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Platelets and cancer: a casual or causal relationship: revisited

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Abstract

Human platelets arise as subcellular fragments of megakaryocytes in bone marrow. The physiologic demand, presence of disease such as cancer, or drug effects can regulate the production circulating platelets. Platelet biology is essential to hemostasis, vascular integrity, angiogenesis, inflammation, innate immunity, wound healing, and cancer biology. The most critical biological platelet response is serving as “First Responders” during the wounding process. The exposure of extracellular matrix proteins and intracellular components occurs after wounding. Numerous platelet receptors recognize matrix proteins that trigger platelet activation, adhesion, aggregation, and stabilization. Once activated, platelets change shape and degranulate to release growth factors and bioactive lipids into the blood stream. This cyclic process recruits and

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aggregates platelets along with thrombogenesis. This process facilitates wound closure or can recognize circulating pathologic bodies. Cancer cell entry into the blood stream triggers platelet-mediated recognition and is amplified by cell surface receptors, cellular products, extracellular factors, and immune cells. In some cases, these interactions suppress immune recognition and elimination of cancer cells or promote arrest at the endothelium, or entrapment in the microvasculature, and survival. This supports survival and spread of cancer cells and the establishment of secondary lesions to serve as important targets for prevention and therapy.

Keywords

Platelet; TCIPA; Metastasis; Thrombosis; Extravasation; CTC

1 Historical perspectives

Twenty years ago when we first reviewed this topic [1], most researchers in the cancer field were skeptical about the concept that platelets played a major role as “first responders” in metastasis, despite our own pioneering work and that of others. In the time between, much of the early work has been corroborated, and remains substantiated as new techniques and investigators enter the field. In this review, we revisit the topic with the latest in accumulated evidence; evidence that clearly points to a role for platelet functions in the hemostatic microenvironment being subverted and employed by tumor cells to facilitate tumor progression, invasion, and metastasis.

1.1 Leeches, light microscopes, “Blutplättchen”, and bone marrow

The vital ebb and flow of blood as a life force has always captivated humankind. Blood was considered to be the first of four essential “humors” by the ancient Greeks. The ancient Mesopotamian, Egyptian, Greek, Roman, Indian, and Chinese physicians practiced bloodletting and often used the application of leeches to treat diseases until the practice reached its height in the 1800s. The use of leeches continues to the present following the Food and Drug Administration-approved use of *Hirudo medicinalis* in modern medicine [2, 3]. The anticoagulant hirudin identified from leech saliva can help relieve venous insufficiency in skin and reattachment surgery [2, 3]. Despite keen medical interest in blood over the ages, the crucial cellular components that contribute to clot formation were not readily identified. Even after the invention of the light microscope in 1590 by Dutch spectacle makers, Zaccharias and Hans Janssen descriptions of blood cells were not immediately forthcoming. It was not until 1658 that Dutch biologist Jan Swammerdam identified red blood cells [4, 5]. Nevertheless, the key subcellular factors were not described until 1842. Within that year, the French physician Alfred Donne first described “particles” in the blood—red globules, white globules, and globulins (little globules) in a lecture to the Academie des Sciences of Paris. A month later, William Addison published hand drawings of platelet–fibrin thrombus referring to them as extremely minute granules [6]. In 1846, Gustav Zimmerman first studied anticoagulated blood by aspirating it into a potassium ferrocyanide solution describing “billions” of *“Elementarkörperchen ”* or “elementary corpuscles”. Subsequently in 1865, Schultze described the tiny spherules of the blood [7]. Then in 1882, Giulio Bizzozero suggested the term *“Blutplattchen ”* or “blood platelets” for

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these corpuscles [8, 9]. Hand-drawn images by Bizzozero from observations using a Hartmack light microscope showed fused platelets with stellate fibrin threads in freshly acquired blood samples as well as observations of their increased stickiness to damaged blood vessels [8, 9]. Rudolf Virchow first described clot formation within the blood vessels of a living animal in 1856 [10]. In 1869, Bizzozero also observed large bodies in bone marrow, irregular giant cells with a diameter of 25 to 65 μm, with a budding central nucleus that were likely platelet-producing megakaryocytes (MK)s, but their biological significance was unknown [11]. Platelet aggregates with bacteria were observed later by Osler and Schäfer in 1873 [12]. Then, although their existence had been known for some time, large rare marrow cells were first given the name “megakaryocyte” by Howell in 1890 and described by detailed *camera lucida* drawings [13]. Later, the connection between blood “plates” and their release from megakaryocytes was made by Wright in 1906 [14, 15]. We now know that mature megakaryocytes are among the scarcest (0.01–0.5 % of nucleated cells) and largest (50–100 μm) cells in human bone marrow and generate platelets (Fig. 1) [16, 17]. Nonetheless, many early hand-drawn observations of platelets and megakaryocytes using simple light microscopy remain remarkable testaments to the powers of keen observation.

1.2 Trousseau, thrombophlebitis, and occult cancer

The first known association between tumors and blood changes was around 1000 BC by the Indian surgeon, Sushruta in the Sanskrit text on surgery, *Sushruta Samhita*. This text describes six main types of tumors. In one, “Raktaja arbuda” is described as a tumor that enters the blood and compresses and constricts blood vessels.

It was not until Armand Trousseau in his 1865 lecture, “Phlegmasia alba dolens”, which is Latin for “inflammation white leg” or “milk leg” due to lack of blood in the extremity caused by deep vein thrombosis, that this condition was associated with cancer [18]. Trousseau’s lecture was the first description of migratory deep vein thrombosis as a prognostic indicator of a “deep-seated concealed cancer” associated with gastric malignancy [18]. It was based on series of case studies including one of his colleagues. Ironically, on 1 January 1867, Trousseau was stricken with the phlebitis syndrome that now bears his name in his upper left extremity [19]. Telling his student, Peter “I am lost, the phlebitis that has just appeared leaves me no doubt about the nature of my illness,” he thereby predicted having his own occult gastric cancer that caused his death a few months later [19].

Later in 1900, Osler and McCrae, among others around that time period, reported on the association of idiopathic phlebitis and gastric cancer [20]. Trousseau’s syndrome found greater acceptance in 1938 following a study by Sproul, where she performed 4,258 autopsies on patients with carcinomas [21]. She discovered multiple thromboses associated with 31.3 % of carcinoma cases in the body and tail of the pancreas, and 9.7 % in the head of the pancreas among other cancer sites [21]. Over the next several decades, the association with thrombophlebitis was discovered for numerous other cancers in case reports and cohort studies [22-39]. Some of these early studies associated thrombophlebitis with mucin-producing carcinomas [27, 31]. Later in 1973, extracts from mucinous adenocarcinoma caused intravascular coagulation and death in rabbits [40]. Thrombophlebitis associated with

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cancer is often migratory or recurrent [34]. Furthermore, affected veins do not consistently show tumor invasion or tumor emboli indicating the presence of cells is not required for thrombus formation [34]. Blood coagulation and fibrinolysis accompanies the development of lung carcinoma and other tumors [41, 34]. In rats, thromboplastic and fibrinolytic properties were observed in a transplantable rat tumor model [42-44]. Causes of hypercoagulability were thought by some to involve increased platelet adhesiveness [45]. Another analysis of 14,000 patient blood smears from patients bearing tumors of different types showed a significant increase in counts above 400,000 platelets/cubic milliliter [46]. The importance of tumor cell–platelet emboli formation and their contribution to the formation of metastasis was recognized in the late 1960s and early 1970s [47-50, 1, 51, 52]. The effects of thrombosis on metastasis were also reported based on early rabbit transplantable tumor models of Brown–Pearce carcinoma and the V2 carcinoma [53-57]. The importance of tumor cell-containing emboli to the formation of metastasis was first quantified using transplantable mouse tumors [58]. Tumor cell-induced platelet aggregation (TCIPA) was found to be critical for heterotypic aggregate formation, but could be inhibited by prostaglandins that prevent antiplatelet aggregation [59-63]. Subsequently, the role of shed tumor cell vesicles, now known as exosomes, on platelet aggregation was found to be influenced by the protease cathepsin B [64]. The light microscope revealed certain aspects of tumor cell–platelet interactions, but certain details would await improvements in technology. The invention of the scanning and transmission electron microscopes helped clarify many of the details of megakaryocyte and platelet structure as well as platelet–tumor cell interactions [65-74].

2 Megakaryocyte and platelet biology

Historically, the light microscope revealed aspects of tumor cell–platelet interactions. However, it was not until the invention of the scanning and transmission electron microscopes that details of megakaryocyte and platelet structure as well as platelet–tumor cell interactions were revealed [65-67, 75, 68, 69, 62, 72]. Unlike cancer cells, megakaryocytes are genetically stable and provide the genetic instructions along with the molecular and structural biomass for platelet genesis (Fig. 1). Platelets lack nuclei and the capacity for genetic adaptation. Intrinsically then, understanding the molecular, cellular, and systemic biology of megakaryocytes and platelets is an important starting point for understanding their role in cancer.

2.1 Megakaryocytes and platelet progenitors

2.1.1 Niches, riches, and bone marrow switches—Systemic, circulating/humoral, and microenvironmental factors in the bone marrow regulate physiologic responses to the body’s demand for platelets [76, 77, 69, 78]. Bone marrow exists in two primary forms, where the yellow bone marrow consists mostly of fat cells, and the red bone marrow contains the hematopoietic cell populations that generate platelets, as well as the red and white cells [79, 80]. Both forms of bone marrow contain extensive numbers of blood vessels and capillaries [81]. Microscopically, osteoblastic niches are spatially separated from vascular niches that provide a systemically enriched oxygen source within the bone marrow [82-85] (Fig. 1). The spatial separation of these niches effectively establishes a decreasing

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oxygen gradient [86, 87], as well as microenvironmental gradients of cytokines and chemokines, growth factors, and calcium in the osteoblastic niche. These gradients collectively regulate megakaryopoiesis or the formation of MK that produce platelets. Gradients of extracellular matrix proteins also provide migratory switches that drive MK toward the microvasculature during the later stages of platelet formation [88-91].

Megakaryopoiesis begins in the bone marrow with hematopoiesis, where hemangioblasts that are present mainly in bone marrow give rise to hematopoietic stem cells (HSCs) and multipotent progenitor cells (MPP) [92, 77, 78, 93]. Along the developmental cascade, a key separation in progenitor lineages occurs between the major lymphoid progenitors along with granulocytic progenitors *versus* the major erythromegakaryocytic progenitor cells [92, 77, 78, 93]. Common myeloid progenitor cells (CMP) give rise to megakaryocyte-erythroid progenitor (MEP) cells that are in turn delineated into megakaryopoietic progenitors (MKP) [94]. Ultimately, MKP undergo a complicated maturation process from immature to mature megakaryocytes before finally generating platelets [92, 77, 69, 95, 93].

3 Transcription factors, microRNAs, and hematopoiesis

Megakaryocyte lineage derivation is mediated by a series of transcription factors. Runt-related transcription factor 1 (RUNX1) is crucial for the entire hematopoietic process. In the case of RUNX1 haploinsufficiency as an example, thrombocytopenia and platelet dysfunction in addition to acute leukemia, are manifest by a fivefold reduction in platelet 12-lipoxygenase and a decrease in platelet production of 12(S)-HETE on agonist stimulation [96]. RUNX1 mutations also impact a number of cancers. For example, RUNX1 can work alone or in cooperation with the Ets transcription factor to promote PSA expression in prostate cancer [97]. The commitment and development of HSCs to form MEP and MK lineages relies on increases in erythroid transcription factor also known as GATA-binding factor 1 (GATA-1) and downregulation of PU.1 [98, 99]. Increased levels of GATA-1 along with Friend of GATA (FOG1) cofactor and the downregulation of *Ikaros* all help to generate MEP lineage cells [100-103]. As GATA-1 levels increase, diploid MKP that proliferate as part of the MEP stop dividing, but retain the capacity for DNA replication and cytoplasmic maturation. Continued RUNX1 production along with the loss of myosin heavy chain 10 (MYH10) expression stimulates the formation of immature MK [76, 104, 105]. The final stages of MK maturation as well as the initiation of platelet formation are stimulated by nuclear factor erythroid-derived 2 (NFE2) production [104, 106, 107]. NFE2 has also been shown to regulate thromboxane synthase (TXAS), a key enzyme in the regulation of platelet activation and aggregation during thrombogenesis, during MK maturation [108]. This complex cascade of changes in these transcription factors tightly regulates platelet genesis while priming them for their biological roles.

Many noncoding RNA molecules are involved in the genesis of megakaryocytes and platelets [109, 110]. In the bone marrow, microRNA (miR) 125a controls the size of the stem cell population by regulating HSC apoptosis [111]. At the MEP stage as well, miR150 negatively regulates c-myb production at the 3′UTR of the mRNA [112, 113]. MYB is a negative regulator of megakaryopoiesis and the resulting loss of protein expression stimulates MEP to differentiate into MK [114-116]. In other studies, loss of miR145 and

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increased expression of Fli-1A were reported in 5q-syndrome MDS, with anemia and thrombocytosis [117]. A variety of other miRNAs are also involved in the pathogenesis of myeloproliferative neoplasms [118, 119]. In contrast, there is likely to be significant potential in miRNA-based platelet therapies [120].

4 Surface receptors and megakaryopoiesis

A number of cell surface receptors serve as lineage markers while functioning to direct hematopoiesis then megakaryopoiesis in preparation for platelet genesis.

Certain cell surface receptors are retained by subpopulations of activated HSC that differentiate into immature and then mature MKP. These markers include stromal-derived factor-1 (SDF-1), CXC chemokine receptor type 12 (CXCR12), along with CXC chemokine receptor type 4 (CXCR4) and the thrombopoietin receptor, which is known as myeloproliferative leukemia virus oncogene (MPL) or cluster of differentiation 110 (CD110) [121]. MPL/CD110 levels significantly increase during the progressive maturation of MK in response to thrombopoietin (THPO/TPO) also known as megakaryocyte growth and development factor (MGDF), which is a key ligand in megakaryopoiesis [122-126].

Early clinical trials revealed that THPO administration caused temporary morphological changes in bone marrow constituents that mimicked chronic myeloproliferative diseases, but that these reversed within 3 months of discontinuing the treatment [127]. Administration of recombinant THPO can increase megakaryopoiesis and platelet production in subjects undergoing radiation and chemotherapy with no apparent impact on platelet aggregation. However, as some individuals developed neutralizing antibodies, this approach was abandoned in pursuit of alternate means to regulate Mpl [128]. With that said, a recent study that examined the effect of the hematopoietic microenvironment on leukemic stem cell survival found a strong correlation between acute myelogenous leukemia (AML) relapse and elevated levels of THPO and Mpl that may impact resistance to daunorubicin [129].

Along with MKP, the fibrinogen receptor known as integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa or CD41) and von Willebrand factor (vWF) expression levels also significantly increase [130-134]. In contrast, CD34, CD45, CD150, Tie-2, and c-kit levels decrease once progenitors commit to the MK lineage [92, 135-138]. These changes in cell surface receptor profiles accompany a decrease in proliferation by MKP prior to the next stage of development involving endomitosis.

4.1 Got DNA? Got cytoplasm?

Endomitosis ensues with the reproduction of nuclear elements that ultimately results in polyploidy [77, 69]. This process involves diploid megakaryocytes that proceed through S phase and progress through mitotic anaphase. Anaphase progresses through the separation of paired sister chromosomes followed by cleavage furrow formation but halts at cytokinesis. Furrow regression then generates a tetraploid cell that reenters G1 arrest. This process continues until MK accumulate a DNA content of 4n, 8n, 16n, 32n, 64n, and even 128n in a single multilobed nucleus and then finally mature before beginning proplatelet formation [139]. This elaborate amplification of nuclear material helps support extensive organelle

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synthesis, as well as dramatic cytoplasmic maturation and expansion needed to produce platelets.

