know about platelets from two, very different experiences: one came in late 1945 when, as a member of the Royal Army Medical Corps, I was one of the first foreign doctors in Hiroshima about 3 months after its destruction by the first atom bomb: innumerable local people were still dying from thrombocytopenic bleeding. The other was in Oxford in 1956 when, working on the storage of amines in cells, I remembered that platelets contained large amounts of 5-hydroxytryptamine and produced evidence for its ionic association with ATP.

Just about 50 years ago came the invention of platelet aggregometry. This method elucidated platelet function underlying haemostasis and thrombosis, initiated the discovery of aggregation inhibitors and permitted the discovery of the release reaction and of its inhibition by aspirin, which was the basis of the drug's 'second life' in the prevention and treatment of coronary thrombosis and stroke. Since then, of course, several aggregation inhibitors with different modes of action have come into effective therapy.

The present is dominated, as almost everywhere in biomedicine, by molecular biology. The origins of this are brilliantly recounted by Francis Crick himself in *What Mad Pursuit* and by Georgina Ferry in her excellent biography of Max Perutz. The molecular mechanisms underlying platelet function appear to be no less complex than those underlying the functions of proper, nucleated cells. So far, however, increasing knowledge of these mechanisms has had little effect in increasing therapeutic possibilities.

As to **the future**, the hope is that this may change and that further elucidation at the molecular level will provide new points for therapeutic attack. Meantime, other work is being done: on putative roles of platelets in inflammation, and in the metastatic spread of cancers.

But there is one deeply troubling thought: to the best of my knowledge, the reason why platelet deficiency is associated with microvascular bleeding is still unexplained. That question concerns the essential function of platelets, and it is extremely unsatisfactory that how platelets are connected with such bleeding remains unknown.

My own main interest now is to provide an **evolutionary explanation** for platelet aggregation by adenosine diphosphate (ADP). This was the first platelet-aggregating agent to be discovered and investigated. ADP induces haemostatic aggregation of platelets in man and other mammals but not of the functionally corresponding cells of other vertebrates including birds and reptiles, nor in those of other animal phyla. Different agents, viz. collagen and thromboxane A₂ (TXA₂), which activate platelets and analogous cells to prevent loss of blood and other body fluids, possess this function much earlier in evolution.

So why ADP, rather than any of the other extracellular transmitters, including Burnstock's ubiquitous extracellular ATP? The answer is suggested by comparing haemostasis in man and other mammals with the corresponding process in other animals. In mammals the blood pressure is high so that blood loss from injury is very rapid. Therefore, haemostasis must be very rapid, a requirement met by platelet aggregation: the activation time of human platelets at 37 °C is less than 100 ms; plasma clotting happens much more slowly. With ATP leaking from damaged cells, the dephosphorylating enzymes on cell surfaces ensure very rapid production of ADP. The further dephosphorylation to ineffective AMP is considerably slower, ensuring a temporary increase in local ADP concentration.

In vivo evidence indicates that ADP does indeed contribute essentially to haemostatic and thrombotic platelet aggregation. As other locally present agents, notably TXA₂, collagen, and thrombin are also effective, the addition of the ADP mechanism in mammals is presumably in the nature of a fail-safe system as employed by engineers. So, as always in science, it is best to end with a question: why not ADP also in birds?

Actually, I cannot do better than end with a quote from my late friend Gwyn Macfarlane, distinguished for discovering the cascade mechanism of blood coagulation: 'I live in the quiet of the country where one hears small things, and the other night I overheard two platelets talking as they drifted in some venous backwater.'

'You know', said one, 'I've got the strangest feeling that I've been here before.'

'What nonsense', said the other. 'Everyone knows that endothelium stretches to infinity. One doesn't go backwards—one just goes on.'

'Yes—until we stop.'

'One doesn't think about that—it's morbid.'

'I can't help it sometimes. One day endothelium will stop passing—everyone else will go on, but I shall be stuck there—dead. I say, I wonder what's on the other side?'

'What do you mean? On the other side of what?'

'On the other side of endothelium, of course.'

'It's a meaningless question. Endothelium encloses everything there is—it's the time-continuum. How can there be another side? It's a logical impossibility!'

‘Well, I met a lymphocyte just now. He’s certain he’s been through the endothelium and has come back to another life on this side.’

‘Lymphocytes! One doesn’t pay any attention to them! All that mystical nonsense about re-incarnation. Next thing, they’ll be saying we’re all part of some Higher Organisation. . .!’

London, UK

Gustav Born

Preface

Blood platelets attract growing interest among clinicians and basic scientists due to the increasing understanding of their role in diverse physiologic and pathologic phenomena and to the uninterrupted discovery of new functions.

The recent characterization of the protein biosynthetic activity of platelets, their complex RNA array, the genetic regulation of several of their functional activities, and their role in tissue repair and in inflammation have prompted a major reconsideration of the role of these cells. However, above all other aspects, it is the central role that platelets play in arterial thrombotic events which has been the object of great attention and of spectacular therapeutic advancements in the last few years.

It seemed thus timely to provide a comprehensive, detailed, and updated handbook for both clinicians and researchers on the basic and clinical aspects of drugs used to modulate platelet function and to review the wide range of anti-platelet drugs currently under development.

The book is divided into three parts, i.e. Pathophysiology, which provides all the basic knowledge on the structure and function of platelets and on their role in physiologic haemostasis and in atherothrombosis; Pharmacology, which discusses in detail the basic pharmacology of all the individual antiplatelet agents currently in use and all of the novel and future antiplatelet approaches; and Therapy, which examines systematically the current therapeutic indications for antiplatelet agents in the different clinical manifestations of ischaemic cardiovascular diseases.

Within these parts, 23 chapters cover extensively all the aspects of antiplatelet therapy, with a view on innovative aspects, like small interfering RNAs, signal transduction pathways as potential targets of antiplatelet therapy, or the pharmacologic modulation of the inflammatory activity of platelets.

A large group of leading experts in platelets and cardiovascular disorders, both clinicians and basic scientists, kindly accepted to contribute to this book thus providing the most updated and authoritative view on the state of the art in antiplatelet therapy. Particular care has been taken by the editors to ensure homogeneity and readability of the book and, to this end, each chapter reports a separate list of the main take-home messages and a table with the most significant

knowledge gaps on the subject. Simple figures and schematic diagrams help to provide an immediate and concise view on complex mechanisms and study results.

