CHAPTER 1

THE STRUCTURE AND PRODUCTION OF BLOOD PLATELETS

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INTRODUCTION

Blood platelets are small, anucleate cellular fragments that play an essential role in hemostasis. During normal circulation, platelets circulate in a resting state as small discs (Fig. 1.1A). However, when challenged by vascular injury, platelets are rapidly activated and aggregate with each other to form a plug on the vessel wall that prevents vascular leakage. Each day, 100 billion platelets must be produced from megakaryocytes (MKs) to maintain the normal platelet count of 2 to 3×10^8/mL. This chapter is divided into three sections that discuss the structure and organization of the resting platelet, the mechanisms by which MKs give birth to platelets, and the structural changes that drive platelet activation.

1. THE STRUCTURE OF THE RESTING PLATELET

Human platelets circulate in the blood as discs that lack the nucleus found in most cells. Platelets are heterogeneous in size, exhibiting dimensions of 0.5×3.0 μm.[1] The exact reason why platelets are shaped as discs is unclear, although this shape may aid some aspect of their ability to flow close to the endothelium in the bloodstream. The surface of the platelet plasma membrane is smooth except for periodic invaginations that delineate the entrances to the open canalicular system (OCS), a complex network of interwinding membrane tubes that permeate the platelet's cytoplasm.[2] Although the surface of the platelet plasma membrane appears featureless in most micrographs, the lipid bilayer of the resting platelet contains a large concentration of transmembrane receptors. Some of the major receptors found on the surface of resting platelets include the glycoprotein receptor for von Willebrand factor (VWF); the major serpentine receptors for ADP, thrombin, epinephrine, and thromboxane A2; the Fc receptor FcγRIIA; and the β3 and β1 integrin receptors for fibrinogen and collagen.

The intracellular components of the resting platelet

The plasma membrane of the platelet is separated from the general intracellular space by a thin rim of peripheral cytoplasm that appears clear in thin sections when viewed in the electron microscope, but it actually contains the platelet's membrane skeleton. Underneath this zone is the cytoplasm, which contains organelles, storage granules, and the specialized membrane systems.

Granules

One of the most interesting characteristics of platelets is the large number of biologically active molecules contained in their granules. These molecules are poised to be deposited at sites of vascular injury and function to recruit other blood-borne cells. In resting platelets, granules are situated close to the OCS membranes. During activation, the granules fuse and exocytose into the OCS.[3] Platelets have two major recognized storage granules: α and dense granules. The most abundant are α granules (about 40 per platelet), which contain proteins essential for platelet adhesion during vascular repair. These granules are typically 200 to 500 nm in diameter and are spherical in shape with dark central cores. They originate from the trans Golgi network, where their characteristic dark nucleoid cores become visible within the budding vesicles.[4] Alpha granules acquire their molecular contents from both endogenous protein synthesis

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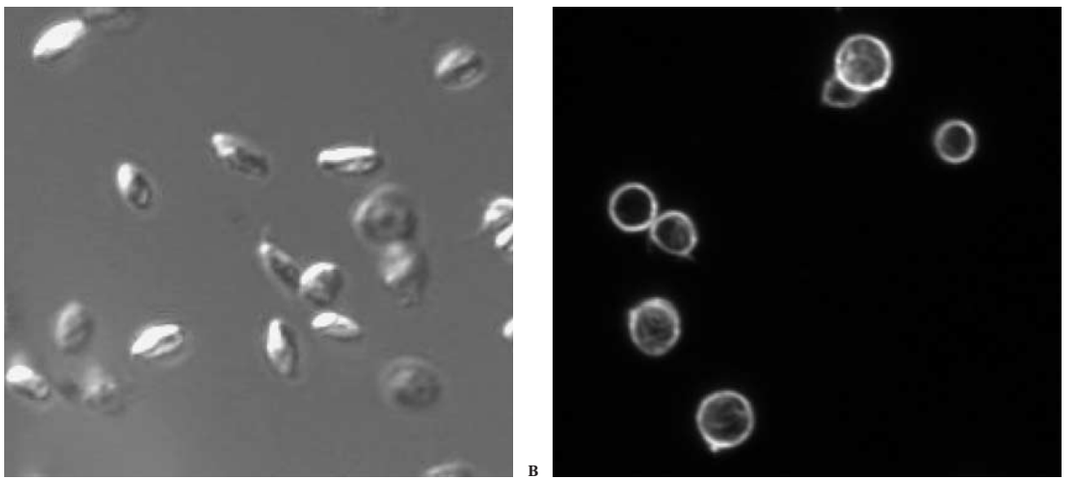

Figure 1.1 The structure of the resting platelet. A. Differential interference contrast micrograph of a field of human discoid resting platelets. B. Immunofluorescence staining of fixed, resting platelets with Alexa 488-antitubulin antibody reveals the microtubule coil. Coils are 1–3 μm in diameter.