Cytoplasmic maturation in MK that accompanies endomitosis is designed to generate the extensive membrane, cytoskeletal, and organelle stores needed to support platelet production [69, 140, 141]. Structurally, this process forms a complex membrane network of tubules and cisternae termed either demarcation membrane system (DMS) or invaginated membrane system (IMS) [142, 143]. Membrane and cytoskeletal remodeling involves a Cdc42-interacting protein 4 (CIP4), which is an F-Bin–amphiphysin–Rvs (F-BAR) protein that localizes to membrane phospholipids through its BAR domain and interacts with Wiskott–Aldrich syndrome protein (WASP) *via* its SRC homology 3 (SH3) domain to coordinate platelet production [144]. Motor protein dynamin 3 (DNM3) participates in membrane rearrangements during megakaryopoiesis [145]. Structural proteins such as spectrin among others, form a two-dimensional lattice that stabilizes the IMS/DMS and becomes an important structural component of platelet precursors known as proplatelets [146].

5 Platelet granule formation

Alpha granule biogenesis leads to the formation of discrete populations as recently observed by three-dimensional electron tomography analysis [147]. These studies revealed that membrane-bound organelles display high variability in morphology, size, and luminal content that fall into three categories (Fig. 2)—(1) spherical granules that have both electron dense and electron lucent zones and 12 nm–vWF tubules; (2) 50-nm-wide tubular organelles containing membrane-bound proteins GLUT3 and $\alpha_{IIb}\beta_3$ integrin along with abundant fibrinogen, albumin, and some thromboglobulin; and (3) a population with 18.4-nm crystalline cross-striations. Other studies suggest that α-granule populations are differentially segregated in a zonal manner [148]. Many of the key granule vesicle trafficking proteins were discovered using samples from patients with α-granule deficiencies [149, 150]. Although the exact mechanism involved in forming the various α-granule subtypes remains unclear, some of the causative genes, NBEAL2, VPS33B, and VPS16B, have recently been identified [151-155].

Dense granule biogenesis, by contrast, involves a specialized mechanism that integrates secretory and the endocytic pathways [156]. Multidrug resistance protein 4 (MRP4) is involved in the transport of nucleotides and other factors into dense granules [157, 158]. Recent data suggest that Rab32 and Rab3 8 transmembrane proteins employ an internal secretory pathway through Golgi complexes and then endosomes to selectively target dense granules. Rab32 and Rab38 define a biosynthetic transport pathway from early endosome-associated tubules to maturing dense granules after they originate from multivesicular bodies. These studies used MEG-01 cells that were analyzed by transmission electron microscopy, spinning-disk confocal fluorescence microscopy of cells immunostained for lysosomal-associated membrane protein-2 (LAMP2) and MRP4, or live MEG-01 cells expressing the dense granule markers VMAT2-Cherry and LAMP2-GFP [156].

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5.1 Getting in the blood

During this maturation process, MKs migrate from the osteoblastic to the vascular niche to elicit the final stages of platelet formation [159-161]. Collagen I is the primary protein in the osteoblastic niche involved in interactions between the extracellular matrix and $\alpha_2\beta_1$ on the surfaces of MK cells [162-164]. These interactions with collagen I facilitate the differentiation of HSC to MK while inhibiting proplatelet production [165]. Maturing MKs migrate to the perivascular surfaces of bone marrow sinusoidal endothelial cells within vascular niches [166, 69, 167, 168]. The perivascular surfaces contain a number of extracellular matrix proteins including, vitronectin, collagen IV, laminin, fibronectin, fibrinogen, and vWF along with pericytes [169-173, 69, 174, 175]. Fibrinogen interactions with integrin $\alpha_{IIb}\beta_3$ receptors on the MK surfaces in particular help govern migration, maturation, and proplatelet formation [176, 177].

Once in contact with the perivascular surfaces of sinusoidal endothelial cells, mature MKs begin platelet genesis. The nucleus of MKs is extruded as platelet formation ensues [178]. MKs extend long-branching processes known as proplatelets into the lumens of bone marrow sinusoidal blood vessels. This process occurs by erosion of the MK pole involved in genesis. Centrosomes disassemble and microtubules made mainly of β1-tubulin translocate to the cell cortex accompanied by pseudopod formation as proplatelets begin forming [69, 78]. Podosomes protrude through basement membrane by degrading the perivascular matrix of sinusoidal vessels [167]. Proplatelet shafts fill with microtubule bundles then elongate to loop around and reenter the shaft, forming buds at the shaft tip [179]. Microtubule sliding drives shaft elongation as they polymerize their free ends in concert with dynein-driven sliding of overlapping microtubules [179, 69]. Microtubule sliding occurs at 4 to 5 μm/min to reach lengths of about 0.5 to 1.0 mm [179, 69]. Microtubule bundles provide physical tracks for the bidirectional movement of mitochondria and granules into proplatelet tips [180]. The formation of microtubule bundles depends upon the β1-tubulin isoform gene Tubb1 and the tubulin binding protein RanBP 10 [181, 173, 182, 183]. Proplatelets *in vitro* or within bone marrow sinusoids appear by intravital microscopy as dumbbell-shaped elongations or globules that shear off into the vascular lumen [181, 184, 69]. Intravascular proplatelets continue their morphogenesis to generate individual platelets aided by intravascular shear forces [185, 184, 69, 186]. The blood flow rate and fluid shear in bone marrow sinusoids is less than other areas of the body and is likely to influence platelet formation [181, 184, 187].

5.1.1 Platelet structural, molecular and cellular biology of mice or men—There are significant differences in resting platelet properties between mice and men [188]. The diameter of human platelets is 1.0 to 2.0 μm as compared to those that are smaller in the mouse, 0.5–1.0 μm [188]. Platelet counts are also significantly different ranging from 150 to 400×10^9/L in humans to 1,000–1,500$\times10^9$/L in mice [188]. Similarly, platelet lifespan in human circulation is from 8 to 10 days whereas mouse platelets last for 3–4 days [188]. Likewise, the platelet alpha and dense granule number per platelet is higher in humans compared to mice [188].

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6 Platelet proteomics

Platelets lack nuclei and robust protein synthesis machinery making them model candidates for proteomic analysis. Proteomic studies have revealed various numbers of proteins in resting platelets [189-194]. One study identified 1,507 proteins present in resting platelets 190 of which were membrane proteins and 262 phosphoproteins [192]. This approach has been useful in evaluating alterations in the proteome of platelet diseases [195, 196]. Comparative analyses of platelet-derived microparticles [197-199] produced upon activation also revealed proteome differences in their profile and product levels depending on the platelet stimulus applied [200]. Variations in the proteomic profiles present in activated platelets also vary depending on the treatment [201-203]. Many recent advances can be found in the “Platelet Web” at http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php.

7 Platelet lipidomics and bioactive lipids

The role of bioactive lipids in platelet biology began with the study of prostaglandin endoperoxides [204]. The focus on platelet eicosanoid biology remained an important area of research throughout the next several decades [205-207]. Platelet activation not only stimulates the production of pro-aggregatory prostaglandin TxA_2 but also various 12/15-lipoxygenase (LOX) products that mediate immune-regulatory activities, namely 12-HETE and 15-HETE [208, 209]. In particular the bioactive arachidonic acid metabolite, 12(S)-HETE, produced by platelets was found to contribute to endothelial cell retraction and extravasation [210, 211].

Lipids also contribute to platelet senescence [212], hemostasis, and other disease states [213-216]. Platelet phospholipids, sphingolipids, lysophosphatidic acid, and platelet-activating factor also play an important role in platelet regeneration therapy that involves platelet-rich plasma injections [217, 214, 218-220].

8 Platelet cytoskeleton and morphology

Ultrastructurally, resting platelets are organized into three zones: (1) a peripheral zone includes a fluffy glycocalyx surface coating, platelet membrane, and membrane cytoskeleton; (2) a sol–gel zone consists of a microtubule system, an open canalicular system (OCS), and the dense tubular system (DTS); (3) an organelle zone contains α-granules, dense granules, lysosomal granules, mitochondria, and glycogen granules [72, 73].

9 Dynamic microtubule rings, discoid shapes, and structural collapse

Morphologically, platelet maturation involves a cytoskeletal network of microtubules, actin, and spectrin among other proteins [221, 222, 181, 78, 74]. In resting platelets, a coil of microtubules forms around the platelet margin into a discoid shape that is modulated by RanBP10 [223, 224, 173, 225, 226] (Fig. 2). At any given time in resting platelets, 50 to 60 % of the total platelet heterodimeric tubulin β1, β2, β4, or β5 subunits are in the polymeric state. Time-lapse fluorescence studies of resting platelets either expressing end-binding protein-green fluorescent protein (EB3-GFP) chimeric fusion protein, or that incorporated

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end-binding protein 1 (EB1), which binds to polymerizing microtubule ends, revealed dynamic microtubule remodeling within the marginal band [225]. The resting platelet marginal band consists of multiple coils of stable and dynamically recycled microtubules that polymerizes in a bipolar fashion and shrinks with age or paclitaxel treatment [225]. Once stimulated by thrombin, adhesion, or other stimuli, the marginal band fragments leading to structural collapse of platelet disks. These fragmented microtubules then repolymerize from multiple nucleation centers, possibly of γ-tubulin rather than the centrosomes typical of nucleated cells [225]. This activation process helps initiate the axial growth of microtubule ends that bind EB1 [225]. Microtubules help form the core structure for the dynamic alterations that occur in the platelet cytoskeleton.

10 Resting platelet cytoskeleton

Along with microtubules, membrane composition and cytoplasmic actin scaffolding tightly regulate the structural morphology of platelets [227, 228] (Fig. 2). Resting platelet surfaces lack projections such as microvilli but are etched with plasma membrane pits that form the OCS of platelets [229, 230, 73]. The OCS contributes additional plasma membrane surface area of up to 420 % to platelets as they spread over exposed wounds or damaged endothelium [231]. The membrane cytoskeleton proteins include spectrin, adducin, filamin (FLN), actin, and vWFR. Around 2,000 tetramers of α and β spectrin (fodrin) molecules assemble into a heterodimeric two-dimensional lattice that is regulated by calpain, where each β spectrin free amino terminus can link to 2–5,000 actin filament ends into a space-filling structural latticework [232]. Nearly 8,000 α and β adducin molecules cap actin filament barbed ends and complex with spectrin in the resting platelet [232]. Similar to spectrin, nearly 12,500 filamin A, B, and C molecules complex with the GP1bα chain of the vWF receptor to strengthen the spectrin–actin network interactions with the plasma membrane [233]. Between 12 and 25,000 vWF receptor, molecules interact with GP1ba and bind 14-3-3 zeta protein, which influences signaling through PI3 kinase, adhesion under shear stress, and apoptosis [234-239]. Studies have suggested that the cytoskeleton is important for maintaining asymmetry in the lipid membranes (*i.e.*, separation of cholinephospholipids from the aminophospholipids between the outer and inner leaflets, respectively). However, even when there is limited spectrin, the separation of phosphatidylserine and phosphatidylethanolamine from phosphatidylcholine is maintained until platelet activation (reviewed in [240]). Furthermore, flavonoids that alter membrane fluidity have been shown to impact platelet aggregation [241] , and the basis for platelet hypersensitivity in diabetics has also been demonstrated to be due to membrane fluidity [242] . In diabetic patients, platelets have increased procoagulant activity, aggregate more, show increased Ca^{2+} mobilization, and are insulin resistant [243]. Metformin is known to affect platelet function [244, 245]. It can reduce platelet aggregation and adhesion in addition to reducing hypercoagulability [246]. There are reports of high metformin doses contributing to bleeding in some patients [247] and platelet mitochondrial dysfunction has been observed *in vitro* [248].

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11 Activated platelet cytoskeleton

The activated platelet undergoes extensive cytoskeletal changes depending upon the stimulus and lipid membrane asymmetry is lost on Ca^{2+} influx [227, 240]. Chloride channels, putatively anoctamin 6 (Ano6), have just been implicated in platelet procoagulant activity by affecting the Ca2+-dependent exposure of phosphatidylserine [249]. Once activated, platelet actin cytoskeleton rapidly disassembles within seconds and collapses resulting in less rigid spheroid morphology. Once disassembled, the actin subunits are rapidly reassembled into a variety of new structures such as filopodia and lamellipodia to dramatically generate new platelet shapes depending upon the external forces, extracellular signals, and physiologic requirements. Disengaging the membrane, cytoskeleton occurs as adducin complexes are released from the barbed ends of actin filaments thereby initiating actin latticework disassembly [232, 250, 251]. Adducin complex release is governed by calcium-calmodulin binding, phosphatidylinositides, Rho kinase, or protein kinase C (PKC)-mediated phosphorylation [232, 250, 251]. Signaling pathway involvement depends upon the initiating stimulus [232, 250, 251]. Concurrently, as actin filaments are disengaged, actin remodeling proteins gelsolin (20,000 molecules/platelet) and cofilin (100,000 molecules/platelet) are activated. Gelsolin is activated seconds after thrombin stimulation by increased cytosolic calcium and severs and caps actin filaments [252, 253]. Cofilin activation begins to plateau within 50 s following phosphorylation at serine 3. This leads to the disassembly of actin cytoskeletal filaments [254, 255]. Reassembly of actin units into new structures initiates within seconds by gelsolin inactivation. The barbed ends of actin fragments, provided by gelsolin severing, creates nucleation centers onto which the actin-related protein (Arp) 2/3 complex binds to initiate new filament genesis [256, 257]. The actin reassembly reaction is stopped by CapZ protein binding to the ends of new barbed filaments [258, 259]. These cytoskeletal responses and reorganization occur within seconds of wound exposure recognition. This is to help establish a physical meshwork of platelets and initiate thrombus formation. Speed is essential since this process must occur in dynamic waves to limit blood loss and begin repair under immense cardiovascular pressure.

12 Platelet degranulation

Microtubule and actin cytoskeleton disassembly that accompanies structural collapse is followed by platelet degranulation or release reaction [260, 261, 74] (Fig. 2). Various subsets of granules are released depending on the platelet activation stimulus [150, 200]. Various combinations of secretory mechanism proteins mediate the selectivity of exocytosis and degranulation [150]. These proteins include soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [149, 262, 263]. The two primary subsets of SNARE proteins that distinguish platelet granules for secretion include synaptosomal-associated protein 23 (SNAP-23) that mediates membrane fusion, and vesicle-associated membrane protein (VAMP) 3, 7, and 8 that decorate subsets of granules [150, 264, 147]. A SNARE chaperone protein known as mammalian uncoordinated-13-4 (Munc13-4) mediates docking between granule and plasma membranes. Docking also involves interactions with Ras-related protein, Rab27a along with synaptotagmin-like protein 4-a/granuphilin-a (Slp4-a) [265, 266]. Syntaxin-11 remains inactive by Munc18b sequestering [267, 268]. Platelet activation causes a conformation change in Munc18b/syntaxin-11 complexes to form a four-

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helical protein bundle with SNAP-23 and VAMP during granule recruitment to platelet membranes and release. Granule release can occur through OCS membranes. Among other methods, time-lapse microscopy of spreading platelets revealed that VAMP-3 and VAMP-8-decorated granules localize to a centralized granulomere [264]. A collapsed microtubule ring surrounds the granulomere prior to granule release. In contrast, granules decorated with VAMP-7 localize at the platelet periphery followed by release [264]. These data illustrate differences in the granule population decoration that separate their release behavior.

