Platelets in pulmonary vascular physiology and pathology

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ABSTRACT

Almost a trillion platelets pass through the pulmonary circulation every minute, yet little is known about how they support pulmonary physiology or contribute to the pathogenesis of lung diseases. When considering this conundrum, three questions jump out: Does platelet production in the lungs occur? Why does severe thrombocytopenia—which undercuts the principal physiological role of platelets to effect hemostasis—not lead to pulmonary hemorrhage? Why does atherothrombosis—which platelets initiate, maintain, and trigger is other critically important arterial beds—not develop in the pulmonary artery? The purpose of this review is to explore these and derivative questions by providing data within a conceptual framework that begins to organize a subject that is largely unassembled.

Key Words: cell signaling, hemostasis, platelets, thrombosis, vascular biology

Platelets are anucleate megakaryocyte fragments. Humans expend an extraordinary amount of energy and raw materials to make platelets and we do so utilizing cell biological processes that are unique. We do this with a purpose: platelets effect physiological hemostasis in the microvasculature, especially of the skin and mucous membranes, and thereby stop bleeding. And it has been speculated that evolutionary engineering of human platelets—including loss of a nucleus—was driven by the adaptive requirement of an improved capacity to prevent bleeding and allow early mammals to survive traumatic injury.[1] It is only recently that human evolution has bumped up against platelet evolution, and that platelets have become antagonistic to human survival: as we live long inactive lives, platelet-dependent atherothrombosis—the etiology of heart attacks and strokes—has emerged as the number one killer of Homo sapiens in the developed world. And while mechanisms of platelet-dependent hemostasis are in large part recapitulated by mechanisms of platelet-dependent thrombosis, understanding their differences is probably the key to elucidating the pathophysiology of platelets, including the pathophysiology of platelets in pulmonary vascular diseases, about which relatively little is known.[2]

PLATELET PRODUCTION

Humans have about one trillion (1×10^{12}) total body platelets. They are small (1-2 μm) discoid, non-nucleated cells that circulate for about 10 days after they are released into the bloodstream by bone marrow megakaryocytes (Fig. 1). Humans produce about 1×10^{11} platelets daily, and production can be increased at least 20-fold in states such as acute hemorrhage, acute hemolysis, or inflammation. Platelet production derives from sequential processes termed megakaryopoiesis and thrombopoiesis, the biological basis of which is an intricate nexus of signaling events specified by cytokines and growth factors and organized temporally and spatially via exquisitely fine-tuned nuclear and cytosolic responses. Thrombopoietin (TPO) is the primary regulator of megakaryopoiesis. In conjunction with other cytokines, including stem cell factor, interleukin (IL)-6, IL-11, and erythropoietin, it promotes megakaryocyte lineage commitment from pluripotent hematopoietic stem cells.[3] Counter-balancing these pro-megakaryopoiesis factors are transforming growth factor β1, platelet factor 4, and IL-4, each of which is considered a negative regulator of platelet production.[3]

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Figure 1: Scanning electron microscope images of resting, partially activated, and fully activated human platelets (from left to right). This article was originally published in the *Journal of Biological Chemistry*. Quantitative analysis of platelet $\alpha v\beta 3$ binding to osteopontin using laser tweezers by Rustem I. Litvinov, Gaston Vilaire, Henry Shuman, Joel S. Bennett, and John Weisel in J Biol Chem 2003;278:51285-51290 (Cover photograph with additional credit to Chandrasekaran Nagaswami and Yevgeniya Baras). © The American Society for Biochemistry and Molecular Biology.

Megakaryocytes mature into unique platelet-producing cells.[4] Thrombopoiesis begins with a shift in compartmentalization as megakaryocytes migrate from the endosteal stem cell compartment to the vascular zone, where they adhere to the marrow sinusoids. These vascular zone megakaryocyes accumulate genetic material and cytoplasm as they maintain cell cycling that never progresses beyond late anaphase, so they fail to undergo nuclear envelope division and cytokinesis, thus resulting in the typically large, irregularly shaped polyploid cells containing up to 64 pairs of chromosomes. As they enlarge, megakaryocytes express a huge surface area derived from an internal reservoir of membrane criss-crossing the cytoplasm—designated the demarcaction membrane system—that permits them to remodel their cytoplasm into a series of microtubule-scaffolded extensions termed proplatelets. Finally, via a series of microtubular, cytoskeletal, and contractile responses modulated by hydrodynamic stimuli, the proplatelet elongates and bifurcates, with individual platelets released into the circulation following their being sheared off by the forces of the flowing blood.[5] TPO is the main regulator of thrombopoiesis and TPO mimetic drugs are effective at improving platelet counts in patients with chronic immune thrombocytopenia, probably because they overcome autoantibody-mediated inhibition of thrombopoiesis.[6]

THROMBOPOIESIS MAY OCCUR IN THE LUNGS

Platelets may be released into the circulation from a pulmonary thrombopoietic compartment. Over 40 years ago, data derived from studies of right atrial blood in humans undegoing cardiac catheterization provided the estimate that up to 50% of bone marrow megakaryocytes exit the marrow cavity, enter the blood, and end up in the lung vasculature, where active thrombopoiesis results in the release from the pulmonary capillary bed of nearly one-fifth of the circulating platelet mass.[7] These clinical data have been corroborated[8] and contradicted,[9] but animal data—principally from the mouse—have consistently shown that megakaryocytes are present and making platelets in the lung.[10] Of note, there are established data that TPO mimetic drugs increase the transit of megakaryocytes from the marrow compartment into the pulmonary microvasculature and that there may be a correlation between this phenomenon and the magnitude of the blood platelet response to the thrombopoietic agent (Fig. 2).[11]

The idea that thrombopoiesis occurs in lungs is particularly interesting when considering that (a) pulmonary hypertension or fibrosis develops in some patients with platelet secretory defects in which unpackaged granule contents leak from the megakaryocyte into the surrounding microenvironment; and (b) pulmonary hypertension occurs in some patients with myeloproliferative disorders (see text below). One might also speculate that platelet production in the lung is important in maintaining hemostasis within the vast pulmonary microvasculature, estimated to contain over a billion capillaries.[12] It is striking that pulmonary hemorrhage is an infrequent manifestation of even extreme disturbances in hemostasis, such as severe underproduction thrombocytopenia or hemophilia.

PLATELETS IN HEMOSTASIS AND THROMBOSIS

Circulating platelets participate in both physiological hemostasis and pathological thrombosis. Primary hemostasis is defined as the platelet/blood vessel interactions that initiate physiological hemostatic plug formation and prevent superficial microvascular hemorrhage. When the trigger is a pathological event, such as a ruptured atherosclerotic plaque, platelet adhesion to the damaged arterial wall leads to their aggregation, resulting in a vasooclusive white thrombus. A platelet-dependent thrombus is the fundamental pathological trigger of arterial ischemia or infarction, such as that causing heart attacks and strokes.

Platelets circulate in an inactivated state. They respond to vessel wall injury, alterations in blood flow, or chemical stimuli with the activation of a functional triad of adhesion, secretion, and aggregation. Activation also results in dramatic cytoskeletal changes that cause platelets to round-up and flatten, and extend loops of cytoplasm as broad lamellopodia
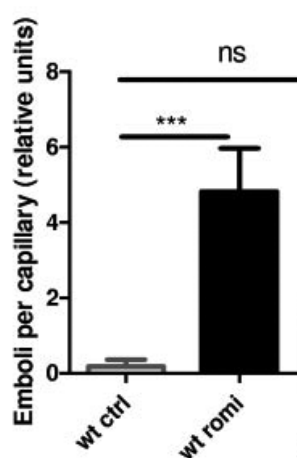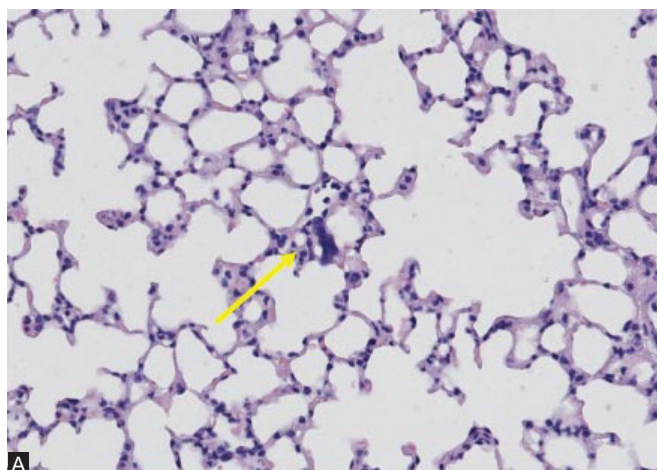
Figure 2: The thrombopoietic agent romiplostim induces an increase in megakaryocytes in the lungs of mice. **(A)** H and E-stained lung tissue of a normal mouse animal treated with romiplostim showing a representative lung megakaryocyte embolus (arrow). **(B)** Quantification of the emboli per capillary in lung tissue. Each bar represents the mean ± SEM of 10 animals. This figure was originally published in *Blood* by the American Society of Hematology (ASH). Romiplostim administration shows reduced megakaryocyte response capacity and increased myelofibrosis in a mouse model of MYH9-RD by Catherine Leon, Katja Evert, Frank Dombrowski, Fabien Pertuy, Anita Eckly, Patricia Laeuffer, Christian Gachet, and Andreas Greinacher in Blood 2012;119:3333-3341. © 2012 Reproduced with permission of ASH in the format republish in a journal via Copyright Clearance Center.

and thin filopodia (Fig. 2). These linked responses occur via a series of carefully coordinated signals that convert extracellular stimuli into intracellular chemical messengers that direct specific enzymatic reactions leading to changes in cell structure, the expression of a new repertoire of functional adhesion receptors, and the secretion of several proaggregatory and growth-promoting substances.

