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The Platelet: Life on the Razor's Edge Between Hemorrhage and Thrombosis

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Introduction

I am truly honored and deeply humbled to receive the 2013 Karl Landsteiner Award. The past recipients of this award are not only stellar members of the transfusion medicine firmament, but also my heroes, and in some cases, my mentors, role models, and friends. I was most fortunate to have my introduction to transfusion medicine during my training in the Clinical Pathology Department at the National Institutes of Health (NIH). Dr. Harvey Alter, Dr. Paul Schmidt, Dr. Paul Holland, Dr. Harvey Klein, and Mary McGinnis were wonderful teachers. Thereafter, I was also fortunate to have Dr. Dennis Galanakis as a colleague at Stony Brook and Dr. Richard Rosenfeld, Dr. Mort Spivack, and Dr. Carolyn Whitsett as colleagues at Mount Sinai.

I feel a close bond to Karl Landsteiner since he joined the Rockefeller Institute in 1923 at the invitation of Dr. Simon Flexner, who knew of his landmark contribution to blood group immunohematology and his discovery of the polio virus, and who also knew that Landsteiner did not have suitable support for his research in Post-World War I Europe. Landsteiner went on to make many additional discoveries at Rockefeller including his work on the Rh antigen. He became an Emeritus Professor in 1929 but stayed active in his lab until 1943 when he suffered a heart attack while still at the bench, pipette in hand, and died 2 days later. The progress we have made since then in preventing and treating heart attacks will be one of the themes of this presentation.

Platelet Physiology

Over the past 60 years we have learned an enormous amount about the molecular basis of platelet physiology and most recently we have been able bring the tools of structural biology to aid us in refining our understanding of the molecular interactions that are responsible for platelet adhesion and aggregation, as well as the release of granule contents.¹ Key elements include the role of the endothelium in producing two inhibitors of platelet activation,

Disclosure

Dr. Collier is an inventor of abciximab (Centocor) and in accord with Federal law and the policies of the Research Foundation of the State University of New York, receives royalties based on the sales of abciximab. Dr. Collier is an inventor of the VerifyNow assays (Accumetrics), and in accord with Federal law and the policies of the Mount Sinai School of Medicine, receives royalties based on the sales of the VerifyNow assays. Rockefeller University has applied for a patent on the RUC compounds.

prostacyclin (PGI₂)² and nitric oxide (NO)^{3,4}. The endothelial membrane also contains the ADPase enzyme CD39, which can digest ADP,^{5,6} one of the platelet activators, leading ultimately to the generation of the platelet inhibitor adenosine. When endothelial cells are damaged or denuded, these platelet inhibitory functions are lost and proteins such as von Willebrand factor, collagen, and perhaps fibrinogen are expressed and/or deposited from plasma at the site of vascular injury. The platelet has multiple receptors for these proteins, including GPIb for von Willebrand factor⁷ and $\alpha 2\beta 1$ and GPVI for collagen,^{8–10} and these can form strong bonds even under conditions of high shear force, resulting in platelet adhesion. Adhesion itself triggers platelet activation and degranulation, ultimately resulting in a major conformational change in the $\alpha \text{IIb}\beta 3$ receptor, which is expressed at very high density on platelets ($\sim 10^5$ per platelet), such that it can bind macromolecular glycoproteins, including fibrinogen and von Willebrand factor that circulate in plasma.¹¹ Since these glycoproteins are multivalent, they can bind to activated $\alpha \text{IIb}\beta 3$ receptors on two different platelets simultaneously, forming bridges that support platelet-platelet interactions, that is, platelet aggregation. The fibrinogen molecule is dimeric and highly extended due to the presence of coil regions, giving it a molecular diameter of more than 450 Å.¹² Its platelet spanning property takes full advantage of its extended structure since the sites on fibrinogen that bind to $\alpha \text{IIb}\beta 3$ are at the very ends of the C-terminal tips of the γ -chains, providing the greatest distance for bridging between platelets.^{13,14} von Willebrand factor (vWF) is made up of varying numbers of identical subunits, ranging up to more than 40, with the largest multimers having molecular weights approaching or exceeding 20,000,000.^{15–18} vWF binds to $\alpha \text{IIb}\beta 3$ via an Arg-Gly-Asp (RGD) sequence in its C4 domain.¹⁷

$\alpha \text{IIb}\beta 3$ is a member of the large integrin family of receptors, each of which is composed of an α and β subunit.¹⁹ These subunits can mix and match and thus there are a total of 24 receptors made from the 18 α subunits and 8 β subunits. $\alpha \text{IIb}\beta 3$ is a member of the $\beta 3$ subfamily, which has two members, $\alpha \text{IIb}\beta 3$ and $\alpha \text{V}\beta 3$. $\alpha \text{V}\beta 3$, which also binds ligands containing the RGD sequence, is expressed on a variety of different cells,^{20–23} whereas $\alpha \text{IIb}\beta 3$ is essentially specific for megakaryocytes and platelets, with just a brief appearance during early hematopoietic differentiation.^{11,24–26} As such, $\alpha \text{IIb}\beta 3$ is highly adapted for its role on platelets.