The book is intended to be a manual and a consultation textbook for all those who are interested in platelets, either for research purposes or for their daily clinical practice.

It is to be expected that blood platelets will continue to surprise us and that new therapeutic developments will take place in the next few years: this book intends to set the stage for the foreseeable future.

An insightful and entertaining foreword by Gustav V.R. Born, one of the fathers of the modern knowledge on platelets, opens the book. The enthusiastic contribution of 30 leading scientists, the editorial assistance of Springer Verlag, as well as the generous assistance of several co-workers at the Editor's institutions have made it possible to complete this ambitious project: we trust that it will be of help and interest to many readers worldwide.

The Editors

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Part I

Pathophysiology

Platelets: Production, Morphology and Ultrastructure

Jonathan N. Thon and Joseph E. Italiano

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Abstract Platelets are anucleate, discoid cells, roughly 2–3 μm in diameter that function primarily as regulators of hemostasis, but also play secondary roles in angiogenesis and innate immunity. Although human adults contain nearly one trillion platelets in circulation that are turned over every 8–10 days, our understanding of the mechanisms involved in platelet production is still incomplete. Platelets stem from large (30–100 μm) nucleated cells called megakaryocytes that reside primarily in the bone marrow. During maturation megakaryocytes extend long proplatelet elongations into sinusoidal blood vessels from which platelets ultimately release. During this process, platelets develop a number of distinguishable structural elements including: a delimited plasma membrane; invaginations of the surface membrane that form the open canalicular system (OCS); a closed-channel network of residual endoplasmic reticulum that form the dense tubular system (DTS); a spectrin-based membrane skeleton; an actin-based cytoskeletal network; a peripheral band of microtubules; and numerous organelles including α -granules, dense-granules, peroxisomes, lysosomes, and mitochondria. Proplatelet elongation and platelet production is an elaborate and complex process that defines the morphology and ultrastructure of circulating platelets, and is critical in understanding their increasingly numerous and varied biological functions.

Keywords Megakaryocyte maturation • Proplatelet production • Preplatelet inter-conversion • Platelet release • Morphology • Ultrastructure

1 Background

Human adults contain nearly one trillion blood platelets in circulation, each with an average lifespan of only 8–10 days. Among their primary functions, platelets serve as the “band-aids” of the bloodstream and respond to blood vessel injury by changing shape, secreting their granule contents, and aggregating to form a platelet clot. Platelets also play secondary roles in helping regulate angiogenesis and innate immunity. To maintain platelet counts of $150\text{--}400 \times 10^9$ platelets per liter of whole blood, roughly 100 billion new platelets must be produced daily from bone marrow megakaryocytes. Megakaryocytes are rare myeloid cells that constitute less than 0.1 % of the cellular population of the bone marrow, and their numbers can be modulated by factors like thrombopoietin (TPO), chemokines, or the localized expression of ligands for megakaryocyte surface receptors (Avecilla et al. 2004; Larson and Watson 2006a).

Megakaryocytes arise from pluripotent hematopoietic stem cells that are mostly restricted to the bone-proximal osteoblastic niche (Avecilla et al. 2004). These develop into burst- and colony-forming precursors, both of which express the CD34 antigen, and which continue to mature along an increasingly restricted lineage that culminates in the formation of precursors that develop into megakaryocytes (Fig. 1) (Ogawa 1993). Differentiation is predominantly driven by TPO signaling through the cMpl receptor, and is supported by additional growth factors such as interleukin-3 (IL-3), stem cell factor (SCF), IL-6, and IL-11 (Kaushansky 2006).

