Platelets, June 2008; 19(4): 239–251

informa
healthcare

REVIEW

Platelets: Developmental biology, physiology, and translatable platforms for preclinical investigation and drug development*

NEAL S. KLEIMAN[1], JANE E. FREEDMAN[2], PAULA B. TRACY[3], BARBARA C. FURIE[4], PAUL F. BRAY[5], SUNIL V. RAO[6], DAVID R. PHILLIPS[7], ROBERT F. STOREY[8], CHRISTOPHER P. RUSCONI[9], PATRICIA A. FRENCH[10], STEVEN R. STEINHUBL[11], & RICHARD C. BECKER[12]

[1]*Methodist DeBakey Heart Center, Houston, Texas, USA,* [2]*Boston University School of Medicine, Boston, MA, USA,* [3]*Department of Biochemistry, University of Vermont, Burlington, USA,* [4]*Division of Hemostasis and Thrombosis, Beth Israel Deaconess Medical Center, Boston, MA, USA,* [5]*Division of Hematology, Jefferson Medical College, Philadelphia, PA, USA,* [6]*Department of Medicine, Duke University Medical Center, Durham, NC, USA,* [7]*Portola Pharmaceuticals, Inc., South San Francisco, CA, USA,* [8]*Cardiovascular Research Unit, University of Sheffield, Sheffield, UK,* [9]*Regado Biosciences, Inc., Durham, NC, USA,* [10]*Left Lane Communications, Chapel Hill, NC, USA,* [11]*Division of Cardiology, University of Kentucky, Lexington, USA, and* [12]*Cardiovascular Thrombosis Center, Duke Clinical Research Institute, Durham, NC, USA*

(Received 17 December 2007; revised 23 January 2008; accepted 25 January 2008)

Downloaded At: 13:58 24 March 2009

Abstract

This paper, developed from the proceedings of the 2007 Platelet Colloquium, considers emerging constructs in platelet biology, preclinical models of thrombosis, and their potential application to the development of platelet-directed pharmacotherapies. Discussed first is the developmental biology of platelets, including megakaryocyte maturation, and the role of apoptotic and growth factors and other proteins in thrombopoiesis. A brief overview of current methods and observations from platelet proteomic analyses is also presented, illustrating the complex interplay of genes, gene expression, protein expression, and protein modification in various atherothrombotic phenotypes. The factor Xa-platelet interface is used as a working model for discussion of anticoagulants as platelet antagonists, highlighting the importance of receptor expression, substrate binding kinetics, platelet subpopulations, and cofactors in thrombosis. Finally, we discuss the use of emerging technologies—such as intravital microscopy and ex vivo perfusion chambers—as translatable platforms for investigating the role of platelets and their pharmacologic inhibition in human health and disease.

Keywords: *Platelet, proteomics, glycoprotein, thrombin, factor Xa, microscopy*

Introduction

Knowledge about the structure, biology, and function of platelets has evolved considerably since 1881, when Giulio Bizzozero linked newly identified, discrete particles in the blood, distinct from red and white blood cells, with the coagulation process [1]. The initial Platelet Colloquium [2–4], held in 2006, discussed the biology of platelet activation, current methods of measuring platelet performance, and how clinical investigations, drug-development programs, and clinical practice are hampered by a recognized disconnect between *in vitro* measures of platelet performance and clinical outcomes.

This paper, developed from the proceedings of the 2007 Platelet Colloquium, examines the fundamental biology of platelets and provides a state-of-the-art summary of emerging constructs for scientific investigation and their translation to the development of platelet-directed pharmacotherapies in human atherothrombotic disorders. These include an overview of megakaryocyte maturation, thrombopoiesis, platelet proteomics, the employment of the factor Xa-platelet interface as a working model for

Correspondence: Richard C. Becker, MD, Duke Clinical Research Institute, 2400 Pratt Street, Terrace Level, Room 0311, Durham, NC 27705. Tel: 919-668-8179. Fax: 919-668-7056. E-mail: richard.becker@duke.edu

*This manuscript reflects presentations given at the 2007 Platelet Colloquium, January 18–20, 2007, Amelia Island, Florida, USA (see Acknowledgments for sponsorship).

ISSN 0953–7104 print/ISSN 1369–1635 online © 2008 Informa UK Ltd.
DOI: 10.1080/09537100801947442

understanding the potential platelet-directed effects of anticoagulant compounds, the use of intravital microscopy in the "real time" study of thrombogenesis, and a road map of technologies that emulate the requisite physiological environment of vascular thrombosis *in vivo* as a means to accelerate the development of antithrombotic agents.

The platelet life cycle

The overall process of platelet production [5] begins with the common hematopoietic stem cell (HSC) (Figure 1). Two distinct types of blood cell lines are derived: *lymphoid*, which includes all types of lymphocytes, and *myeloid*, which includes granulocytes, monocytes, red blood cells and platelets. Polyploid megakaryocytes are the immediate progenitors of platelets.

Megakaryocytes regenerate in human bone marrow at the rate of about 10^8 cells per day [5]; each megakaryocyte, in turn, can generate more than 5000 platelets. Production of such a number of cells, each with a relatively short life span (7–10 days), likely offers a teleological advantage in terms of speed and adaptability to hemostatic challenges.

Thrombopoeitin—a heavily glycosylated, 332-amino-acid protein synthesized primarily in the liver, and to a lesser extent in the kidney and bone marrow stroma—is a particularly important cytokine, stimulating megakaryocyte proliferation and differentiation [6]. Formerly known as the c-Mpl ligand [7], thrombopoeitin selectively induces maturation and release of platelets, supports the survival and expansion of all cells in the megakaryocyte line, and enhances α-granule release and thrombin-induced platelet aggregation [5]. Its receptor has been identified, and platelet production declines by about 85% when this receptor is blocked [5]. Of interest and biological relevance, there are only 25–100 copies of the thrombopoietin receptor per platelet [8].

The megakaryocyte undergoes a series of morphological changes during the 4- to 10-hour process of platelet production (Figure 2) [9]. Nuclear endomitosis and organelle synthesis occurs first, along with expansion of the cytoplasm. An array of microtubules assembled from $\alpha\beta$-tubulin dimers emerges from centrosomes, which then migrate to the cell periphery. Aided by sliding of the microtubules, the megakaryocyte cytoplasm then develops multiple thick pseudopods in preparation for formation of 5–10 *proplatelets*. Organelles and granules migrate along the microtubules to the developing, elongating proplatelet ends, where new platelets will form. Proplatelets are 250–500 µm long on average and, through extensive, actin-dependent branching along their lengths, can produce 100–200 platelets each. The proplatelets are then released from the cell into the vascular sinus, often appearing paired in a dumbbell shape. The nucleus is ejected from the mass of proplatelets, and individual platelets are later released or "budded off" from proplatelet ends.