13 Platelet granules

Platelet degranulation releases three major forms of storage granule products. (A) Alpha-granules are most numerous at 50–80 per human platelet and take up 10 % of the platelet volume [78] (Fig. 2). They also contain the greatest abundance of factors including the following: (1) adhesion molecules—vWF, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, P—selectin, thrombospondin, fibrinogen, and fibronectin; (2) coagulation factors—prothrombin, fibrinogen, factor V, and factor VIII; (3) fibrinolytic factors—α2 macroglobulin, plasminogen, PAI-1, SERPINE1, and urokinase plasminogen activator (uPA); (4) growth factors—PDGF, BFGF, EGF, HGF, IGF1, TGFβ, VEGF-A, and VEGF-C; (5) pro-angiogenic and anti-angiogenic factors—angiopoietin-1, angiostatin, and sphingosine-1-phosphate (S1P); (6) tissue remodeling matrix metalloproteinases—MMP-1, MMP-2, MMP-3, MMP-9, MT1-MMP (MMP-14), their tissue inhibitor of metalloproteinases (TIMPs; TIMP-1, TIMP-2, and TIMP-4), and a disintegrin and metalloproteinases (ADAMs; ADAM-10, ADAM-17, and ADAMTS-13) [269]. (7) Platelet α-granules contain a wide range of proinflammatory mediators such as C-X-C motif chemokines, including CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α) [270]. Among these, CXCL4 and CXCL7 are the most abundant [271, 272]. Platelet α-granule proinflammatory factors also include chemokine C-C motif ligands CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES), CCL7 (MCP-3), and CCL17 (TARC) along with IL1-β, platelet-activating factor (PAF), acetylhydrolase, and lysophosphatidic acid (LPA) [149]; (8) immunologic molecules—C1 inhibitor and IgG; and (9) other proteins—albumin, α1-antitrypsin, growth arrest-specific 6 (Gas6), high-molecular-weight kininogen (HMWK) [273, 150]. (B) Dense granules by contrast are fewer in number at three to eight per human platelet and primarily contain small molecules and far fewer factors including the following: (1) ions—calcium, magnesium, phosphate, and pyrophosphate; (2) nucleotides—adenosine triphosphate (ATP), guanosine-5′-triphosphate (GTP), adenosine diphosphate (ADP), and guanosine diphosphate (GDP); (3) membrane proteins—tetraspanins and LAMP2; (4) transmitters—5-HT, epinephrine, and histamine; and (5) protease inhibitors—tissue factor pathway inhibitor (TFPi) [273, 150]. Both α-granule and dense granule release help to amplify secondary platelet responses and initiate wound repair. (C) Lysosome function in platelet biology and hemostasis is not well characterized. They release phospholipase A, protease, and glycohydrolase enzymes that may suggest a role in platelet responses and dissolution of clots [274-277]. As first responders, successive waves of platelet activation, adhesion, aggregation, and stabilization activate specific subsets of cytoskeletal changes depending on the stimulus and physiologic need to recognize, initiate, and secure vascular lesions.

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14 Nitric oxide synthase

Endothelial cells express nitric oxide synthase (eNOS), which produces nitric oxide (NO) and maintains platelets in a resting state. Once produced, NO acts on NO-sensitive guanylyl cyclase (sGC) to produce cyclic GMP (cGMP) [278, 279]. cGMP then activates phosphodiesterase (PDE)2A and PDE5A, causing the degradation of cyclic AMP (cAMP) and cGMP, in contrast to inhibiting PDE3A. The phosphorylation of PDE5 also involves cGMP-dependent protein kinase (protein kinase G [PKG1]), which further activates the enzyme [280]. The net effect of cyclic nucleotide production, nitric oxide, and protein kinase activity is to maintain platelets in a resting state, which is in contrast to G protein-coupled receptor (GPCR)-mediated platelet activation.

15 Platelet G protein-coupled receptors

The activation signals that initiate Rho GTPase pathways among other biological responses by platelets involve multiple receptors and ligands (Fig. 3). These molecules fall into at least seven different families of receptors including GPCR, leucine repeat receptors (LRR), immunoglobulin (Ig) superfamily, integrins, C-type lectin receptors, tyrosine kinase (TK) receptors, and glycoproteins. These receptors act in concert to strike a balance between the initiation *versus* suppression of activation, aggregation, adhesion, amplification, coagulation, stabilization, and recruitment of platelets while executing their biological responses.

Platelet GPCRs initiate key biological responses [281-283]. This receptor family consists of seven-transmembrane domain receptors [281-283]. Small GTPase proteins are bound to the cytoplasmic tails of inactive GPCR including (1) $G_{q\ alpha}$ or $G_{q/11}$, which activates various phospholipase C isoforms such as, $PLC_{\gamma 2}$ or PLC_{β}, which in turn catalyzes production of second messengers diacyl glycerol (DAG)/inositol triphosphate (IP3) followed by stimulation of PKC, and, finally, increases intracellular calcium [284, 285]; (2) $G\alpha_{12/13\ alpha}$ activates RhoA family cytoskeletal remodeling proteins [286]; (3) $G_{\alpha i\ alpha}$ inhibits the production of cAMP from ATP [287]; or (4) $G_{s\ alpha}$ stimulates the cAMP pathway by activating protein kinase A (PKA) [287, 288]. Both G_{α} and G_{γ} protein subunits are post-translationally modified by the N-terminal covalent attachment of lipids, including being myristolated, palmitoylated, or prenylated, which enables them to anchor to the inner leaflet of the plasma membrane [289]. In resting platelets, GDP is bound to G_{α} subunit maintaining it in the inactive conformation. After ligand binding, GPCRs become activated through conformational changes [281-283]. This causes the disassociation of the G_{α}-subunit from its heterotrimeric G_{β}, G_{γ} regulatory G protein complex [290, 291]. The G_{α}-subunit is then free to interact with a variety of guanine exchange factor (GEF) proteins that exchange GTP for GDP on the target. After downstream activation of a specific pathway, the target proteins interact with GTPase-activating proteins (GAP), which initiates hydrolysis of GTP to GDP and entry into a new signaling cycle. Platelet GPCRs include: (1) thrombin receptors—PAR1, PAR2, PAR3 (mouse), and PAR4; (2) ADP nucleotide receptors—$P2Y_1$ and $P2Y_{12}$; (3) prostaglandin receptors (PG)—thromboxane A_2 (TxA_2/prostanoid thromboxane receptor (TP)), prostacyclin (prostaglandin I_2 (PGI_2)/IP), and prostaglandin E_2 (PGE_2/EP_1, EP_3, and EP_4); (4) lipid receptors—PAF and, lysophospholipid receptor (LPL-R); (5) chemokine receptors—C-X-C CXCR-4, CC chemokine receptors, CCR1, CCR3, and CCR4; (6) other

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receptors—vasopressin receptor (V_{1a}), adenosine receptor (A_{2a}), β2 adrenergic receptor (epinephrine, norepinephrine, and dopamine receptor), and serotonin (5-hydroxytryptamin, 5-HT2A) receptor [292, 293]. Specificity of downstream signaling depends on which combinatorial association of the G_α, G_β, or G_γ heterotrimeric regulatory G protein complexes occurs with a given GPCR. This process is context-specific and typically involves crosstalk with other receptors and signaling pathways to elicit a specific platelet response. This is particularly true regarding the context and extent of platelet adhesion receptor engagement with a variety of receptors for extracellular matrix, other platelets, or specific cells. As a potential example, it is known that serotonin is elevated in breast cancer and that serotonin modulates breast involution through tight junction disruption, which becomes deranged in breast cancer [294]. Furthermore, exogenous addition of arachidonic acid (AA) can disrupt tight junctions [295]. In other systems, there is precedent for PLA2 stimulation and AA release after 5HTA or 5HT2A activation [296, 297]. Therefore, the potential exists for a continuous feedback loop where serotonin from breast cancer cells may activate platelets through 5HTA and this in turn may promote platelet release of AA and other metabolites that further disrupt tight junctions. These reactions then may influence angiogenesis as both serotonin and thromboxane have been shown to induce the shedding of platelet exosomes that induce smooth muscle proliferation [298].

16 Resting platelet G protein-coupled receptors

Platelets exhibit numerous GPCR-mediated checkpoints that maintain the resting state and involve cyclic nucleotide production and protein kinase activity (Fig. 3a). The most prominent of these is the prostacyclin receptor (IP receptor), which binds prostacyclin or PGI_2 [299]. PGI2 is a metabolic derivative of the 20-carbon essential fatty acid AA. AA is converted primarily by the cyclooxygenase 2 (COX-2) to prostaglandin G_2 and then H_2 followed by conversion to prostacyclin by PGI_2 synthase (PTGIS) in vascular endothelial cells [300]. PGI_2 is one of the most potent inhibitors of platelet aggregation, a potent vasodilator, and moderator of anti-atherogenic control of vascular smooth muscle cell proliferation–migration–differentiation [301, 302]. IP receptors in resting platelets act through Gαs by stimulating adenylate cyclase (AC) to synthesize cAMP from ATP. Platelets express a number of different membrane-bound AC isoforms, including AC3, AC6, and AC7 [303, 304]. Provided that cAMP is not broken down by PDE, it activates either PKA [305, 288, 306] or exchange proteins activated by cAMP (Epac) [307, 308]. Platelet PKA exists as a tetramer of two regulatory subunits bound to catalytic subunits, RIα/β-C or RIIβ-C, that are inactive in resting platelets. The tetramer disassociates after cAMP binds to the regulatory subunits releasing the kinase to catalyze target protein phosphorylation. PKA phosphorylates Ras-related protein 1B (Rap1B) [309] among other proteins such as Rap1GAP2, TRPC6, glycoprotein Ib (GPIb)β, IP_3RI-IRAG, and $G\alpha_{13}$ to inhibit G-protein activity and Ca^{2+} release [288]. PKA also phosphorylates inhibitors of protein phosphatases PP1 and PP2A. This prevents the dephosphorylation of PKA and non-PKA targets. In addition, Epac proteins replace GTP for GDP in Rap to modulate kinase signaling.

Platelets exhibit numerous GPCR-mediated checkpoints that maintain the resting state and involve cyclic nucleotide production, nitric oxide, and protein kinase activity [310]. Resting platelets maintain basal levels of cAMP through AC [311, 312].

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17 Activating G protein-coupled receptors

Many robust GPCR-mediated responses were first described by studying platelet agonists (Fig. 3b). The ADP response was the first studied in platelets [313]. Thrombin stimulation was also described early in platelets [314, 305]. The discovery of prostaglandin H_2 (PGH_2) [315] and TxA_2 responses was also initially described in platelets [316]. We now know that ADP induces $P2Y_1$, and thromboxane A_2 stimulates TP, whereas thrombin acts through PAR1 or PAR4 [317]. In the case of thrombin activation, one of the primary initiators is tissue factor (TF), particularly in the case of tumor cells [318, 319, 317]. These platelet GPCRs, bind alpha regulatory subunits, $G_{q\ alpha}$ or $G_{q/11}$. Upon the downstream activation of $PLC_{\gamma 2}$, or PLC_{β}, the production of second messengers DAG/IP_3 stimulates PKC along with an increase in intracellular calcium. These events help the subsequent activation of Rap1 [309, 320]. This occurs through the combined activity of diacylglycerol-regulated guanine nucleotide exchange factor 1 (CalDAG-GEF1) together with Rap1-interacting adapter molecule (RIAM), a Rap1GTP adaptor protein [309, 320-323].

ADP can induce $P2Y_{12}$ activation, which acts through inhibitory $G_{\alpha i}$ subunits. $G_{\alpha i}$ suppresses the generation of cAMP by AC in the plasma membrane. In some instances, $G_{\alpha i}$-mediated inactivation of AC can block activation by $G_{\alpha s}$-dependent GPCRs. The resting state of platelets is also influenced by PKA and PKG activation states, as well as by CalDAG-GEF 1 and Rap 1GTP adaptor protein status, and free Ca^{2+} levels [324, 320, 325].

18 Rho-induced morphological changes

Different morphological changes in the platelet cytoskeleton and degranulation predominantly involve specific Rho family GTPases [290, 291, 326-328] (Fig. 3b). Following activation, platelet RhoA influences actin contractility and shape change along with thrombus generation [329-331]. RhoA acts on multiple cytoskeletal proteins. In its GTP-bound form, $G\alpha_{13}$ activates p115RhoGEF, thereby activating RhoA through its $G\alpha_{13}$ switch region 1 (SRI) [332, 333]. This switch region of $G\alpha_{13}$ interacts with p115RhoGEF activating Rho and then Rho-associated protein kinase (ROCK) [332, 333]. Once active, ROCK phosphorylates and inactivates the myosin light chain (MLC) phosphatase [334-336]. This increases the net MLC phosphorylation at serine 19 by myosin light chain kinase (MLCK) [337-339]. ROCK activation can also stimulate LIM-kinase (LIMK) to phosphorylate cofilin thereby regulating actin polymerization [340-342]. The end result of RhoA activation by GPCRs is cytoskeletal reorganization.

19 Cdc42-induced filopodia

Another Rho family protein, Cdc42 along with WASP and neuronal N-WASP induces Arp2/3-mediated actin branching to form filopodia and granule secretion [343-346]. In the absence of pre-existing actin filaments, IRSp53 initiates this process *via* its I-BAR domain by bending the membrane outward and binding Cdc42 along with its target proteins. These Cdc42 targets include a formin protein, mDia2, along with WASP/N-WASP. The resulting complexes stimulate actin polymerization. Filopodia also form from pre-existing lamellipodial actin filaments by clustering myosin X along WASP/Arp2/3-nucleation sites. The extension of filopodia follows by the addition of actin monomers (G-actin) onto

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polymerizing actin filaments (F-actin). As the filopodial process matures, vasodilator-stimulated phosphoprotein (VASP), myosin X, and mDia2 are localized to the tip. This occurs along with the dynamic movement of myosin X to facilitate delivery of proteins to the growing tip. The polymerization of actin occurs at mDia2 nucleation sites in cooperation with actin monomer delivery by VASP. Profilin binding presents actin monomers directly to mDia2. Process formation is catalyzed by Cdc42 and Rif stimulation of mDia2-facilitated actin polymerization. Cdc42 also initiates WASP/Arp2/3-driven polymerization. Platelet filopodia enhance interactions between other circulating platelets or cells, fibrin networks, and extracellular matrix proteins.

20 Raci-induced lamellipodia

Platelet spreading and lamellipodia formation are initiated by Rac1 [347, 346, 348, 320]. The physiologic demand for platelet coverage of exposed extracellular matrix among other triggers drives Rac1-mediated responses. These responses generally begin with cofilin-mediated severing of actin filaments, exposing barbed ends. Exposed filament barbs serve as nucleation sites for Arp2/3 binding and the formation of new filaments. During the extension process, Arp2/3 nucleation sites generate branches within the actin filament network. Rac1 GTPases then activate Arp2/3 along with WASP-family verprolin-homologous (WAVE) complexes at the membrane followed by formin-mediated actin complex extension. G-actin monomers are presented to formin by profilin during filament polymerization. The anticapping protein, VASP, also contributes to actin filament extension. In the absence Arp2/3 complexes, formin helps generate unbranched filaments. The respective events triggering RhoA, Cdc42, or Rac1 activity involves crosstalk with integrin, GPCR, LRR, or Ig receptors to initiate the specific platelet actin cytoskeletal morphological changes necessary to support either adhesion, aggregation, secretion, or thrombus formation.