and by the uptake and packaging of plasma proteins via receptor-mediated endocytosis and pinocytosis.[5] Endogenously synthesized proteins such as PF-4, β thromboglobulin, and von Willebrand factor are detected in megakaryocytes (MKs) before endocytosed proteins such as fibrinogen. In addition, synthesized proteins predominate in the juxtanuclear Golgi area, while endocytosed proteins are localized in the peripheral regions of the MK.[5] It has been well documented that uptake and delivery of fibrinogen to α granules is mediated by the major membrane glycoprotein $\alpha_{IIb}\beta_3$.[6,7,8] Several membrane proteins critical to platelet function are also packaged into alpha granules, including $\alpha_{IIb}\beta_3$, CD62P, and CD36. α granules also contain the majority of cellular P-selectin in their membrane. Once inserted into the plasma membrane, P-selectin recruits neutrophils through the neutrophil counter receptor, the P-selectin glycoprotein ligand (PSGL1).[9] Alpha granules also contain over 28 angiogenic regulatory proteins, which allow them to function as mobile regulators of angiogenesis.[10] Although little is known about the intracellular tracking of proteins in MKs and platelets, experiments using ultrathin cryosectioning and immunoelectron microscopy suggest that multivesicular bodies are a crucial intermediate stage in the formation of platelet α granules.[11] During MK development, these large (up to 0.5 μm) multivesicular bodies undergo a gradual transition from granules containing 30 to 70 nm internal vesicles to granules containing predominantly dense material. Internalization kinetics of exogenous bovine serum albumin–gold particles and of fibrinogen position the multivesicular bodies and α granules sequentially in the endocytic pathway. Multivesicular bodies contain the secretory proteins VWF and β thromboglobulin, the platelet-specific membrane protein P-selectin, and the lysosomal membrane protein CD63, suggesting that they are a precursor organelle for α granules.[11] Dense granules (or dense bodies), 250 nm in size, identified in electron micrographs by virtue of their electron-dense cores, function primarily to recruit additional platelets to sites of vascular injury. Dense granules contain a variety of hemostatically active substances that are released upon platelet activation, including serotonin, catecholamines, adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), and calcium. Adenosine diphosphate is a strong platelet agonist, triggering changes in the shape of platelets, the granule release reaction, and aggregation. Recent studies have shown that the transport of serotonin in dense granules is essential for the process of liver regeneration.[12] Immunoelectron microscopy studies have also indicated that multivesicular bodies are an intermediary stage of dense granule maturation and constitute a sorting compartment between α granules and dense granules.

Organelles

Platelets contain a small number of mitochondria that are identified in the electron microscope by their internal cisternae. They provide an energy source for the platelet as it circulates in the bloodstream for 7 days in humans. Lysosomes and peroxisomes are also present in the cytoplasm of platelets. Peroxisomes are small organelles that contain the enzyme catalase. Lysosomes are also tiny organelles that contain a large assortment of degradative enzymes, including β-galactosidase, cathepsin, aryl sulfatase, β-glucuronidase, and acid phosphatases. Lysosomes function primarily in the break down of material ingested by phagocytosis or pinocytosis. The main acid hydrolase contained in lysosomes is β-hexosaminidase.[13]

Membrane systems

Open canalicular system

The open canalicular system (OCS) is an elaborate system of internal membrane tunnels that has two major functions. First, the OCS serves as a passageway to the bloodstream, in which the contents can be released. Second, the OCS functions as a reservoir of plasma membrane and membrane receptors. For example, approximately one-third of the thrombin receptors are located in the OCS of the resting platelet, awaiting transport to the surface of activated platelets. Specific membrane receptors are also transported in the reverse direction from the plasma membrane to the OCS, in a process called downregulation, after cell activation. The VWF receptor is the best studied glycoprotein in this respect. Upon platelet activation, the VWF receptor moves inward into the OCS. One major question that has not been resolved is how other proteins present in the plasma membrane are excluded from entering the OCS. The OCS also functions as a source of redundant plasma membrane for the surface-to-volume ratio increase occurring during the cell spreading that accompanies platelet activation.

Dense tubular system

Platelets contain a dense tubular system (DTS),[14] named according to its inherent electron opacity, that is randomly woven through the cytoplasmic space. The DTS is believed to be similar in function to the smooth endoplamic reticular system in other cells and serves as the predominant calcium storage system in platelets. The DTS membranes possess Ca^{2+} pumps that face inward and maintain the cytosolic calcium concentrations in the nanomolar range in the resting platelet. The calcium pumped into the DTS is sequestered by calreticulin, a calcium-binding protein. Ligand-responsive calcium gates are also situated in the DTS. The soluble messenger inositol 1,4,5 triphosphate releases calcium from the DTS. The DTS also functions as the major site of prostaglandin and thromboxane synthesis in platelets.[15] It is the site where the enzyme cyclooxygenase is located. The DTS does not stain with extracellular membrane tracers, indicating that it is not in contact with the external environment.

The cytoskeleton of the resting platelet

The disc shape of the resting platelet is maintained by a well-defined and highly specialized cytoskeleton. This elaborate system of molecular struts and girders maintains the shape and integrity of the platelet as it encounters high shear forces during circulation. The three major cytoskeletal components of the resting platelet are the marginal microtubule coil, the actin cytoskeleton, and the spectrin membrane skeleton.