Megakaryocytes are found adjacent to the bone marrow stroma, and proplatelets extend into the vascular sinus side of the marrow. Occasionally, the proplatelet spans two separate spaces, with at least one observation, in the setting of acute severe thrombocytopenia, showing platelet release in both

Downloaded At: 13:58 24 March 2009

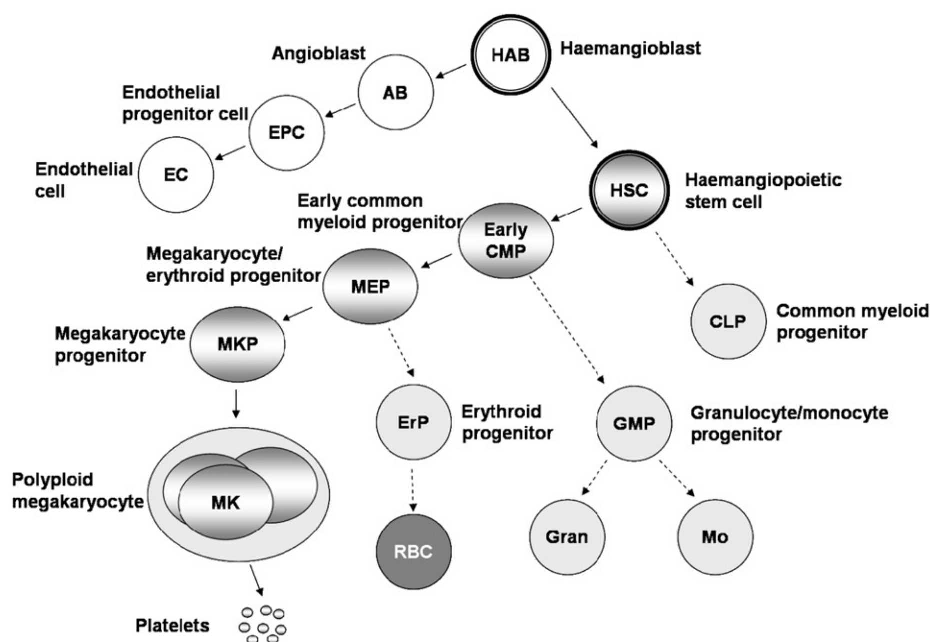

Figure 1. Overall scheme of platelet production sequence. Gran, granulocyte progenitor cell; Mo, monocyte progenitor cell; RBC, red blood cell. Reprinted from Deutsch et al. [5], with permission.

the deep marrow compartment and the vascular sinus [10]. It is unknown what effect differing environments (and circulating compounds) might have on newly formed platelets, but at least some platelets found within the deep marrow may be sequestered from the influence of large molecules [10].

Apoptosis (programmed cell death) plays a pivotal role in platelet formation and release. New megakaryocytes express proteins that inhibit apoptosis, such as Bcl-2 and Bcl-xL. Overexpression of these factors inhibits megakaryocyte differentiation into proplatelets [11, 12]. In contrast, mature platelets do not express Bcl-2, and older megakaryocytes do not express Bcl-xL. Megakaryocytes also express proapoptotic factors including caspases, nitric oxide, transforming growth factor (TGF-1), and SMAD proteins [13]. Activated caspases-3 and -9 are involved in proplatelet formation in megakaryocytes [11], but mature platelets contain only caspase-3 [14]. Caspase-12 is also found in megakaryocytes but not in platelets [15]. Nitric oxide can induce both platelet formation and apoptosis in mature megakaryocytes and may act in synergy with thrombopoeitin to induce platelet release [16, 17]. Thus megakaryocytes and platelets have differing, compartmentalized mechanisms for apoptosis, and the selective expression and downregulation of apoptotic factors serves as a biological "thermostat" for the proplatelet–platelet developmental process [18].

Finally, several proteins of fundamental importance are selectively expressed during various stages of platelet development. For example, commitment of a haematopoietic precursor cell to the megakaryocyte line is indicated by the expression of CD61 (β3) and increased CD41 expression [19]. Reticulated platelets—those that have a high mRNA and granule content and are newly released from the bone marrow—express much more cyclooxygenase (COX)-2 than do mature platelets [20, 21] and may

Downloaded At: 13:58 24 March 2009

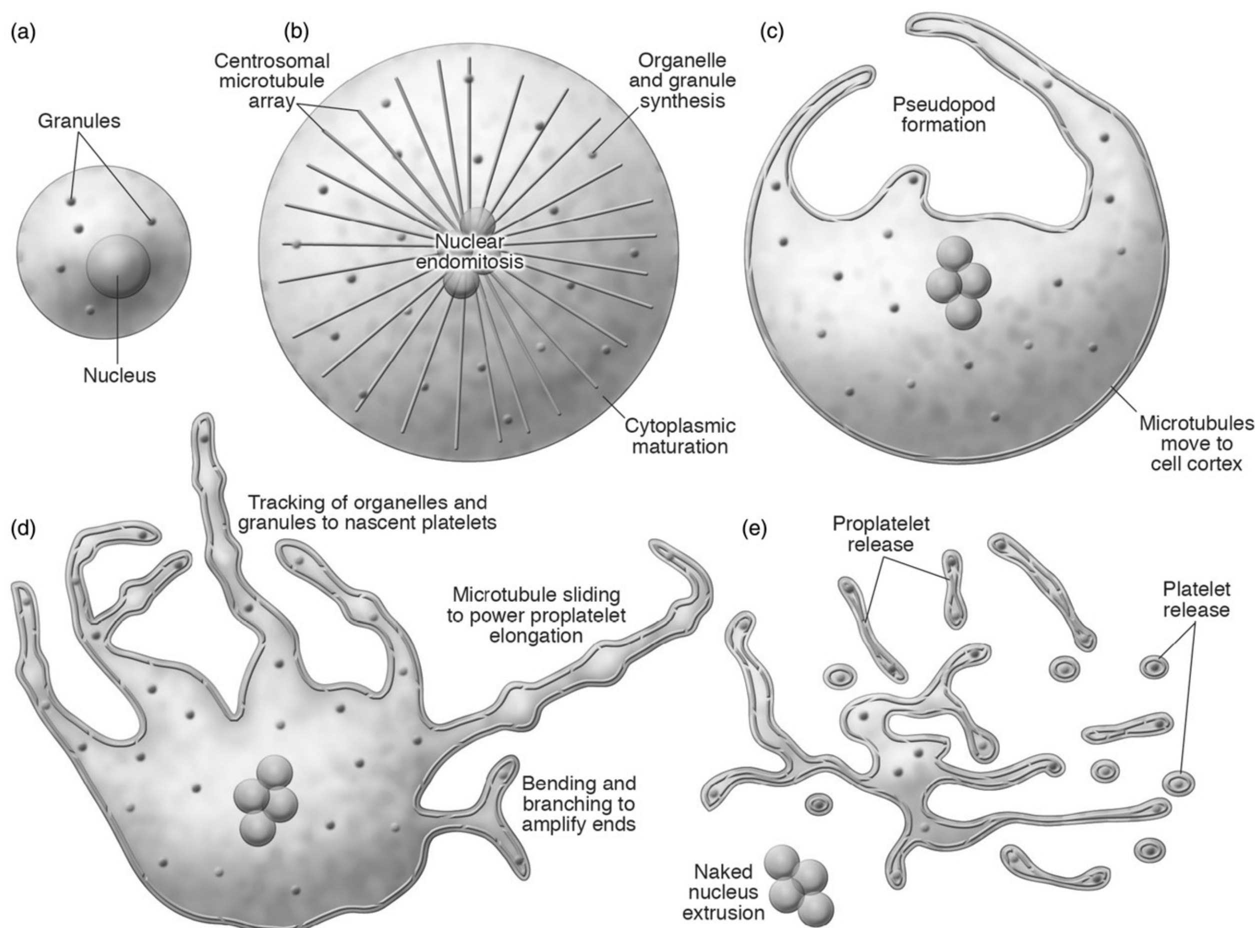

Figure 2. Overview of megakaryocyte production of platelets. Immature megakaryocytes (a) first undergo (b) nuclear endomitosis, organelle synthesis, and cytoplasmic maturation and expansion, while a microtubule array merges from centrosomes. (c) Before proplatelet formation, centrosomes disassemble and microtubules migrate to the cell cortex. Proplatelet formation begins with the development of pseudopods. (d) Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends, where nascent platelets assemble. Proplatelet formation/expansion continues throughout the cell, while bending and branching amplify existing proplatelet ends. (e) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the proplatelet mass, and individual platelets are released from proplatelet ends. Reprinted from Patel et al. [9], with permission.

play a prominent role in the response to antiplatelet drugs [22]. Increased expression of protease-activated receptors (PARs), which are involved in thrombin-mediated platelet activation, has been reported during megakaryocyte differentiation and maturation [23, 24]. Expression of $\alpha_{IIb}\beta_3$ precedes expression of glycoproteins (GPs) Ib-alpha, Ib-beta, V, and IX and, by promoting binding to sinusoidal matrix constituents, may regulate both proplatelet formation and platelet release [25]. $P2Y_1$ and $P2Y_{12}$ also are believed to be functional in the early megakaryocyte developmental stages. The expression, localization, modification, structure, function, and activity profiles of these proteins are summarized in the next section.