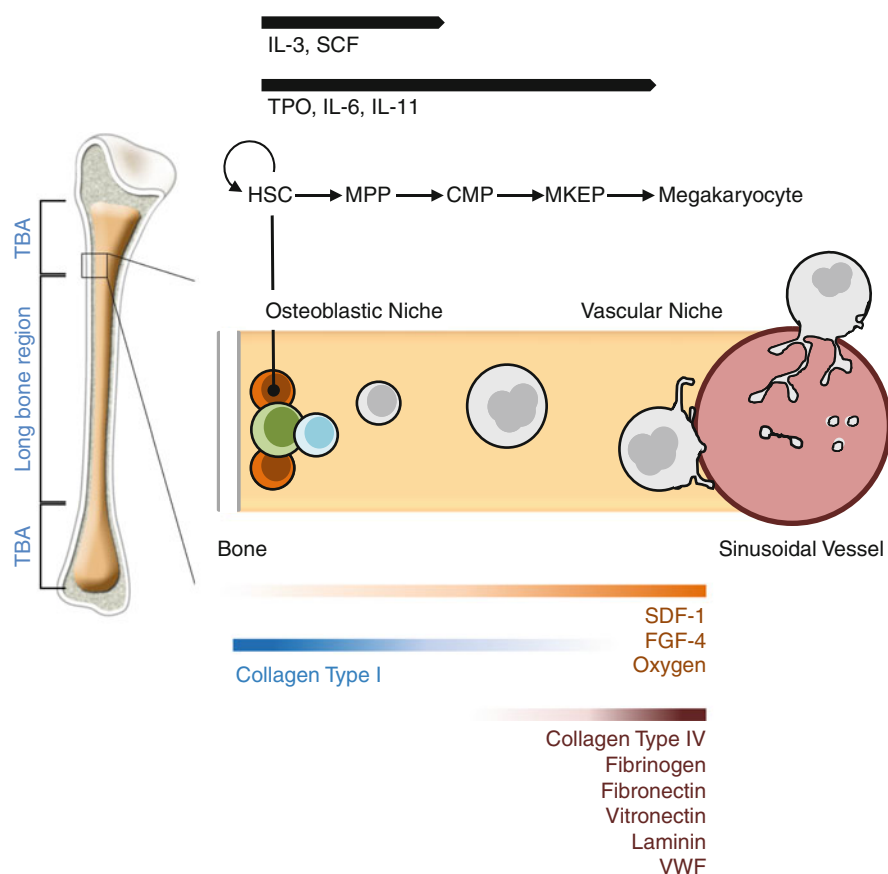


Fig. 1 Summary of megakaryocyte maturation and platelet production. Hematopoietic stem cells residing next to the endosteal bone surface produce progenitors that migrate to blood vessels at the center of the bone marrow cavity. Upon each division, a single daughter cell leaves the bone to proliferate and differentiate into various possible lineages. Differentiation down the megakaryocytic lineage is driven predominantly by TPO, and is supported by additional growth factors such as IL-3, SCF, IL-6, and IL-11. This process is assisted by the extracellular matrix proteins type I and type IV collagen, fibrinogen, fibronectin, vitronectin, laminin, and VWF. Soluble factors such as SDF-1 and FGF-4, as well as the presence of intact sinusoids in the bone marrow help target megakaryocytes to the vascular niche during maturation. An elaborate intracellular program of nuclear amplification and protein production in maturing megakaryocytes precedes the mechanical extension of proplatelet elongations into the sinusoidal blood vessels of the bone marrow. Released proplatelets continue to mature in the vasculature and ultimately release individual platelets from their tips. *CMP* common myeloid progenitor, *HSC* hematopoietic stem cell, *IL* interleukin, *MKEP* megakaryocyte erythroid progenitor, *MPP* multipotent progenitor stem cell, *SCF* stem cell factor, *TBA* trabecular bone area, *TPO* thrombopoietin. Adapted from Yin and Li (2006)

Thrombopoietin is the principal regulator of thrombopoiesis and primate platelet counts can be reduced with an inhibitor against TPO without impairment of primary hemostasis (Kaushansky 2006; Tucker et al. 2010). These results suggest that suppressing platelet production without interfering with the hemostatic function

of platelets may offer a safe alternative to current therapies for prevention of stroke and heart attacks triggered by blood clotting (Tucker et al. 2010).

During maturation megakaryocytes undertake several rounds of chromosomal duplication without cell division (up to 64-fold) called endomitosis. This results in a single polylobulated nucleus as the megakaryocyte proceeds through G1, S, and G2 phases in succession, but fails to complete M phase, re-entering the G1 phase instead (Ravid et al. 2002). Megakaryocyte cytoplasmic volume increases proportionately with ploidy, which is used to direct protein and lipid synthesis in order to support platelet production (Bluteau et al. 2009). This increases the size of the megakaryocyte substantially (up to an approximate diameter of 100 μm) and fills it with platelet-specific organelles, granules, cytoskeletal proteins, and an extensive surface-connected internal membrane labyrinth called the invaginated membrane system. This specialized structure formerly called the demarcation membrane system (DMS) is continuous with the plasma membrane and is thought to constitute an extensive internal membrane reservoir that may facilitate platelet production.

As they mature, megakaryocytes migrate from the osteoblastic niche to a region proximal to the blood vessels of the bone marrow cavity, called the vascular niche. Such niches, while incompletely characterized, represent dynamic biological scaffolds that adapt to physiological requirements for rapid platelet production by regulating megakaryocyte maturation. The current model of platelet formation recognizes that upon reaching maturity bone marrow megakaryocytes will extend, through junctions in the lining of blood sinuses, long branching processes designated proplatelets. These dynamic intermediate structures are composed of tandem arrays of slender tubular projections with platelet-sized swellings at their ends. Released proplatelets continue to mature in the vasculature and ultimately release individual platelets from their tips (Italiano et al. 1999, 2007). A single megakaryocyte can give rise to 1,000–3,000 platelets (Stenberg and Levin 1989) before the residual nuclear material is eliminated by macrophage-mediated phagocytosis (Radley and Haller 1983). Indeed, mice lacking distinct hematopoietic transcription factors (such that they fail to produce proplatelets in culture) have severe thrombocytopenia, underscoring the correlation to platelet biogenesis in vivo (Lecine et al. 1998; Shivdasani et al. 1995; Shivdasani 2001). Because of the dramatic morphological changes that occur during thrombocytopoiesis, a better understanding of the processes that drive megakaryocyte maturation, proplatelet production, and platelet release is paramount.

2 Gaps in the Field

Our understanding of the molecular basis of platelet production has progressed substantially in recent years. But despite this progress, many questions remain: What mechanisms determine platelet size? How do specific genetic disorders result in unusually large or small platelets? What signals stimulate proplatelet production? Where does platelet biogenesis occur? Are there humoral factors that regulate the final stages of platelet production? And how do we grow functional platelets

in vitro on a large scale? Understanding these basic questions may lead to the translation of basic megakaryocyte and platelet biology into major clinical advances.

3 Mechanics of Proplatelet Elongation

Proplatelet production begins with the erosion of one pole of the megakaryocyte to generate large pseudopod-like structures that elongate, thin, and branch to yield slender tubular projections of uniform diameter (2–4 μm) (Fig. 2). This process is highly dependent upon a complex network of protein filaments that represent the molecular struts and girders of the cell. Tubulin and actin are both major components of this cytoskeletal network (Italiano et al. 1999). Megakaryocytes also contain a dense tubular network, and an expansive and interconnected membranous network of cisternae and tubules (the invaginated membrane system), which may represent a channeled system for granule release (Nakao and Angrist 1968). While the mechanism of membrane reorganization remains largely unknown, the invaginated membrane system is also thought to supply membrane during proplatelet formation and is largely supported by a spectrin-based membrane skeleton (Patel-Hett et al. 2011).

Microtubules and actin filaments are formed from thousands of molecules that assemble into linear polymers and play complementary roles in platelet production (Tablin et al. 1990). Proplatelet elongation, for example, is a microtubule-driven process (Tablin et al. 1990) which unfolds over a period of 4–10 h. Just prior to proplatelet formation, microtubules consolidate in a mass just beneath the cortical plasma membrane (Italiano et al. 1999). Proplatelet formation begins when these microtubules align into bundles and fill the cortex of the first blunt processes extended by megakaryocytes. Proplatelet shafts continue to become filled with thick bundles of (hundreds of) microtubules that undergo a thinning phase (to ~20 microtubules) and loop around within the proplatelet to reenter the shaft forming buds at the proplatelet tip (Hartwig and Italiano 2006). Proplatelet extension is due to the continuous polymerization of tubulin bundles at their free plus ends, and dynein-powered sliding of overlapping microtubules (Patel et al. 2005b). Mature megakaryocytes (like platelets) predominantly express the hematopoietic line-specific beta-1 tubulin isoform which plays a specialized role in platelet synthesis, structure, and function. The localization of beta-1 tubulin to the proplatelet marginal band is necessary for proplatelet production and beta-1 tubulin^{-/-} mice exhibit thrombocytopenia resulting from a defect in generating proplatelets (Schwer et al. 2001).