The state of platelet activation is regulated dynamically by the actions of a diverse array of excitatory and inhibitory extracellular stimuli.[13] Platelets are equipped with specific plasma membrane receptors that organize these various stimuli and transform them into biological responses (Fig. 3). This transformation occurs via transmembranous

signaling that results in the generation of intracellular second messengers. The major activation pathways in platelets are stimulated by von Willebrand factor binding to GpIb-IX-V; collagen binding to GpVI (coupled to an ITAM-containing FcRγ) and α2β1; thrombin binding to protease receptors (PAR)1 and PAR4, and thromboxane binding to the prostanoid receptor TP; adenosine diphosphate binding to the purinergic receptors (P) P2Y12 and P2Y1; and epinephrine binding to the α2 adrenergic receptor. The primary signal relay downstream of PAR, P2Y, and TP receptors is the stimulation of heterotrimeric G-protein-coupled activation of phospholipase (PL) C, which cleaves membrane phosphatidylinositol 4,5,-bisphosphate (PIP2) to diacylglycerol (which activates protein kinase C) and inositol 1,4,5 trisphosphate (which leads to elevated cytoplasmic ionized calcium). Other functionally important signaling pathways include phosphatidylinositol 3-kinase, which phosphorylates PIP2 at the 3 position, leading to PI3,4,5P3, and PLA2, which hydrolyzes arachidonic acid (AA) from the 2 position of membrane phosphatidylcholine. The major activation pathways in platelets are stimulated by collagen, von Willebrand factor, thrombin, thromboxane, adenosine diphosphate, and epinephrine. Pathways that are activated downstream of the receptors for these stimuli include the following: phospholipase (PL) C, which cleaves membrane phosphatidylinositol 4,5,-bisphosphate (PIP_2) to diacylglycerol (which activates protein kinase C); inositol 1,4,5 trisphosphate (which leads to elevated cytoplasmic ionized calcium); phosphatidylinositol 3-kinase, which phosphorylates PIP_2 at the 3 position, leading to PI3,4,5P$_3$; and PLA$_2$, which hydrolyzes arachidonic acid (AA) from the 2 position of membrane phosphatidylcholine. AA is rapidly converted by platelet cyclooxygenase (COX-1) to the proaggregatory and vasoconstricting (and therefore prothrombotic) product thromboxane A$_2$. Platelet signaling involves other switches as well, including tyrosine kinases, small GTP-binding proteins, tyrosine/serine/threonine phosphatases, calcium-dependent proteases, and structural elements such as cytoskeletal and adapter proteins. This transmembranous signaling apparatus is considered to be adaptive, highly redundant, and fine-tunable, and it converges on two principle functional responses: the secretion of preformed granule contents (some of which are proaggregatory and vasoconstricting) and the activation of integrin αIIbβ3 to a conformation that engages cohesive ligands that bridge platelets and causes aggregation.

The major inhibitory signaling pathways are activated by prostacyclin (PGI$_2$) and nitric oxide (NO), two constitutively released endothelial cell products that serve to maintain blood fluidity principally by maintaining platelets in their basal quiescent state. Prostacyclin binds to specific cell surface receptors to activate adenylyl cyclase, while NO diffuses through the platelet plasma membrane to activate cytosolic (or soluble) guanylyl cyclase. These convert adenosine and guanosine triphosphate (ATP and GTP)
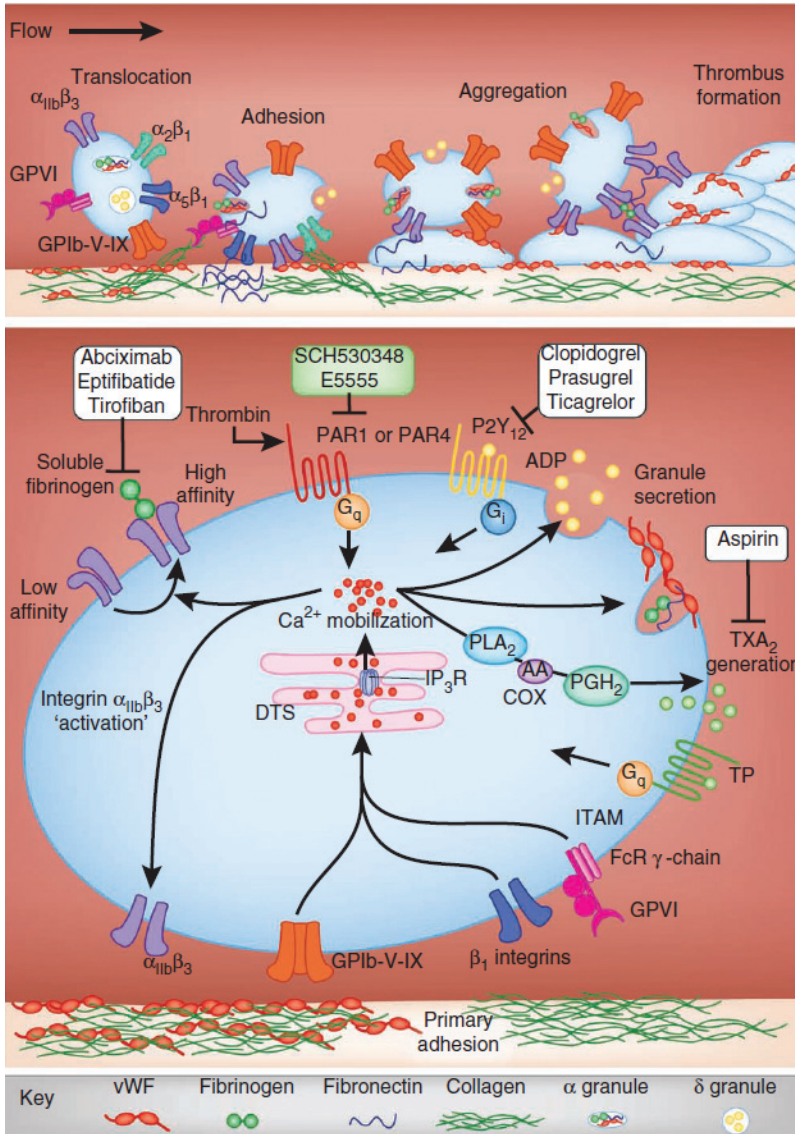
Figure 3: Adhesion and activation mechanisms supporting the hemostatic and prothrombotic function of platelets. Major ligands and receptors mediating platelet adhesion and activation at sites of vascular injury. Platelets are captured in the injured vessel wall from flowing blood through the specific interaction of the platelet GpIb-IX-V complex with collagen-bound VWF exposed on the subendothelium (top). This ligand-receptor interaction has a rapid on-off rate that supports platelet translocation at the vessel wall. Stable platelet adhesion occurs through the binding of platelet GPVI to fibrillar collagen as well as ligation of collagen to α2β1. Once firmly adherent, platelets undergo a series of biochemical changes that induce integrin αIIbβ3 activation, leading to the high affinity interaction with adhesion proteins including vWF, fibrinogen, and fibronectin. These adhesive interactions are indispensable for platelets to form stable aggregates with other activated platelets and promote thrombus growth. Activated platelets release or locally generate soluble agonists, including ADP, TXA2, and thrombin, that can induce autocrine or paracrine platelet activation (bottom). Each agonist activates specific G protein-coupled receptors on the platelet surface, including ADP-P2Y1 or ADP-P2Y12, TXA2-TP, and thrombin-PAR1 or PAR4, stimulating intracellular signaling events that include cytosolic calcium mobilization. This second messenger has a key role in promoting integrin αIIbβ3 activation, TXA2 generation, granule secretion, and the procoagulant function of platelets. This figure also shows the point of antagonism of several FDA-approved anti-platelet drugs (abciximab, eptifibatide, tirofiban, clopidogrel, prasugrel, ticagrelor, and aspirin) and two experimental PAR1 inhibitors (SCH530348 and E5555). Abbreviations: GP – glycoprotein, PAR–protease activated receptor, P – purinergic, ADP – adenosine diphosphate, TXA – thromboxane, TP–TXA2 receptor, G –heterotrimeric GTP – binding protein, FcRγ – Fc receptor gamma chain, ITAM – immunoreceptor tyrosine-based activation motif, vWF – von Willebrand factor, PLA2 – phospholipase A2, COX – cyclooxygenase, AA – arachidonic acid, PGH2 – prostaglandin H2, IP3R – inositol-1,4,5-trisphosphate receptor, DTS – dense tubular system. This figure was originally published in *Nature Medicine* by Nature Publishing Group. Arterial thrombosis–insidious, unpredictable and deadly by Shaun P. Jackson in Nature Medicine 2011;11:1423-1436. Reproduced with permission of Nature Publishing Group in the format reuse in a journal via Copyright Clearance Center.

into cAMP and cGMP, respectively. cAMP and cGMP cause activation of cAMP- and cGMP-dependent protein kinases, which serve to inhibit platelet activation through pleiotropic downstream effectors.