Platelet proteomics

As described above, platelets are fragments of megakaryocytic cytoplasm that contain no genomic DNA. They do maintain a small amount of cytoplasmic RNA, however, in addition to the proteins and organelles needed for translation (protein synthesis) [26]. This section discusses early observations and existing limitations of platelet proteomic analyses.

Currently available techniques for proteomic analysis have several limitations. First, each yields unique sets of proteins, making cross-technique comparisons difficult. Typically, only abundant proteins are identified, although some membrane proteins of lower abundance have been identified. In the gel-based systems, about 60% of cell membrane proteins are lost during processing, many of which are of higher molecular weight. In antibody-based arrays, analysis is limited to the available and developed antibodies, which target only specific proteins and have the potential for cross-reactivity. There are also limitations in the purification technique for each method; thus the potential for sample contamination must always be considered. Finally, protein precipitation methods can contribute to variance in either the presence or concentration of specific proteins (i.e. platelet activation will change the protein profile).

About 1000 platelet proteins have been identified using various proteomic approaches, representing only a fraction of what might be predicted based on the results of whole genome expression arrays performed with RNA from megakaryocytes (~10 000 transcripts) [27] and platelets (3000–4000 + transcripts) [25, 28, 29]. One explanation for the observed discrepancy is a requirement for confirmation using a separate method. Another is an inability to capture less abundant proteins. The method of preparation also can affect which proteins are found, although a more recent analysis found good agreement between two different proteomic methods [30]. This same study also functionally classified the 107 proteins identified in the analysis [20]. Most of these proteins were involved with intracellular signalling, protein processing, and regulation of the platelet cytoskeleton.

Although limited, most platelet proteomic research has focused on three areas: cataloguing the spectrum that makes up a "normal" human platelet proteome [30, 31], characterizing proteins released by activated platelets [32], or identifying phosphoproteins [or posttranslational modification (PTM)] generated by platelet stimulation [33, 34]. Studies that have attempted to identify the corresponding proteins encoded by novel megakaryocyte/platelet transcripts have generally been successful, suggesting that the false-positive discovery rate in the transcriptome studies can be controlled by rigorous statistical analysis. This suggests that a large fraction of the protein repertoire present in platelets is not detected by current proteomics studies, for reasons outlined before.

Although most studies have provided only descriptive data, one study of platelet proteomics identified a new platelet receptor [35]. This receptor, G6b, is a member of the immunoglobulin superfamily and has an immunoreceptor tyrosine-based inhibitory motif, only the second such receptor discovered. It undergoes extensive alternate splicing and tyrosine phosphorylation and is associated with the tyrosine phosphatase SHP-1. Through this association, G6b might play an important role in limiting platelet activation by the collagen receptor GP VI and the low-affinity immune receptor FcγRIIA [36]. Further studies are needed to determine the potential of the G6b receptor as a viable target for platelet inhibition.

Several studies have compared the relationship between the platelet transcriptome and its proteome [25, 37, 38]. The results of selected comparisons are shown in Table I [25]. McRedmond and colleagues [25] identified 82 proteins from activated platelets and compared them with 2928 messages in the transcriptional profile. The proteome correlated well with the transcriptome—with 69% of secreted proteins detectable at the mRNA level—and with data derived from two published datasets [37, 38]. Proteomic analysis confirmed the expression of many genes previously unreported in platelets. The transcriptome, which was very specific for platelets, showed an excess of the signal transduction, receptor, ion channel, and membrane gene ontology categories and a lack of protein synthesis categories. Transcriptional analysis also predicted the presence of new proteins in the platelet and holds promise in elucidating novel mechanisms for thrombotic disorders.

Relatively few studies have assessed the link between differential gene expression within platelets and the common phenotypes of coronary artery disease (CAD); however, several potentially important observations have been made [39–41]. When genes found to be associated with CAD risk

Downloaded At: 13:58 24 March 2009

Table I. Comparison of platelet proteomic and transcriptomic studies.

Study	Distinct proteins, *n*	No match on array, *n* (%)	Matching array, *n*	Present in transcriptome, *n* (%)	Absent from transcriptome, *n* (%)
Secreteome	82	13 (16)	70	48 (69)	22 (31)
O'Neill (2002)	124	4 (3)	120	82 (68)	38 (32)
Marcus (2000)	122	58 (48)	64	44 (69)	20 (31)
Total	–	–	206	136 (66)	70 (34)

Reprinted from McRedmond et al. [25], with permission.

Downloaded At: 13:58 24 March 2009

have been grouped into functional clusters, interesting patterns have emerged—the clusters most often represented within the platelet transcriptome reflect processes related to inflammation [39–42], apoptosis or cell proliferation [39, 42, 43], cell migration or adhesion [39, 41–44], and lipid metabolism [39, 41]. These studies offer many avenues for targeted research and potential for both diagnostic testing and risk assessment.

The underlying goal of platelet proteomics and genomics in the clinical setting is to understand and ultimately translate the complex interplay of genes, gene expression, and protein expression/modification to the clinical arena of thrombotic disorders, defining thrombotic risk for specific individuals, communities, and whole populations. A secondary goal is to incorporate knowledge into drug development so as to improve drug safety, tolerance, and efficacy. Ongoing efforts to create state-of-the-art technologies that can more comprehensively characterize the platelet genome, transcriptome, proteome, and metabolome as an integrated biological system will greatly aid in this effort.

The factor Xa-platelet interface: a model for anticoagulants as platelet antagonists

When factor Xa, a pivotal serine protease, binds to membrane-bound factor Va in the presence of calcium ions, the prothrombinase complex is formed [45], facilitating the conversion of prothrombin (via cleavage of two peptide bonds) to α-thrombin. Physiologically relevant and functional prothrombinase complexes can form on the membrane surface of adherent activated platelets, on activated platelets being recruited into a growing platelet thrombus [46], and possibly on macrophages participating in inflammatory reactions [47].

The end product of this process, α-thrombin, possesses diverse procoagulant, anticoagulant, and profibrinolytic properties. Its procoagulant effects include cleavage of fibrinogen, activation of cofactors (factors V and VIII), activation of zymogens (factors VII, XI, and XIII), and activation of platelets via PAR-1 and PAR-4. Thrombin is also important for establishing endothelial surface anticoagulation reactions through its binding to thrombomodulin and for stimulating release of tissue plasminogen activator and urokinase-like plasminogen activator from vascular endothelial cells. Inhibition of factor Xa would influence each of these processes to a variable degree.

Figure 3 shows how the individual constituents regulate factor Xa function in the prothrombinase complex [48]. Factor Va and the activated platelet membrane are essential constituents of the complex. The calcium-dependent interactions of factor Xa with platelet-bound factor Va, as well as the activated platelet itself, to form the prothrombinase complex increases the rate at which thrombin is produced by about five orders of magnitude [49]. Removal of factor Va from the complex leads to a 10,000-fold decrease in the rate of thrombin generation [48], whereas assembly of the prothrombinase complex in the absence of the activated platelet surface leads to a minimum 1000-fold decrease in the rate of thrombin formation [48]. Thus factor Va and the activated platelet membrane are essential for the formation of a physiologically relevant complex.