Integrin receptors are notable for their ability to undergo bidirectional signaling.¹⁹ $\alpha \text{IIb}\beta 3$ undergoes activation via inside-out signaling in response to one or more agonists, leading to the conformational change that results in high affinity ligand binding. After ligand binding, the $\alpha \text{IIb}\beta 3$ ectodomain transmits signals back inside the platelet, resulting in cytoskeletal rearrangements and granule exocytosis.²⁷ Platelet activation can be initiated by the binding of one or more of the agonists for which platelets have specific receptors, including among others, those for thrombin (PAR-1 and PAR-4),²⁸ ADP (P2Y₁ and P2Y₁₂),^{29,30} thromboxane A₂,³¹ collagen (GPVI, $\alpha 2\beta 1$),^{8–10,32} von Willebrand factor (GPIb/IX/V complex),³³ and serotonin (5-HT_{2a}).³⁴

The Platelet's Role in Hemostasis

The most compelling evidence for the importance of platelets in normal hemostasis came from the classic transfusion medicine study by Duke in 1910.³⁵ Confronted with a 20 year

old patient with normal coagulation assays, profound thrombocytopenia (platelet count = 6,000/ μ l), and massive hemorrhage (more than a pint of epistaxis) at the Massachusetts General Hospital on May 11, 1909, Dr. F. T. Murphy decided to attempt a direct whole blood transfusion. "Donor selection" consisted of choosing an "Armenian friend of about the same age" as the 29 year old Armenian patient. The volume transfused could not be measured because it was a direct donation, but they concluded that it was a "large amount" because the donor's pulse increased. In fact, the platelet count in the recipient increased to 123,000/ μ l six hours after the transfusion, confirming that indeed a large volume of blood was transfused! The transfusion was a great success, halting the patient's epistaxis and other bleeding manifestations almost immediately and normalizing the bleeding time assay that Duke developed, in which an incision is made in the patient's earlobe, from 90 minutes to 3 minutes (Fig. 1).

Less than a decade after Duke's report, Eduard Glanzmann, a Swiss pediatrician, reported that some patients with normal numbers of platelets have a serious bleeding disorder as a result of having "weak" platelets.³⁶ This report of the archetypical disorder now known as Glanzmann thrombasthenia established the field of qualitative platelet disorders. Studies of the platelets of patients with this disorder beginning in the mid-1960s led to the discovery that these patient's platelets either lack α Ib β 3 and/or have α Ib β 3 receptors that cannot be activated or bind ligand regardless of the agonist used to induce activation.^{11,37} Their platelets are capable of adhering to damaged blood vessels and to von Willebrand factor and collagen, but they cannot aggregate one with another, and thus cannot form an effective hemostatic plug. These data reinforced that the α Ib β 3 receptor represents the final common pathway via which all platelet activating agents produce platelet aggregation.

The Role of Platelets in Thrombosis

The role of platelets in coronary artery thrombosis was appreciated much later than their role in hemostasis, in large part because there were no specific antiplatelet agents with which to test the hypothesis. Early studies with aspirin were equivocal as a result of weaknesses in trial design,³⁸ and so it was not until the ISIS-2 study in 1988 demonstrated that aspirin alone could reduce the odds of death from myocardial infarction by 23% and that adding aspirin to streptokinase enhanced the odds reduction of death from 25 to 42% in the more than 17,000 patients enrolled in the study that the role of platelets in the process was broadly accepted.³⁹ A meta-analysis of the value of anti-platelet agents, most notably aspirin, in the prevention of recurrent vascular events published in the same year,⁴⁰ provided support for a prophylactic role of antiplatelet therapy. Studies conducted at approximately the same time with ticlopidine, an antiplatelet agent without a known molecular target, in combination with aspirin in patients undergoing coronary artery balloon angioplasty and stent placement,⁴¹ lent further empiric evidence that inhibiting platelets could prevent ischemic complications of thrombotic disease.