Less is known about the role of actin in proplatelet production. Repeated actin-dependent bending and branching that bifurcates the proplatelet shaft is common, and serves to increase the number of free proplatelet ends from which platelets are thought to release (Hartwig and Italiano 2006). Branching occurs when a portion of the proplatelet shaft becomes bent, from which some of the microtubules within the loop separate from the bundle to form a new bulge in the shaft. Elongation/sliding of these microtubules then generates a new daughter proplatelet process.

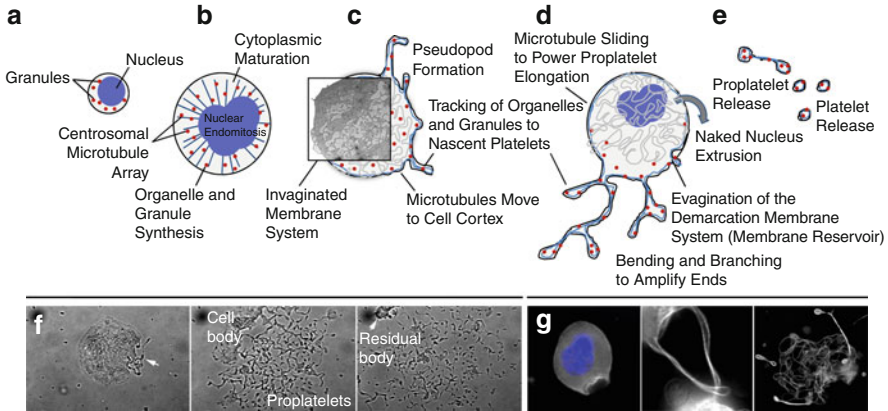


Fig. 2 Cytoskeletal mechanisms of proplatelet production and platelet release. A systematic series of events occurs as megakaryocytes transition from immature cells (a) to released platelets (e). (b) Immature megakaryocytes will undergo repeated cycles of nuclear endomitosis for the purpose of supporting organelle synthesis, and dramatic cytoplasmic maturation and expansion. (c) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. A demarcation membrane system that is continuous with the surface membrane of the cell provides a reservoir of membrane for growth of the proplatelet processes. Thick bundles of microtubules fill the shafts and cortex of broad pseudopodia that are subsequently extended by the megakaryocyte. (d) Proplatelet production commences when the megakaryocyte cytoplasm starts to erode at one pole. Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. Once the bulk of the megakaryocyte cytoplasm has been converted into proplatelets, the entire process ends in a rapid retraction that separates the released proplatelets from the residual cell body (nuclear extrusion). (e) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. As proplatelets elongate, their microtubule bundles twist bringing opposing bundles in contact, and allowing them to become zipped together in the proplatelet shaft. This forms loops at the ends of the proplatelets where granules and organelles become trapped. Sliding movements by microtubules in the shaft elongate released proplatelets further, and separate the ends from the shaft, mediating platelet release. (f) Differential interference contrast microscopy time-lapse sequence of a maturing megakaryocyte, showing the events that lead to elaboration of proplatelets *in vitro*. (g) Immunofluorescence microscopy sequence of the microtubule cytoskeleton and polylobulated nucleus of a proplatelet-producing megakaryocyte. Adapted from Hartwig and Italiano (2006) and Patel et al. (2005a)

In megakaryocytes, multivesicular bodies (MVBs) represent a developmental stage in α -granule and dense-granule maturation (Heijnen et al. 1998; Youssefian and Cramer 2000). Indeed, differences in intra-granular protein distribution of MVBs, α -granules, and dense-granules have been reported (van Nispen Tot Pannerden et al. 2010), and may reflect effective sorting and/or retention mechanisms in their formation. Microtubule bundles in the proplatelet shaft serve as tracks along which these granules move individually (Blair and Flaumenhaft 2009; Richardson et al. 2005). This process is sequential and bidirectional, culminating at the proplatelet tips where the cargo becomes trapped (Italiano et al. 2003). Defects of α -granule formation have been described in both humans

and mice, and often result from mutation or deletions in genes encoding vesicular trafficking proteins (Blair and Flaumenhaft 2009). Of these, SNAREs (soluble NSF attachment protein receptors) represent the core of the fusion machinery, and the distribution and association of vSNAREs (vesicular SNAREs, associated with granules) with tSNAREs (target SNAREs, associated with the plasma membrane) provides the basis for α -granule localization and secretion (Blair and Flaumenhaft 2009).

4 Regulation of Platelet Production

It is not surprising, given that megakaryocytes will migrate from the osteoblastic niche to the vascular niche, that the bone marrow stroma should contribute to megakaryocyte maturation (Avecilla et al. 2004; Larson and Watson 2006a). Indeed, the osteoblastic niche contains high levels of extracellular factors that prevent proplatelet formation (Fig. 1) (Avecilla et al. 2004). Adherence of megakaryocytes to PA6 stromal cells of the osteoblastic niche, for example, has been shown to keep megakaryocytes from producing proplatelets in vitro while promoting megakaryocyte growth and proliferation (Nagahisa et al. 1996). Likewise, soluble factors such as stromal derived factor-1 (SDF-1) and fibroblast growth factor-4 (FGF-4), as well as the presence of intact sinusoids in the bone marrow help target megakaryocytes to the vascular niche where they encounter extracellular factors that promote proplatelet production (Avecilla et al. 2004).