PLATELET-COAGULATION FACTOR INTERACTIONS

Activated platelets are very important in regulating the generation of insoluble fibrin. They release a variety of prothrombotic substances—such as fibrinogen, von Willebrand factor, factor XI, and factor V—and they express receptors for factor XI that enhance its activation by thrombin.[14] Through the "flipping" of phosphatidylserine from the inner to outer plasma membrane, activated platelets express assembly sites for soluble clotting factors and thereby promote critically important coagulation reactions: the activation of factor X by TF/factor VIIa and factor IXa/factor VIIIa; and the activation of prothrombin by the factor Xa/factor Va complex. In addition to these reactions, activated platelets shed "microparticles" that may promote and disperse procoagulant responses. Activated platelets also regulate natural anticoagulant mechanisms by promoting the inactivation of factor Va by activated protein C and by releasing two proteins that inhibit factor XIa.[15] Finally, activated platelets release polyphosphates that activate factor XII to initiate prothrombotic and proinflammatory responses.[16]

PLATELET-LEUKOCYTE INTERACTIONS

Platelet-dependent hemostasis and thrombosis are directly coupled to leukocyte recruitment and activation.[16] Activated platelets express P-selectin, which engages P-selectin

glycoprotein ligand-1 (PSGL-1) on neutrophils and monocytes. This results in leukocyte adherence and activation on and within the developing platelet thrombus. In the leukocyte, PSGL-1-mediated responses are coupled to the upregulation of Mac-1—which binds to platelet GpIbα—and the activation of CD11b/CD18—which binds to fibrinogen attached to platelet αIIbβ3. This process of "secondary capture" is often followed by leukocyte-endothelial cell (EC) interactions, the release of inflammatory and procoagulant factors by the platelet-leukocyte aggregates, and amplification of platelet and leukocyte activation, leading to pathological changes in EC permeability and function.[17] Activated platelets also express and secrete CD40 ligand. It engages CD40 on leukocytes and stimulates several important immune responses, such as chemokine release by macrophages, maturation of dendritic cells, isotype switching of B lymphocytes, and enhanced activation of cytotoxic T lymphocytes.[14] Of note, eliminating platelet-neutrophil interactions in a mouse model of acute lung injury protects against hypoxemia and increase survival;[18] furthermore, enhanced platelet-neutrophil interactions may play a role in a peculiar range of human diseases—from sudden infant death syndrome[19] to cystic fibrosis[20] to transfusion-related lung injury (see text below).

Platelet-lymphocyte interactions are directed by chemokines. Megakaryocytes synthesize several chemokines, and these molecules are stored in platelet a granules and released upon activation. Platelets contain the α-chemokines platelet factor IV, growth-regulating oncogene α, platelet basic protein (which is cleaved to β thromboglobulin), epithelial neutrophil activating protein-78, IL-8, β-chemokines inflammatory peptide-1α, RANTES (regulated on activation normal T cell expressed and secreted), monocyte chemotactic protein-3, and thymus and activation-regulated chemokine. These effect immune responses by binding to specific receptors on lymphocytes, monocytes, and NK cells designated CXCR (for the a-chemokines) and CCR (for the b-chemokines). Platelet-released chemokines stimulate helper and cytotoxic T cells, B cells, monocytes, and NK cells. Platelets themselves express CXCR4, CCR1, CCR3, and CCR4, and these receptors transduce functionally important "priming" signals when they engage their specific chemokine ligands.[21]

PLATELET SECRETION

Platelets store or synthesize many other bioactive compounds capable of regulating platelet, coagulation protein, leukocyte, and vascular wall responses. The most prominent of these are pharmacological targets: aspirin-inhibitable thromboxane A2 (TXA2), which is proaggregatory and vasoconstricting; and thienopyridine-inhibitable adenosine diphosphate (ADP), which is proaggregatory. TXA2 is

synthesized from membrane arachidonic acid by platelet COX-1, and ADP is stored in dense granules and released upon platelet stimulation.

In addition, megakaryocytes synthesize and platelets store a variety of growth factors, cytokines, and adhesive proteins, each of which is capable of influencing short- and long-term tissue responses. Platelet α-granules contain platelet-derived growth factor (PDGF), transforming growth factor-β, and basic fibroblast growth factor, and their release into the local microenvironment may cause profound pathological changes, including myelofibrosis and perhaps even pulmonary fibrosis (see below). Platelets also store vascular endothelial growth factor-A (VEFG-A) along with a "panoply of endothelial trophogens."[22] In the steady state, they comprise the tools by which platelets accomplish their task as "guardians of the microvasculature." When they are deficient, such as in thrombocytopenic states, it may not be possible to maintain hemostatic EC junctions, and the break in these junctions results in mucocutaneous bleeding. When they are secreted in excess, they may trigger neovascularization and angiogenesis, and thereby contribute to tissue repair and remodeling, and perhaps tumor cell growth.[23]

PLATELET-COMPLEMENT INTERACTIONS

The complement system provides another link between thrombosis and inflammation. Platelets are primed by several complement proteins and are activated to secrete and aggregate by C1q, C3a, and C5b-9.[24-26] Conversely, activated platelets activate the complement system.[26,27] Complement-activated platelets are a rich source of procoagulant microparticles,[25] which appear to feed-forward activate the complement system.[27] Mutations in complement regulatory proteins, most commonly Factor H, result in a thrombotic microangiopathy known as atypical hemolytic uremic syndrome that almost always involves the kidneys and almost never involves the lungs.[28] The role of complement in other TMAs and the basis for sparse pulmonary vasculature involvement in the aHUS and other TMAs[29] are uncertain.

PLATELETS IN INNATE IMMUNITY

Innate immunity is an organism's first defense against infection. Human innate immunity involves biological systems that began evolving over 400 million years ago, when both immunity and coagulation were effected by a single cascade of reactions mediated by proteins activated by limited proteolysis and sharing similar domain architectures, including an identical serine-histidine-asparagine amino

acid sequence in their active sites. The best living vestige of this is the horseshoe crab, which is protected against blood loss and microbial invasion by the same series of catalytic serine proteases, the building blocks of which are considered to have divergently evolved into human coagulation and complement systems.[30] The horseshoe crab also uses a single circulating cell, the hemocyte, for immune and hemostatic functions. The hemocyte is the evolutionary precursor of both leukocytes and platelets, so it is not surprising that human platelets serve an innate immune function by recognizing, binding, and contributing to the killing of invasive microbes. They do this through direct microbial-platelet interactions[31,32] and indirectly through interactions with extracellular histones generated by activated neutrophils.[33,34]

Platelets have docking sites on their surface glycoproteins (for example, the GpIb complex and integrin $\alpha IIb\beta 3$ [GpIIb-IIIa]) for many Staphylococcus species of bacteria and are considered to be a "first responder" to bacterial wound invasion. The bacterial/platelet interaction leads to platelet aggregation, which may sequester the microbe and stop additional bacterial influx through the damaged vasculature. Platelets also express Toll-like receptors (TLR) 2 and 4 and when they encounter specific bacterial pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), which release a variety of platelet microbicidal proteins (PMPs).[35] There are five families of PMPs that affect extracellular killing: the PF4; platelet basic protein; RANTES; thymosinβ4; and fibrinopeptides families. PMPs include several chemokines that are designated "kinocidins" because of their versatility; bacterial killing is coupled to PMP-induced leukocyte recruitment and PMPs decorate bacteria and enhance phagocytosis and killing by leukocytes.

Platelets also help defend against microbial invasion through their contribution to the construction of neutrophil extracellular traps (NETs). NETs are comprised of a tangled meshwork of neutrophil DNA and histones released actively (by LPS over minutes) or through apoptosis (Staphylococcus aureus causes this over hours). NETs capture invading microbes and sequester them with a variety of highly concentrated microbicidal proteins that kill both bacteria and fungi. Histones H3 and H4 activate platelets via TLR2 and TLR4, leading to secretion of PMPs, P-selectin expression, platelet aggregation, prothrombinase activity, and fibrin deposition (Fig. 4).[35] These responses cause an invading microbe to be trapped and killed within a platelet-fibrin clot, and thereby prevent local and systemic dissemination. It is hypothesized, but not proven, that Staphyococcus and LPS-induced sepsis may in part cause thrombocytopenia, coagulopathy, microvascular thromboses, and multiorgan failure—including ARDS—through mechanisms that
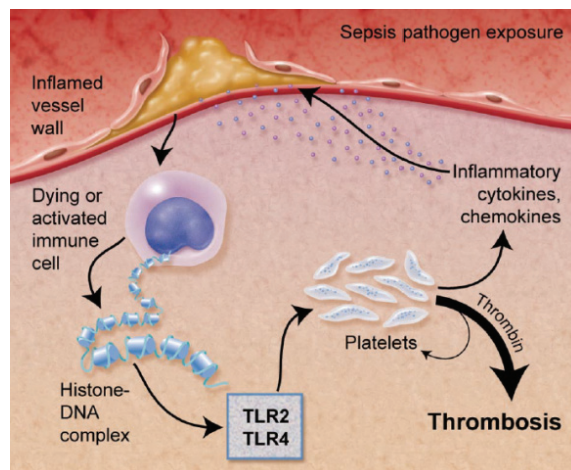
Figure 4: Histone-DNA complexes are released from leukocytes and activate platelets by binding to their toll-like receptors TLR2 and TLR4. Activated or apoptotic neutrophils release histones that bind to specific platelet toll-like receptors. This results in platelet activation leading to several important antimicrobial, proinflammatory, and prothrombotic responses, including the secretion of platelet microbicidal proteins, P-selectin expression, platelet aggregation, and the expression on the platelet surface of prothrombinase activity leading to fibrin production and deposition. This figure was originally published in *Blood* by the American Society of Hematology (ASH). Inflammation and the platelet histone trap by Lea M. Beaulieu and Jane E. Freedman in Blood 2011;118:1714-1715. © 2012 Reproduced with permission of ASH in the format republish in a journal via Copyright Clearance Center.

involve intravascular and systemic histone-platelet TLR2/4-induced NET formation.