The requirement for complex formation has at least three main physiological consequences. First, it localizes reactions to the site of injury, limiting procoagulant effects to the relevant area. Second, the thrombin generated amplifies the coagulation response at the injured site. Third, complex formation modulates its own function by ensuring that physiological inhibitors or inactivators of complex constituents are ineffective. For example, factor Xa is protected from antithrombin III inhibition, and factor Va is protected from activated protein C-catalyzed inactivation.

Factor Xa function via assembly of the platelet prothrombinase complex is regulated by PAR-1 and PAR-4 activation. These receptors function somewhat independently in humans [22]. PAR-1 is activated at low concentrations of thrombin, whereas PAR-4 requires much higher concentrations of thrombin because it does not express the fibrinogen-binding exosite (exosite-I). Maximal PAR-1 activation is not sufficient for generation of full prothrombinase activity, however. When compared with thrombin as an agonist, only 75–80% of maximal activity is achieved with PAR-1, and its effects can be blocked by an appropriate antibody [50].

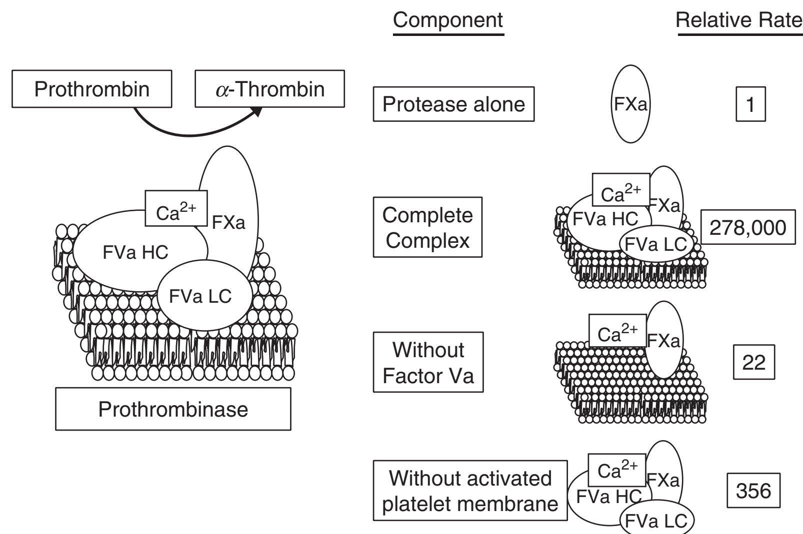

Figure 3. Physiological regulation of factor Xa (FXa) function. Ca^{2+}, calcium; Fva, factor Va; HC, heavy chain; LC, light chain. Data from Nesheim et al. [48], with permission.

Downloaded At: 13:58 24 March 2009

PAR-4 activation can yield full prothrombinase activity, but only at high concentrations of thrombin. Thus platelets are able to respond differently to their own thrombin-generating potential, depending upon the thrombin concentration that acts as an agonist. Synergism between PAR-1 and PAR-4 also has been shown [50].

Thrombin, as a platelet agonist, highly regulates prothrombinase complex assembly on the activated platelet membrane. In assessing the effects of increasing concentrations of thrombin agonism on the binding of factors Va and Xa to human platelets, Bouchard and colleagues [51] showed that, whereas platelet activation is required to mediate their binding, the binding of each ligand is distinct and correlates with the ''level'' of platelet activation. Low concentrations of thrombin afford complete saturation of factor Va binding sites, which are not capable of supporting factor Xa binding. Rather, factor Xa binding is achieved only with additional platelet activation. Thus activation-dependent events independently regulate both factor Va and factor Xa binding.

Of interest, when platelets are activated with thrombin at concentrations producing maximal prothrombinase activity (as defined by kinetic assessment), and a saturating concentration of plasma-derived factor Va is added, three distinct platelet populations emerge: activated platelets that bind no factor Va, activated platelets that bind an intermediate level of factor Va, and platelets that show significant or ''high'' factor Va binding [52]. When the experiment is repeated using factor Xa, only two platelet populations emerge: activated platelets that bind no factor Xa and those that show significant or ''high'' factor Xa binding. The population of ''high factor Va binding'' platelets was the same platelet population that effected factor Xa binding. Thus some platelets appear to be unable to bind factor Xa even though they have successfully bound factor Va.

Two pools of factor V are present in humans [53]. After synthesis in the liver, factor V is released into plasma. About 20–25% is taken up by endocytosis into platelet precursors (megakaryocytes) and later appears in the α-granules of circulating platelets [54, 55]. Platelet-derived factor Va appears to play a larger relative role in hemostasis, given that platelets congregate at the site of vascular injury, and the functional characteristics of plasma- and platelet-derived factor Va also differ substantially. The platelet-derived molecule, for example, is released as a functional cofactor already expressing nearly 50% of the fully activated receptor [56]. It is also resistant to activated protein C-catalyzed inactivation [57]. In addition, platelet-derived factor Va expresses greater and more sustained procoagulant activity after it is cleaved by catalytic amounts of plasmin [58].

In summary, factor Xa function via the platelet prothrombinase complex is regulated by several factors, including PAR-1 and PAR-4–mediated platelet activation, the overall level of platelet activation achieved, the influence of distinct platelet subpopulations with different binding capabilities, and the presence of a physically and functionally distinct platelet-derived cofactor pool [59], which appears to undergo differential proteomic processing in the megakaryocyte. These factors may offer potential targets for intervention in the development of antithrombotic therapies.

Intravital microscopy in the preclinical study of thrombosis

Recent advances in technology have established new platforms for determining the role of platelets in

human health and disease. One unique system permits the "real time" investigation of thrombus development in live mice using a wide-field fluorescence microscope fitted with a confocal head (Figure 4) [60]. An argon laser is used to induce localized injury on an exposed cremasteric or mesenteric arteriole or venule. An intensifier then captures digital images on a charge-coupled device (CCD) camera. The images are stored for later analysis on a computer workstation.

The kinetics of thrombus formation (platelet accumulation) after laser injury to a venule are shown in Figure 5, as evidenced by accumulation of platelets labelled with an anti-CD41 fluorescent antibody [61]. There is a rapid accumulation of platelets within the first minute after injury, then decreased accumulation over the next 90 seconds, with stabilization occurring within 5 minutes after injury. The generation of fibrin shows the same initial rapid increase after injury, but there is no decrease in generation before stabilization at about 100 seconds after injury.

This system has been used to assess the pharmacodynamic effects of antiplatelet compounds *in vivo*. In one experiment, thrombus formation was studied in mice that express human tissue factor (TF), in the absence and presence of a chimaeric murine-human anti-human TF monoclonal antibody (Furie, unpublished data). In the absence of the inhibitory antibody, platelet accumulation and fibrin generation occurred rapidly and followed the usual pattern seen after vascular injury. With anti-TF antibody inhibition, peak platelet accumulation was decreased by nearly 75%, and fibrin generation was decreased by nearly 90%.

The laser-induced injury model also has been used to study the contributions of platelet receptors to thrombus formation in genetically modified mice. A series of studies performed by Nieswandt and colleagues compared thrombosis in wild-type mice and mice lacking the Fc receptor, gamma subunit (FcRγ), which does not express the collagen-signalling receptor GP VI on the platelet surface [62]. Using a ferric chloride model of arterial injury, this strain of mutant mice exhibited impaired thrombus initiation and propagation [63]. After laser-induced injury, however, platelet thrombi were indistinguishable between the wild-type and mutant mice [63]. The explanation for this discrepancy lies in the extent of collagen exposure with each method. In the laser-injury model, platelet thrombus is generated without detectable exposure of collagen. In the ferric chloride model, substantial amounts of collagen are exposed, and the thrombus overlays the site of collagen exposure [63]. Accordingly, these two experimental models appear to reflect different pathways for platelet activation.