The marginal band of microtubules

One of the most distinguishing features of the resting platelet is its marginal microtubule coil (Fig. 1.1B).[16,17] Alpha and β tubulin dimers assemble into microtubule polymers under physiologic conditions; in resting platelets, tubulin is equally divided between dimer and polymer fractions. In many cell types, the α and β tubulin subunits are in dynamic equilibrium with microtubules, such that reversible cycles of microtubule assembly–disassembly are observed. Microtubules are long, hollow polymers 24 nm in diameter; they are responsible for many types of cellular movements, such as the segregation of chromosomes during mitosis and the transport of organelles across the cell. The microtubule ring of the resting platelet, initially characterized in the late 1960s by Jim White, has been described as a single microtubule approximately 100 μm long, which is coiled 8 to 12 times inside the periphery of the platelet.[16] The primary function of the microtubule coil is to maintain the discoid shape of the resting platelet. Disassembly of platelet microtubules with drugs such as vincristine, colchicine, or nocodazole cause platelets to round and lose their discoid shape.[16] Cooling platelets to

4°C also causes disassembly of the microtubule coil and loss of the discoid shape.[17] Furthermore, elegant studies show that mice lacking the major hematopoietic β-tubulin isoform (β-1 tubulin) contain platelets that lack the characteristic discoid shape and have defective marginal bands.[18] 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; this appears to be due to defective marginal bands with fewer microtubule coilings. Whereas normal platelets possess a marginal band that consists of 8 to 12 coils, β-1 tubulin knockout platelets contain only 2 or 3 coils.[18,19] A human β-1 tubulin functional substitution (AG>CC) inducing both structural and functional platelet alterations has been described.[20] Interestingly, the Q43P β-1-tubulin variant was found in 10.6% of the general population and in 24.2% of 33 unrelated patients with undefined congenital macrothrombocytopenia. Electron microscopy revealed enlarged spherocytic platelets with a disrupted marginal band and structural alterations. Moreover, platelets with this variant showed mild platelet dysfunction, with reduced secretion of ATP, thrombin-receptor-activating peptide (TRAP)–induced aggregation, and impaired adhesion to collagen under flow conditions. A more than doubled prevalence of the β-1-tubulin variant was observed in healthy subjects not undergoing ischemic events, suggesting that it could confer an evolutionary advantage and might play a protective cardiovascular role.

The microtubules that make up the coil are coated with proteins that regulate polymer stability.[21] The microtubule motor proteins kinesin and dynein have been localized to platelets, but their roles in resting and activated platelets have not yet been defined.

The actin cytoskeleton

Actin, at a concentration of 0.5 mM, is the most plentiful of all the platelet proteins with 2 million molecules expressed per platelet.[1] Like tubulin, actin is in a dynamic monomer-polymer equilibrium. Some 40% of the actin subunits polymerize to form the 2000 to 5000 linear actin filaments in the resting cell.[22] The rest of the actin in the platelet cytoplasm is maintained in storage as a 1 to 1 complex with β-4-thymosin[23] and is converted to filaments during platelet activation to drive cell spreading. All evidence indicates that the filaments of the resting platelet are interconnected at various points into a rigid cytoplasmic network, as platelets express high concentrations of actin cross-linking proteins, including filamin[24,25] and α-actinin.[26] Both filamin and α-actinin are homodimers in solution. Filamin subunits are elongated strands composed primarily of 24 repeats, each about 100 amino acids in length, which are folded into IgG-like β barrels.[27,28] There are three filamin genes on chromosomes 3, 7, and X. Filamin A (X)[29] and filamin B (3)[30] are expressed in platelets, with filamin A being present at greater than 10-fold excess to filamin B. Filamin is now recognized to be a prototypical scaffolding protein that attracts binding partners and positions them adjacent to the plasma membrane.[31] Partners bound by filamin members include the small GTPases, ralA, rac, rho, and cdc42, with ralA binding in a GTP-dependent manner[32]; the exchange factors Trio and Toll; and kinases such as PAK1, as well as phosphatases and transmembrane proteins. Essential to the structural organization of the resting platelet is an interaction that occurs between filamin and the cytoplasmic tail of the GPIbα subunit of the GPIb-IX-V complex. The second rod domain (repeats 17 to 20) of filamin has a binding site for the cytoplasmic tail of GPIbα 33, and biochemical experiments have shown that the bulk of platelet filamin (90% or more) is in complex with GPIbα.[34] This interaction has three consequences. First, it positions filamin's self-association domain and associated partner proteins at the plasma membrane while presenting filamin's actin binding sites into the cytoplasm. Second, because a large fraction of filamin is bound to actin, it aligns the GPIb-IX-V complexes into 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 filamin-GPIbα connection is essential for the formation and release of discoid platelets by MKs, as platelets lacking this connection are large and fragile and produced in low numbers. However, the role of the filamin-VWF receptor connection in platelet construction per se is not fully clear. Because a low number of Bernard-Soulier platelets form and release from MKs, it can be argued that this connection is a late event in the maturation process and is not per se required for platelet shedding.