PLATELET PHARMACOLOGY

Only a small group of antithrombotic drugs target platelets (Fig. 3).[36] They are used for the primary and secondary prevention of coronary, cerebral, and peripheral arterial thrombosis, or to improve outcomes in acute coronary syndromes with or without percutaneous coronary interventions.

Aspirin is a nonselective cyclooxygenase (COX) inhibitor that irreversibly acetylates serine 529 in the catalytic pocket of platelet COX-1 (constitutive COX) and inhibits thromboxane A2 synthesis for the life of the platelet. Clopidogrel and prasugrel are thieneopyridine compounds that require metabolism through the hepatic cytochrome P450 system to products that irreversibly bind to and block one of the three platelet ADP receptors—the $P2Y_{12}$ receptor. Ticagrelor is a small molecule inhibitor that binds reversibly to the platelet $P2Y_{12}$ receptor. Each of these drugs alone imparts only a relatively small degree of platelet inhibition for an obvious reason: each inhibits only one of the many pathways that signal platelet activation leading to secretion and aggregation. They are therefore associated only with incremental therapeutic effects and only a small risk of bleeding.[36]

In contrast, the aIIbb3 blockers eliminate all platelet-platelet interactions and are extremely efficacious when used for brief periods to treat persons with acute coronary syndromes. The three drugs available in the US are abciximab, eptifibatide, and tirofiban. Abciximab is slowly reversible, and epitifibatide and tirofiban are rapidly reversible. Each binds and inhibits ligand recognition by aIIbb3, leading to platelet paralysis; aggregation in response to every physiological agonist is eliminated. Their extreme potency to inhibit platelet aggregation translates into extreme benefits and risks, leading to reductions in short-term mortality of ACS at the cost of increased bleeding, including a small risk of severe bleeding. Their extreme potency for inhibiting platelet aggregation translates into extreme benefits and risks, leading to reductions in short-term mortality of ACS at the cost of increased bleeding, including a small risk of severe bleeding.[36]

PULMONARY VASCULAR ANATOMY AND RHEOLOGY

The pulmonary artery is relatively unaffected by atherothrombosis. It is also relatively resistant to bleeding in the face of extreme hemostatic challenge. The basis for these clinical observations is not clear, but it is likely to emanate from the biophysical properties of the pulmonary vasculature. The pulmonary arteries diverge by 15 branch orders into a vast network of capillaries whose endothelial cells abut alveolar epithelial cells organized into deformable structures that most closely resemble polyhedrons when the alveolar sac is fully inflated.[37] These capillaries, filled with oxygen-rich blood, re-converge about 15 times into increasingly larger pulmonary veins. Vessels of successive orders are connected in series, and vessels within each order are connected in parallel. This circuitry design maintains low resistance (high capacitance) and low pressure in the face of high pulsatile blood flow (the entire right ventricular output) moving through a vast circuit estimated to comprise 300 million arteries, 300 million veins, and several billion capillaries.[12]

Afferent arterioles are defined as 100 µm descending to <10 µm in diameter, with little subendothelium separating the luminal endothelium from the vascular smooth muscle cell layer. The capillary bed is comprised of an extensive collateral network of tiny (~6 µm) thin-walled vessels that function in gas and solute exchange. The pulmonary capillaries are comprised of thin, (100-200 nm) continuous (without fenestrations) endothelial cells. There is one capillary per interalveolar septum.[37] Capillaries then converge into widening branches of the efferent venules, which expand in size from tens to hundreds of microns in diameter. Pulmonary venules lack two features found

in almost all other venules, and the lack of these features undoubtedly reflects important hemostatic function. The pulmonary venules lack fenestrae—that permit egress of cells and proteins adluminally in other circuits—and valves—that prevent backflux into the capillary network. Rather than valves, pulmonary venules and veins possess a nearly continuous layer of smooth muscle cells surrounding the endothelium. This smooth muscle layer buttresses the barrier function of the return circuit and actively maintains blood flow out of alveolar interstitium all the way back to the left atrium through sequential sphincter-like contractions.[38] It is reasonable to speculate that these features are anatomic elements essential for protecting the lungs against hemostatic challenges that typically lead to hemorrhage from the venular compartment in other tissues and organs.

VIRCHOW'S TRIAD AND THE PULMONARY VASCULATURE

Rheological principles govern pulmonary vascular biology, physiological hemostasis, and pathological thrombosis, but blood flow is only one component of a complex nexus of variables that modulate pro-hemostatic and pro-thrombotic responses. Our understanding of these complexities has been organized for over one and one-half centuries according to the model of the eminent German pathologist Rudolph Virchow. Virchow's triad reminds us that hemostasis and thrombosis are regulated by the simultaneous interactions between blood (cells and soluble constituent), blood vessel (endothelium, subendothelium, and smooth muscle), and blood flow (related to diameter, branching, turbulence, and obstruction). In considering the variables that regulate platelet-dependent hemostasis and thrombosis in the lungs, one can begin by teasing apart Virchow's triad to see what it looks like within the pulmonary vascular compartment.

BLOOD FLOW

Shear stress is defined as the force per-unit area exerted on blood by blood flowing in layers of differential velocity. Blood flow in tubular structures results in highest velocity and lowest shear stress in the central stream and with lowest velocity and highest shear stress at the vessel wall. Platelet-dependent reactions occur in vascular compartments with high wall shear stress, while the soluble coagulation protein reactions assemble and generate fibrin under low shear conditions. The healthy pulmonary circuit maintains relatively low shear stress throughout its expanse because of its ability to accommodate increased flow without changing its intraluminal pressure. This happens because of vasodilation of both arterial and venous elements, and because of the recruitment of capillary

reservoirs during episodes of increased alveolar ventilation. It is only in diseased vessels, such as those in pulmonary hypertension, that blood flow in the pulmonary artery is accompanied by elevated pressures and the generation of pathologically elevated wall shear stress.

The healthy pulmonary artery and its branches maintain shear stresses below 30 dyne/cm^2. Shear stresses may rise to 60 dynes/cm^2 in "feed" arterioles which bifurcate into branch arterioles at ~200 μm intervals, with declining flow velocities and shear stress as branches narrow (for example, a first branch of 20 dynes/cm^2 shear stress → a fourth branch shear stress of 9 dynes/cm^2). At branch points there is turbulence, increased resistance, and even backflux as cyclical blood flow overcomes afferent arteriolar autoregulatory vasoconstriction triggered with every cardiac diastolic relaxation. As blood enters the extensively branched capillary bed, shear stress decreases further. There are very few published data that provide one with precise measurements of shear stress—or other rheological parameters—in capillaries, but estimates based on viscosity calculations and flow and pressure measurements leading into and out of capillaries indicate that physiological capillary blood flow is low flow velocity and generates low shear stresses. The branching post-capillary venules then converge into efferent vessels of increasing diameter and capacitance and gradually increasing—but still low—shear stress (<5 dynes/cm^2). In the pulmonary system, venular backflux is prevented and centripetal flow maintained by smooth muscle "sphincters" in venules 50-100 μm in diameter. Flow turbulence decreases and flow velocity increases as smaller venular branches come together into larger venules and, eventually, form the pulmonary veins.

BLOOD

von Willebrand factor

Pathological shear stress-dependent platelet adherence in arteries and arterioles is triggered by platelet GpIbα binding to plasma or vessel wall von Willebrand factor (VWF). VWF is synthesized by vascular endothelium and by megakaryocytes. It is constitutively released adluminally (into the subendothelium) and abluminally (into the blood) by the endothelium, and it is also stored and secreted following cellular activation (stored in endothelial cell Weibel-Palade bodies and in platelets' α-granules). It is a multimeric protein built up of tens to hundreds of disulfide-bonded multivalent protomeric units. Larger multimers appear to have greater hemostatic and prothrombotic properties.