In addition to providing a suitable surface for attachment, extracellular matrices of the bone marrow can also regulate proplatelet production through specific matrix-receptor signaling (Larson and Watson 2006b). Type I collagen, the most abundant extracellular protein of the osteoblastic niche, completely suppresses proplatelet formation while promoting megakaryocyte spreading (Nilsson et al. 1998). This effect is thought to be regulated by the interaction of integrin $\alpha_2\beta_1$ with type I collagen, and the subsequent activation of the small GTPase Rho and the Rho-kinase ROCK (Chen and Shivdasani 2009), and could be overcome by the myosin IIA antagonist blebbistatin (Balduini et al. 2008). Phosphorylation of the regulatory light chain of myosin IIA by ROCK significantly attenuates the ATPase activity of myosin and suppresses proplatelet formation in both human and mouse megakaryocytes (Chen et al. 2007). Conversely, loss of myosin IIA function through dominant inhibitory mutations, targeted gene disruption, or manipulation of cultured megakaryocytes does not inhibit, and may indeed accelerate, proplatelet formation (Chen et al. 2007). As the megakaryocyte migrates toward the vascular niche, this attenuation is believed to disappear and proplatelet production is permitted to commence. Myosin IIA, the non-muscle myosin heavy chain product of the *MYH9* gene, has therefore been implicated in restraining proplatelet formation in developing megakaryocytes through this pathway until they are able to achieve terminal maturity in the bone marrow vascular niche (Chen et al. 2007). In the case of *MYH9*-related disorders, defective myosin IIA could promote premature proplatelet

formation in the osteoblastic niche through loss of attenuation, or result in defective proplatelet formation and reduced or abnormal platelet release in the vasculature (Chen and Shivdasani 2009). Indeed, patients with autosomal dominant inherited *MYH9*-related disorders (e.g., May-Hegglin anomaly) generally exhibit macrothrombocytopenia as well as variable degrees of hearing loss, nephritis, and cataracts (Chen and Shivdasani 2009).

Extracellular matrix proteins are a major component of the vascular niche as well, which is known to comprise type IV collagen, fibrinogen, fibronectin, vitronectin, laminin, and VWF (Larson and Watson 2006b). Type IV collagen, fibrinogen, and vitronectin have all been shown to promote proplatelet production, and antagonists directed against GPs Ib α , IIb, and IIbIIIa will inhibit this process when added to cultured megakaryocytes (Larson and Watson 2006a).

Src family kinases (SFKs) have also been demonstrated to play a critical role in integrin-induced megakaryocyte spreading, migration, and activation of PLC γ 2 in primary bone marrow-derived megakaryocytes (Mazharian et al. 2011a, b). Megakaryocytes treated with an inhibitor of SFKs are unable to spread or migrate towards a gradient of SDF1 α (Mazharian et al. 2011a, b). Interestingly, Dasatinib—a potent ATP-competitive inhibitor of multiple tyrosine kinases (such as SFKs) that is widely used for the treatment of Imatinib-resistant chronic myelogenous leukemia (CML)—was recently shown to cause thrombocytopenia in mice to a similar level observed in humans (Mazharian et al. 2011a, b). This effect was reversible and could be attributed to an impairment of megakaryocyte migration and proplatelet formation.

Despite these recent advances, it is still unclear how signaling through integrins by extracellular matrix proteins mediate terminal cytoskeletal changes that ultimately drive proplatelet production. Nevertheless, these represent attractive drug targets that can be exploited to regulate platelet levels without interfering with their hemostatic functions.

5 Terminal Stages of Platelet Release

Although the mechanics of proplatelet production have been studied, the terminal stages of platelet release remain poorly understood. This is partly due to significant limitations in the field, such as the asynchronous maturation of HSCs in culture, and our inability to synchronize proplatelet production. Megakaryocyte cultures always contain a complex mix of HSCs, immature megakaryocytes, proplatelet-producing megakaryocytes, released proplatelets and platelets which can range dramatically in size and shape (Behnke and Forer 1998; Italiano et al. 2007), and whose relative distribution changes during cell culture. As there are currently no methods available to isolate the multiple intermediate stages in platelet release, most studies to date have focused on the qualitative aspects of megakaryocyte maturation. Nevertheless, proplatelets have been identified both in vitro and in vivo (Leven 1987; Tablin et al. 1990), and proplatelet-producing megakaryocytes yield platelets that are

structurally and functionally similar to blood platelets (Choi et al. 1995; Italiano et al. 1999). Many important questions therefore remain concerning platelet formation, such as how and where are platelets actually released, what cytoskeletal forces are involved in this process, are platelets released in an intermediate “proplatelet” form, and what signals regulate each step of megakaryocyte maturation and platelet formation.

The repeated observation of proplatelets in the bone marrow, the correlation of proplatelet formation and platelet counts, and the elegant mechanisms of proplatelet packaging imply that proplatelets are essential intermediates in platelet production (Larson and Watson 2006a). Nevertheless, micrographs of proplatelet-producing megakaryocytes *in situ* or *in vitro* images of isolated megakaryocytes in culture have yielded only static snapshots and resulted in competing mechanistic models of platelet release. *In vivo* approaches, such as the use of live imaging with multiphoton intravital microscopy (Junt et al. 2007), have validated the proplatelet model of platelet production and suggest that platelets are formed upon the further fragmentation of cytoplasmic processes extended and released into the sinusoids of bone marrow by extravascularly located megakaryocytes (Behnke 1969; Choi et al. 1995; Radley and Scurfield 1980). It should be noted that trans-sinusoidal migration of whole megakaryocytes in these studies was rare, and the bone marrow sinusoidal diameter was observed to impose size constraints on released megakaryocyte fragments *in vivo*. Moreover, the presence of proplatelets in the peripheral blood of the CD41-EYFP^{ki/+} mice implies that it is proplatelets, not megakaryocytes, that are directly responsible for individual platelet release (Junt et al. 2007). Indeed, almost all megakaryocyte fragments identified were 10–100 times as large as circulating platelets. As many of the shed megakaryocyte fragments were unbranched, and most exceeded platelet dimensions, this may be indicative of an intermediate stage in platelet development (Junt et al. 2007). Megakaryocytes routinely released heterogeneous particles with properties resembling immature proplatelets into bone marrow microvessels, suggesting proplatelet morphogenesis continues in peripheral blood to create individual platelets (Junt et al. 2007). This process is possibly assisted by intravascular shear forces in pulmonary arterioles and is consistent with observations that proplatelet counts are higher in pre-pulmonary vessels than in post-pulmonary vessels (Handagama et al. 1987), whereas platelet counts are higher in the latter (Howell and Donahue 1937).