My Early Platelet Studies

My studies of platelets began while I was a medical student with Dr. Marjorie Zucker at New York University School of Medicine. Dr. Zucker was a leader in studying platelet

physiology⁴² and under her mentorship I studied the retention of platelets when passed through a column of glass beads, an assay that was sensitive to the defect in patients with von Willebrand disease.⁴³ Later, in Dr. Harvey Gralnick's laboratory at the National Institutes of Health (NIH), I studied von Willebrand factor binding to platelets.^{44–52} I made use of the recent discovery that the antibiotic ristocetin, which was removed from clinical use because it caused thrombocytopenia, could induce platelet agglutination by a mechanism dependent on the presence of von Willebrand factor.^{53,54}

After setting up my lab at the State University of New York at Stony Brook, I began to study the interaction of fibrinogen to platelets.⁵⁵ In 1980 I confirmed the results of others that the platelets of patients with Glanzmann thrombasthenia could not bind fibrinogen and went on to show that this finding was a result of a qualitative or quantitative defect in the receptor for fibrinogen rather than a defect in receptor activation.⁵⁶ I reasoned that the then new technology of monoclonal antibodies could provide important additional information about platelet-fibrinogen interactions and so devised an assay to screen for antibodies that could block the interaction of platelets with fibrinogen.⁵⁷ We initially worked with the 10E5 antibody, which was a potent inhibitor of platelet aggregation, essentially recapitulating the platelet aggregation defect found in Glanzmann thrombasthenia patients' platelets when added to normal platelets. Working with Dr. Robert Jordan's group at Centocor, Dr. Uri Seligsohn's group in Israel, and Dr. Reuben Mibashan's group in King's College, London, we went on to show that: 1.) 10E5 bound to α IIb β 3 (then termed GPIIb/IIIa),⁵⁷ 2.) there are about 100,000 α IIb β 3 receptors per platelet,⁵⁸ 3.) the platelets of patients with Glanzmann thrombasthenia bound virtually no 10E5 molecules,^{59,60} 4.) the platelets of obligatory heterozygous carriers of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations in Israel bound ~50–60% of the normal number of 10E5 molecules,⁶⁰ and 5.) the 10E5 monoclonal antibody binding assay could be used for prenatal diagnosis using fetal blood for families that wanted the information for family planning.⁶¹ When α IIb and β 3 were cloned and their nucleotide sequences determined, we collaborated with Drs. Fangping Chen, Deborah French, Peter Newman, Mortimer Poncz, and Uri Seligsohn in studies to identify the molecular biologic basis of the disorder in different groups^{62–67} and then used this information to provide prenatal diagnosis by DNA-based polymerase chain reaction determinations and linkage studies.^{63,68} Studies using chorionic villus samples made it possible to return information to families at ~11 weeks of gestation rather than the ~18–20 weeks required for fetal blood sampling.

Using Monoclonal Antibodies to Prevent and Treat Thrombosis

Since thrombotic ischemic coronary artery disease was, and remains, a major cause of morbidity and mortality,⁶⁹ we wondered whether the monoclonal antibodies we made that could essentially eliminate platelet aggregation might potentially be used therapeutically. We worked with monoclonal antibody 7E3⁷⁰ because, unlike 10E5, it cross-reacts with dog platelets,⁷¹ and many of the thrombotic disease models were performed in dogs. We did not use the intact antibody since we expected that it would lead to platelet clearance via the interaction of the antibody's Fc domain with cells containing Fc receptors in the monocyte/macrophage system. Using the F(ab)₂ fragment of 7E3, we could inhibit platelet thrombus formation to a greater extent than aspirin in a number of different animal models.^{72–75} In

collaboration with the scientists at Centocor, we then redesigned the original murine monoclonal antibody into the drug abciximab, a recombinant chimeric Fab fragment composed of the murine 7E3 variable regions, which constitute the binding site, and human IgG1 constant regions.⁷⁶ Based on a pivotal study in high risk patients undergoing primary balloon angioplasty in which abciximab reduced ischemic events when added to aspirin and heparin,⁷⁷ the FDA approved abciximab for human use in 1994 and it gained regulatory approval in Europe, Scandinavia, and parts of Asia at about the same time. Abciximab proved particularly effective in reducing the ischemic complications of coronary artery stent placement, which invariably initiates platelet aggregation by exposing the platelet-reactive proteins in the atherosclerotic lesions.⁷⁸