21 Platelet extracellular matrix receptors

Platelets encounter various subendothelial proteins following vascular injury (Fig. 4). These factors include proteoglycans, laminin, fibronectin, and vitronectin along with various isoforms of collagen. vWF binds to exposed collagen I, III, and VI fibers. Additional vWF is recruited into the matrix network to form tethering fiber strands [349-353]. Platelet surface multimeric GPIb complexes form catch bond interactions with exposed tethering fiber strands [349-352, 354, 353]. The initiation of catch–slip tethering causes platelets to begin rolling behavior under the fluid shear stress caused by circulating blood [349-353]. Functional GPIb receptor complexes consist of four transmembrane proteins that include, from the outside in to the center, two 20-kDa GPIX subunits, two 26-kDa GPIbβ subunits, two 135-kDa GPIbα subunits, and one central 82-kDa GPV subunit [355, 354]. The GPIb receptor subunits belong to the LRR receptor family of proteins and are almost exclusively expressed in platelets [355, 354]. The loss of GPIb expression leads to an inherited bleeding disorder known as Bernard–Soulier syndrome [356]. Glycosphingolipid-enriched microdomains (GEM) support clustering of GPIb-IX-V multimeric complexes that interact with vWF multimers [357]. Interactions with vWF along with filamin and cytoskeletal proteins trigger multimeric GPIb-IX-V complex formation within GEM regions [357]. Outside-in signal transduction occurs through the interaction between cytoplasmic tails and

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calmodulin, filamin, 14-3-3ζ, PI3 kinase, actin-binding protein (ABP), and F-actin filaments [358, 234, 359, 238]. Src/Lyn family kinase activation by receptor protein-tyrosine phosphatase (RPTP), results in downstream signaling through spleen tyrosine kinase (Syk). Independent activation of Fc receptors FcγRIIA and FcR γ-chain stimulate Syk activity. Signals that converge on Syk can phosphorylate and activate p38 mitogen-activated protein kinase (p38MAPK). p38MAPK in turn can activate cytosolic phospholipase $A\gamma_2$ ($cPLA\gamma_2$) and stimulate TxA_2 synthesis [358, 234, 359, 238]. TxA_2 or Syk can directly activate phospholipase Cγ2 (PLCγ2). IP_3/DAG initiates the release of Ca^{2+} or PKC activation [358, 234, 359, 238]. Ca^{2+} release along with PKC stimulation, in turn, initiates crosstalk that causes inside-out activation of integrins to stabilize interactions with the exposed vessel wall. Tethering can also occur with ultralarge von Willebrand factor (ULVWF) and endothelial cells to attract platelets to these protein strands along with other cells such as leukocytes and potentially tumor cells [360-362].

22 Platelet integrin receptors, inside-out and outside-in signals

Integrin-mediated inside-out and outside-in signaling and/or crosstalk plays an essential role in the biologic responses of platelets (Fig. 3b). Platelets encounter numerous circulating ligands, cells, pathogens, and extracellular matrix ligands in the bloodstream depending upon context. In order to immediately respond, platelets express a wide variety of integrin receptors that engage context-specific ligands. Functional integrin receptors exist as heterodimers consisting of transmembrane glycoprotein α and β subunits. Resting platelets express low affinity or closed conformation integrins containing ligand-binding sites that are unexposed in a bent over conformation. Following activation, the α and β subunits project upright and shift to a high affinity or open state that efficiently binds ligands. Once rolling behavior is initiated by multimeric GPIb–complex interactions with vWF, crosstalk through release of Ca^{2+} or PKC activates a key stabilization integrin $\alpha_{IIb}\beta_3$ [363-366]. This inside-out activation process shifts $\alpha_{IIb}\beta_3$ integrins from a closed to an open state. In the open state, transmembrane 148-kDa subunit α_{IIb} and 95-kDa subunit β_3 heterodimers are very promiscuous and bind multiple RGD-containing ligands [363-366]. The list of RGD ligands includes fibrin, fibrinogen, fibronectin, vitronectin, thrombospondin, or vWF complexes. Active-state $\alpha_{IIb}\beta_3$ integrin complexes can subsequently recruit additional platelets, initiate platelet aggregation, stabilize adhesion to extracellular matrix, and promote thrombus formation. Abnormal $\alpha_{IIb}\beta_3$ integrin receptor expression is involved in Glanzmann's thrombasthenia [367, 368]. This bleeding disorder was first described in 1918 by the Swiss pediatrician, Eduard Glanzmann [369]. Patients with this disorder exhibit hemorrhagic symptoms and "weak platelets" [369]. In addition to the promiscuous $\alpha_{IIb}\beta_3$, an array of more selective ligand binding integrins also stabilizes interactions with the vascular microenvironment. For example, the collagen receptor $\alpha_2\beta_1$ is a key matrix-stabilizing integrin. The $\alpha_2\beta_1$ heterodimer complex consists of a 150-kDa α_2 chain and a 130-kDa β1 chain. Once activated, $\alpha_2\beta_1$ stabilizes direct adhesive contacts to collagen that prompts lamellipodia formation and platelet spreading. In contrast, when platelets encounter circulating or extracellular matrix fibronectin they engage $\alpha_5\beta_1$ integrin heterodimers. The $\alpha_5\beta_1$ integrin response depends on context and the platelet activation state. The α_5 (125-kDa subunit) and β_1 (130-kDa subunit) heterodimer integrin complex binds more selectively to

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fibronectin RGD peptide sites in static conditions. Activation of $\alpha_5\beta_1$ promotes platelet tyrosine phosphorylation, changes in calcium levels, and/or lamellipodia formation. Platelets also express $\alpha_6\beta_1$ integrin receptors that selectively bind to laminin in the basement membrane. The laminin-binding 120-kDa α_6 subunit combines with β_1 subunits as an integrin complex. Adherence to laminin triggers PI3K and Cdc42 signaling pathways, which induce filopodia formation. The activation of $\alpha_6\beta_1$ integrin receptors typically involves crosstalk with platelet collagen receptor glycoprotein VI (GPVI) to achieve stable adhesion.

23 Immunoglobulin receptor superfamily

A key platelet transmembrane GPVI belongs to the Ig receptor superfamily [370, 371] (Fig. 4). This 63-kDa glycoprotein is found exclusively in platelets and has two Ig extracellular domains that bind collagen. As a major collagen receptor, it recognizes glycine-proline-hydroxyproline-10-repeat sequences and the quaternary structure of collagen, which contributes to platelet-activating functions [372, 373]. Platelet recognition and activation involves GPVI monomers that shift from low affinity and signal transduction potential to multimers that gain high affinity and signal initiation potential for the periodic structure of collagen [357, 374]. GPVI associates with FcRγ homodimer adapter proteins containing an immunoreceptor tyrosine-based activation motif (ITAM) consisting of tandem YxxL amino acid motifs that are phosphorylated by Fyn and Lyn resulting in platelet activation [375-377]. Syk, through its SH2 domains, interacts with tyrosine-phosphorylated FcRγ. The downstream signaling pathway involves scaffold proteins linker for activation of T cells (LAT), and SH2-containing leukocyte protein of 76-kDa (SLP76), which organize signaling molecules into multiprotein complexes [357, 374]. These signaling complexes recruit Vav1/3, Bruton's tyrosine kinase (BTk), PI3 kinase α/β, and other signaling molecules culminating in activation of PLCγ-2. PLCγ-2 in turn elevates intraplatelet Ca^{2+} levels and calcium and diacylglycerol regulated guanine exchange factor I (CalDAG-GEFI) activation [357, 374]. PLCγ-2 can also initiate DAG-mediated activation of PKC and signaling through the purinergic receptor P2Y12 [357, 374]. Both PLCγ-2, kinetically distinct, pathways converge on the small GTPase Rap1 that can initiate TxA_2 synthesis, integrin activation, and granule secretion.

24 Platelet C-type lectin receptors

Platelet C-type LECtin-like receptor (CLEC-2/Aggrus) is another platelet receptor in addition to GPVI and GPIb-IX-V that initiates platelet activation through ITAM signaling and molecular multimerization [375, 357, 374] (Fig. 4). CLEC-2 binds to mucin glycoprotein podoplanin [378-380]. Podoplanin is expressed on cells of the lymphatic endothelia, type I lung epithelia, choroid plexus epithelia, kidney podocytes, lymph node stroma, along with cancer-associated fibroblasts (CAF) and tumor cells where it is found at the leading edge and is thought to potentiate migration and invasion [381, 378, 382, 383, 379, 380, 384]. Podoplanin is a heavily *O*-glycosylated type-1 transmembrane sialomucin glycoprotein [378, 357, 385, 386]. Multiple podoplanin molecules may interact with platelet CLEC-2 receptors triggering signal transduction [357, 387]. Two molecules of CLEC-2 are also known to bind to tetramers of the snake venom peptide rhodocytin to form a multimeric

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complex that stimulates signal transduction [388, 386, 389]. Multimerization of CLEC-2 activates the same ITAM-Syk-PLCγ-2 signaling cascade as GPVI [375, 357, 374].

25 Platelet P-selectin receptors

Platelet P-selectin was first described as membrane glycoprotein GMP-140 [390, 391] (Fig. 4). P-selectin/GMP-140 contains a “lectin” region, an “EGF” domain, nine complement binding protein-like tandem repeats, a transmembrane domain, and a short cytoplasmic tail. P-selectin in turn binds to P-selectin glycoprotein ligand (PSGL)-1 [392], neutrophil leukocyte-endothelial cell adhesion molecule 1 (LECAM-1) [393], endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) [394], and sialyl Lewis(x) oligosaccharide [395]. P-selectin interaction with a variety of immune cells and endothelial cells is important in mediating disease states such as inflammation, autoimmunity, pre-eclampsia, and wound healing [396-398]. The proinflammatory lipid metabolite, 12(S)-HETE, elevates the expression of platelet P-selectin [399]. Significant evidence supports the notion that platelet P-selectin also mediates tumor metastasis and thrombotic complications in cancer patients such as exemplified by Trousseau’s syndrome [400-403]. P-selectins along with Land E-selectins may initiate tethering and rolling of cells flowing past the luminal surfaces during the initial phases of intravascular adhesive interactions that are stabilized by other receptors [400, 401, 404-406, 402, 403].

26 Platelet activation, thrombus formation, and vascular shear

Thrombus formation involves multiple “first responder” platelet-mediated steps including rolling, adherence, spreading, migration, aggregation, and stabilization (Fig. 5). Fluid shear along with the discoid shape and the small size of platelets causes them to collect and contact the luminal walls of blood vessels. As they contact, normal intact endothelial cells that cover the luminal surfaces, various factors such as soluble nitric oxide, PGI_2, and ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1/CD39)-mediated reduction of local purine nucleotides help maintain the resting state of circulating platelets [278, 407, 302, 279, 408]. Once injury-induced exposure of subendothelial matrix occurs, collagen fibrils cooperate in forming transient tethering bonds between matrix-bound vWF and platelet GPIb-IX-V receptor complexes [349-352, 354, 353]. These tethers are formed and broken to support rolling and slow platelet movement. Once movement slows, more intimate interactions between collagen fibers and platelet GPVI surface receptors triggers inside-out activation that shifts integrins from a closed to an open state [370, 371]. Activated $\alpha_2\beta_1$ interactions with collagen and $\alpha_{IIb}\beta_3$ binding to fibrinogen initiate outside-in signaling that prompts platelet shape change and degranulation [363-366]. Degranulation releases α-granules and dense granules, which amplifies secondary platelet responses and initiates tissue repair. Platelets migrate over the subendothelial matrix isolating the exposed surfaces from the flowing blood, or into vascular beds, and initiate tissue repair [409-413]. A blanket of platelets begins to accumulate, but may form and release microemboli as reactive and protective surfaces simultaneously build up. Activated platelets serve as nucleation sites as build up proceeds. Nucleation is amplified through the release of prothrombotic granule contents and tissue factor-initiated coagulation [414]. The synthesis and release of TxA_2, thrombin (FIIa), ADP, among other factors, recruits and activates additional platelets. This

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extends the platelet blanket. Platelets interact with adjacent platelets through $\alpha_{IIb}\beta_3$ receptors by binding fibrinogen and fibrin fibers [363-366]. Successive cycles of platelet activation, adherence, spreading, migration, aggregation, and stabilization form a thrombus that becomes enmeshed within a growing fibrin network and entraps other blood cells. Thrombotic plug formation is stabilized and counterbalanced by the disaggregation of platelets and fibrinolysis. Recruitment of fibroblasts and immune cells through platelet-initiated angiogenic repair mechanisms stimulates wound healing and resolution [410]. Exposure of subendothelial matrix along with the presentation of atherosclerotic plaques, foreign bodies, abnormal angiogenesis, or pathologic cells such as tumor cells in the circulation serve as robust triggers of platelet activation.

27 The cancer challenge to hemostasis

Cancer can exert significant challenges to all of the molecular, cellular, and biologic properties thus far described for platelets as “first responders”. Primary tumors produce many byproducts that stimulate angiogenesis and can enter the circulation (Fig. 6). Platelets can recognize and respond to circulating tumor by-products and initiate the coagulation cascade in the absence of intact cells. The system is challenged further as extracellular matrix is exposed during tumor cell invasion or as circulating cancer cells interact directly with platelets. As platelets fulfill their normal hemostatic function in the presence of cancer, they tend to initiate thrombotic events that can facilitate cancer progression.

28 Tumor by-products and thrombogenesis

The two major conduits for systemically distributing throughout the body the by-products and cells originating from tumors are the lymphatic and cardiovascular systems [415-417] (Fig. 6). These two vascular systems are interconnected through anastomoses at high endothelial venules (HEVs) in lymph nodes along with the larger collecting vessels of the thoracic duct and the jugular vein [415-417]. Tumor formation typically induces neoangiogenesis in both vascular systems that generally form abnormal vessels, which are leaky [418, 419, 406, 420]. Leaky vessels, in turn, facilitate the entry of tumor by-products into the cardiovascular circulation that can initiate venous thromboembolus (VTE) formation linked to Trousseau’s syndrome [317]. Tumors produce factors that activate the coagulation cascade to produce thrombin and activate PAR receptors on both platelets and tumor cells. Cancer procoagulants include thrombin produced by cancer cell-stimulated host cells, tumor tissue damage, inflammatory factors, tumor necrosis, mucin production, surgical trauma, chemotherapy-induced toxicity, and foreign bodies. Also, tissue factor on cancer cell surfaces binds to coagulation factor VIIa (FVIIa) and coagulation factor X (FX), activating the clotting pathway [406, 421, 317]. Once platelets and the clotting cascade are activated, thrombin serine protease activity alters fibrinogen to form a fibrin meshwork [406, 421, 317].

29 Thrombocytosis

Cancer-induced thrombocytosis significantly increases the number of circulating platelets [422, 1, 46]. This constitutes an important link to Trousseau’s syndrome, but the mechanisms responsible remained poorly understood until recently [423](Fig. 6). A

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multicenter study involving 619 patients with epithelial ovarian cancer was conducted to test associations between platelet counts and disease outcome [423]. Thrombocytosis along with elevated plasma levels of thrombopoietin and interleukin-6 (IL-6) is associated with advanced disease and shortened survival in ovarian cancer patients [423]. In accompanying orthotopic mouse models of ovarian cancer, tumor-derived IL-6 stimulated hepatic thrombopoietin synthesis and paraneoplastic induction of thrombocytosis [423]. These studies helped establish an important paracrine link between cancer and thrombocytosis. It has also been reported that there is an increase in the number of CD63-positive platelet microparticles in the circulation of ovarian cancer patients compared to those with benign tumors [424]. However, while these did reflect a procoagulant phenotype [425, 426], they did not, in and of themselves, discriminate benign from malignant tumors. Interestingly, the same group reported that menopause/climacteric was associated with fewer circulating platelet exosomes/microparticles [427]. Healthy individuals reportedly have a microvesicle concentration of 5–50 μg/ml [428].