The discovery of a new intermediate stage in platelet production (the preplatelet) and the demonstration that proplatelets are direct precursors to platelets *in vitro*/*in vivo* confirms this new paradigm (Thon et al. 2011). The model of platelet production proposed by our lab predicts that megakaryocytes will release proplatelets into sinusoidal blood vessels where they undergo successive rounds of abscission along their midbody and at their ends to produce smaller barbell-shaped proplatelets and individual platelets (Fig. 2). This process is mediated by the formation of a cleavage furrow at the point of division and results in platelet release from the proplatelet ends at an increasing rate in time as more ends become available after each abscission event. Barbell-shaped proplatelets of ~30–50 μm -perimeter reversibly convert into preplatelets (anucleate discoid particles 2–10 μm across that have the

capacity to convert reversibly into elongated proplatelets by twisting microtubule-based forces). This process may represent a novel mechanism of microtubule reorganization and granule redistribution after each abscission event. During barbell proplatelet formation, dynamic and bidirectional assembly and reorganization of microtubule coils mediate platelet cytoskeleton remodeling as α - and dense-granules track to distal proplatelet tips. Platelets ultimately release from barbell-shaped proplatelet ends after a final abscission event at the proplatelet midsection. Interestingly, the release of platelets from the ends of proplatelets is potentiated by shear, and corresponds with earlier reports of higher platelet counts in post-pulmonary vessels.

6 Platelet Morphology and Ultrastructure

Platelets are small (2–3 μm), anucleate, disc-shaped cells of which two-thirds are present in the general circulation with the remaining third reversibly sequestered in the spleen. Platelets contain a number of distinguishable structural elements including: a delimited plasma membrane; invaginations of the surface membrane that form the open canalicular system (OCS); a closed-channel network of residual endoplasmic reticulum that form the dense tubular system (DTS); a spectrin-based membrane skeleton; an actin-based cytoskeletal network; a peripheral band of microtubules; and numerous organelles including α -granules, dense-granules, peroxisomes, lysosomes, and mitochondria (Fig. 3) (White 1968, 1972).

7 The Plasma Membrane

The plasma membrane is composed of a phospholipid bilayer in which cholesterol, glycolipids, and glycoproteins are embedded. Unlike erythrocytes which retain these molecules in the inner leaflet of their plasma membrane, platelets present these molecules on their surface, which may denote their multiple (and varied) functions in the body. Sodium- and calcium-adenosine triphosphate pumps in the plasma membrane control the intracellular ionic environment of the platelet, while the asymmetrical organization of phospholipids between the inner and outer leaflets regulates coagulation. Negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol, for example, are predominantly expressed in the inner leaflet of the plasma membrane which maintains the platelet surface in a non-procoagulant state. Phospholipid asymmetry is maintained by ATP-dependent aminophospholipid translocases (flippases) that actively move negatively charged phospholipids from the outer to the inner leaflet and may also be supported by phospholipid interactions with cytoskeletal or cytoplasmic elements in the platelet. On platelet activation aminophospholipids become exposed on the platelet surface by ATP-dependent floppases and scramblases, triggering cell-surface-based coagulation reactions (Heemskerk et al. 2002).

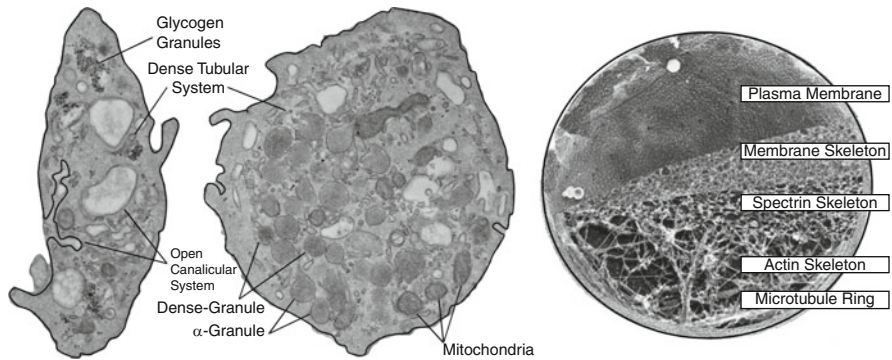


Fig. 3 Ultrastructural features observed in thin section electron micrographs of discoid platelets cut in the cross section and equitorial planes. Components include the open canalicular and dense tubular membrane systems, glycogen granules, α -granules, dense-granules, mitochondria, plasma membrane, membrane skeleton, spectrin skeleton, actin skeleton, and microtubule ring. Adapted from Hartwig et al. (1999)

The plasma membrane also contains dynamic, cholesterol- and sphingolipid-rich microdomains called lipid rafts that play important roles in signaling and intracellular trafficking in platelets. While notably devoid of caveolin, lipid rafts contain the protein markers flotillin (1 and 2) and stomatin, as well as the ganglioside GM₁. Other protein components of resting platelets include CD36, CD63, CD9, GP IIb/IIIa, and GLUT-3. On platelet activation GP VI, Fc γ RIIa, GP Ib/IX/V, c-SRC, phosphatidic acid, and phosphoinositol 3-kinase products also partition into lipid rafts, which may mediate receptor clustering and regulate their activation. Indeed, the platelet membrane is densely packed with highly specific surface receptors that finely regulate signal-dependent platelet activation and may tailor α -granule release to coagulation, inflammation, atherosclerosis, antimicrobial host defense, angiogenesis, wound repair, or malignancy (Blair and Flaumenhaft 2009).

8 The Open Canalicular System

The surface-connected OCS comprises an elaborate series of plasma membrane-contiguous indentations and conduits that tunnel throughout the interior of the platelet (Behnke 1970). While its role in the cell remains somewhat controversial, the OCS is largely thought to serve three major functions; The first is as a mechanism for entry of external elements into the platelet, as well as a potential route for the release of granule contents to the platelet exterior. Indeed, upon platelet activation select α -granule subpopulations will either move to the center of the platelet or localize to the platelet periphery; denoting a possible mechanism for differential granule release (*see Alpha-Granules*). Secondly, the OCS may constitute an extensive internal membrane reservoir that may facilitate filopodia formation and spreading following platelet adhesion to an activating surface. This

process results in a dramatic increase in plasma membrane surface area compared to that of a resting platelet, and may be assisted by α - and dense-granules fusion to the plasma membrane upon granule release. Lastly, the OCS may function as a storage site for plasma membrane glycoproteins. GP Ib/IX, for example, becomes selectively sequestered to the OCS upon thrombin (and possibly plasmin) activation of the platelet (Michelson 1992). Conversely, membrane proteins of the OCS may become recruited to the platelet surface with activation; although α -granule fusion with the plasma membrane is likely a much larger contributor.