To examine the state of activation of platelets incorporated into thrombi after laser-induced injury, Dubois and colleagues isolated platelets from donor mice of various genotypes, loaded them with fura-2, a fluorochrome that is sensitive to calcium concentration, and infused the labelled platelets into a recipient mouse of the same genotype [64]. Platelet activation during laser-induced thrombus formation was examined by measuring intracellular calcium flux. Though platelet activation was not required for recruitment to the growing thrombus, it was a prerequisite for incorporation into the thrombus; platelets that did not become activated dissociated from the thrombus. Thus calcium mobilization appears to be required for stable platelet incorporation into a developing thrombus but not for initial recruitment to the thrombus.

In mice that are deficient in von Willebrand factor (VWF –/– mice), the kinetics of platelet thrombus formation after laser injury are essentially the same as in wild-type mice, but the maximal thrombus size is decreased [64]. Despite a diminution in thrombus size, platelet activation, as monitored by calcium flux per platelet, is normal. Thus in the VWF knockout thrombosis model, VWF is not required for platelet activation. Similarly, experiments in FcRγ –/– mice, which lack GP VI on the platelet surface, indicate that GP VI is not required for platelet activation during laser-induced thrombus formation (Figure 6) [64].

In mice lacking PAR-4 (PAR-4 –/– mice), laser injury leads to *initial* thrombus formation that is similar to that in wild-type mice, but the thrombus reaches maximal size at about 15–20 seconds [65]. *Maximal* thrombus size in PAR-4 –/– mice is 400-fold smaller than in wild-type mice. As measured by P-selectin exposure, PAR-4 –/– platelets are activated more slowly within the platelet thrombus. Thus initial platelet thrombus formation after laser injury does not require thrombin, and delayed activation of platelets can occur in a thrombin-independent process. In contrast, fibrin production is similar in wild-type and PAR-4 –/– mice [65]. There are at least two alternative explanations for these results. Only a subset of the accumulated platelets might be necessary and sufficient for the level of thrombin generation required to achieve maximal fibrin deposition in this model. Alternatively, other membrane surface components of the injured vessel or early thrombus might support thrombin generation, accounting for normal fibrin generation [65]. These results challenge the long-standing model of blood coagulation in which platelet activation and exposure of anionic phospholipids, primarily phosphatidylserine, on the activated platelet membrane allow for assembly of the prothrombinase complex.

Considered collectively, studies of thrombus formation in live animals challenge traditional models of platelet and soluble coagulation factor participation in thrombus formation.

Downloaded At: 13:58 24 March 2009

Downloaded At: 13:58 24 March 2009

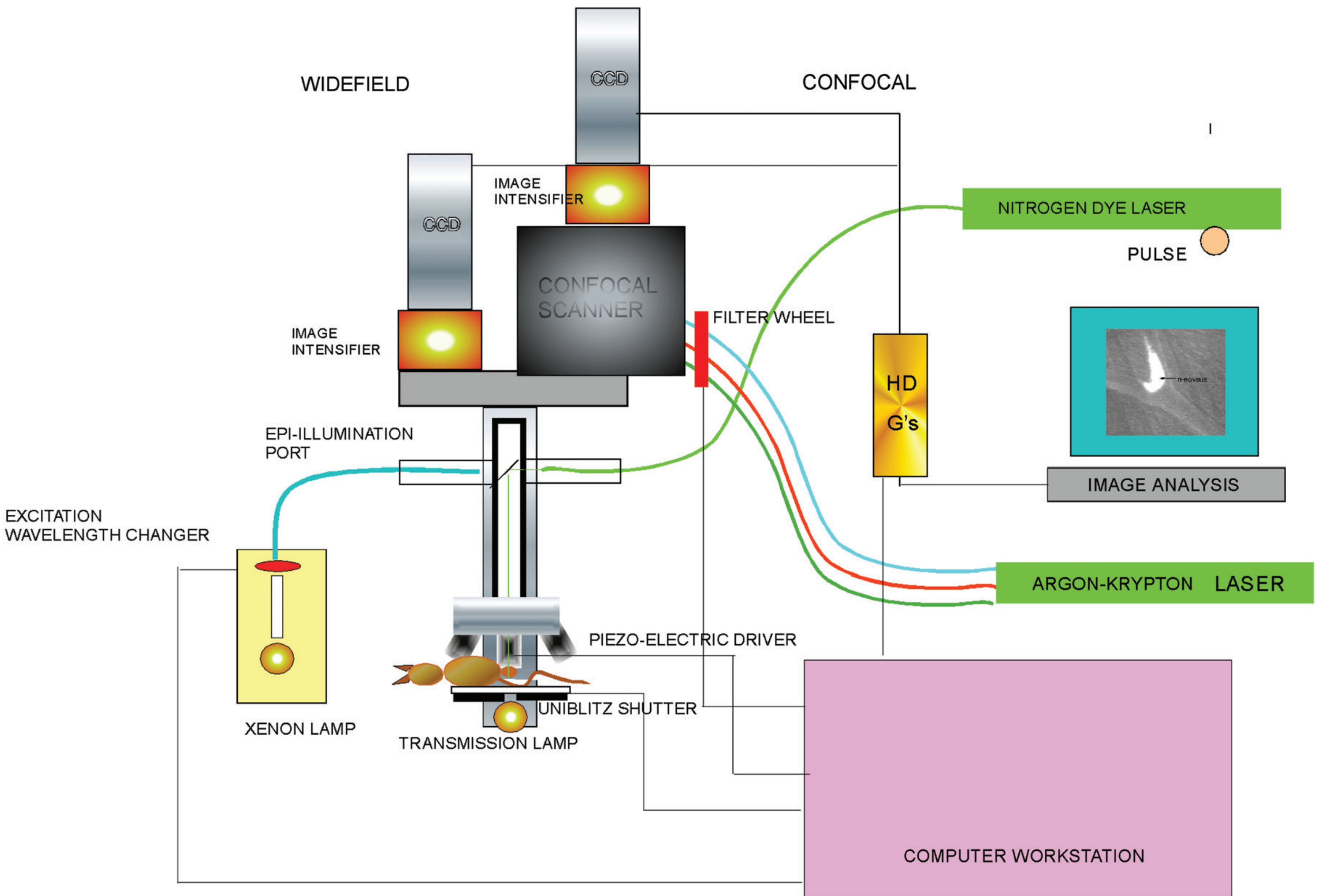

Figure 4. Schematic of high-speed intravital confocal, widefield microscopy. This system allows the examination of thrombus formation and development in real time in living mice. CCD, charge-coupled device camera. Reprinted from Falati et al. [60], with permission.

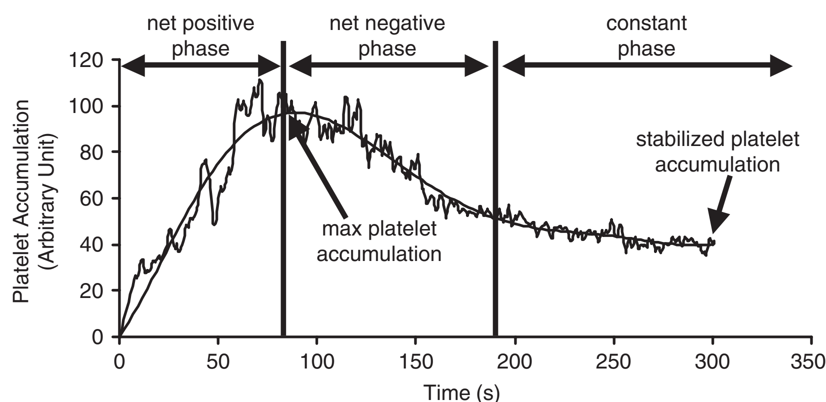

Figure 5. Time course of platelet accumulation *in vivo* in mice after laser injury to the vessel wall. Graph represents the median results for 80 experiments. Reprinted from Sim et al. [61], with permission.