The protomeric subunit is comprised of two disulfide-linked mature VWF monomers, each one of which is divided structurally and functionally into several domains: the

A, B, C, and D domains. The A domains are of particular importance because the A1 domain forms the primary GpIbα recognition site and binds to type VI collagen; the A2 domain contains the recognition sequence for degradation by the VWF multimer-cleaving protease ADAMTS 13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif); and the A3 domain binds to fibrillar collagens type I and III found in arterial subendothelium, thus allowing soluble VWF to tack down onto the subendothelium of ruptured atherosclerotic plaques. Two other domains of VWF are also important for hemostasis and thrombosis: the C2 domain contains a RGD integrin recognition domain essential for VWF binding to platelet αIIbβ3; and an N-terminal D domain binds factor VIII.[39]

Platelet GpIb complex

The GpIb complex is made up of four, type I transmembranous proteins: GpIbα is disulfide-bound to GpIbβ and GpIbα/β is non-covalently bound to GpIX. Two GpIbα/β/IX complexes are non-covalently bound to a single molecule of GpV. The VWF binding site is on a region of the N-terminal extracellular domain of GpIbα. The conformation of this region of GpIbα is altered by shear stress into a ligand-receptive conformation.[40] Upon ligand binding under high shear stress conditions, the cytoplasmic domain of GpIbα signals secretion and αIIbβ3 activation, possibly through cytoskeletal-based mechanotransduction.[41]

VWF binding to GpIbα is required for microvascular hemostasis, which appears to be triggered in the injured arteriole—a high shear stress microenvironment—by soluble and subendothelial VWF alone, or bound to collagen (type VI may be the predominant microvascular collagen) attaching to platelets via GpIbα, with the subsequent activation of αIIbβ3 to a ligand-receptive conformation. The high shear stress in the arteriole limits fibrin deposition and leukocyte recruitment, and neither soluble coagulation factors nor leukocyte number or function contribute in any clinically important manner to microvascular hemostasis in the epithelium (like skin, mucous membranes, and the urinary and GI tracts). Only the GpIb complex, which forms catch bonds with VWF of very high tensile strength and therefore capable of withstanding the disrupting effects of elevated shear forces, can capture, adhere, and accrue platelets in arterioles or stenotic arteries.[42]

As shear stress in the occlusive hemostatic plug falls to venular levels, fibrinogen binding to activated αIIbβ3 is important for inter-platelet cohesion. The importance of fibrinogen is emphasized by clinical observations that fibrinogen or αIIbβ3 deficiency causes a severe hemostatic defect. Regional regulation of the VWF-triggered hemostatic plug induced by a bleeding time wound is remarkably fine-tuned, as the platelet-rich thrombi accrue only at the

mouths of the transected arterioles, and little or no platelet accumulation occurs within the adjacent arteriole lumen.

VWF-dependent arteriolar platelet thrombosis is the hallmark of thrombotic thrombocytopenia purpura (TTP) and the hemolytic-uremic syndrome (HUS). In TTP, the primary pathogenetic event may be an acquired autoantibody-mediated deficiency of ADAMTS-13, resulting in persistent "ultra-large" VWF multimers—on endothelial cell (EC) surfaces and in the blood—effecting GpIb-IX-V-dependent platelet adhesion, secretion (predominantly dense granules which contain co-stimulatory adenosine diphosphate), $\alpha IIb\beta 3$ activation, and aggregation. In diarrhea-associated HUS, the primary pathophysiology may involve an enterotoxin-mediated over-stimulation and under-degradation of "ultra-large" VWF multimers from arteriolar endothelial Weibel-Palade bodies. This bolus of large VWF multimers remains attached to the EC surface and thereby recruits passing platelets through the shear-dependent binding of platelet GpIbα to the EC-associated VWF.[43] A similar effect may occur in low shear stress post-capillary venules when rapid EC Weibel-Palade body release is stimulated by calcium ionophore or histamine, suggesting that there may be clinical conditions associated with venular inflammation in which part of the pathophysiological process is caused by VWF/GpIb-IX-V-mediated platelet recruitment within a low shear stress microenvironment.

Co-receptors

While the GpIb-IX-V complex is required for the initiation of microvascular hemostasis and is a primary factor contributing to microvascular thrombosis in TTP and HUS, several additional platelet receptors are involved in both microvascular physiology and pathology.[13,15] In every case, the co-receptor functions to support platelet-mediated microvascular responses after GpIb-IX-V engages ligands (the co-receptor supports post-adhesion responses), and in some cases the co-receptor requires activation or expression downstream of VWF/GpIb-IX-V interactions ($\alpha IIb\beta 3$ and $\alpha 1\beta 2$ are "activated," while P-selectin is expressed following α-granule secretion). Observations of human and/or mouse deficiencies of $\alpha IIb\beta 3$ (the human defect is called Glanzmann's thrombasthenia) reveal that it causes a severe bleeding disorder but protects against macrovascular arterial thrombosis. Similar observations have been made with the protease-activated receptors that mediate thrombin-induced platelet activation; the thromboxane A_2 receptor, and the P2Y$_{12}$ receptor that binds adenosine diphosphate (ADP), is the target of thienopyridine anti-platelet drugs and appears to be indispensable to platelet aggregation triggered by VWF binding to GpIb-IX-V. Similar but lesser effects on hemostasis and macrovascular thrombosis are seen in mice lacking the P2Y$_1$ receptor or P-selectin. In contrast,

human deficiencies of the collagen receptors—GpVI or integrin $\alpha 2\beta 1$—result in a mild bleeding diathesis, while GpVI- or $\alpha 2\beta 1$-deficient mice may have only a small or no hemostatic defect, respectively. Either GpVI or $\alpha 2\beta 1$ deficiency, or pharmacological perturbation, results in delayed and decreased *ex vivo* thrombus formation on type 1 collagen under both arteriolar and low shear stress conditions, indicating that GpVI and $\alpha 2\beta 1$ (probably to a lesser extent in comparison to GpVI) are important but secondary mediators of microvascular thrombosis. The prototypical example of a platelet co-receptor that appears to effect hemostasis and thrombosis paradoxically is platelet endothelial cell adhesion molecule (PECAM)-1, which is expressed by the microvascular endothelium and platelets. Mice deficient in PECAM-1 have a hemostatic defect not because of a loss of platelet expression, but because they lack homotypic interactions between PECAM-1 on adjacent ECs needed to regulate endothelial integrity. In fact, PECAM-1-deficient platelets are actually hyper-responsive to both VWF and collagen because PECAM-1 is a negative regulator of GpIb-IX-V- and GpVI-dependent signaling.[44]

ADAMTS-13

ADAMTS-13 is the VWF multimer-cleaving protease. It is synthesized in the liver, circulates in blood, and may attach to the vascular endothelium. While the regional distribution of ADAMTS-13 activity is not yet understood, based on the end-organ pathology of TTP it appears to play an important role in processing "ultra-large" VWF multimers—released constitutively and secreted following EC stimulation—in the cerebral, mesenteric, myocardial, splenic, renal, pancreatic, and adrenal arterioles. It also appears that arteriole-level shear stress is required for ADAMTS-13-mediated cleavage of "ultra-large" VWF multimers: higher levels of shear stress open up or untangle the multimers and thereby expose the ADAMTS-13 cleavage site in the VWF monomer A2 domain.

The mechanism by which ADAMTS-13 deficiency leads to TTP is pretty well understood.[45] Under physiological conditions, ADAMTS-13 is synthesized, homes to the arteriole EC, and, under arteriole levels of shear stress, breaks down prothrombotic "ultra-large" VWF multimers. This maintains blood flow within the microvasculature. When ADAMTS-13 is deficient, arteriolar-level shear stress triggers platelet GpIbα binding to the unprocessed multimers, thus causing the arterioles to become occluded with both EC-attached platelets and platelet clumps that exceed the diameter of the narrowing arteriolar branches. This leads to ischemia and infarction of many organs.

Of note, the lungs are usually spared from the thrombotic complications of TTP, HUS, or other thrombotic microangiopathies despite that the pulmonary artery and its branches are lined by endothelial cells that synthesize VWF. It may be that ADAMTS-13 is not involved in pulmonary

vascular hemostasis and therefore a systemic deficiency of ADAMTS-13 has no impact in the lungs. ADAMTS-13 is not expressed in pulmonary tissues,[46] but the absence of lung involvement in TTP suggests that ADAMTS-13 produced by the liver is sequestered by the pulmonary vasculature.

Red blood cells

Platelet GpIb-IX-V-dependent hemostasis is mainly a physiological microvascular response to injury. In addition to the soluble factors that interact directly with platelets, cellular blood elements modify platelet/vascular interactions indirectly. The most important of these are red cells. Under tubular flow conditions, red cells flow centrally and push the platelet stream peripherally toward the vessel wall. Because blood flow in a tubular arteriole is parabolic and comprised of an infinite number of infinitesimal laminae sliding against one another, the centrifugal movement of platelets exposes them to flow laminae of lowest velocity and highest shear stress, slowing them and making wall collisions more frequent and efficient (or sticky). As arterioles narrow and branch into capillaries, their luminal diameter narrows, red cells are excluded from the central stream (and eventually the entire stream), flow velocity falls, and platelets become evenly dispersed throughout the blood stream.

If the average pulmonary capillary diameter is calculated to be 5.8 μm, and the size of a platelet is 3 μm, and a red cell is 7 μm, it becomes intuitively apparent that platelets flow relatively better (e.g., faster) than red cells through these capillaries. This facilitates red cell-mediated oxygen uptake in the pulmonary alveoli and delivery everywhere else. And it also keeps platelets from slowing and sticking because platelet/capillary interactions are minimized simply because more platelets are in the central stream as they pass around red cells squeezing their way through the microvascular network. One can speculate that pulmonary capillary beds may be relatively protected against platelet-induced thrombosis because these rheological factors, which are a direct consequence of capillary diameter and red cell size, shape, and deformability, keep platelets flowing through the thin central stream of capillary blood. The calculated velocity of platelet flow through a pulmonary capillary is ~500 μm per second.