The spectrin membrane skeleton

The OCS and plasma membrane of the resting platelet are supported by an elaborate cytoskeletal system. The platelet is the only other cell besides the erythrocyte whose membrane skeleton has been visualized at high resolution. Like the erythrocyte's skeleton, that of the platelet membrane is a self-assembly of elongated spectrin strands that interconnect through their binding to actin filaments, generating triangular pores. Platelets contain approximately 2000 spectrin molecules.[22,35,36] This spectrin network coats the cytoplasmic surface of both the OCS and plasma membrane systems. Although considerably less is known about how the spectrin–actin network forms and is connected to the plasma membrane in the platelet relative to the erythrocyte, certain differences between the two membrane 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.[22] These ends arrive at the plasma membrane originating from filaments in the cytoplasm. Hence, the spectrin lattice is assembled into a continuous network by its association with actin filaments. Second, tropomodulins are not expressed at sufficiently high levels, if at all, to have a major role in the capping of the pointed ends of the platelet actin filaments; instead, biochemical experiments have revealed that a substantial number (some 2000) of these ends are free in the resting platelet. Third, although little tropomodulin protein is expressed, adducin is abundantly expressed and appears to cap many of the barbed ends of the filaments composing the resting actin cytoskeleton.[37] Adducin is a key component of the membrane skeleton, forming a triad complex with spectrin and actin. Capping of barbed filament ends by adducin also serves the function of targeting them to the spectrin-based membrane skeleton, as the affinity of spectrin for adducin-actin complexes is greater than for either actin or adducin alone.[38,39,40]

MEGAKARYOCYTE DEVELOPMENT AND PLATELET FORMATION

Megakaryocytes are highly specialized precursor cells that function solely to produce and release platelets into the circulation. Understanding mechanisms by which MKs develop and give rise to platelets has fascinated hematologists for over a century. Megakaryocytes are descended from pluripotent stem cells and undergo multiple DNA replications without cell divisions by the unique process of endomitosis. During endomitosis, polyploid MKs initiate a rapid cytoplasmic expansion phase characterized by the development of a highly developed demarcation membrane system and the accumulation of cytoplasmic proteins and granules essential for platelet function. During the final stages of development, the MKs cytoplasm undergoes a dramatic and massive reorganization into beaded cytoplasmic extensions called proplatelets. The proplatelets ultimately yield individual platelets.

Commitment to the megakaryocyte lineage

Megakaryocytes, like all terminally differentiated hematopoietic cells, are derived from hematopoietic stem cells, which are responsible for constant production of all circulating blood cells.[41,42] Hematopoietic cells are classified by their ability to reconstitute host animals, surface markers, and colony assays that reflect their developmental potential. Hematopoietic stem cells are rare, making up less than 0.1% of cells in the marrow. The development of MKs from hematopoietic stem cells entails a sequence of differentiation steps in which the developmental capacities of the progenitor cells become gradually more limited. Hematopoietic stem cells in mice are typically identified by the surface markers Lin-Sca-1+c-kit^{high}.[43,44,45] A detailed model of hematopoiesis has emerged from experiments analyzing the effects of hematopoietic growth factors on marrow cells contained in a semisolid medium. Hematopoietic stem cells give rise to two major lineages, a common lymphoid progenitor that can develop into lymphocytes and a myeloid progenitor that can develop into eosinophil, macrophage, myeloid, erythroid, and MK lineages. A common erythroid-megakaryocytic progenitor arises from the myeloid lineage.[46] However, recent studies also suggest that hematopoietic stem cells may directly develop into erythroid–megakaryocyte progenitors.[47] All hematopoietic progenitors express surface CD34 and CD41, and the commitment to the MK lineage is indicated by expression of the integrin CD61 and elevated CD41 levels. From the committed myeloid progenitor cell (CFU-GEMM), there is strong evidence for a bipotential

progenitor intermediate between the pluripotential stem cell and the committed precursor that can give rise to biclonal colonies composed of megakaryocytic and erythroid cells.[48,49,50] The regulatory pathways and transcriptional factors that allow the erythroid and MK lineages to separate from the bipotential progenitor are currently unknown. Diploid precursors that are committed to the MK lineage have traditionally been divided into two colonies based on their functional capacities.[51,52,53,54] The MK burst-forming cell is a primitive progenitor that has a high proliferation capacity that gives rise to large MK colonies. Under specific culture conditions, the MK burst-forming cell can develop into 40 to 500 MKs within a week. The colony-forming cell is a more mature MK progenitor that gives rise to a colony containing from 3 to 50 mature MKs, which vary in their proliferation potential. MK progenitors can be readily identified in bone marrow by immunoperoxidase and acetylcholinesterase labeling.[55,56,57] Although both human MK colony-forming and burst-forming cells express the CD34 antigen, only colony-forming cells express the HLA-DR antigen.[58]