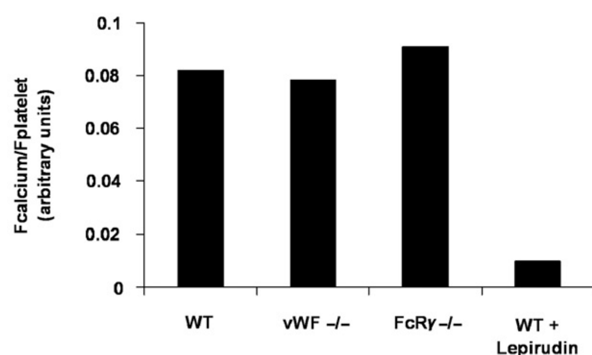

Figure 6. Platelet activation as assessed by calcium mobilization in various mouse phenotypes. Mice studied were wild type (WT), von Willebrand factor (vWF)-deficient, Fc receptor, γ-subunit–deficient, and WT given lepirudin. Reprinted from Dubois et al. [64], with permission.

Downloaded At: 13:58 24 March 2009

Further research is needed to elucidate the physiological and translational implications of these findings, taking into account all of the complex, integrated mechanisms involved in thrombosis.

The importance of experimental models in establishing platforms for drug discovery

There are two primary objectives in the realm of antiplatelet drug discovery: 1) to identify and develop lead compounds with increased safety and efficacy, and 2) to establish more reliable methods for pharmacodynamic analysis that foster proper dosing of these agents.

All current antithrombotic drugs, including aspirin, represent a compromise between efficacy and safety—between reducing the incidence of thrombosis in a given patient population and causing bleeding. Many otherwise effective drugs ultimately fail development because optimal dosing (the best compromise) cannot be established. Perhaps the greatest limitation in this regard is the inappropriate use of pharmacodynamic assays in the development of antithrombotics. The development of eptifibatide, a platelet GP IIb/IIIa inhibitor, can be used to illustrate the pitfalls of using pharmacodynamic measurements during the drug-development process.

In 1972, Phillips et al. identified GP IIb and GP IIIa on the platelet surface, finding that they exist as a heterodimeric complex that is a direct receptor for fibrinogen [66, 67]. The investigators later succeeded in cloning GP IIb/IIIa, and the gene sequence was used in conjunction with other data to establish the integrin family of adhesion receptors [68]. Coller and colleagues raised a monoclonal antibody to this receptor, and showed that it blocked thrombosis in animal models [69]. In 1987, Huang et al. discovered that trigramin, an RGD-containing peptide found in snake venom, competitively inhibited this and other integrins [70]. Subsequently, Scarborough and colleagues found that barbourin, another snake venom peptide that contained the KGD sequence, was a specific inhibitor of GP IIb/IIIa. They used this compound as a model to create eptifibatide, a synthetic heptapeptide that retained the integrin specificity of barbourin [71]. The antithrombotic activity of eptifibatide was established by Hanson et al. in a baboon model of thrombosis [72].

However, an early study of eptifibatide in patients undergoing percutaneous coronary intervention (PCI), IMPACT-II, yielded disappointing results. The incidence of the primary endpoint (death, myocardial infarction, unplanned surgical or repeat percutaneous revascularization, or coronary stent implantation for abrupt closure) did not differ significantly by treatment in an intention-to-treat analysis, and endpoint events did not show a dose-response relationship (9.9 vs. 9.2% for lower-dose eptifibatide and 11.4% for placebo) [73]. It was hypothesized that citrate anticoagulation removed ionized calcium from the GP IIb/IIIa receptor and enhanced the apparent inhibitory activity of eptifibatide *in vivo* [74]. This overestimation of eptifibatide's platelet-directed inhibitory activity in the earliest studies led to suboptimal drug dosing

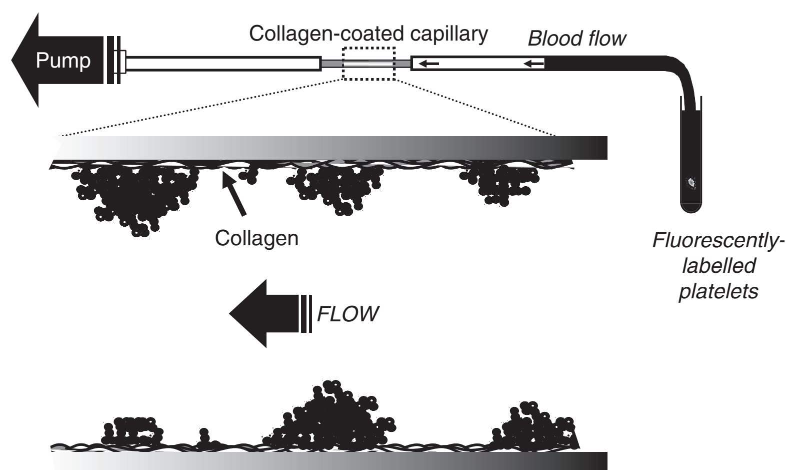

Figure 7. Schematic representation of perfusion chamber assessment of the thrombotic process. Adapted from Andre et al. [77], with permission.

Downloaded At: 13:58 24 March 2009

in subsequent clinical trials. And in fact, the next two large trials of eptifibatide, ESPRIT and PURSUIT, which used higher doses, did show significant benefits of treatment for patients undergoing PCI and those with acute coronary syndromes, respectively [75, 76].

This case illustrates the hazards of relying on pharmacodynamic methods that do not reflect the true physiological environment. For antithrombotic agents, the choice of anticoagulant used in blood collection clearly affects the resulting pharmacodynamic measurements. In addition, the low-shear environment of an *in vitro* aggregation assay bears little resemblance to high-shear, *in vivo* thrombosis in coronary arteries. Other methods may provide a more accurate picture of a drug's antithrombotic effects and thus make discovery and development more efficient.

One such method is perfusion chamber technology, which allows thrombosis profiling in real time (Figure 7) [77]. This technique has been used to investigate the respective roles of platelet receptors and their inhibitors in the thrombotic process.

Andre and colleagues assessed the role of the $P2Y_{12}$ receptor by comparing the thrombotic phenotypes of wild-type mice, mice that lacked the $P2Y_{12}$ receptor, and mice that were heterozygous for the receptor [78]. In addition to perfusion-chamber experiments, they also assessed the thrombotic response to ferric chloride-induced vascular injury. Thrombi were generated much more quickly in the wild-type and heterozygous mice than in the $P2Y_{12}$-deficient mice, but these thrombi tended to be transient. The time to occlusion of the mesentery artery after induced injury was significantly longer in the deficient mice than in the wild-type animals. When the investigators repeated the perfusion-chamber experiment using human platelets inhibited by various concentrations of 2-methyl-thio AMP, a direct antagonist of $P2Y_{12}$, similar patterns of transient thrombosis were observed [78]. Thus, continued ADP signalling through $P2Y_{12}$ appears to be necessary to maintain a stable thrombus.

In terms of drug discovery, then, targeting factors that affect not only thrombus growth but also their stabilization may offer fruitful avenues for research. Platelet coreceptors also may play an important role in this regard. The signalling reactions involved in forming stable platelet aggregates concomitantly induce the expression or secretion of factors affecting thrombus propagation, such as prothrombinase and factor Xase; the secretion or expression of proteins from platelet α-granules; or the engagement of platelet coreceptors [79].