9 Dense Tubular System

First characterized histochemically by the presence of peroxidase activity, the DTS is a closed-channel network of residual endoplasmic reticulum primarily thought to sequester ionized calcium (Ca^{2+}) similar to the sarcotubules of the skeletal muscle (White 1972). The calcium binding protein calreticulin is likely responsible for calcium sequestration in the DTS, which is regulated by a number of downstream messenger molecules produced during platelet activation. Platelet activation with thrombin, for example, results in the phosphorylation of the $G\alpha$ subunit of the G protein-coupled receptor PAR-1, which is responsible for activating the membrane-associated signal generating enzymes phospholipase C and phospholipase A_2 (Brass et al. 1993). Phospholipase C, in turn, generates the second messenger inositol 1,4,5 triphosphate (IP3), which binds IP3 type II receptors on the DTS membrane to stimulate Ca^{2+} release. The release of intracellular Ca^{2+} from the DTS results in GP IIb/IIIa redistribution, cytoskeletal reorganization, and granule release; this makes regulation of intracellular Ca^{2+} stores in the DTS a useful target for anti-platelet drugs.

10 The Resting Platelet Cytoskeleton

The disc shape of the resting platelet is maintained by a well-defined and highly specialized cytoskeleton. This intricate system of molecular struts and girders preserves the shape and integrity of the platelet as it is exposed to high shear forces in the bloodstream. The three major cytoskeletal components of the resting platelet are (1) the spectrin-based membrane skeleton, (2) the actin cytoskeleton, and (3) the marginal microtubule coil.

11 The Spectrin-based Membrane Skeleton

The plasma membrane and OCS of the resting platelet are supported by an elaborate cytoskeletal network. The platelet is the only other cell besides the red blood cell whose membrane skeleton has been visualized at high resolution. Like the red

blood cell, the platelet membrane skeleton is also self-assembled of elongated spectrin strands that interconnect through actin filament binding, generating triangular pores. Platelets contain approximately 2,000 spectrin molecules (Hartwig and DeSisto 1991). This spectrin network laminates the cytoplasmic surface of both the OCS and plasma membrane systems. Although considerably less is understood about how the spectrin–actin network assembles and is attached to the plasma membrane in the platelet relative to the red blood cell, certain differences between the two skeletons have been defined. First, the spectrin strands composing the platelet membrane skeleton interconnect using the ends of long actin filaments instead of short actin oligomers (Hartwig and DeSisto 1991). These ends appear at the plasma membrane originating from filaments in the cytoplasm. Therefore, the spectrin lattice is assembled into a continuous network by its association with actin filaments. Second, tropomodulins are not present at sufficiently high levels to have a major role in the capping of the pointed ends of actin filaments; instead, experiments have demonstrated that a significant number (~2,000) of these ends are free in the resting platelet. Third, adducin is abundantly expressed and appears to cap most of the barbed ends of actin filaments in the resting cytoskeleton (Barkalow et al. 2003). Adducin is a key component of the membrane skeleton forming a triad complex with actin and spectrin. Capping of barbed filament ends by adducin also targets them to the spectrin-based membrane skeleton, as the affinity of spectrin for adducin–actin complexes is greater than for either adducing or actin alone.

12 The Actin Cytoskeleton

Actin, at a concentration of 0.5 mM, is the most abundant of all the platelet proteins with over two million molecules per platelet (Nachmias and Yoshida 1988). Similar to tubulin, actin is in a dynamic monomer–polymer equilibrium. Forty percent of the actin subunits assemble to form the 2,000–5,000 linear filaments present in the resting platelet (Hartwig and DeSisto 1991). The rest of the actin in the cytoplasm is maintained in storage as a 1-to-1 complex with β 4-thymosin and is converted to filaments during platelet activation to drive cell spreading (Safer and Nachmias 1994). Evidence indicates that the filaments of the resting platelet are crosslinked at various points into a stiff cytoplasmic network, as platelets express high concentrations of actin crosslinking proteins including filamin and α -actinin (Rosenberg et al. 1981). Both filamin and α -actinin are homodimers in solution. Filamin subunits are elongated strands composed primarily of 24 repeats, each ~100 amino acids in length that are folded into IgG-like β -barrels. There are three filamin genes on chromosomes 3, 7, and X. Filamin A (on X) and filamin B (on 3) are expressed in platelets with filamin A present at greater than tenfold excess to filamin B. Filamin is recognized to be a prototypical scaffolding protein that attracts binding partners and positions them adjacent to the plasma membrane. Proteins bound by filamin members include the small GTPases, rho, rac, ralA, and cc42 with

ralA binding in a GTP-dependent manner; the exchange factors Trio and Toll; kinases such as PAK1; phosphatases; and transmembrane proteins. Critical to the structural organization of the resting platelet is the connection between filamin and the cytoplasmic tail of the GPIb α subunit of the GPIb-IX-V complex. Biochemical experiments have demonstrated that the majority of filamin (>90 %) in platelets is in complex with GPIb α (Kovacsovics and Hartwig 1996). This interaction has three consequences. First, it places filamin's self-association domain and corresponding partner proteins at the plasma membrane at the same time as presenting filamin's actin binding sites into the cytoplasm. Second, since a large fraction of filamin is bound to actin, it arranges the GPIb-IX-V complexes into linear rows on the surface of the platelet over the underlying filaments. Third, because the filamin linkages between actin filaments and the GPIb-IX-V complex pass through the pores of the spectrin lattice, it restrains the molecular movement of the spectrin strands in this lattice and holds the lattice in compression. The FLN-GPIb α connection is critical for the formation and release of discoid platelets by megakaryocytes as platelets lacking this connection are fragile and large and produced in low numbers. However, the role of the filamin-vWfR connection in platelet construction per se is not fully understood.