Various classification schemes based on morphologic features, histochemical staining, and biochemical markers have been used to categorize different stages of MK development. In general, three types of morphologies can be identified in bone marrow. The promegakaryoblast is the first recognizable MK precursor. The megakaryoblast, or stage I MK, is a more mature cell that has a distinct morphology.[59] The megakaryoblast has a kidney-shaped nucleus with two sets of chromosomes (4N). It is 10 to 50 μm in diameter and appears intensely basophilic in Romanovsky-stained marrow preparations due to the large number of ribosomes, although the cytoplasm at this stage lacks granules. The megakaryoblast displays a high nuclear-to-cytoplasmic ratio; in rodents, it is acetylcholinesterase-positive. The promegakaryocyte, or Stage II MK, is 20 to 80 μm in diameter with a polychromatic cytoplasm. The cytoplasm of the promegakaryocyte is less basophilic than that of the megakaryoblast and now contains developing granules.

Endomitosis

Megakaryocytes, unlike most other cells, undergo endomitosis and become polyploid through repeated cycles of DNA replication without cell division.[60,61,62,63] At the end of the proliferation phase, mononuclear MK precursors exit the diploid state to differentiate and undergo endomitosis, resulting in a cell that contains multiples of a normal diploid chromosome content (i.e., 4N, 16N, 32N, 64N).[64] Although the number of endomitotic cycles can range from two to six, the majority of MKs undergo three endomitotic cycles to attain a DNA content of 16N. However, some MKs can acquire a DNA content as high as 256N. Megakaryocyte polyploidization results in a functional gene amplification whose likely function is an increase in protein synthesis paralleling cell enlargement.[65] The mechanisms that drive endomitosis are incompletely understood. It was initially postulated that polyploidization may result from an absence of mitosis after each round of DNA replication. However, recent studies of primary MKs in culture indicate that endomitosis does not result from a complete absence of mitosis but rather from a prematurely terminated mitosis.[65,66,67] Megakaryocyte progenitors initiate the cycle and undergo a short G1 phase, a typical 6- to 7-hour S phase for DNA synthesis, and a short G2 phase followed by endomitosis. Megakaryocytes begin the mitotic cycle and proceed from prophase to anaphase A but do not enter anaphase B or telophase or undergo cytokinesis. During polyploidization of MKs, the nuclear envelope breaks down and an abnormal spherical mitotic spindle forms. Each spindle attaches chromosomes that align to a position equidistant from the spindle poles (metaphase). Sister chromatids segregate and begin to move toward their respective poles (anaphase A). However, the spindle poles fail to migrate apart and do not undergo the separation typically observed during anaphase B. Individual chromatids are not moved to the poles, and subsequently a nuclear envelope reassembles around the entire set of sister chromatids, forming a single enlarged but lobed nucleus with multiple chromosome copies. The cell then skips telophase and cytokinesis to enter G1. This failure to fully separate sets of daughter chromosomes may prevent the formation of a nuclear envelope around each individual set of chromosomes.[66,67]

In most cell types, checkpoints and feedback controls make sure that DNA replication and cell division are synchronized. Megakaryocytes appear to be the exception to this rule, as they have managed to deregulate this process. Recent work by a number of

laboratories has focused on identifying the signals that regulate polyploidization in MKs.[68] It has been proposed that endomitosis may be the consequence of a reduction in the activity of mitosis-promoting factor (MPF), a multiprotein complex consisting of Cdc2 and cyclin B.[69,70] MPF possesses kinase activity, which is necessary for entry of cells into mitosis. In most cell types, newly synthesized cyclin B binds to Cdc2 and produces active MPF, while cyclin degradation at the end of mitosis inactivates MPF. Conditional mutations in strains of budding and fission yeast that inhibit either cyclin B or Cdc2 cause them to go through an additional round of DNA replication without mitosis.[71,72] In addition, studies using a human erythroleukemia cell line have demonstrated that these cells contain inactive Cdc2 during polyploidization, and investigations with phorbol ester–induced Meg T cells have demonstrated that cyclin B is absent in this cell line during endomitosis.[73,74] However, it has been difficult to define the role of MPF activity in promoting endomitosis because these cell lines have a curtailed ability to undergo this process. Furthermore, experiments using normal MKs in culture have demonstrated normal levels of cyclin B and Cdc2 with functional mitotic kinase activity in MKs undergoing mitosis, suggesting that endomitosis can be regulated by signaling pathways other than MPF. Cyclins appear to play a critical role in directing endomitosis, although a triple knockout of cyclins D1, D2, and D3 does not appear to affect MK development.[75] Yet, cyclin E–deficient mice do exhibit a profound defect in MK development.[76] It has recently been demonstrated that the molecular programming involved in endomitosis is characterized by the mislocalization or absence of at least two critical regulators of mitosis: the chromosomal passenger proteins Aurora-B/AIM-1 and survivin.[77]