The introduction of abciximab, followed in 1998 by two other α IIB β 3 antagonists (eptifibatide and tirofiban)^{79,80} that are patterned on the R(K)GD cell binding sequence, provided powerful new antiplatelet agents that reduced the mortality and morbidity of percutaneous coronary interventions (PCI). Hospitals that adopted these agents rapidly had in-hospital mortality rates for myocardial infarction that were approximately one-half of those hospitals that did not adopt them.⁸¹ A 2013 Cochrane review of these agents found that their use in association with PCI in 43 randomized trials involving 31,744 patients was associated with a reduced 30 day odds ratio of 0.79 for death (95% CI 0.64–0.97), and that their use in association with PCI in 44 studies involving 31,880 patients was associated with a 30 day odds ratio for death or myocardial infarction of 0.66 (95% CI; 0.60–0.72).⁸² They were, however, associated with an increased risk of hemorrhage, especially at the site of vascular access, and with thrombocytopenia in a small percentage of patients; thrombocytopenia is most common with abciximab.^{82,83} We collaborated with Dr. Timothy Springer's group, which has pioneered in the application of x-ray crystallography to understand the atomic basis of ligand binding to α IIB β 3,¹⁴ to define the mechanism by which eptifibatide and tirofiban bind to α IIB β 3⁸⁴ and we inferred the site of abciximab binding to the β 3 subunit by site-directed mutagenesis.⁸⁵

To provide some historical context for appreciating the remarkable advances that have been made in treating acute myocardial infarction, it is interesting to compare the treatment given then President Dwight D. Eisenhower in 1955⁸⁶ with the treatment given to Vice President-elect Dick Cheney in 2000. Eisenhower's physician was with him when he developed chest pain while on a trip to Denver, but he didn't perform an ECG until the next day because the treatment at that time was pain relief with morphine, anticoagulants to prevent venous thrombosis, and 6 weeks of bed rest to "rest" the heart. The mortality at that time was ~30%. In contrast, Cheney was in the catheterization lab within several hours and was treated with angioplasty and stent placement, accompanied by aspirin, enoxaparin, and abciximab. He was able to walk out of the hospital in less than two days and was back at work within a week.^{87,88} The mortality had decreased to ~5%.

Since their approval, the indications for α IIB β 3 antagonists have narrowed as new anticoagulants and new antiplatelet agents have been introduced, including bivalirudin^{89,90} and the successors to ticlopidine that inhibit the P2Y₁₂ ADP receptor (clopidogrel,⁹¹ prasugrel,⁹² and ticagrelor⁹³). The use of radial artery vascular access rather than femoral

artery access dramatically reduces access site bleeding, one of the major complications of α IIB β 3 antagonists, thus improving the benefit-to-risk ratio of these agents.

Experimental Use of Abciximab Early After Onset of Symptoms of Myocardial Infarction

One experimental use of α IIB β 3 antagonists that is of particular interest is their use very early after the onset of the symptoms of ST segment-elevation myocardial infarction (STEMI). Studies with abciximab indicate that such use has the potential to not only increase blood flow and reduce short term mortality and damage to the myocardium, but also protect against the later development of congestive heart failure.^{94–96} In fact, it has been estimated that more than half of the deaths from STEMI occur in the pre-hospital period and that the greatest opportunity for improving the treatment of STEMI depends on earlier therapy.^{97,98} In contrast to the dramatic advances in improving in-hospital mortality of STEMI, there has been much less progress in improving pre-hospital therapy.⁹⁷ The potential effect of early treatment of STEMI with abciximab on heart function^{96,99} is especially important because the dramatic decline in hospital mortality of myocardial infarction over the past four decades (Fig. 2)¹⁰⁰ has been painfully mirrored by an increase in hospital admissions for congestive heart failure (Fig. 3).¹⁰¹