30 Circulating tumor cells

The initial steps of hematogenous metastasis formation typically involve invasion of premalignant/cancer cells through the basement membrane and entry into blood vessels [429, 430]. The detection of circulating tumor cells (CTCs) in patient blood samples is associated with disease burden, metastatic relapse, and/or reduced survival in a variety of cancers [431] . Once in circulation, CTCs may encounter platelet-derived microparticles that harbor microRNA [428]. Likewise, platelets may encounter exosomes, or “oncosomes” [432] , derived from CTCs that harbor bioactive lipids such as 12(S)-HETE [433], which may serve to activate the platelets and begin the “ships passing in the night” cell-to-cell exchange as a result of extracellular vesicles [434] that leads to metastasis. The clinical outcome of these encounters remains to be determined, and offers a wealth of possibilities for therapeutic intervention.

Recent technological advances have enabled the isolation of CTCs from patients’ blood samples [435]. When coupled to new sequencing methods, these approaches can help better understand the metastatic process [436]. Understanding and tracing the lineage of cells that successfully enter the blood stream will help refine our ability to better target metastasis.

31 Tumor cell-induced platelet aggregation

CTCs activate and aggregate platelets, which correlate with their metastatic potential [48, 437, 1, 438] (Fig. 5). The heterotypic emboli that form following TCIPA enhance metastatic cell survival and dissemination. When CTCs become enveloped with platelets, they can evade the immune system as well as TNF-α-mediated cytotoxicity. Platelet coatings also shield CTCs from high fluid shear forces at the vascular wall. As heterotypic CTC emboli grow, entrapment in the microvasculature enhances their arrest rate. This process is facilitated by cell surface receptors on endothelial cells and a variety of immune cells. Adhesion of tumor emboli to the vascular endothelium as well as immune cells stimulates cytokine and growth factor production among other cellular responses.

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32 Eicosanoids and tumor cell-induced platelet aggregation

Similar to wound site recognition, TCIPA triggers the production of TxA_2 and platelet activation [439, 60, 440] (Fig. 5). Paclitaxel exerts an antiplatelet effect by interfering with TxA_2 synthase [441]. In TCIPA, MRP4 moves from the dense granules to the platelet plasma membrane and transports TxA_2 outside platelets [157, 158] (Fig. 3b). The release of TxA_2 into the microenvironment activates TP receptors on platelets [442, 439, 60, 440, 443, 444]. Activation by Walker 256 carcinosarcoma cells, for example, induces arachidonic acid release followed by 12-lipoxygenase-mediated-conver-sion to 12(S)-HETE that further stimulates TCIPA [207]. TCIPA is inhibited by PGI_2-mediated stimulation of IP receptors [445, 61, 440, 63]. PGI_2-mediated inhibition of TCIPA is more effective than other classes of PGs including PGE_1 or PGD_2 as well as the PGI2 metabolite 6-keto-$PGF_{1\alpha}$ [63]. In contrast, the proinflammatory/prooncogenic PGE_2 and its enzymatic metabolite 13,14-dihydro-15-ketoPGE_2 prevent PGI_2 inhibition of TCIPA [63, 446]. PGI_2-mediated signaling is also strong enough to arrest and reverse partial TCIPA. This inhibition serves as a potent antimetastatic agent [60, 445, 61, 440, 63]. The balance of eicosanoid metabolism in the vascular system is critical to the formation of tumor cell–platelet emboli and can be influenced by targeted therapy [447].

33 Tumor proteases

A variety of protease molecules such as thrombin are produced directly by tumor cells and stimulate PARs that initiate TCIPA [448-450] (Figs. 3b, 5, and 6). Thrombosis can also be triggered by neo-adjuvant and chemotherapy [451-453]. Other factors such as tissue factor act indirectly through thrombin generation, but tumor cells can also produce cancer procoagulant (CP) protein, which also stimulates TCIPA [454-456]. Another enzyme, cathepsin B is a cysteine protease that can be induced by 12(S)-HETE, and it also induces TCIPA when released from tumor cells [64]. MMPs produced by tumor cells can also act on platelet surface receptors and trigger activation [457-459]. In experiments using HT-1080 and MCF-7 cells, MMP-2 was released from both tumor cells and platelets that enhanced TCIPA [460, 459]. Further evaluation revealed that TCIPA involved proMMP-2 conversion to MMP-2 followed by MMP-14 activation [460]. Platelet and tumor activity is influenced by reciprocal feedback. For example, platelet granule products as well as 12(S)-HETE synthesized by platelets stimulate MMP-9 production by tumor cells and increase invasion [461, 462]. The regulation of MMP activity is modulated further by TIMPs that are produced by both tumor cells and released by platelets [463, 464]. Additional regulation of protease activity involves PAF [465, 466].

34 Tumor cell adhesion receptors

Tumor cell adhesion receptors play a vital role during the initiation and stabilization of plasma membrane interactions with platelets (Fig. 4). Similar to the initial platelet interactions with wounded blood vessels, GPIb-IX-V on both platelet and MCF7 tumor cell surfaces are among the first receptors engaged and can be upregulated on the tumor cell surfaces [460, 467] . Whether tethering *via* plasma vWF is involved during GPIb-IX-V-mediated platelet and MCF7 interactions remains unclear. A role for vWF in tethering is

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likely since the purified protein supports TCIPA induced by HT-1080 cells [468], which is further supported by TCIPA inhibition using anti-vWF antibody treatment [469, 467]. Also analogous to wounding, interactions between $\alpha_{IIb}\beta_3$ integrins on both platelet and tumor cell surfaces can help stabilize adhesive interactions [470, 75, 1, 469, 467]. Furthermore, RGD-containing proteins may act as a linker between the two plasma membranes [471-473]. Unlike collagen-platelet interactions, those with $\alpha_{IIb}\beta_3$ integrin may occur in the absence of platelet GPVI receptor engagement unless collagen fragments are present. Other integrin receptors are also likely to be involved in these heterotypic membrane interactions. The $\alpha_v\beta_3$ vitronectin receptor, for example, is very abundant on many cancer cell surfaces, but lower on platelets [474]. In the presence of plasma RGD linker proteins such as fibrinogen or vitronectin, $\alpha_v\beta_3$ membrane receptors can support tumor cell–platelet aggregate formation [474]. Another subset of heterotypic receptor interactions can potentially contribute to TCIPA when mucin-producing tumor cells are involved. In some instances, platelet CLEC-2 can bind to tumor cell podoplanin during heterotypic aggregate formation [378, 382, 383, 379, 380]. In other cases, P-selectins are also involved when mucin-producing tumor cells enter the blood stream and initiate TCIPA [475-479].

35 Tumor cell-induced platelet degranulation

The release of platelet α-granules in response to tumor cell stimuli profoundly influences biology in the cardiovascular system (Fig. 2). The release of vWF, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, P-selectin, thrombospondin, and fibronectin from platelet α-granules stimulates and strengthens adhesive interactions between tumor cells and exposed subendothelial extracellular matrix components. These adhesive interactions are accompanied by the release of α-granule prothrombin, fibrinogen, factor V, and factor VIII, contents that promote coagulation. Fibrinolytic factors that include A2 macroglobulin, plasminogen, PAI-1, SERPINE1, and uPA are also released from α-granules to counter fibrin meshwork formation and remodel fibrin clots.

Numerous growth factors, including PDGF, BFGF, EGF, HGF, IGF1, TGFβ, VEGF-A, and VEGF-C are released from α-granules during the formation of tumor cell–platelet emboli that stimulate tumor cell growth and angiogenesis. Additional α-granule contents such as angiopoietin-1 (ANGPT1), promote angiogenesis, and stabilization of endothelial cell junctions, which is counterbalanced by the release of antiangiogenic factors such as angiostatin. Platelet release of S1P depends on thromboxane [480] and has pleiotropic effects on angiogenesis, including the inhibition of sprouting angiogenesis through its action on the sphingosine-1-phosphate receptor (S1PR1).

As a part of their normal tissue remodeling properties, platelet α-granules release matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9, MT1-MMP, and MMP-14), their TIMPs (TIMP-1, TIMP-2, and TIMP-4), and ADAMs (ADAM-10, ADAM-17, ADAMTS-13). Platelet α-granules along with pathologic contributions of these same classes of molecules by tumor cells, promote vascular remodeling, and invasion at secondary metastatic sites [269].

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The simultaneous release of proinflammatory factors from platelet α-granules and those produced by tumor cells attracts a variety of immune cells to form tumor cell–platelet emboli [149]. Platelet α-granule release of C-X-C motif chemokines including CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), and CXCL12 (SDF-1α), initiate and regulate microenvironmental and systemic immune responses during the formation of tumor cell–platelet emboli [270]. So also, MCP-1, CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), and CCL17 (TARC), along with IL1-β3 and PAF acetyl hydrolase, released from platelet α-granules, initiate, amplify, or modulate immune responses to tumor cells [149, 270]. The concurrent release of lipids such as LPA stimulates platelet aggregation and metastasis [481]. Similarly, other molecules like C1 inhibitor, IgG, as well as albumin, α1-antitrypsin, Gas6, and HMWK also influence the inflammatory process during the formation of tumor cell emboli [273, 150].

The release of dense granules following tumor cell-induced platelet degranulation by contrast is less well understood. The release of platelet-dense granule components influences tumor cell-induced biological changes in the vascular microenvironment. The release of calcium and magnesium ions can promote platelet activation and aggregation. The release of nucleotides such as ADP activates platelets through $P2Y_1$ and $P2Y_{12}$ receptors [273, 150]. The dense granule release or engagement of membrane tetraspanins, CD9, CD63, CD151, Tspan9, and Tspan32, regulates cell surface interactions [482, 483]. CD9 is the most abundant platelet tetraspanin and engages Fcγ RIIA, a low-affinity receptor for IgG or GPVI collagen receptors during platelet interactions [482-484]. Tetraspanin CD63 interactions among others influence metastasis formation [485]. LAMP-2 is also present in platelet-dense granule membranes and has a similar distribution to CD63 [486]. The release of neurotransmitters 5-HT, epinephrine, and histamine can potentiate ADP-induced platelet activation, TCIPA, and aggregation [273, 150, 487]. Both a-granule and dense granule release helps to amplify both primary and secondary platelet responses as they interact with tumor cells. Tumor cells initiate successive waves of platelet activation, adhesion, aggregation, and degranulation during the formation of tumor cell platelet emboli [70].

36 Rabbit ears, ear chambers, V2 carcinoma, and tumor cell platelet emboli

The earliest accounts of cancer cell-containing thrombi found in circulation being associated with the spread of tumors were described in surgical pathology lectures by Billroth in 1878 [488]. Using the rabbit ear chambers to actively observe V2 carcinoma, Wood observed the dynamic arrest and formation of platelet thrombi along with the active migration of tumor cells from the vascular space to the perivascular space [54, 57]. The dynamic *in vivo* observations of Wood [54, 57] did not agree with those previously reported by Baserga and Safi-Otti in 1955 [489]. In their case, Baserga and Safi-Otti observed the intravascular arrest and proliferation of anaplastic carcinoma (T1SO) cells prior to extravasation. Both studies revealed tumor cell arrest in small arterioles in conjunction with thrombus formation [489, 54, 57]. As a primary difference, Baserga and Safi-Otti observed intravascular proliferation of arrested tumor cells [489]. This tumor growth occurred over several days and resulted in the destruction of the vessel, and subsequent tumor cell extravasation.

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37 Tissue grinders, rat tumor cells, thromboemboli, and ultrastructure

Many details about the biology involved in tumor cell platelet emboli formation *in vivo* were revealed by ultrastructural analyses. Jones *et al.* first observed the formation of tumor cell platelet emboli along with formation of fibrin using electron microscopy and immunofluorescence after tail vein injection into Sprague–Dawley rats [490]. This is an allogeneic system that was described as free from immunologic effects during the short time frames examined. In follow-up studies using the same model system, early-stage emboli formation revealed the presence of neutrophils and lymphocytes as early as 2 min post-injection [491]. At 15 min, fibrin formation was present in emboli and remained from 3 to 6 h after injection even after the disappearance of platelets [491]. Similar observations were made by Warren and Vales [52]. This group also observed the release of platelet vesicles and adhesion to vessel walls *in vitro* [492]. The studies by Warren also described tumor cell migration through the endothelial layer from the adherent embolus that was restricted by the basement membrane of the endothelium [493]. Likewise, the injection of rat AH-130 ascites hepatoma cells also formed tumor cell platelet emboli in microvessels of the lung [68]. Arrest and extravasation of AH-130 cells was observed in lung arterioles and capillaries [68]. These model systems generally utilized tumor cells in suspension that were harvested from homogenized tumor sections. Residual connective tissue fragments may have contributed to promoting emboli formation, but by and large there was no evidence of collagen fragment or matrix protein association with tumor cell surfaces at the ultrastructural level.

38 Elutriation, mouse tumor cells thromboemboli, and more ultrastructure

Concerns over the homogeneity of tumor cell preparations led to the use of counterflow centrifugal elutriation to isolate pure tumor cell preparations of uniform size for tail vein injections [70]. Enzymatic digestion of minced tumor fragments using collagenase and trypsin inhibitors generates a heterotypic cell suspension. Subsequent counterflow centrifugal elutriation involves a specialized rotor chamber, where centrifugal forces generated outward are counterbalanced by drag forces from fluid flowing in the opposite direction to clean and purify cell preparations [494]. Elutriated tumor cells prepared in this fashion are more likely to retain the representative receptor expression profile and other intrinsic properties that are closer to the native tumor state (Figs. 7 and 8a-c). Tumor cells that are routinely passaged using trypsin over time are stripped of their surface components and are less likely to provide a true representation of the endogenous tumor. Tail vein injections of these pure tumor cell preparations into syngeneic mice enabled accurate characterization of the resulting intravascular biology *in vivo* [65, 70] (Figs. 7 and 8d, e). In these experiments, platelet-aggregating activities of Lewis lung carcinoma, B16 amelanotic melanoma, and 16C mammary adenocarcinoma resulted in tumor cell platelet emboli formation in the lungs [70]. Platelets began aggregating with tumor cells as early as 2 min followed by biphasic association with arrested tumor cells that peaked at 10–30 min and 4–24 h [70]. Ultrastructurally, tumor cells exhibited newly formed processes that became interdigitated with the platelet aggregate. Such processes were restricted to areas of contact with platelets and not endothelial cells or other blood elements (*i.e.*, erythrocytes and polymorphonuclear leukocytes). Numerous tumor cell mitochondria were concentrated in

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the areas of greatest platelet-tumor cell process activity. At early time intervals (2–10 min), intravascular platelet degranulation occurred primarily in platelets associated with tumor cell processes. Tumor cells also engulfed platelet fragments *in vivo* [70]. Follow-up studies using the same models focused on tumor cell interactions with endothelial cells and the subendothelial matrix [67]. Platelet aggregation occurred from 1 to 4 h until a thrombus formed [67]. Around the 4-h time point, endothelial cells retracted exposing the subendothelial matrix in conjunction with the extension of tumor cell processes [67]. At about the 24-h time interval, thrombotic elements dissipated leaving the remaining attached tumor cells exposed to a reestablished circulation [67]. Mitoses were observed after 24 h with cell division and the development of intravascular tumor nodules. The final step in the extravasation sequence was dissolution of the basement membrane by the attached tumor cells [67]. In other mouse studies, platelet adhesive glycoprotein receptors and their counterparts expressed by tumor cells participated in TCIPA and served as an early step in the development of metastatic lesions [495]. These models are representative of experimental metastasis utilizing bolus tail-vein injection approaches. Spontaneous metastasis models are more representative of actual tumor cells shedding, which occurs a few cells at a time, into the blood stream. However, for practical reasons, it is difficult to examine the timing and progression of these thromboembolus formation steps using spontaneous metastasis models.