Cytoplasmic maturation

During endomitosis, the MK begins a maturation stage in which the cytoplasm rapidly fills with platelet-specific proteins, organelles, and membrane systems that will ultimately be subdivided and packaged into platelets. Through this stage of maturation, the MK enlarges dramatically and the cytoplasm acquires its distinct ultrastructural features, including the development of a demarcation membrane system (DMS), the assembly of a dense tubular system, and the formation of granules. During this stage of MK development, the cytoplasm contains an abundance of ribosomes and rough endoplasmic reticulum, where protein synthesis occurs. One of the most striking features of a mature MK is its elaborate demarcation membrane system, an extensive network of membrane channels composed of flattened cisternae and tubules. The organization of the MK cytoplasm into membrane-defined platelet territories was first proposed by Kautz and DeMarsh,[78] and a high-resolution description of this membrane system by Yamada soon followed.[79] The DMS is detectable in early promegakaryocytes but becomes most prominent in mature MKs where—except for a thin rim of cortical cytoplasm from which it is excluded—it permeates the MK cytoplasm. It has been proposed that the DMS derives from MK plasma membrane in the form of tubular invaginations. [80,81,82] The DMS is in contact with the external milieu and can be labeled with extracellular tracers, such as ruthenium red, lanthanum salts, and tannic acid.[83,84] The exact function of this elaborate smooth membrane system has been hotly debated for many years. Initially, it was postulated to play a central role in platelet formation by defining preformed "platelet territories" within the MK cytoplasm (see below). However, recent studies more strongly suggest that the DMS functions primarily as a membrane reserve for proplatelet formation and extension. The DMS has also been proposed to mature into the open canalicular system of the mature platelet, which functions as a channel for the secretion of granule contents. However, bovine MKs, which have a well-defined DMS, produce platelets that do not develop an OCS, suggesting the OCS is not necessarily a remnant of the DMS.[84]

Platelet formation

The mechanisms by which blood platelets are produced have been studied for approximately 100 years. In 1906, James Homer Wright at Massachussetts General Hospital began a detailed analysis of how giant precursor MKs give birth to platelets. Many theories have been suggested over the years to explain how MKs produce platelets. The demarcation membrane system (DMS), described in detail by Yamada in 1957, was initially proposed to demarcate preformed "platelet territories" within the cytoplasm of the MK.[79] Microscopists recognized that maturing

MKs become filled with membranes and platelet-specific organelles and proposed that these membranes form a system that defines fields for developing platelets.[85] Release of individual platelets was proposed to occur by a massive fragmentation of the MK cytoplasm along DMS fracture lines located between these fields. The DMS model proposes that platelets form through an elaborate internal membrane reorganization process.[86] Tubular membranes, which may originate from invagination of the MK plasma membrane, are predicted to interconnect and branch, forming a continuous network throughout. The fusion of adjacent tubules has been suggested as a mechanism to generate a flat membrane that ultimately surrounds the cytoplasm of an assembling platelet. Models attempting to use the DMS to explain how the MK cytoplasm becomes subdivided into platelet volumes and enveloped by its own membrane have lost support because of several inconsistent observations. For example, if platelets are delineated within the MK cytoplasm by the DMS, then platelet fields should exhibit structural characteristics of resting platelets, which is not the case.[87] Platelet territories within the MK cytoplasm lack marginal microtubule coils, one of the most characteristic features of resting platelet structure. In addition, there are no studies on living MKs directly demonstrating that platelet fields explosively fragment or shatter into mature, functional platelets. In contrast, studies that focused on the DMS of MKs before and after proplatelet retraction induced by microtubule depolymerizing agents suggest that this specialized membrane system may function primarily as a membrane reservoir that evaginates to provide plasma membrane for the extensive growth of proplatelets.[88] Radley and Haller have proposed that DMS may be a misnomer, and have suggested "invagination membrane system" as a more suitable name to describe this membranous network.

The majority of evidence that has been gathered supports the proplatelet model of platelet production. The term "proplatelet" is generally used to describe long (up to millimeters in length), thin cytoplasmic extensions emanating from MKs.[89] These extensions are characterized by multiple platelet-sized beads linked together by thin cytoplasmic bridges and are thought to represent intermediate structures in the megakaryocyte-to-platelet transition. The actual concept of platelets arising from these pseudopodia-like structures occurred when Wright recognized that platelets originate from MKs and described "the detachment of plate-like fragments or segments from pseudopods" from MKs.[90] Thiery and Bessis[91] and Behnke[92] later described the morphology of these cytoplasmic processes extending from MKs during platelet formation in more detail. The classic "proplatelet theory" was introduced by Becker and De Bruyn, who proposed that MKs form long pseudopod-like processes that subsequently fragment to generate individual platelets.[89] In this early model, the DMS was still proposed to subdivide the MK cytoplasm into platelet areas. Radley and Haller later developed the "flow model," which postulated that platelets derived exclusively from the interconnected platelet-sized beads connected along the shaft of proplatelets[88]; they suggested that the DMS did not function to define platelet fields but rather as a reservoir of surface membrane to be evaginated during proplatelet formation. Developing platelets were assumed to become encased by plasma membrane only as proplatelets were formed.