A similar rheological environment is found in venules, where the hemostatic response is compartmentalized: until red cells that have squeezed their way through the capillary bed gradually queue back into concentric central stream laminae, platelets remain randomly dispersed throughout the venular lumen even as the flowing blood accelerates up to velocities three to four times greater than those found in the capillaries. This means that platelet and venule wall collisions leading to attachment are relatively rare. This is the main reason why defects in platelet-dependent hemostasis in most tissues are manifested by post-capillary venular bleeding. Why does not this occur in the lungs? Perhaps because lung venules are relatively protected from red cell leakage by smooth muscle cells that wrap around the endothelium to create a hemostatic barrier that can be enhanced by vasoconstriction.

The pulmonary venule barrier may be a double-edged sword, however, as the pulmonary post-capillary venule is the site where non-deformable sickled red cells cause vasoocclusion leading to the acute chest syndrome in sickle cell diseases.[47] In fact, the major defect resulting from red cell sickling that leads to vasoocclusion is a perturbation in the natural anticoagulant properties of the intact vascular endothelium. Sickled cells trapped in the post-capillary venules undergo hemolysis and release free hemoglobin, which scavenges nitric oxide (NO) and stimulates arginase, thereby decreasing arginine, which is the substrate for endothelial cells nitric oxide synthase. The sum result is that the pulmonary post-capillary venule suffers a deficiency of vasodilatory and anti-platelet NO, leading to ischemia from imbalanced vasoconstriction and platelet activation. In the long-term, these events lead to pathological remodeling of the pulmonary microvasculature associated with pulmonary hypertension in ~ 20% of patients with sickle cell disease.[47] A similar effect may be the basis for the development of pulmonary hypertension in thalassemia.[48]

BLOOD VESSEL

The vasculopathy of sickle cell disease is an example of the importance of an intact vascular endothelium in actively maintaining blood fluidity. A bleeding time wound shows platelet thrombus formation only at the site of arteriolar transection because the adjacent arteriolar, capillary, and venular ECs constitutively secrete or express on their surface molecules that prevent platelet adhesion, secretion, and aggregation. These include PGI_2 and nitric oxide (both of which inhibit platelet adhesion and activation), the ectoADPase CD39 (which breaks soluble ADP down), thrombomodulin and heparin sulfates (which bind and inactivate thrombin), and urokinase-type plasminogen activator (uPA), and tissue plasminogen activator (tPA; which generate plasmin capable of degrading VWF and fibrinogen).

The capillary endothelium is particularly richly endowed with tissue factor pathway inhibitor (TFPI) which is both secreted into the blood and retained on the capillary endothelium, where it binds to and inactivates the tissue factor/Factor VIIa/ Factor Xa complex, thereby eliminating thrombin generation. This suggests that the capillary may be an important gate preventing the initiation of coagulation despite continuous low-level exposure to pro-thrombotic stimuli.

Only uPA or tPA deficiency is associated with spontaneous microvascular thromboses, but a deficiency of any one of the vessel-derived natural anticoagulants leads to exaggerated responses to thrombotic stimuli. The exception to this is CD39 deficiency, which causes a severe bleeding diathesis due to compromised hemostasis because elevated blood levels of adenine nucleotides lead to $P2Y_1$—mediated platelet desensitization. This is a useful reminder of the dynamic nature of the complex interactions that occur over time within Virchow's triad.

Subendothelial VWF is most abundant in the macrovasculature (most large veins > pulmonary artery > cerebral arteries > aorta > coronary arteries > renal arteries > hepatic arteries > pulmonary vein). It is generally less abundant in microvascular subendothelium, and its distribution in the microvasculature is noteworthy: venules > arterioles > capillaries, with very little or no VWF observed in any embryo capillary bed (in mice) and with relatively little VWF observed in adult myocardium (in pigs).

PATHOLOGICAL INTERPLAY BETWEEN PLATELETS AND THE PULMONARY CIRCULATION

As the pathogenesis of pulmonary vascular diseases becomes increasingly illuminated, one can find many shadows of platelets, but the links that could direct innovations in diagnosis and treatment are missing. This probably reflects normal physiology; the lungs are endowed with innate protection against several platelet-dependent disorders, such as atherothrombosis, microvascular thrombosis, and hemorrhage from severe thrombocytopenia or functional platelet disorders. And it is clear that the pulmonary vascular elements within Virchow's triad that are most important in effecting this protection are its unique rheology and vessel wall anatomy. Therefore, to examine the role of platelets in pulmonary vascular pathobiology, one must begin by shining a light on them to see what they are doing within Virchow's triad in different disease states.[49,50]

HEMORRHAGIC INTERPLAY

Alveolar hemorrhage
Diffuse alveolar hemorrhage (DAH) develops in stem cell transplant (SCT) recipients. Its overall incidence is estimated at 1-21% and it occurs equally frequently in both auto- and allo-SCT.[51] In one series, DAH was the principal diagnosis in 39% of SCT patients referred for bronchoscopy; nearly half of these patients died from respiratory failure or multiorgan failure.[52] While DAH is clearly due to severe pulmonary microvascular

derangements from radiotherapy, chemotherapy, graft-versus-host disease, and superimposed infection, thrombocytopenia is an important contributing factor and a standard therapeutic target.[52]

DAH also develops in association with connective tissue diseases, Goodpasture's syndrome (where it leads to the "pulmonary-renal syndrome"), antiphospholipid antibodies, and many other vasculitides.[53-55] DAH is also part of a rare, congenital syndrome of "idiopathic pulmonary hemosiderosis"[56] and a similar acquired disorder sometimes associated with primary pulmonary hypertension designated "pulmonary capillary hemangiomatosis."[57]

In most cases of DAH, the primary insult is to the microvasculature and presumably the post-capillary venules. This insult leads to venular leakage of red cells despite normal hemostatic function (i.e., normal platelets and coagulation activity). Of note, hemoptysis in mitral stenosis is often from DAH, indicating that elevated pulmonary venous pressure is transduced back to the microvasculature and causes EC junction failure despite normal platelets and normal peristaltic function of pulmonary venules and veins to move blood centipetally. While there is little evidence of a role for platelet-dependent hemostasis in most cases of DAH outside of the SCT setting, cases of severe and often lethal DAH have been associated with platelet $\alpha IIb\beta3$ (GpIIb-IIIa) inhibitors given to patients with acute coronary syndromes.[58]

THROMBOTIC INTERPLAY

Pulmonary embolism
Pulmonary embolism (PE) is not a primary pulmonary vascular disease. Nonetheless, a massive PE can cause acute right ventricular collapse from pulmonary hypertension and unrecognized or recurrent PEs causes chronic thromboembolic pulmonary hypertension (see below). A PE is a fibrin-rich thrombus that almost always develops in the low flow venous circuit. Platelets become a constituent of the thrombus secondarily, and their role in the pathophysiology and treatment of PE is considered small.[59]

There are experimental[60] and clinical[61] data demonstrating that platelets are activated in venous thromboembolism (VTE). There are also many clinical data demonstrating a small but significant effect of antiplatelet therapy on the primary prevention of VTE.[59] Aspirin is less effective than other pharmacological interventions, and it also causes less bleeding. Because of its ease-of-use, inexpensiveness, and safety, aspirin has been and is currently being examined in two large prospective placebo-controlled

randomized trials of secondary VTE prevention in patients with unprovoked VTE at high risk of recurrence. The WARFASA trial showed positive results (~40% reduction in recurrences in comparison to placebo[62]), and the ASPIRE trial (an Australian trial of 3,000 persons examining if 100 mg aspirin prevents recurrent VTE after standard anticoagulation therapy) is anxiously awaited.[63] It will be interesting if ASPIRE confirms the WARFASA results, and if one is able to examine these cohorts to determine if inhibiting cyclooxygenase affects long-term pulmonary vascular responses to venous thromboembolism, such as the development of chronic thromboembolic pulmonary hypertension.

Thrombotic microangiopathies

Pulmonary vascular endothelial cells (EC) synthesize, store, and release VWF, perhaps more robustly than EC in other circuits. Yet the regulation of VWF processing in the lungs is largely unknown, and VWF-induced platelet-dependent pulmonary vascular thrombosis is considered rare. The paradigm for a VWF and platelet-dependent thrombotic diathesis is thrombotic thrombocytopenic purpura (TTP) which, as previously described above, does not typically involve the lungs. There are, however, reported cases of lung involvement in primary or de novo TTP due to acquired ADAMTS-13.[29] The pathology of the lungs reveals microvascular hyaline thromboses—containing platelets and fibrin—in the terminal arterioles and capillaries.[64] This is associated in some cases with "non-cardiogenic" pulmonary edema from venular capillary leak. It is interesting to note that there are cases of diffuse alveolar hemorrhage due to microvascular thromboses in transplanted lungs of patients who developed post-transplant TTP due to ADAMTS-13 deficiency, an uncommon cause of post-transplant TTP.[65] This is a reminder that the clinical expression of ADAMTS-13 deficiency in any particular organ or tissue depends on other perturbations to Virchow's triad that develop in that vascular compartment.