13 The Marginal Band of Microtubules

One of the most distinguishing features of the resting platelet is its marginal microtubule coil (White 1968). Alpha-beta tubulin dimers polymerize into microtubules under physiologic conditions, and in resting platelets, tubulin is equally separated between dimer and polymer fractions. The $\alpha\beta$ tubulin subunits are in a dynamic equilibrium with microtubules such that reversible cycles of assembly-disassembly of microtubules are observed. Microtubules are long polymers, 24 nm in diameter, responsible for many types of cellular movements, such as the transport of organelles within the cell and the segregation of chromosomes during mitosis. The microtubule coil of the resting platelet, initially described in the late 1960s by James White, has been described as a single microtubule approximately 100 nm long and is coiled 8–12 times inside the periphery of the platelet (White 1968). The principal function of the microtubule coil is to maintain the disc shape of the resting platelet. Disassembly of platelet microtubules with drugs such as nocodazole, colchicine, or vincristine cause platelets to round and lose their discoid shape (White 1968). Chilling platelets to 4 °C also causes depolymerization of the microtubule coil and loss of the disc shape (White 1968). Furthermore, transgenic studies show that mice lacking the major hematopoietic-specific β -tubulin isoform (β 1 tubulin) have platelets that lack the hallmark disc shape and contain defective marginal coils (Schwer et al. 2001). Genetic elimination of β 1 tubulin in mice results in thrombocytopenia with mice having circulating platelet counts below 50 % of normal. Beta 1 tubulin-deficient platelets are spherical in shape and this appears to be due to defective marginal bands with reduced microtubule coilings. Whereas normal

platelets possess a marginal band consisting of 8–12 coils, $\beta 1$ tubulin knockout platelets contain only 2–3 coils (Italiano et al. 2003; Schwer et al. 2001). A human $\beta 1$ tubulin base pair substitution (AG $\rightarrow$ CC) resulting in a Q43P mutation has been identified, and demonstrated to cause both functional and structural platelet alterations (Freson et al. 2005). The Q43P $\beta 1$ -tubulin variant was found in 10.6 % of the general population and in 24.2 % of 33 unrelated patients with undefined congenital macrothrombocytopenia. Ultrastructural studies revealed large spherical platelets with a disrupted microtubule coil and structural alterations. Interestingly, platelets with the Q43P $\beta 1$ -tubulin variant showed a mild platelet dysfunction, with reduced adenosine triphosphate (ATP) release, thrombin-receptor-activating peptide (TRAP)-induced aggregation, and inhibited adhesion to collagen under flow. A more than doubled prevalence of the $\beta 1$ -tubulin variant was observed in healthy subjects not undergoing ischemic events, indicating it could confer an evolutionary advantage and protective cardiovascular role. The microtubules that make up the coil are decorated with proteins that modulate polymer stability (Kenney and Linck 1985). Kinesin and cytoplasmic dynein, the major microtubule motors, have been localized to platelets, but their roles in resting and activated platelets are not well understood.

14 Alpha-Granules

One of the most interesting characteristics of platelets is the large number of biologically active molecules that are stored in their granules. These molecules are poised to be precisely delivered at sites of vascular injury and function to recruit other cells in the blood. In resting platelets, granules are positioned close to the OCS membranes. During activation, the granules fuse and secrete into the OCS (Flaumenhaft 2003). Platelets have two major storage granules, α - and dense-granules. The most abundant are α -granules (~40–80 per platelet), which contain proteins essential for platelet adhesion during vascular repair (e.g., GPIIb/IIIa, Fibrinogen, VWF). These granules are typically 200–500 nm in diameter and possess spherical shapes with a dark central core. They originate from the trans-Golgi network, where their characteristic dark nucleoid core becomes visible within the budding vesicles (Jones 1960). Alpha-granules obtain their molecular contents from both endogenous protein synthesis and by uptake and packaging of exogenous plasma proteins by receptor-mediated endocytosis and pinocytosis. Endogenously synthesized proteins such as β -thromboglobulin, platelet factor 4, and von Willebrand factor are detected in megakaryocytes before endocytosed proteins such as fibrinogen. In addition, synthesized proteins predominate in the Golgi complex, while endocytosed proteins are located in the peripheral regions of the cell. It has been established that uptake and delivery of fibrinogen to α -granules is mediated by the major membrane glycoprotein IIb/IIIa (Coller et al. 1991). Numerous membrane proteins essential to platelet function are also packaged into α -granules, including GP IIb/IIIa, P-selectin (CD62P), and CD36 (Blair and

Flaumenhaft 2009). Alpha-granules also have the bulk of cellular P-selectin in their membrane. Once inserted into the plasma membrane, P-selectin recruits neutrophils via the neutrophil counter receptor, the P-selectin glycoprotein ligand (PSGL1) (Diacovo et al. 1996). Alpha-granules also contain over 30 angiogenesis regulatory proteins, which allow them to function as mobile regulators of new blood vessel growth (Folkman et al. 2001). A body of experimental data suggests that specific classes of proteins may be differentially packaged into platelet α -granules, providing a mechanism for the differential release of proteins (Italiano et al. 2008).

Although there is very little known about the intracellular tracking of proteins in megakaryocytes and platelets, experiments using ultrathin cryosectioning and immunoelectron microscopy suggest that MVBs are an essential intermediate stage in the assembly of platelet α -granules (Heijnen et al. 1998). During development of megakaryocytes, these large (up to 0.5 μ m) MVBs undergo a gradual transition from granules containing 30–70 nm internal vesicles to granules containing predominantly dense material. Studies examining the internalization kinetics of exogenous bovine serum albumin-gold particles and of fibrinogen, place the MVBs and α -granules sequentially in the endocytic pathway. MVBs contain the secretory proteins β -thromboglobulin and vWF, the platelet-specific membrane protein P-selectin, and the lysosomal membrane protein CD63, suggesting they are a precursor organelle for α -granules (Heijnen et al. 1998).

15 Dense-Granules

Dense-granules (or dense-bodies) are 250 nm in size, are identified in electron micrographs by virtue of their electron-dense cores, and function predominantly to recruit additional platelets to sites of vascular damage. Dense-granules house a variety of hemostatically active molecules that are secreted upon platelet activation, including catecholamines, serotonin, calcium, adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP). ADP is a weak platelet agonist, triggering platelet shape change, granule release, and aggregation. Experiments have demonstrated that the transport of serotonin in platelet dense granules is essential for the process of liver regeneration (Lesurtel et al. 2006). Immunoelectron microscopy studies have also indicated that MVBs are an essential intermediate stage of dense-granule maturation and constitute a sorting compartment between α -granules and dense-granules (Youssefian and Cramer 2000).

16 Organelles

Platelets contain a relatively small number of mitochondria that are recognized in the electron microscope by their internal cristae. Mitochondria provide an energy source for the platelet as it circulates in the blood for 7–10 days in humans.