39 Mouse tumor cells, platelet dynamics, and intravital microscopy

In contrast to rabbit ear chambers, intravital fluorescence microscopy performed on mice has revealed dynamic platelet-melanoma cell interactions [496]. Platelet depletion significantly reduced melanoma cell adhesion to the injured vascular wall *in vivo* [496]. Using a mouse model of hematogenous metastasis to the lung, platelet interactions and metastasis of B16 melanoma cells were decreased after treatment with mAb blocking the $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ integrin [496].

40 OK, so where to from here?

Collectively, these findings clearly illustrate the historical, predictive, and functional importance of platelets and hemostasis to cancer biology. Thrombocytosis and thrombogenesis are vital biologic and prognostic characteristics of cancer. These findings also illustrate how critical platelet-tumor cell thromboemboli formation is to metastasis. Many diagnostic as well as clinical and preventive translational targets have been identified. With these givens noted, numerous intervention points and unanswered questions remain open.

40.1 Detecting and preventing thrombogenesis?

Cancer is a heterogeneous disease with a significant risk of venous thrombosis depending on cancer types and stages, treatment measures, individual patient factors, and the specific population factors [497]. Once leaky vessels have been formed by aberrant angiogenesis in tumors, it is difficult to prevent the entry of tumor by-products into the circulation as associated with VTE and Trousseau’s syndrome. Early detection of tumor-specific or prothrombogenic biomarkers in the blood may be among various other options for routine

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screening, particularly in high-risk patients [498]. Aberrant TF expression is a characteristic of human cancer, metastatic potential, and neoangiogenesis. Similarly, uPA and urokinase plasminogen activator receptor (uPAR) can indicate proteolysis of the extracellular matrix in tumors. Measurement of TF and uPAR as biomarkers of cancer or neoangiogenesis in plasma may serve as diagnostic or prognostic risk factors [498].

Therapeutic management options for VTE in cancer patients remain limited [499]. Heparin monotherapy followed by long-term therapy with a vitamin K antagonist may be one option, but various clinical questions remain [500, 499]. Optimizing the duration of anticoagulant treatment for primary or recurrent VTE in conjunction with managing bleeding abnormalities is a key to success [499]. A consensus on clinical treatment recommendations is lacking due to the paucity of randomized controlled trials focused on cancer-associated thrombosis. The impact of new thrombin and factor Xa inhibitors in this setting remains to be established [500, 499].

40.2 Megakaryocytes as translational targets?

Influencing the biology of megakaryocytes or the production of platelets is a potential intervention point. Considering the potential link between tumor-derived IL-6 capacity to stimulate hepatic thrombopoietin synthesis and paraneoplastic induction of thrombocytosis, this is a potential intervention point [423]. Directly inhibiting IL-6 levels using anti-IL-6 therapy may be one option. These approaches have been explored in the treatment of chronic inflammatory disorders such rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) [501, 502]. Tocilizumab is one IL-6 inhibitor (IL-6i) licensed for RA and JIA, and other agents targeting either IL-6 or its receptor remain under investigation [502].

Directly targeting thrombopoietin synthesis or the development of thrombopoietin receptor (TPO-R) antagonists may be another approach. TPO-R agonists are available for treating thrombocytopenia. Two of these new agents, romiplostim and eltrombopag, are for use in patients with primary immune thrombocytopenia that is unresponsive to conventional treatments [503, 504]. No TPO-R antagonists are currently available.

There may be other intervention points for limiting IL-6 production. To some extent, IL-6 production may involve a feedback loop through the production of PGE_2 in the tumor microenvironment since generation of the latter induces IL-6 synthesis [505]. Thus, nonsteroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 inhibitors (COXIBs) may help suppress thrombocytosis by preventing PGE_2 synthesis [446].

Genetic interventions might be another possible approach in high-risk populations. However, to impact platelet biology requires molecular modification of megakaryocytes. Megakaryocytes are genetically more stable than tumor cells and therefore more reliable targets. Genetic interventions of this nature might include autologous bone marrow transplants. One could envision a potential scenario involving the extraction of patient bone marrow, isolation of MEP cells, and genetic manipulation prior to their delineation into MKP. Since platelets act as “first responders” and can actually migrate into the subendothelial matrix, this presents a useful targeting approach. Enhanced expression and packaging of shRNAs that suppress oncogene or enhance suppressor gene expression could

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then seek out circulating cancer cells or tumors. One foreseeable advantage of using autologous MEPs includes the terminal differentiation to MKPs that results in their loss once platelets are formed giving clinicians some control over biologic dosing.

41 NSAIDs and COXIBs inhibit cancer

NSAIDs and COXIBs effectively inhibit cancer formation and progression [446]. Aspirin covalently inactivates COX-1 more selectively than COX-2 but causes GI toxicity [446]. Population and clinical studies indicate that regular intake of aspirin and various other NSAIDs reduce the risk and incidence of various cancers [506-512]. Prospective cohort studies that involved 82,911 women enrolled in the Nurses' Health Study [513] or 47,363 male health professionals [514] showed that regular long-term use of aspirin significantly reduced incidence of CRC. In other prospective studies, daily use of aspirin significantly reduced the incidence of colorectal adenomas, breast cancer, and lung cancer [515-520]. A recent international consensus statement indicates the benefits of aspirin use [521]. The advantage of using COX-1 selective agents, particularly aspirin, is the attenuation of TxA_2 production by platelets. This approach prevents all of the platelet-mediated effects associated with their activation, aggregation, degranulation, and tumor cell platelet emboli formation. This reduces cardiovascular risk but unfortunately does not alleviate the GI toxicity or effectively prevent the production of proinflammatory/prooncogenic PGE_2 [446].

In contrast, COXIBs are competitive and selective inhibitors of COX-2 but are associated with cardiovascular toxicity [446]. A number of case/control studies support the use of COXIBs in preventing colon cancer with fewer GI side effects [522, 515, 523-526]. However, the discovery of increased cardiovascular risk overshadowed these findings [522, 515, 523-526]. Similarly, lung cancer and prostate cancer incidence is also reduced through the use of COXIBs [527, 528]. Collectively, these studies illustrate the potential benefits associated with the use of COXIBs, particularly a reduction in cancer incidence in high-risk populations. Recently, Dovizio *et al.* showed that the induction of COX-2 overexpression by platelet-tumor cell cross-talk could be inhibited by disrupting galectin-3 function or collagen receptor-mediated platelet adhesion. Their study suggested that blocking physical interactions by targeting collagen binding sites could be an alternate means of affecting COX-2 [529].

In contrast to aspirin, the advantage of COXIBs is their suppressive effect on proinflammatory/prooncogenic PGE_2 production by tumor cells. This reduces GI toxicity, but regrettably enhances cardiovascular risk by suppressing PGI_2 production in endothelial cells, and by reducing the antiplatelet aggregation effects [446]. Since PGE_2 and its enzymatic metabolite 13,14-dihydro-15-ketoPGE_2 prevent PGI_2 inhibition of TCIPA, this underscores the need for selective targeting of the PGE_2 pathway [63, 446]. Although attempts have been made to selectively target the PGE2 pathway, more effective agents are needed [530, 531]. These new drugs could be used in conjunction with low-dose aspirin to eliminate the genesis and spread of many different cancers.

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Ultimately, more targeted and selective approaches are needed to eliminate the proinflammatory, prooncogenic, and prometastatic effects of platelets in cancer biology while maintaining their important hemostatic role as “first responders”.

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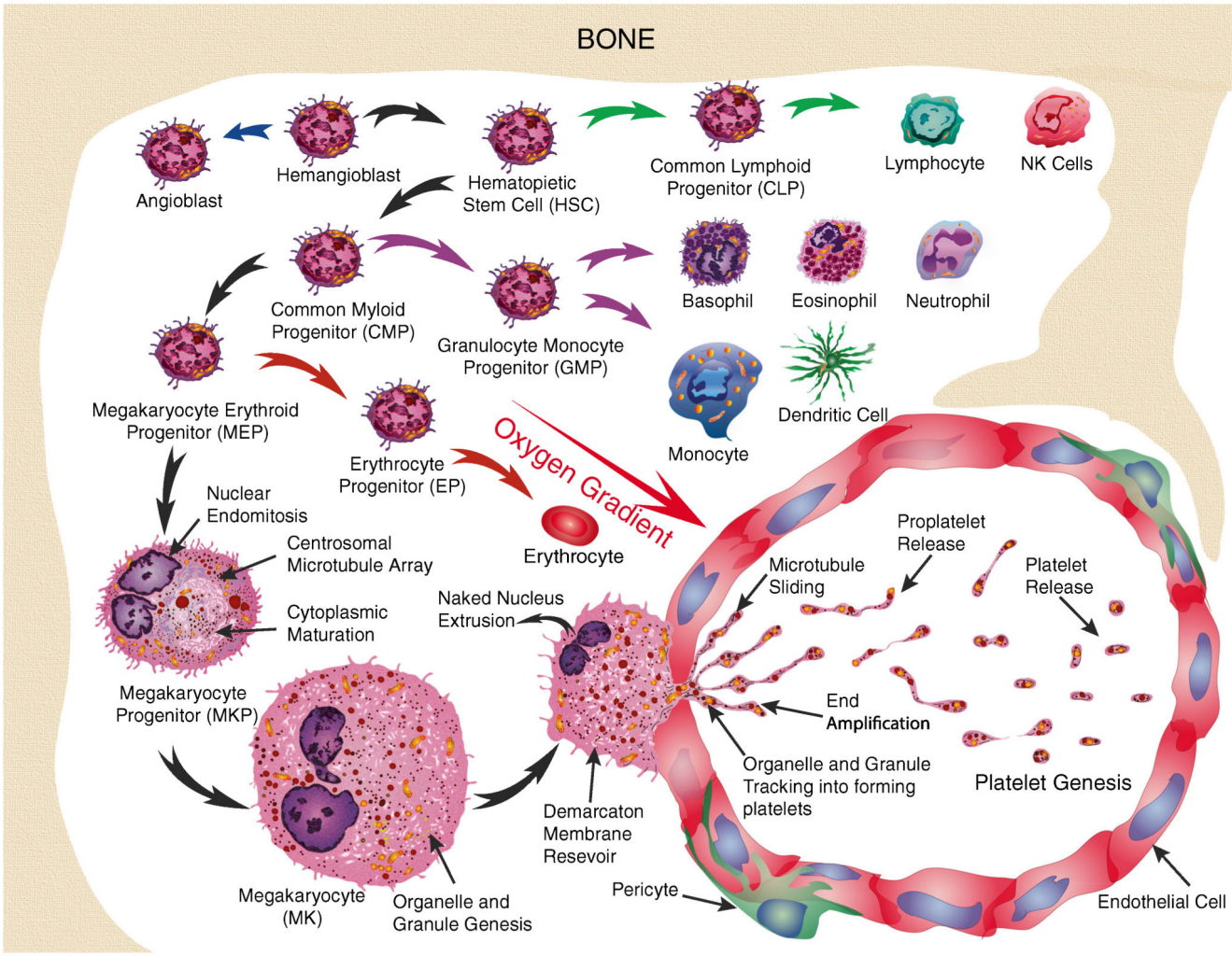

Fig. 1.

Platelet genesis occurs in the bone marrow. Hemangioblasts initially undergo divergence to form two primary lineages of cells, either angioblasts or hematopoietic stem cells (HSC). HSC subsequently form common lymphoid progenitor (CLP) or common myeloid progenitor (CMP) cells. CLP give rise to lymphocytes and other lymphoid lineage cell types while CMP generate myeloid cell types. Granulocyte monocyte progenitor (GMP) lineages include basophils, eosinophils, neutrophils, monocytes, and dendritic cells. In contrast, megakaryocyte erythroid progenitors (MEP) give rise to megakaryocytes and erythroid cells. Megakaryocyte progenitor (MKP) cell progression involves nuclear endomitosis leading to polyploidy to as high as 128n. These changes are accompanied by centrosomal microtubule array formation and cytoplasmic maturation. The increase in DNA, cytoplasm, granule, and organelle formation significantly increases the size of megakaryocytes (MK) up to 65 μm in diameter in preparation for platelet genesis. MK migrates along an oxygen gradient and send platelet-generating processes into the lumen of bone marrow capillaries that fill along sliding microtubule tracks with organelles and granules. Platelets are formed by cytoplasmic end amplification followed by proplatelet release and maturation

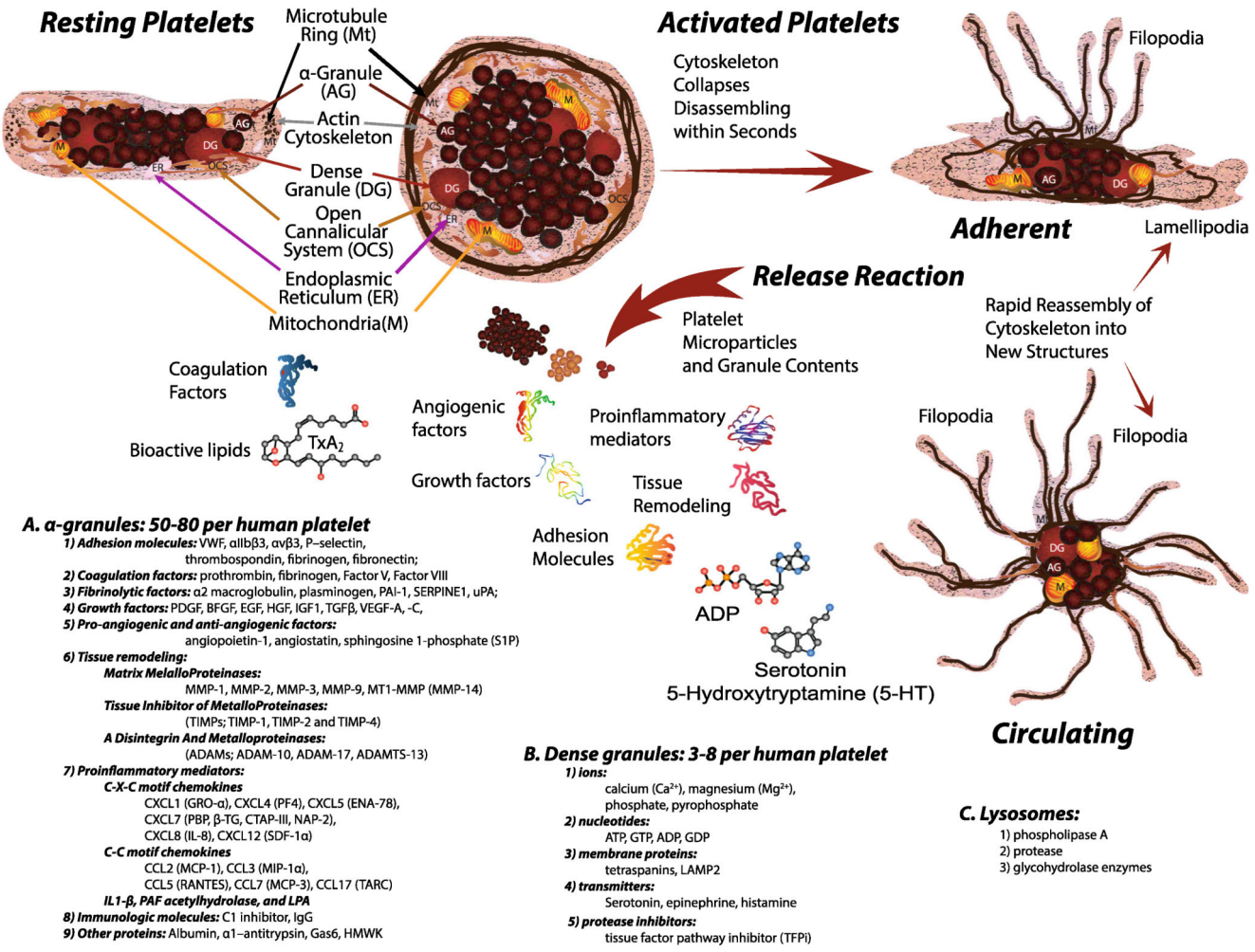

Fig. 2.