The bulk of experimental evidence now supports a modified proplatelet model of platelet formation. Proplatelets have been observed (1) both in vivo and in vitro, and maturation of proplatelets yields platelets that are structurally and functionally similar to blood platelets[93,94]; (2) in a wide range of mammalian species, including mice, rats, guinea pigs, dogs, cows, and humans[95,96,97,98,99]; (3) extending from MKs in the bone marrow through junctions in the endothelial lining of blood sinuses, where they have been hypothesized to be released into circulation and undergo further fragmentation into individual platelets[100,101,102]; and (4) to be absent in mice lacking two distinct hematopoietic transcription factors. These mice fail to generate proplatelets in vitro and display severe thrombocytopenia.[103,104,105] Taken together, these findings support an important role for proplatelet formation in thrombopoiesis.

The discovery of thrombopoietin and the development of MK cultures that reconstitute platelet formation in vitro has provided systems to study MKs in the act of forming proplatelets. Time-lapse video microscopy of living MKs reveals both temporal and spatial changes that lead to the formation of proplatelets (Fig. 1.2).[106] Conversion of the MK cytoplasm concentrates almost all of the intracellular contents into proplatelet extensions and their platelet-sized particles, which in the final stages appear as beads

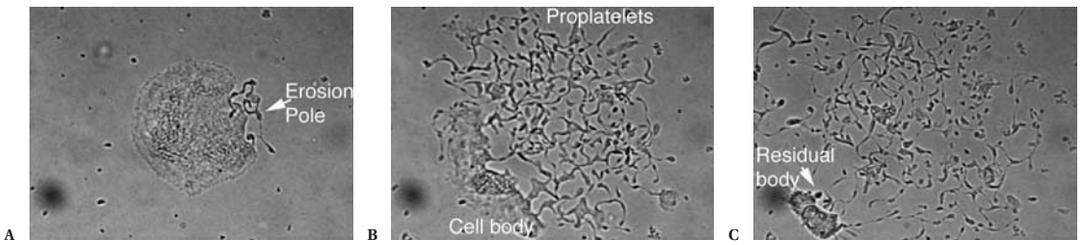

Figure 1.2 Formation of proplatelets by a mouse megakaryocyte. Time-lapse sequence of a maturing megakaryocyte (MK), showing the events that lead to elaboration of proplatelets *in vitro*. (A) Platelet production commences when the MK cytoplasm starts to erode at one pole. (B) The bulk of the megakaryocyte cytoplasm has been converted into multiple proplatelet processes that continue to lengthen and form swellings along their length. These processes are highly dynamic and undergo bending and branching. (C) Once the bulk of the MK cytoplasm has been converted into proplatelets, the entire process ends in a rapid retraction that separates the released proplatelets from the residual cell body (Italiano JE *et al.*, 1999).

linked by thin cytoplasmic strings. The transformation unfolds over 5 to 10 hours and commences with the erosion of one pole (Fig. 1.2B) of the MK cytoplasm. Thick pseudopodia initially form and then elongate into thin tubes with a uniform diameter of 2 to 4 μm. These slender tubules, in turn, undergo a dynamic bending and branching process and develop periodic densities along their length. Eventually, the MK is transformed into a "naked" nucleus surrounded by an elaborate network of proplatelet processes. Megakaryocyte maturation ends when a rapid retraction separates the proplatelet fragments from the cell body, releasing them into culture (Fig. 1.2C). The subsequent rupture of the cytoplasmic bridges between platelet-sized segments is believed to release individual platelets into circulation.

The cytoskeletal machine of platelet production

The cytoskeleton of the mature platelet plays a crucial role in maintaining the discoid shape of the resting platelet and is responsible for the shape change that occurs during platelet activation. This same set of cytoskeletal proteins provides the force to bring about the shape changes associated with MK maturation.[107] Two cytoskeletal polymer systems exist in MKs: actin and tubulin. Both of these proteins reversibly assemble into cytoskeletal filaments. Evidence supports a model of platelet production in which microtubules and actin filaments play an essential role. Proplatelet formation is dependent on microtubule function, as treatment of MKs with drugs that take apart microtubules, such as nocodazole or vincristine, blocks proplatelet formation. Microtubules, hollow polymers assembled from α and β tubulin dimers, are the major structural components of the engine that powers proplatelet elongation. Examination of the microtubule cytoskeletons of proplatelet-producing MKs provides clues as to how microtubules mediate platelet production (Fig. 1.3).[108] The microtubule cytoskeleton in MKs undergoes a dramatic remodeling during proplatelet production. In immature MKs without proplatelets, microtubules radiate out from the cell center to the cortex. As thick pseudopodia form during the initial stage of proplatelet formation, membrane-associated microtubules consolidate into thick bundles situated just beneath the plasma membrane of these structures. And once pseudopodia begin to elongate (at an average rate of 1 μm/min), microtubules form thick linear arrays that line the whole length of the proplatelet extensions (Fig. 1.3B). The microtubule bundles are thickest in the portion of the proplatelet near the body of the MK but thin to bundles of approximately seven microtubules near proplatelet tips. The distal end of each proplatelet always has a platelet-sized enlargement that contains a microtubule bundle which loops just beneath the plasma membrane and reenters the shaft to form a teardrop-shaped structure. Because microtubule coils similar to those observed in blood platelets are detected only at the ends of proplatelets and not within the platelet-sized beads found along the length of proplatelets, mature platelets are formed predominantly at the ends of proplatelets.