The physiology of pulmonary vascular ADAMTS-13 is (to our knowledge) unknown. It is likely that ADAMTS-13 is synthesized and secreted by the liver, enters the circulation though the hepatic vein, and then enters the lungs through the pulmonary artery. Once in the lungs, ADAMTS-13 must home to the vascular endothelial compartment; the sites of it homing, its recognition and binding motifs, and how its activity is regulated—in short, almost everything about its presence and function in the pulmonary circulation—however, remain a mystery. It is likely that elucidating the biology and pathology of pulmonary vascular ADAMTS-13 will teach us a lot about protecting the lung from thromboses and perhaps even provide potential therapeutic insight into common pulmonary inflammatory processes, such as pneumonia and asthma.[66,67]

Sickle cell disease

There is systemic platelet activation in sickle cell crises. This is a secondary phenomenon of uncertain pathophysiological consequences. It is due in part to shear-induced platelet activation by flow across a regurgitant tricuspid valve (due to pulmonary hypertension), hemolysed red cell-derived ADP-induced platelet activation, and the decreased endogenous antithrombotic properties of the vascular endothelium due to nitric oxide scavenging by free hemoglobin.[68] VWF also participates in the pathogenesis of sickle cell vasoocclusion, but its effect is independent of platelets and ADAMTS-13 deficiency.[69] Freshly secreted large VWF multimers are involved in the adherence of young red cells ("neocytes"), but not sickled red cells, to the microvascular endothelium.

The two most important pulmonary complications of sickle cell disease are the acute chest syndrome and chronic pulmonary hypertension. Both are common (affect ~30%), due to many different factors (such as infection and necrotic marrow fat embolism) and are not simply the short- and long-term consequences of vaso-occlusion.[70] Within this complex pathophysiological milieu, it is difficult to rank the importance of platelet-dependent thrombosis. One unfavorable prognostic indicator of the acute chest syndrome is a drop in platelet count, but this finding does not appear to represent pulmonary sequestration of platelets or any associated platelet thromboses. There are no data available to assess an effect of antiplatelet therapy in the acute chest syndrome and there appears to be no effect of antiplatelet agents on the natural history of sickle cell disease, including the frequency of the acute chest syndrome and the development of pulmonary hypertension.[71-73]

Pulmonary fibrosis

In the United States, idiopathic pulmonary fibrosis (IPF) affects about 10 persons per 100,000, and the median survival for those affected is between 3 and 6 years.[74] Its pathogenesis—including the role of platelets, VWF, and ADAMTS-13—is not understood, but it is generally not considered to be a primary pulmonary vascular disease.

There are, however, two theoretical reasons to examine platelets in IPF: (1) platelets store and secrete upon activation several cytokines and growth factors allegedly involved in the fibrotic response, such as transforming growth factor-β, fibroblast growth factor, and platelet-derived growth factor;[75] and (2) "secondary" pulmonary fibrosis is associated with inherited storage pool defects due to abnormal packaging of platelet α- and dense granules.

Congenital α-granule deficiency is a very rare bleeding disorder designated the "gray platelet syndrome." The human mutation leading to it has been identified (a gene coding for a trafficking protein), its inheritance and

penetrance are variable, and the platelet phenotype and disease expression are thoroughly cataloged.[76,77] Circulating platelets do not have α-granules due to failure of proteins synthesized by the megakaryocyte (such as growth factors) or endocytosed by megakaryocytes or platelets (such as fibrinogen) to traffic into the secretable pool of α-granule constituents. Most patients with the gray platelet syndrome have myelofibrosis caused by the constitutive release into the bone marrow microenvironment of megakaryocyte-derived growth and fibrosing factors that would normally be packaged into α-granules. There is one case of pulmonary fibrosis reported out of a total of 18 well-characterized gray platelet syndrome families.[78]

Pulmonary fibrosis is more common in the inherited platelet storage pool deficiency designated "Hermansky-Pudlak syndrome" (HPS). This is another rare bleeding disorder due to homozygous deficiency of one of seven HPS genes involved in the biogenesis of several storage organelles, such as platelet dense granules and lysosomes. The clinical expression varies with the specific gene affected, but generally the bleeding disorder is accompanied by oculocutaneous albinism and pulmonary disease. The most common variant is due to a mutation in the HPS1 gene on chromosome 10. A 16-base-pair duplication in exon 15 affects over 400 persons in a small region of Northwest Puerto Rico, and other mutations in HPS1 are found in similarly affected people in Europe and Japan. Almost all of these persons develop pulmonary fibrosis by their fourth decade, and pulmonary fibrosis is lethal in at least 50% by the fifth decade. None develop myelofibrosis.

Mechanisms of pulmonary fibrosis in HPS are not known. One model posits that pulmonary alveolar type II pneumocytes fail to secrete surfactant and that unpackaged lipids accumulate as waxy ceroid deposits in the air spaces. These deposits evoke macrophage-induced lung injury, especially the release of cathepsin L, which stiffens lungs by degrading collagen and elastin.[79]

The role of platelets in HPS-associated pulmonary fibrosis has not been investigated, and mechanisms of platelet-mediated pulmonary fibrosis in the gray platelet syndrome are uncertain; the paradigm of myelofibrosis in the gray platelet syndrome, however, should not be overlooked. It is possible that pulmonary fibrosis develops in both HPS and the gray platelet syndrome because the dysfunctional granule biogenesis directly affects megakaryocytes within a pulmonary vascular compartment. In HPS, leakage of unpackaged lysosomal contents may lead to airway and alveolar damage, and leakage of dense granule serotonin may lead to smooth muscle cell contraction, proliferation, and neointima formation.[80] In the gray platelet syndrome, leakage of mitogenic, angiogenic, and permeability factors may lead to fibrosing airway and alveolar damage.

There is additional evidence in support of a connection between abnormal platelet production and pulmonary pathophysiology. For example, biopsy-proven pulmonary interstitial inflammation accompanies immune thrombocytopenia (ITP) and remits along with the ITP.[81] This is germane because megakaryocytopoiesis is almost always abnormal in ITP, with the bone marrow typically showing megakaryocyte hyperplasia, and it is possible that more brisk megakaryocytopoiesis in the lungs leads to the inflammatory response. As a second example, there is evidence that patients with idiopathic myelofibrosis develop secondary pulmonary hypertension[82] and that patients with primary pulmonary hypertension develop secondary myelofibrosis.[83] The next section will examine these phenomena and attempt to illuminate where platelets fit into this unexpected bidirectional linkage between the pulmonary vasculature and abnormal hematopoiesis.

Pulmonary hypertension

The example that underlies the basis for considering platelets relevant in the pathogenesis of pulmonary hypertension (PH) derives from a rare case of a poorly understood, inherited isolated dense granule deficiency (i.e., not associated with lysosomal or melanosomal abnormalities) associated with PH.[84] The observations that this patient's platelets leaked serotonin and that there were elevated levels of plasma serotonin are important components of the foundation for the "serotonin hypothesis" of PH.[85] And while the role of platelets in PH appears less certain today than when these observations were reported in 1990, data on their potential mechanisms-of-effect in this disease shed some light on how platelets and pulmonary vascular diseases might be linked.[80]

Pulmonary hypertension is associated with thrombocytosis. In many cases, it is a reactive thrombocytosis secondary to lung inflammation developing during the natural history of PH.[85,86] But pulmonary hypertension also occurs in patients with post-splenectomy thrombocytosis[87] and myeloproliferative disease-associated thrombocytosis,[88] and it commonly develops in myelofibrosis with myeloid metaplasia.[82] How do these clinical conditions, each of which imposes unique changes on several elements within Virchow's triad, lead to pulmonary hypertension?

Post-splenectomy or MPD-associated thrombocytosis may contribute to PH through thrombocyte elaboration of growth factors, such as platelet-derived VEGF-A and PDGF. In fact, platelet-derived VEGF-A and PDGF are proposed as mediators of vascular changes developing in idiopathic PH.[89] In post-splenectomy PH, red cells are also important in the pathogenesis of PH. When a spleen is absent there is abnormal red cell processing which causes many bizarrely shaped, less-deformable red cells (poikilocytes) to circulate. Poikilocytes are trapped, ripped up, and hemolysed

within the pulmonary microvasculature. Resulting free hemoglobin shuts down the endogenous NO antithrombotic property of the pulmonary vascular endothelium while free ADP stimulates the platelets, resulting in a prothrombotic imbalance within Virchow's triad.[87]

In the myeloproliferative diseases (MPD)—including myelofibrosis—extramedullary hematopoiesis may also contribute to PH. It develops as stem cells leave the dysfunctional marrow compartment and colonize new compartments, including the lungs. This leads to functional changes similar to those in idiopathic PH, such as arteriolar and capillary obstruction. It also leads to the elaboration of cytokines and growth factors that cause histopathological changes typical of idiopathic PH, including intimal hyperplasia, vascular smooth muscle proliferation, and neovascularization. In support of the extramedullary hematopoiesis model, there is one series of patients with the MPD chronic myelogenous leukemia in which 10/21 autopsies showed pulmonary vascular megakaryopoiesis and 2 out of the 10 with pulmonary megakaryopoiesis had accompanying pulmonary fibrosis.[90] In support of the humoral model of cytokine and growth factor effects, there is one case of MPD-associated PH in which blood levels of platelet factor 4 (a bioactive platelet a-granule constituent) were elevated,[87] and one fairly large series in which 22 patients with myelofibrosis and PH were evaluated and determined to have a "distinctive angiogenic phenotype" characterized by increased circulating endothelial progenitor cells, increased serum VEGF, and increased microvascular density in the marrow compartment.[82]