Resting platelets maintain their discoid shape using a structural ring of microtubules (Mt) and actin cytoskeleton. The plasma membrane is connected to an internal membrane reservoir called the open canalicular system (OCS). Platelets also contain organelles including endoplasmic reticulum (ER), and mitochondria (M). Resting platelets carry stores of bioactive contents in a granules (**a**), dense granules (**b**), and lysosomes (**c**) that are released following activation. Once activated, the platelet cytoskeleton collapses followed by extensive shape change depending upon the trigger stimulus. Lamellipodia are formed when in contact with flat surfaces such as extracellular matrix, and facilitate platelet migration. Filopodia that promote contact with other platelets or cells are formed by adherent and circulating platelets in suspension

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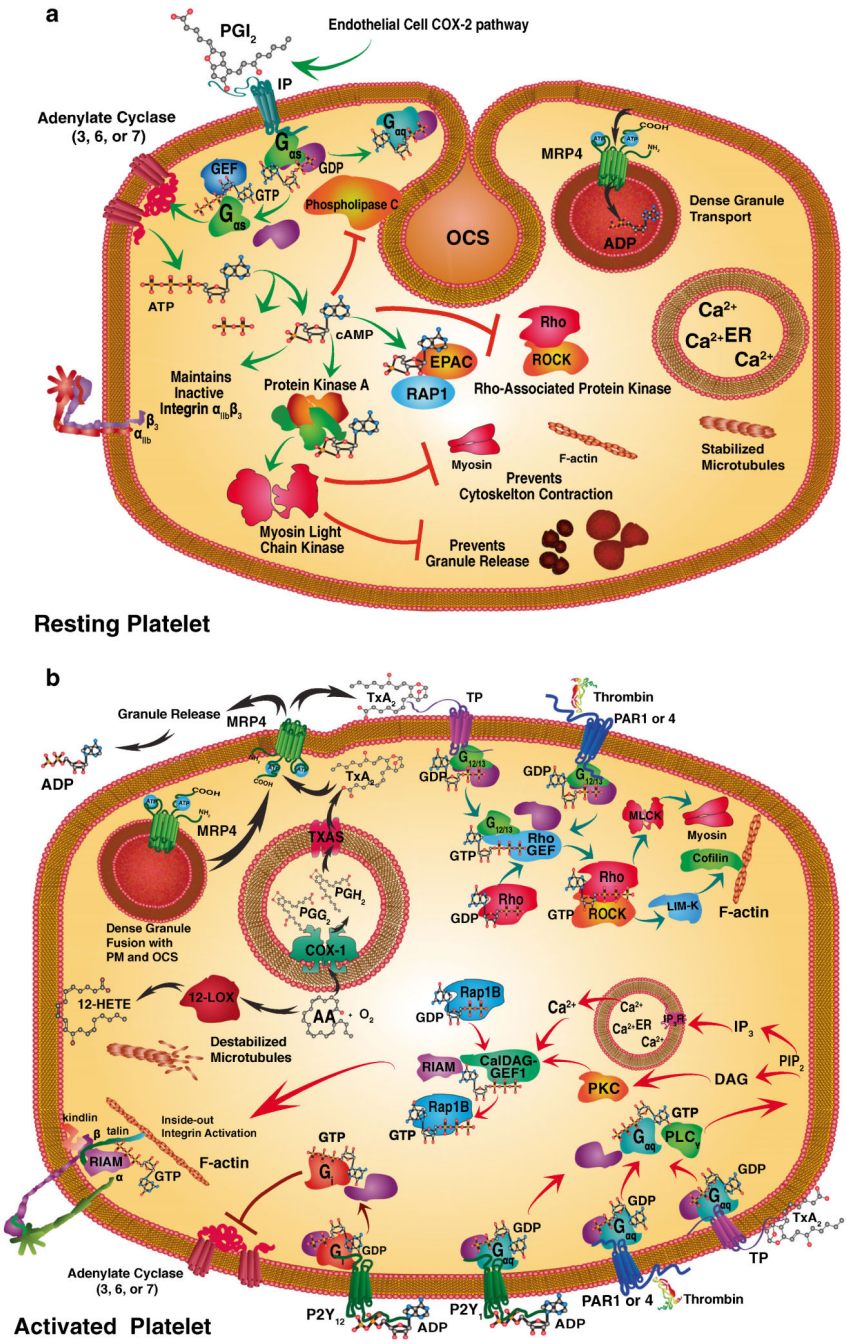

Fig. 3.

Specific signaling pathways maintain the resting platelet state (**a**) or initiate platelet activation (**b**). Prostacyclin (PGI_2) is primarily produced *via* the COX-2 pathway by endothelial cells and binds to stimulatory $G_{\alpha s}$-coupled and GTP-GDP exchange factor (GEF) proteins through prostacyclin receptors (IP, *green arrows*). The resting platelet state (**a**) is maintained by IP-mediated $G_{\alpha s}$-protein coupled stimulation through the synthesis of cyclic-AMP (cAMP) by adenylate cyclase (AC 3, 6, or 7). cAMP elicits a variety of effects including: inhibition of phospholipase C_γ (PLC_γ) and Rho/Rho-associated kinase (ROCK)

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pathway. cAMP also directly stimulates exchange protein directly activated by cAMP (EPAC) and Ras-related protein 1 (Rap1) signaling, along with PKA. Protein kinase A, in turn, phosphorylates cAMP myosin light chain kinase (MLCK) that maintains an inactive actin cytoskeleton and prevents granule release as well as maintaining integrins in an inactive state. Resting platelets also accumulate ADP in dense granules *via* granule membrane multidrug resistance protein 4 (MRP4). Calcium stores and microtubules also remain stabilized in resting platelets. Activating platelets by contrast can involve a variety of different signaling pathways depending on the stimulus (**b**). Thromboxane A_2 is a potent activator of platelets through the $G_{12/13}$ protein coupled thromboxane receptor (TP; *teal arrows*). Likewise, thrombin activates protease-activated receptors (PAR) 1 and 4 through $G_{12/13}$ coupled proteins. Both $G_{12/13}$ coupled receptors activate Rho/ROCK kinase activity leading to MLCK phosphorylation and thereby myosin activation, and LIM domain kinase (LIM-K) that phosphorylates cofilin, which triggers actin filament disassembly. These cytoskeletal changes are accompanied by the destabilization of microtubules. TP and $PAR_{1,4}$ along with ADP activation of the purinergic G-protein coupled receptor (P2Y) act upon G_{aq} proteins (*red arrows*). This activity stimulates PLC_{γ} and the release of phosphatidylinositol 4,5-bisphophonate (PIP_2) and activation of IP_3 in combination with diacyl-glycerol (DAG). These events trigger calcium release and protein kinase C (PKC) activation. Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEF-I) catalyzes GDP for GTP exchange in Rap1B. These events activate Rap1-GTP-interacting adapter molecule (RIAM) that stimulates talin and kindlin interactions between integrin β subunits and F-actin and integrin activation. A different inhibitory Gi protein coupled receptor, P12Y, is activated by ADP and inhibits AC 3, 6, or 7. ADP is released from platelet-dense granule fusion with the plasma membrane or OCS MRP4 that transports TXA_2 and ADP. These activation events initiate the synthesis of 12-hydroxyeicosatetraenoic acid (12(S)-HETE) or TXA_2 from arachidonic acid (AA). TXA_2 is synthesized after conversion of AA and oxygen (O_2) by cyclooxygenase 1 (COX-1) to prostaglandin G_2 and then H_2 (PGG_2, PGH_2) followed by the final conversion by thromboxane A synthase (TXAS)

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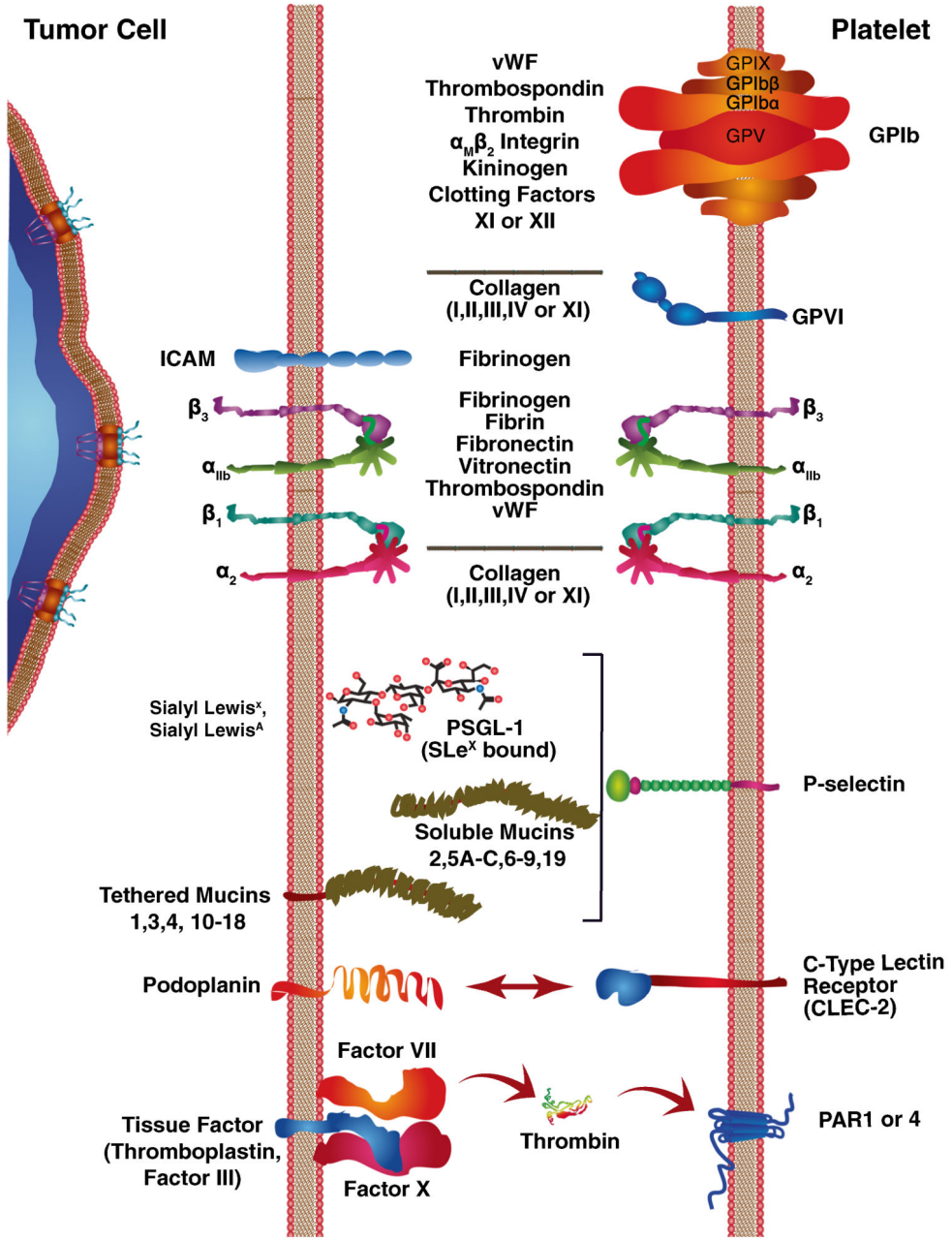

Fig. 4.

Tumor cells interact with platelets through a variety of receptors. Interactions between tumor cells and platelets can involve diverse types of cell–cell and extracellular matrix (ECM) proteins as intermediaries. Tumor cell invasion into the blood stream can expose extracellular matrix or trigger the expression of ultralarge von Willebrand factor (ULVWF) that trigger platelet rolling through tethering to collagen and other ECM proteins or endothelial cells. Platelet GPIb complexes recognize vWF tethers, along with thrombospondin, thrombin, $\alpha_M\beta_2$ integrin, kininogen, and clotting factors XI or XII. The GPIb complex consists of two sets of GPIX, GPIbβ, and GPIbα along with a centrally situated GPV protein. Subsequent stabilization of adhesive contacts with collagen exposed

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by tumor cells is mediated by GPVI. Tumor cell interactions with fibrinogen can also occur through intracellular adhesion molecule-1 (ICAM-1). Integrin heterodimers mediate numerous interactions with a variety of ECM proteins. The $\alpha_{IIb}\beta_3$ integrin is the most promiscuous and binds to fibrinogen, fibrin, fibronectin, vitronectin, thrombospondin, and vWF. Interactions with a variety of collagen molecules are mediated by $\alpha_2\beta_1$. Interactions between tumor cells and cell surface carbohydrates can involve selectins. P-selectin binds to Sialyl Lewis^x or Sialyl Lewis^{xA}; these interactions can also involve either tethered or soluble mucins. Similarly, carbohydrate interactions can involve C-type LECtin receptor-2 (CLEC-2) on platelets and podoplanin on tumor cells. Tissue factor expression by tumor cells can bind coagulation factor VII or X and trigger thrombin generation that activates PAR 1 or 4

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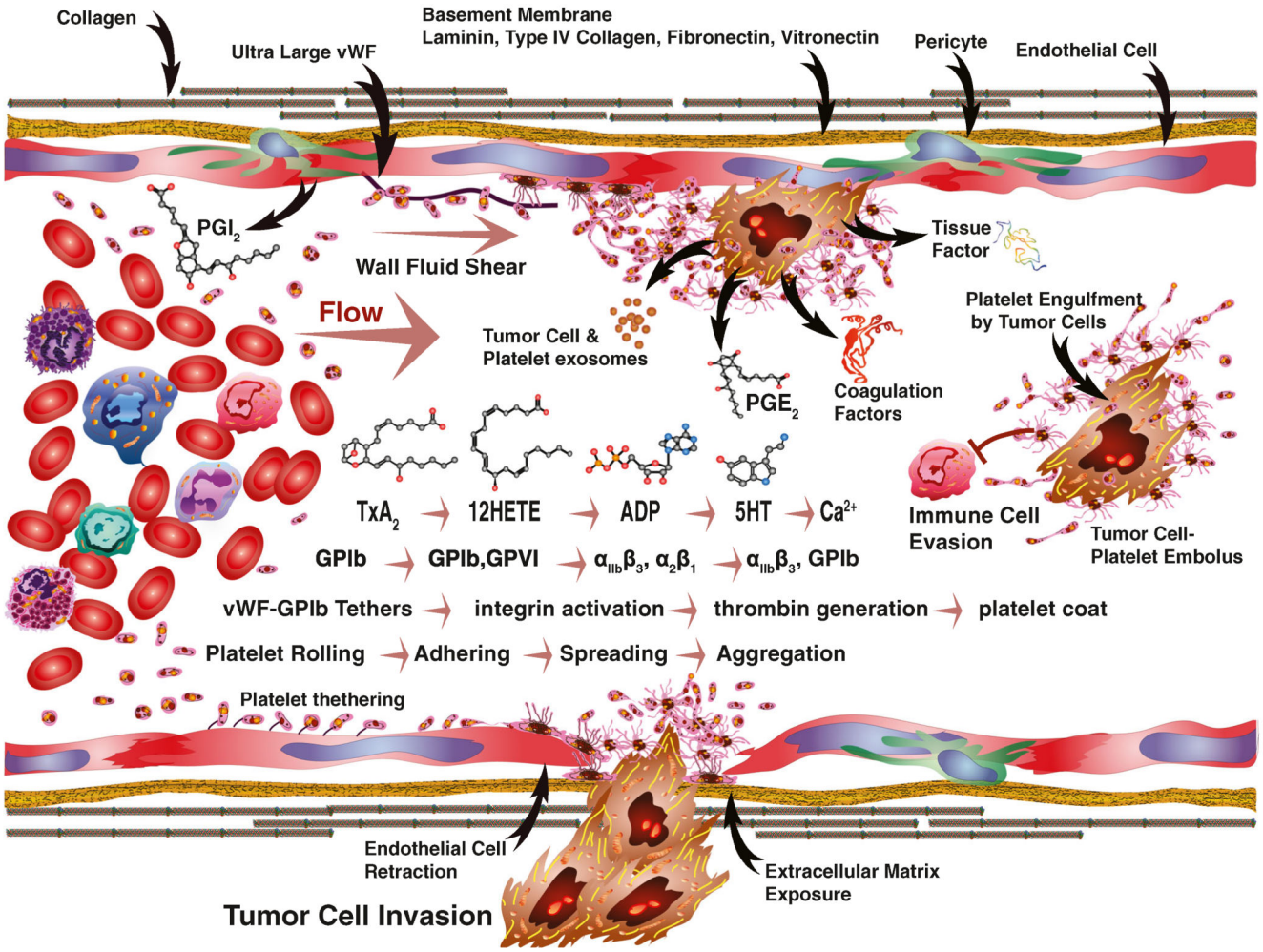

Fig. 5.