In recent studies, direct visualization of microtubule dynamics in living MKs using green fluorescent protein (GFP) technology has provided insights into how microtubules power proplatelet elongation.[108]

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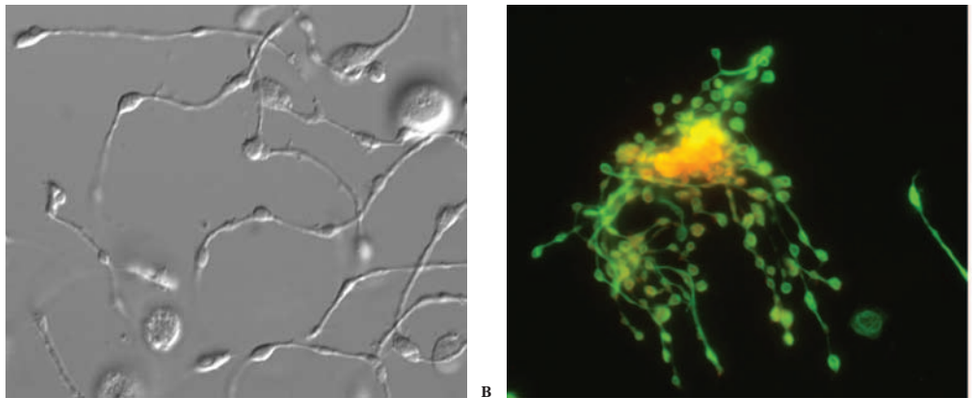

Figure 1.3 Structure of proplatelets. (A) Differential interference contrast (DIC) image of proplatelets elaborated by mouse megakaryocytes in culture. Proplatelets contain platelet-sized swellings that decorate their length giving them a beads-on-a-string appearance. (B) Staining of proplatelets with Alexa 488-anti-tubulin IgG reveals the microtubules to line the shaft of the proplatelet and to form loops at the proplatelet tips.

End-binding protein three (EB3), a microtubule plus end-binding protein associated only with growing microtubules, fused to GFP was retrovirally expressed in murine MKs and used as a marker to follow microtubule plus end dynamics. Immature MKs without proplatelets employ a centrosomal-coupled microtubule nucleation/assembly reaction, which appears as a prominent starburst pattern when visualized with EB3-GFP. Microtubules assemble only from the centrosomes and grow outward into the cell cortex, where they turn and run in parallel with the cell edges. However, just before proplatelet production begins, centrosomal assembly stops and microtubules begin to consolidate into the cortex. Fluorescence time-lapse microscopy of living, proplatelet-producing MKs expressing EB3-GFP reveals that as proplatelets elongate, microtubule assembly occurs continuously throughout the entire proplatelet, including the swellings, shaft, and tip. The rates of microtubule polymerization (average of 10.2 μm/min) are approximately 10-fold faster than the proplatelet elongation rate, suggesting polymerization and proplatelet elongation are not tightly coupled. The EB3-GFP studies also revealed that microtubules polymerize in both directions in proplatelets (e.g., both toward the tips and cell body), demonstrating that the microtubules composing the bundles have a mixed polarity.

Even though microtubules are continuously assembling in proplatelets, polymerization does not provide the force for proplatelet elongation. Proplatelets continue to elongate even when microtubule polymerization is blocked by drugs that inhibit net microtubule assembly, suggesting an alternative mechanism for proplatelet elongation.[108] Consistent with this idea, proplatelets possess an inherent microtubule sliding mechanism. Dynein, a minus-end microtubule molecular motor protein, localizes along the microtubules of the proplatelet and appears to contribute directly to microtubule sliding, since inhibition of dynein, through disassembly of the dynactin complex, prevents proplatelet formation. Microtubule sliding can also be reactivated in detergent-permeabilized proplatelets. ATP, known to support the enzymatic activity of microtubule-based molecular motors, activates proplatelet elongation in permeabilized proplatelets that contain both dynein and dynactin, its regulatory complex. Thus, dynein-facilitated microtubule sliding appears to be the key event in driving proplatelet elongation.

Each MK has been estimated to release thousands of platelets.[109,110,111] Analysis of time-lapsed video microscopy of proplatelet development from MKs grown in vitro has revealed that ends of proplatelets are amplified in a dynamic process that repeatedly bends and bifurcates the proplatelet shaft.[106] End amplification is initiated when a proplatelet shaft is bent into a sharp kink, which then folds back on itself, forming a loop in the microtubule bundle. The new loop eventually elongates, forming a new proplatelet shaft branching from the side of the original proplatelet. Loops lead