How else might platelets contribute to pulmonary hypertension? There are ambiguous data about systemic platelet activation in idiopathic pulmonary hypertension and pulmonary hypertension associated with congenital heart diseases causing right-sided overload.[91] More consistent elevations of platelet activation are observed in anorectic medication (like fenfluramine)-induced pulmonary hypertension. In these cases, the common denominator may be cellular activation—leading to serotonin release by platelets and pulmonary vascular smooth muscle cell contraction—by drug-induced blockade of a voltage-sensitive potassium channel.[92-94] Consistent with the idea that platelets are involved in anorectic drug-induced PH, effective PH treatment with the prostaglandin PGI_2 usually leads to decreased platelet activation.[95] In all of these cases, it is impossible to determine if platelet activation is a cause or an effect of pulmonary vascular changes in flow and structure, but it is most likely an effect of uncertain consequence. Similarly, elevations of plasma VWF in association with both primary and secondary PH are probably epiphenomena of no pathophysiological significance.[96] Compelling data show that PH is associated with increased platelet-derived TXA_2

and soluble CD40 ligand, and also decreased endothelial PGI_2. But the pathophysiological importance of these data seems negligible in the face of other data showing that treatment with aspirin or a thienopyridine has no effect on the severity or natural history of PH, and that there is no evidence that PH is associated with an endothelial cell-specific cyclooxygenase 2 inhibitor, such as rofecoxib or celocoxib, which selectively depletes PGI_2.[92,97]

Myelofibrosis in pulmonary hypertension

The connection between bone marrow and pulmonary vascular compartments is as intriguing as it is opaque. This is because it is bidirectional; just as primary myelofibrosis leads to PH, primary PH leads to myelofibrosis.[83,98,99] The association is very strong: when first reported, all 17 primary PH patients who were evaluated had myelofibrosis, including 11 designated as having severe fibrosis.[98] Myelofibrosis is a reactive process (not due to a clonal proliferation of myeloid elements) and it may be clinically important because it is accompanied by anemia and/or thrombocytopenia in the majority of patients.[83] The cause of myelofibrosis is unknown, and there are no pathophysiological elements that are known to bridge lung and marrow fibrosis. Speculation focuses on pathological angiogenesis, and there is one study showing a paucity of microvascular pericytes in the bone marrow of PH patients with secondary myelofibrosis.[99] This finding implies that myelofibrosis is related to aberrant angiogenesis and suggests the possibility that this aberrancy also underlies idiopathic PH. The role of megakaryocytes and platelets has not been explored. One study shows that thrombopoietin is elevated in the right ventricle, pulmonary artery, and left ventricle of patients with primary PH.[100] This could reflect decreased pulmonary thrombopoiesis (platelets regulate blood TPO levels by binding it; they are a TPO "sink") as changes in the vascular compartment in PH drive platelet precursors from the lungs into the bone marrow compartment.

Lung metastasis

Decades of experimental evidence indicate that platelets contribute to the spread of cancer cells, including spread to the lungs. This involves direct tumor cell-platelet interactions and tumor cell-platelet-endothelial cell interactions.[101,102] Platelet elements directing homing of metastases to the lung include the cell surface proteins GpIbα,[103] GpVI,[104] αIIbβ3,[102] and P-selectin.[102] Platelet elements that induce tumor cell changes that promote migration and vascular invasion resulting in hematogenous spread to the pulmonary vascular compartment include the platelet surface receptor CLEC-2[105] and the α granule constituents TGFβ[106] and thrombin.[107] Pulmonary metastasis can be considered to also involve perturbations in other components of Virchow's triad, and there is evidence that endothelial injury[101] and endothelial expression of the vitronectin receptor (αvβ3)[102]

enhance the docking of blood-borne platelet-cancer cell aggregates to the pulmonary vascular endothelium and thereby promote metastasis. Of note, while there is evidence that aspirin may decrease long-term mortality in several solid tumors including lung cancer,[108] there are no data implicating platelet inhibition as the mechanism of this effect.

Miscellaneous lung diseases

Platelets may be significant elements in the pathogenesis of several pulmonary diseases, including some that are not obviously vascular diseases.

Chronic thromboembolic pulmonary hypertension (CTEPH) is due to progressive vasoocclusion of the pulmonary artery developing after venous thromboembolism.[109] Type 2 is proximal- and type 3 is distal-branch intimal thickening and fibrosis, and type 4 is a thrombotic microangiopathy characterized by extensive neovascularization.[110] There is no evidence that platelets are involved in these pathophysiological changes to the pulmonary artery, but one could speculate that types 2 and 3 recapitulate platelet-induced atherothrombotic changes in other arteries,[111] and type 4 results from shear-induced platelet activation causing their release of inflammatory and angiogenic proteins.[41]

The antiphospholipid syndrome (APS) is a thrombotic disorder caused by endothelial inflammation, triggered by anti-phospholipid antibodies.[112] It often involves the pulmonary arteries indirectly as a consequence of VTE, but sometimes thrombosis occurs in the pulmonary arteries in situ,[112] possibly due to platelet-dependent thrombosis.[113] The catastrophic antiphospholipid syndrome is a severe thrombotic microangiopathy leading to multiorgan failure that is deadly in at least one-half of those affected. The pulmonary vasculature is frequently involved.[114,115] The therapeutic anti-C5 antibody eculizumab works in refractory catastrophic APS, suggesting that this could be the paradigm of complement-platelet-pulmonary vascular pathobiology.[116]

Pulmonary venoocclusive disease (VOD) is an uncommon complication of stem cell transplant and an uncommon toxicity of certain chemotherapies, especially bleomycin, mitomycin, and carmustine.[117] It is triggered by post-capillary venular inflammation causing thrombosis. This leads to pulmonary hypertension and right-heart failure. Pulmonary VOD rarely leads to pulmonary capillary hemangiomatosis (PCH), which is an angioproliferative response to post-capillary venular occlusion.[118] For pulmonary VOD and PCH, pathogenetic factors downstream of venular damage are unknown. The role of platelets in thrombosis and in maintaining post-capillary venular integrity, and the fact that platelets are rich sources of cytokine and growth factors, suggests the possibility of their involvement. There is no evidence, however, that antiplatelet therapy (or any therapy) favorably affects the natural history of pulmonary VOD or PCH.

Acute lung injury (ALI) is a vague term for a common condition encompassing pneumonia, aspiration, sepsis, trauma, and even massive transfusion; its most severe manifestation is known as "ARDS."[119] The heterogeneity of causes defines multiple pathogenetic mechanisms, some of which includes platelet-dependent pathology. For example, experimental data demonstrate that platelets are essential for establishing leukocyte-capillary endothelial interactions and subsequent alveolar inflammatory responses in animals with acid- or sepsis-induced of ALI.[18,120] These data introduce that hypothesis that platelet P-selectin is a potential therapeutic target in ALI.[119] As another example, there is evidence that platelet lipid debris accumulating during blood bank storage may contribute to transfusion-associated acute lung injury (TRALI).[121] Most cases of TRALI are from donors' antibodies binding to recipient leukocytes resulting in activated host neutrophils being sequestered in the pulmonary microcirculation. Ten percent of TRALI cases, however, are not associated with leukocyte antibodies, and it is believed that most of these cases are due to the release of biologically active lipids from platelets during storage.[120] There is evidence of platelet and leukocyte activation and circulating leukocyte/platelet aggregates in humans with asthma,[122] R-L shunting of proplatelets, activated platelets in clubbing,[123] and even hyper-reactive platelets, activated platelets, and circulating leukocyte/platelet aggregates in patients with cystic fibrosis.[124] Of note, however, is that there is no evidence for a beneficial effect of antiplatelet therapy with aspirin or a thieneopyridine on ALI, TRALI, asthma, clubbing, or cystic fibrosis. Chronic high-dose ibuprofen may slow the progression of cystic fibrosis, but this is probably not a platelet-mediated effect.[125]

SUMMARY

There is an impressive breadth and depth of information about platelets and how they function within Virchow's triad to effect physiological hemostasis and pathological thrombosis. A similarly impressive information base encompasses pulmonary vascular biology and pathophysiology. Less is known, however, about how platelets function in the lungs under normal and pathological conditions, or about how the lungs affect platelets in hemostasis and thrombosis. But we may be closer to formulating answers to the questions posed in the introduction: Does platelet production in the lungs occur? (almost certainly); Why does severe thrombocytopenia not lead to pulmonary hemorrhage? (the vascular and rheological microenvironments of the

pulmonary capillaries are more hemostatic than the microenvironments of epithelial tissues); and Why does atherothrombosis not develop in the pulmonary artery? (it probably does occur in chronic thromboembolic pulmonary hypertension but is generally absent because flow in the healthy pulmonary artery generates shear stresses below the threshold for triggering VWF-mediated platelet adhesion and activation). New derivative questions inevitably arise: Is lung platelet production related to the fundamental pathophysiology of pulmonary vascular diseases of unknown etiology, such as idiopathic pulmonary hypertension, idiopathic pulmonary fibrosis, or type IV CTEPH? Is there a major molecular mediator of pulmonary hemostasis that could be translated into treatment for patients with refractory thrombocytopenia and intractable bleeding? Why do vasoocclusive lesions of CTEPH develop over the course of months rather than years, as occurs in atherothrombosis of the coronary and cerebral arteries? Someday these questions will be answered. And while we seek answers, please consider that they are likely to be gathered quicker and with greater validity if we examine them after illuminating Virchow's triad within the pulmonary vascular compartment. Illumination will permit us to identify key individual pathophysiological elements that are mechanistically relevant. We can then focus on their contribution to diseases affecting the pulmonary vasculature and try to determine if such pathobiology applies to diseases affecting other tissue compartments.

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