Tumor cell interactions with platelets and other circulating factors under fluid flow are complex. Fluid shear increases from the center of blood vessels toward the vessel walls. Prostacyclin (PGI_2) produced by endothelial cells inhibits platelet activation. Various molecules produced by tumor cells or other sources can activate platelets as part of a cascade of events. Progressive stimulation by TxA_2-12(S)-HETE-ADP-5HT and calcium release are events that fall within the small molecule cascade. The formation of vWF-GpIb tethers support cell rolling or ultralarge vWF molecule formation, attracts platelets, and promotes their binding. Along the same cascade, GPIb-GPVI begin stabilizing adhesion and triggering $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ along with other activation followed by spreading, aggregation, and invasion. Within the same cascade, thrombin generation promotes platelet coat formation and embolus formation that can enable tumor cells to evade cell-mediated immunity. Tumor cell products include exosomes, PGE2, tissue factor, and coagulation factors that act as triggers for platelet activation. Endothelial cell retraction associated with tumor cell invasion exposes those basement membrane components such as laminin, type IV collagen, fibronectin, and vitronectin involved in the tumor cell–platelet interaction cascade

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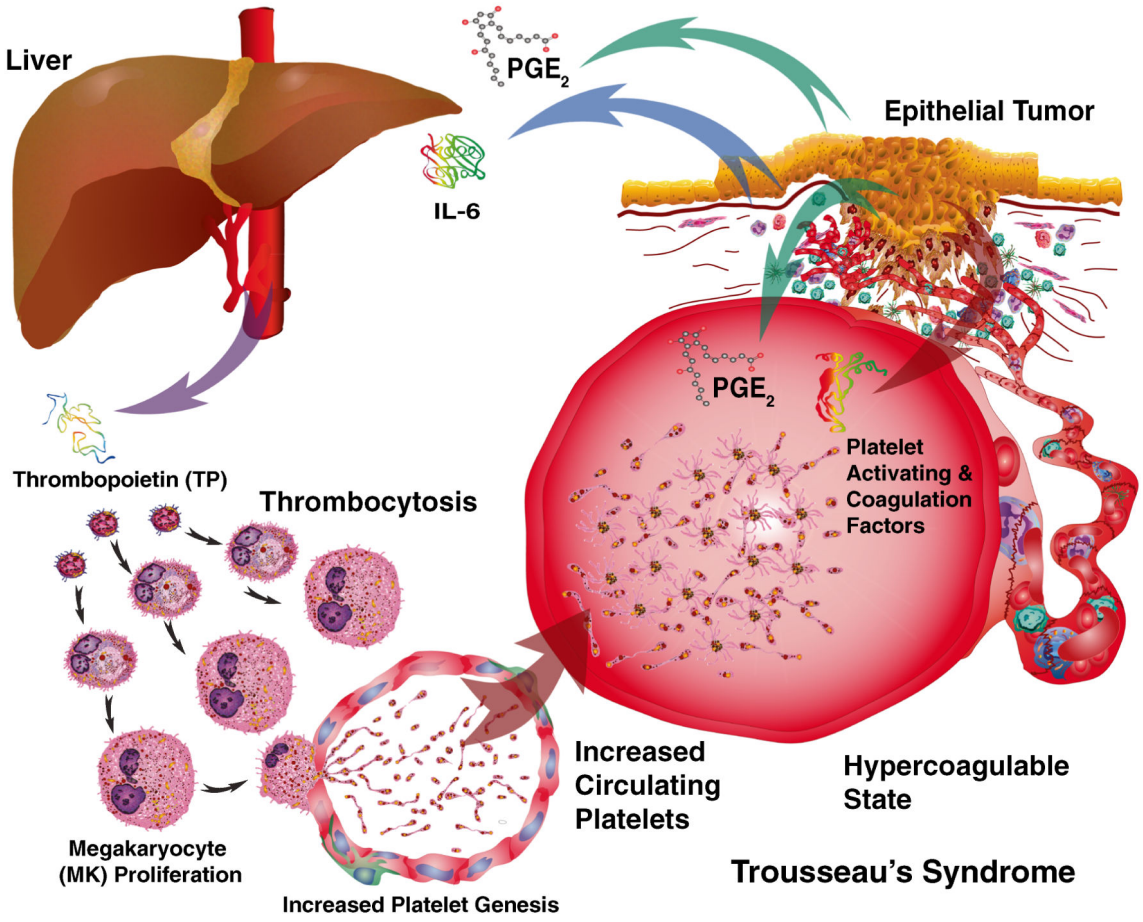

Fig. 6.

Trousseau's syndrome is characterized by a hypercoagulable state. The production of IL6 by tumors stimulates the production of thrombopoietin by the liver. Thrombopoietin (THPO), in turn, stimulates thrombocytosis. The production of PGE_2 by tumors also heightens the responsiveness of platelets. Platelet-activating and thrombin-stimulating factors produced by tumors also elevate the potential for thrombosis. The combination of increased numbers of circulating platelets along with circulating prothrombogenic factors creates the hypercoagulable state first described by Trousseau

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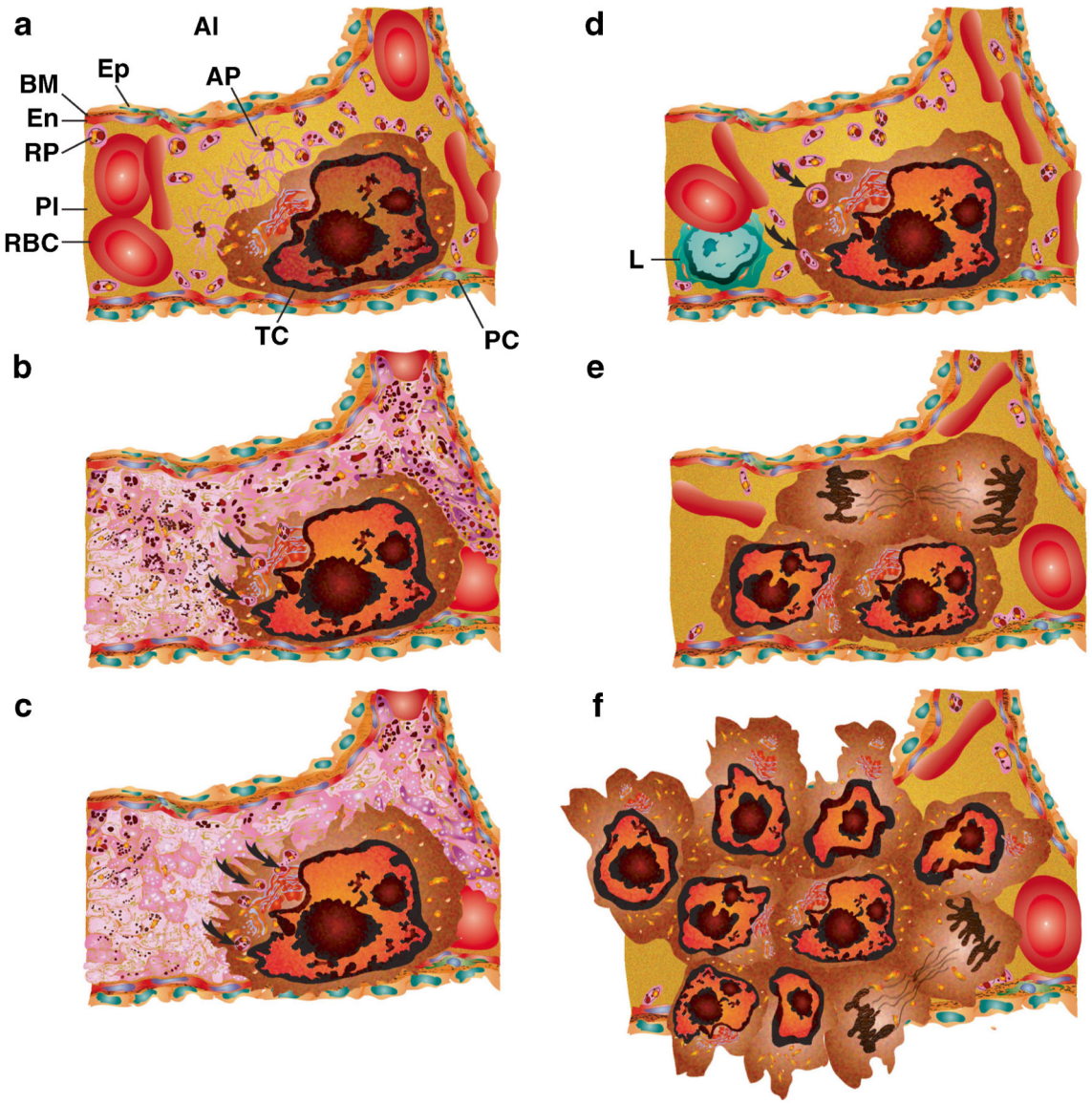

Fig. 7.

Sequence of events involved in tumor cell–platelet interactions during the arrest and extravasation of tumor cells. **a** The initial arrest often involves close contact between the plasma membranes of endothelial cell–platelets–tumor cells. Resting platelets (RP) associate with inactive endothelial cells (En) that are maintained in conjunction with pericytes (PC) while platelets are activated by interaction with tumor cells or their by-products in circulating plasma (Pl). **b** Tumor cell–platelet contacts initiate extensive platelet activation (AP) and aggregation with formation of tumor cell cytoplasmic extensions into platelet thrombus and uptake of platelet fragments by tumor cells. These interactions encompass the entire lumen of microvessels to the exclusion of other cells such as red blood cells (RBC). Platelet degranulation initially occurs in platelets closely associated with tumor cell surfaces. **c** Platelet degranulation becomes more extensive accompanied by additional platelet fragment engulfment by tumor cells and by endothelial cell retraction as tumor cells attach

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to subendothelial matrix and begin matrix degradation. **d** Platelet aggregates begin to disappear possibly by additional tumor cell uptake of platelet fragments. **e** Tumor cells begin to proliferate in capillaries. **f** Additional tumor cell proliferation occurs in conjunction with basement membrane (BM) degradation and invasion into the surrounding lung alveoli (Al)

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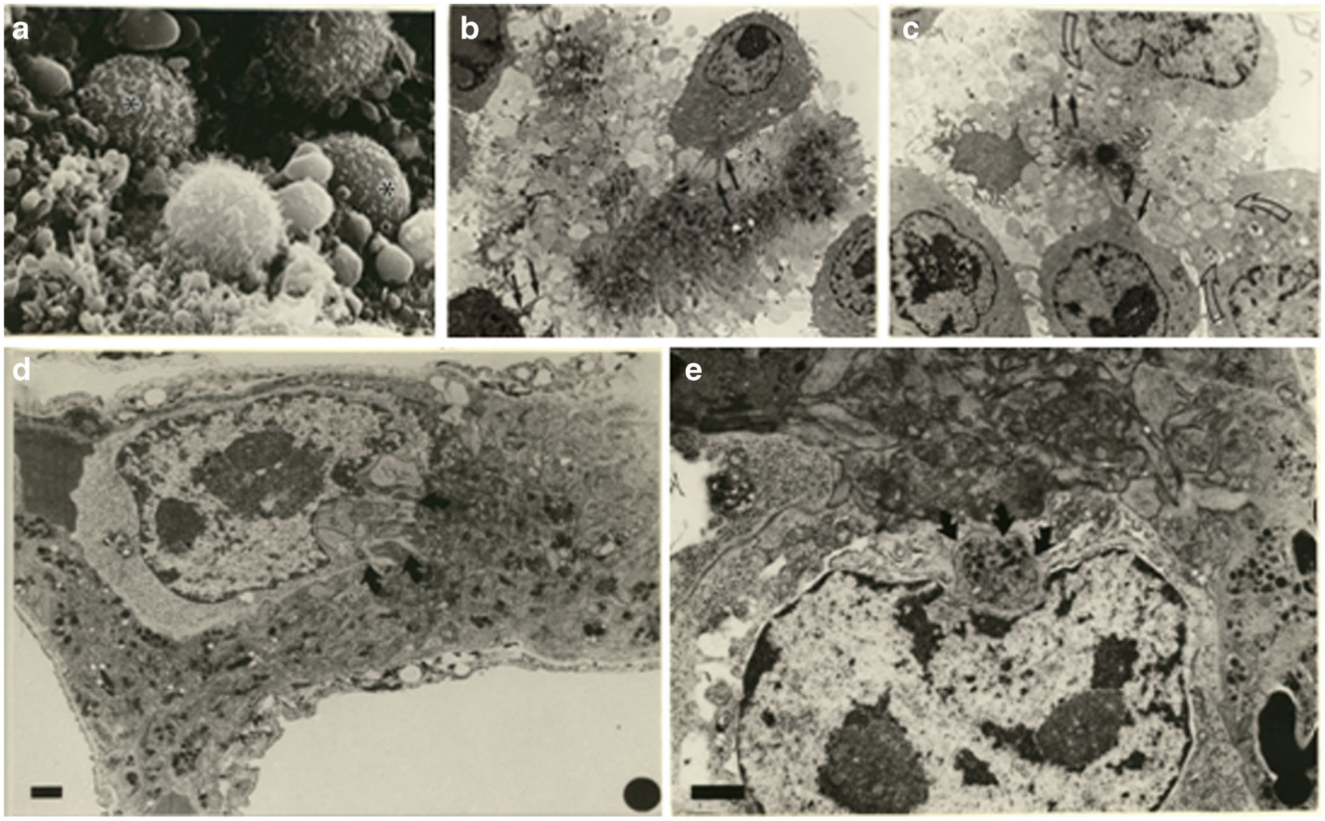

Fig. 8.

Electron micrographs of early events in platelet–tumor cell interactions. **a** Elutriated W256 tumor cells in the plateau phase interacting with platelets *in vitro* at 10 min. *Asterisked cells* have lost surface microvilli (scanning electron micrograph; magnification, ×3,900). **b** Elutriated W256 tumor cells beginning shallow process formation (*arrows*) into platelets at midphase, and **c** deeper into platelet emboli at plateau phase (*arrows*) with engulfment of platelet fragments (*open arrows*) (transmission electron micrographs; magnifications—**b** ×3,000 and **c** ×4,300). **d** Interaction with and process penetration (*arrows*) of Lewis lung tumor cells into C57Bl/6 platelets 2 min post tail-vein injection, and **e** engulfing platelet fragments (*arrows*) at the plateau phase (transmission electron micrographs; magnifications—**d** ×6,800 and **e** ×12,000, *scale bars* 1 μm). **a**–**c** Reprinted from [59] with permission from S. Karger AG, Basel; **d, e** courtesy of Menter