A plethora of potential therapeutic targets exists on platelets. Better inhibitors of $P2Y_{12}$ might be developed that could affect the central role of ADP in platelet thrombus stability. A focus on aggregation coreceptors also might provide more targeted inhibition of aggregation-induced secondary reactions of platelets, as well as increased inhibition of thrombosis with less bleeding risk. Continued evolution in perfusion chamber methods and other technologies that reflect the physiological environment more accurately should facilitate the development of targeted antithrombotic agents. It might even be possible in the future to develop agents that are specific to the vascular microenvironment, such that cerebrovascular and cardiovascular thrombosis could be treated or prevented with the use of discrete sets of antithrombotic drugs.

Conclusion

Our understanding of the generation, structure, proteomic processing, and function of platelets and their subcomponents continues to expand.

Advances in proteomic, transcriptomic, and metabolomic analysis will lead to new avenues of investigation in the quest for targeted antithrombotic therapies. Similarly, the evolution of live animal imaging and ex vivo perfusion chamber technology and other models that accurately reflect the true physiological environment operating in human thrombosis will hasten the development of new antithrombotic agents and their availability for clinical use in patients with and those at risk for thrombosis.

Acknowledgments

The 2007 Platelet Colloquium was funded by grants from AstraZeneca Pharmaceuticals LP, Wilmington, Delaware, USA; Bristol-Myers Squibb, New York, NY, USA/sanofi-aventis, Bridgewater, NJ, USA; Daiichi Sankyo, Inc., Parsippany, NJ, USA/Eli Lilly & Co., Indianapolis, Indiana, USA; Regado Biosciences, Inc., Durham, NC, USA; Schering-Plough Corporation, Kenilworth, NJ, USA; Scios Inc., Mountain View, California, USA; and The Medicines Company, Parsippany, NJ, USA. The authors also acknowledge the valued contributions of Robert R. Fenichel, MD, to both the Colloquium and the development of this manuscript.

Downloaded At: 13:58 24 March 2009

References

1. Bizzozero G. Su di un nuovo elemento morfologico del sangue dei mammiferi e della sue importanza nella trombosi e nella coagulazione. L'Ossevatore 1881;17:785–787.
2. Gurbel PA, Becker RC, Mann KG et al. Platelet function monitoring in patients with coronary artery disease. J Am Coll Cardiol 2007;50:1822–1834.
3. Smyth SS, Monroe DM, Wysokinsi WE, et al. Platelet activation and its patient-specific consequences. Thromb Res 2007 Oct 17; [Epub ahead of print].
4. Steinhubl SR, Schneider DJ, Berger PB, et al. Determining the efficacy of antiplatelet therapies for the individual: Lessons from clinical trials. J Thromb Thrombolysis 2007 Nov 1; [Epub ahead of print].
5. Deutsch VR, Tomer A. Megakaryocyte development and platelet production. Br J Haematol 2006;134:453–466.
6. Pang L, Weiss MJ, Poncz M. Megakaryocyte biology and related disorders. J Clin Invest 2005;115:3332–3338.
7. Wendling F, Maraskovsky E, Debili N et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. Nature 1994;369:571–574.
8. Broudy VC, Lin NL, Sabath DF, Papayannopoulou T, Kaushansky K. Human platelets display high-affinity receptors for thrombopoietin. Blood 1997;89:1896–1904.
9. Patel SR, Hartwig JH, Italiano JE Jr. The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest 2005;115:3348–3354.
10. Poujol C, Durrieu-Jais C, Larrue B et al. Accessibility of abciximab to megakaryocytes and endothelial cells in the bone marrow compartment: Studies on a patient receiving antithrombotic therapy. Br J Haematol 1999;107:526–531.
11. De Botton S, Sabri S, Daugas E et al. Platelet formation is the consequence of caspase activation within megakaryocytes. Blood 2002;100:1310–1317.
12. Kaluzhny Y, Yu G, Sun S et al. BclxL overexpression in megakaryocytes leads to impaired platelet fragmentation. Blood 2002;100:1670–1678.
13. Kim JA, Jung YJ, Seoh JY et al. Gene expression profile of megakaryocytes from human cord blood CD34+ cells ex vivo expanded by thrombopoietin. Stem Cells 2002;20:402–416.
14. Clarke MC, Savill J, Jones DB et al. Compartmentalized megakaryocyte death generates functional platelets committed to caspase-independent death. J Cell Biol 2003;160:577–587.
15. Battinelli E, Loscalzo J. Nitric oxide induces apoptosis in megakaryocytic cell lines. Blood 2000;95:3451–3459.
16. Battinelli E, Willoughby SR, Foxall T et al. Induction of platelet formation from megakaryocytoid cells by nitric oxide. Proc Natl Acad Sci USA 2001;98:14458–14463.
17. Kaluzhny Y, Ravid K. Role of apoptotic processes in platelet biogenesis. Acta Haematol 2004;111:67–77.
18. Rocca B, Secchiero P, Ciabattoni G et al. Cyclooxygenase-2 expression is induced during human megakaryopoiesis and characterizes newly formed platelets. Proc Natl Acad Sci USA 2002;99:7634–7639.
19. Shim MH, Hoover A, Blake N et al. Gene expression profile of primary human CD34+/CD38lo cells differentiation along the megakaryocyte lineage. Exp Hematol 2004;32:638–648.
20. Tanaka N, Sato T, Fujita H et al. Constitutive expression and involvement of cyclooxygenase-2 in human megakaryocytopoiesis. Arterioscler Thromb Vasc Biol 2004;24:607–612.
21. Guthikonda S, Lev EI, Patel R et al. Reticulated platelets and uninhibited COX-1 and COX-2 decrease the antiplatelet effects of aspirin. J Thromb Haemost 2007;5:490–496.
22. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 2005;3:1800–1814.
23. Cupit LD, Schmidt VA, Gnatenko DV et al. Expression of protease activated receptor 3 (PAR3) is upregulated by induction of megakaryocyte phenotype in human erythroleukemia (HEL) cells. Exp Hematol 2004;32:991–999.
24. Macaulay IC, Tijssen MR, Thijssen-Timmer DC et al. Comparative gene expression profiling of *in vitro* differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. Blood 2007;109:3260–3269.
25. McRedmond JP, Park SD, Reilly DF et al. Integration of proteomics and genomics in platelets: A profile of platelet proteins and platelet-specific genes. Mol Cell Proteomics 2004;3:133–144.
26. Macaulay IC, Carr P, Gusnanto A, Ouwehand WH, Fitzgerald D, Watkins NA. Platelet genomics and proteomics in human health and disease. J Clin Invest 2005;115:3370–3377.
27. Tenedini E, Fagioli ME, Vianelli N et al. Gene expression profiling of normal and malignant CD34-derived megakaryocytic cells. Blood 2004;104:3126–3135.
28. Gnatenko DV, Dunn JJ, McCorkle SR et al. Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood 2003;101:2285–2293.
29. Kerrigan SW, Gaur M, Murphy RP et al. Caspase 12: A developmental link between G-protein-coupled receptors and integrin alphaIIbeta3 activation. Blood 2004;104:1327–1334.
30. Tyan YC, Liao JD, Jong SB et al. Proteomic profiling of platelet proteins by trypsin immobilized self-assembled monolayers digestion chip and protein identification using electrospray ionization tandem mass spectrometry. J Biomed Mater Res A 2004;71:90–97.
31. Moebius J, Zahedi RP, Lewandrowski U et al. The human platelet membrane proteome reveals several new potential membrane proteins. Mol Cell Proteomics 2005;4:1754–1761.
32. Coppinger JA, Cagney G, Toomey S et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. Blood 2004;103:2096–2104.