A New α IIB β 3 Antagonist for Pre-hospital Therapy of STEMI

To address the unmet medical need for effective pre-hospital therapy of STEMI we first performed a high throughput screen of more than 33,000 compounds and identified one (RUC-1) that inhibited α IIB β 3-mediated adhesion of human platelets to fibrinogen.^{102–104} We went on to make derivatives of RUC-1 and found RUC-2, which is ~100-fold more potent and has a unique mechanism of action that involves competing for a carboxyl group on glutamic acid residue in the β 3 subunit (E220) that is crucial for binding the divalent cation Mg^{2+} in a specialized domain termed the Metal Ion Dependent Adhesion Site (MIDAS).^{105,106} Ligands, including fibrinogen and von Willebrand factor, bind to α IIB β 3 in part by having an aspartic acid carboxyl group oxygen coordinate the MIDAS Mg^{2+} . Since RUC-2 displaces the Mg^{2+} from the MIDAS, the receptor cannot bind fibrinogen or other ligands. When ligands normally bind to α IIB β 3, moreover, the negatively charged ligand carboxyl not only coordinates the MIDAS Mg^{2+} , but also stabilizes a conformation in which an adjacent β 3 loop moves toward the MIDAS Mg^{2+} , setting in motion a series of additional major conformational changes in the receptor that shift it into a high affinity ligand binding state and expose new sites on the receptor. This ligand-induced conformational change has been implicated in contributing to the thrombocytopenia associated with eptifibatide, and tirofiban.¹⁰⁷ Moreover, the conformational change to a high affinity ligand binding state has been hypothesized to contribute to the paradoxically increased mortality rate found in patients treated chronically with experimental oral α IIB β 3 antagonists that were patterned, like eptifibatide and tirofiban, on the R(K)GD sequence that contains an aspartic acid carboxyl that coordinates the MIDAS Mg^{2+} .^{108,109} Since RUC-2 displaces the MIDAS Mg^{2+} , there is no Mg^{2+} to bind ligand via a carboxyl group that could induce the conformational change. As a result, unlike eptifibatide and tirofiban, RUC-2 does not activate the receptor when it is preincubated with the receptor and then washed away.¹⁰⁵

Thus, RUC-2 has many positive features for the pre-hospital therapy of STEMI, including the potency of an $\alpha\text{IIb}\beta 3$ antagonist and freedom from inducing the conformational changes associated with activation of the receptor and exposing neoepitopes that may be the basis for some forms of thrombocytopenia. Intramuscular administration (IM) by autoinjector is desirable in the pre-hospital setting since oral antiplatelet agents are poorly absorbed in patients with STEMI^{110–112} and intravenous administration is more technically challenging. RUC-2 does not require intravenous administration and in animal models it can completely inhibit platelet aggregation induced by ADP within 5 minutes of intraperitoneal administration. Its effects begin to wear off after several hours, which fits well with its use being limited to the pre-hospital phase since it would allow the physicians in the receiving hospital broad options in deciding on additional therapy. What RUC-2 lacks, however, is the high aqueous solubility required for delivery by autoinjector since an adult dose needs to be contained in no more than ~1 ml. As a result, we made modifications in RUC-2 and produced RUC-4, which differs only in substituting a nitrogen for a carbon in one of the phenyl rings, but is much more soluble.¹⁰⁶ RUC-4 has many of the same features of RUC-2, and in addition it is rapidly effective in inhibiting platelets after IM administration in non-human primates. It is currently undergoing additional preclinical testing to assess its therapeutic potential.

Conclusion

I have had the great good fortune during my career to observe firsthand how our increasingly sophisticated understanding of platelet physiology and thrombotic cardiovascular disease has led to substantial clinical advances. The serendipitous discovery of aspirin's antiplatelet effects led the way, followed by the introduction of the P2Y₁₂ antagonist ticlopidine, which was identified by mass screening. The development of $\alpha\text{IIb}\beta 3$ antagonists represented a major conceptual turning point because it was the first time that agents were designed to inhibit a specific target that was selected based on understanding platelet physiology. As a result, it ushered in the modern era of rational antiplatelet drug design, which has been reinforced by the recent FDA approval of the drug vorapaxor, a PAR-1 thrombin receptor agonist developed based on the pioneering studies of Dr. Shaun Coughlin,^{113,114} for secondary prophylaxis of vascular disease.¹¹⁵ RUC-4 and other agents under development represent yet another level of scientific sophistication, in which an atomic level of understanding of both the receptor and the antagonist can be used to identify agents that have the most favorable properties for a specific clinical indication.

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Figure 1A

Figure 1B

Figure 1.

Figure 1A. The clinical course of a patient reported by Duke.³⁵ The transfusion was performed at 2 a.m. on May 12.

Figure 1B. Bleeding time results from a patient described by Duke in 1910.³⁵ Initial platelet count 3,000/ μ l. The blots in Series A were taken immediately after the ear was pricked; Series B, 20 minutes; C, 40 minutes; D, 60 minutes, and E, 80 minutes later. The bleeding time at this time was 90 minutes. Series F, showing a normal bleeding time, was taken after the transfusion at a time when the platelet count was 110,000/ μ l. (Reprinted with permission from reference 35. Copyright 1910 American Medical Association. All rights reserved).

Figure 2. Hospital case-fatality rates for acute myocardial infarction, U.S., 1970–2009. [Reproduced from NHLBI Fact Book 2012 (reference 100)].

Figure 3. Hospital discharges for heart failure by sex, U.S., 1980–2010. Source: National Hospital Discharge Survey/National Center for Health Statistics and National Heart, Lung and Blood Institute. (Reprinted with permission from reference 69.)