33. Garcia A, Prabhakar S, Hughan S et al. Differential proteome analysis of TRAP-activated platelets: Involvement of DOK-2 and phosphorylation of RGS proteins. Blood 2004; 103:2088–2095.
34. Garcia A, Senis YA, Antrobus R et al. A global proteomics approach identifies novel phosphorylated signaling proteins in GPVI-activated platelets: Involvement of G6f, a novel platelet Grb2-binding membrane adapter. Proteomics 2006; 6:5332–5343.
35. Senis YA, Tomlinson MG, Garcia A et al. A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel ITIM protein. Mol Cell Proteomics 2007;6:548–564.
36. Newland SA, Macaulay IC, Floto AR et al. The novel inhibitory receptor G6B is expressed on the surface of platelets and attenuates platelet function *in vitro*. Blood 2007;109:4806–4809.
37. O'Neill EE, Brock CJ, von Kriegsheim AF et al. Towards complete analysis of the platelet proteome. Proteomics 2002;2:288–305.
38. Marcus K, Immler D, Sternberger J et al. Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix assisted laser desorption/ ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. Electrophoresis 2000; 21:2622–2636.
39. Archacki SR, Angheloiu G, Tian XL et al. Identification of new genes differentially expressed in coronary artery disease by expression profiling. Physiol Genomics 2003;15:65–74.
40. Satterthwaite G, Francis SE, Suvarna K et al. Differential gene expression in coronary arteries from patients presenting with ischemic heart disease: Further evidence for the inflammatory basis of atherosclerosis. Am Heart J 2005; 150:488–499.
41. Papaspyridonos M, Smith A, Burnand KG et al. Novel candidate genes in unstable areas of human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2006;26:1837–1844.
42. Deng DX, Tsalenko A, Vailaya A et al. Differences in vascular bed disease susceptibility reflect differences in gene expression response to atherogenic stimuli. Circ Res 2006;98:200–208.
43. Rossi ML, Marziliano N, Merlini PA et al. Different quantitative apoptotic traits in coronary atherosclerotic plaques from patients with stable angina pectoris and acute coronary syndromes. Circulation 2004;110:1767–1773.
44. Randi AM, Biguzzi E, Falciani F et al. Identification of differentially expressed genes in coronary atherosclerotic plaques from patients with stable or unstable angina by cDNA array analysis. J Thromb Haemost 2003;1:829–835.
45. Mann KG, Nesheim ME, Church WR et al. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 1990;76:1–16.
46. Bouchard BA, Butenas S, Mann KG et al. Interactions between platelets and the coagulation system, In: AD Michelson, editor, Academic Press, San Diego; 2007, pp 377–402.
47. Bouchard BA, Tracy PB. The participation of leukocytes in coagulant reactions. J Thromb Haemost 2003;1:464–469.
48. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine factor V and factor Va to the activity of prothrombinase. J Biol Chem 1979;254:10952–10962.
49. Nesheim ME, Kettner C, Shaw E, Mann KG. Cofactor dependence of factor Xa incorporation into the prothrombinase complex. J Biol Chem 1981;256:6537–6540.
50. Heger CD, Moseley CN, Tracy PB. Activation of either PAR1 or PAR4 on human platelets can elicit expression of maximum platelet procoagulant activity. Blood 2001; 98(Suppl 1):518a, (Abstr 2163).
51. Bouchard BA, Catcher CS, Thrash BR et al. Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation. J Biol Chem 1997;272:9244–9251.
52. Feng P, Tracy PB. Not all platelets are equivalent procoagulants. Blood 1998;92(Suppl 1):350a, (Abstr 1441).
53. Tracy PB, Eide LL, Bowie EJW et al. Radioimmunoassay of Factor V in human plasma and platelets. Blood 1982; 60:59–63.
54. Bouchard BA, Williams JL, Meisler NT et al. Endocytosis of plasma factor V by megakaryocytes occurs via a clathrin-dependent, receptor-mediated event. J Thromb Haemost 2005;3:541–551.
55. Camire RM, Pollak ES, Kaushansky K et al. Secretable human platelet-derived factor V originates from the plasma pool. Blood 1998;92:3035–3041.
56. Monkovic DD, Tracy PB. Functional characterization and activation of human platelet released factor V by factor Xa and thrombin. J Biol Chem 1990;265:17132–17140.
57. Camire RM, Kalafatis M, Simioni P et al. Platelet-derived factor Va and factor Va^{Leiden} cofactor activity are sustained on the surface of activated platelets despite the presence of activated protein C. Blood 1998;91:2818–2829.
58. Conlon SJ, Camire RM, Kalafatis M et al. Cleavage of platelet-derived factor Va by plasmin results in increased and sustained cofactor activity on the thrombin-activated platelet surface. J Thromb Haemost 1997;77(Suppl. 1):616, (Abstr 2507).
59. Gould WR, Silveira JR, Tracy PB. Unique *in vivo* modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor: Characterization of purified platelet-derived factor V/Va. J Biol Chem 2004;279:2383–2393.
60. Falati S, Gross P, Merrill-Skoloff G et al. Real-time *in vivo* imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. Nat Med 2002;8:1175–1181.
61. Sim DS, Merrill-Skoloff G, Furie BC et al. Initial accumulation of platelets during arterial thrombus formation *in vivo* is inhibited by elevation of basal cAMP levels. Blood 2004;103:2127–2134.
62. Nieswandt B, Bergmeier W, Schulte V et al. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain. J Biol Chem 2000;275:23998–24002.
63. Dubois C, Panicot-Dubois L, Merrill-Skoloff G et al. Glycoprotein VI-dependent and -independent pathways of thrombus formation *in vivo*. Blood 2006;107:3902–3906.
64. Dubois C, Panicot-Dubois L, Gainor JF et al. Thrombin-initiated platelet activation *in vivo* is vWF independent during thrombus formation in a laser injury model. J Clin Invest 2007;17:953–960.
65. Vandendries ER, Hamilton JR, Coughlin SR et al. Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. Proc Natl Acad Sci USA 2007;104:288–292.
66. Phillips DR. Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. Biochemistry 1972;11:4582–4588.
67. Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. Blood 1988;71:831–843.
68. Hynes RO. Integrins: A family of cell surface receptors. Cell 1987;48:549–554.
69. Coller BS, Folts JD, Scudder LE et al. Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model. Blood 1986;68:783–786.

Downloaded At: 13:58 24 March 2009

70. Huang TF, Holt JC, Lukasiewicz H et al. Trigramin. A low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb-IIIa complex. J Biol Chem 1987;262:16157–16163.
71. Scarborough RM, Rose JW, Hsu MA et al. Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of *Sistrurus m. barbouri*. J Biol Chem 1991;266:9359–9362.
72. Scarborough RM. Eptifibatide. Drugs Future 1998; 23:585–590.
73. IMPACT-II Investigators. Randomised placebo-controlled trial of effect of eptifibatide on complications of percutaneous coronary intervention. Lancet 1997;349:1422–1428.
74. Phillips DR, Teng W, Arfsten A et al. Effect of Ca2+ on GP IIb/IIIa interactions with Integrilin: Enhanced GP IIb/IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. Circulation 1997;96:1488–1494.
75. ESPRIT Investigators. Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): A randomised, placebo-controlled trial (erratum in: Lancet 2001;357:1370). Lancet 2000;356:2037–2044.
76. PURSUIT Trial Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. N Engl J Med 1998;339:436–443.
77. Andre P, Arbeille B, Drouet V et al. Optimal antagonism of GPIIb/IIIa favors platelet adhesion by inhibiting thrombus growth. An ex vivo capillary perfusion chamber study in the guinea pig. Arterioscler Thromb Vasc Biol 1996;16:56–63.
78. Andre P, Delaney SM, LaRocca T et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest 2003;112:398–406.
79. Nanda N, Andre P, Bao M et al. Platelet aggregation induces platelet aggregate stability via SLAM family receptor signaling. Blood 2005;106:3028–3034.

Downloaded At: 13:58 24 March 2009